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14. ABSTRACT

Macrophages are a major inflammatory cell in malignant pleural mesothelioma and may help maintain tumor resistance to therapy. In this final year, we have completed the key tasks that confirm the importance of the macrophage in the inflammatory cell environment of mesothelioma. We have confirmed that it is possible to alter the polarization of macrophages to either an M1 (anti-tumor) or M2 (pro-tumor) phenotype and that a shift toward the Th1 phenotype or depletion of macrophages increases mesothelioma cell apoptosis and chemosensitivity. In our orthotopic murine mesothelioma model, the depletion of macrophages increases the mesothelioma response to chemotherapy. We have recently used a non-toxic CSF1 receptor inhibitor already in clinical trials to manipulate macrophages and shown that the tumors become more responsive to chemotherapy regimens used in patients with mesothelioma. At the end of our three year award, we have convincing evidence that manipulation of macrophages can alter the response of mesothelioma to standard therapy. Our major publications on these findings are in preparation and an RO1 application with a clinical trial included is planned for submission this fall.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:

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I. INTRODUCTION

Inflammation is an early and consistent feature of mesothelioma [1, 2], an incurable tumor with a high level of resistance to chemotherapy [3]. Within this inflammatory milieu, we have discovered that there is a large population of macrophages, in a higher percentage than we have found in other thoracic malignancies. The macrophage is perhaps the earliest cell recruited to the asbestos fibers that are the cause of mesothelioma. After the tumor forms, macrophages are also, as we have found, a constant feature of mesothelioma. The association of macrophages appears thus to be a feature of mesothelioma during its initiation as well as its progression over four decades.

Macrophages, often known as tumor-associated macrophages, are found, albeit to a lesser extent, throughout many solid tumors. Increasingly, they are recognized as a key factor enhancing the malignant features of the tumor [4]. In separate tumors, a greater extent of macrophage infiltration in a tumor correlates with worse prognosis [5, 6]. Macrophages are now understood to have different phenotypes, directed by their cellular and cytokine environment [7]. The classic macrophage phenotype, termed M1 in analogy to the Th1 phenotype of lymphocytes, is directed to phagocytosis and antibacterial actions. This classic activation is seen in the pro-inflammatory environment, where LPS or interferons are prominent. More recently, an M2 phenotype was described that is directed by other cytokines, namely IL4, TGF beta. This alternatively activated macrophage is considered to be important at for the resolution of inflammation, dampening the inflammatory response and orchestrating a healing or tissue remodeling phase [8].

We wish to understand the mechanisms by which macrophages influence mesothelioma cells and whether we can manipulate the tumor-associated macrophages within the tumor by deletion or by alteration of the macrophage phenotype to sensitize tumors to apoptosis. To address these questions, we have several in vitro, ex vivo and in vivo approaches to investigate the mechanisms of macrophage-mesothelioma cell interactions. Our studies are designed to test the hypothesis that macrophages influence mesothelioma cell survival and can be manipulated to enhance mesothelioma cell apoptosis and response to chemotherapy.

In this final year of our grant-funded research, we have completed the key aims. We also are poised to continue this research by extending these findings into a new in vivo model, the MexTAG mouse [9, 10]. With substantial progress, we are preparing an RO1 together with a renowned oncologist with significant expertise in mesothelioma, Dr. Hedy Kindler, to propose a clinical trial testing GW2580, an anti-CSF1R, to inhibit the mesothelioma macrophages and enhance the chemoresponsiveness of the tumor.

II. RESEARCH ACCOMPLISHMENTS

Task 1. To determine the functional significance of macrophage phenotype on mesothelioma cell survival.

a. Elucidate the percentage of immune cells (CD45+) in human mesothelioma tumors and correlate immune cell infiltration with histopathologic subtype (months 1-6).

COMPLETE: Results were reported in the 2010 Annual Progress Report, have been presented at several meetings (see Appendix), and are in preparation in manuscript form.
b. Determine the macrophage population (CD14+) as a percentage of the total immune cell population by flow cytometry (months 1-36).

COMPLETE: Results were reported in the 2011 Annual Progress Report, were presented at several meetings this year (see Appendix) and are in preparation in manuscript form.

c. Determine the profile of other immune cells within the microenvironment of the mesothelioma tumor using a panel of cell surface markers (months 1-36).

COMPLETE: Mesothelioma tumors (n=19), both chemotherapy naïve and chemotherapy treated, along with normal pleura (n=8) were studied by FACS for the profile of all immune cells. There is a clear difference in the macrophage infiltration in the tumors, whether previously treated or not treated with chemotherapy. There is a trend toward an increase in percentage of the T cells, CD4 and CD8. There is also a decrease in the percentage of neutrophils.

\[
\begin{array}{c}
\text{Normal Pleura} \\
\text{MPM CTX-naive} \\
\text{MPM CTX-treated}
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FACS analysis of leukocytes as a % of total CD45+ cells in normal pleura, CTX-naïve MPM, and CTX-treated MPM. ** p < 0.01 compared to normal pleura, *** p < 0.001 compared to normal pleura by Mann-Whitney test.

d. Confirm macrophage percentages by immunodetection of the same mesothelioma in fixed tissues (CD68+) (months 1-36).

COMPLETE: Results presented in the 2010 Annual Progress Report have been confirmed using immunohistochemistry of paraffin-embedded mesothelioma. On average, using both immunohistochemistry and flow cytometry, macrophages constitute between 30-70% of the CD45+ (inflammatory) cell population in mesothelioma. We expanded this task to determine whether prior treatment with chemotherapy altered the percentage of macrophages. In the panels below, we can report that there is no difference in these two groups of mesothelioma.


(A) Representative hematoxylin and eosin (H&E), CD45, and CSF-1R staining of normal pleura and malignant pleural mesotheliomas (MPM) resected from patients who were chemotherapy (CTX) naïve, or who had received neoadjuvant CTX prior to surgery (MPM + CTX). (B) Quantitation of CD45 and CSF-1R staining performed by Aperio software analysis. *** p < 0.001 by Mann-Whitney test.

e. Isolate macrophages from human mesothelioma disaggregated into single cells by flow cytometry for use in co-culture spheroids with mesothelioma cells (1-12).

COMPLETE: As presented in preliminary form in the 2010 Annual Progress Report, we have shown that human macrophages from mesothelioma can be harvested from the tumor and co-cultured with mesothelioma cells in culture. In parallel experiments, results with the mesothelioma macrophages and with the macrophages derived from peripheral blood are equivalent.

f. Determine macrophage functional properties in mesothelioma using fixed tissues, by tissue microarray, and by immunohistochemistry for protein expression to define their M1 or M2 microenvironmental status (months 1-12).

COMPLETE: Staining of fixed tissues using immunohistochemistry failed to show consistent patterns. Another limitation was the comparison of staining with normal controls: normal pleura is a thin tissue with significant edge effect, making clearcut immunohistochemical staining difficult to interpret. For these reasons, we moved instead to use PCR to determine the functional status of the macrophages in mesothelioma tissue.

g. Determine the M1 or M2 gene expression signature of macrophages by commercial global chip assay for RNA from tumor tissue or cultures of cells/spheroids for gene profile (months 1-12).

COMPLETE: We have now completed our analysis of 20 tumors with the addition of 5 normal pleural samples, obtained from lungs otherwise normal that were not used for lung transplantation. The recruitment of the normal samples was a slow process, dependent on close coordination with another laboratory and on the availability of normal lungs. With this optimal normal control, we can now show upregulation of message for several inflammatory cytokines, including CCL3, a key macrophage-produced inflammatory chemokine, and CSF-1, a macrophage stimulating and recruitment factor. There is elevation of IL4 indicating an M2/Th2 phenotype.
Human malignant pleural mesotheliomas exhibit increased mRNA expression of macrophage markers compared to normal pleura. Quantitative PCR analysis performed on whole tissues to determine mRNA gene expression of cytokines and immune cell markers in normal pleura (white bar), CTX naïve malignant mesothelioma (black bar), and CTX-treated malignant mesothelioma (red bar). * p < 0.05 compared to normal pleura, ** p < 0.05 compared to CTX naïve by Mann-Whitney test. Statistically significant changes were found for the macrophage markers CD14 and CSF1R, and the TH2 cytokine IL4.

h. Analyze cytokines produced by mesothelioma tumor fragments by commercial cytokine bead assay from human mesothelioma grown as tumor fragments (months 1-12).

COMPLETE: A variety of inflammatory cytokines have been detected in the supernatant of tumor fragment spheroids, which parallel the RNA message described in g above. In particular, IL-4 is present along with the CSF1 growth factor.

i. Analyze cytokines produced by multicellular spheroids made from either mesothelioma cells alone or mesothelioma cells plus macrophages (THP-differentiated) (months 1-12).

COMPLETE: As presented in the 2010 Annual Progress Report, we have completed studies with hybrid spheroids showing that we are able to polarize the mesothelioma + macrophage hybrid spheroids to an M1 or M2 phenotype. Expression of M1 cytokines (TNF-alpha, IL12 and IL6) were elevated following polarization with interferon gamma plus endotoxin to an M1 phenotype; expression of M2 cytokines (particularly IL-10) was elevated following polarization toward an M2 phenotype using IL-4. The polarization was dependent on the presence of macrophages; no change in cytokine expression was seen in spheroids containing mesothelioma cells alone. Thus, the response of these tumor models to the polarization environment requires the presence of the macrophage population.

j. Analyze changes in cytokine expression when spheroids (mesothelioma only or mesothelioma plus macrophage) are treated with TRAIL or TRAIL plus gemcitabine (months 1-12).

COMPLETE: We have determined that the polarization state or cytokine profile of the hybrid spheroids is constant after treatment with TRAIL plus gemcitabine (T+G) and after carboplatin plus pemetrexed, the standard chemotherapeutic regimen given to patients with mesothelioma.
Task 2. To determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro.

a. Confirm ability of clodronate-liposomes to deplete macrophages from multicellular spheroids made either with THP-1 differentiated macrophage cells or with primary mesothelioma-derived macrophages and determine optimal dose and timing (months 9-14).

COMPLETE: As described in our previous progress reports, we have moved from toxic clodronate to CSF1R inhibitors, either the Plexxikon compound PLX-3397 or currently GW2580, both of which inhibit the CSF1 receptor. We have determined that the PLX-3397 and the GW2580 compounds both induce loss of macrophage viability, either with macrophages grown in culture and in hybrid spheroids. By qPCR, we can also find that GW2580 alters the polarization by decreasing Th2 markers; there is a trend suggesting an increase in Th1.

GW2580 decreases the viability of Th2-polarized macrophages: PBMC derived macrophages were polarized towards Th1 or Th2 with appropriate cytokines, were treated without or with GW2580 (1µM) for 3 days, harvested and stained with trypan blue. GW2580 significantly decreases the macrophages viability when compared to Th1-polarized macrophages. n=3 * p<0.01

GW2580 also reprograms the macrophages: PBMC derived macrophages were polarized towards Th1 or Th2 as above and treated without or with GW2580 (1µM) for 3 days, harvested for RNA extraction and analyzed by qPCR. GW2580 significantly increased the expression of Th2 markers (see MRC1). Th1 markers (see IL12A) showed a trend toward an increase. (n=3, p<0.05)
b. Confirm ability of clodronate-liposomes to deplete macrophages from mesothelioma tumor fragment spheroids with dose and timing established above (months 12-24).

COMPLETE: The two compounds also deplete macrophages from tumor fragment spheroids (see figure below).

GW2580 decreases the number of macrophages in TFS: Unpolarized TFS were treated without or with GW2580 (1µM) for 3 days, harvested for paraffin fixation and labeled for CD68, a marker of macrophages by immunohistochemistry (marked brown). GW2580 decreases the number of macrophages in the TFS (representative of 3 different tumors).

c. Analyze effect of macrophages and of macrophage depletion on apoptosis of mesothelioma cells to treatment with TRAIL or TRAIL plus gemcitabine using multicellular spheroids either with no macrophages, macrophage-depleted or with macrophages (months 12-24).

COMPLETE: We have now shown that the inhibition and relative depletion of macrophages in hybrid spheroids leads to an enhanced chemoresponsiveness of the mesothelioma cells. The increased chemoresponsiveness has been mediated by either of two CSF1R antagonists, PLX3397 or GW2580 (shown below) and can be seen with either TRAIL plus gemcitabine or with carboplatin plus pemetrexed.

Macrophages and mesothelioma cells form hybrid spheroids. This spheroid contains 10,000 mesothelioma cells (green, anti-cytokeratin) and 500 (5%) macrophages (red, anti-CD69) (see arrows). By fluorescent microscopy, the macrophages are found to be distributed throughout the spheroid. Spheroid is approximately 0.5 mm in diameter.
d. Analyze the effect of macrophages and of macrophage depletion on apoptosis of mesothelioma cells in tumor fragment spheroids to treatment with TRAIL or TRAIL plus gemcitabine (months 12-24).

COMPLETE: Similarly to the effect in hybrid spheroids, we have now exposed tumor fragment spheroids from 5 different patients and exposed them to GW2580 prior to treatment with carboplatin plus pemetrexed. This finding confirms the results in cultured macrophages (see Task 2a) and hybrid spheroids (Task 2c) that the Th2 phenotype is most sensitive to blockade of the CSF1R by GW2580.

GW2580 enhances the apoptotic response to chemotherapy in Th2-polarized spheroids: TFS were polarized in presence of appropriate cytokines, pretreated without or with GW2580, exposed to chemotherapy, harvested for paraffin fixation and double immunostained for cytokeratin (CK), a marker of mesothelioma cells, and cleaved caspase 3 (CASP3), a marker of apoptosis. GW2580 enhances the apoptotic response to chemotherapy in Th2-polarized tumor fragment spheroids (representative of 5 different tumors, p<0.05).
e. Confirm ability of M1 cytokines to polarize macrophages to a strong M1 phenotype by exposing multicellular spheroids with macrophages (primary mesothelioma or THP-1) to M1 polarizing agents (interferon gamma plus LPS) and confirming with cytokine and gene expression assays for classics M1 or M2 polarization (months 12-24).

COMPLETE: As described in previous progress reports, we have confirmed that polarization is effective in reprogramming macrophages to an M1/Th1 or M2/Th2 phenotype. We continue to see no significant difference in the cytokine expression of the different subsets of M2: M2a and M2c.

![Graph showing cytokine expression]

Human macrophages derived from peripheral blood monocytes can be polarized to an M1 or M2 phenotype. Peripheral blood monocytes were matured to macrophages and exposed for 24 h to IFNgamma plus LPS (to stimulate toward a TH1 or M1 phenotype), IL-4 (toward TH2 or M2a) or IL-10 (toward TH2 or M2c). RNA was harvested and analysed by qPCR for expression of message for M1 or M2 marker cytokines.

f. Polarize macrophages in tumor fragment spheroids using the approach found best above (months 12-24).

COMPLETE: We were able to confirm polarization of tumor fragment spheroids to Th1 or Th2, with the upregulation of characteristic mRNA for Th1 or Th2 markers. Immunohistochemical staining showed that the expression of iNOS, a marker for Th1, was increased in cultured macrophages and in tumor fragment spheroids polarized toward Th1; expression of the CD206 marker for Th2 was increased when polarized toward Th2.

g. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in multicellular spheroids (grown with primary mesothelioma macrophages or THP-1 macrophages) when treated with TRAIL plus gemcitabine (months 12-24).

COMPLETE: We have extensive data on the effect of repolarization on the apoptotic responses of the mesothelioma cells. As shown in the figure below and reported in the prior progress reports, we have data with multiple different mesothelioma cell lines, a variety of sources of macrophages (from primary mesothelioma, from peripheral blood,
from THP cells) and with different apoptotic regimens (TRAIL containing, or carboplatin plus pemetrexed), that the M1/Th1 phenotype leads to an increased apoptotic response of the mesothelioma cells. The mechanism is still under investigation and may depend on the intracellular cytokine assays planned to show the cytokines responsible. Early studies have implicated TNF for part of the pro-apoptotic response, although other cytokines may be playing an additional role.

h. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in tumor fragment spheroids (months 12-24).

COMPLETE: We have completed these studies showing that polarization to a Th1 phenotype leads to significant increases in chemosensitivity to carboplatin plus pemetrexed (see figure below). The spheroids are first polarized toward an M1 or M2 phenotype (or a no polarization, or M0 condition) and are then exposed to chemotherapy for 48 h (carboplatin plus pemetrexed). Following fixation and double fluorescent staining for mesothelioma cells (cytokeratin positive) and active apoptosis (cleaved caspase 3), we have found significantly increased apoptosis in spheroids polarized to M1. These findings are similar to those in the hybrid spheroids above and indicate a pro-apoptotic effect of the M1 polarization state of macrophages. However, in the tumor fragment spheroids, the M1 phenotype itself tends to increase apoptosis.

In 5 experiments in each cell line, polarization increases the apoptotic sensitivity of mesothelioma cells, but only when macrophages are present. This effect is seen for TRAIL plus gemcitabine (not shown) and also for the most clinically relevant therapeutic regimen, carboplatin plus pemetrexed, the treatment considered the standard for patients and the treatment now being used in the mice studies. * p<0.03 n=5

A Untreated
Tumor fragments were exposed to nothing (M0) or to a polarization schedule for 48 h to induce an M1 or an M2 (Th1 or Th2) phenotype and stained for apoptosis (A) without further treatment or (B) after exposure to carboplatin plus pemetrexed for another 48 h. The tumor fragments were fixed, embedded, sectioned and stained for cytokeratin (green) and cleaved caspase 3 (red). The merged color (yellow) shows the presence of apoptotic mesothelioma cells. Apoptosis is increased in tumor cells within Th1-polarized TFS, particularly when treated. No difference is seen in untreated TFS (not shown) (Green=cytokeratin indicating mesothelioma cells, red=cleaved caspase 3; yellow=apoptotic mesothelioma cells)

**Human Tumor Fragment Spheroids are Sensitized to Chemotherapy by Th1 Polarization**

In tumor fragments derived from 5 different patients, polarization to a Th1 phenotype enhanced apoptosis by itself and also enhanced the apoptotic response to carboplatin plus pemetrexed treatment for 48 h (C+P). (n=5, *p<0.05)
Task 3. Define functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

a. Characterize immune cell profile of the murine mesothelioma model induced by intraperitoneal asbestos injections in the NF2+/- mouse model (months 0-24).

COMPLETE: Full immunophenotyping of the immune cells have been carried out by multicolor FACS in the orthotopic mesothelioma model, in which syngeneic mesothelioma murine cells (40L) are introduced into the peritoneum of the mice and form into mesothelioma tumors that closely resemble human mesothelioma. At baseline, without treatment, the predominant inflammatory cell recruited to the tumor is the macrophage, in a similar pattern to that seen in humans.

Blockade of CSF-1/CSF-1R reprograms the mesothelioma tumor immune microenvironment. Fluorescence- Activated Cell Sorting (FACS) analysis was performed on single cell suspensions isolated from 40L orthotopic mesothelioma tumors from mice treated with: 1) vehicle or IgG; 2) vehicle or IgG + CTX (carboplatin + pemetrexed); 3) GW2580 + CTX; or 4) anti-CSF-1 + CTX. Colored bars represent the proportion of each cell population identified out of the total CD45+ cells.

As reported in our last progress report, the asbestos-injected de novo tumor model NF2+/- had significant limitations. The two major limitations were the low and unpredictable number of mice that develop mesothelioma and the time required. After 1 year following multiple injections of asbestos, less than 30% of our mice were shown to have mesothelioma. However, we have used this period to advance several techniques that will help us evaluate mice for early development of mesothelioma and we have arranged, by collaboration with Dr. Richard Lake of Perth Australia, to obtain an improved de novo mouse mesothelioma model, the MexTag model [9, 10]. This mouse has a mesothelin promoter that induces SV40 large T antigen expression in the mesothelial cells; only a few injections of asbestos lead to mesothelioma leading to mesothelioma in all mice, with the development between 20-40 weeks. Such a model has many advantages: almost 100% development of mesothelioma, the 1-2 injections of asbestos and the more rapid development. This model will be much better for the planned treatment studies.

As a result of our difficulties with the NF2+/- mice, we have also developed techniques to screen mice for mesothelioma: 1) cytology of peritoneal lavage, which demonstrated mesothelioma cell clusters (e.g. spheroids) in otherwise normal appearing mice that later were proved to have mesothelioma by autopsy and 2) Doppler ultrasound, which was able to demonstrate pre-morbid mesothelioma in several mice due to the vascular enhancement of the peritoneal wall thickened by tumor. Thus, we have new tools to
evaluate these new mice to determine the earliest appearance of mesothelioma so that clinical studies can be initiated. The MexTag mice will be used for the studies that continue following this award.

b. Deplete in vivo macrophages with intraperitoneal clodronate-liposomes in mice without mesothelioma to establish protocol (months 4-12).

COMPLETE: We have completed the clodronate studies and followed the in vivo protocol for the use of intraperitoneal clodronate for macrophage depletion. In GFP-macrophage mice, the clodronate was able to eliminate the GFP signal and all cells costaining with two macrophage markers, CD11b+ and F4/80.

However, as mentioned, we have pursued clinically-relevant studies of macrophage inhibition using compounds that are nearing clinical use, non-toxic reagents such as the PLX-3397 or the GW2580 inhibitors of the CSF1 receptor. These studies have begun with the PLX3397 compound and show that PLX3397 does alter the macrophage population in the mouse. These studies have been carried out in mice with orthotopic luciferase-labeled intraperitoneal mesothelioma, not in normal mice, because we wished to go quickly to the relevant model.

The general treatment schedule is shown:
**PLX3397 decreases macrophage number in orthotopic mesothelioma.** In mice with orthotopic 40L luciferase-labeled intraperitoneal mesothelioma, the PLX3397 compound was injected intraperitoneally with or without chemotherapy (carboplatin (10 mg/kg) plus pemetrexed (50 mg/kg) given in 4 doses over 2 weeks). By flow cytometry, the presence of macrophage population in the tumors was significantly reduced. The reduction in macrophage number was seen whether or not the chemotherapy was given.

As described above, we have moved to the orally available and clinically relevant CSF1R antagonist, GW2580. The studies were repeated with GW2580 in the mouse chow. Both PLX3397 (above) and GW2580 (below) were able to deplete macrophage number in the orthotopic mesothelioma tumors.

**The CSF-1R tyrosine kinase inhibitor GW2580 enhances the antitumor effects of cytotoxic chemotherapy in a syngeneic orthotopic murine model of mesothelioma.** (A) Schematic of experimental design. Wild-type C57BL/6 mice were i.p. injected with syngeneic 40L mesothelioma tumor cells at day 0. Tumor-bearing mice were randomized into one of four experimental groups at day 21. Mice were sacrificed at day 35. Cytotoxic chemotherapy (CTX) consisted of carboplatin (50 mg/kg) and pemetrexed (100 mg/kg) administered i.p. GW2580 was incorporated into chow at 800 mg/kg which mice consumed ad libitum. Experiment was performed twice with a minimum of 5 mice per treatment group in each experiment. (B) Effect of CTX and GW2580 on tumor macrophage infiltration. Quantitation of CD11b+ F4/80+ Ly6C Ly6G MHCII+ cells (macrophages) as a percentage of total CD45+ cells by FACS analysis of whole mesenteric tumors. * p < 0.05, ** p < 0.01 by Mann-Whitney test.

Thus, in this orthotopic model, both of the two different CSF1 receptor antagonists are able to decrease macrophage populations in mesothelioma.

c. Deplete in vivo macrophages in mice with mesothelioma to confirm depletion, to establish effect on other immune cell populations, and to confirm lack of toxicity on the mice (months 12-24).

COMPLETE: In the orthotopic 40L mesothelioma mice, the PLX3397 treatment in the schedule as shown above was also evaluated for several different immune populations by flow cytometry. It was found that the reduction of macrophages was accompanied by an increase in CD8 positive T cells (figure below, see *). Such a result parallels findings in breast cancer and may indicate that CD8 T cells will be shown to mediate cytotoxicity against the tumor.
The CSF-1R tyrosine kinase inhibitor PLX3397 exhibits anti-tumor activity in an syngeneic orthotopic model of malignant mesothelioma. (A) Schematic of experimental design. Wild-type C57BL/6 mice were i.p. injected with syngeneic 40L mesothelioma tumor cells at day 0. Tumor-bearing mice were randomized into experimental groups at day 14. Mice were sacrificed at day 35. PLX3397 was incorporated into chow at 290 mg/kg which mice consumed ad libitum. The experiment was performed twice with a minimum of 10 mice per treatment group in each experiment. (B) Effect of PLX3397 on tumor immune cell infiltration by FACS analysis. * p < 0.05 by Mann-Whitney test.

In these studies, the PLX3397 compound can be seen to alter the inflammatory cellular balance within the tumor microenvironment. By reducing macrophages and increasing CD8+ T cells, the PLX3397 compound may be fortifying the anti-tumor forces within the tumor.

d. Determine whether macrophage depletion in mice with mesothelioma enhances apoptotic and therapeutic response of mice with mesothelioma to TRAIL plus gemcitabine over 1-4 weeks after treatment (months 24-36).

COMPLETE: As described above, we have relied for these studies on the orthotopic model of mesothelioma in immunocompetent mice produced by the IP injection of a syngeneic murine mesothelioma line (40L) in C57/Bl/6 mice (as described above). In this model, intraperitoneal mesothelioma forms 3-4 weeks after the ip injection of 2 x 10^6 40L cells. This model was developed by Dr. Agnes Kane, who kindly provided this cell line and a second line (7) for these studies [11].

The early results were described in the 2010 Annual Progress Report and showed that macrophage depletion using liposomal clodronate enhanced the response of mesothelioma to treatment with TRAIL plus gemcitabine. These exciting results led us to move to a more clinically relevant treatment regimen, using carboplatin plus pemetrexed, and a more clinically relevant macrophage inhibition regimen, using PLX-3397. The 40L cells have also been transfected with a luciferase construct so that can be monitored by luciferase optical imaging. We have found however that luciferase assays are most useful for screening for the increase in tumor size; the luciferase is not quantitative so actual tumor volume must be measured at postmortem.
In these studies, using the protocol shown in Task 3b above but without the use of chemotherapy, we have now shown that PLX3397 reduces the mesothelioma tumor volume when used alone, even without any chemotherapy (figure below).

Monotherapy with PLX3397 reduces the burden of mesothelioma in an orthotopic mouse mesothelioma model.

In addition, we were able to show that the PLX3397 monotherapy also increased the presence of apoptosis in the orthotopic mesothelioma cells, suggesting that survival of the mesothelioma cells was dependent on CSF1, presumably via macrophage effects on tumor cell survival (see figure below).

Monotherapty with PLX3379 led to an increase in apoptosis of the mesothelioma cells, as shown by an increase in cleaved caspase-3. There was no significant change in the proliferation of the tumor cells, as shown by a constant level of BrdU staining. Quantitation was performed on 5 fields of view from 5 independent mouse tumors per treatment group by Aperio ImageScope software. * p < 0.05 by Mann-Whitney test.

e. Repolarize macrophages in vivo by injections of interferon gamma plus LPS and assaying cytokines of peritoneal ascites and gene expression of peritoneal macrophages obtained by lavage to confirm method of repolarization, and to select best tolerated method for the mice (months 12-24).

Efforts to repolarize the macrophages intraperitoneally were less successful and much less likely to be clinically relevant than the inhibition of macrophages. With the new agents that became available during this award, e.g. PLX3397 and GW2580, we elected
to concentrate our efforts and our mice experiments with the CSF1R antagonists. Such agents are already entering the clinical trials for malignancy and our studies were adapted to move more quickly toward a clinically useful treatment strategy.

f. Determine whether the depletion or repolarization of macrophages in vivo enhances the apoptotic and therapeutic response of mice with mesothelioma to TRAIL plus gemcitabine over 1-4 weeks after treatment (months 24-36).

COMPLETE: These studies have been very successful. GW2580 to inhibit macrophages was effective in enhancing the chemosensitivity of the mesothelioma cells to chemotherapy. Mice with mesothelioma treated with GW2580 showed an increase in apoptotic response to the chemotherapy and a decrease in tumor burden. These studies are consistent with our earlier studies using clodronate to deplete macrophages or using PLX3397 to inhibit macrophages. These studies provide confidence that the macrophage is a promising target in mesothelioma.

The CSF-1R tyrosine kinase inhibitor GW2580 depletes macrophages in the orthotopic model. (A) Schematic of experimental design. Wild-type C57BL/6 mice were i.p. injected with syngeneic 40L mesothelioma tumor cells at day 0. Tumor-bearing mice were randomized into one of four experimental groups at day 21. Mice were sacrificed at day 35. Cytotoxic chemotherapy (CTX) consisted of carboplatin (50 mg/kg) and pemetrexed (100 mg/kg) administered i.p. GW2580 was incorporated into chow at 800 mg/kg which mice consumed ad libitum. Experiment was performed twice with a minimum of 5 mice per treatment group in each experiment. (B) Effect of CTX and GW2580 on tumor macrophage infiltration. Quantitation of CD11b⁺ F4/80⁺ Ly6C⁻ Ly6G⁻ MHCI⁺ cells (macrophages) as a percentage of total CD45⁺ cells by FACS analysis of whole mesenteric tumors. * p < 0.05, ** p < 0.01 by Mann-Whitney test.
The CSF-1R tyrosine kinase inhibitor GW2580 enhances the antitumor effects of cytotoxic chemotherapy in a syngeneic orthotopic murine model of mesothelioma. (C) Effect of CTX and GW2580 on tumor burden, tumor cell apoptosis, and proliferation. Representative gross images (upper panel) and H&E, cleaved caspase-3 and BrdU stained histological sections of mesenteric tumors. Tumor burden quantitation was performed on 3 serial H&E stained sections 90 microns apart. Cleaved caspase-3 and BrdU quantitation was performed on 5 fields of view from 5 independent mouse tumors per treatment group by Aperio ImageScope software. * p < 0.05 by Mann-Whitney test.

g. Determine whether the depletion or repolarization of macrophages enhances survival of mice with mesothelioma after treatment with vehicle, TRAIL or TRAIL plus gemcitabine (months 24-36).

Survival studies were postponed until the MexTag mice could be introduced into the study. A cohort of these mice has been injected with asbestos intraperitoneally and is being readied for inclusion in a survival study with four conditions: no treatment, GW2580 alone, carboplatin plus pemetrexed alone and GW2580 plus carboplatin plus pemetrexed. The results from these studies, along with complete characterization of the MexTag mice, will be included in a manuscript being prepared for publication.
III. KEY RESEARCH ACCOMPLISHMENTS

Task 1. To determine the functional significance of macrophage phenotype on mesothelioma cell survival.

a. Elucidate the percentage of immune cells (CD45+) in human mesothelioma tumors and correlate immune cell infiltration with histopathologic subtype (months 1-6).

   Months 1-12
   • Acquired 71 fixed and paraffin-embedded mesothelioma tumor samples
   • Prepared mesothelioma tumor tissue microarrays for immunohistochemistry
   • Determined the pathologic subtype of each tumor in concert with the thoracic pathologist, Dr. Steven Nishimura
   • Quantified each sub-population as a percentage of the total inflammatory cell population using digital imaging software, the Aperio system, optimized for either membrane or nuclear staining
   • Correlated presence of inflammatory cell populations with mesothelioma pathologic subtype
   • Obtained normal pleural controls by a collaboration with a pulmonary laboratory of Dr. Michael Matthay to obtain normal human pleura from human donor lungs rejected for transplantation

b. Determine the macrophage population (CD14+) as a percentage of the total immune cell population by flow cytometry (months 1-36).

   Months 1-12
   • Optimized disaggregation protocol for mesothelioma tumors
   • Established that the percentage of CD14+ cells in the mesothelioma inflammatory population exceeds that of the other thoracic tumors, lung and esophagus
   • Expanded flow cytometry studies to a new high density 14 color system
   • Confirmed the high percentage of macrophages using high density flow
   • Analyzed 40 tumors so far using 14 color, high density flow cytometry
   • Initiated collection of peripheral blood samples from the patients with mesothelioma to compare activation status of cells in tumor compared to periphery
   • Included mesothelin in panel of cell surface markers to quantify percentages of mesothelioma cells in tumors

c. Determine the profile of other immune cells within the microenvironment of the mesothelioma tumor using a panel of cell surface markers (months 1-36).

   Months 1-12
   • Performed immunohistochemical analysis on 71 tumors to identify inflammatory cell infiltrates, e.g. CD45, CD68, CD8, CD4 and CD20

   Months 12-24
   • Using flow cytometry to evaluate several activation markers on inflammatory cells

d. Confirm macrophage percentages by immunodetection of the same mesothelioma in fixed tissues (CD68+) (months 1-36).

   Months 1-12
   • Determined the percentages of macrophages in mesothelioma using same tumors studied in immunohistochemistry (by CD68+) and in flow cytometry (by CD14+)

e. Isolate macrophages from human mesothelioma disaggregated into single cells by flow cytometry for use in co-culture spheroids with mesothelioma cells (1-12).

   Months 1-12
• Isolated macrophages from fresh mesothelioma tumors with high viability (80-90%) and in sufficient numbers to study
• Produced hybrid spheroids with macrophages isolated from human mesothelioma tumors

Month 13-24
• Determined that macrophages from human mesothelioma acted the same as the peripheral blood monocytic cells (PBMC) so will rely on the PBMC for future studies

f. Determine macrophage functional properties in mesothelioma using fixed tissues, by tissue microarray, and by immunohistochemistry for protein expression to define their M1 or M2 microenvironmental status (months 1-12)

Month 1-24
• Stored all incoming mesothelioma tissues in tissue bank for these studies while technique is being tested in laboratory, once the technique is perfected it will be straightforward to analyze the stored tissues

Month 24-36
• Used the tissues preferentially for PCR which was more consistent and did not require staining of the normal pleura, a structure so thin as to be a poor control for immunohistochemistry

g. Determine the M1 or M2 gene expression signature of macrophages by commercial global chip assay for RNA from tumor tissue or cultures of cells/spheroids for gene profile (months 1-12).

Month 1-12
• Quantified cytokine message in 7 snap frozen mesothelioma tumors
• Compared message to 4 normal pleural samples

Month 13-24
• Extended studies to 25 tumors
• Refined technique of obtaining normal pleural samples
• Extending studies in normal pleura in order to complete statistically valid comparison
• Concluded that expression signature of human mesothelioma compared to normal pleura contains predominantly macrophage markers and Th2 markers

h. Analyze cytokines produced by mesothelioma tumor fragments by commercial cytokine bead assay from human mesothelioma grown as tumor fragments (months 1-12).

Month 1-24
• Determined that cytokines were secreted by tumor fragment spheroids, but were too dilute for accuracy

Month 25-36
• Reduced volume of supernatant until cytokine signal was improved; cytokines produced by tumor fragment spheroids included Th2 cytokines such as IL-4, consistent with genetic expression signature described above

i. Analyze cytokines produced by multicellular spheroids made from either mesothelioma cells alone or mesothelioma cells plus macrophages (THP-differentiated) (months 1-12).

Month 1-12
• Quantified expression of key M1 or M2 cytokines in multicellular spheroids e.g. M1 (TNF, IL-12, IL-6) or M2 (IL-4, IL-10, TGF) compared to housekeeping gene TBP
• Determined that the polarization protocol does polarize hybrid spheroids to an M1 or M2 phenotype
j. Analyze changes in cytokine expression when spheroids (mesothelioma only or mesothelioma plus macrophage) are treated with TRAIL or TRAIL plus gemcitabine (months 1-12).

Months 1-12
- Determined that treatment with TRAIL or TRAIL plus gemcitabine has no significant effect on the M1 or M2 phenotype

**Task 2. To determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro.**

a. Confirm ability of clodronate-liposomes to deplete macrophages from multicellular spheroids made either with THP-1 differentiated macrophage cells or with primary mesothelioma-derived macrophages and determine optimal dose and timing (months 9-14).

Months 1-12
- Obtained and prepared clodronate-liposomes for in vivo use
- Use in vitro systems not initiated in months 1-12

Months 13-24
- Have moved to use of CSF1 receptor antagonists
- PLX3397, a CSF1 receptor inhibitor, reduces the survival of macrophages in vitro
- PLX3397 also reduces the number of macrophages in hybrid spheroids

Months 25-36
- Both PLX3397 and GW2580 reduce the viability of cultured macrophages, primarily those polarized toward a Th2 phenotype.

b. Confirm ability of clodronate-liposomes to deplete macrophages from mesothelioma tumor fragment spheroids with dose and timing established above (months 12-24).

Months 1-12
- Obtained and prepared clodronate-liposomes for in vivo use
- Use in vitro systems not initiated in months 1-12

Months 13-24
- GW2580 over 3 days reduces the number of macrophages found in tumor fragment spheroids

c. Analyze effect of macrophages and of macrophage depletion on apoptosis of mesothelioma cells to treatment with TRAIL or TRAIL plus gemcitabine using multicellular spheroids either with no macrophages, macrophage-depleted or with macrophages (months 12-18).

Months 1-12
- Derived macrophages from peripheral blood and THP monocyte-like cells
- Produced hybrid spheroids with two different mesothelioma cell lines (REN, M28) combined with macrophages derived from 4 different sources (THP, peripheral blood, banked blood, mesothelioma tumors)
- Established an imaging protocol to show that macrophages were viable and well distributed within spheroids
- Determined that macrophages alone (without polarization) had no consistent effect on the mesothelioma cell apoptotic response to treatment

Months 13-24
- Shown that macrophages are necessary for the effect of polarization on apoptotic response

Months 25-35
• Demonstrated that both PLX3397 and GW2580 increased the apoptotic response of mesothelioma cells to carboplatin plus pemetrexed when the mesothelioma cells were in hybrid spheroids that had been polarized toward Th2, again confirming that these inhibitors of macrophage viability and function target the Th2 macrophage.

d. Analyze the effect of macrophages and of macrophage depletion on apoptosis of mesothelioma cells in tumor fragment spheroids to treatment with TRAIL or TRAIL plus gemcitabine.

Month 1-12
  • Not initiated in months 1-12. Plan to use optimal protocol worked out in Task 2c in multicellular, hybrid spheroids

Month 13-24
  • Planning these studies using the PLX3379 compound in next 12 months

Month 25-36
  • Have demonstrated that PLX3397 and GW2580 (shown) increase the apoptotic response of the mesothelioma cells within tumor fragment spheroids when the spheroids are polarized to the Th2-phenotype; as in the hybrid spheroids, the CSF1R blockade appears to target the Th2-polarized macrophage leading to an increased chemoresponsiveness of the mesothelioma cells.

e. Confirm ability of M1 cytokines to polarize macrophages to a strong M1 phenotype by exposing multicellular spheroids with macrophages (primary mesothelioma or THP-1) to M1 polarizing agents (interferon gamma plus LPS) and confirming with cytokine and gene expression assays for classics M1 or M2 polarization (months 12-24).

Months 1-24
  • Determined that M1 stimulation (IFN gamma plus LPS) does polarize macrophages to an M1 phenotype (e.g. expressing TNF, IL12 and IL6)
  • Determined that M2 stimulation (IL4 - M2a or IL10 - M2c) does polarize macrophages to an M2 phenotype (e.g. expressing TGF beta, IL10 and IL4)

f. Polarize macrophages in tumor fragment spheroids using the approach found best above (months 12-24).

Months 1-12
  • Not initiated in months 1-12.

Months 13-24
  • Polarized macrophages within tumor fragment spheroids from 4 human tumors
  • Confirmed with PCR the effective M1 vs M2 polarization of the tumor fragments

g. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in multicellular spheroids (grown with primary mesothelioma macrophages or THP-1 macrophages) when treated with TRAIL plus gemcitabine (months 12-24).

Months 1-12
  • Determined that the presence of M1 polarized macrophages consistently enhances mesothelioma cell apoptotic responses to TRAIL plus gemcitabine
  • Showed that this conclusion does not depend on the source of macrophages although peripheral blood monocyte appear most consistent
  • Showed, in expanded studies, that this pro-apoptotic effect was mediated by soluble factors and could be reproduced by exposing the mesothelioma cells to media conditioned by M1 polarized macrophages
  • Determined that the cytokine TNF (an M1 cytokine), but not IL-10 (an M2 cytokine), contributed to the macrophage pro-apoptotic effect
h. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in tumor fragment spheroids (months 12-24).

Months 1-12
- Not initiated in months 1-12

Months 13-24
- Determined that polarization significantly enhances apoptosis induced by carboplatin plus pemetrexed in tumor fragment spheroids from 4 human mesothelioma patients
- Determined that polarization to an M1 phenotype may also increase apoptosis without treatment

Months 25-36
- Determined that Th1 polarization consistently increases the apoptotic response of mesothelioma cells to chemotherapy

Task 3. Define functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

a. Characterize immune cell profile of the murine mesothelioma model induced by intraperitoneal asbestos injections in the NF2+/- mouse model (months 0-24).

Months 1-12
- Established an NF2+/- mouse cohort of 20 mice that have completed the required 8 every 3 weeks intraperitoneal asbestos injections for production of mesothelioma
- Initiated a further cohort of 30 mice that are in process of receiving the 8 asbestos ip injections
- Established a second immunocompetent mouse model of mesothelioma using the 40L murine mesothelioma cell line obtained from Dr. Agnes Kane
- Carried out one experiment in the 40L mesothelioma model showing that mesothelioma develops in 4 weeks, as reported by Dr. Kane
- Characterization of immune cell population in these two models (asbestos induced NF2+/- and syngeneic orthotopic 40L) are planned for months 12-36

Months 13-24
- Determined techniques of pleural lavage cytology and Doppler ultrasound to try to identify mesothelioma in the NF2+/- mice
- Concluded that the incidence of mesothelioma in these mice was too unusual and too random to allow useful treatment trials
- Developed a collaboration with Dr. Richard Lake of Perth Australia in order to use the MexTag mesothelin-SV40 mice which develop mesothelioma earlier (20-40 weeks) after only 1-2 intraperitoneal injections of asbestos
- Ordered these mice

Months 25-36
- MexTag mice were injected with asbestos intraperitoneally and are now starting to develop de novo mesothelioma
- Completed the immunophenotyping by 14 multicolor FACS on the immune influx in the orthotopic 40L mouse mesothelioma model; macrophages are the predominant inflammatory cell

b. Deplete in vivo macrophages with intraperitoneal clodronate-liposomes in mice without mesothelioma to establish protocol (months 4-12).

Months 1-12
- Depleted intraperitoneal macrophages successfully using clodronate-embedded liposomes from GFP-macrophage labeled mice by showing loss of GFP signal and loss of cells co-staining with F4/80 and CD11b+

Months 13-24
• Depleted intraperitoneal macrophages using PLX-3397 in the orthotopic 40L mesothelioma model

Months 25-36
• Use both PLX3397 and GW2580 and show that they deplete macrophages in in vivo tumor

c. Deplete in vivo macrophages in mice with mesothelioma to confirm depletion, to establish effect on other immune cell populations, and to confirm lack of toxicity on the mice (months 12-24).

Months 1-12
• Used clodronate-liposomes in mice with 40L orthotopic mesothelioma
• Collected tissues after clodronate treatment to determine whether clodronate used in this schedule depleted all tumor-associated macrophages

Months 13-24
• Found that PLX-3397 is non-toxic to mice over 2 weeks, given in chow
• Determined that PLX-3397 increased CD8 and CD4 positive T cells

Months 25-36
• Showed that PLX3397 depleted tumor associated macrophages in the orthotopic mouse mesothelioma model
• Showed that PLX3397 also increased CD8+ T cells
• Confirmed that PLS3397 was nontoxic to mice, as shown by normal behavior and lack of alteration of key serum biomarkers

d. Determine whether macrophage depletion in mice with mesothelioma enhances apoptotic and therapeutic response of mice with mesothelioma to TRAIL plus gemcitabine over 1-4 weeks after treatment (months 24-36).

Months 1-12
• Carried out one experiment in which mice implanted ip with 40L syngeneic mesothelioma cells were first given clodronate-liposomes (or PBS or PBS-liposomes) intraperitoneally and then treated with nothing, TRAIL alone, gemcitabine alone or TRAIL plus gemcitabine
• Showed that clodronate-treatment itself was associated with a lower tumor burden
• Found that clodronate-treatment increased the efficacy of the treatment with TRAIL plus gemcitabine
• Collected ascites and tissues for determining presence of macrophages, other immune cells, tumor burden and apoptotic cells

Months 13-36
• Found that PLX-3397 when given alone without chemotherapy is able to reduce the burden of mesothelioma
• Found that PLX-3397 when given alone without chemotherapy is able to increase the apoptosis of the tumor cells without altering the proliferation

e. Repolarize macrophages in vivo by injections of interferon gamma plus LPS and assaying cytokines of peritoneal ascites and gene expression of peritoneal macrophages obtained by lavage to confirm method of repolarization, and to select best tolerated method for the mice (months 12-24).

• Not initiated in months 1-36 due to focus on the clinically relevant approach of CSF1 receptor antagonism

f. Determine whether depletion or repolarization of macrophages in vivo enhances the apoptotic and therapeutic response of mice with mesothelioma to TRAIL plus gemcitabine over 1-4 weeks after treatment (months 24-36).

• Not initiated in months 1-24 due to focus on the clinically relevant approach of CSF1 receptor antagonism
Months 24-36
- Showed that GW2580 itself decreased macrophage infiltration but did not reduce tumor burden
- Showed that GW2580 enhanced the effect of chemotherapy on tumor burden
- Showed that GW2580 significantly increased the apoptotic response to chemotherapy

g. Determine whether the depletion or repolarization of macrophages enhances survival of mice with mesothelioma after treatment with vehicle, TRAIL or TRAIL plus gemcitabine (months 24-36).
  - Carrying out survival studies using the newly acquired MexTag mice which develop asbestos-induced mesothelioma

IV. REPORTABLE OUTCOMES
A. MANUSCRIPTS (Provided in Appendix B)

Months 1-12

Months 13-24
  Featured in:

Months 25-36:


B. ABSTRACTS
Months 1-36 (Provided as Appendix C in this Progress Report)

C. PRESENTATIONS

Symposia and Workshops: International


Symposia and Workshops: National

2009  Broaddus, V. Courtney. *Role of macrophages in apoptotic resistance of mesothelioma*. In Workshop on Preclinical Drug and Target Discovery Pipeline. International Mesothelioma Program, Brigham and Womens Hospital, Harvard Medical School, Boston, MA, USA.

2010  Coussens, Lisa M. PLENARY LECTURE. *Regulation of protumor immunity and cancer development*. 2010 Annual Meeting of the American Association for Cancer Research, Washington DC USA.


2010  Coussens, Lisa M. *Regulation of protumor immunity and cancer development*. In: 3rd Annual Wyeth Discovery Frontiers in Human Disease Symposium, New York, NY USA

2010  Coussens LM. *Inflammation and cancer: reprogramming the immune microenvironment as an anti-cancer therapeutic strategy*. NCI, Immunity Inflammation and Cancer Conference, Bethesda, MD.


2011  Broaddus VC. Malignant mesothelioma: what is the role of radiotherapy? Radiation Oncology Grand Rounds, UCSF.
2012  Broaddus VC. Spheroids and 3D insights into apoptotic resistance; mesothelioma and lung cancer. Harvard Lung Conference, Boston, MA.

Invited Lectures/Seminars: International

2010  Broaddus VC. Invited speaker, International Mesothelioma Interest Group, Kyoto, JAPAN.


2012  Coussens LM. Inflammation and Cancer: reprogramming the immune microenvironment as an anti-cancer therapeutic strategy. International Symposium of the Collaborative Research Center, Bad Neuenahr Ahrweiller, Germany.

Invited Lectures/Seminars: National

2010  Coussens, Lisa M.  *Inflammation and cancer: polarized immune responses regulate cancer development*. Cold Spring Harbor Laboratory, CSH NY USA

2010  Broaddus, V. Courtney.  *Current studies under the DOD grant mechanism: macrophages and their contribution to mesothelioma*. Mesothelioma Applied Research Foundation, Washington, DC, USA


2012  Broaddus VC. Manipulating the core apoptotic machinery in mesothelioma. 2nd International Symposium on Lung-sparing Therapies for Malignant Mesothelioma, UCLA, Santa Monica, CA.


2012  Broaddus VC. Asbestos and its toxic relationship with the pleura: update and future concerns. Medical Grand Rounds, Massachusetts General Hospital, Boston, MA.

Presentations by Coussens or Broaddus Lab Members:


2010  Blakely CM. Targeting macrophages in a preclinical model of mesothelioma. Hematology-Oncology Scientific Retreat, UCSF.

2011  Blakely CM. Macrophage-depletion as a novel therapeutic approach for malignant pleural mesothelioma. Poster presentation at the American Association of Cancer Research (AACR), Orlando, FL.

2011  Battula S. Th1-polarized macrophages enhance the apoptotic response to chemotherapy in mesothelioma. Pulmonary Research Retreat, UCSF.

2011  Blakely CM. CSF1 receptor blockade by the tyrosine kinase inhibitor PLX3397 reprograms malignant mesothelioma tumor microenvironments and decreases tumor growth. AACR-NCI-EORTC International Conference, San Francisco, CA.


2012  Battula S. Macrophages can be manipulated to enhance the apoptotic response to chemotherapy in mesothelioma. Oral presentation, International Mesothelioma Interest Group, Boston, MA.

D. PATENTS AND LICENSES: None

E. DEGREES OBTAINED: None

F. REAGENT DEVELOPMENT:
- Preparation of tumor tissue microarrays prepared with 71 mesothelioma tumors
- Preparation of tumor tissue microarrays stained for CD68, CD4, CD8 and a multitude of inflammatory markers
- Generation of hybrid multicellular spheroids with macrophages derived from THP cell lines
- Generation of hybrid multicellular spheroids with macrophages derived from peripheral blood monocytes
- Generation of hybrid multicellular spheroids with macrophages derived from macrophages isolated from fresh human mesothelioma
- Collection of peripheral blood from patients with mesothelioma
- Collection of frozen mesothelioma tissue from patients at the time of surgery to be used for RNA extraction and qPCR

G. FUNDING APPLIED FOR BASED ON WORK SUPPORTED BY THIS FUNDING:

AWARDED
- Melissa Wheeler (UCSF Pharm D student in Coussens’ lab)
  NIH/NCRR/OD UCSF-CTSI Grant Number TL1 RR024129
- Sailaja Battula, PhD (Postdoctoral student in Broaddus’ lab)
  NIH Training Grant Postdoctoral Fellowship (2011-2013)
PENDING
• Barbone D. SPORE Mechanism Career Development Award. 2012

IN PREPARATION
• Coussens LM, Kindler H, Broaddus VC. NIH / NCI RO1, Multi-PI

H. EMPLOYMENT/RESEARCH OPPORTUNITIES APPLIED FOR:
• Kolhatkar, Nikita. Accepted to a postdoctoral research program in immunology at The University of Washington, Seattle, WA. 2010
V. CONCLUSION

Inflammation is now recognized as promoting solid tumor growth and survival. The inflammatory cells release cytokines and soluble mediators that directly promote growth and survival of the malignant cells, and indirectly support the tumor by inducing angiogenesis or by suppressing effective cytotoxic T-cell functions. Each tumor now appears to have a unique inflammatory cell milieu. From our studies to date, mesothelioma appears to have an innate inflammatory profile, with a predominance of macrophages and neutrophils, whereas the other thoracic malignancies studied have a more adaptive profile.

The macrophage is the major inflammatory cell type in mesothelioma and, as such, may play a powerful role in mesothelioma development, maintenance and resistance to chemotherapy. Macrophages from these tumors retain plasticity and can be polarized to an M1 or an M2 phenotype. Macrophages, when polarized to an M1 phenotype, alter the sensitivity of the mesothelioma cells to therapy. This sensitization to chemotherapy does not require contact but can be reproduced by conditioned media from the macrophages. Thus, polarizing the tumor microenvironment to a pro-apoptotic (M1) phenotype is a possible therapeutic strategy.

Another strategy is to deplete or inhibit the tumor-associated macrophages, which at baseline are in a pro-tumor (M2) phenotype. These studies have made progress in the in vivo setting, in which clodronate, and now CSF1 receptor antagonists, are able to reduce macrophages and increase apoptosis. These early in vivo studies show the exciting potential for manipulation of the macrophage in the tumor environment. If depletion of the macrophages can produce a significant improvement in mesothelioma treatment in this one model, then polarization to an M1 phenotype may have even a greater benefit.

At the end of our final funding cycle, we have achieved most of our key aims and plan to continue in this promising area. We have confirmed our initial findings in our application and extended them significantly toward developing new therapeutic strategies targeting the tumor-associated macrophage.

FUTURE DIRECTIONS & PLANS

Dr. Lisa Coussens, Dr. Broaddus and Dr. Battula are continuing the work into the mechanisms of macrophage enhancement of chemosensitivity and in the therapeutic possibilities of anti-CSF1R antagonism. We aim to complete experiments using the asbestos-induced mesothelioma in MexTag mice to confirm our findings in the orthotopic mesothelioma model and carry out survival studies to show that the effect of anti-CSF1R reagents such as GW2580 on decreasing tumor burden also translate to increased survival. A major publication on our findings is in preparation. We also are currently writing a multi-PI NIH R01 grant application to enable more basic, animal and clinical studies; for the clinical trial design and supervision, we have engaged Dr. Hedy Kindler, Medical Oncologist at the University of Chicago who brings extensive experience in mesothelioma clinical trials.
VI. BIBLIOGRAPHY


VII. APPENDICES
A. Complete academic curriculum vitae for Dr. V. Courtney Broaddus and Dr. Lisa Coussens
B. Publications for months 1-36
C. Abstracts for months 1-36
University of California San Francisco

CURRICULUM VITAE

Name: V. Courtney Broaddus

Position: Professor of Medicine, Step 3
Department of Medicine
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EDUCATION:

1971-1975 Duke University, Durham, NC B.S. Summa Cum Laude, Zoology
1975-1979 University of Pennsylvania M.D. Medicine
1979-1980 University of Pennsylvania Intern Medicine
1980-1982 University of Pennsylvania Resident Medicine
1983-1986 University of California, San Francisco Fellow Pulmonary/Critical Care Medicine

2001-2002 Comprehensive Cancer Center, UCSF Sabbatical Laboratory of Gerard Evan, Ph.D.
2007 Leadership Development for Physicians in Academic Health Centers Harvard School of Public Health

LICENSES, CERTIFICATION:

1982 Medical License, California G-049379
1982 Internal Medicine American Board of Internal Medicine
1986 Pulmonary Disease American Board of Internal Medicine

PRINCIPAL POSITIONS HELD:

1986-1988 University of California, SF Instructor in Residence Medicine
1988-1995 University of California, SF Assistant Professor in Residence Medicine
1995-1997 University of California, SF Associate Professor in Residence Medicine
1997-2001 University of California, SF Associate Professor Medicine
2001-now University of California, SF Professor Medicine

OTHER POSITIONS HELD CONCURRENTLY:

1998-now Dept of Medicine Chief, Division of Pulmonary and Critical Care Medicine, SFGH
HONORS AND AWARDS:

1974      Phi Beta Kappa, Junior Year
1975      Summa Cum Laude
1978      Alpha Omega Alpha, Junior Year
1979      Janet M. Glasgow Memorial Award and Citation
1985      Individual National Research Service Award, NIH
1987      Clinical Investigator Award, NHLBI
1988      American Physiological Society, Elected
1991      Distinction in Teaching Award, Academic Senate, UCSF
1991      Associate, Scientific Staff, Cardiovascular Research Institute, Elected
1992      Pre-tenure Award, UCSF
1999-2002 President, International Mesothelioma Interest Group
2003      Nomination for Most Outstanding Teacher, UCSF Women In Medicine
2005-2012 Best Doctors in America, Selected
2005      Faculty of the Year Award, SFGH Association of Business Officers' Group
2006      Western Society of Clinical Investigation, Elected
2007 & 2008 Nomination for Subspecialist Consultant of the Year Award, SFGH
2008      Nomination for Wagner Award, International Mesothelioma Interest Group
2010      Michael S. Stulbarg Outstanding Teaching Award, UCSF Pulmonary
2010      Pioneer Award, The Mesothelioma Applied Research Foundation
2012      John F. Murray Distinguished Professorship, Inaugural Recipient

KEYWORDS/AREAS OF INTEREST:
Apoptosis, mesothelioma, macrophages, synergy, 3-dimensional models, TRAIL (TNF-related apoptosis-inducing ligand), pleural disease, pleural effusions, asbestos.

PROFESSIONAL ACTIVITIES

CLINICAL

Attending, Medical Intensive Care Unit, SFGH: I attend 1 month of the year on the ICU, 7 days a week, supervising 8 senior residents and interns.

Attending, Pulmonary Consult Service, SFGH: I attend 1 month each year seeing inpatients with pulmonary problems, 5 days a week, supervising 2 fellows and 1-2 residents-medical students.

PROFESSIONAL ORGANIZATIONS

Memberships
Lung Biology Center, San Francisco General Hospital
Thoracic Oncology Research Group, UCSF Cancer Center
Cardiovascular Research Institute, University of California, San Francisco
Western Society of Clinical Investigation
American Thoracic Society
California Thoracic Society
International Mesothelioma Interest Group
Mesothelioma Applied Research Foundation
International Association for the Study of Lung Cancer (IASLC)

Service to Professional Organizations
1988-1992 ALA/American Thoracic Society Research Fellowship Review Committee
1991-1992 American Thoracic Society Program Committee,
1994-1995 American Thoracic Society Women's Affairs Committee
1994-1997 American Thoracic Society Program Committee, Respiratory Cell and Molecular Biology
1995-1997 California Lung Association Research Fellowship Committee
1995-1997 American Thoracic Society Program and Budget Committee
1995-1999 Am Federation for Med Research UCSF Representative
1997-2003 American Lung Association of CA Research Administrative Committee
1999-2002 Intl Mesothelioma Interest Group President
1999-2001 American Thoracic Society Chair, Long Range Planning Committee, Respiratory Cell and Molecular Biology
2003-2004 American Thoracic Society Education Committee
2003-2007 American Thoracic Society Scientific Advisory Committee
2005-now Intl Mesothelioma Interest Group Member, Board of Directors Scientific Organizing Committee
2006-now Intl Mesothelioma Interest Group Chair, Website Subcommittee
2007-2009 American Thoracic Society Chair, Nominating Committee, Respiratory Cell and Molecular Biology
2008-2011 Mesothelioma Applied Research Foundation Member, Scientific Advisory Board
2012-now International Association for the Study of Lung Cancer (IASLC) Member, Core Program Committee 15th World Conference, Sydney, Australia
2011-now Intl Mesothelioma Interest Group Chair, Organizing Committee 11th International Conference, Boston, MA

SERVICE TO PROFESSIONAL PUBLICATIONS
1990-now Ad hoc referee for:
Oncogene (2 papers in last year)
American Journal of Pathology (1 paper in last year)
New England Journal of Medicine (2 papers in last year)
American Journal of Respiratory and Critical Care Medicine (4 papers in 2 years)
American Journal of Respiratory Cell and Molecular Biology (6 papers in 2 years)
2012- Editor-in-Chief, Murray & Nadel's Textbook of Respiratory Medicine, 6th Edition
2005-now Principal Editor, Website for Murray and Nadel's Textbook of Respiratory Medicine
www.expertconsult.com
1996-1998 Associate Editor, American Journal of Respiratory Cell and Molecular Biology
INVITED PRESENTATIONS

INTERNATIONAL (Selected)
1995 International Chemokine Symposium, Bath, England (Invited speaker)
1996 International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles, Lake Placid, NY (Platform)
1999 International Meeting of the Formosan Medical Association, Taiwan, 1999 (Platform)
2008 Centre for Cancer Research and Cell Biology, Queen’s University, Belfast, No. Ireland
2010 International Mesothelioma Interest Group, Kyoto Japan (Invited Speaker)
2011 IASLC/World Conference on Lung Cancer, Amsterdam, Netherlands (Invited Speaker)
2012 2nd Canadian Symposium on Malignant Mesothelioma, Vancouver, CA 2012
2012 2nd International Symposium on Lung-Sparing Therapies for Malignant Pleural Mesothelioma, Los Angeles, CA

American Thoracic Society International Conference. 1986 (Plenary talk); 1988 (Invited speaker, Course on the Academic Pulmonary Physician); 1989 (Invited speaker, Forum on Training and Transition); 1992 (Co-Chair, Poster Symposium); 1993 & 1994 (Co-Chair, Poster Discussion Symposium, Symposium); 1994 (Invited Speaker, Symposium); 1995 (Invited Speaker, Symposium; Meet the Professor Seminar); 1995 & 1997 (Chair, Speaker, Mini-symposium); 1996 (Co-chair, Speaker, Minisymposium); 1997 (Featured speaker, Minisymposium); 1999 (Invited speaker); 2000 (Speaker); 2001 (Co-chair, speaker, Mini-symposium); 2003 (Chair, Symposium); 2004 (Co-chair, Symposium; Meet the Professor Seminar); 2005 (Invited plenary speaker); 2006 (Invited Speaker, Participant in Expert Clinician Panel); 2007 (Co-Chair, Symposium; Presenter, Trudeau Award; Participant in Master Clinician Panel); 2008 (Participant in Master Clinician Panel; Co-Chair Symposium; Presenter); 2009 (Invited Clinical Expert in Pleural Disease; Participant in Master Clinician Panel; Co-Chair, Minisymposium); 2010 (Co-Chair, Minisymposium, Invited Presenter); 2012 (Co-Chair, Minisymposium)

International Mesothelioma Interest Group. San Francisco, 1993 (Invited Speaker); Paris, 1995 (Co-organizer, speaker); United Kingdom, 1999 (Speaker); Brescia, Italy, 2003 (Co-organizer, Speaker); Chicago, 2006 (Organizer Apoptosis Satellite Session, Co-chair, speaker). 2008 (Invited Plenary Speaker, Co-chair of symposium), 2009 (Program Planning Executive Committee, Invited Speaker, Chair of Symposia); 2012 (Program Planning Executive Committee, Invited Speaker, Tumor Microenvironment; Invited Speaker, Apoptosis Resistance).

NATIONAL
1988 Advances in Internal Medicine, San Francisco, CA
1989 Meet the Professor, American College of Physicians, San Francisco, CA
1989 University of Oklahoma Health Science Center, Oklahoma City, OK
1989 St. Luke’s-Roosevelt Hospital Center, Columbia University, New York, NY
1990 National Institutes of Health Workshop, Bethesda, MD
1990 Invited Speaker, Scientific Conference on Acute Lung Injury, American Heart Association, Dallas, TX
1990 Advances in Internal Medicine, San Francisco, CA
1991 Advances in Internal Medicine, San Francisco, CA
1994 Chair and Invited Speaker, Cambridge Health Institute Conference on Inflammatory Cytokine Antagonists, Philadelphia, PA
1994 Invited Speaker, University of Pennsylvania, Philadelphia, PA
1994 Gordon Conference on Chemotactic Chemokines
1994 University of Pennsylvania, Philadelphia, PA
1995 Texas Thoracic Society, Austin, TX
1996  Selected Participant, Professional Development Seminar for Senior Women in Medicine, Association of American Medical Colleges, Washington DC
1997  Visiting Pulmonary Scholar, Duke-UNC-NCSU-NIEHS-EPA-CIIT, NC
1998  Visiting Speaker, St. Thomas Hospital, Vanderbilt Univ, Nashville, TN
1998  Invited Speaker, First Annual Symposium on the Pleura, St. Thomas Hospital, Vanderbilt, TN.
1998  Visiting Professor, Medical Grand Rounds, University of Texas at Tyler, TX.
2000  Visiting Professor, Stanford University, Palo Alto, CA
2000  Invited Participant and Co-Chair, American Thoracic Society- National Institute of Environmental Health Science (ATS-NIEHS) Workshop-Toronto, Canada
2002  Visiting Professor, University of Southern California, Los Angeles, CA
2002  Visiting Professor, Yale University, New Haven, CT
2003  Visiting Professor, University of Montana, Center for Environmental Health Sciences, Missoula, MT
2004  Visiting Professor, University of Pennsylvania, Philadelphia, PA
2006  Co-Chair, Minisymposium, Experimental Biology, San Francisco, CA
2006  Invited Participant and Co-Chair, International Mesothelioma Interest Group, Apoptosis Satellite Symposium, Chicago, IL
2006  Invited Speaker, University of Chicago School of Medicine
2006  Visiting Professor, Northwestern University School of Medicine
2007  Invited Speaker, International Mesothelioma Program, Harvard Medical School/Brigham & Women’s Hospital, Boston, MA
2008  Invited Speaker, International Mesothelioma Interest Group, Amsterdam
2009  Invited Speaker, NIH Mesothelioma Symposium, Bethesda, MD.
2009  Invited Speaker, ATS/Hawaii Thoracic Society State of the Art Course, Maui, HI
2009  Invited Outside Reader, PhD Dissertation, Bonnie Lau, Dept of Pathology, Brown University, Providence, RI
2009  Invited Speaker, International Mesothelioma Program, Workshop on Preclinical Drug and Target Discovery, Brigham and Womens Hospital, Boston, MA
2010  Invited Speaker, Mesothelioma Applied Research Foundation, Washington, DC.
2012  Visiting Professor, Harvard Combined Pulmonary & Critical Care Fellowship Program, Boston, MA
2012  Invited Speaker, Medical Grand Rounds, Massachusetts General Hospital, Boston, MA.

REGIONAL AND OTHER INVITED PRESENTATIONS
1988  Medical Grand Rounds, SFGH
1993  Research Conference on Lung Injury, Genentech, So SF
1994  SFGH Cellular and Molecular Medicine Seminar
1995  Medical Grand Rounds, VAMC/ SFGH
1996  Medical Grand Rounds, Moffitt/UCSF
1996  Selected Participant, Senior Women's Conference, University of California San Francisco
1996  Panel Discussant, UCSF Department of Medicine.
1996  Medical Grand Rounds, SFGH
1997  Invited Speaker, UCSF Pulmonary Retreat, Asilomar, CA.
1998  Invited Speaker, UCSF Pulmonary Retreat, Asilomar, CA.
1998  Invited Speaker, Thoracic Oncology Conference, UCSF/Stanford/Mt. Zion
2000  Medical Grand Rounds, Stanford University
2000  Invited speaker, Division of Pulmonary and Critical Care, Stanford Univ.
2000  Invited Speaker, Pulmonary Research Retreat, UCSF
2003  Medical Grand Rounds, SFGH
2003  Invited Speaker, Recent Advances in Pulmonary and Critical Care Medicine
2003  Invited Speaker, Pulmonary Research Retreat, UCSF
2005  Invited Speaker, Thoracic Oncology Research Group, UCSF
2005  Invited Speaker, Radiation Oncology Grand Rounds, UCSF and Mt Zion
2006  Invited Speaker, Thoracic Oncology Research Group, UCSF
2006  Invited Speaker, Pulmonary Research Retreat, UCSF
2008  Invited Speaker, Dean's Seminar Series, SFGH
2009  Invited Speaker, Pulmonary Research Retreat, UCSF
2011  Invited Speaker, Cancer, Immunity & Microenvironment Program, UCSF
2011  Invited Speaker, Radiation Oncology Grand Rounds, UCSF

GOVERNMENT and OTHER PROFESSIONAL SERVICE:
1994  Ad Hoc Member, Comparative Medicine Review Committee, NIH
1999  Ad Hoc Reviewer, National Heart, Lung, and Blood Institute
2001  Ad Hoc Reviewer, National Cancer Institute
2002  Invited Member, NIH Center for Scientific Review, Special Emphasis Panel, Experimental Therapeutics Panel (ZRG1 ET-1).
2004  Site Reviewer, Program Project Grant Review Committee, NCI. MGH/Brigham and Women's, Boston, MA
2005  Invited Member, National Asbestos Research Working Group
        National Health and Medical Research Council, Australia
2006  Invited Participant, NHLBI Strategic Plan for the Division of Lung Diseases
2009  Reviewer, FY09 Peer-reviewed Medical Research Program, AIBS,
        US Army Medical Research and Materiel Command
2009  Expert Panel Member, NIEHS Asbestos Mechanism of Action Workshop
2010  Ad Hoc Reviewer, Special Emphasis Panel,
        Respiratory Integrative Biology and Translational Research Study Section

UNIVERSITY AND PUBLIC SERVICE
UNIVERSITY SERVICE
UCSF CAMPUS-WIDE
1996-1998  Member, Chancellor's Award for the Advancement of Women Committee
2003-now  Member, Academic Senate Committee on Research, UCSF
2005  Chair, Academic Senate Task Force Reviewing
        University of California Policy on Human Subject Injury
2006-2007  Member, Reconvened SFGH Subcommittee of the
        Chancellor's Advisory Committee on the
        Long Range Planning Amendment
2006 & 2007  Faculty Presenter, Inaugural Faculty Welcoming Week,
        Chancellor's Council on Faculty Life, "Building a Research Career"
2007  Small group leader, Junior faculty retreat,
        Striving & Thriving in the Academic World,
        Chancellor's Advisory Committee on the Status of Women

SCHOOL OF MEDICINE
1988-1989  Internship Selection Committee, Univ. of Calif, San Francisco
2005  Member, Stewardship Review Committee,
        Chair of Dermatology, UCSF
2005 Member, Ad Hoc Subcommittee on Faculty Misconduct, Office of Academic Affairs
2006-2007 Member, Search Committee for Chair, Department of Medicine
2006-2007 Member, Select Subcommittee of Search Committee for Chair, Department of Medicine, UCSF
2007 Invited speaker, The Senior Faculty Career Challenge, Dean's Office of School of Medicine. “Strategies for revitalizing divisional goals/function”
2007-2010 Member, Scholarships and Awards Committee, UCSF School of Medicine

DEPARTMENT OF MEDICINE
1988-1989 Member, Bylaws Committee, San Francisco General Hospital
1989 Member, Program Planning Committee
Recent Advances in Pulmonary & Critical Care Medicine (8th Annual), University of California, San Francisco
1989-2000 Member, Critical Care Committee, San Francisco General Hospital
1990-1991 Chair, Program Planning
Recent Advances in Pulmonary & Critical Care Medicine (9th and 10th Annual), Univ of California, San Francisco
1990-present Member, Steering Committee of the Pulmonary Research Group, University of California, San Francisco (elected)
1993-present Member, Pulmonary Fellowship Selection Committee, University of California, San Francisco
1994-present Member, Pulmonary Research Group Retreat Planning Committee
1994-1996 Member, Search Committees for Chief of Surgical Research and Chief of Rheumatology, San Francisco General Hospital
1996-1998 Member, Task Force on Diversity, University of California, SF
1996-1999 Member, Search Committee for Joint Appointment in Radiology/Pulmonary and Critical Care Medicine, San Francisco General Hospital
1996-2002 Member, Promotions Subcommittee, Academic Senate, University of California, San Francisco
1996-1997 Member, Executive Committee, Department of Medicine, SFGH
1996-2002 Board Member, UCSF/Macy's Center for Creative Therapies, San Francisco General Hospital
1997 Member, Search Committee, first Associate Chair for Biomedical Research, Department of Medicine, UCSF
1997-1998 Member, Search Committee for Chief of Cardiology, SFGH
1998-1999 Member, Search Committee for Chief of Gastroenterology, SFGH
1998-1999 Member, Committee, Chief, Pulmonary and Critical Care Med, UCSF
1998-2000 Member, General Clinical Research Center Advisory Committee
1999-2000 Member, Search Committee for Thoracic Surgeon, SFGH
2000 Member, Search Committee for Chief of Radiology, SFGH
2000 Member, Search Committee for Manager, Department of Medicine, SFGH
2003-2004 Chair, Search Committee, Chief of Pulmonary & Critical Care Medicine, Veteran's Administration Medical Center, UCSF
2005-2006 Member, Search Committee for Chest Radiologist, SFGH
2005 Member, John Carbone Chair Nominating Committee
2006- Member, Search Committee for Scientist, Surgical Research Laboratory
2006-2007 Member, Search Committees for Gastroenterology FTE
2006-current Chair, Committee to Establish John F. Murray Distinguished Professorship
2008-now Member, Search Committee for Lung Biology Center Physician-Scientist
2008-now Member, Search Committee for Chief,
Division of Pulmonary & Critical Care Medicine, UCSF
2009-now Chair, Search Committee for Pulmonary Faculty Member, SFGH/UCSF.
2009-now Member, Recruitment and Retention Workgroup, Department of Medicine Strategic Plan
2011 Member, Search Committee for Associate Chair of Research
2011 Chair, Search Committee for Director, Medical ICU, SFGH
2011 Member, Search Committee for Hospital Director of Critical Care Medicine, SFGH

PUBLIC SERVICE
1997 Presenter on Asbestos-Related Diseases, Gloria R. Davis Academic Middle School Asbestos Exposure & Risk Assessment, S.F. Department of Public Health
1997 Coordinator, Cigarette Smoke Demonstration, Take Your Daughters to Work Day
2005-7 Lecturer on Avian and Mammal Lungs, San Francisco Day School
2009-11 Class Captain, Annual Fund, International High School, San Francisco.

TEACHING AND MENTORING

Other Courses
1990-now Pulmonary Physiology Seminars (2 hours/year)
1990 Faculty Leader, Preparing Interns for Residency
1990-now Medical Service Conferences
1991 Women’s Medical Student Association Retreat
1993-2003 Lecturer to Medical Residents (Parnassus, SFGH, VAMC)
1987-2002 Speaker, Recent Advances in Pulmonary & Critical Care Medicine
1998-now Summer Seminar Series and Practical Sessions on Pleural Disease
2004 Faculty Coach and Presenter for Junior Faculty, Mid-Term Appraisal for Faculty at UCSF
2004 Annual PIBS/BMS Course, Ethics and the Responsible Conduct of Research
2005 Lecturer to Medical Residents (Parnassus, SFGH, VAMC)
2005-2007 Workshop Leader, Mid-term Appraisal for Faculty at UCSF

Predoctoral Students Supervised

<table>
<thead>
<tr>
<th>Dates</th>
<th>Name</th>
<th>Position while supervised</th>
<th>Current position</th>
</tr>
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<tbody>
<tr>
<td>1995-97</td>
<td>Sudha Rani Narasimha</td>
<td>Medical Student</td>
<td>Medical Resident, UCLA</td>
</tr>
<tr>
<td>1997-99</td>
<td>Jack Wu</td>
<td>Undergraduate</td>
<td>Medical resident</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cook County, Chicago, IL</td>
</tr>
<tr>
<td>2002-04</td>
<td>Kevin Lee</td>
<td>Undergraduate</td>
<td>Medical student</td>
</tr>
<tr>
<td>2009-10</td>
<td>Nikita Kolhatkar</td>
<td>Graduate</td>
<td>current</td>
</tr>
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</table>

Graduate Students Supervised

<table>
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<tr>
<th>Dates</th>
<th>Name</th>
<th>Position while supervised</th>
<th>Current position</th>
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</thead>
<tbody>
<tr>
<td>2005-6</td>
<td>Dario Barbone</td>
<td>Graduate student</td>
<td>Postdoctoral scholar, UCSF</td>
</tr>
<tr>
<td>2008-9</td>
<td>Bonnie Lau</td>
<td>Graduate student</td>
<td>MD/PhD Brown University</td>
</tr>
</tbody>
</table>

Postdoctoral Fellows Supervised

<table>
<thead>
<tr>
<th>Dates</th>
<th>Name</th>
<th>Position while supervised</th>
<th>Current position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1989-91</td>
<td>Alice M. Boylan, MD</td>
<td>Pulmonary research fellow</td>
<td>Associate Professor of Medicine Medical Univ of South Carolina</td>
</tr>
<tr>
<td>Dates</td>
<td>Name</td>
<td>Position While Mentoring</td>
<td>Role</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------------</td>
<td>--------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>1991-1993</td>
<td>Rex Yung, MD</td>
<td>Pulmonary research fellow</td>
<td>Associate Professor of Medicine Johns Hopkins Univ.</td>
</tr>
<tr>
<td>1993-1995</td>
<td>Hans Folkesson, PhD</td>
<td>Postdoctoral research fellow</td>
<td>Associate Professor of Physiology Northeastern Ohio University, OH</td>
</tr>
<tr>
<td>1995-1996</td>
<td>Jamie Bigelow, MD</td>
<td>Pulmonary research fellow</td>
<td>Pulmonologist St. Francis Hospital, SF</td>
</tr>
<tr>
<td>1996-1997</td>
<td>Evaldo Marchi, MD</td>
<td>Visiting research fellow</td>
<td>Professor of Surgery Sao Paolo, Brazil</td>
</tr>
<tr>
<td>1998-2000</td>
<td>Tom Geiser, MD</td>
<td>Postdoctoral research fellow</td>
<td>Associate Professor of Medicine University Hospital, Bern, SW</td>
</tr>
<tr>
<td>2000-2001</td>
<td>Masa Ishigaki, MD PhD</td>
<td>Visiting research fellow</td>
<td>Associate Professor, Japan</td>
</tr>
<tr>
<td>2000-2003</td>
<td>Claire Vivo, PhD</td>
<td>Postdoctoral research fellow</td>
<td>Research Scientist Ordway Research Institute, NY</td>
</tr>
<tr>
<td>2003-2004</td>
<td>Ki Up Kim, MD</td>
<td>Visiting research fellow</td>
<td>Professor of Medicine, Chief of Pulmonary Division Soon Chun Hyang University Hosp. Seoul, South Korea</td>
</tr>
<tr>
<td>2004-2005</td>
<td>Lorriana Leard, MD</td>
<td>Pulmonary research fellow</td>
<td>Assistant Prof of Medicine, UCSF Associate Director, UCSF Fellowship Program</td>
</tr>
<tr>
<td>2003-2006</td>
<td>Keith Abayasiriwardana, PhD</td>
<td>Postdoctoral research fellow</td>
<td>Senior Scientist, Vaccine Research, Pfizer</td>
</tr>
<tr>
<td>2006-2008</td>
<td>Tsung-Ming Yang, MD</td>
<td>Postdoctoral research fellow</td>
<td>Assistant Professor, Chiayi Chung-Gung Memorial Hosp. Chiayi, Taiwan</td>
</tr>
<tr>
<td>2008-on</td>
<td>Eunice Kim, MD</td>
<td>Pulmonary Fellow</td>
<td>Pulmonary Research Fellow</td>
</tr>
<tr>
<td>2008-on</td>
<td>Denitza Blagev, MD</td>
<td>Pulmonary Fellow</td>
<td>Pulmonary Research Fellow</td>
</tr>
<tr>
<td>2008-on</td>
<td>Joyce Lee, MD</td>
<td>Pulmonary Fellow</td>
<td>Pulmonary Research Fellow</td>
</tr>
<tr>
<td>2009-on</td>
<td>Joshua Galanter, MD</td>
<td>Pulmonary Fellow</td>
<td>Pulmonary Research Fellow</td>
</tr>
<tr>
<td>2006-now</td>
<td>Dario Barbone, PhD</td>
<td>Postdoctoral Research Fellow</td>
<td>Postdoctoral Research Fellow</td>
</tr>
<tr>
<td>2010-now</td>
<td>Sailaja Battula, PhD</td>
<td>Postdoctoral Research Fellow</td>
<td>Postdoctoral Research Fellow</td>
</tr>
</tbody>
</table>

PROTEINFORMAL TEACHING
1987-now: Attending Rounds, Pulmonary Consult Service, SFGH
(1 month/year with 2 fellows, 1 medical student and/or resident)
1987-now: Attending Rounds, Medical ICU Service, SFGH
(1.5 month/year with 4 3rd year residents and 4 interns/ informal and formal teaching)

FACULTY MENTORING
I have selected some representative examples of mentoring relationships from recent years.

<table>
<thead>
<tr>
<th>Dates</th>
<th>Name</th>
<th>Position While Mentoring</th>
<th>Role</th>
<th>Current Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000-2002</td>
<td>David Morris, MD</td>
<td>Division chief</td>
<td>Informal advisor</td>
<td>Head Respiratory Research Roche, Palo Alto, CA</td>
</tr>
<tr>
<td>2000-2005</td>
<td>Robert Jasmer, MD</td>
<td>Division chief</td>
<td>Advisor</td>
<td>Physician, private practice</td>
</tr>
<tr>
<td>2004-now</td>
<td>Payam Nahid, MD</td>
<td>Division chief</td>
<td>Advisor, Reviewed grants</td>
<td>Assoc Prof, UCSF</td>
</tr>
<tr>
<td>2004-now</td>
<td>Mary Gray, MD</td>
<td>LBC Assoc Director</td>
<td>Recruited into LBC, collaborator/advisor</td>
<td>Assoc Prof, UCSF</td>
</tr>
<tr>
<td>2002-now</td>
<td>Laura Koth, MD</td>
<td>LBC Assoc. Dir.</td>
<td>Career advisor</td>
<td>Asst Prof, UCSF</td>
</tr>
<tr>
<td>2004-now</td>
<td>Lorriana Leard, MD</td>
<td>Lab head/Div.chief</td>
<td>Advisor, formal mentor</td>
<td>Asst Prof, UCSF</td>
</tr>
<tr>
<td>2005-2007</td>
<td>Dana McClintock, MD</td>
<td>Career committee</td>
<td>Selected formal mentor</td>
<td>Physician, academic practice</td>
</tr>
</tbody>
</table>
### TEACHING AWARDS AND NOMINATIONS:

- **1991**: Distinction in Teaching Award, Academic Senate, UCSF
- **2003**: Nomination for Most Outstanding Teacher, UCSF Women In Medicine
- **2007 & 2008**: Nominations for Subspecialist Consultant of the Year Award, SFGH Dept of Medicine
- **2010**: Michael S. Stulbarg Outstanding Teaching Award, Pulmonary & Critical Care Medicine, UCSF

### SUMMARY OF TEACHING HOURS:

- **2009-2010**: Total anticipated hours of teaching: 540
  - Formal class or course teaching hours: 20
  - Informal teaching hours: 250
  - Mentoring hours: 270
RESEARCH AND CREATIVE ACTIVITIES
RESEARCH AWARDS AND GRANTS

ACTIVE:

Supporting Agency: Peer-Reviewed Medical Investigator-Initiated Research Program – Department of Defense
Grant Title: The Role of Macrophage-induced Inflammation in Mesothelioma.
Role on project: Principal Investigator
Time Commitment to Project: 3 Calendar Months Effort (25%)
Total Funding Period: 07/01/2009 – 6/30/2012
Direct Dollars: / yr 1
/ yr 1-3

Project Overlap: None
Goals: To demonstrate the functional significance of macrophages as promoters of tumor cell survival in mesothelioma and determine whether tumor-associated macrophages can be repolarized to enhance mesothelioma cell apoptosis.
Specific Aims: 1. To determine the functional significance of macrophage phenotype in mesothelioma. 2. To determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro. 3. To define the functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

Supporting Agency: Simmons Mesothelioma Foundation
Total Funding Period: 7/1/2012 – 6/30/14
Grant Title: The Role of Macrophage-induced Inflammation in Mesothelioma
Role on project: Principal Investigator
Time Commitment to Project: 30%
Direct Dollars: / yr

Program Overlap: None
Goals: To demonstrate the functional significance of macrophages as promoters of tumor cell survival in mesothelioma and determine whether tumor-associated macrophages can be repolarized to enhance mesothelioma cell apoptosis.
Specific Aims: 1. To determine the functional significance of macrophage phenotype in mesothelioma. 2. To determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro. 3. To define the functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

Past
NIH Institutional National Research Service Award (HL07185) 1983-1985
NIH Individual National Research Service Award (HL07271) 1985-1986
Academic Senate Committee on Research Grant 1986
Origin of pleural effusions in volume-loaded sheep
Academic Senate Committee on Research Grant 1988
Origin of pleural effusions in hydrostatic pulmonary edema
NHLBI Pulmonary Vascular SCOR (HL19155) (PI) 1986-1991
Dynamics of pleural liquid turnover in health and disease.
American Lung Association Research Grant, 1987-1989
Formation of pleural effusions in pleural inflammation.
Clinical Investigator Award, NHLBI (KO8 HL01893) 1987-1992
Comparative physiology of the normal and inflamed pleura.
NHLBI Pulmonary Vascular SCOR (HL19155) (Co-Investigator) 1991-1993
Mechanisms of acute pleural and lung injury.
Pretenure Award, University of California, San Francisco 1992-1993
   Mechanisms of interaction of asbestos and mesothelial cells.
   The role of rabbit IL-8 in neutrophil-mediated inflammation.
Genentech (PI) 1993-1994
   The role of rabbit IL-8 in neutrophil-mediated inflammation.
RO1 ES06331 (NIEHS) (PI) 1994-1998
   Molecular interactions of asbestos and mesothelial cells.
Genentech (PI) 1995-1997
   The role of rabbit IL-8 in sepsis.
Research Evaluation and Allocation Committee Grant, UCSF 1996-1997
   The role of fiber internalization in mediating the toxic effects of
   asbestos on mesothelial cells.
Principal Investigator, Tobacco-related Disease Research Program 1998-2001
   Programmed cell death in cigarette-induced lung disease.
RO1 ES08985 (NIEHS) (PI) 1997-2002
   Protective role of apoptosis in asbestos pleural injury.
RO1 ES08985 (NIEHS) (PI) 2000-2002
   Supplement for microarray studies.
Peterson Family Foundation (PI) 2005-2007
   Role of Akt/mTOR in mesothelioma.
Buzzi Foundation, Italy 2005-2007
   Role of PI3K/Akt/mTOR pathway in resistance to apoptosis.
RO1 CA95671 (NIH/NCI) (PI) 2003-2009
   Amplification of TRAIL-induced apoptosis in mesothelioma.
Mesothelioma Applied Research Foundation Award. 2007-2009
   Antibody development against mesothelioma.
PEER REVIEWED PUBLICATIONS:


(* equal contributors as senior author)


(* With accompanying editorial)


IN REVIEW

Barbone D, Fennell D, BROADDUS VC. Vorinostat eliminates multicellular resistance of mesothelioma 3D spheroids via restoration of Noxa expression. In review.


Yang T-M, Barbone D, BROADDUS VC. Hypoxia inducible factor-1 alpha contributes to apoptotic resistance in lung cancer multicellular spheroids. In review.
NON-PEER REVIEWED PUBLICATIONS AND OTHER CREATIVE ACTIVITIES:

EDITORIALS


WORKSHOP OR MEETING SUMMARIES


REVIEWS


**CHAPTERS**


RESEARCH PROGRAM
DESCRIPTION OF CURRENT RESEARCH

My research program continues to focus on the apoptotic resistance of tumors, malignant mesothelioma and lung cancer. We have focused particularly on mesothelioma, as a highly refractory and chemoresistant tumor, and are applying our findings now to lung cancer. We are investigating the apoptotic signaling involved in bypassing resistance and inducing apoptosis, specifically by combining agonists of the two major apoptotic pathways: the death receptor pathway and the DNA damage/mitochondrial pathway. We have shown that, while each pathway alone fails to induce apoptosis in the tumor cells, the combination will induce synergistic apoptosis. We continue to explore this phenomenon, searching for non-toxic means of stimulating these pathways such as heat stress and JNK stimulators such as anisomycin.

We have incorporated 3-dimensional models in our study of resistance, both of the mesothelioma cell lines grown as multicellular spheroids and of the human mesothelioma tumor itself grown as tumor fragment spheroids. Both these models allow us to test the resistance in a more clinically relevant system. We have found that a major survival pathway, the PI3K/Akt/mTOR pathway, contributes to resistance in these 3-dimensional structures. Ultimately however, we believe that the resistance is manifested at the mitochondria by an altered repertoire of anti- and pro-apoptotic molecules. In an ongoing collaboration with Dr. Dean Fennell of Belfast, No. Ireland and Drs. Raphael Bueno and David Sugarbaker of Brigham & Women's, Boston, we are now exploring the mitochondria as a central integrator of apoptotic signaling. Most recently, we are using histone deacetylator inhibitors to restore expression of pro-apoptotic proteins that reverse multicellular resistance and performing connectivity analysis of the key genes upregulated in 3D to identify drugs that can reverse multicellular resistance.

As an exciting direction using 3-dimensional models, we are collaborating with Dr. Lisa Coussens in studying the interaction of tumor-associated macrophages with the tumor cells. We will use our models, 3D multicellular spheroids and human tumor fragments, to investigate the contribution of macrophages to the apoptotic resistance of the tumor cells. We have found that mesothelioma contains a high number of macrophages, far more than in lung cancer or other tumors; thus, if these macrophages can be eliminated or manipulated to change from a supportive role to an anti-tumor role, this could be of significant therapeutic benefit for this currently incurable tumor. Our current findings are that macrophages (polarized from their M2-protumor phenotype to an M1-antitumor phenotype) can enhance the chemosensitivity of the mesothelioma cells. In vivo studies are ongoing to explore this approach in mice with orthotopic and de novo mesothelioma.
# CURRICULUM VITAE

Lisa M. Coussens, Ph.D.
Professor and Chair, Department of Cell & Developmental Biology

Oregon Health & Sciences University
Richard Jones Hall, Room 5508
3181 SW Sam Jackson Park road
Portland, OR 97239-3098

Voice: (503) 494-7811
Fax: (503) 494-4253
Email: coussenl@ohsu.edu

## I. EDUCATION:

<table>
<thead>
<tr>
<th>Year</th>
<th>Institution</th>
<th>Degree</th>
<th>Major</th>
</tr>
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<tbody>
<tr>
<td>1976-1980</td>
<td>San Francisco State University</td>
<td>B.A.</td>
<td>Biology</td>
</tr>
<tr>
<td>1988-1993</td>
<td>University of California, Los Angeles</td>
<td>Ph.D.</td>
<td>Biological Chemistry</td>
</tr>
<tr>
<td>1993-1997</td>
<td>University of California, San Francisco</td>
<td>Post-Doctoral Fellow</td>
<td>Cancer Biology</td>
</tr>
</tbody>
</table>

## II. PRINCIPAL POSITIONS HELD:

<table>
<thead>
<tr>
<th>Year</th>
<th>Institution</th>
<th>Position</th>
<th>Department/Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997-1999</td>
<td>Univ. of California, San Francisco</td>
<td>Assistant Research Biochemist</td>
<td>Hormone Research Inst.</td>
</tr>
<tr>
<td>1999-2004</td>
<td>Univ. of California, San Francisco</td>
<td>Assistant Professor, In Residence</td>
<td>Cancer Research Inst. &amp; Dept of Pathology</td>
</tr>
<tr>
<td>2004-2006</td>
<td>Univ. of California, San Francisco</td>
<td>Associate Professor, In Residence</td>
<td>Cancer Research Inst. &amp; Dept of Pathology</td>
</tr>
<tr>
<td>2006-2007</td>
<td>Univ. of California, San Francisco</td>
<td>Associate Professor</td>
<td>Dept. of Pathology &amp; Cancer Research Inst.</td>
</tr>
<tr>
<td>2007-2011</td>
<td>Univ. of California, San Francisco</td>
<td>Professor</td>
<td>Dept. of Pathology &amp; Cancer Research Inst.</td>
</tr>
<tr>
<td>2011-2012</td>
<td>Univ. of California, San Francisco</td>
<td>Professor</td>
<td>Dept. of Pathology</td>
</tr>
<tr>
<td>2012-present</td>
<td>Univ. of California, San Francisco</td>
<td>Adjunct Professor</td>
<td>Dept. of Pathology</td>
</tr>
<tr>
<td>2011-present</td>
<td>Oregon Health &amp; Sciences University</td>
<td>Professor and Chair</td>
<td>Cell &amp; Developmental Biology</td>
</tr>
<tr>
<td>2011-present</td>
<td>Oregon Health &amp; Sciences University</td>
<td>Associate Director for Basic Research</td>
<td>Knight Cancer Institute</td>
</tr>
</tbody>
</table>

## OTHER POSITIONS HELD CONCURRENTLY:

<table>
<thead>
<tr>
<th>Year</th>
<th>Institution</th>
<th>Position</th>
<th>Department/Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1989-1992</td>
<td>Whittier College, Whittier, CA</td>
<td>Lecturer</td>
<td>Biology Dept</td>
</tr>
<tr>
<td>1992</td>
<td>Genentech, Inc., South San Francisco</td>
<td>Scientific Consultant</td>
<td>Dept. of Legal Affairs</td>
</tr>
<tr>
<td>2000-2012</td>
<td>Helen Diller Family Comprehensive Cancer Center, UCSF</td>
<td>Co-Director</td>
<td>Mouse Pathology Core</td>
</tr>
<tr>
<td>2007-2009</td>
<td>CANCER RESEARCH</td>
<td>Senior and Deputy Editor</td>
<td>Tumor Microenvironment Section</td>
</tr>
<tr>
<td>2009-2012</td>
<td>Helen Diller Family Comprehensive Cancer Center, UCSF</td>
<td>Co-Leader</td>
<td>Program in Cancer Immunity &amp; Microenvironment Breaking Advances</td>
</tr>
<tr>
<td>2009-2012</td>
<td>CANCER RESEARCH</td>
<td>Deputy Editor</td>
<td></td>
</tr>
</tbody>
</table>

## III. HONORS AND AWARDS:

<table>
<thead>
<tr>
<th>Year</th>
<th>Award</th>
<th>Institution/Grant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1985</td>
<td>Recognition Award</td>
<td>Genentech, Inc.,</td>
</tr>
<tr>
<td>1986</td>
<td>Recognition Award</td>
<td>Genentech, Inc.,</td>
</tr>
<tr>
<td>1988</td>
<td>Recognition Award</td>
<td>Genentech, Inc.,</td>
</tr>
<tr>
<td>2000-02</td>
<td>Hellman Family Award For Early Career Faculty</td>
<td>Univ. of Calif., San Francisco</td>
</tr>
<tr>
<td>2000-01</td>
<td>V Foundation Scholar</td>
<td>The V Foundation for Cancer Research</td>
</tr>
<tr>
<td>2002</td>
<td>Gertrude B. Elion Cancer Research Award</td>
<td>Am. Assoc. for Cancer Research</td>
</tr>
<tr>
<td>2006-11</td>
<td>Era of Hope Scholar Award</td>
<td>Dept. of Defense, Breast</td>
</tr>
</tbody>
</table>
### IV. PROFESSIONAL ACTIVITIES

<table>
<thead>
<tr>
<th>Year</th>
<th>Role</th>
<th>Organization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999 - 2012</td>
<td>Member</td>
<td>Graduate Program in BioMedical Sciences (BMS)</td>
</tr>
<tr>
<td>1999 - 2012</td>
<td>Member</td>
<td>Helen Diller Family Comprehensive Cancer Center</td>
</tr>
<tr>
<td>2000 - 2012</td>
<td>Member</td>
<td>Graduate Program in Biological Sciences (PIBS)</td>
</tr>
<tr>
<td>2001 - 2012</td>
<td>Co-Director</td>
<td>Mouse Pathology Core</td>
</tr>
<tr>
<td>2004 – 2012</td>
<td>Member</td>
<td>Program in Immunology</td>
</tr>
<tr>
<td>2004 – 2007</td>
<td>Senior Editor</td>
<td>Cancer Research (Cell, Tumor and Stem Cell Biology Section)</td>
</tr>
<tr>
<td>2007 – 2009</td>
<td>Deputy Editor</td>
<td>Cancer Research (general)</td>
</tr>
<tr>
<td>2007 – 2009</td>
<td>Senior Editor</td>
<td>Cancer Research (Tumor Microenvironment Section)</td>
</tr>
<tr>
<td>2007 - present</td>
<td>Member</td>
<td>External Scientific Advisory Board</td>
</tr>
<tr>
<td>2007 – 2011</td>
<td>Member</td>
<td>External Scientific Advisory Board, U54: Aging, Tumor Microenvironment and Prostate Cancer; P.I. Steve Plymate, Univ. of Washington, HMC.</td>
</tr>
<tr>
<td>2008 – 2011</td>
<td>Member</td>
<td>Board of Directors (elected)</td>
</tr>
<tr>
<td>2009 - present</td>
<td>Member</td>
<td>External Scientific Advisory Board; Neuroblastoma Program Project Grant</td>
</tr>
<tr>
<td>2009 – 2012</td>
<td>Deputy Editor</td>
<td>Cancer Research (Breaking Advances section)</td>
</tr>
<tr>
<td>2009 - 2012</td>
<td>Co-Director</td>
<td>Program in Cancer Immunity and Microenvironment</td>
</tr>
<tr>
<td>2010 - 2012</td>
<td>Council Member</td>
<td>Women in Cancer Research, Council (elected)</td>
</tr>
<tr>
<td>2009 - present</td>
<td>Member</td>
<td>External Advisory Committee, P01CA100324, Program in Motility and Invasion, John Condeelis, Ph.D., PI, Program Director</td>
</tr>
<tr>
<td>2011</td>
<td>Member</td>
<td>Scientific Review Board</td>
</tr>
<tr>
<td>2012 - present</td>
<td>Faculty member</td>
<td>Program in Molecular &amp; Cellular Biosciences</td>
</tr>
</tbody>
</table>

**2011 - 16** Era of Hope Scholar Expansion Award  
**2011 - 16** KOMEN Promise Award  
**2012** AACR-Women in Cancer Research Charlotte Friend Memorial Lectureship  
**2012** Mildred Scheel Memorial Lectureship (Inaugural)
<table>
<thead>
<tr>
<th>Year</th>
<th>Position</th>
<th>Organization</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012-present</td>
<td>Member</td>
<td>OHSU Knight Cancer Institute, Program in Cancer Biology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oregon Health &amp; Sciences University</td>
</tr>
<tr>
<td>2012-present</td>
<td>Member</td>
<td>Scientific Advisory Board</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Koch Inst, for Integrated Cancer Research, Massachusetts Inst. of Tech.</td>
</tr>
<tr>
<td>2012-present</td>
<td>Member</td>
<td>Editorial Board</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cancer Cell</td>
</tr>
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**Local, National and International Meetings Organized:**

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>5th Annual Timberline Symp. on Epithelial Cell Biology, <em>Intrinsic and Microenvironmental Regulation of Epithelial Cancer</em>, Co-Organizer with Dr. Harold Moses (Vanderbilt University, TN, USA), Timberline, OR, USA</td>
</tr>
<tr>
<td>2006</td>
<td>Co-Organizer (with Dr. Lewis Lanier), UCSF HDFCCC Annual Symposium, <em>Inflammation &amp; Cancer: Bench to Bedside</em></td>
</tr>
<tr>
<td>2007</td>
<td>Keystone Symposia, <em>Inflammation and Cancer</em>, Co-Organizer with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA); Santa Fe, New Mexico, USA</td>
</tr>
<tr>
<td>2008</td>
<td>International Society for Biological Therapy of Cancer (ISBTo), <em>2008 Workshop on Inflammation in Cancer Development</em>, Co-Organizer with Drs. Michael Karin, (UCSD), Steven Dubinett (UCLA), and George Weiner (WU); San Diego CA USA</td>
</tr>
<tr>
<td>2010</td>
<td>Co-Organizer (with Dr. Lewis Lanier), UCSF HDFCCC Program in Cancer Immunity and Microenvironment Symposium</td>
</tr>
<tr>
<td>2011</td>
<td>AACR Special Conference: <em>Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy</em>, Co-Organizer with Drs. Yves DeClerck (USC, Children’s Hospital) and Melody Swartz (EPFL)</td>
</tr>
</tbody>
</table>

**V. PROFESSIONAL ORGANIZATIONS**

**Memberships**

<table>
<thead>
<tr>
<th>Year</th>
<th>Membership</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000 - 2009</td>
<td>American Society for Matrix Biology</td>
</tr>
<tr>
<td>2000 – present</td>
<td>American Association for Cancer Research</td>
</tr>
<tr>
<td>2001 – 2008</td>
<td>American Society for Cell Biology</td>
</tr>
<tr>
<td>2004 – present</td>
<td>American Society for Investigative Pathology</td>
</tr>
<tr>
<td>2004 - 2009</td>
<td>International Protease Society</td>
</tr>
</tbody>
</table>

**Service to Professional Organizations**

**American Association for Cancer Research**

<table>
<thead>
<tr>
<th>Year</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003</td>
<td>Subsection Co-chair (Tumor Progression, Invasion and Metastasis) Cellular, Molecular and Tumor Biology Subcommittee, AACR Program Committee for <em>94th Annual Meeting</em>.</td>
</tr>
<tr>
<td>2003</td>
<td>Chair and organizer, Educational Session (Proteases: Successes and Failures): <em>94th Annual Meeting</em>, Washington D.C., USA</td>
</tr>
<tr>
<td>2004 - 2006</td>
<td>Member, Grants Committee</td>
</tr>
<tr>
<td>2005</td>
<td>Minisymposium Co-Chair (Inflammation, Microenvironment and Tumor Progression): <em>96th Annual Meeting</em>, Anaheim, CA USA</td>
</tr>
<tr>
<td>2005</td>
<td>Session Chair (Inflammation): <em>AACR Special Conference: Cancer, Proteases and the Microenvironment</em>, Bonita Springs, Florida, USA</td>
</tr>
<tr>
<td>2006</td>
<td>Subsection Co-chair (Tumor Progression, Invasion and Metastasis) of the Tumor Biology Subcommittee, AACR Program Committee for <em>97th Annual meeting</em></td>
</tr>
<tr>
<td>2006</td>
<td>Minisymposium Co-Chair (Inflammation and Cancer): <em>97th Annual Meeting</em>, Washington DC, USA</td>
</tr>
<tr>
<td>2006 - 2010</td>
<td>Steering Committee Member: AACR Tumor Microenvironment Working Group (TME/AACR).</td>
</tr>
</tbody>
</table>
2007 Organizer, Education session (Inflammation and Cancer), 98th Annual Meeting, Los Angeles, USA
2007 Minisymposium Co-Chair (Tumor Microenvironment): 98th Annual Meeting, Los Angeles, CA USA
2007 Co-Chairperson, Program Committee: 2008 99th Annual Meeting of the AACR. April 12-16, 2008, San Diego, CA, USA
2008 Program Committee Member, Tumor Microenvironment Subcommittee for 99th Annual Meeting of the AACR. April 12-16, 2008, San Diego, CA, USA
2007 - 2010 Member, AACR Special Conferences Committee
2008 Co-Organizer Special Conference: Inflammation and Cancer, with Drs. Michael Karin and Larry Marnett. Oahu, Hawaii, USA.
2008 - 2011 Member, Board of Directors (elected)
2009 Organizer and Chair: Inflammation and Cancer: Novel Mechanisms Regulating Protumor Immunity Major Symposium, 2009 100th AACR Annual Meeting, Denver, CO, USA
2009 Organizer and Chair: Education Session, Aspects of the Tumor Microenvironment that Regulate Solid Tumor Development, 2009 100th AACR Annual Meeting, Denver, CO, USA
2010 Co-Chairperson, Program Committee: 2010 101st Annual Meeting of the AACR, April 17-21, 2010, Washington, DC USA
2009 - 2010 Member, Scientific Review Committee for Stand Up to Cancer Innovative Research Grants
2009 - 2010 Member, Selection Committee: 2010 Pezcoller Foundation-AACR International Award for Cancer Research
2010 - 2011 Council Member, Women in Cancer Research Council (elected)
2010 Co-Chair, Minisymposium ‘The Tumor Microenvironment and Therapeutic Strategies” 2010 101st Annual Meeting of the AACR, April 17-21, 2010, Washington, DC USA
2010 - 2011 Member, Selection Committee: 2010-2011 AACR Award for Lifetime Achievement in Cancer Research
2011 Co-Chair, Minisymposium ‘Tumor Microenvironments” 2011 102nd Annual Meeting of the AACR, April 3-6, 2010, Orlando, FL USA
2011 Co-Organizer with Drs. Yves DeClerck (USC, Children’s Hospital) and Melody Swartz (EPFL); AACR Special Conference: Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy, November 2011, Orlando FL USA
2012 Co-Chairperson, Program Committee: 2012 103rd Annual Meeting of the AACR, April 3-6, 2012, Chicago, IL USA
2012 Chair, Plenary session: “Tumor Heterogeneity: Challenges and Therapeutic Opportunities” 2012 103rd Annual Meeting of the AACR, April 3-6, 2012, Chicago, IL USA
2012 Chair, Education session: “Tumor Microenvironment” 2012 103rd Annual Meeting of the AACR, April 3-6, 2012, Chicago, IL USA
2012 Speaker, 2012 AACR Meet the Research Pioneer, 2012 103rd Annual Meeting of the AACR, April 3-6, 2012, Chicago, IL USA

American Society for Cell Biology
2000 American Society for Cell Biology, photo credits in ‘Exploring the Cell” Ed. W. Wells
2001 Table Leader, Career Discussion Lunch, Women in Cell Biology and Education Committee, 40th Annual Meeting, Washington, DC, USA
2001 Co-chair and Co-organizer, Mini-symposium (Microenvironment/Extracellular Matrix in Development and Disease): 40th Annual Meeting, Washington, DC, USA
2003 Table Leader, Career Discussion Lunch, Women in Cell Biology and Education Committee of the ASCB, 42nd Annual Meeting, San Francisco, CA, USA
2006 Co-Chair Minisymposium (Cancer Mechanisms): 46th Annual Meeting, San Diego CA, USA
American Cancer Society
1999 14th Annual Excalibur Round Table, San Francisco, CA, USA
2000 San Mateo County Annual Volunteer Meeting, San Mateo, CA, USA

International Society for Preventive Oncology
2002 Session Chair (Chemoprevention): 6th Annual Meeting, Pasteur Institute, Paris, France.

International Proteolysis Society
2007 Member, International Scientific Advisory Committee, 5th General Meeting of the International Proteolysis Society, Rion-Patras, GREECE.
2011 Member, Organizing Committee, 9th General Meeting of the International Proteolysis Society, San Diego CA, USA

International Society for Biological Therapy of Cancer
2008 Co-Organizer, 2008 Workshop on Inflammation in Cancer Development, San Diego CA, USA

Service to Professional Publications:
2003 - 2005 Associate Editor, Cancer Research
2005 – 2007 Editorial Board, Carcinogenesis
2004 – 2007 Senior Editor, Cancer Research (Cell, Tumor and Stem Cell Biology Section)
2007 – 2009 Senior Editor, Cancer Research (Tumor Microenvironment Section)
2007 – 2009 Deputy Editor, Cancer Research
2007 Guest Editor, PNAS Editorial Board
2008 Guest Editor (with Tyler Jacks), Current Opinion in Genetics & Development
2008 – 2010 Editorial Board, Cancer Microenvironment
2009 – 2012 Deputy Editor for Breaking Advances, Cancer Research
2012 – present Editorial Board, Cancer Cell

Ad hoc reviewing
1994 Oncogene;
1995 Am J Pathology; Matrix Biology; J Cell Biology
1999 Am J Pathology; Cancer Letters; Nature Medicine; Nature; PNAS; Cell Motility & the Cytoskeleton; Cancer Research
2000 Am J Pathology; Cancer Research; Genes & Development; Int. J Cancer
2001 J Cell Biology; Int. J of Cancer; EMBO; Neoplasia; Cancer Research
2002 Cancer Research; Am J Pathology; Int. J Cancer; Biological Chemistry; Cancer Cell; Cancer Letters
2003 PNAS; Cancer Research; Int. J of Cancer; J Molecular Medicine; Biological Chemistry; Science; Cancer Cell; Nature Medicine; J Leukocyte Biology; Neoplasia; Am J Pathology
2004 Lancet; Cancer Cell; Cancer Research; American J Pathology; J Cell Biology; Nature Reviews Immunology; Nature Reviews Cancer; PNAS; J Biological Chemistry; Nature; J Exp Med; Int J Cancer
2005 Nature Medicine, Cancer Cell, Cancer Research; Am J Pathology; Cell; Nature; Nature Reviews Immunology; Nature Reviews Cancer; Carcinogenesis
2007 Cell; Nature; PNAS: J Cell Biology; Cancer Research; J Exp Med; Breast Cancer Research
2008 Cancer Cell; PNAS; J Immunology; Nature; J Exp Med; Trends in Genetics; Current Opinions in Investigational Drugs
2009 Cancer Cell; Cell; Nature; J Exp Med; J Clin Invest, Cancer Research; Int J Cancer; Oncogene, J Immunology
2011 Nature; Nature Medicine; Cancer Research; Cancer Cell; J Clin Invest; Breast Cancer Research; PlosOne, PNAS; Oncogene; J Exp Med; Oncogene;
2012 PNAS; Can Res; Oncogene; Trends in Immunology; Nature; Clin Can Res; J Exp Med; Cancer Discovery; Immunity; J Cell Physio; JoVE; Immunity; J Clin Invest;
### VI. INVITED PRESENTATIONS

#### Symposia and Workshops: International

<table>
<thead>
<tr>
<th>Year</th>
<th>Title</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>2nd Annual International Protease Society</td>
<td>Freising, Germany.</td>
</tr>
<tr>
<td>2002</td>
<td>6th International Symposium on Predictive Oncology &amp; Intervention Strategies</td>
<td>Pasteur Institute, Paris, France</td>
</tr>
<tr>
<td>2002</td>
<td><strong>KEYNOTE LECTURE</strong>, Dutch Cancer Society Annual Symposium</td>
<td>Luntern, The Netherlands</td>
</tr>
<tr>
<td>2003</td>
<td>2nd Annual International Symposium on Epithelial Biology</td>
<td>Timberline, Oregon USA</td>
</tr>
<tr>
<td>2004</td>
<td>10th International Congress of the Metastasis Research Society</td>
<td>Genoa Italy</td>
</tr>
<tr>
<td>2005</td>
<td>2005 International Consortium Meeting of the Children’s Tumor Foundation: Molecular Biology of NF1, NF2 and Schwannomatosis</td>
<td>Aspen, CO, USA</td>
</tr>
<tr>
<td>2005</td>
<td>International Symposium on Systems Genome Medicine - Bench to Bedside</td>
<td>Institute of Medical Sciences University of Tokyo, Tokyo, Japan</td>
</tr>
<tr>
<td>2005</td>
<td>Immunotherapy of Cancer, XI Annual Symposium of the Danish Cancer Society, Copenhagen, Denmark</td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>4th General Meeting of the International Proteolysis Society, Quebec City, Canada</td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>Centro Nacional de Investigaciones Oncológicas (CNIO) Cancer Conference: Inflammation and Cancer, Mardid SPAIN</td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>18th Annual Pezoller Symposium ‘Tumor Microenvironment: Heterotypic Interactions’, Trento ITALY</td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>European Association for Cancer Research (EACR) 1st Annual Meeting, Budapest HUNGARY</td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>XXXIVth Meeting of the International Society for Oncodevelopmental Biology and Medicine (ISOBM: Tumor Biology, Detection and Therapy, Pasadena, CA, USA</td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>37th International Symposium of the Princess Takamatsu Cancer Research Fund ‘Cancer Cells and Their Microenvironment’, Tokyo, JAPAN</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>4th International Conference on Tumor Microenvironment, Florence, ITALY</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>2nd International Symposium on Cancer Metastasis and the Lymphovascular System: Basis for Rational Therapy, San Francisco CA USA</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td><strong>KEYNOTE LECTURE</strong>, 7th International Symposium on Hodgkin Lymphoma, Cologne, GERMANY</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>CANDLEIGHT LECTURE, Inflammation and Cancer: From molecular links to bed side; Inaugural meeting for the Istituto Clinico Humanitas, Milan ITALY</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>7th Annual International Congress on the Future of Breast Cancer, Kauai, Hawaii USA</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td><strong>CANCER RESEARCH UK LECTURE</strong>, NCRI Cancer Conference, Birmingham UNITED KINGDOM</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>21st Lorne Cancer Conference, Lorne AUSTRALIA</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>6th International Symposium on the Intraductal Approach to Breast Cancer, Santa Monica CA USA</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>19th Annual BioCity Symposium, “Tumor Microenvironment in Cancer Progression”, Tirkku FINLAND</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td><strong>KEYNOTE LECTURE</strong>, European Association of Cancer Research, Special Conference on Inflammation and Cancer, Berlin GERMANY</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>7th International Symposium on Minimal Residual Cancer, Athens, GREECE</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>Tri-Society Annual Conference of the Society for Leukocyte Biology, International Cytokine Society, and the International Society for Interferon and Cytokine Research, Lisbon, Portugal</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>5th International Conference on Tumor Microenvironment, Versailles, FRANCE</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td><strong>PRESIDENT’S PLENARY LECTURE</strong>: Italian Cancer Society Annual Meeting, Milano ITALY</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>PLENARY LECTURE, CHUV Research Day, University hospital (CHUV) and the Faculty of Biology and</td>
<td></td>
</tr>
</tbody>
</table>
Medicine, Lausanne, SWITZERLAND.

2010  NATURE - CNIO Cancer Symposium on Frontiers in Tumour Progression, Madrid SPAIN
2011  Curie Institute Symposium, Breast Cancer from Biology to Clinics, Paris FRANCE
2011  EPFL Inaugural Symposium, Hallmarks and Horizons in Cancer, Lausanne SWITZERLAND
2011  Joint meeting of the International Cytokine Society and the International Society for Interferon and Cytokine Research, Florence, ITALY
2011  41st Australian Society for Immunology (ASI), Adelaide, South AUSTRALIA
2012  International Symposium of the Collaborative Research Center (ISCRC), Molecular Basis and Modulation of Cellular Interactions in the Tumor Microenvironment, Cologne, Germany
2012  Keystone Symposium on The Role of Inflammation During Carcinogenesis, Dublin, IRELAND
2012  Federation of Clinical Immunology Societies (FOCIS) 2012, Improving Human Health Through Immunology, Vancouver, BC, CANADA

UPCOMING INVITATIONS
2012  25th International IGB Workshop, CNR, Capri Island, Naples, ITALY
2012  Annual Meeting of the (French) National Institute of Cancer, Plenary session on “Cancer Immunity and Inflammation”, Paris FRANCE
2012  25th International IGB Workshop, organized by the Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, Naples, ITALY
2013  9th AACR-Japanese Cancer Association International Conference; February 21-25, 2013, Maui, Hawaii, USA
2013  Cancer Research Center of Lyon (CRCL), First International CRCL Symposium: A Focus on Tumor Escape. Lyon FRANCE
2013  International Conference on Immunochemotherapy, Paris FRANCE

Symposia and Workshops: National
1994  Current Transgenic Technology, B & K Universal, San Mateo, CA, USA
1997  Biology of Proteolysis, Cold Spring Harbor Laboratory, NY, USA
1997  Molecular Biology & Pathology of Neoplasia, AACR, Keystone, CO, USA
1997  Matrix Metalloproteinases, Gordon Research Conference, Proctor Academy, New London, NH, USA
1998  Proteolysis, Gordon Research Conference, Colby-Sawyer College, New London, NH, USA
1998  Cellular Targets of Viral Carcinogenesis, AACR Special Conference. Dana Point, CA, USA
1998  Mechanisms of Tumor Growth & Invasion Mediated by Proteolysis, UCSF-Molecular Design Institute. San Francisco, CA, USA
1999  Tumor Microenvironment, Education Session, AACR Annual Meeting. Philadelphia, PA, USA
1999  Matrix Metalloproteinases, Gordon Research Conference, Colby-Sawyer New London, NH, USA.
2000  Epithelial-Stromal Interactions & Tumor Progression Workshop, National Cancer Inst., Bethesda, MD, USA
2000  10th National Conference of the Inflammation Research Association, Hot Springs, VA, USA
2001  ‘Meet-the-Expert’ Sunrise Session, AACR Annual Meeting, New Orleans, LA, USA
2002  Chemotherapy of Experimental & Clinical Cancer, Gordon Research Conference, Colby Sawyer College, New London, NH, USA
2002  Proteolytic Enzymes & their Inhibitors, Gordon Research Conference, Colby Sawyer, New London, NH, USA
2002  From the Cancer Cell to a Tumor - Tumors as Outlaw Organs, Schilling Research Conference, The American Cancer Society, Aptos CA, USA
2002  Cancer Intervention 2002, Van Andel Research Institute, Grand Rapids, Michigan USA
2002  Pathobiocemistry B Study Section Workshop, Natl. Cancer Institute, Hilton Head, SC, USA
2002  Proteases, Extracellular Matrix and Cancer, AACR Special Conference, Hilton Head Island, SC, USA
2002  ECM and Cancer, Minisymposium, ASCB Annual Meeting, San Francisco, CA, USA
2003  Matrix Metalloproteinases, Gordon Research Conference, Big Sky, Montana, USA
2003  Angiogenesis & Microcirculation, Gordon Research Conference, Salve Regina, Newport R.I., USA
2003  Inflammatory Cells and Cancer, Symposium, American Society of Hematology 2003 Annual Meeting, San Diego, CA, USA
2003 Validation of a Causal Relationship: Criteria to Establish Etiology, National Cancer Institute, Cancer Etiology Branch, Washington, DC, USA.

2003 Functional Imaging of Proteolysis, Special Session, ASCB Annual Meeting, San Francisco, CA, USA

2004 Scleroderma Research Foundation Annual Scientific Workshop, San Francisco, CA, USA

2004 Systems Biology of Cancer: The Tumor as an Organ, Symposium, 95th AACR Annual Meeting. Orlando, FL, USA

2004 Inflammation and Cancer, Symposium, 95th AACR Annual Meeting. Orlando, FL, USA

2004 Remarkable Role of the Microenvironment in Development and Disease Pathogenesis, Symposium; Experimental Biology 2004, Sponsored by: the Assoc. of Anatomy, Cell Biology and Neurobiology, Washington, D.C., USA.

2004 Molecular and Cellular Basis of Disease: Structure and Function of the Extracellular Matrix in Disease: Novel Roles and Regulation of MMPs and TIMPs in Disease, Symposium; Experimental Biology 2004, Sponsored by: the Am. Society of Investigative Pathology, the American Society for Matrix Biology and the North American Vascular Biology organization. Washington, D.C., USA.

2004 Pacific Coast Protease Workshop, Half Moon Bay, CA, USA.


2005 Keystone Symposia, The Role of Microenvironment in Tumor Induction and Progression (J5), Banff, Alberta CANADA

2005 Keystone Symposia, Inflammation and Cancer (B8), Breckenridge, CO, USA

2005 Symposium on Inflammation, Repair and Carcinogenesis in Liver, Pancreas and Colon. UCSF Liver Center and the Program in Gastrointestinal Cancer of the UCSF Cancer Center, Rohnert Park, CA, USA

2005 In the Forefront of Advances in Cancer Research, Symposium, 96th AACR Annual Meeting. Anaheim, CA, USA

2005 Macrophage Symposium, AMGEN, Seattle, WA, USA

2005 Immune Response to Cancer Symposium, 41st Annual Meeting, American Society Clinical Oncology (ASCO), Orlando. FL, USA

2005 Phagocyte, Gordon Research Conference, New London, CT, USA

2005 Mouse Models of Human Cancer Consortium, Annual Steering Committee Meeting, New Brunswick, NJ USA

2005 Matrix Metalloproteinases, Gordon Research Conference, Big Sky, Montana, USA

2005 Annual Buffalo Regional Conference on Immunology, Buffalo, NY, USA

2005 2005 Montagna Symposium on 'Tissue repair - molecular mechanisms and clinical challenges’, Salishan Lodge, OR, USA

2005 4th Annual AACR Conference on Frontiers in Cancer Prevention Research, Baltimore MD, USA

2005 AACR Special Conference, Cancer, Proteases and the Microenvironment, Bonita Springs, Florida, USA

2006 Timberline Annual Symposium on Epithelial Biology, Intrinsic and Microenvironmental Regulation of Epithelial Cancer’, Timberline Lodge, Oregon, USA

2006 Keystone Symposium, Molecular Targets for Cancer Prevention, Granlibakken Resort, Tahoe City, CA, USA

2006 Inflammation and Cancer, Symposium, 97th AACR Annual Meeting. Washington, D.C., USA

2006 Lineberger Cancer Center’s 30th Annual Scientific Symposium, University of North Carolina, Chapel Hill, North Carolina, USA

2006 KEYNOTE LECTURE, Vanderbilt-Ingram Cancer Center Retreat 2006, Vanderbilt University, Nashville TN, USA

2006 TUMOR BIOLOGY PLENARY LECTURE, Advances in Neuroblastoma Research 2006, Los Angeles, CA, USA

2006 Genetic, Cellular and Microenvironmental Determinants of Tumor Progression and Metastasis: A ‘TPM’ Workshop Honoring Martin L. Padarathsingh, Ph.D. TPM Study Section Workshop, Natl. Cancer Institute, Georgetown, VA, USA

2006 ASCO/Federation of European Societies Symposium: Inflammation in Cancer Progression, 2006 ASCO Annual Meeting, Atlanta, GA, USA
2006 AACR Special Conference, *Tumor Immunology: An Integrated Perspective*, Miami, FL, USA
2007 7th AARC-Japanese Cancer Association Joint Conference: *In the Forefront of Basic and Translational Cancer Research*, Waikoloa, Hawaii, USA
2007 Keystone Symposium *Inflammation and Cancer*, Santa Fe, NM, USA
2007 AAAS Annual Meeting, *Healthy Aging: Inflammation and Chronic Diseases*’ Symposium, San Francisco, CA USA
2007 Tumor Microenvironment and Tumor-Stromal Interactions Workshop: Sponsored by Biogen Idec Inc., Oncology Discovery Research, San Diego CA USA
2007 22nd Aspen Cancer Conference: Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy, Aspen CO, USA
2007 Gordon Research Conference, *Epithelial Differentiation & Keratinization*, Bryant University, Smithfield, RI, USA
2007 National Cancer Institute Workshop, ‘*Profiling of Immune Response to Guide Cancer Diagnosis, Prognosis and Prediction of Therapy*’, Bethesda, MD, USA
2008 47th Midwinter Conference of Immunologists, ‘*Meeting the challenge: Immunobiology in health and disease*’, Asilomar, CA USA
2008 AACR-TREC-NCI Conference on *Energy Balance and Cancer: Mediators and Mechanisms*, Lansdowne, VA USA
2008 Keystone Joint Symposium, ‘*Cell Death in the Immune System / Cell Death and Cellular Senescence*’, Beaver Run Resort in Breckenridge, CO, USA
2008 Keystone Symposium, *Inflammation, Microenvironment and Cancer*, Snowbird Resort in Snowbird, Utah, USA
2008 THE JOHN F. ANDERSON MEMORIAL LECTURE IN MEDICINE, ‘The Linkage between Inflammation and Cancer’, University of Virginia, Charlottesville VA, USA
2008 Tumor Microenvironment Symposium, Stony Brook University, Stony Brook. NY. USA
2008 KEYNOTE LECTURE, Fox Chase Cancer Center 13th Annual Postdoctoral Fellow and Graduate Student Symposium, Philadelphia, PA USA
2008 AACR Centennial Conference: *Translational Cancer Medicine 2008: Cancer Clinical Trials and Personalized Medicine*; Hyatt Regency Monterey in Monterey, CA USA
2008 University of Michigan Comprehensive Cancer Center 2008 Fall Symposium, Ann Arbor MI, USA
2008 AACR Special Conference, *Chemical and Biological Aspects of Inflammation and Cancer*, Ko Olina Hawaii, USA
2008 International Society for Biological Therapy of Cancer (iSBTc), Workshop on Inflammation in Cancer Development, Westin Horton Plaza San Diego, CA USA
2008 Skirball Symposium, New York University School of Medicine, New York, NY USA
2008 AACR Special Conference in Cancer Research, *Tumor Immunology: New Perspectives*; Miami FL, USA
2009 1st Conference on Regulatory Myeloid Suppressor Cells, Clearwater, FL USA
2009 Keystone Symposium, *Extrinsic Control of Tumor Genesis*, Vancouver, British Columbia CANADA
2009 *Inflammation and Cancer: Novel Aspects of Protumor Immunity*, Major Symposium, 100th Annual Meeting AACR, Denver CO USA
2009 2nd Annual Retreat of the CCR-NCI Cancer and Inflammation Program, Gettysburg, PA USA
2009 24th Annual Aspen Cancer Conference, Aspen, CO, USA
2009 AACR Special Conference, *Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications*, San Diego CA USA
2009 NCI’s National Tumor Microenvironment Network, Nashville TN USA
2010 Joint Keystone Symposia, Role of Inflammation in Oncogenesis/Molecular and Cellular Biology of Immune Escape in Cancer, Keystone CO USA
2010 3rd Annual Wyeth Discovery Frontiers in Human Disease Symposium, New York, NY USA
2010 PLENARY LECTURE, 2010 Annual Meeting of the American Association for Cancer Research, Washington DC USA
2010 10th Annual Oncology Research Symposium at MIT’s Koch Institute for Integrative Cancer Research, Boston MA USA
2010 Metastasis and the Tumor Microenvironment, Short Course, Eppley Institute for Cancer Research, Univ of Nebraska, Omaha, NB USA
2010 Cancer Cell Biology and Signaling Workshop, ImClone Systems/Eli Lilly, New York NY, USA
2010 Center for Excellence in Immunology of the National Cancer Institute Symposium, Bethesda MD, USA
2010 25th Annual Critical Issues in Tumor Microenvironment, Angiogenesis and Metastasis, Boston MA, USA
2010 Metastasis Research Society-AACR Joint Conference on Metastasis and the Tumor Microenvironment, Philadelphia, PA USA
2010 J. WALTER JUCKETT DISTINGUISHED LECTURE, University of Vermont Cancer Center Clinical and Translational Research Symposium, Inflammation & Cancer, Burlington VT, USA
2010 KEYNOTE LECTURE, 2010 Saban Research Institute Annual Symposium, Honoring Yves DeClerck, University of Southern California and Children's Hospital Los Angeles, Los Angeles CA, USA
2010 PLENARY LECTURE, 2010 American College of Veterinary Pathologists and American Society for Veterinary Clinical Pathology, Concurrent Annual Meetings, Baltimore MD, USA
2011 KEYNOTE LECTURE, 11th Annual Meeting of NANT Consortium Investigators. Biology and Therapy of High Risk Neuroblastoma, Redondo Beach CA, USA
2011 2nd International Conference on Immunochemotherapy, entitled “Immunochemotherapy: Correcting Immune Escape in Cancer”, Philadelphia PA USA
2011 The Biology of Cancer: Microenvironment, Metastasis & Therapeutics, Cold Spring Harbor Laboratory Meeting Series. Cold Spring Harbor, NY USA
2011 2nd NCI Tumor Microenvironment Network Junior Investigator Meeting, Cambridge, MA USA
2011 AACR Special Conference: Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy, Orlando Florida USA
2011 PLENARY LECTURE, San Antonio Breast Cancer Conference, San Antonio, Texas USA
2012 51st Midwinter Conference of Immunologists, Asilomar, CA USA
2012 PLENARY SESSION “Tumor Heterogeneity: Challenges and Therapeutic Opportunities” 2012 103rd Annual Meeting of the AACR, April 3-6, 2010, Chicago, IL USA
2012 AACR Special Conference on Pancreatic Cancer, Lake Tahoe NV, USA

UPCOMING INVITATIONS
2012 CELL Symposium, Hallmarks of Cancer, San Francisco CA USA
2012 AACR Special Conference, Tumor Immunology: Multidisciplinary Science Driving Basic and Clinical Advances, Miami FL, USA
2013 Society of Leukocyte Biology, Regulators of Innate Cell Plasticity Effects in Host Defense” Newport RI, USA
2013 AMERICAN CANCER SOCIETY BASIC SCIENCE LECTURE, Society of Surgical Oncology Annual Meeting, Washington D.C., USA

Invited Lectures/Seminars: International
2000 Medical Genome Center, Division of Molecular Medicine, Australian National University, Canberra, A.C.T. AUSTRALIA.
2001 German Cancer Center, Heidelberg, GERMANY.
2001 MERCK Pharmaceutical, Damstedt GERMANY.
2003 University of Toronto, Ontario Cancer Institute & Princess Margaret Hospital, Toronto, Ontario, CANADA
2004  Cancer Research UK, Barts & The London Queen Mary’s School of Medicine & Dentistry, John Vane Science Center, Charterhouse Square, London, UNITED KINGDOM
2004  Cancer Research UK, London Research Institute, Lincoln’s Inn Fields Laboratories, London, UNITED KINGDOM
2004  University of British Columbia, Department of Biochemistry and Molecular Biology, Vancouver, British Columbia, CANADA
2007  Angiogenesis and Tumor Targeting Research Unit & Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, ITALY
2008  Institute of Cell Biology, ETH Zurich Switzerland
2008  Institute of Cancer and the CR-UK Clinical Centre, Barts & The London School of Medicine and Dentistry, London UNITED KINGDOM
2009  University of South Hampton, UNITED KINGDOM
2009  The Netherlands Cancer Institute, Amsterdam, THE NETHERLANDS
2010  DISTINGUISHED GUEST LECTURER, Institute of Cancer, Barts & London School of Medicine. London UNITED KINGDOM
2012  Excellence in Genetics and Immunology Lecture Series, Complex Traits Group at McGill University, Montreal, Qc, CANADA
2012  MILDRED SCHEEL LECTURESHIP (INAUGURAL), German Cancer Aid, and Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, GERMANY

UPCOMING INVITATIONS
2013  Cambridge Research Institute Distinguished Lecture, Cambridge UK.

Invited Lectures/Seminars: National
1997  Biologic Therapy Research Conference. Univ. of Pittsburgh Medical Center, Pittsburgh, PA, USA
1997  Immunology Seminar Series. Univ. of Pittsburgh Medical Center, Pittsburgh, PA, USA
1999  Axys Pharmaceuticals, South San Francisco, CA, USA
1999  Berlex Pharmaceuticals, Emeryville, CA, USA
1999  Axys Pharmaceuticals, La Jolla, CA, USA
1999  14th Annual Excalibur Round Table, American Cancer Society, San Francisco, CA, USA
1999  Colloquium in Microbiology, Cell and Molecular Biology. San Francisco State Univ., San Francisco, CA, USA
2000  Chiron Corporation, Emeryville, CA, USA
2000  Oral and Pharyngeal Cancer Branch/NIDCR, National Institutes of Health, Bethesda, MD, USA
2000  Fibrogen, Inc., South San Francisco, CA, USA
2000  Scios Inc., Sunnyvale, CA, USA
2000  Molecular Biology Department, University of Southern California, Los Angeles, CA, USA
2001  Dept. of Pediatric Hematology and Oncology, Children’s Hospital Los Angeles, Univ. of Southern California, Los Angeles, CA, USA
2001  Jonnson Comprehensive Cancer Center, Univ. of Calif., Los Angeles, Los Angeles, CA, USA
2002  Institute for Engineering and Medicine, Univ. of Pennsylvania, Philadelphia, PA, USA
2002  Oncology Grand Rounds, Univ. of Missouri, Columbia, MO.
2002  Cancer Center, Univ. of California, Davis, Davis CA, USA
2002  AstraZeneca, Waltham, MA USA
2002  Pharmacology Seminar Series, Dept. of Pharmacology, Wayne State Univ., Detroit, MI, USA
2003  Dept. of Biology, Univ. of Calif., San Diego, San Diego, CA USA
2003  Tularik, Inc., South San Francisco, CA USA
2003  Dept. of Cancer Biology's Cancer Metastasis Research Program Seminar Series, M.D. Anderson Cancer Center, Univ. of Texas, Houston, TX, USA
2003  Dept. of Cancer Biology, Stanford University, Stanford, CA, USA
2004  Burnham Cancer Institute, San Diego, CA, USA
2004  The Wistar Cancer Institute, Philadelphia, PA, USA
2004  Regeneron Pharmaceuticals, Inc. Tarrytown, New York, USA
2004  Keynote Lecture: Vanderbilt University Digestive Disease Research Center Retreat, Vanderbilt University, Nashville, TN, USA
2004  Dana Farber Cancer Center, Harvard Medical School, Boston MA, USA
2004 Indiana University, Herman B. Wells Center for Pediatric Research and Clinical Cancer Center, Indianapolis IN, USA
2004 Immunology Graduate Program Seminar, Stanford University, Stanford, CA, USA
2005 Dept. of Nutritional Sciences & Toxicology, Univ. of Calif., Berkeley, Berkeley, CA USA
2005 Rigel, Inc., South San Francisco, CA USA
2005 Dept of Pathology & Lab Medicine, Univ. of California, Los Angeles, Los Angeles, CA USA
2006 Division of Cancer Biology and Angiogenesis in the Department of Pathology at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA USA
2006 Department of Molecular and Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA USA
2007 Lymphoma and Myeloma Conference, M.D. Anderson Cancer Center, Houston, TX, USA
2007 University of Minnesota, Dept. of Lab Medicine and Pathology, Minneapolis, MN, USA
2007 Memorial-Sloan Kettering Cancer Center, Program in Cancer Biology and Aging, New York NY, USA
2007 Abramson Family Cancer Research Institute and Univ. of Pennsylvania, Division of Hematology-Oncology, Philadelphia, PA USA
2007 Albert Einstein College of Medicine, New York NY, USA
2007 Oncology Division Research, Biogen Idec Inc., San Diego, CA USA
2007 Genentech, Inc. Immunology Program, South San Francisco, CA USA
2007 University of Iowa Carver College of Medicine, Dept of Pathology, Pathology Grand Rounds, Iowa City, Iowa, USA
2007 Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA
2007 University of Michigan, Program in Immunology and Cancer Research Series, Ann Arbor, MI USA
2008 Department of Pathology/UCLA School of Medicine Seminar, Los Angeles CA USA
2008 ANNUAL KEYNOTE LECTURE, Dept of Cancer Biology, Meharry Medical College, Nashville, TN USA
2008 University of California, Davis Cancer Center, Sacramento, CA USA
2008 Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
2008 Cancer Biology Series, Ben May Cancer Center, University of Chicago, Chicago, IL, USA
2008 National Cancer Institute Center for Cancer Research Grand Rounds Series in Clinical and Molecular Oncology, Bethesda MD, USA
2009 University of Michigan, Oral Health Sciences Program and Biomedical Engineering Seminar Series, Ann Arbor, MI USA
2009 Department of Pharmacology, Wayne State University, Detroit, MI USA
2009 Molecular Biology Seminar Series, Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Aurora, CO USA
2009 National Institutes of Health/National Cancer Institute, Vascular Biology Seminar Series, Bethesda MD, USA
2009 Genentech, Inc., Molecular Oncology Program, South San Francisco, CA USA
2009 Breast Cancer Network of Strength, California Breast Cancer Organizations, Northern California Affiliate, David CA USA
2009 Fred Hutchinson Cancer Center, Seattle WA USA
2010 Cold Spring Harbor Laboratory, CSH NY USA
2010 Albert Einstein College of Medicine, New York, NY USA
2010 Department of Cell Biology & Physiology Washington University, St Louis, MO USA
2010 Cancer Center Seminar Series at Burnham Institute for Medical Research, San Diego CA, USA
2010 Oncology Seminar Series, MedImmune, Gaithersburg, MD, USA
2010 Immunology Institute Seminar Series, Mt Sinai School of Medicine, NY, NY USA
2010 San Francisco State University, Fall Seminar Series, San Francisco CA USA
2011 McArde Seminar in Cancer Biology series, Univ of Wisconsin-Madison, USA
2011 Duke University Medical Center, Durham, North Carolina USA
2011 BORNTREE DISTINGUISHED LECTURE, Immunology and Infectious Disease Program, Dept of Veterinary and Biomedical Sciences, Pennsylvania State Univ. University Park, PA USA
2011 HUCK DISTINGUISHED LECTURE, The Huck Institute, University Park, Pennsylvania State Univ. University Park, PA USA
2011 Weill Cornell Medical College of Cornell University, Center for Vascular Biology, NY USA
2011 **CHARLES I. SIEGAL MEMORIAL LECTURE**, Dana-Farber Cancer Institute and the Dana-Farber Cancer Institute, Boston MA, USA

2011 Tulane Cancer Center, Hematology & Medical Oncology, Tulane Univ. School of Medicine, New Orleans, LA USA

2011 Northwestern University Breast Cancer Research Program and Breast Cancer Research Seminar Series, Northwestern University, Chisago IL, USA

2011 Novartis Institutes for Biomedical Research, Emeryville CA USA

2011 FivePrime Therapeutics, South San Francisco, CA USA

2011 Abbott Biotherapeutics, Redwood City CA USA

2012 Brown Foundation Institute of Medicine, Univ. of Texas, Health Science Center at Houston, Houston TX USA

2012 Baylor College of Medicine, Houston TX USA

2012 Harvard Medical School’s (HMS) Committee on Immunology Seminar Series

2012 Massachusetts General Hospital’s (MGH) Seminar Series

2012 University of Rochester, Department of Microbiology and Immunology, Rochester, NY, USA

2012 Becton, Dickinson and Company, San Jose CA, USA

**UPCOMING INVITATIONS**

2012 Huntsman Cancer Institute of University of Utah, Salt Lake City UT, USA

2012 Eisai, Inc. Andover MA, USA

**Invited Lectures/Seminars: OHSU**

2012 OHSU Knight Cancer Biology Research Group Meeting, OHSU

2012 OHSU School of Medicine, TEDMED 2012 Live Simulcast

**UPCOMING INVITATIONS**

2012 OHSU MD/PhD Annual Retreat, McMenamins Edgefield, Troutdale, OR.

2012 OHSU PMCB Annual Retreat, OR

**Invited Lectures/Seminars: UCSF**

1997 Breast Cancer SPORE Seminar. UCSF

1999 Cancer Research Institute Retreat, Tomales Bay, CA

2000 Chemistry and Cancer: How Chemistry-Based Tools Are Helping Solve Today’s Serious Health Problems, Dev. & Alumni Relations, UCSF

2000 Oncology Grand Rounds, Department of Hematology and Oncology, UCSF

2000 PIBS-Cell Biology Seminar Series, UCSF

2000 Pathology and Lab Medicine Grand Rounds, UCSF

2000 BMS Student Pizza Talk, UCSF

2000 Cell Cycle & Dysregulation Club, Comprehensive Cancer Center, UCSF

2000 Comprehensive Cancer Center Retreat, Granlibakken, Tahoe City, CA

2001 BMS Student Pizza Talk, UCSF

2001 Pathology and Lab Medicine Grand Rounds, Departments of Medicine and Pathology, UCSF

2001 UCSF, Cell Biology Retreat, Wilbur Hot Springs, CA, USA

2001 UCSF TETRAD Retreat, Granlibakken, Lake Tahoe, CA, USA

2001 UCSF Cancer Research Institute/BMS Retreat, Granlibakken, Lake Tahoe, CA, USA

2002 Current Topics in Medical Science, UCSF Medical Scientist Training Program (M170.09)

2002 Mouse Models of Human Cancer Program, Comprehensive Cancer Center, UCSF

2002 Cancer Research Institute Retreat, Santa Cruz, CA

2003 PIBS Student Pizza Talk, UCSF

2003 Breast Oncology Program, Comprehensive Cancer Center, UCSF

2003 Comprehensive Cancer Center Faculty Retreat: Identification and Functional Assessment of Cancer Effectors, Golden Gate Club, San Francisco CA

2004 BMS Graduate Program Retreat, Granlibakken Tahoe City, CA

2005 BMS Student Pizza Talk, UCSF

2006 *Introduction to Research*, Department of Pathology, UCSF

2008 Division of Experimental Medicine, Divisional Seminar Series, UCSF

2009 Immunology Program, UCSF
VII. GOVERNMENT AND OTHER PROFESSIONAL SERVICE:

GOVERNMENT SERVICE


2003 Division of Cancer Biology, National Cancer Institute: Microenvironment Think Tank Participant and Reporter

2003 Division Cancer Etiology, National Cancer Institute: Validation of A Causal Relationship: Criteria to Establish Etiology Think Tank Invited speaker and Participant

2004 National Institutes of Health, National Cancer Institute Subcommittee C (05/2004) – Basic & Preclinical NCI Initial Review Group, NCI-C RPRB (T2) Angiogenesis

2005 National Institutes of Health, National Cancer Institute Subcommittee D (02/2005) – Clinical Studies NCI Initial Review Group, NCI-D RPRB Tumor Pathology

2005 National Institutes of Health, Center for Scientific Review-Oncology Special Emphasis Panel (SEP); ZRG1 ONC (03) M, Developmental Therapeutics

2010 National Institutes of Health, Center for Scientific Review-Neuroscience Special Emphasis Panel (SEP)/Scientific Review Group 2010/05 ZNS1 SRB-R (47)

2010 National Institutes of Health, Center for Scientific Review-Neuroscience Special Emphasis Panel (SEP)/Scientific Review Group 2011/01 ZRG1 DTCS-A (81) 6th Era of Hope conference Technical Planning Committee (TPC)

2011 Department of Defense (DOD), Breast Cancer Research Program (BCRP) Ad hoc reviewer (06/2011), Cancer Immunotherapy & Immunology (CII) Study Section, Oncology 2 - Translational Clinical IRG (OTC) Division of Translational and Clinical Sciences

OTHER PROFESSIONAL SERVICE

1999 Arkansas Science & Technology Authority Ad hoc Grant Review


2000 Division of Cancer Biology, NCI: Epithelial-Stromal Interactions & Tumor Progression Workshop Invited speaker and Participant

2001 Department of Veterans Affairs Ad hoc Grant Review, Oncology Review Board

2001 Research Grants Council of Hong Kong Ad hoc Grant Review

2003 Danish Cancer Society, DENMARK Ad hoc Grant Review

2004 Division of Gastroenterology and Digestive Disease Research Center, Vanderbilt University, Nashville TN, USA ‘H. pylori-induced Inflammation and Gastric Adenocarcinoma’, PO1 External Advisory Panel

2004 Cancer Research Ireland, Irish Cancer Ad hoc grant review
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<tr>
<th>Year</th>
<th>Position and Details</th>
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<tbody>
<tr>
<td>2004</td>
<td>Society Dutch Cancer Society Ad hoc grant review</td>
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<tr>
<td>2004</td>
<td>Vanderbilt University, Nashville TN, USA; SPORE in GI Cancer Ad hoc reviewer for SPORE Developmental Research Program</td>
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<tr>
<td>2005</td>
<td>Keystone Symposia, Inflammation and Cancer Co-organizer (with Dr. Ray DuBois, Vanderbilt Univ, TN), Breckinridge, CO, USA</td>
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<tr>
<td>2005</td>
<td>5th Annual Timberline Symp, on Epithelial Cell Biology, 'Intrinsic and Microenvironmental Regulation of Epithelial Cancer' Co-Organizer (with Dr. Harold Moses, Vanderbilt University, TN, USA), Timberline, OR, USA</td>
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<td>2006</td>
<td>Keystone Symposia Cancer Study Group for 2009 programming Study group member</td>
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<td>2006</td>
<td>Keystone Symposia, Inflammation and Cancer Organizer (with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA) Santa Fe, New Mexico, USA</td>
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<tr>
<td>2007</td>
<td>Keystone Symposia Inflammation and Cancer External Scientific Advisory Board Member</td>
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<td>2007</td>
<td>Masonic Cancer, University of Minnesota Center; Douglas Yee, M.D., Director</td>
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<tr>
<td>2007 - 2011</td>
<td>University of Washington, Seattle WA, USA Member, External Scientific Advisory Board, CA U54 TMEN: Significance of Microenvironment for Prostate Cancer Initiation and Progression; P.I. Stephen R Plymate, Univ. of Washington School of Medicine.</td>
</tr>
<tr>
<td>2008</td>
<td>International Society for Biological Therapy of Cancer (iSBTc), 2008 Workshop on Inflammation in Cancer Development Co-Organizer (with Drs. Michael Karin, Steven Dubinett, George Weiner)</td>
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<td>2009</td>
<td>GlaxoSmith Kline Member, Tykerb Post-ASCO KOL Advisory Board</td>
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<td>2009</td>
<td>University of Southern California, Children's Hospital Member, External Scientific Advisory Board, Neuroblastoma, Program Project grant (P01), PI: Robert Seeger, M.D.,</td>
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<td>2009 - 2010</td>
<td>Cancer Prevention and Research Institute of Texas (CPRIT) Member, Scientific Review Committee; Basic Cancer Biology Review Committee</td>
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<td>2011</td>
<td>Starr Cancer Consortium Scientific Review Board Member, Scientific Review Committee</td>
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<tr>
<td>2011</td>
<td>AACC Special Conference on ‘Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy’ Co-Organizer (with Drs. Yves DeClerck and Melodie Shwartz)</td>
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<tr>
<td>2012 - present</td>
<td>Koch Institute for Integrated Cancer Research, Massachusetts Inst. of Tech. Member, External Advisory Board</td>
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<tr>
<td>2012 - present</td>
<td>Melvin and Bren Simon Cancer Center, Indiana University Member, External Advisory Board</td>
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</table>

VIII. UNIVERSITY AND PUBLIC SERVICE

UNIVERSITY SERVICE

Oregon Health and Sciences University

2012 Member, Search Committee, Dept. of Urology and Knight Cancer Institute
2012 Chair, Search Committee, Depts of Cell & Developmental Biology, Molecular and Microbial Immunology
2012 Co-Chair, Search Committee, Dept. of Cell and Developmental Biology and Knight Cancer Institute
2012 Co-Chair, Search Committee, Dept of Medicine. Division of Hematology & Oncology and Knight Cancer Institute
2012 Chair, V Foundation Scholar nomination committee

University of California; System wide
1992-1993 Graduate Student Representative, Dept. of Biological Chemistry Faculty Council, UCLA
2004 ad hoc Member External Advisory Panel; Jonsson Comprehensive Cancer Center, University of California, Los Angeles, Los Angeles CA, USA
2009 Member, Site Visit Programmatic Review Group, Department of Pathology & Laboratory Medicine, UCLA School of Medicine. Graduate Council of the UCLA Academic Senate.

University of California, San Francisco; CAMPUS-WIDE
1997 Presentation, Donor Seminar, UCSF Development Office
1998 Presentation, Donor Seminar, UCSF Development Office
2000 – 2004 Member, Steering Committee, Ovarian Cancer Program Project Grant
2000 – 2005 Member, Scholarships and Awards Committee, Academic Senate, School of Medicine
2002 – present Member, BioMedical Sciences Graduate Program (BMS) Executive Committee
2002 – 2004 Member, Medical Scientist Training Program Executive Committee
2004 - 2006 Member, Search Committee, Director of Molecular Imaging, Dept. of Radiology, Committee Chair: Ron Arenson, M.D. no successful recruitment
2004 – 2006 Member, BioMedical Sciences Graduate Program (BMS); Admissions Committee
2004 Organizer, BioMedical Sciences Graduate Program Retreat, Granlibakken, N. Lake Tahoe, CA USA
2005 - 2006 Member, Tissue Engineering Ladder-rank Faculty Search Committee, Dept. of Surgery. Committee Chair: Nancy Boudreau, Ph.D. Successful recruitment of Valerie Weaver, Ph.D.
2005 - 2009 Member, Ethel and Jane Sokolow Memorial Cancer Endowment Lectureship Committee.
2006 Member, Cancer Faculty Search Committee, Anatomy Dept., Committee Chair: Zena Werb, Ph.D. Successful recruitment of Jeroen Roose, Ph.D.
2006 Member, Faculty Advisory Committee for 2007 Journalist Seminar on Inflammation and Disease. Sponsored by Associate Vice Chancellor Barbara J. French
2007 Member, committee to select recipient of Dean’s Postdoctoral Prize Lecture.
2007 Member, Faculty Search Committee for Restorative Neurosurgery and Stem Cell Neurobiology, VA Medical Center/UCSF NeuroSurgery. Committee Chair: Linda Noble, Ph.D.; Status: not filled.
2009 Member, Committee to choose 1st Bonnie J. and Anthony Addario Endowed Chair in Thoracic Oncology, School of Medicine, UCSF. Recipient: Thierry Jahon, M.D.
2010 Member, 2010 Selection Committee for the Hellman Family Early-Career Faculty Awards.

University of California, San Francisco, Helen Diller Family Comprehensive Cancer Center
1999 Member, Cancer Center Research Building Space Review Policy Committee
1999 – 2002 Member, Mt Zion Animal Barrier Facility Committee
1999 – 2005 Member, Cancer Center Friday Seminar Series Committee
2000 Organizer and Chair, MZ Cancer Center Research Building Annual Retreat
2001 Member, ‘Star Performance Award’ selection committee
2001 Presentation, Evelyn Herman Reception, UCSF Development Office
2001 – 2002 Member, Cancer Center Research Building, ‘Cancer Center Faculty Working Group’
2001 – 2006 Member, Mouse Models of Human Cancer Working Group
2002 – 2003 Member, UCSF Mt Zion campus, Animal Protocol Review Committee
2002 Member, ACS IRG grant review committee
2002 – 2006 Steering Committee Member, Mouse Models of Human Cancer
2003 Member, Review Committee, UCSF Comprehensive Cancer Center Stewart Trust Award
2003 – 2009 Chair, UCSF Mt Zion Campus Animal Protocol Review Committee
2003 Member, Search Committee: Associate Director for Administration, UCSF Comprehensive Cancer Center (Erica Weber, recruited)

2004 Member, Review Committee, UCSF Comprehensive Cancer Center Stewart Trust Award

2006 Co-Organizer, UCSF CCC Annual Symposium, 'Inflammation & Cancer: Bench to Bedside'.

2008 Chair, Committee to nominate Postdoctoral scholar for AACR 2008 Annual Meeting, Inaugural "Future Leaders, New Directions" Special Symposium. Nominee: Laura Soucek, Ph.D. (awarded)

University of California, San Francisco, Cancer Research Institute

2001 – 2002 Member, Cancer Research Institute Membership Subcommittee

University of California, San Francisco, Department of Pathology

2003 Member, Committee to recommend faculty for the Robert E. Smith Endowed Chair in Experimental Pathology

2004 Member, Search Committee, Ladder rank faculty, Physician-Scientist, Anatomic Pathology. Successful recruitment of Jay Debnath, M.D., Ph.D.

2007 Member, Search Committee, Ladder-rank faculty, Physician-Scientist, Pathology and Neuropathology. Committee Chair: Michael D Prados, M.D.; Status: open.

2008 Member, Search Committee, Ladder-rank faculty, Physician-Scientist, Experimental Pathology. Committee Chair: Benedict Yen, M.D.; Status: open

2009-2011 Member, Academic Merit and Promotions Committee; Pathology Dept.

University (other)

2002 Guest Instructor, Graduate Oncology, University of Missouri, Columbia, Missouri USA

2003 Guest Instructor, Cancer Biology, Stanford University, Stanford, CA USA

2004 Guest Instructor, Immunology, Stanford University, Stanford, CA USA

2008 Guest Instructor, Exploring the Tumor microenvironment, Postgraduate course, ISREC, Lausanne University's Biochemistry and Biology Departments, and the Lausanne Branch of the Ludwig Institute, Lausanne Switzerland. Course Organizers, Ivan Stamenkovic and Michel Aguet

PUBLIC SERVICE:

1990 Lecturer, Science Academy Of Whittier, Summer Institute. Whittier College, Whittier, CA


1993 Lecturer, Joslyn Community Center. Claremont, CA.

1994 Provided elementary educators with science-related supplies (photos, slides, fixed tissue samples).

1995 Co-Coordinator Hormone Research Institute, 'Take Our Daughters To Work Day', Univ. of Calif., San Francisco


2006 Interviewed for “Expert Commentary” by BreastLink.org, on article “Association Between Circulating White Blood Cell Count and Cancer Mortality,” Archives of Internal Medicine, January 23, 2006; 166:188-194.

http://www.breastlink.org/index.php?module=announce&ANN_user_op=view&ANN_id=208

2007 UCSF Research Perspectives 2007 – Inflammation as Cause and Consequences of Disease, Media
## IX. TEACHING AND MENTORING

### Formal Scheduled Classes for OHSU Students:

<table>
<thead>
<tr>
<th>Qtr</th>
<th>Academic Yr</th>
<th>Course No. &amp; Title</th>
<th>Teaching Contribution</th>
<th>Units</th>
<th>Class Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>2011/12</td>
<td>CELL 616; Advanced Topics in Cancer Biology</td>
<td>Lecture; <em>Tumor Microenvironment</em></td>
<td>3</td>
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</tbody>
</table>

### Formal Scheduled Classes for UCSF Students:

<table>
<thead>
<tr>
<th>Qtr</th>
<th>Academic Yr</th>
<th>Course No. &amp; Title</th>
<th>Teaching Contribution</th>
<th>Units</th>
<th>Class Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>1997/98</td>
<td>IDS 100; Histology Laboratory</td>
<td>Neoplastic Skin Histopathology; Laboratory lecture &amp; instruction</td>
<td>10</td>
<td>150</td>
</tr>
<tr>
<td>W</td>
<td>1998/99</td>
<td>IDS 100; Histology Laboratory</td>
<td>Neoplastic Skin Histopathology; Laboratory lecture &amp; instruction</td>
<td>10</td>
<td>150</td>
</tr>
<tr>
<td>W</td>
<td>1999/00</td>
<td>IDS 100; Histology Laboratory</td>
<td>Neoplastic Skin Histopathology; Laboratory lecture &amp; instruction</td>
<td>10</td>
<td>150</td>
</tr>
<tr>
<td>S</td>
<td>1999/00</td>
<td>BMS 297A; Molecular Biology &amp; Pathology of Neoplasia</td>
<td>Animal Models of Cancer Laboratory; Laboratory lecture &amp; instruction</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>S</td>
<td>2000/01</td>
<td>BMS 297A; Molecular Biology &amp; Pathology of Neoplasia</td>
<td>Animal Models of Cancer Laboratory; Laboratory lecture &amp; instruction</td>
<td>3</td>
<td>15</td>
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<tr>
<td>W</td>
<td>2000/01</td>
<td>BMS 225; Tissue and Organ Biology</td>
<td>Lecture and laboratory instruction</td>
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<tr>
<td>S</td>
<td>2000/01</td>
<td>BMS 260; Cell Biology</td>
<td>Discussion group leader</td>
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<tr>
<td>F/W</td>
<td>2001/02</td>
<td>IDS 101; Prologue</td>
<td>Laboratory Instructor</td>
<td>9</td>
<td>30</td>
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<tr>
<td>W</td>
<td>2001/02</td>
<td>BMS 225; Tissue and Organ Biology</td>
<td>Lecture and laboratory instruction</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>W</td>
<td>2001/02</td>
<td>IDS 103; Cancer Block</td>
<td>Invasion &amp; Metastasis; Lecturer</td>
<td>7</td>
<td>150</td>
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<tr>
<td>S</td>
<td>2001/02</td>
<td>BMS 260; Cell Biology</td>
<td>Discussion group leader</td>
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<td>7</td>
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<tr>
<td>F</td>
<td>2002/03</td>
<td>BMS 260; Cell Biology</td>
<td>Discussion group leader</td>
<td>1</td>
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<tr>
<td>W</td>
<td>2002/03</td>
<td>IDS 103; Cancer Block</td>
<td>Invasion &amp; Metastasis; Lecturer</td>
<td>7</td>
<td>150</td>
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<tr>
<td>F/W</td>
<td>2002/03</td>
<td>IDS 101; Prologue</td>
<td>Laboratory Instructor</td>
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<td>30</td>
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<tr>
<td>F</td>
<td>2003/04</td>
<td>BMS 260; Cell Biology</td>
<td>Discussion group leader</td>
<td>1</td>
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<tr>
<td>S</td>
<td>2003/04</td>
<td>BMS 225B, Tissue and Organ Biology</td>
<td>Lecturer and Laboratory Instructor</td>
<td>1.5 - 5</td>
<td>tbd</td>
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<tr>
<td>W</td>
<td>2003/04</td>
<td>Biochem 297; Molecular Biology &amp; Pathology of Neoplasia</td>
<td>Angiogenesis: Lecturer</td>
<td>3</td>
<td>30</td>
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<tr>
<td>W</td>
<td>2003/04</td>
<td>BMS 297A Molecular Biology &amp; Pathology of Neoplasia Laboratory</td>
<td>Lecturer and Laboratory Instructor, <em>Animal Models of Neoplasia</em></td>
<td>1</td>
<td>10</td>
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<tr>
<td>S</td>
<td>2003/04</td>
<td>BMS 225B; Tissue &amp; Organ Biology</td>
<td>Lecturer: Cancer I &amp; Cancer II</td>
<td>1.5 - 5</td>
<td>16</td>
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<tr>
<td>F</td>
<td>2004/05</td>
<td>BMS 260; Cell Biology</td>
<td>Discussion group leader</td>
<td>1</td>
<td>6</td>
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<tr>
<td>F</td>
<td>2005/06</td>
<td>BMS 260; Cell Biology</td>
<td>Discussion group leader</td>
<td>1</td>
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<tr>
<td>W</td>
<td>2006/07</td>
<td>Biochem 297; Molecular Biology &amp; Pathology of Neoplasia</td>
<td>Inflammation and Cancer: Lecturer</td>
<td>3</td>
<td>30</td>
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<td>W</td>
<td>2008/09</td>
<td>BMS230; Cellular &amp; Molecular Biology of</td>
<td>Course Co-Director</td>
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### Postgraduate and Other Courses:

<table>
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<tr>
<th>Year</th>
<th>Course Details</th>
<th>Institution</th>
<th>Lecture Details</th>
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<tbody>
<tr>
<td>1989</td>
<td>M204, Biochemistry Lab</td>
<td>Univ. of Calif., Los Angeles</td>
<td>Student Teaching Assistant for quarter long course (100 medical students)</td>
</tr>
<tr>
<td>1989</td>
<td>Biology 250, Human Heredity</td>
<td>Dept. of Biology, Whittier College, Whittier CA</td>
<td>Organized and taught entire lecture-based course (30 undergraduate students)</td>
</tr>
<tr>
<td>1990</td>
<td>Biology 350 &amp; 350L, Molecular Genetics</td>
<td>Dept. of Biology, Whittier College, Whittier CA</td>
<td>Organized and taught entire lecture and laboratory course (16 undergraduate students)</td>
</tr>
<tr>
<td>1990</td>
<td>M204, Biochemistry Lab</td>
<td>Univ. of Calif., Los Angeles</td>
<td>Student Teaching Assistant for quarter long course (100 medical students)</td>
</tr>
<tr>
<td>1990</td>
<td>Biology 250, Human Heredity</td>
<td>Dept. of Biology, Whittier College, Whittier CA</td>
<td>Organized and taught entire lecture-based course (30 undergraduate students)</td>
</tr>
<tr>
<td>1992</td>
<td>Biology 350 &amp; 350L, Molecular Genetics</td>
<td>Dept. of Biology, Whittier College, Whittier CA</td>
<td>Organized and taught entire lecture and laboratory course (16 undergraduate students)</td>
</tr>
<tr>
<td>2003</td>
<td>Graduate Oncology</td>
<td>University of Missouri, Columbia, MS, USA</td>
<td>Invited Guest Lecturer: Lecture syllabus &amp; delivered 2-hr lecture for course (15 students, graduate, medical &amp; postgraduate fellows)</td>
</tr>
<tr>
<td>2003</td>
<td>Graduate Program in Cancer Biology</td>
<td>Stanford Univ., Stanford, CA USA</td>
<td>Invited Guest Lecturer: Delivered 1-hr lecture to graduate students in Cancer Biology Graduate program</td>
</tr>
<tr>
<td>2004</td>
<td>Graduate Program in Immunology</td>
<td>Stanford Univ., Stanford, CA USA</td>
<td>Invited Guest Lecturer: Delivered 1-hr lecture to graduate students in Immunology Graduate program</td>
</tr>
<tr>
<td>2005</td>
<td>UCSF Dermatology residents' Basic Science Seminar Series</td>
<td>Invited Guest Lecturer: Delivered 1-hr lecture to UCSF Dermatology Residents (11 M.D. and M.D., Ph.D. Residents)</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>ISREC, Lausanne University's Biochemistry and Biology Dept, and Lausanne Branch of the Ludwig Institute</td>
<td>Guest Instructor: Exploring the Tumor microenvironment, postgraduate course. (20 PhD students, 3 hours of instruction)</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>OOA Course: Tumor Microenvironment</td>
<td>The Netherlands Cancer Institute</td>
<td>Guest Faculty: (4.5 hours of instruction, 25 PhD students)</td>
</tr>
<tr>
<td>2010</td>
<td>25th Annual Harvard Tumor Course: Critical Issues In Tumor Microenvironment, Angiogenesis &amp; Metastasis: From Bench to Bedside &amp; Back</td>
<td>Faculty member: (2 hours of instruction, 100 students)</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>Eppeley Institute for Research in Cancer</td>
<td>Univ. of Nebraska Medical Center. Short Course in Cancer Biology: Metastasis and the Tumor Microenvironment</td>
<td>Faculty member: (3 hours of instruction, 166 students)</td>
</tr>
<tr>
<td>2010</td>
<td>San Francisco State University, Dept of Biology Seminar Series</td>
<td>Guest Faculty: (1 1/2 hour of instruction, 75 students)</td>
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<tr>
<td>2011</td>
<td>26th Annual Harvard Tumor Course: Critical Issues In Tumor Microenvironment, Angiogenesis &amp; Metastasis</td>
<td>Faculty member: (2 hours of instruction, 100 students)</td>
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</table>
High School and Undergraduate Students Supervised or Mentored:

<table>
<thead>
<tr>
<th>Dates</th>
<th>Name</th>
<th>Program or School</th>
<th>Faculty Role</th>
<th>Current position</th>
</tr>
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<tbody>
<tr>
<td>1998</td>
<td>Christopher Tinkle</td>
<td>Undergraduate, Univ. of Texas, Austin, TX, USA</td>
<td>Summer Research Training Program Supervisor</td>
<td>Ph.D. awarded 2008; M.D. awarded 2010; Resident, Rad/Onc, UCSF</td>
</tr>
<tr>
<td>2000</td>
<td>Adam Zucker</td>
<td>Undergraduate, Oberlin College, Ohio USA</td>
<td>Supervised Summer work</td>
<td>unknown</td>
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<tr>
<td>2000</td>
<td>Ashkan Hirari</td>
<td>Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA</td>
<td>Supervised Summer work</td>
<td>unknown</td>
</tr>
<tr>
<td>2001</td>
<td>Jason Reuter</td>
<td>Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA</td>
<td>Supervised Summer work</td>
<td>unknown</td>
</tr>
<tr>
<td>2002</td>
<td>Destinee Cooper</td>
<td>Undergraduate, Univ. of Calif., Davis USA</td>
<td>Summer Research Training Supervisor</td>
<td>unknown</td>
</tr>
<tr>
<td>2006</td>
<td>Sunum Mobin</td>
<td>UCSF Science &amp; Health Education Partnership: High School Intern Program</td>
<td>Summer Research Training Supervisor</td>
<td>unknown</td>
</tr>
<tr>
<td>2008-2009</td>
<td>Julia Lam</td>
<td>Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA</td>
<td>Independent study (199), Mentor</td>
<td>B.S. awarded 2009</td>
</tr>
<tr>
<td>2010</td>
<td>Scott Keil</td>
<td>Undergraduate, The University of Glasgow, Scotland</td>
<td>Summer Research Training Supervisor</td>
<td>The University of Glasgow, Scotland</td>
</tr>
<tr>
<td>2010-2011</td>
<td>Heather Chen</td>
<td>Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA</td>
<td>Summer Research Training Supervisor</td>
<td>Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA</td>
</tr>
<tr>
<td>2010</td>
<td>Amy Desalazar</td>
<td>Cupertino High School Cupertino, CA USA</td>
<td>Summer Research Training Supervisor</td>
<td>Cupertino High School</td>
</tr>
<tr>
<td>2010-2011</td>
<td>Nikhil Wadhwani</td>
<td>Undergraduate, Sarah Lawrence College, Bronxville, NY USA</td>
<td>Summer Research Training Supervisor</td>
<td>Sarah Lawrence College, Bronxville, NY</td>
</tr>
<tr>
<td>2010</td>
<td>Sharfa Junaid</td>
<td>Undergraduate, Univ. of Calif., San Diego, San Diego CA USA</td>
<td>Summer Research Training Supervisor</td>
<td>Undergraduate, Univ. of Calif., San Diego, San Diego CA USA</td>
</tr>
<tr>
<td>2010-2011</td>
<td>Jon Lau</td>
<td>University of Nevada, Reno, Reno, Nevada USA</td>
<td>Summer Research Training Supervisor</td>
<td>University of Nevada, Reno, Reno, Nevada USA</td>
</tr>
<tr>
<td>2011</td>
<td>Kara Wang</td>
<td>Undergraduate, Pomona College, Claremont, CA USA</td>
<td>Summer Research Training Supervisor</td>
<td>Undergraduate, Pomona College, Claremont, CA USA</td>
</tr>
<tr>
<td>2011</td>
<td>Graham Litchman</td>
<td>Undergraduate, San Francisco State Univ, San Francisco CA USA</td>
<td>Summer Research Training Supervisor</td>
<td>Masters student, Univ. of Nevada, Reno</td>
</tr>
<tr>
<td>2011</td>
<td>Jessica Wignall</td>
<td>Smith College, Northampton, MA USA</td>
<td>Summer Research Training Supervisor</td>
<td>Smith College, Northampton, MA USA</td>
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Predoctoral Students Supervised or Mentored:

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<tr>
<th>Dates</th>
<th>Name</th>
<th>Program or School</th>
<th>Faculty Role</th>
<th>Current position</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>Jin-Sae Rhee</td>
<td>UCSF MSTP/BMS, graduate student</td>
<td>Rotation Supervisor</td>
<td>PhD awarded 2003, M.D. awarded 2005</td>
</tr>
<tr>
<td>2000 - 2003</td>
<td>Jin-Sae Rhee</td>
<td>UCSF M.D., Ph.D.</td>
<td>Ph.D. supervisor</td>
<td>Pediatrician, Private Sector</td>
</tr>
<tr>
<td>Year</td>
<td>Student Name</td>
<td>Institution/Program</td>
<td>Role/Supervisor</td>
<td>Degree Awarded</td>
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<tr>
<td>2000</td>
<td>Maria Christophorou</td>
<td>UCSF BMS, graduate student</td>
<td>Faculty coach, BMS 297</td>
<td>Ph.D. awarded 2006</td>
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<tr>
<td>2001</td>
<td>Leslie Chu</td>
<td>UCSF BMS, graduate student</td>
<td>Rotation Supervisor</td>
<td>Ph.D. awarded 2005</td>
</tr>
<tr>
<td>2001</td>
<td>Rayna Takaki</td>
<td>UCSF BMS, graduate student</td>
<td>Rotation Supervisor</td>
<td>Ph.D. awarded 2006</td>
</tr>
<tr>
<td>2002</td>
<td>Lucy Lebedeva</td>
<td>UCSF PIBS, graduate student</td>
<td>Faculty coach, BMS 297</td>
<td>Ph.D. awarded 2005</td>
</tr>
<tr>
<td>2002</td>
<td>Leslie Chu</td>
<td>UCSF BMS, graduate student</td>
<td>Ph.D. Orals committee</td>
<td>Ph.D. awarded 2005</td>
</tr>
<tr>
<td>2002</td>
<td>Andre Whitkin</td>
<td>MSTP student, Cornell University USA</td>
<td>Supervised Summer work</td>
<td>unknown</td>
</tr>
<tr>
<td>2002</td>
<td>Karin de Visser</td>
<td>The Netherlands Cancer Institute, The Netherlands</td>
<td>Ph.D. Thesis Reading Committee</td>
<td>Ph.D. awarded 2002</td>
</tr>
<tr>
<td>2003</td>
<td>Cathy Collins</td>
<td>UCSF MSTP student</td>
<td>MSTP Advisor</td>
<td>PhD awarded 2009; M.D. awarded 2011</td>
</tr>
<tr>
<td>2004</td>
<td>Eric Tamm</td>
<td>University of British Columbia, Canada</td>
<td>Doctoral Dissertation External Examiner</td>
<td>Ph.D. awarded 2004</td>
</tr>
<tr>
<td>2004</td>
<td>Annie Hsieh</td>
<td>University of Södertörn, Sweden</td>
<td>Masters Thesis Supervisor</td>
<td>unknown</td>
</tr>
<tr>
<td>2005</td>
<td>Geoff Benton</td>
<td>UCSF TETRAD/PIBS, graduate student</td>
<td>Ph.D. Orals committee</td>
<td>Ph.D. awarded 2011</td>
</tr>
<tr>
<td>2006</td>
<td>Morgan Truitt</td>
<td>UCSF BMS, graduate student</td>
<td>Rotation Supervisor</td>
<td>UCSF BMS PhD graduate student</td>
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<tr>
<td>2006</td>
<td>Danielle Shin</td>
<td>UCSF MSTP student</td>
<td>Rotation Supervisor</td>
<td>Ph.D. awarded 2011</td>
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<tr>
<td>2006-08</td>
<td>Celeste Rivera</td>
<td>SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student</td>
<td>M.S. research advisor</td>
<td>M.S. awarded 2010; Research Technician, EPFL Lausanne Switzerland</td>
</tr>
<tr>
<td>2007-09</td>
<td>Leslie Vasquez</td>
<td>SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student</td>
<td>M.S. research advisor</td>
<td>M.S. awarded 2009; PharmD student, UCSF</td>
</tr>
<tr>
<td>2008</td>
<td>Ashley Martin</td>
<td>UCSF BMS, graduate student</td>
<td>Rotation Supervisor</td>
<td>UCSF BMS PhD graduate student</td>
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<tr>
<td>2009</td>
<td>Kay Wiebrands</td>
<td>Master’s Student Utrecht University, the Netherlands</td>
<td>Masters Thesis Internship Supervisor</td>
<td>M.S. awarded 2009</td>
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<tr>
<td>2009-10</td>
<td>David Tawfik</td>
<td>Medical Student III, UCSF</td>
<td>MSIII break year. Dean’s Quarterly Research Fellowship; PACCTR Fall Quarter Fellowship;</td>
<td>M.D., awarded 2011; Resident in Internal Medicine, UCLA Harbor Hospital</td>
</tr>
<tr>
<td>2009-present</td>
<td>Renee Vanderlaan</td>
<td>UCSF BMS, graduate student</td>
<td>Chair: Thesis Committee</td>
<td>UCSF BMS graduate student, Lab of Matthias Hebrook, Ph.D.</td>
</tr>
<tr>
<td>2009-11</td>
<td>A. Preethi Ganessan, M.D., PhD.</td>
<td>Ph.D. Graduate Student (Cancer Research UK, Univ of Southampton)</td>
<td>Ph.D. supervisor</td>
<td>Pediatric Hematology Resident, Univ of Southampton, UK</td>
</tr>
<tr>
<td>2010-present</td>
<td>Lucia Cottone</td>
<td>Ph.D. graduate student (San Raffaele Institute, Milan Italy)</td>
<td>Ph.D. supervisor and 2nd supervisor del candidato</td>
<td>Ph.D. student, San Raffaele Institute, Milan Italy</td>
</tr>
<tr>
<td>2011-present</td>
<td>Conny Hainer</td>
<td>Master’s student, Technical University of Berlin, Berlin, Germany</td>
<td>MS supervisor</td>
<td>Master’s student, Technical University of Berlin, Berlin, Germany</td>
</tr>
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### Postdoctoral Fellows and Residents Directly Supervised or Mentored

<table>
<thead>
<tr>
<th>Dates</th>
<th>Name</th>
<th>Position &amp; Funding</th>
<th>Faculty Role</th>
<th>Current Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000-2002</td>
<td>Leon Van Kempen, Ph.D.</td>
<td>Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship</td>
<td>Research Supervisor</td>
<td>Assoc. Prof., Univ. of Nijmegen, Dept. of Pathology, The Netherlands</td>
</tr>
<tr>
<td>2002-2005</td>
<td>Robert Diaz, Ph.D.</td>
<td>Post-Doc Researcher; Coussens R01</td>
<td>Research Supervisor</td>
<td>Scientist, Roche Pharmaceuticals</td>
</tr>
<tr>
<td>2003-2005</td>
<td>Stephen Robinson, Ph.D.</td>
<td>Post-Doc Researcher; Coussens R01</td>
<td>Research Supervisor</td>
<td>Private sector, United Kingdom</td>
</tr>
<tr>
<td>2003-2004</td>
<td>H. Jennifer Shen, Ph.D.</td>
<td>Post-Doc Researcher; Coussens R01</td>
<td>Research Supervisor</td>
<td>unknown</td>
</tr>
<tr>
<td>2005-2010</td>
<td>David DeNardo, Ph.D.</td>
<td>Post-Doc Researcher; 1) NGA: 5T32CA09043 Pl: BISHOP; Molec. Analysis of Tumor Viruses; 2) Am Cancer Society Fellowship 2007-2010</td>
<td>Research Supervisor</td>
<td>Assistant Professor, Molecular Oncology and Immunology, Washington University, St Louis, St Louis MS USA</td>
</tr>
<tr>
<td>2005-2007</td>
<td>Nor Eddine Sounni, Ph.D.</td>
<td>Post-Doc Researcher; Coussens R01</td>
<td>Research Supervisor</td>
<td>Research Scientist, Univ. of Liege, Belgium</td>
</tr>
<tr>
<td>2006-2007</td>
<td>Tingting Tan, M.D., Ph.D.</td>
<td>Post-Doc Researcher; Coussens R01</td>
<td>Research Supervisor</td>
<td>Hem/Onc Fellow, Fox Chase Cancer Center, Phil., PA</td>
</tr>
<tr>
<td>2006-2010</td>
<td>Magnus Johansson, Ph.D.</td>
<td>Post-Doc Researcher; Swedish Cancer Society Fellowship 2006-08</td>
<td>Research Supervisor</td>
<td>MBA candidate, Stanford Univ., Palo Alto CA</td>
</tr>
<tr>
<td>2007-2010</td>
<td>Pauline Andreu, Ph.D.</td>
<td>Post-Doc Researcher; Cancer Research Institute Postdoctoral Fellowship 2008-2011</td>
<td>Research Supervisor</td>
<td>Research Funding Agency in Private Sector, France</td>
</tr>
<tr>
<td>2008-present</td>
<td>Brian Ruffell, Ph.D.</td>
<td>Post-Doc Researcher; Dept of Defense Postdoctoral fellowship 2009-2012</td>
<td>Research Supervisor</td>
<td>Post-Doctoral fellow, Coussens Lab, UCSF, OHSU</td>
</tr>
<tr>
<td>2009-2011</td>
<td>Stephen Shiao, M.D., Ph.D.</td>
<td>UCSF Radiation Oncology Resident</td>
<td>Research Supervisor</td>
<td>UCSF Radiation Oncology, Chief Resident</td>
</tr>
<tr>
<td>2010-2012</td>
<td>Collin Blakeley, M.D.</td>
<td>UCSF Hematology</td>
<td>Research Supervisor</td>
<td>UCSF Hematology</td>
</tr>
</tbody>
</table>
### FACULTY MENTORING

<table>
<thead>
<tr>
<th>Dates</th>
<th>Name</th>
<th>Position while Mentored</th>
<th>Mentoring Role</th>
<th>Current Position</th>
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<tr>
<td>2001 – 2004</td>
<td>Ernst Lengyel, M.D., Ph.D.</td>
<td>Asst. Adjunct Professor</td>
<td>Research Mentor</td>
<td>Assoc. Prof., Dept. Gyn. &amp; Oncology, Univ. of Chicago, Chicago, IL</td>
</tr>
<tr>
<td>2002 – 2007</td>
<td>Darya Soto, M.D.</td>
<td>Asst. Adjunct Professor, K08 Research Mentor</td>
<td>Private Practice</td>
<td>Private Practice, Burlingame, CA</td>
</tr>
<tr>
<td>2005 – 2007</td>
<td>Runi Chattopadhyay, M.D.</td>
<td>Clinical Instructor and Clinical Fellow</td>
<td>Basic Science Mentor, K12</td>
<td>Private Practice, San Francisco, CA</td>
</tr>
<tr>
<td>2006 – 2011</td>
<td>Limin Liu, Ph.D.</td>
<td>Assistant Professor</td>
<td>Member, Mentoring Committee</td>
<td>Dept. of Microbiology &amp; Immunology, Sandler Center for Basic Research in Asthma, UCSF</td>
</tr>
<tr>
<td>2010 - 2011</td>
<td>Jaynata Debnath, M.D., Ph.D.</td>
<td>Assistant Professor</td>
<td>Faculty Mentor</td>
<td>Associate Professor, Dept. of Pathology, UCSF</td>
</tr>
</tbody>
</table>

Sabbatical Visitors:
1999 - 2000  Yves DeClerck, M.D.  Professor, Univ. of Southern Calif. & Children’s Hospital of Los Angeles

### SUMMARY OF TEACHING HOURS

<table>
<thead>
<tr>
<th>Academic Year</th>
<th>Teaching/Mentoring Summary</th>
<th>Hours</th>
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<tr>
<td>1997/98</td>
<td>Total hours of teaching/mentoring:</td>
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<td>Formal class or course teaching hours:</td>
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<tr>
<td></td>
<td>Informal teaching hours including prep time:</td>
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<td>Mentoring hours:</td>
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<td>1998/99</td>
<td>Total hours of teaching/mentoring:</td>
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<td>Informal teaching hours including prep time:</td>
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<td></td>
<td>Mentoring hours:</td>
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<td>1999/00</td>
<td>Total hours of teaching/mentoring:</td>
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<td>Informal teaching hours including prep time:</td>
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<td>Mentoring hours:</td>
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<td>2000/01</td>
<td>Total hours of teaching/mentoring:</td>
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<td>Formal class or course teaching hours:</td>
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<td></td>
<td>Informal teaching hours including prep time:</td>
<td>9</td>
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<tr>
<td></td>
<td>Mentoring hours:</td>
<td>105</td>
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</table>
X. RESEARCH AND CREATIVE ACTIVITIES

RESEARCH AWARDS AND GRANTS:
CURRENT

BCRF (PI: Rugo) 10/01/12-09/30/13
Source: Breast Cancer Research Foundation directs/yr

Title: Cellular mechanisms of resistance to antiangiogenic and the impact of immune therapy, and molecular characterization of circulating tumor cells (CTCs) in patients with advanced breast cancer
The major goal of this study is to 1) evaluate significance of immune cells in primary mammary carcinomas and lung metastases in the context of response and relapse/resistance to anti-angiogenic and/or immune modulating therapy, 2) Investigate the impact of inhibiting immune cell infiltration alone or in combination with antiangiogenic agents or chemotherapy on the growth of primary tumors and metastases, and 3) characterize the expression of a specific set of genes and gene copy number in circulating tumor cells (CTCs) in patients with ABC, including markers of response and resistance to specific types of therapy.
Role: subcontract PI (Aim 1 and 2)
Source: NIH/NCI  directs/yr
Title: Leukocyte Biomarkers for Predicting Human Breast Cancer Outcomes
The major goal of this study is to identify myeloid- and lymphoid-based biomarkers representing either functional mediators of immune cell phenotype or instead reflecting leukocyte composition, and which of these in turn represent predictive variables for predicting breast cancer response to CTX +/- macrophage-depletion therapy
Role: multi P.I.

W81XWH-10-BCRP-EOHS-EXP (PI: Coussens, LM) 09/30/11 – 09/29/16
Era of Hope Scholar Expansion Award
Source: DOD/U.S. Army Medical Research & Materiel Command  directs/yr
Title: Modulating Immune Response to Improve Therapy for Breast Cancer
The major goal of this study is to test the hypothesis that the immune microenvironment in breast cancer can be effectively manipulated therapeutically to limit breast cancer recurrence and extend overall survival
Role: P.I.

R01 CA155331 (PI: Coussens, LM) 05/01/11 – 03/31/16
Source: NIH/NCI  directs/yr
Title: Regulating the Immune Microenvironment in Breast Cancer
The major goal of this study is to evaluate the efficacy of Th2-blockade as a therapy for reprogramming pro-tumor immune cells in mouse models of mammary carcinogenesis.
Role: P.I.

KG111084 (multiPI: Coussens, LM; Hwang S, Rugo H) 07/01/11 – 06/31/16
Source: Komen Foundation, PROMISE Grant  directs/yr
Title: Enhancing Efficacy of Chemotherapy in Triple Negative/Basal-like Breast Cancer by Targeting Macrophages.
The major goal of this study is to test the hypothesis that that macrophages in triple negative (TN)/basal-like breast cancer potentiate late-stage disease progression and limit long-term survival, and that by either minimizing tumor associated macrophages (TAM) recruitment or reprogramming TAM bioactivity, outcomes for patients with TN/basal-like breast cancer will significantly improve.
Role: multi P.I.

KG110560 (multiPI: Hwang S; Coussens, LM) 07/01/11 – 06/31/14
Source: Komen Foundation, IDEA Award  directs/yr
Title: Immune and Collagen Basis of Breast Cancer Risk
The major goal of this study is to establish whether immune and collagen status is correlated with density-associated breast cancer risk.
Role: multi P.I.

R01CA140943 (multiPI: Coussens, Boudreau, Daldrup-Link) 07/01/09 – 06/30/13
Source: NIH/NCI  directs/yr
Title: Improved Imaging and Drug Delivery Using Novel Approaches to Regulate Tissue Perfusion
The major goal of this project is to examine how short-term inhibition of ALK5 in vivo alters hemodynamics and tissue perfusion in mouse models of cancer.
Role: multi P.I.

RO1 CA130980 (PI: Coussens, LM) 07/01/08-06/30/13
Source: NIH/NCI  directs/yr
Title: Regulation of Inflammation-Associated Epithelial Cancer Development
The goal of this study is to determine regulatory programs activating chronic inflammation during squamous carcinogenesis
Role: P.I.

RO1 CA132566 (multiPI: Coussens, LM; Jablons DM) 05/01/08-04/30/13
Source: NIH/NCI  directs/yr
Title: Inflammation and Lung Carcinogenesis
The goal of this study is to determine how inflammation and Wnt signaling regulate stem cell niche
autonomy during lung carcinogenesis  
**Role:** multi P.I.

<table>
<thead>
<tr>
<th>Grant ID</th>
<th>Title</th>
<th>Source</th>
<th>Duration</th>
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</thead>
<tbody>
<tr>
<td>1S10OD010348-01</td>
<td>Vevo 2100 Ultrasound System</td>
<td>NIH directs/yr</td>
<td>06/01/2012 – 05/31/2013</td>
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<tr>
<td>PREVIOUS</td>
<td>Title: Regulation of junB Gene Expression by TGF-Beta</td>
<td>NIH/UCLA directs/yr</td>
<td>07/01/89 – 06/30/92</td>
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<tr>
<td></td>
<td>Competitive Pre-Doctoral award to study transcription factor junB.</td>
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<td></td>
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<tr>
<td></td>
<td>Title: Effects of E1A on TGF-Beta-inducible junB Expression</td>
<td>NIH/UCSF directs/yr 1-3</td>
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<tr>
<td></td>
<td>Competitive Pre-Doctoral award to study transcription factor junB.</td>
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<tr>
<td></td>
<td>Title: Molecular Analysis of Tumor Viruses</td>
<td>NIH/UCSF directs/yr 1-3</td>
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<tr>
<td></td>
<td>Post-Doctoral fellowship to study mouse model of epithelial carcinogenesis.</td>
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<tr>
<td></td>
<td>Title: Metalloproteinases and Malignant Progression of Squamous Epithelium in K14-HPV16 Transgenic Mice</td>
<td>NIH/UCSF directs/yr 1-2</td>
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<tr>
<td></td>
<td>Competitive Post-Doctoral fellowship to study proteases and tumor development</td>
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<td>Title: Proteases in Cancer Biology and Drug Development</td>
<td>NIH/NCI directs/yr 1-5</td>
<td>06/10/97 – 06/30/03</td>
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<tr>
<td></td>
<td>Project 3 – Proteases in Models of Tumor Initiation/Progression</td>
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<tr>
<td></td>
<td>The major goal of this project is to study the role of proteases in cancer biology.</td>
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<td>Core C – Transgenic Animal Models</td>
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<td></td>
<td>Director (year 4 and 5)</td>
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<td></td>
<td>The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.</td>
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<tr>
<td></td>
<td>Title: Proteases and Genomics in a Mouse Model of Epithelial Cancer</td>
<td>American Cancer Society</td>
<td>07/01/99-06/30/00</td>
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<tr>
<td></td>
<td>principal Investigator</td>
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<tr>
<td></td>
<td>Pilot project tested role of proteinases as effectors of genomic instability.</td>
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<td></td>
<td>Title: Epithelial Neoplastic Progression and Degradation of Type I Collagen</td>
<td>UCSF Comprehensive Cancer Center, Intramural</td>
<td>02/01/00-01/31/01</td>
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<tr>
<td></td>
<td>Principal Investigator</td>
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<tr>
<td></td>
<td>Pilot project assessed functional significance of type I collagen metabolism during epithelial carcinogenesis.</td>
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<tr>
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<td>Title: Research Evaluation &amp; Allocation Committee</td>
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<td>07/01/00-06/30/01</td>
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<td>Principal Investigator</td>
<td></td>
<td></td>
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<tr>
<td>Source:</td>
<td>UCSF Academic Senate</td>
<td>directs/yr 1</td>
<td></td>
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<tr>
<td>Title:</td>
<td>Role of Gelatinase B in Maintenance of Genomic Instability</td>
<td>directs/yr 1</td>
<td></td>
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<tr>
<td>Role:</td>
<td>Principal Investigator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pilot project tested the role of MMP9 as an indirect regulator of genomic instability.</td>
<td></td>
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</table>

| Title: | Regulation of Intracellular Signaling Pathways by Gelatinase B/MMP-9 |
| Role: | Principal Investigator |
| Pilot project to study signal transduction pathways regulated by MMP-9. |

| Title: | Gelatinase B and Epithelial Cancer Development |
| Role: | Principal Investigator |
| Pilot project to study role of MMP9 during epithelial carcinogenesis. |

| Title: | Functional Role of MMP-2 During Epithelial Carcinogenesis |
| Role: | Principal Investigator |
| Pilot project to study role of MMP-2 during epithelial carcinogenesis. |

| Title: | Paracrine Regulation of Epithelial Carcinogenesis by MMP-9 |
| Role: | Principal Investigator |
| Pilot project to identify matrix molecules regulated by MMP-9. |

| Title: | Regulation of epithelial cancer by gelatinase B/MMP-9 |
| Role: | Principal Investigator |
| Pilot project to determine how MMP-9 regulates proliferation, VEGF bioavailability and angiogenesis during epithelial carcinogenesis. |

| Title: | Type I Collagen Remodeling and Mammary Carcinogenesis |
| Role: | Principal Investigator (Developmental Project) |
| The overall goal of this pilot project was to explore the role of collagen metabolism during mammary carcinogenesis. |

| Title: | Therapeutic Radionuclide Tumor-targeting Strategy for Breast Cancer |
| Role: | Co-Investigator |
| The specific aim of this project is to develop a radionuclide delivery molecule (RDM) that specifically targets cancer cells that express matrix-metalloproteinase-14 (MMP-14) on their surface and demonstrate delivery of radiolabeled RDM to MMP-14 expressing cells in vitro and in vivo. |
**R01 DK067678** (PI: Cher, M; Wayne State University)  07/01/03-06/30/06  
**Source**: NIH/NIDDK  directs/yr 1  
**Title**: *Proteases in Prostate Cancer Bone Metastasis*  directs/yr 1-4  
**Role**: Subcontract Principal Investigator  
The major goal of this subcontract is to assist with the planned experiments by providing mice (protease deficient) of defined genotype for proposed studies to analyze proteases during prostate metastasis to bone *in vivo*.

**Opportunity Award, Sandler Family** (PI: Coussens, LM; UCSF)  02/15/05 -02/14/07  
**Source**: UCSF Intramural  directs yr 1  
**Title**: *B Lymphocytes as Targets for Cancer Prevention*  total directs  
**Role**: Principal Investigator  
The major goal of this project is to investigate the efficacy of targeting B cells for chemoprevention.

**DAMD17-02-1-0693** (PI: Sloane, B; Wayne State University)  08/01/02-07/31/06  
**Source**: Department of Defense  directs/yr 1-4  
**Breast Cancer Center of Excellence**  directs/yr 1  
**Title**: *Validation of Proteases as Therapeutic Targets in Breast Cancer Functional Imaging of Protease Expression, Activity and Inhibition*  
**Role**: Subcontract Principal Investigator  directs/yr 1-4  
The goal of this program is to validate proteases as therapeutic targets in breast cancer by functional imaging of protease expression, activity and inhibition.

**R01 CA94168** (PI: Coussens, LM; UCSF)  04/01/02-06/31/07  
**Source**: NIH/NCI  directs/yr 1  
**Title**: *Regulation of Epithelial Cancer by MMP-9/gelatinase B*  directs/yr 1-5  
**Role**: Principal Investigator  
The goal of this project is to identify molecules that mediate proliferative and cellular pathways activated by MMP-9.

**U54 RR020843** (PI: Smith, J; Burnham Institute)  09/30/04-07/31/06  
**Source**: NIH/National Center for Research Resources  directs yr 1-5  
**Title**: Center on Proteolytic Pathways  
**Role**: Principal Investigator (Driving Biological Problem #1)  directs/yr  
**DBP#1 Proteolytic Pathways in Acute Vascular Response**

**P01 CA72006** (PI: Werb, Z; UCSF)  07/07/03 – 06/30/08  
**Source**: NIH/NCI  directs yr 6  
**Title**: *Proteases in Cancer Biology and Drug Development*  directs/yr 6-11  
**Project 3 - Proteases in Models of Tumor Initiation/Progression*  directs/yr 6  
**Role**: Co-Investigator, Project 3  directs/yr 6-11  
The major goal of this project is to study the role of proteases in cancer biology.  
**Core C - Transgenic Animal Models**  directs/yr 6  
**Role**: Director  directs/yr 6-11  
The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.

**R01 CA98075** (PI: Coussens, LM; UCSF)  07/01/03-06/30/09  
**Source**: NIH/NCI  directs/yr 1  
**Title**: *Microenvironmental Regulation of Tumor Progression*  directs/yr 1-5  
**Role**: Principal Investigator  
The overall goal of this grant is to determine the role of collagen metabolism on epithelial carcinogenesis.

**P50 CA58207** (van ’t Veer; UCSF)  08/01/92–11/30/12  
**Source**: NIH/NCI  Bay Area Breast Cancer SPORE  
**Career Development and Developmental Research Award** Multi Project PI: Weaver, Hwang, Coussens  
**Title**: Risk to Malignancy and Immune and Collagen Status
The goals of this project are to 1) Determine whether immune infiltrate and collagen heterogeneity exist between and within multiple regions of breast tissue, 2) Evaluate whether malignant progression is associated with a distinct immune infiltrate and if that is reflected by physical state of collagen, and 3) Determine relationship between immune infiltrate, collagen, radiographic density and clinical measures of cancer risk.

**Role:** Multi P.I.

**P50 CA58207** (van ’t Veer; UCSF) 08/01/92–11/30/12

**Source:** NIH/NCI (project expenses only)

**Title:** Bay Area Breast Cancer SPORE

Career Development and Developmental Research Award, Multi Project PI: Boudreau N; Coussens LM (5/1/10-04/30/11)

Title: Macrophage-Mediated Delivery of the Breast Tumor Suppressor HoxD10 via Autologous Transfer to Breast Tumors. The aims of this project are to 1) establish function and optimize introduction of the engineered HoxD10 protein into macrophages and/or monocytes; 2) visualization of modified monocyte/macrophage accumulation in mammary tumors in vivo and 3) analysis of the impact of monocyte/macrophage delivered HoxD10 on breast tumor growth, progression and metastasis in MMTV-PyMT mouse model of mammary carcinogenesis.

**Role:** Multi P.I.

**BC051640 Era of Hope Scholar Award** (PI: Coussens, LM; UCSF) 06/01/06 – 05/31/11

**Source:** DoD, U.S. Army Medical Research and Materiel Command

**Title:** Microenvironment Regulation of Mammary Carcinogenesis

The goal of this Scholar Award is to identify leukocytes and their proteases that modify breast carcinogenesis and to develop noninvasive imaging reagents targeting leukocytes to image inflammation.

**Role:** P.I.

**W81XWH-08-PRMRP-IIRA** (multiPI: Broaddus, C; Coussens, LM) 07/01/09 – 06/30/12

**Source:** DoD, U.S. Army Medical Research and Materiel Command

**Title:** Role of Macrophage-induced Inflammation in Mesothelioma

The goals of this project are 1) to determine the functional significance of macrophage phenotype in mesothelioma, 2) to determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro and 3) to define the functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

**Role:** multi P.I.

**XI. PEER-REVIEWED PUBLICATIONS**


Featured in:


• Nature Reviews Cancer (2011) 11:3056. ‘Bad company’, by N. McCarthy


Manuscripts Submitted or In Revision:

• Affara NI, Ruffell B, Johansson M, Fujikawa K, DeNardo DG, Chen HI, Wadhani N, Steinhoff M, Truitt M,
XII. PATENTS

   Title: Novel Indications for Transforming Growth Factor-Beta Regulators.
   Inventors: Lisa M. Coussens and Zena Werb
   Application published on August 28, 2008 as U.S. Patent Publication #2008-0206219-A1


XIII. NON-PEER REVIEWED PUBLICATIONS AND EDITORIALS:

Review Articles, Symposium Proceedings and Editorials


Book Chapters


Published Abstracts:


**XIV. RESEARCH PROGRAM:**

The Coussens lab focuses on the role of immune cells and their mediators as critical regulators of cancer (squamous cancer of the skin, non small cell lung cancer, mesothelioma, breast and pancreas cancer)
development. During the early development of cancer, many physiological processes occur in the vicinity of 'young tumor cells' that are similar to processes that occur during embryonic development and to healing of wounds in adult tissue, e.g., leukocyte recruitment and activation (inflammation), angiogenesis (development of new blood supply) and tissue remodeling. During tumor development; however, instead of initiating a 'healing' response, activated leukocytes provide growth-promoting factors that help tumors grow. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. To address these issues, we have taken several approaches to investigate mechanisms involved in: i. induction and maintenance of chronic inflammatory microenvironments in premalignant, malignant and metastatic tissues, ii. role of leukocyte in regulating tissue remodeling, angiogenesis, immune suppression and cancer development, and iii. development of novel non-invasive imaging reagents to monitor immune response in tissues/tumors. The long-term goal of this work is to translate basic observations made in the mouse, toward rational design of novel therapeutics whose aim will be to block and/or alter rate-limiting events critical for solid tumor growth, maintenance or recurrence in humans. Currently, we are actively utilizing transgenic mouse models of solid tumor development (non-small cell lung cancer, non-melanoma squamous and breast cancer, pancreatic adenocarcinoma, and mesothelioma) to reveal the functional roles of adaptive and innate leukocytes during tumor development, and to identify new targets for anti-cancer therapy. These experimental studies are conducted in parallel with evaluation of representative human cancer specimens to affirm that mechanisms revealed in the experimental setting represent fundamental parameters of multi-stage cancer development in humans.

XV. MOST SIGNIFICANT PUBLICATIONS


Role: This research was the first to demonstrate a protumor role for B cells and humoral immunity in solid tumor development. Using the K14-HPV16 mouse model of squamous carcinogenesis, we reported that adoptive transfer of B lymphocytes or serum from HPV16 mice into T and B cell-deficient/HPV16 mice reinstated necessary parameters for full malignancy, e.g., chronic inflammation, angiogenic vasculature, hyperproliferative epidermis. These findings support a model in which acquired immunity is essential for establishing chronic inflammatory states that promote de novo carcinogenesis. This research initiated a paradigm shift as it provided the first evidence linking humoral immunity and solid tumor development, thus revealing provocative new targets for anti-cancer therapy. This manuscript was the “featured article” in its issue of Cancer Cell, and was the subject of several invited review articles (Houghton et al., Cancer Cell 2005; Montavani, Nature, 2005), as well as being featured in 'Research Highlights' in Nature Reviews Cancer and Nature Reviews Immunology (Minton, 2005). 100% of the research supporting this manuscript was conceived and conducted in my laboratory. Dr. de Visser wrote drafts of the manuscript under Dr. Coussens’ direct supervision.


Role: Infiltration of T lymphocytes in human breast cancers has been recognized by pathologist for decades, however their functional role has been underdetermined. We utilized a transgenic mouse model of mammary carcinogenesis and demonstrated a tumor-promoting role for Treg-CD4+ T lymphocytes that elicit pro-tumor, as opposed to cytotoxic bioactivities of tumor-associated macrophages and enhancement of pro-metastatic epidermal growth factor receptor signaling programs in malignant mammary epithelial cells. This work revealed a novel pro-tumor regulatory program involving components of the acquired and cellular immune systems that effectively collaborate to promote pulmonary metastasis of mammary adenocarcinomas, and identified new cellular targets, namely CD4+ T effector cells and IL-4 for anti-cancer therapy. This manuscript appeared as the “featured article” in the August 4th 2009 issue of Cancer Cell, and was the subject of an invited “Preview” article (Pardoll, Cancer Cell 2009), and was featured in the research highlights section of Nature Reviews Cancer (McCarthy, 2009). In 2011, this manuscript was ranked as a “Top Cancer Papers” between 2008-2011, Nature Medicine, 17;262-265; Nature Medicine 17: 278-279; Nature Medicine, 17:295. 100% of the research supporting this manuscript was conceived and conducted in my laboratory. Dr. DeNardo wrote drafts of the manuscript under Dr. Coussens’ direct supervision.

**Role:** This work presented novel findings demonstrating functional significance of FcRγ receptors and humoral immunity as potentiators of squamous carcinogenesis. While myeloid cells and some T cell subsets have been implicated in neoplastic progression and cancer development, the tumor-promoting capabilities of B lymphocytes have remained unclear. Using the HPV16 transgenic mouse model of inflammation-associated squamous carcinogenesis, we previously reported that B and T cell-deficient HPV16 mice failed to progress beyond a benign hyperplastic state due to deficient activation of chronic inflammatory programs in early neoplastic skin (deVisser et al, *Cancer Cell* 2005). In Andreu et al., we revealed that B cells potentiate squamous carcinogenesis via humoral immunity, where immunoglobulins (Ig) in the form of immune complexes (IC) activate FcRγ receptor-mediated signaling pathways on resident and recruited myeloid cells. Activation of these programs on resident mast cells initially leads to peripheral blood leukocyte recruitment into neoplastic skin and activation of angiogenic vasculature. The subsequent chronic inflammation that ensues in part maintains angiogenic support but also supports neoplastic keratinocyte hyperproliferation and progression to dysplastic/carcinoma in situ states and subsequent malignant conversion and carcinoma development. These novel findings have clinical significance in that they imply that anti-cancer strategies targeting B cells, Ig or FcRγ may harbor therapeutic efficacy in limiting risk of malignant conversion in patients suffering from chronic inflammatory diseases, or in patients harboring premalignant lesions whose molecular and/or immunologic characteristics favor tumor development. This manuscript appeared as the “featured article” in the March 2010 issue of *Cancer Cell*, and was the subject of an invited “Preview” article (Mantovani, *Cancer Cell* 2010), and was featured in the research highlights section of *Nature Reviews Immunology* (Byrd, 2010). In 2011, this manuscript was *Ranked as a “Top Cancer Papers”* between 2008-2011 by Nature Medicine (17:262-265; 17: 278-279), and subsequently reviewed as a ‘News and Views in *Nature Medicine* (2011), 17(3) 285-286. ‘B Cells and Macrophages in Cancer: Yin and Yang’, by A. Mantovani. 100% of the research supporting this manuscript was conceived and conducted in my laboratory. Drs. Andreu, Johansson and Affara wrote drafts of the manuscript under Dr. Coussens’ direct supervision.


**Role:** In patients with locally advanced solid tumors, first-line treatment is often neo-adjuvant or pre-operative chemotherapy, which helps shrink tumors before surgery, allowing for more conservative surgical approaches and reducing the potential for developing systemic disease. However, despite aggressive chemotherapy, long-term survival for many patients remains poor, in part owing to limitations with the targeting and accumulation of cytotoxic drugs in tumor tissue. The vasculature of solid tumors is abnormal, both in terms of vessel architecture and the dynamics of blood flow. Permeable heterogeneous vessel walls allow the leakage of proteins and fluid that, coupled with the inefficiency of lymphatic drainage, could be exploited to develop novel, enhanced drug delivery strategies that are therapeutically selective and improve clinical outcome. This work describes a previously unappreciated role for transforming growth factor beta (TGFβ) in regulating vascular stability and vessel permeability in solid tumors. Using mouse models, we demonstrated an endogenous pathway that regulates normal vascular permeability, which is controlled by perivascular collagen, the metalloproteinase enzyme MMP14, and TGFβ. In wild-type mice, inhibitors of either MMP14 or TGFβ signaling induce blood vessel permeability. Conversely, enhanced MMP14 or TGFβ activity in the mouse epidermis decreases leakage across cutaneous vessels. This pathway remains functional during tumor progression, as acute blockade of either MMP14 or TGFβ signaling transiently alters vessel stability, ‘opening’ vascular beds and promoting intravenous delivery of high molecular weight compounds to the tumor. This implying that delivery of standard therapeutic agents or diagnostic molecular imaging agents to tumor tissue may be enhanced by transient blockade of the TGFβ pathway. If so, this could advance disease therapy and/or diagnostic imaging, not only in cancer medicine, but also in fibrotic disorders such as scleroderma and kidney failure. Research in support of this manuscript spanned over 7 years and many post doctoral fellows and technicians. Dr. Coussens, who wrote the manuscript with input from Drs. Boudreau and Werb, conceived of the initial hypothesis and ideas towards successful completion of this project.

Role: Local control of malignant breast tumors by cytotoxic therapies is critical for long-term survival of breast cancer patients. This study is the first to provide evidence that common cytotoxic agents induce alterations in the immune microenvironment, including recruitment of immune-suppressive macrophages that facilitate resistance to chemotherapy and promote metastasis. These data provide rationale for selective blockade of CSF1R signaling pathways in combination with cytotoxic therapies to combat breast cancer, and potentially other tumor types where macrophages foster tumor development. This work provided the foundation for a KOMEN Promise grant award that enables ongoing mouse modeling, as well as an investigator-initiated phase Ib/II clinical trial to evaluate a CSF1R kinase inhibitor in combination with taxol-based chemotherapy in women with recurrent triple negative breast cancer. This manuscript was the subject of an invited “Preview” article (De Palma and Lewis, Nature 2011), and was featured in the research highlights section of Nature Reviews Cancer (McCarthy, 2011). 100% of the research supporting this research was conceived and conducted in Dr. Coussens’ laboratory. Dr. DeNardo wrote drafts of the manuscript under Dr. Coussens’ supervision.
Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity

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Abstract Tumor-associated myeloid cells have been implicated in regulating many of the “hallmarks of cancer” and thus fostering solid tumor development and metastasis. However, the same innate leukocytes also participate in anti-tumor immunity and restraint of malignant disease. While many factors regulate the propensity of myeloid cells to promote or repress cancerous growths, polarized adaptive immune responses by B and T lymphocytes have been identified as regulators of many aspects of myeloid cell biology by specifically regulating their functional capabilities. Here, we detail the diversity of heterogeneous B and T lymphocyte populations and their impacts on solid tumor development through their abilities to regulate myeloid cell function in solid tumors.

Keywords Cancer · Inflammation · Lymphocyte · Macrophage · Metastasis

1 Introduction

Virchow first described leukocyte infiltration of solid tumors in the 1800s; however, only recently have we begun to understand the diverse regulatory roles played by immune cells during cancer development. Historically, leukocytes found in and around developing tumors were thought to represent an attempt by the host to eradicate neoplastic cells. Indeed, some leukocytes, including CD8+ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, do play a critical role in restraining tumor development [1]. However, we now appreciate that the significance of these anti-tumor programs can be thwarted by other subsets of leukocytes that instead foster tumor development [2–5]. Immune-competent mouse models of human cancer have enabled a detailed evaluation of the tumor-promoting capacity of several subsets of myeloid cells, including mast cells (MCs), monocytes, granulocytes/neutrophils, and macrophages, as well as some subsets of lymphocytes [6, 7]. However, depending on their differentiation status and immune microenvironment, subpopulations of these same cells can also support tumor rejection and response to anti-cancer therapy [2, 8, 9], thus indicating that pro- and anti-tumor programming of leukocytes is dynamic. In this review, we discuss recent insights into the role of B and T lymphocytes as “gatekeepers” of myeloid cell bioactivity (Fig. 1) and how recognition of these dynamic interactions reveals novel opportunities for anti-cancer therapy.

2 Paradoxical role of CD4+ T lymphocytes in solid tumor development

In contrast to CD8+ CTLs that play well-defined roles in hindering cancer development, the functional significance of CD4+ T lymphocytes in tumor progression appears more paradoxical. For example, retrospective evaluation of colon and lung carcinomas revealed that extensive infiltration of tumors by CD4+ T cells correlates with favorable clinical outcome, whereas in breast and renal cancers exhibiting similar infiltrations instead correlates with decreased overall
neity of CD4+ T cell subsets that accumulate in tissues may either pro- or anti-tumor immune programs. The heterogeneity of CD4+ T cell subsets include TH1 and TH2 lineages that are characteristically fostered by exposure to interleukin (IL)12 and IL4, respectively. Following activation, CD4+ T cells assuming the TH1 fate secrete interferon (IFN)γ, tumor necrosis factor (TNF)α, IL2, and IL12 [20]. Through the production of these cytokines, TH1 cells regulate immune surveillance programs by up-regulating antigen processing and presentation on major histocompatibility complex (MHC) I and II molecules by professional antigen presenting cells (APCs) and as such can regulate the duration and magnitude of CD8+ CTL responses [21]. In addition, following strong antigen-specific activation, TH1 cells can directly kill tumor cells by releasing high levels of IFNγ, TNFα and cytolytic granules. Thus, when present, TH1 responses can directly and indirectly regulate anti-tumor programs that restrain cancer development.

In contrast, TH2 CD4+ T cells express high levels of IL4, -5, -6, -10, and -13 that, together, alter adaptive immunity by inducing T cell anergy, inhibiting T cell-mediated cytotoxicity as well as fostering humoral immune responses directed by B cells [22, 23]. The TH2 cytokines IL4 and IL13 are important mediators of CD4+ T cell functionality in vitro. TH2 cells inhibit apoptosis and induce proliferation of breast carcinoma cells; in vivo, IL4 emanating from CD4+ T cells fosters breast cancer growth [24, 25]. Consistent with these findings, a high ratio of TH2+ to TH1+ cells correlates with parameters of clinical disease progression, such as increased tumor size and grade and lymph node metastasis of breast cancers [26].

Adding to this TH1 versus TH2 paradigm, CD4 lines have recently expanded to include a TH17 subset that is differentiated by a combination of IL6 and transforming growth factor (TGF)β and mediate their effects through secretion of IL17, -21, and -22, [27–29]. TH17 cells are thought to play an important role in protection against some extracellular pathogens and in regulating auto-immune disease [30, 31]. As such, TH17 cells have been implicated in the development of inflammation-associated colonic tumors in response to pathogenic bacteria [32]. TH17 cell infiltration has also been observed in patients with colon, ovarian, prostate, and hepatocellular carcinoma where high numbers of IL17-producing cells correlates with poor prognosis [33–35]. In mouse models of non-small cell lung cancer (NSCLC), IL17 enhanced tumor growth by promoting development of angiogenic vasculature [36, 37]. In contrast, in a B16 melanoma model, IL17 depletion rendered mice more susceptible to metastasis [38], a phenotype that was blocked by adoptive transfer of tumor-specific T(H)17 cells that fostered immune surveillance by CD8+ CTLs and dendritic cells (DCs) [38]. Together, these experimental findings indicate that the role

3 CD4+ T lymphocyte heterogeneity

CD4+ T cells represent a highly heterogeneous population of cells that develop along different functional lineages depending upon polarizing cytokine signals during activation by antigens [19]. Classically, CD4+ T lymphocyte subsets include TH1 and TH2 lineages that are characteristically fostered by exposure to interleukin (IL)12 and IL4, respectively. Following activation, CD4+ T cells assuming the TH1 fate secrete interferon (IFN)γ, tumor necrosis factor (TNF)α, IL2, and IL12 [20]. Through the production of these cytokines, TH1 cells regulate immune surveillance programs by up-regulating antigen processing and presentation on major histocompatibility complex (MHC) I and II molecules by professional antigen presenting cells (APCs) and as such can regulate the duration and magnitude of CD8+ CTL responses [21]. In addition, following strong antigen-specific activation, TH1 cells can directly kill tumor cells by releasing high levels of IFNγ, TNFα and cytolytic granules. Thus, when present, TH1 responses can directly and indirectly regulate anti-tumor programs that restrain cancer development.

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of TH17 cells in regulating aspects of cancer development may also be context dependent.

In addition to TH1, TH2, and TH17 CD4+ effectors, populations of CD4+FoxP3+ T regulatory (Treg) cells are also thought to play a considerable role in regulating tumor immunity. In human cancers, increased prevalence of CD4+FoxP3+ Treg cells correlates with increased survival for follicular lymphoma [39], while it instead correlates with poor prognosis in pancreatic ductal carcinoma [40], non-small cell lung cancer [41], renal cell carcinoma [11], and breast carcinoma [42]. Suppression of the anti-tumor activities of CD8+ CTLs, NKs, and DCs is at the heart of how Treg cells control tumor development [43, 44]; however, the multitude of mechanisms whereby they regulate anti-tumor programs suggest the existence of distinct tissue-specific subpopulations of Treg cells, each endowed with or capable of various bioeffector activities [45–47]. Mechanistic studies have revealed that Treg cells support pro-tumor immunity not only by increasing local levels of immunosuppressive cytokines including TGFβ, IL35, and IL10 but also by direct cytolytic effects through production of perforin and granzyme. In addition, Treg cells can disrupt the metabolic activity of cyclic adenosine monophosphate (cAMP) transfer, as well as inhibit APC function by inducing binding of CTLA-4 to CD80/86 [44, 48].

Thus, while it is now clear that a spectrum of CD4+ T cell subtypes are present in human tumors of essentially all types, the role they play in promoting or repressing tumor development likely has to do with the type of CD4+ T cell subtype that is either recruited to or accumulates within each distinct tumor microenvironment. These in turn regulate anti-tumor programs by professional cytotoxic cells (CD8+ TCTLs and NK cells), as well as regulating pro-tumor properties of a diverse array of myeloid cell subtypes as discussed below.

4 Myeloid heterogeneity and tumor development

Innate immune cells of myeloid origin, e.g., granulocytes (neutrophils, basophils, and eosinophils), DCs, macrophages, NK cells, and MCs, are also prominent components of pre- and malignant tissues and functionally contribute to cancer development by releasing a myriad of cytokines, chemokines, matrix metalloproteinases, serine proteases, DNA-damaging molecules (reactive oxygen species), histamine, and other bioactive mediators that regulate tissue remodelling and angiogenesis [49–53], suppress anti-tumor immunity [54–56], and enhance tumor cell survival, migration, and metastasis [57, 58].

Nucleated hematopoietic cells that have been directly implicated in tumor angiogenesis include MCs [51], tumor-associated macrophages (TAMs) [5, 23, 59], Tie2-expressing monocytes [50, 60], neutrophils [52], DC precursors [61], and myeloid immune suppressor cells [62, 63]. Other hematopoietic cell types, such as platelets [64], eosinophils [65], and hematopoietic progenitors [66], also participate in angiogenic processes, but it remains to be established whether they can directly promote tumor angiogenesis, rather than having a broader function in supporting tissue inflammation and remodelling.

In contrast, these same myeloid cell lineages also foster tumor rejection by inducing angiostatic programs, enhancing CTL and NK responses, and directly inducing tumor cell death [13]. As an example of these paradoxical roles, studies from several laboratories have reported that TAMs enhance angiogenesis and metastasis of malignant mammary tumors [25, 67, 68]. In contrast, TAMs exposed to toll-like receptor (TLR) ligands and/or IFNγ directly lyse mammalian tumor cells, increase antigen presentation, and secrete angiostatic proteins such as CXCL10 and 11 [8, 9, 69]. These distinct bioactivities are mirrored in neutrophils, MCs, and DCs and may be due to the inherent plasticity of myeloid lineage cells regulated by local factors present in distinct tissue and/or organ microenvironments.

The bioactive states of macrophages, as well as other myeloid cells, have been classified according to TH1 and TH2 nomenclature, referred to as M1 (classical) or M2 (alternative) activation, respectively [2, 70, 71] (Fig. 1). M1 macrophages are regulated by TH1 cytokines including IFNγ, TNFα, and granulocyte monocyte-colony stimulating factor (GM-CSF) that enhance macrophage cytotoxic activity, production of pro-inflammatory cytokines, and antigen presentation capacity [70, 71]. In contrast, tissue macrophages can achieve various alternatively activated M2 states following exposure to TH2 cytokines, including IL4 or IL13 (M2a), potentiation by immune complexes and TLR ligands (M2b) or immunosuppressive cytokines including IL10 or TGFβ and/or glucocorticoid hormones (M2c) [70].

The general hallmarks of M2 macrophages include high levels of IL10, IL1Ra, IL1 decoy receptor CCL17 and CCL22 secretion, high expression of mannose, scavenger and galactose-type receptors, low expression of IL12, as well as poor APC capability. Intriguingly, although these alternative activation states (M2a, b, and c) share many phenotypic characteristics, they are distinct and induce individual context-dependent environmental responses. For example, induction of an M2c phenotype by IL10 results in highly immune suppressive macrophages that can also produce matrix components such as versican or PTX3. In contrast, Th2 cytokine induction of M2a TAMs induces expression of fibronectin, as well as catabolism of L-arginine by arginase that in turn leads to increased collagen synthesis and matrix remodeling [70, 72]. Our own work has revealed that IL4 and/or IL13 activation of macro-
phages induces production of growth factors including epidermal growth factor, TGFβ, and basic fibroblast growth factor that together regulate invasive, angiogenic, and immune-suppressive programs [25] (unpublished data). Both M2a and M2b macrophages down-regulate the pro-inflammatory cytokines IL1, IL6, and TNFα [73], whereas induction of M2b macrophages by immune complexes induces these same inflammatory cytokines in addition to IL10 and also likely enhances vascular responses such as endothelial migration and vessel dilation [74]. Thus, M2-polarized cells promote scavenging of debris, angiogenesis, and remodeling and repair of wounded/damaged tissues. Parallel and non-redundant activity states have also been defined for DCs (i.e., DC1, DC2) [75] and neutrophils (i.e., N1, N2) [76] (Fig. 1).

To address pro- versus anti-tumor capabilities of these opposing states, Hagemann and colleagues demonstrated that by “reprogramming” M2 TAMs through deletion of IKKβ macrophage phenotype could be switched from immunosuppressive to actively promoting immune surveillance, as reflected by decreases in IL10 and arginase-1 expression and increased IL12 and nitric oxide production together resulting in decreased ovarian tumor growth through recruitment and activation of NK cells [67, 77]. Again, organ specificity and/or etiology may play a role in regulating how reprogramming can be achieved. In a mouse model of squamous carcinogenesis, we recently reported that FcγR signaling in myeloid cells directly regulates whether myeloid cells enhance or repress cancer development, which correlate with their unique gene expression signatures that reflect M1 versus M2 and DC1 versus DC2 programs [74]. These data indicate the significance of reprogramming myeloid cell phenotypes to affect tumor outcome. The major question that arises with regards to this capability then becomes what are the cellular and molecular programs in tissues and/or tumors that regulate the bioactive state of these important myeloid cells and how recognition of these can be translated into anti-cancer therapy.

5 T lymphocytes as regulators of anti-tumor macrophages

Establishment of an immune reaction in homeostatic tissue typically involves activation of NK cells in response to stress signals or infectious agents, whom by their production of IFNγ in turn prime macrophages towards an M1 state, culminating in enhanced presence of macrophages with cytotoxic capability [71]. However, production of IFNγ by NK cells is generally transient and therefore insufficient to sustain M1 macrophage polarization; thus, IFNγ-producing TIL cells are critical for immune responses requiring sustained M1 macrophage bioactivities. In tumors, studies by Corthay and colleagues demonstrated that TIL1 cell regulation of locally activated M1 macrophages were significant and fostered rejection of myeloma and lymphomas in the absence of CTL responses [69, 78]. Moreover, expression of IL12 and IFNγ by TIL1 cells can combine to enhance anti-tumor responses by NK and NK-T cells by up-regulating expression of NK receptors such as NKG2D (in response to IL12) and expression of NK receptor ligands such as RAE1 on target cells (in response to IFNγ) [79]. TIL1 responses then in turn favor anti-tumor NK and macrophage responses that eliminate neoplastic cells. However, while TIL1 cells are antigen specific, tumoricidal macrophages exert indiscriminate cell killing activity. Thus, multiple immunosuppressive programs have evolved to eliminate the adverse autoimmune pathologies, such as rheumatoid arthritis, that are associated with over-activation of these M1 responses [80]; unfortunately, many of these immunosuppressive programs are usurped by developing cancers.

6 T lymphocytes as regulators of pro-tumor myeloid cells

In contrast to induction of tumor-immune surveillance programs by TIL1 cells, TREG and TIL2 cells have the capacity to induce alternative activation states of macrophages, DCs, and neutrophils involved in promoting cancer development. Studies of human TREGs have demonstrated their ability to block classical activation of macrophages and instead foster immunosuppressive myeloid phenotypes through the production of IL10 and TGFβ [81]. Similar biology may apply to neutrophils, as recently reported by Fridlender and colleagues who found that loss of TGFβ signaling through ALK4/5 inhibition resulted in recruitment of N1-polarized neutrophils with tumoricidal bioactivities [76]. While TGFβ in the tumor microenvironment is produced by multiple cell types, these data may indicate that TREG cells suppress N1 tumoricidal responses through production of TGFβ and, as such, favor pro-tumor N2 or immature monocyte (IMC) phenotypes. Our own work has demonstrated that CD4+ TIL2 cells in mammary tumors promote M2 responses in TAM and IMCs that in turn enhance pulmonary metastasis [25]. Together, these data indicate that the balance of TIL1 versus TIL2/TREG responses regulates the pro- versus anti-tumor programming of tumor-associated myeloid cells.

7 B lymphocytes as regulators of myeloid cells during cancer development

B lymphocytes constitute a central component of humoral immunity and not only serve in antibody production but also in antigen presentation and cytokine secretion. In
particular, B lymphocyte expression of MHC and costimulatory molecules as well as secretion of pro-inflammatory cytokines are critical for regulating CD4+ and CD8+ T cell activation, expansion, antigenic spreading, and memory T lymphocyte formation. The heterogeneity of B lymphocyte responses has been recently recognized and diverse B cell subtypes with either pro-immune or regulatory properties have been identified in vivo. Precisely, regulatory B lymphocytes (BREG), which include various subtypes of IL10-producing cells, have been identified in the context of autoimmune diseases and exert anti-inflammatory functions [82, 83]. However, a role for BREG cells in cancer has not been fully elucidated.

B lymphocytes in general have only recently gained recognition for representing significant components of tumor immunity [84]. B cell involvement in solid tumor development was initially described in syngeneic allograft murine tumor models in combination with genetic or antibody-mediated B cell depletion. In these studies, B cell-deficient mice (μMT) exhibited resistance to several types of syngeneic tumors, including EL4 thymoma, MC38 colon carcinoma, and B16 and D5 melanoma [85, 86], whereas partial B cell depletion resulted in significantly reduced tumor burden in a transplantable model of colorectal cancer [87]. A tumor-promoting role for B cells in solid tumor development was also revealed in transgenic mice expressing tumor necrosis factor (TNF)-receptor-associated factor 3 (TRAF3) in lymphocytes [88]. TRAF3+ lymphocytes induce humoral immune responses leading to chronic inflammation and a significantly elevated incidence of squamous cell carcinomas [88]. These experimental findings indicate that, in the absence of an initiating oncogenic event, B lymphocyte-mediated chronic inflammation is sufficient to foster solid tumor formation. In contrast, an opposite and anti-tumor immune surveillance role for B lymphocytes has also been demonstrated in a syngeneic melanoma model where deletion of mature B cells by anti-CD20 IgG significantly enhanced tumor growth and metastasis [80], suggesting that the role of B lymphocytes in tumor progression, like CD4+ T lymphocytes, may be context dependent and driven by individual B lymphocyte subtype specificity.

Mechanistically, B cells and humoral immunity can act to modulate solid tumor development by regulation of diverse effector pathways involving secretion of pro-inflammatory, as opposed to regulatory, cytokines, e.g., IL10, TGFβ, inhibition of CTL activity [89], perturbation of Th1/Th2 CD4+ T cell lineages [90, 91], as well as differential recruitment and activation of innate immune cells [89, 92].

Recently, using a transgenic mouse model of inflammation-associated carcinogenesis, i.e., K14-HPV16 mice [93], we revealed a novel pathway by which B lymphocytes enhance squamous carcinogenesis and demonstrated the significance of the B cell/immunoglobulin/FcγR signaling axis. We found that B cells and humoral immunity fostered cancer development by activating Fcγ receptors on resident and recruited myeloid cells [74]. Stromal accumulation of autoantibodies in premalignant skin, through their interaction with activating FcγRs, regulated recruitment, composition, and bioeffector functions of leukocytes, in particular subsets of tumor-promoting polarized myeloid cells in neoplastic tissue which in turn enhanced neoplastic progression and subsequent carcinoma development [74]. A similar pro-tumor role for B cells was recently reported by Ammirante and colleagues who found that B cells are critical for growth of castration-resistant prostate cancer metastasis, not through production of immunoglobulins or regulation of FcγR signaling but instead by delivery of lymphotoxin that in turn activates IKK-α and STAT3 in prostate cancer cells and subsequently stimulates metastasis by an NF-κB-independent, cell-autonomous mechanism [94]. These findings together with other experimental studies support a model in which B cells, through various mechanisms, including humoral immunity, activating FcγRs and IKK, are required for establishing chronic inflammatory programs that promote de novo carcinogenesis.

8 Conclusions

While many factors regulate the propensity of immune cells to promote or repress solid tumor development, polarized adaptive immune responses by B and T lymphocytes can specifically regulate multiple pro-tumor properties of myeloid cells that in turn control many of the “hallmarks” of cancer development [95, 96]. Thus, recognition of the soluble molecules that mediate these important paracrine interactions may represent critical targets to evaluate for anti-cancer therapy. Importantly, targeting of pro-tumor pathways that neutralize M2-type macrophage and/or Th2-type CD4+ T cell responses and therein foster M1 or Th1-type immunity may enhance sensitivity to cytotoxic therapies, including chemo- and radiation therapy, whose durability may be limited by the longevity of the anti-tumor immune responses that they induce.

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References


The Bcl-2 repertoire of mesothelioma spheroids underlies acquired apoptotic multicellular resistance

D Barbone, JA Ryan, N Kolhatkar, AD Chacko, DM Jablons, DJ Sugarbaker, R Bueno, AG Letai, LM Coussens, DA Fennell and VC Broaddus

Three-dimensional (3D) cultures are a valuable platform to study acquired multicellular apoptotic resistance of cancer. We used spheroids of cell lines and actual tumor to study resistance to the proteasome inhibitor bortezomib in mesothelioma, a highly chemoresistant tumor. Spheroids from mesothelioma cell lines acquired resistance to bortezomib by failing to upregulate Noxa, a pro-apoptotic sensitizer BH3-only protein that acts by displacing Bim, a pro-apoptotic Bax/Bak-activator protein. Surprisingly, despite their resistance, spheroids also upregulated Bim and thereby acquired sensitivity to ABT-737, an inhibitor of anti-apoptotic Bcl-2 molecules. Analysis using BH3 profiling confirmed that spheroids acquired a dependence on anti-apoptotic Bcl-2 proteins and were ‘primed for death’. We then studied spheroids grown from actual mesothelioma. ABT-737 was active in spheroids grown from those tumors (5/7, ~70%) with elevated levels of Bim. Using immunocytochemistry of tissue microarrays of 48 mesotheliomas, we found that most (33, 69%) expressed elevated Bim. In conclusion, mesothelioma cells in 3D alter the expression of Bcl-2 molecules, thereby acquiring both apoptotic resistance and sensitivity to Bcl-2 blockade. Mesothelioma tumors ex vivo also show sensitivity to Bcl-2 blockade that may depend on Bim, which is frequently elevated in mesothelioma. Therefore, mesothelioma, a highly resistant tumor, may have an intrinsic sensitivity to Bcl-2 blockade that can be exploited therapeutically.

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Subject Category: Cancer

Resistance to apoptosis may underlie the chemoresistance of tumors. Apoptotic resistance and chemoresistance, however, may not be fully reflected in two-dimensional (2D) cell cultures (monolayers). Indeed, when cancer cells are grown as three-dimensional (3D) spheroids, they acquire multicellular resistance that mimics the chemoresistance observed in vivo and can effectively recapitulate some of the complexity of solid tumors.

Understanding multicellular resistance may provide key insights into effective therapies for recalcitrant solid tumors such as human malignant mesothelioma, which usually presents at a late stage as a thick tumor mass. At this time, few effective therapeutic options are available for mesothelioma patients and survival remains poor. Our group and others have found that mesothelioma spheroids acquire multicellular resistance to a variety of treatments. We decided to focus on resistance to bortezomib (PS-341, Velcade), an agent that has shown promise in pre-clinical studies of mesothelioma and is now being evaluated in clinical trials. Although it is not yet known whether mesothelioma will exhibit resistance to bortezomib, bortezomib resistance, while rare in hematologic cancers, has been a common problem in solid tumors.

Bortezomib, a proteasome inhibitor, is thought to induce apoptosis via the alteration of expression of Bcl-2 proteins. Thus, resistance to bortezomib may also manifest itself in changes in the balance of the pro- and anti-apoptotic Bcl-2 molecules. If so, new experimental approaches, such as Bcl-2 homology domain 3 (BH3)-profiling, may be used to uncover the anti-apoptotic barriers mediating resistance. With BH3-profiling, the cellular dependence on anti-apoptotic proteins can be decoded based on mitochondrial sensitivity to a panel of BH3 peptides. In addition, using mechanistic approaches such as these, it has been seen that anti-apoptotic resistance mechanisms may coexist with a high level of pro-apoptotic potential, a situation termed ‘primed for death’. Tumors identified as ‘primed for death’ may respond to inhibition of the anti-apoptotic defenses with small molecules such as ABT-737, an inhibitor of Bcl-2/XL/w. Probing of the anti-apoptotic strategies of 3D spheroids may reveal vulnerabilities that can be sought in tumors as well.

Here, we have investigated the multicellular resistance and the apoptotic priming that develops in mesothelioma cells grown in 3D spheroids. By using Bcl-2 inhibitors and BH3-profiling, we have probed the anti-apoptotic defenses of multicellular spheroids and also of human mesothelioma.

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Abbreviations: TFS, tumor fragment spheroid; 2D, two-dimensional; 3D, three-dimensional; EPP, extrapleural pneumonectomy; TMA, tissue microarray; R8, 8 arginine-repeat peptide; BH3, Bcl-2 homology domain 3

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grown as tumor fragment spheroids (TFS). This study presents for the first time key insights into the apoptotic repertoire of 3D spheroids and proposes ways to detect the pro-apoptotic potential within mesothelioma and to exploit it therapeutically.

Results

Mesothelioma cells acquire resistance to bortezomib when grown as 3D spheroids. We first studied two mesothelioma cell lines, M28 and REN, previously reported to be sensitive to bortezomib-induced apoptosis and known to form spheroids. Monolayers and spheroids were treated with increasing doses of bortezomib (1–100 nM) for 48 h. Monolayers displayed an IC50(48h) of approximately 18 nM (M28) and 9 nM (REN), whereas spheroids exhibited resistance, with an approximate IC50(48h) of 100 nM (M28 and REN) (Figure 1a).

Resistance was not due to differences of proteasome activity at baseline or after bortezomib. We found that the proteasomal chymotryptic activity of monolayers and spheroids of REN cells was equal at baseline and equally inhibited following exposure to bortezomib or to another proteasome inhibitor, MG-132, for 4 h (Figure 1b).

Resistance was not a function of high cell density, as has been proposed. We found that completely confluent monolayers did not acquire resistance to bortezomib (Figure 1c), but were as responsive to bortezomib as the subconfluent monolayers.

In spheroids, pro-apoptotic Noxa is not upregulated by bortezomib whereas Bim is high. The proteasome recycles >80% of the total protein content of cells and thereby mediates the activity of key cellular functions such as apoptosis. Bortezomib-induced apoptosis has been attributed to the accumulation of the pro-apoptotic BH3-only protein Bim and the induction of other pro-apoptotic sensitizers such as Noxa. We asked whether bortezomib might alter these apoptotic triggers differently in spheroids and, if so, whether such differences might underlie spheroids’ acquired multicellular resistance.

Using immunoblotting, we analyzed the expression of key Bcl-2 proteins in monolayers and spheroids before and after treatment with bortezomib (Figure 2). In monolayers exposed to bortezomib, Noxa was strongly upregulated. In spheroids exposed to bortezomib, however, Noxa failed to increase.

In addition, spheroids upregulated Bim expression at baseline and following bortezomib (Figure 2). Intriguingly, spheroids, despite their increased apoptotic resistance, expressed more pro-apoptotic Bim than did monolayers. Because Noxa functions indirectly by displacing Bim from anti-apoptotic Mcl-1, we asked whether addition of Noxa to the spheroids could restore apoptotic sensitivity.

A Noxa peptide reduces multicellular resistance acquired by spheroids. To confirm that Noxa contributed to the apoptosis induced by bortezomib in the monolayers, we reduced Noxa expression by siRNA. Silencing successfully reduced bortezomib-induced Noxa upregulation, without alteration of other Bcl-2 molecules, Mcl-1 or Bim (Figure 3a). The reduction in Noxa significantly reduced apoptosis in monolayers following bortezomib, suggesting an important role for Noxa in mediating bortezomib-induced apoptosis (Figure 3a).

We then attempted to restore Noxa in spheroids using a NoxaBH3 peptide bound to 8 arginine residues (R8-NoxaBH3), a modification that aids diffusion of the peptide across cell membranes. Although an R8 control peptide had no activity, R8-NoxaBH3 enhanced the apoptotic response of the spheroids, thereby significantly reducing the multicellular resistance of spheroids to bortezomib (Figure 3b).

In addition, R8-NoxaBH3 alone induced apoptosis in REN spheroids. To determine whether R8-NoxaBH3 peptide had off-target effects, we ablated Mcl-1 by siRNA. Because Noxa activity requires Mcl-1, we would expect R8-NoxaBH3 to have no activity in the absence of Mcl-1. Without Mcl-1, R8-NoxaBH3 lost all apoptotic activity indicating that, at
least in these cells at the concentrations used, Noxa peptide activity was specifically targeted to Mcl-1 (Supplementary Figure 1).

The activity of the Noxa peptide suggested that the lack of Noxa upregulation in spheroids contributed to the multicellular resistance to bortezomib. Nevertheless, Noxa, as a BH3-only sensitizer, depends on the presence of a pro-apoptotic Bax/Bak-activator such as Bim. The high expression of Bim in the spheroids suggested that the spheroids were primed for apoptotic death and thus vulnerable to other approaches designed to release Bim.

**ABT-737 reduces multicellular resistance of spheroids.** We asked whether ABT-737, a small molecule that inhibits Bcl-2/XL/w, would be effective in spheroids by unmasking the pro-apoptotic potential revealed by the R8-NoxaBH3 peptide. Indeed ABT-737 (1 μM) increased the apoptotic response to bortezomib of spheroids as well as of monolayers and reversed the multicellular resistance of spheroids (Figure 4). Moreover, ABT-737 alone induced apoptosis of both M28 and REN spheroids, confirming that spheroids are ‘primed for death’ and dependent on their anti-apoptotic defenses for survival. To confirm the role of Bim in the activity of ABT-737, we reduced Bim expression using siRNA. Knockdown of Bim abolished the ability of ABT-737 by itself to induce apoptosis in spheroids and significantly reduced its ability to enhance bortezomib-induced apoptosis (Figure 5a).

Unlike Bcl-2/XL/w, Mcl-1 is not inhibited by ABT-737 and is known to mediate resistance to ABT-737. We ablated Mcl-1 using siRNA to determine its contribution to resistance in spheroids. Ablation of Mcl-1 increased the apoptotic responses to ABT-737 and to bortezomib (Figure 5b). In fact, inhibition of both Bcl-2/XL/w (by ABT-737) and Mcl-1 (by siRNA) increased the apoptosis in M28 and REN spheroids, to 100 and 65% of total cells, respectively, even in the absence of bortezomib.
ABT-737 has been shown in some settings to alter levels of Bcl-2 proteins,\textsuperscript{25–27} including Mcl-1. To address this issue, we grew REN cells as monolayers and spheroids and exposed them to ABT-737 alone or to ABT-737 together with bortezomib for 6h. During that time, selected to avoid the changes induced by apoptosis, we found no significant change in the levels of two Bcl-2 proteins, Mcl-1 and Bim (Supplementary Figure 2).

To determine whether ABT-737 or bortezomib acts on the benign counterpart of mesothelioma, we studied normal mesothelial cells obtained from benign human ascites. Because benign mesothelial cells \textit{in vivo} exist as single cell monolayer \textit{and in vitro} do not form spheroids as readily as do malignant cells, we studied them as monolayers. Normal mesothelial cells did not undergo apoptosis following bortezomib, ABT-737 alone or bortezomib plus ABT-737 (Supplementary Figure 3A). The normal cells also did not express Bim, even following bortezomb treatment for 6h (Supplementary Figure 3B).

**Spheroids are ‘primed for death’**. Mesothelioma cells in 3D spheroids thus appeared to acquire apoptotic resistance along with an underlying sensitivity to apoptosis. We used BH3-profiling to confirm these findings in four cell lines (two epithelioid lines, M28 and REN and two sarcomatous lines, SARF and VAMT). For these studies, cells from monolayers and spheroids were disaggregated identically to single cells for flow cytometry and probed with a panel of BH3 peptides or small molecules such as ABT-737 (1 μM), either alone or in combination, for 24 h. ABT-737 eliminated the acquired multicellular resistance of spheroids to bortezomib. Interestingly, when given alone, ABT-737 was able to induce apoptosis in spheroids but not in monolayers. (*P < 0.05 compared with bortezomib alone; \(++ P < 0.05\) spheroids versus monolayers, \(n = 3\)).

\begin{figure}
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\caption{ABT-737 eliminates multicellular resistance to bortezomib. M28 and REN monolayers and spheroids were treated with bortezomib (25 nM) and ABT-737 (1 μM) either alone or in combination, for 24 h. ABT-737 eliminated the acquired multicellular resistance of spheroids to bortezomib. Interestingly, when given alone, ABT-737 was able to induce apoptosis in spheroids but not in monolayers. (*\(P < 0.05\) compared with bortezomib alone; \(++ P < 0.05\) spheroids versus monolayers, \(n = 3\)).}
\end{figure}

ABT-737 has activity against human mesothelioma grown as TFS. The sensitivity of multicellular spheroids to Bcl-2 blockade with ABT-737 raised the intriguing possibility that human mesothelioma tumor cells in their 3D environment might also be sensitive. Using a human tumor
fragment model we previously developed for ex vivo studies,\textsuperscript{5} we tested spheroids grown from seven mesothelioma tumors for responsiveness to ABT-737. In tumor fragments from five of the tumors, ABT-737 did sensitize the mesothelioma cells to bortezomib-induced apoptosis (Figures 8a and b). In two cases, there was no response to ABT-737 (\( \leq 5\% \) apoptosis), either when ABT-737 was given alone or together with bortezomib. We then asked whether the level of Bim expression could explain the different responses to ABT-737 in these tumors. Using immunohistochemistry of a tissue microarray (TMA) comprising the original tumor samples from which the TFS were derived, we found that the responsive tumors showed high Bim expression compared with normal pleura, whereas the unresponsive tumors expressed Bim at low or undetectable levels, comparable to normal pleura (Figure 8c). The possibility that Bim could represent a predictive biomarker for ABT-737 activity in mesothelioma prompted us to investigate the frequency of Bim overexpression in mesothelioma tumors. Using immunohistochemistry of 48 mesothelioma samples and 5 normal pleural samples included on a tumor TMA and assessed using an image analysis system, we found that 33 (69\%) of mesothelioma tumors expressed Bim at levels higher than in the normal tissues. Elevated Bim expression was found in most tumors of the epithelioid (20 of 22) and mixed subtype (12 out of 21) but was uncommon in the sarcomatous subtype (only 1 of 5).
Discussion

Understanding how cancer cells attain chemoresistance can provide clues for designing new therapeutic strategies. Because cancer cells in 3D cultures acquire a multicellular resistance that resembles the chemoresistance seen in solid tumors in vivo, 3D models may be a useful platform for investigating novel therapeutic approaches. In this study, as in previous studies in our laboratory, we investigated resistance mechanisms using 3D models generated from cell lines that we then tested for relevance in tumor grown ex vivo. 8,28 We were particularly interested in applying this approach to...
bortezomib, a therapy that has shown less efficacy in solid tumors than in non-solid tumors such as multiple myeloma, perhaps because of resistance mechanisms seen only in 3D. Although 2D models have been used to elucidate resistance to bortezomib, here we show for the first time the additional multicellular resistance mechanisms that develop in 3D. Compared with the same cells in 2D, mesothelioma cells in 3D express a different balance of Bcl-2 pro- and anti-apoptotic molecules and acquire a dual nature: on the one hand, they become resistant to bortezomib; on the other hand, they become dependent on anti-apoptotic Bcl-2 defenses and therefore sensitive if the anti-apoptotic mechanisms are neutralized. In the ex vivo human mesothelioma tumor model, we found that blockade of the Bcl-2 anti-apoptotic molecules enhanced the apoptotic response to bortezomib, at least in tumors with overexpression of the pro-apoptotic BH3-only molecule Bim. The response of the in vitro and ex vivo models to inhibition of anti-apoptotic Bcl-2 members supports a new, clinically relevant approach to this highly chemoresistant tumor.

Mesothelioma cells in 3D acquired a resistance to bortezomib that was initially identified by an inability to upregulate Noxa, the pro-apoptotic BH3-only molecule that interacts with Mcl-1 to release Bim. In many systems, Noxa has been shown to mediate bortezomib activity, a role we confirmed in the mesothelioma monolayers. In spheroids, the addition of a R8-NoxaBH3 peptide was able to restore bortezomib-induced apoptosis to the same level as in the monolayers, suggesting that the lack of Noxa upregulation contributed to the bortezomib resistance in 3D. Because Noxa acts indirectly by releasing Bim, we suspected that the effect of Noxa depended on Bim and, as expected, the ablation of Bim decreased the activity of Noxa. However, we were surprised at the high level of Bim in the 3D spheroids, especially given their chemoresistance. It suggested that the Bim was sequestered and held in check by anti-apoptotic buffering and that, despite apoptotic resistance, spheroids would be poised for apoptosis if the Bim could be released. Indeed, the small molecule, ABT-737, an inhibitor of Bcl-2/XL/w, was able to undermine bortezomib-induced apoptosis in the monolayers, suggesting that the lack of Noxa upregulation contributed to the bortezomib resistance in 3D. Because Noxa acts indirectly by releasing Bim, we suspected that the effect of Noxa depended on Bim and, as expected, the ablation of Bim decreased the activity of Noxa. However, we were surprised at the high level of Bim in the 3D spheroids, especially given their chemoresistance. It suggested that the Bim was sequestered and held in check by anti-apoptotic buffering and that, despite apoptotic resistance, spheroids would be poised for apoptosis if the Bim could be released. Indeed, the small molecule, ABT-737, an inhibitor of Bcl-2/XL/w, was able to undermine the bortezomib resistance of spheroids and, in some cell lines, to induce apoptosis in spheroids by itself. In fact, when used as single agents in the resistant spheroids, ABT-737 was more effective than bortezomib.

By BH3-profiling, we confirmed that the cells disaggregated from the spheroids had acquired sensitivity to ABT-737 and to peptides that displace Bim from Bcl-2 and Bcl-XL. Of interest, the apoptotic priming was acquired as the malignant cells moved to 3D, a feature that has not previously been described. Analogous to what has been proposed for cancer in general, spheroids acquired resistance to apoptosis along with a latent pro-apoptotic potential that could be harnessed therapeutically.

Mcl-1 can confer resistance to ABT-737 as well as to bortezomib, and can be a major resistance factor in 3D spheroids, as we have previously shown in lung cancer. Interestingly, in our studies in mesothelioma, the presence of Mcl-1 did not prevent the response to ABT-737 although the reduction of Mcl-1 (by R8-NoxaBH3 or by Mcl-1 siRNA) did increase the response to ABT-737, as has been previously shown. Thus, Mcl-1 appeared to blunt, but not block, the activity of ABT-737 and the combined inactivation of both Mcl-1 and the Bcl-2/XL/w was more effective than inhibition of either one alone. Mcl-1 also appeared to have a smaller role when the increase in Bim levels was lower. For example, R8-NoxaBH3 was less effective than ABT-737 in the cell line with the lowest increase in Bim (M28). Therefore, we speculate that Bim may be preferentially sequestered by Bcl-2/XL/w, as has been described, with additional buffering by Mcl-1 as Bim increases. Nonetheless, Mcl-1 represents a potential barrier to the use of ABT-737 clinically. Thus, the effectiveness of ABT-737 in combination with bortezomib or other therapies may be enhanced by agents that reduce Mcl-1.

Why Bim increases in the 3D spheroids is not yet known; nonetheless, the finding that Bim increases in mesothelioma cells in 3D represents a unique and potentially important observation. Bim was essential for the response to ABT-737, as demonstrated by the Bim siRNA experiments, and the level of Bim correlated with sensitivity to ABT-737 in the multicellular spheroids and in the tumor fragments. Bim may thus be a useful predictive biomarker for the response of mesothelioma to ABT-737 together with bortezomib. Almost 70% of mesotheliomas were found to express Bim at levels higher than in normal tissue. Interestingly, Bim overexpression was frequent in the more chemosensitive subtype (epithelioid, 90%) and uncommon in the more chemoresistant subtype (sarcomatoid, 20%). Unleashing the pro-apoptotic molecule Bim using small molecule inhibitors such as ABT-737 may enhance the effectiveness of current chemotherapy in this recalcitrant tumor.

TFS were developed to investigate therapeutic approaches in a clinically relevant ex vivo setting. When tested in TFS, ABT-737 was also effective, but only in spheroids derived from tumors with a high expression of Bim. Thus, by virtue of studies in these complementary 3D models, we have identified a potential therapy, ABT-737, and identified a possible biomarker for predicting response to ABT-737 in this tumor. Bim. Further studies will be necessary to confirm the validity of these predictions. Nonetheless, we think that agents able to upregulate Bim, such as vorinostat, could be potent adjuncts to current chemotherapies.

There are potential clinical benefits to targeting tumors at the level of their anti-apoptotic defenses. Most targeted therapies have focused on impairing signaling pathways on which cancers depend for survival. However, the efficacy of these approaches may be impaired by intracellular signaling redundancy, crosstalk, adaptation and differing levels of activating/silencing mutations. Direct inhibitors of anti-apoptotic defenses could bypass the need to inhibit multiple pathways by moving to a distal level at which multiple signals are integrated. Our data suggest that such an approach may be effective in the different mesothelioma subtypes, against a variety of apoptotic therapies, and without injury to normal tissues.

In sum, using 3D culture models, we have shown that multicellular resistance to bortezomib is mediated by alterations in the Bcl-2 repertoire, which provides a druggable target specific to the malignant cells. Inhibition of the anti-apoptotic buffers in association with chemotherapy represents a promising strategy for the treatment of mesothelioma.
APPENDIX B

Bcl-2 blockade undermines multicellular resistance

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Materials and Methods
Cell cultures and reagents. The human mesothelioma cell lines M28, REN, VATM and SARC were cultured in DMEM supplemented with 10% FBS and 100 IU/ml penicillin-streptomycin in a 37 °C humidified incubator with 5% CO2 (full DMEM). Primary human mesothelial cells were cultured from ascites fluid from patients without infection or malignancy according to a protocol approved by the UCSF Committee on Human Research. TFS were generated as previously described. Tumor samples were obtained from extrapleural pneumonectomy (EPP) or pneumonectomy procedures performed by JS and RB at Brigham and Women’s Hospital in Boston, MA, USA. R8-Noxa[559685] (RRRRRRRR-VECATOLRFFDGKINFRQKL) and R8 (RRRRRRRR) peptides were from Genscript (Piscataway, NJ, USA). Bortezomib (Velcade) was from Millennium Pharmaceuticals (Cambridge, MA, USA). ABT-737 was generously provided from Abbott Pharmaceuticals (Abbott Park, IL, USA).

Generation and treatment of spheroids
Multicellular spheroids. Multicellular spheroids were generated in non-adherent round-bottomed 96-well plates. The 96-well plate was coated with a 1:24 dilution of polyHEMA (120 mg/ml) (AP3932, Sigma-Aldrich, St. Louis, MO, USA) in 95% ethanol and dried at 37 °C for 48 h. Before use, plates were sterilized by UV light for 30 min. For generation of multicellular spheroids, 10,000 cells were added into each well of polyHEMA-coated 96-well plate. The plates were briefly spun at 500 rpm and then placed in a 37 °C humidified incubator with 5% CO2 for 24 h. For generation of monolayers, 180,000 cells were added into each well of six-well plates.

Tumor fragment spheroids. TFS were generated from seven tumor samples obtained from surgical resection performed at the Brigham and Women’s Hospital (Boston, MA, USA). A part of the tumor was fixed in 10% formalin (Fisher Scientific, Fair Lawn, NJ, USA) and embedded in paraffin. For spheroid culture, tumor tissue was diced finely with scalpels to pieces smaller than 1 mm in diameter that were suspended in medium in 10-cm plates coated with 0.8% agar (Agar Noble, #A5431, Sigma-Aldrich) in full DMEM. The volume of overlay media was 15 ml, and half the volume of the overlay media was changed twice a week. The agar-coated plates were regularly observed using an inverted phase microscope during the incubation period, up to 4 weeks. Spheroids were collected at different time points, treated as described in figure legends, fixed in 10% formalin and embedded in paraffin for immunohistochemical staining.

Treatment. Before treatment, 18 multicellular spheroids or 20–30 TFS were transferred to each well of a polyHEMA-coated 24-well plate to match the numbers of cells plated as monolayers (180,000 cells per well). The spheroids and monolayers were treated with apoptotic agents in full DMEM with or without inhibitors (and the appropriate DMSO vehicle control) for 24 h.

Immunoblotting. After treatment, monolayers and spheroids were lysed in boiling lysis buffer (2.5% SDS, Tris-HCl 250 mM, pH 7.4). The concentration of total protein was evaluated with a colorimetric assay (DC protein assay from Bio-Rad, Hercules, CA, USA). In all, 50 μg of cell lysates were loaded in reducing conditions (0.2 M Tris, pH 6.8, 5% SDS, 5% glycerol, 0.01% bromophenol blue and 200 mM boiling lysis buffer (2.5% SDS, Tris-HCl 250 mM pH 7.4)). The concentration of total protein was evaluated with a colorimetric assay (DC protein assay from Bio-Rad, Hercules, CA, USA). The cells were lysed in boiling lysis buffer (2.5% SDS, Tris-HCl 250 mM, pH 7.4). After separation in SDS-PAGE (5 to 15% acrylamide) and transfer to PVDF (Immobilon, Millipore, Billerica, MA, USA), membranes were blocked with a protein-free TBS blocking buffer (Pierce, Rockford, IL, USA) and gently agitated with antibodies diluted in 5% non-fat dry milk or 5% BSA, as appropriate, at 4 °C overnight. Secondary antibodies were from Amersham (Piscataway, NJ, USA). Chemiluminescence was detected with the enhanced SuperSignal West Pico Substrate (Pierce). The antibodies against Bcl-2 (#2872) and Bcl-X1 (#2764) were from Cell Signaling Technology (Beverly, MA, USA). The Mcl-1 (sc-819) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the Bim antibody (455965) was from BD Pharmingen (San Diego, CA, USA), the Noxa antibody was from Calbiochem (#OP180, San Diego, CA, USA), and the alpha-tubulin antibody (AT-6074) was from Sigma-Aldrich.

Proteasome activity assay. Proteasome activity in REN spheroids and monolayers was measured using a commercial 20S proteasome activity assay kit (APT280, Millipore Corporation). In brief, spheroids and monolayers were lysed with proteasome activity buffer (50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl and 1% Triton X-100). The lysates were incubated with the fluorogenic substrate LLVY-AMC, a fluorophore 7-amino-4-methylcoumarin (AMC) bound with LLVY peptide, at 37 °C for 2 h. LLVY is a substrate recognized and cleaved by the 20S proteasome. After LLVY chromogenic cleavage by the 20S proteasome, AMC is released and emits fluorescence that can be read by using a 380 nm excitation and a 460 nm emission filter in a fluorometer.

RNA interference. Cells (4 × 10^4) were pelleted and resuspended in 100 μl of nucleofection buffer (solution T, Amx Biosystems, Cologne, Germany) with 1.5 μg of the appropriate siRNA duplex (Ambion, Austin, TX, USA). A non-targeting siRNA sequence was utilized as control in all experiments. This suspension was transferred to a sterile cuvette and nucleofected using program T20 on a Nucleofector II device (Amx Biosystems). After 30-min recovery in complete DMEM medium, the cells were plated and allowed to grow for 24 h. Cells were then trypsinized, counted and plated as monolayers and spheroids for 24 h, and exposed to apoptotic stimuli. The siRNA sequences were: Bim (5'-ACUUAUCAGCACAUGUGUCGct-3'), Mcl-1 (5'-CCUGAUAUCUUCUAAAtt-3') and Noxa (5'-GAAuUGUUCAUAAUAcGt-3'), non-targeting control (5'-GCAcUcUcUGUAUGuGnt-3').

BH3 profiling. Experiments were performed at the Dana Farber Cancer Institute (Boston, MA, USA) by JA Ryan and AG Lea as previously published. Monolayers and spheroids formed from equal numbers of cells were disaggregated in an identical fashion to single cells. The cells were gently permeabilized as described and exposed to BH3 peptides (100 nM) for 90 min before JC1 was added for 30 min and depolarization measured as a percentage of the total.

Hoechst staining for measurement of apoptosis in multicellular spheroids. Monolayers and spheroids were disaggregated by exposure to trypsin for the same period of time, washed with ice-cold PBS, and then fixed with 2.5% glutaraldehyde (Sigma-Aldrich). The cells were then stained with 8 μg/ml of Hoechst 33342 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and placed on slides. For each condition, at least 300 cells were counted in triplicate by investigators blinded to the experimental conditions. Cells with distinctive signs of nuclear condensation were considered apoptotic.

Confocal analysis for measurement of apoptosis in TFS. In all, 5 μm paraffin sections were deparaffinized with Xylene (2 × 5 min), 100% EIOH (2 × 2 min), 95% EIOH (2 × 2 min), 75% EIOH (2 × 2 min), 50% EIOH (1 × 2 min) and ddH2O (2 × 2 min). Endogenous peroxidases were blocked with a solution of 250 ml MeOH + 5 ml 30% H2O2 for 20 min. Antigens were retrieved in citrate buffer (Citra #HK087-5K, Biogenex, Fremont, CA, USA) in a microwave oven set to high for 7–8 min. Sections were blocked with a 5% normal goat serum, 2.5% BSA in PBS for 30–45 min in humidified chamber at RT. Primary antibodies for cleaved caspase 3 (1:100, #AB3623, Chemicon, Billerica, MA, USA) and pan-cytokeratin (1:100, Progen, Heidelberg, Germany; clone GP14) were incubated in a humidified chamber at 4 °C overnight. The secondary antibodies donkey anti-rabbit AlexaFluor 488 (Pierce; #31281) and anti-guinea pig AlexaFluor 633 (Invitrogen; #RA-21105), both 1:200, were incubated for 30–45 min at RT in a humidified chamber. Slides were washed three times in PBS for 3 min and mounted with Vectashield. In a blinded fashion, the investigators examined images of doubly stained slides. Apoptotic mesothelioma cells were considered cells that had merged red (pan-cytokeratin) and green (cleaved caspase 3) and were expressed as a percentage of the total cells (DAPI-stained nuclei). For each condition, 3–10 spheroids were counted until a total of 300 DAPI-stained cells were visualized.

Bim immunohistochemistry of TMAs. Two TMAs were studied. One included the seven mesothelioma tumors that were studied as TFS for their apoptotic response and two normal pleural samples. The second included 48 mesothelioma tumors and 5 normal pleural samples. All samples were obtained without identifiers from surgeries performed at the Brigham and Women’s Hospital and at UCSF Medical Center and were fixed in formalin and assembled in a TMA. Each sample was embedded in triplicate. The histopathology was determined on separate formalin-fixed, hematoxylin- and eosin-stained sections of the tumor. In all, 5 μm paraffin sections of the TMA were processed as described for the confocal analysis of apoptosis. Bim antibody (1:150, 4 °C overnight) was visualized with a HRP/DAB Envision + Kit (Dako, Carpinteria, CA, USA; #K4010). Intensity of Bim staining was measured by the Positive Pixel Count algorithm on a ScanscopeXT system (Aperio Technologies, Inc., Vista, CA, USA), which determined the average intensity of pixels within the tissue region of interest.

Statistical analysis. Data are expressed as mean ± S.D. of at least three different experiments. Statistical significance was evaluated by one- or two-way
analysis of variance, and Tukey's test was performed to detect where the differences lay (GraphPad Prism v 4.0, GraphPad Software, Inc., La Jolla, CA, USA). A P-value < 0.05 was considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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NON-NEOPLASTIC AND NEOPLASTIC PLEURAL ENDPOINTS FOLLOWING FIBER EXPOSURE

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Exposure to asbestos fibers is associated with non-neoplastic pleural diseases including plaques, fibrosis, and benign effusions, as well as with diffuse malignant pleural mesothelioma. Translocation and retention of fibers are fundamental processes in understanding the interactions between the dose and dimensions of fibers retained at this anatomic site and the subsequent pathological reactions. The initial interaction of fibers with target cells in the pleura has been studied in cellular models in vitro and in experimental studies in vivo. The proposed biological mechanisms responsible for non-neoplastic and neoplastic pleural diseases and the physical and chemical properties of asbestos fibers relevant to these mechanisms are critically reviewed. Understanding mechanisms of asbestos fiber toxicity may help us anticipate the problems from future exposures both to asbestos and to novel fibrous materials such as nanotubes. Gaps in our understanding have been outlined as guides for future research.

TRANSLOCATION AND RETENTION OF FIBERS IN THE PLEURA

Anatomy and Physiology of the Pleura

The parietal pleura lines the chest wall and the superior surface of the diaphragm and the visceral pleura covers the lungs (Figure 1). The pleural space in humans contains a small amount of fluid (0.1–0.2 ml/kg body weight) that is a filtrate from the underlying systemic circulation (Owens & Milligan, 1995; Broaddus et al., 1988). This space (10–20 μm wide) is lined by a single layer of mesothelial cells resting on a basement membrane and underlying connective tissue and blood vessels. The major routes of drainage of fluid, protein, particulates, and cells from the pleural space are through the lymphatic stomata that open between mesothelial cells on the parietal pleural lining (Hammar, 1994; Wang, 1975; Broaddus, 2008). Effusions, an accumulation of excess liquid in the pleural space, are common features of a multitude of diseases. Transudative effusions, those not associated with inflammation or injury, usually develop due to increased...
hydrostatic pressure. In congestive heart failure, the most common cause of transudative effusions, increased pulmonary venous pressure leads to fluid accumulation in the interstitial spaces of the lung; the fluid then moves toward the lower pressure pleural space and leaks across the visceral pleura into the pleural space (Broaddus et al., 1990; Owens & Milligan, 1995). In the setting of inflammation or injury of the lung, pleura, or other organs, exudative effusions may form; these effusions contain elevated levels of protein due to the increased leakage across capillaries with increased permeability (Mutsaers et al., 2004). Excess fluid in any part of the body may find its way to the pleural space via the interstitial tissues along pressure gradients and by moving across the permeable pleural membranes. The normal and pathological paths by which liquid, cells, and particles enter and exit the pleural space suggest pathways by which asbestos fibers may also enter and exit or fail to exit the pleural space. The study of the physiology of the pleural space is challenging; even when using laboratory animal studies, analyses of the pleural space are limited by the narrowness of the space and the difficulty in sampling without inducing inflammation or injury.

**Pathways Leading to Translocation of Fibers to the Pleura**

The route of translocation of fibers from the lungs to the visceral pleura, into the pleural space, and to the parietal pleura is unknown. It is postulated that asbestos fibers may migrate to the lung interstitium and visceral pleura by a paracellular route or by direct penetration across injured alveolar epithelial cells (Miserocchi et al., 2008). Fibers may be transported to the pleural space via the lymphatics and bloodstream (Oberdörster et al., 1983). Fibers may translocate by themselves or within macrophages. Although studies of asbestos fiber movement have not been possible due to technical limitations, it is likely that asbestos fibers translocate to the pleural space passively in the same manner as interstitial fluid. This process may be enhanced by lung inflammation induced by asbestos fibers or by mixed dust exposures that increase interstitial fluid accumulation and thus fluid movement along the interstitial spaces to the pleural space (Miserocchi et al., 2008).

There are thus few studies that investigated the translocation of fibers from the lung into the pleural space. Even in the few existing studies, data from animal studies may have limited...
relevance for humans because of the different visceral pleural anatomy in rodents. In the rodent, the visceral pleura is "thin," consisting mostly of a mesothelial layer and basement membrane lying directly over the alveoli. There is little submesothelial connective tissue and no pleural vasculature. In sheep and humans and other large mammals, the visceral pleura is "thick" and has a significant submesothelial connective tissue space, containing nerves and systemic blood vessels (Figure 1). In contrast to the visceral pleura, the parietal pleura in different species has a constant and similar anatomy (Figure 1; Light & Broaddus, 2010). Thus, due to differences in the visceral pleura, one might postulate a difference between rodents and humans in the movement of the fibers into the pleural space. Due to similarities in the parietal pleura, one might suggest that the localization, accumulation, and actions of fibers in the parietal pleura might be similar.

These questions have been almost impossible to address using current technology but it is hoped that new tools and imaging techniques such as nuclear magnetic resonance (NMR) spectroscopy or two-photon microscopy can be developed to provide data on (1) how fibers distribute in the lungs and pleura, (2) the ultimate destination of fibers, (3) how fiber movement is enhanced, and (4) whether fibers are translocated and retained differently in animals and humans. Such techniques could be invasive, using labeled fibers that could be traced, for use in animal studies, and noninvasive for clinical studies of those exposed to asbestos. This imaging information on fiber localization would enhance diagnosis and follow-up of subjects exposed to asbestos, such as in directing where to sample tissues to assess fiber dosimetry, and how to determine non-neoplastic and neoplastic pleural diseases.

**Pleural Fiber Dosimetry in Rodents**

Translocation and retention of fibrous particulates from initial sites of pulmonary deposition to extrapulmonary sites are believed to be important aspects of their potential toxicity (Dodson et al., 2003; Suzuki & Kohyama, 1991). Pathologic tissue responses such as edema, inflammation, or fibrosis might potentially affect translocation and retention of particulates in the body, as well as properties of particles themselves including dose, dimensions and biopersistence. Although similarities exist between animal models and humans concerning physiological processes such as interstitial fluid dynamics and lymphatic flow, there are also anatomical differences such as in visceral pleural thickness (Tyler, 1983), as well as physiological differences such as of macrophage size and function, that need to be taken into account when comparing across animal species and when extrapolating from animals to humans (Jarabek et al., 2005; Maxim & McConnell, 2001). Rodents and humans also differ in particle respirability (Mossman et al., 2011) and this limits the use of rodent models for human risk assessment based on fiber dimensions (Lippmann & Schlesinger, 1984; Lippmann et al., 1980).

Biopersistence of fibers in the lung parenchyma also influences the fiber dose that is ultimately translocated to the pleura. Biopersistence in the lung is dependent on (1) site and rate of deposition, (2) pulmonary clearance parameters, (3) solubility in lung fluids, (4) breakage rate and patterns, and (5) rates of fiber translocation and retention. Surface chemistry and diameter are important determinants of solubility. Much of the knowledge base concerning the role of biopersistence is actually derived from studies of synthetic vitreous fibers (Bernstein, 2007; Oberdörster, 2000).

Fiber characteristics also affect clearance from the lung and translocation to the pleura. Macrophage-mediated particle clearance in the lung is likely to influence translocation of particles to interstitial sites. There are important interspecies differences in particle clearance, as well as in biological effects of high pulmonary concentrations of particles, in humans and in different animal species (Bermudez et al., 2002; Oberdörster, 2002). In addition, the method of dose administration in experimental
animals is shown to influence pleural pathology outcomes following particle exposure. In silica-exposed rats, pleural granulomas developed in animals following inhalation, but not after instillation, and the different response was likely due to differences in kinetics of particle delivery and lymphatic clearance (Henderson et al., 1995).

The effects of asbestos may be altered when asbestos is mixed with other particulates, a situation common in occupational and environmental exposures. Studies by Davis and colleagues (1991) showed that coexposure of rats to amosite asbestos and to quartz increased the incidence of amosite-induced pleural mesothelioma, presumably by elevation in fiber dosimetry and translocation through the visceral pleura. Recent studies by Bernstein and colleagues (2008) demonstrated that coexposure of chrysotile asbestos together with nonfibrous particulates decreased fiber retention in the lungs of rats, perhaps by increasing macrophage recruitment and macrophage-mediated clearance or by inducing more inflammatory fluid movement to the pleura.

Fiber translocation in rodents appears to be rapid and may be responsible in some cases in particular for pathologic outcomes. In rats, short chrysotile asbestos fibers are found in the pleural space within a week following intratracheal instillation (Viallat et al., 1986). Similarly, crocidolite fibers were detected in the pleural space 1 wk following inhalation (Choe et al., 1997). In another study in rats, short fibers (<5 μm length) were found 5 d after inhalation exposure to a synthetic vitreous fiber (refractory ceramic fiber) aerosol (Gelzleichter et al., 1996). In studies in rats and hamsters involving chronic inhalation of synthetic vitreous fibers as well as of amosite and chrysotile asbestos used as reference materials, significant interspecies differences in pleural pathology were seen (Mast et al., 1994; McConnell, 1994). Subsequent short-term mechanistic studies of translocation showed that the Syrian golden hamster, a species prone to development of pleural fibrosis and mesothelioma following synthetic vitreous fiber exposure, displayed greater translocation of fibers to the pleura than did similarly exposed rats (Gelzleichter et al., 1999). The greater translocation in the Syrian golden hamster may thus have accounted for its greater susceptibility to fiber-induced toxicity.

**Pleural Fiber Dosimetry in Humans**

There is virtually no knowledge of the kinetics of fiber translocation and retention in the human pleura and there are few pleural fiber burden studies in occupationally exposed workers. In addition, due to loss of anatomical orientation after ashing or digestion of target tissues, it is not known where fibers reside intracellularly or extracellularly. Fibers are identified within mesothelial cells (Davis, 1974; Fasske, 1986; Lee et al., 1993) but, due to technical limitations, no comprehensive studies have quantified intracellular fiber burden at the microscopic level. Although analytical transmission electron microscopy (TEM) with x-ray energy-dispersive analysis is the gold standard for quantitation and identification of asbestos fibers in tissue, in vivo studies would be enhanced greatly by nondestructive imaging approaches that could detect the presence of fibers, their chemical composition, or even the cellular response without destroying anatomical relationships.

A major data gap in understanding mechanisms of asbestos-related pleural disease is the paucity of information available to determine the dose of asbestos fibers that is deposited and retained in the pleural membranes. This overview describes the technical complexity and limitations associated with quantitation of human lung and pleural fiber burdens, as well as summarizing available data.

Roggli (1990, 1992), Roggli and Sharma (2004), and Dodson and Atkinson (2006) reviewed the numerous variables and technical considerations associated with quantitation of tissue fiber burdens in general. Their major conclusions and caveats include:
1. The source of tissue samples ranged from pleural biopsies obtained during diagnostic thoracoscopy (Boutin & Rey, 1993); to surgical specimens including needle biopsies, wedge biopsies, or pneumonectomy or pleural decortication samples; and to pleural and lung tissues obtained during autopsy examination (Roggli & Sharma, 2004).

2. Regardless of the source of tissue, sampling is a potential source of error since there is significant variation in anatomical distribution of fibers, especially in the parietal pleura (Roggli, 1992; Boutin et al., 1996; Mitchev, et al., 2002).

3. Tissues may be contaminated during surgical resection or at autopsy due to fibers present in fixatives, in specimen containers, on surgical gloves, or on dissecting instruments (Roggli & Sharma, 2004).

4. Light microscopy is inadequate for identification and counting of asbestos fibers. Dodson and Atkinson (2006) recommend analytical transmission electron microscopy in combination with x-ray energy-dispersive analysis and selected area diffraction techniques for specific mineralogical identification. Both coated and uncoated fibers, as well as particulates, should be analyzed and quantitated (Dodson & Atkinson, 2006).

5. A systematic approach to counting fibers of all dimensions and analysis of lung fiber burdens needs to be used, as described by the European Respiratory Society (DeVuyst et al., 1988).

6. Appropriate control populations need to be used because there is significant variability in human lung fiber burdens (Roggli, 1990). A systematic analysis of lung asbestos fiber burdens in workers with asbestos-related disease, people with asbestos exposure in households or in buildings, and control cases revealed a wide range of counts with considerable overlap between workers, other asbestos-exposed cases, and controls (Roggli & Sharma, 2004).

7. The criteria used to define and count asbestos fibers need to be stated explicitly. Some investigators only count fibers longer than 5 \( \mu \text{m} \); however, the majority of asbestos fibers in human tissue samples are shorter than 5 \( \mu \text{m} \) (Dodson & Atkinson, 2006).

8. Tissue preparation techniques may introduce artifacts due to tissue drying or traumatic disruption of fiber bundles (Dodson & Atkinson, 2006).

Finally, although quantitation of human lung and pleural asbestos fiber burden is the only technique available to assess the dose delivered to and retained at the target tissue, there are additional considerations in interpretation of these data. Tissue fiber burden depends on the time since cessation of exposure. In addition, the fiber burden and the types of fibers in the lung may not reflect the fiber burden in the pleura. For example, shorter uncoated fibers are more readily cleared from the lungs; however, while these fibers may be decreasing in the lungs, they may be accumulating in the pleura and extrapulmonary sites (Holt, 1981) and be associated with development of disease at these sites (Dodson & Hammer, 2006; Dodson & Atkinson, 2006).

It is important to note that the lungs of normal control cases evaluated at autopsy contain significant numbers of commercial and non-commercial asbestos fibers, as well as other particulate and fibrous minerals. This is noteworthy especially in lungs from those who resided in urban settings (Table 1). By comparing lung fiber burdens between those with pleural mesothelioma and those without, investigators showed that, although there is overlap, there is an increased risk for mesothelioma, with an elevated lung burden of certain fibers, such as crocidolite, amosite, and tremolite; due to its lower biopersistence, chrysotile may not be reliably analyzed by autopsy studies (Table 2).

In contrast to these and other studies of fiber burdens in the lung, only a few studies have reported asbestos fiber burdens in the pleura. In those few studies that analyzed pleural fiber burden, the results from lung and
TABLE 1. Asbestos Fiber Content in Lung Tissue of an Urban Population

<table>
<thead>
<tr>
<th>Fiber type</th>
<th>Fiber number/g wet lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysotile asbestos fibers</td>
<td>$130.0 \times 10^3$</td>
</tr>
<tr>
<td>Antigorite</td>
<td>$2.5 \times 10^3$</td>
</tr>
<tr>
<td>Noncommercial amphiboles:</td>
<td></td>
</tr>
<tr>
<td>Tremolite</td>
<td>$15.0 \times 10^3$</td>
</tr>
<tr>
<td>Actinolite</td>
<td>$5.1 \times 10^3$</td>
</tr>
<tr>
<td>Anthophyllite</td>
<td>$3.7 \times 10^3$</td>
</tr>
<tr>
<td>Commercial amphiboles:</td>
<td></td>
</tr>
<tr>
<td>Amosite and crocidolite</td>
<td>$1.1 \times 10^3$</td>
</tr>
</tbody>
</table>

Note. Analysis of 21 urban cases using analytical transmission electron microscopy with analysis of all fibers longer than 1 μm revealed these average fiber numbers/g wet lung. (Churg & Warnock, 1980).

TABLE 2. Lung Fiber Burdens in Malignant Mesothelioma Patients

<table>
<thead>
<tr>
<th>Fiber type</th>
<th>Percent of patients with fibers</th>
<th>Percent of controls with fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysotile</td>
<td>80</td>
<td>67</td>
</tr>
<tr>
<td>Tremolite</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Crocidolite</td>
<td>59</td>
<td>16</td>
</tr>
<tr>
<td>Amosite</td>
<td>81</td>
<td>40</td>
</tr>
<tr>
<td>Other:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mullite</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>Iron</td>
<td>88</td>
<td>65</td>
</tr>
<tr>
<td>Rutile</td>
<td>83</td>
<td>79</td>
</tr>
<tr>
<td>Muscovite</td>
<td>61</td>
<td>65</td>
</tr>
<tr>
<td>Silica</td>
<td>55</td>
<td>65</td>
</tr>
</tbody>
</table>

Note. In a study of young persons (age 50 yr or less at the time of diagnosis), the lungs of 69 patients who had died with malignant pleural mesothelioma and 57 controls selected from the national work-related disease surveillance system in the United Kingdom were analyzed by electron microscopy for fiber distribution. Increased odds ratios for mesothelioma were found for crocidolite, amosite, and tremolite; the contribution of chrysotile was less clear due to low biopersistence. Nonasbestos fibers probably made no contribution to mesothelioma in this study (McDonald et al., 2001).

pleura differed, perhaps due to the technical problems described earlier, and appeared to indicate that pleura has a predominance of short chrysotile fibers. Sebastien et al. (1980) concluded that lung fiber burden could not be used as an accurate reflection of pleural fiber burden. In their parietal pleural samples, most of the asbestos fibers were short chrysotile fibers. Gibbs et al. (1991) also reported lower asbestos counts in the visceral pleura than in matched lung samples from the same patients and found mostly short chrysotile asbestos fibers.

Dodson et al. (1990) analyzed lung tissue, lymph nodes, and pleural plaques obtained at autopsy from eight shipyard workers in Italy. Data showed both chrysotile and amphibole asbestos fibers in the lungs; however, chrysotile asbestos fibers were the most frequent type of asbestos found in pleural plaques. Most fibers in the lymph nodes and pleural plaques were shorter than 5 μm, although some fibers longer than 8 μm were present at these sites. More recently, Suzuki and his coworkers (2005) compared asbestos fiber burdens of human mesothelioma tissues obtained following bulk tissue digestion orashing of 25-μm tissue sections using high-resolution analytical electron microscopy. The majority of fibers were ≤5 μm long and 92.7% were ≤0.25 μm wide. Chrysotile asbestos fibers were identified most frequently in a total of 168 cases of human malignant mesotheliomas obtained from biopsy or autopsy specimens (Suzuki et al., 2005).

In an earlier study, Suzuki and Yuen (2001) detected only short, thin chrysotile asbestos fibers in 25.7% of the lungs and in 77.4% of the mesothelial tissues of patients with malignant mesothelioma. These tissue samples were obtained from cases throughout the United States that were sent to Dr. Suzuki for pathological review and were systematically analyzed using histology, immunohistochemistry, and electron microscopy, in some cases, over a 15-yr period. As summarized succinctly by Dumortier et al. (1998) in a letter to the editor in 1998, the size and type of asbestos fibers associated with development of diffuse malignant pleural mesothelioma remain controversial (Mossman et al., 2011; Case et al., 2011; Aust et al., 2011).

One possible explanation for the confusion in pleural sampling came from a pioneering study carried out by Boutin et al. (1996). Using video-assisted fiber-optic thoracoscopy in eight asbestos-exposed patients and six unexposed cases, Dr. Boutin and colleagues (1996) sampled specific anatomic regions of the parietal pleura identified as collecting spots for inorganic particulates and fibers that
translocate to the pleural spaces. These regions are called “black spots” due to localized accumulation of carbon particles and are sites of lymphatic drainage located in the lower coastal regions of the parietal pleura and on the superior dome of the diaphragm. Using transmission electron microscopy, Boutin et al. (1996) identified numerous amphibole as well as chrysotile fibers at black spots, and 22.5% were ≥5 μm long. The mean asbestos fiber concentration in the 8 exposed cases was 12.4 ± 9.8 × 10^6 fibers/g dry lung tissue, 4.1 ± 1.9 × 10^6 fibers/g black spots on the parietal pleura, and 0.5 ± 0.2 × 10^6 fibers/g normal parietal pleura, using bleach digestion of lung tissue and low-temperature ashing of pleural tissue samples. Evidence indicated that asbestos fibers accumulate in focal areas of the parietal pleura and that these “black spots” are the most likely anatomic origin of diffuse malignant mesothelioma. In a subsequent study of black spots analyzed from 150 consecutive autopsies of urban residents in Brussels, Belgium, the histopathological appearance of black spots showed chronic inflammation with lymphocytes, plasma cells, and macrophages with a variety of particulates and fibers both intracellularly and extracellularly (Mitchev et al., 2002). Of note, there was no anatomic relationship between black spots and parietal pleural plaques. Black spots were present in 92.7% of these cases; these lesions were more numerous in older cases and in males. Evidence indicated that these cases may have had greater exposure to coal dust used for home heating and in industry. In this case series, asbestos bodies >1000/g dry lung were found in 15 of 97 cases studied; unfortunately, pleural samples were not analyzed for the presence of asbestos fibers (Mitchev et al., 2002). The discrepancy between studies of pleural fiber burden and disease may thus be explained by the inhomogeneity of fiber deposition in the parietal pleura. Since this important observation of the localization of pleural fibers in black spots, almost no studies addressed pleural fiber burden to clarify which fibers are present and which fibers are associated most closely with asbestos-induced pleural disease, whether neoplastic or non-neoplastic.

**Knowledge and Data Gaps in Fiber Translocation and Dosimetry**

In considering the data existing on the subject of fiber translocation and dosimetry, there are numerous gaps in the knowledge base that may be amenable to newer methods.

(a) There is a significant lack of understanding of the contributions of the various potential routes of fiber translocation, including direct interstitial transport, macrophage-mediated transport, lymphatic transport, and hematogenous transport. There is little known about which fibers move out from the lung to the pleura and at what rate, and which accumulate in the pleura. There is a need to improve our understanding of the kinetics of fiber translocation and pathogenic pleural responses following mixed asbestos fiber exposure and with coexposure to other particulates.

(b) There are gaps in understanding the role of fiber dose, dimension, and type in the induction of pleural lesions. There is a lack of understanding of the relationship between pleural fiber burden and disease in mixed fiber dust exposure. To date, experimental animal studies only examined relatively limited size fractions of fibers, due to limited respirability in rodent studies (Lippmann & Schlesinger, 1984; Lippmann et al., 1980).

(c) There is a need to study the role of short, thin fibers in the induction of pleural lesions. Most pleural disease is believed to be due to amphibole exposure (Churg, 1982; Roggli et al., 2002), and most disease was ascribed to long, thin fibers, but there is still much uncertainty concerning the contributions to disease of short, thin fibers that predominate in pleural fiber burden studies (Dodson et al., 2003; Suzuki et al., 2005; Mossman et al., 2011; Aust et al., 2011; Case et al., 2011). While the preponderance of evidence shows that long, thin fibers are the most pathogenic, there
is little understanding of how dose, surface properties, and biopersistence of short, thin fibers affect the exposure-response relationship with respect to non-neoplastic pleural outcomes in mixed exposures. This need is made more urgent with recent findings concerning the pleural effects of engineered fibrous nanomaterials such as instilled (Poland et al., 2008) and inhaled (Ryman-Rasmussen et al., 2009) carbon nanotubes in mice.

(d) There is a significant lack of information correlating kinetics with pathological outcomes in the pleura following experimental fiber exposures in laboratory animals. Maxim and McConnell (2001) suggested that humans and rats are similar in pathological responses to fibers with respect to pulmonary fibrosis outcomes; as yet, there are no comparable data for pleural fibrosis.

(e) There is need to develop fiber size separation methods to enable mechanistic studies of characterized fiber preparations. This will allow an understanding of the role of fiber size and dimension on cellular targets of pleural disease.

(f) In general, it is not understood how inhalation of fibers leads ultimately to pleural disease. To date there have been few inhalation studies with well-characterized aerosols of different asbestos fiber types in experimental animals. Most inhalation bioassays have been long-term hazard assessment studies in animal models; otherwise, studies have relied on short-term instillation studies in rodents or in vitro studies. The cost, complexity, and specialized requirements of inhalation studies with fibers have not allowed routine state-of-the-art fiber inhalation exposures in support of mechanistic studies.

INTERACTION OF FIBERS WITH TARGET CELLS IN THE PLEURA

Cellular Interactions

For reasons yet to be fully elucidated, fibrous particulates have an unusual affinity for the visceral and parietal pleura, and these tissues are sites for inflammatory, fibroproliferative, and neoplastic diseases in humans and in experimental animals. Non-neoplastic asbestos-associated diseases of the pleura in humans include benign asbestos-related pleural effusion, pleural plaques, diffuse pleural thickening, and rounded atelectasis (Chapman et al., 2003; Nishimura & Broaddus, 1998). Pleural fibrotic and inflammatory lesions develop in rodents in response to inhaled fibers, but these have not been categorized into separate lesion types as is the case in humans and often have not been described separately from pulmonary parenchymal fibrosis by toxicologic pathologists. It is noteworthy that significant pleural lesions similar to human pleural fibrotic lesions were found in chronic rodent inhalation bioassays with synthetic vitreous fibers as well as with asbestos fibers (McConnell et al., 1999).

Mesothelial cells, resident and elicited inflammatory cells, and pleural fibroblasts are believed to be important effector cells in the pathogenesis of asbestos-induced non-neoplastic pleural diseases. Mesothelial progenitor cells may also participate in pleural repair and disease (Herrick & Mutsaers, 2004). There have been numerous studies both in vivo in experimental animals and in vitro using human and animal cell culture systems that review the cellular interactions in pleural tissues and in the pleural space (Chapman et al., 2003; Mutsaers et al., 2004, 2006; Robledo & Mossman, 1999). It is known that following inhalation and instillation of asbestos fibers into the lung, there are rapid alterations in both resident and elicited populations of pleural inflammatory cells and mesothelial cells. Pleural inflammatory cell changes were produced in rats in association with translocation of asbestos fibers (Choe et al., 1997) or following particulate-induced pulmonary inflammation itself (Lehnert et al., 1985). Changes were noted in mesothelial cells of the visceral pleura at early time points following asbestos instillation and inhalation (Adamson, 1997; Dodson & Ford, 1985), and interactions between pleural inflammatory cells and
mesothelial cells are believed to be important in the development of fiber-induced pleural injury and disease. Rat and rabbit pleural mesothelial cells are known to release chemoattractants for inflammatory cells following exposure to asbestos (Boylan et al., 1992; Hill et al., 2003; Tanaka et al., 2000), and pleural macrophage-derived mediators can modulate mesothelial cell function (Baumann et al., 1993, 1996).

**Mesothelial Cell Biology and Fiber Interactions**

During the past two decades, there has been a great increase in our knowledge of the importance of the mesothelial cell in fiber-induced pleural disease. It has become apparent that these cells play a central dynamic role in the control of injury and repair processes that take place in the pleural and other serosal tissues (Mutsaers et al., 2004). Mesothelial cells are a unique cell type originating from mesoderm and vested with a number of important specialized functions including release of pro- and anti-inflammatory and other immunomodulatory mediators; secretion of factors that promote deposition and clearance of fibrin; and synthesis of growth factors and extracellular matrix proteins that aid in serosal repair (Jantz & Antony, 2008). Asbestos may injure pleural mesothelial cells either by direct or indirect mechanisms, including (1) injury by free radicals, (2) inflammasome activation, (3) alterations of intracellular signaling pathways, (4) release of cytokines and chemokines, (5) physical disruption of chromosomes, (6) alterations in growth factors, and (7) changes in coagulation and fibrinolysis pathways (Manning et al., 2002; Mutsaers et al., 2004; Robledo & Mossman, 1999). Mesothelial cells internalize the fibers via integrins or other receptors, and uptake of the fibers was found in some studies to be necessary for adverse effects of the fibers such as reactive oxygen species (ROS) generation, DNA damage, and apoptosis (Liu et al., 2000). Reactive oxygen species derived directly from the surface chemistry of fibers themselves (Fubini, 1997) as well as from cellular responses are believed to be important in both neoplastic and non-neoplastic asbestos-associated pleural disease (Janssen-Heininger et al., 2008; Shukla et al., 2003a). Although the limitations of cell culture systems for particulate studies have been well described, it is recognized that much of the mechanistic understanding of how fibers interact with mesothelial cells and produce fiber-induced effects derives from in vitro experiments (Donaldson et al., 2009).

**Knowledge and Data Gaps Concerning Pleural Cell Biology and Asbestos Fiber Exposure**

(a) Fibers may translocate to the pleural space and are postulated to induce pleural disease by direct interaction with pleural cells. However, some pleural conditions such as inflammation or fibrosis may be influenced by fibers and their actions in the neighboring lung. The relative contribution of direct fiber exposure versus indirect signaling effects on mesothelial cells is not known.

(b) There is still much to know about how fiber mineralogy, dimensions, physicochemical properties, and biopersistence contribute to induction and progression of pleural lesions.

(c) There is need for better development of biomarkers of pleural disease and assessment of pleural changes in experimental animal models. Although bronchoalveolar lavage fluid (BALF) analysis has been routinely utilized in experimental studies of the lung, pleural lavage has not been routinely used to assess changes in the pleural space. Advancing understanding of pleural disease will require better use of pleural endpoints in acute and chronic studies of fiber exposure.

(d) The major target of asbestos in the pleural space is thought to be the mesothelial cell. The contribution of the inflammatory pleural cells including macrophages is less well understood. In addition, there is a need to develop additional understanding of possible mesothelial progenitor cells.
in asbestos-associated injury and disease (Herrick & Mutsaers, 2004).

(e) The thoracoscopic study by Boutin et al. (1996) demonstrating the focal accumulation of fibers in "black spots" of the parietal pleura provided important new insights. Similar studies might advance the understanding of pleural fiber burden and disease. By obtaining biopsy samples from those undergoing thoracoscopy or thoracotomy, one could determine the locations of fibers and the genetic changes at sites of asbestos deposition, and investigate biomarkers of asbestos toxicity. A systematic analysis using analytical transmission electron microscopy could quantitate the dimensions and types of mineral particles and fibers that are translocated to and retained in the pleura (both visceral and parietal) of control individuals and patients with asbestos-related pleural diseases. Such studies may also help identify whether fibers are located predominately intracellularly or extracellularly, and identify the target cells.

(f) The role of the specific arms of the inflammatory response can now be studied using mice with genetically engineered deletion of specific cell types or inflammatory cytokines. Such studies can be used to indicate the role of inflammation in producing the various fiber-induced pleural diseases and whether particular inflammatory mechanisms might be a therapeutic target.

(g) Noninvasive techniques for assessing fiber burden or the tissue reaction to fibers would be of inestimable value in investigating the natural history of pleural reactions in animals and in humans over the decades of tumor development. Novel imaging techniques could ultimately serve as a tool for following those at risk and testing strategies for intervention.

Biological Mechanisms Responsible for Non-Neoplastic Pleural Disease

It has generally been accepted from studies of animal models of asbestos fiber exposure that inflammatory changes in the lung and pleura precede subsequent fibroproliferative and mesothelial cell proliferative responses. The early pioneering intracavitary instillation and implantation studies of Freidrich Pott and coworkers (Pott, 1980; Pott et al., 1974) and Merle Stanton and colleagues (Stanton et al., 1969, 1977, 1981) revealed that the physical properties of fibers such as dimension are important in the pathogenesis of asbestos-associated disease of the serosal tissues (Case et al., 2011; Aust et al., 2011).

Since those initial studies, much has been learned about other physicochemical properties of inhaled particles believed to be important in their disease-inducing abilities such as the surface properties relevant for oxidant generation (Fubini, 1997) and the chemical properties relevant for biopersistence (Bernstein et al., 2001, 2005; Bernstein, 2007; Mossman et al., 2011).

It is noteworthy that up until now biopersistence studies focused on the lung parenchyma. There are few pleural fiber burden or pleural biopersistence investigations in either experimental animals or humans.

Fiber Type and Potency

In rodent studies in which high concentrations of fibers were instilled or implanted in the pleural space, all mineralogical forms of asbestos fibers were produced pleural fibrosis and malignant mesothelioma. In an inhalation study in rats using well-characterized aerosols and state-of-the-art methods to assess retained lung burdens, Bernstein et al. (1995) found that chrysotile exposure failed to induce pleural lesions despite producing severe pulmonary fibrosis (asbestosis) and lung tumors (Mast et al., 1994). This appears to correlate with human epidemiologic studies because recent analysis suggests that most asbestos-associated mesotheliomas are due to amphibole exposure (Berman & Crump, 2008; Mossman et al., 2011).

While there is a general lack of understanding of comparative pleural potency of different asbestos fiber types, a reanalysis of previous asbestos fiber inhalation studies in
rats compared size, shape, and mineralogy with lung tumor and mesothelioma outcomes (Berman et al., 1995). In this study, multivariate measures of exposure were identified that described the lung tumor responses in 13 previous asbestos (chrysotile, amosite, crocidolite, tremolite) inhalation experiments in AF/HAN rats. Due to limitations in the characterization of asbestos fiber dimensions in the original studies, new exposure measures were developed from samples of the original dusts that were regenerated and analyzed by transmission electron microscopy using a direct transfer technique. Structures contributing to lung cancer risk appeared to be long (≥20 μm) and thin (≤0.4 μm) fibers. The analysis did not find significant mineralogical differences in potency across asbestos types for pulmonary tumors but noted that amphibole asbestos was more potent than chrysotile in induction of malignant mesothelioma.

**Proposed Mechanisms for Asbestos-Associated Non-Neoplastic Pleural Lesions in Rodents**

Although the rodent visceral pleura differs markedly from that of humans in thickness and anatomy (Tyler, 1983), the rodent parietal pleura and the resident pleural inflammatory cells are similar to those of humans (Everitt et al., 1997; Gelzleichter et al., 1996). In rodents, asbestos and synthetic vitreous fiber exposure by inhalation or instillation resulted in pleural inflammatory and fibrotic changes (McConnell et al., 1999). In animal models of asbestos-induced lung cancer and mesothelioma, inflammation and fibrosis always preceded the development of oncogenic outcomes (Greim et al., 2001), and these processes may share some mechanistic underpinnings. Similarly, it is worthy of note that in the rodent fiber inhalation bioassays conducted to date, pleural inflammatory and fibroproliferative lesions were accompanied by pulmonary parenchymal changes. Recently, there have been a number of chronic studies that suggested that the Syrian golden hamster may be particularly susceptible to the development of pleural mesothelioma as well as of pleural fibroproliferative changes following asbestos and synthetic vitreous fiber inhalation (Everitt et al., 1997; Gelzleichter et al., 1999; Mast et al., 1994; McConnell et al., 1999).

As described earlier, mesothelial cell responses to translocated fibers and/or responses to inflammatory mediators released from lung parenchymal and pleural cells are believed to be important in the pathogenesis of pleural fibrosis and asbestos-associated pleurisy (Mutsaers et al., 2004; Robledo & Mossman, 1994). Mesothelial cells are known to phagocytize asbestos fibers (Boylan et al., 1995), a step that may then lead to oxidant-induced signaling pathways, altered cell proliferation, apoptosis (Liu et al., 2000; Shukla et al., 2003b), necrosis (Yang et al., 2010), and release of chemokines and cytokines that mediate pleural inflammation (Jantz & Antony, 2008). A variety of growth factors are associated with pleural fibrosis, especially transforming growth factor (TGF)-β1 (Decologne et al., 2007; Mutsaers et al., 2006).

**Proposed Mechanisms for Asbestos-Associated Non-Neoplastic Pleural Disease in Humans**

The nonmalignant manifestations of asbestos in the pleura include benign asbestos pleurisy, pleural plaques, diffuse pleural fibrosis, and rounded atelectasis (ATS Official Statement, 2004; Nishimura & Broaddus, 1998). Although these nonmalignant pleural diseases may themselves produce symptoms, especially diffuse pleural fibrosis, these are important clinically because the symptoms identify people who have had significant exposure to asbestos and often mimic and require diagnostic workups to exclude malignancy. These diseases are also important for research by giving insight into fiber toxicology and pathogenesis and by identifying groups for which development of biomarkers and early intervention for diagnosis or treatment are warranted.

Benign asbestos pleurisy may develop as early as 10 yr after exposure (ATS, 2004). Because it usually produces no apparent
symptoms and is often detected incidentally, the incidence is unclear. The benign pleural effusion may be bloody, thus leading to concern for underlying malignancy. The effusion may last for months, may be unilateral or bilateral, and may recur. The effusion may antedate diffuse pleural fibrosis (Lillis et al., 1988), although the reason for this association is not known.

Pleural plaques are the most common pleural manifestations of asbestos exposure and represent evidence of clinically significant exposure, retention, and biologic response to fibers. In general, plaques develop 20–30 yr after initial exposure (Nishimura & Broaddus, 1998; ATS, 2004). Plaques are usually located on the parietal pleura or on the dome of the diaphragm and appear as circumscribed areas of collagen deposition without inflammation. Plaques may be associated with decreases in lung function and symptoms of dyspnea, but most individuals with pleural plaques alone display no apparent symptoms and no obvious impaired lung function. Although localized and unilateral pleural thickening may have other causes such as prior tuberculosis, trauma, or talc instillation, multiple and bilateral pleural plaques, particularly when calcified, are considered to be pathognomonic for asbestos or erionite exposure (Nishimura & Broaddus, 1998). Of note, those subjects without plaques may also have significant asbestos exposure; it is not known why some exposed individuals form plaques and others do not.

Plaques are biomarkers for asbestos or erionite exposure and of elevated fiber burden in the lung (Churg, 1982; Kishimoto et al., 1989; Roggli & Sanders, 2000). It is not known how pleural plaques correlate with pleural fiber burden. Different fiber types may play a role in plaque formation: Plaques have been associated with the presence of high aspect ratio amphiboles in the lung (Churg, 1982, 1983, 1994), but in at least one study, only chrysotile fibers were found in the plaques themselves (Churg, 1982).

The biologic response to asbestos fibers in individuals with pleural plaques may differ from those without plaques. In animal studies pleural plaques were found to be a consequence of the cellular inflammatory response to asbestos (Sahn & Antony, 1984). In any case, pleural plaques represent a marker of exposure to asbestos and therefore a marker of increased risk for asbestos-related disease, and perhaps may be used to select patients for focused clinical trials to assess other biomarkers of risk and to identify prevention strategies. In general, understanding more about the formation of pleural plaques and their significance as a biomarker of exposure and of enhanced risk would provide insight into pathological mechanisms and suggest possible interventions in exposed populations.

Rounded atelectasis is thought to be a consequence of any type of pleuritis and pleural fibrosis and represents a folding of the lung within a region of pleural thickening. It may resemble a mass and thus raise concern for lung cancer. Little is known regarding the pathogenesis of this entity (Hillerdal, 1989).

Diffuse pleural thickening is a diffuse, not circumscribed, thickening of the pleura that develops approximately 30 yr following exposure (Nishimura & Broaddus, 1998; ATS, 2004). Unlike pleural plaques, diffuse thickening mostly affects the visceral pleura. Diffuse pleural thickening is associated with clinically significant ventilatory impairment, pulmonary restriction, and low lung volumes. Diffuse pleural thickening may coexist with pleural plaques, and may be associated with a higher fiber burden than is found with pleural plaques alone (Stephens et al., 1987). The relationship of diffuse pleural thickening to asbestos-induced fibrosis of the lungs is not known. The types of asbestos fibers likely to produce diffuse pleural thickening are not known. In one study, the fibers found in the pleura were short chrysotile fibers, while the fibers in the lungs were longer and thinner amphiboles (Gibbs et al., 1991). As with other non-neoplastic asbestos-induced pleural disease, diffuse pleural thickening raises concerns for underlying mesothelioma. If lung function is severely compromised, the patient may undergo decortication or removal of the pleura; nevertheless, removal of the thickened pleura may not improve lung function.
due to accompanying fibrosis of the underlying lung.

These non-neoplastic pleural pathologies are particularly common in those exposed to amphibole fibers in Libby, MT (Peipins et al., 2003), suggesting that these fibers may exert unique toxicity for the pleura. Libby amphibole is a mixture of winchite, richterite, and tremolite, in decreasing order of abundance (Meeker et al., 2003). In 1980, a morbidity study was carried out on workers who had used Libby vermiculite as an inert carrier for various types of lawn-care products (Lockey et al., 1984). Libby vermiculite was found to contain asbestiform minerals. In the workers exposed to Libby vermiculite, workplace exposures were associated with bloody pleural effusions and localized pleural thickening. A follow-up study of the worker cohort 25 years after discontinuation of Libby vermiculite mining in 1980 demonstrated an elevated prevalence of pleural changes, increasing from 2% in 1980 to 29% (80 of 280 workers) in 2005 (Rohs et al., 2005). Of workers with a low lifetime cumulative fiber exposure (CFE) of only $<2.2$ fibers/cc-yr, as many as 20% displayed pleural changes. A significant CFE response relationship was demonstrated between percent pleural changes, which ranged from 7 to 54%, and the lowest to the highest CFE quartile. The mean CFE (SD) related to localized pleural thickening, diffuse pleural thickening, and interstitial fibrosis in vermiculite workers with no historical exposure to commercial asbestos was 3.45 (4.95), 8 (5.32), and 11.37 (6.82) fiber-cc/yr, respectively (Rohs et al., 2005). This relationship was confirmed by Whitehouse (2004), who demonstrated progressive loss of lung function in Libby residents with and without reported occupational exposure who had predominantly pleural changes. Studies of Libby miners and millers demonstrated an association between Libby amphibole exposure and increased incidence of nonmalignant respiratory disease mortality at a CFE of less than 4.5 fiber/cc-yr (Sullivan, 2007). In summary, studies of workers exposed to the Libby amphibole indicate the propensity for these amphiboles to induce pleural disease and nonmalignant respiratory morbidity and mortality at relatively low lifetime cumulative fiber exposure levels.

### Knowledge and Data Gaps in Nonmalignant Pleural Disease in Humans

Unanswered questions regarding nonmalignant pleural disease include:

(a) Pleural plaques have been associated with long amphibole fibers in the lung and with short chrysotile fibers in the pleura. Thus, it is not known which types of asbestos fibers induce pleural plaques and how pleural plaques correlate with pleural (not lung) fiber burden.

(b) The fiber burden and fiber types in the pleura of those with pleural disease have not been documented. Fiber burden in the lung may not correlate with that in the pleura: Low counts in the lung may be associated with high counts in the pleura if fibers have translocated to the pleura; fiber types found in the lung may be the ones that are retained at this site, whereas different fibers may translocate to the pleura and induce disease there. Autopsy studies might be used to compare lung and pleural fiber burdens and relative distribution of different fiber types and sizes in these different locations.

(c) Most studies of pleural fiber burden reported the presence of short chrysotile fibers, and yet the role of these short chrysotile fibers in pleural disease has not been established. Because most pleural disease has been attributed to high aspect ratio amphibole fibers, it is not known whether the short chrysotile fibers are pathogenic, either alone or by enhancing the toxicity of longer amphibole fibers, or whether they are acting as bystanders. These fibers may be located outside the area of interest, corresponding to the “black spots” where pathogenic fibers are located. Further animal studies using well-characterized short chrysotile fibers in the pleural space would be valuable in addressing this important issue.
(d) Although the incidence and severity of pleural disease following exposure to Libby amphibole is high, it is not yet known whether it is actually higher than after exposure to other asbestos types. If Libby amphibole is particularly toxic for the human pleura, the mechanism is not known; perhaps Libby fibers are more readily translocated and retained in the pleura or the Libby fibers that reach the pleura are particularly toxic. In vitro and in vivo studies using Libby amphibole can address these important questions.

(e) The genetic determinants of the individual responses to asbestos are not known—genetic factors may determine susceptibility to non-neoplastic and neoplastic pleural disease following asbestos exposure. Similarly, it is not known whether genetic differences explain why some individuals develop a fibrotic response while others have a neoplastic response. Genetic studies of affected individuals and their families would be valuable to address this issue.

(f) New mechanistically oriented, short-term testing strategies need to be developed to assess pathogenicity of fiber and particulate preparations, not only with respect to carcinogenicity but also for fibrotic and inflammatory changes in the pleura. The reason for the propensity of the Syrian golden hamster to develop pleural disease needs further investigation.

(g) There is a need to compare nanoparticle-induced lung and pleural changes with asbestos-associated pleural diseases to identify specific physicochemical determinants of toxicity.

(h) The role of inflammation in the development of pleural fibrosis is not understood. Studies are needed in the role of inflammatory cells and of profibrotic cytokines such as TGF-beta. The role of specific receptors such as the Nalp3 inflammasome and its contribution to chronic inflammatory states (Dostert et al., 2008) need additional investigation.

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**BIOLOGICAL MECHANISMS RESPONSIBLE FOR NEOPLASTIC PLEURAL DISEASE**

**Fiber Type and Potency**

The most potent risk factors for diffuse malignant mesothelioma are environmental or occupational exposure to erionite, asbestos fibers, and vermiculite that contains noncommercial amphiboles (Institute of Medicine, 2006). Based on a recent meta-analysis of the epidemiologic evidence (Berman & Crump, 2008), amphibole asbestos is more potent than chrysotile asbestos in inducing diffuse malignant mesothelioma. This difference in potency was attributed to the greater biopersistence of amphibole asbestos in lungs in comparison with chrysotile asbestos (Bernstein & Hoskins, 2006). Development of diffuse malignant mesothelioma following exposure to chrysotile asbestos is attributed to contamination of some chrysotile deposits with tremolite, a naturally occurring amphibole (Institute of Medicine, 2006).

Biopersistence in the lungs is a key physicochemical property of crystalline mineral fibers and is associated with induction of fibrosis, lung cancer, and malignant mesothelioma in rodent models (ILSI, 2005). Biopersistence in the pleura has not been studied extensively. Boutin and Rey (1993) recovered asbestos fibers in parietal pleural samples of asbestos workers during thoracoscopy. It is likely that long asbestos fibers accumulate at the parietal pleural membrane because they cannot be efficiently cleared through lymphatic stomata. Earlier studies reported low pleural asbestos fiber burdens in asbestos workers (Gibbs et al., 1991). More recent studies recovered large numbers of short asbestos fibers from the lungs and pleural tissues of asbestos-exposed patients (Dodson et al., 2003, 2005, 2007; Suzuki et al., 2005). Mechanistic studies conducted in cell culture associated exposure to long asbestos fibers with activation of the EGF receptor and intracellular signaling pathways leading to cell proliferation (Pache et al., 1998; Mossman et al., 2011). Asbestos fibers were
also found to interfere physically with the mitotic apparatus (Hei et al., 2000; Huang et al., 2011).

Fibers may be altered secondarily in the lungs or pleura. Depending on their chemical composition, surface area, and crystalline structure, asbestos fibers may leach, split, or break (ILSI, 2005). The kinetics of fiber alteration and clearance from the pleural space has not been investigated. Secondary modification of surface properties including binding of phospholipids, acquisition or depletion of cations, and protein adsorption in the pleura may also modify toxicity (Fubini & Mollo, 1995).

### Proposed Mechanisms for Asbestos-Induced Mesothelioma

Asbestos and erionite fibers were shown to induce genotoxicity directly. Chronic rodent studies established an association between persistent inflammation and carcinogenicity induced by inhalation of crystalline mineral fibers (ILSI, 2005). Chronic inflammation triggered in response to biopersistent fibers may amplify the genotoxicity of asbestos fibers in pleural target cells (Figure 2). Following internalization by phagocytosis, asbestos fibers trigger macrophage activation and generation of reactive oxygen and nitrogen species, leading

![Diagram](image)

**FIGURE 2.** Proposed mechanisms for asbestos-induced mesothelioma. Asbestos fibers are thought to lead to mesothelioma via mechanisms as outlined in this algorithm. Asbestos fibers enter the pleural space, where they interact with pleural macrophages and mesothelial cells and induce an influx of inflammatory cells. These early interactions result in release of reactive oxygen and nitrogen species (ROS, RNS), cytokines, and growth factors that may mediate indirect effects on mesothelial cells. The fibers may also act directly on mesothelial cells by inducing DNA damage, interrupting chromosomal segregation, or inducing apoptosis or necrosis. Such direct and indirect actions lead to chronic stimulation and injury of the mesothelium that may proceed over decades by a multistep path to cancer. Key steps in the development of cancer include genetic and epigenetic alterations leading to sustained cell proliferation, resistance to apoptosis, and inactivation of tumor suppressor genes.
to tissue injury. Recent studies in genetically engineered mice suggest a central role for the NALP3 inflammasome in rapid release of active IL-1β (Cassel et al., 2008; Dostert et al., 2008), a cytokine that triggers recruitment of additional inflammatory cells and release of cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-6, IL-8) that perpetuate inflammation in response to biopersistent asbestos fibers (Shukla et al., 2003a). TNF-α also activates the nuclear factor (NF)-κB pathway in mesothelial cells, allowing these cells to survive and proliferate in the presence of asbestos-induced DNA damage (Yang et al., 2006).

Amphibole asbestos fibers contain surface redox-active iron (Fe) that generates ROS leading to lipid peroxidation, protein oxidation, and DNA damage in lung and pleural target cells (Manning et al., 2002). Erionite fibers may secondarily acquire Fe that catalyzes generation of ROS (Hardy & Aust, 1995; Aust et al., 2011). Secondary deposition of endogenous Fe may enhance redox activity or disrupt Fe homeostasis in the lungs or pleura producing oxidative stress (Ghio et al., 2008). In response to chronic oxidative stress, intracellular signaling pathways trigger activation of transcription factors, stimulation of cell proliferation, and resistance to apoptosis (Albrecht et al., 2004; Mossman et al., 2011).

Asbestos in cell culture (Broaddus et al., 1996; Berube et al., 1996) and in some animal studies (Marchi et al., 2000) was found to induce apoptosis in mesothelial cells, and ROS may contribute to this early apoptosis (Broaddus et al., 1996); at later times, DNA or chromosomal damage may also trigger apoptosis. Presumably, it is the cells that have inherent resistance to apoptosis or acquire resistance that will survive the initial and ongoing damage to initiate multistep acquisition of genetic abnormalities that characterize tumor development (Broaddus, 1997). Mesothelial cells (or other progenitor cells) may acquire resistance by inherent overexpression of anti-apoptotic molecules or more likely by upregulation of these molecules, e.g., the Bcl-2 or the IAP family. Mesothelial cells with other preexisting abnormalities in DNA damage-induced signaling or in mitochondrial function may not undergo apoptosis and may persist despite asbestos-induced toxicity (Upadhyay & Kamp, 2003).

Mesothelial cells may have inherent or acquired activation of prosurvival pathways, from either the exposure to asbestos with upregulation of growth factor receptors (Pache et al., 1998) or downstream pathways (MAPK, ERK, Akt/mTOR) or another survival mechanism (Jimenez et al., 1997; Altomare et al., 2005), as proposed with SV40 infection (Kroczyńska et al., 2006). Inflammation may also initiate an environment that itself promotes prosurvival mechanisms. Inflammation may also be induced by the fibers themselves, by an influx of cells of the innate immune system or by asbestos-induced necrosis (Yang et al., 2010). The microenvironment including the presence of inflammatory cells, endothelial cells, and fibroblasts, along with the formation of a three-dimensional shape itself, supports the resistance to apoptosis (Barbone et al., 2008; Daubiari et al., 2009). Critically important survival mechanisms that inhibit apoptosis, if understood, might be a target for intervention (Heintz et al., 2010).

This chronic inflammatory environment may contribute to acquired, heritable genetic, or epigenetic alterations leading to inactivation of tumor suppressor genes, activation of oncogenes, and altered regulation of cell cycle and DNA repair pathways (Kratzke and Gazdar, 2005). Specific genetic, epigenetic, and chromosomal alterations are characteristic of diffuse malignant mesothelioma (Murthy and Testa, 1999; Apostolou et al., 2005). Oxidants generated directly by redox-active asbestos fibers or indirectly following phagocytosis may also induce DNA and chromosomal damage (Jaurand, 1996; Hei et al., 2000; Huang et al., 2011). An indirect mechanism associated with persistent inflammation was proposed for altered gene methylation profiles characteristic of human malignant pleural mesotheliomas (Christensen et al., 2009). These genetic and epigenetic alterations may select for mesothelial cells that are able to survive and proliferate.
in a chronic inflammatory environment (Huang et al., 2011).

**KNOWLEDGE AND DATA GAPS FOR THE BIOLOGIC MECHANISMS FOR NEOPLASTIC PLEURAL DISEASE**

(a) Workers are usually exposed to mixed dusts contaminated with asbestos fibers. It is unknown whether asbestos-related pleural diseases are potentiated by exposure to other dusts such as vermiculite, crystalline silica, or metals in the occupational environment.

(b) The role of SV40 virus as a cofactor with asbestos fibers in the development of diffuse malignant mesothelioma is controversial (Gazdar et al., 2002). Mechanistic studies in cell cultures and in rodents suggest that SV40 viral oncoproteins induce mesothelial cell transformation and diffuse malignant mesothelioma, although human epidemiological studies do not support a causal association (Weiner & Neragi-Miandoab, 2009). Additional epidemiological studies using specific serological markers for SV40 virus infections are needed (Kean et al., 2009).

(c) The physical and chemical properties of mineral fibers associated with carcinogenicity include surface chemistry and reactivity, surface area, fiber dimensions, and biopersistence. The relative importance of these different properties with respect to carcinogenic potency is uncertain and may depend on the geological source of the fibrous mineral and its associated contaminants. Commercial asbestos fibers and erionite fibers have been most widely studied. Noncommercial amphibole fibers, other naturally occurring asbestiform fibers, and newly engineered fibrous nanomaterials (Jaurand et al., 2009; Sanchez et al., 2009) need to be well characterized and their potential for translocation and persistence in the pleura must be determined.

(d) The potential for any natural or engineered fibrous material with physicochemical properties similar to asbestos fibers to induce persistent inflammation and promote the development of diffuse malignant mesothelioma needs to be investigated before the fibers are widely used.

(e) Various direct and indirect mechanisms were proposed for the induction of diffuse malignant mesothelioma by asbestos fibers. These mechanisms may interact at multiple stages during the long latent period associated with this malignancy. The relative importance of these different mechanisms in tumor development and progression is unknown. The ability of different fiber types to induce specific genetic and epigenetic alterations characteristic of diffuse malignant mesothelioma needs to be determined (Andujar et al., 2007).

(f) Chronic rodent inhalation assays are expensive, technically demanding, and not suitable for mechanistic studies because only a minority of rats develop diffuse malignant mesothelioma following inhalation. Current screening assays for fiber toxicity use short-term in vitro or in vivo assays; however, it is difficult to extrapolate from acute, high-dose exposures to chronic or repeated, low-dose exposures in vivo. A new toxicologic screening strategy needs to be developed and validated to assess potential carcinogenicity of naturally occurring mineral fibers and engineered fibrous nanomaterials.

(g) Mesothelial cells are mobile and appear to be able to detach and relocate at other sites in the pleural space (Foley-Comer et al., 2002). Mesothelioma is also associated with mobile spheroids, clumps of malignant cells floating in the pleural fluid; such spheroids appear to remain viable and resistant to apoptosis (Barbone et al., 2008; Daubriac et al., 2009). It is not known whether this mobility may allow preneoplastic cells to move from areas of asbestos accumulation to other areas where mesothelioma may develop. If so, and if new technologies are developed that will allow one to distinguish malignant mesothelial cells from reactive benign mesothelial cells, pleural fluid could be sampled for preneoplastic mesothelial
cells in order to identify individuals at risk for developing diffuse malignant mesothelioma.

(h) New immunohistochemical and molecular markers of preneoplastic and neoplastic lesions would improve early diagnosis and therapy of diffuse malignant mesothelioma (Husain et al., 2009).

(i) Populations exposed to asbestos or asbestiform fibers from Libby MT, workers in certain trades, those exposed on 9/11 at Ground Zero, or those with known high exposure (e.g., those with bilateral pleural plaques) need to be followed in epidemiologic studies that include noninvasive studies of biomarkers and imaging with the potential for more invasive studies using pleuroscopy or pleural lavage. In this way, knowledge can be gained about the natural history of asbestos-induced pleural disease in order to understand preneoplastic changes and ultimately permit early diagnosis or prevention studies.

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Leukocytes in Mammary Development and Cancer

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The Mammary Gland as an Experimental Model (19 articles)
Leukocytes in Mammary Development and Cancer

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Leukocytes, of both the innate and adaptive lineages, are normal cellular components of all tissues. These important cells not only are critical for regulating normal tissue homeostasis, but also are significant paracrine regulators of all physiologic and pathologic tissue repair processes. This article summarizes recent insights regarding the trophic roles of leukocytes at each stage of mammary gland development and during cancer development, with a focus on Murids and humans.

Mammary gland development can be divided into discrete phases. An initial anlage is laid down from the milk-line during embryonic development resulting in a minimal ductal structure emanating from the nipple. Development of this anlage into a ductal tree is reactivated postnatally by exposure to the female sex steroid hormone estradiol-17β (E2), whose synthesis begins upon entry into puberty. In mice, this occurs at about 3 wk of age and is characterized by the formation of terminal end buds (TEB) at the ends of the ducts. These TEBs are clublike multilamine epithelial structures that are the proliferative engines that drive mammary development. These structures also contain the mammary stem cells whose progeny differentiate into luminal and myoepithelial cells. The TEB structures disappear on their encounter with the edge of the fat pad and turn into terminal end-ducts (TED) that cease proliferation and which are bilaminar. As the primary branches grow out through the fat pad, secondary branches form to generate the mature tree that in mice is completed about 8 wk of age coincident with sexual maturity. At each estrus cycle thereafter, there is further development of the secondary branches and dependent on mouse strain, a degree of lobuloalveolar development. The next major phase of growth is during pregnancy in response to progesterone and prolactin when there is significant secondary branching morphogenesis, and the generation of the milk producing lobuloalveolar structures sprouting from these branches. At the end of the process, the gland is filled with ducts and alveolar structures with
a commensurate loss of adipocytes. After birth and on suckling, lactation occurs with its effect on the secretory structure of alveoli that flatten to surround a milk-filled lumen. Weaning terminates the lactational process and the gland involutes to re-form a virgin-like structure to begin the cycle again during the next pregnancy (Daniel and Silberstein 1987; Richert et al. 2000; Neville et al. 2002). Every stage of mammary epithelial development is accompanied by changes in the surrounding stroma. This stroma is populated by many immune cells particularly those of the innate system. Although these cells undoubtedly have a role in immunological responses especially during lactation (Paape et al. 2002; Atabai et al. 2007), this review will concentrate on the trophic roles of these hematopoietic cells at each stage of development and during cancer development, with a focus on Murids and humans.

PUBERTAL MAMMARY DEVELOPMENT

In early postnatal development, classical experiments revealed that instructive signals arise from stromal cells that define the identity of the mammary epithelial structures (Sakakura 1987). In mice, the rudimentary mammary ductal tree begins to develop with the formation of the multilaminate club-shaped TEBs at their distal end. These TEBs grow out through the fatty stroma, bifurcating to generate the primary ductal tree (Richert et al. 2000).

The stroma of the developing mammary gland is dominated by adipocytes (Neville et al. 1998). However, although these cells are required for mammary epithelial development, they do not appear to define its identity (Landskroner-Eiger et al. 2010). Instead adipocytes provide structural support and their secreted adipokines that influence ductal development. Macrophages are found abundantly adjacent to the nipple area and rudimentary ductal structures at 2 wk of age before mammary development commences (Gouon-Evans et al. 2000). Co-incident with the initiation of development the newly formed TEBs is surrounded by a complex stroma containing fibroblasts, macrophages, mast cells, and eosinophils (Gouon-Evans et al. 2000, 2002; Lilla and Werb 2010) (Figs. 1–4). In contrast, neither basophils nor T and B cells can be detected in the vicinity of the TEBs (Gouon-Evans et al. 2002).

The macrophages have a tendency to accumulate around the shaft of the club and in this vicinity they move rapidly along the sheaf of collagen fibrils that align along the axis of the TEB (Ingman et al. 2006) (Fig. 3). Eosinophils are preferentially located around the head of the TEB and they also concentrate in the cleft that forms as the TEBs bifurcate (Gouon-Evans et al. 2000) (Figs. 1, 4). Mast cells are found in a scattered pattern at the invasive front of the TEBs (Atabai et al. 2007; Lilla and Werb 2010) (Fig. 2). Macrophages and eosinophils persist in these locations through development but disappear as soon as the TEBs turn into TEDs (Gouon-Evans et al. 2000; Lilla and Werb 2010). Thereafter, eosinophils, macrophages and mast cells are not present in significant numbers adjacent to the epithelia until development is restarted during pregnancy (Pollard and Hennighausen 1994; Szewczyk et al. 2000; Gouon-Evans et al. 2002; Lilla and Werb 2010). However, macrophages are found throughout the adipose tissue during this and other stages of mammary development (Gouon-Evans et al. 2000; Schwertfeger et al. 2006a).

The mechanisms whereby these innate immune cells are recruited to the mammary epithelial structures have not been fully elucidated, although the process is clearly triggered by estrogen. Genetic depletion of the chemo-attractant eotaxin completely eliminates the recruitment of eosinophils to the mammary gland even though circulating numbers in bone marrow and peripheral blood are normal (Gouon-Evans et al. 2000). Eotaxin is induced in the mammary gland at puberty coincident with eosinophil recruitment strongly suggesting that this is the estrogen-regulated chemoattractant (Gouon-Evans et al. 2000). Studies of mice in which colony stimulating factor-1 (CSF-1) is depleted owing to homozygosity for the Csf1 null mutation, osteopetrotic, (Csf1°°) have shown that many tissues but not all are severely depleted of macrophages. The mammary gland is one of these severely affected tissues indicating...
an essential requirement for CSF-1 (Pollard and Stanley 1996; Gouon-Evans et al. 2000). However, although CSF-1 is expressed by the mammary epithelium (Ryan et al., 2001), transplantation experiments indicate that this epithelial expression is not required for macrophage recruitment during development (Van Nguyen and Pollard, 2002). This suggests that another epithelial-derived chemoattractant recruits the macrophages to the TEB and that

![Figure 1. Macrophage and eosinophil recruitment to the terminal end buds of mice. H&E longitudinal sections of terminal end buds at 5 wk of age. Sections were first stained with H&E (A–C) and then destained and immunostained using anti-F4/80 antibody followed by a peroxidase detection system for positive signal (brown; D,E,F). Note the presence of a dense stroma particularly around the shaft and beginning of the TEB head that consists of fibroblasts and abundant immune cells. This stroma isolates the epithelial compartment from the adipocytes of the fat pad but is sparser at the growing tip. B,C, and E,F are high-powered views of A and D, respectively, and the lower panels boxed in A and D are shown in B and E whereas the upper panels are in C and F. The immunostain indicates the F4/80 positive macrophages (arrows) and eosinophils (filled arrow heads) the latter recognized by their eosinophilic granules and polymorphonuclear structures in C. Note the distinct but overlapping localization of macrophages and eosinophils around the bulbous head and shaft of the TEB. (Figure adapted from Gouon-Evans et al. [2000] and reprinted here with permission from The Company of Biologists © 2000.)

Figure 2. Distribution of mast cells around the terminal end bud of mice. Shown are formalin fixed, paraffin embedded sections stained with toluidine blue in which mast cells are identified by an enzymatic reaction to detect chymase a defining enzyme of these cells. (A) Mast cells (red arrows) shown in the fatty stroma in front of an invading terminal end bud (TEB). (B) Many mast cells (red arrows) adjacent to developing mammary epithelium of mice at 5 wk of age. Black arrow shows a mast cell degranulating. (Panel A reprinted from Lilla and Werb [2010] and reprinted here with permission from Elsevier © 2010; panel B kindly provided by Dr. Zena Werb, USCF). Bar 50 μm.

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CSF-1 is necessary for their lineage development systemically and for their development from monocytes within the tissue. Interestingly, eosinophils situated at the TEB specifically express the monocyte chemoattractant, CCL-6 (C10), suggesting across-talk between these eosinophils and macrophages (Gouon-Evans et al. 2002). However, it is unknown whether CCL-6 is required for macrophage recruitment at this site.

Human mammary development is much less well understood and appears to be less defined as its processes are rather sporadic (Howard and Gusterson 2000). Sexual dimorphism in contrast to the embryonic specification in mice, in humans is initiated by sex hormone secretion at puberty. In females proliferative end budlike structures form and ductal outgrowth occurs that results in the formation of terminal ductal lobular units. According to the individual, variable and extensive branching occurs through puberty that ultimately leads to a structure where primary ducts lead from the nipple to a complex branching pattern of subsidiary ducts that in turn lead to segmental ducts and smaller subsegmental ducts. These subsegmental ducts in turn lead to terminal ducts that lead to blind ended acini. The collection of acini embedded in a complex stroma is referred to as the terminal duct lobular alveolar unit (TDLU). This is thought to be the functional unit of the breast and also thought to be the site of tumor initiation. Strikingly there is great variation in proliferation rates in different TDLUs suggesting the predominance of local factors (Howard and Gusterson 2000). Little is known about hematopoietic cells in human mammary gland during development. Extra-medullary hematopoiesis has been described adjacent to the ductal structures in infant breasts until four months of age (Anbazhagan et al. 1991). Macrophages are abundant cells in nipple aspirates from reproductive age women (King et al. 2002). Intriguingly macrophages in the human breast express aromatase suggesting that they may be a local source of estradiol (Mor et al. 1998). A variation in macrophage density therefore could possibly explain localized differences in growth of the TDLUs.

Mechanistic studies performed in mice strongly argue for important roles for hematopoietic cells in mammary ductal development. Postnatal ablation of bone marrow cells

Figure 3. Association of macrophages and collagen fibers with the terminal end bud. Multiphoton imaging of frozen sections of terminal end buds with nuclei stained with DAPI in which the collagen fibers are shown by second harmonic residence and pseudo-colored in green while macrophages are shown by expression of GFP from the CsIr-promoter (data from Sasmono et al. 2003) pseudocolored to red. In A and B, TEBs from mice heterozygous (+/−) for the Csf1r allele, whereas C is from mice homozygous (−/−) for this allele. Note the sheafing of the TEB with collagen 1 containing fibers and the association of macrophages with these fibers. The tubular structure with the visible collagen sheaf running laterally across the image in B is a blood vessel. In C, the collagen fibers are more disorganized and the TEB is rounder in structure (data from Ingman et al. 2006).
by irradiation before mammary development begins, blocks subsequent mammary development. This developmental block can be overridden by restoration of the bone marrow by transplantation of the irradiated mice, a treatment that results in a complete rescue of the mammary tree. This indicates that irradiation does not irrevocably damage mammary stem cells (Gouon-Evans et al. 2000). In the few mice that survive irradiation without transplantation but who are severely depleted of leukocytes, the mammary gland also fails to develop to any significant extent (Gouon-Evans et al. 2000).

Mice homozygous for the Csf1 null mutation, osteopetrotic, (Csf1<sup>-/-</sup>) have inhibited mammary development characterized by fewer numbers of TEBs, reduced branching, and diminished ductal length compared to wild-type mice. Thus in these macrophage-deficient mice, although a ductal tree eventually develops that fills the fat pad, the resulting gland is atrophic (Gouon-Evans et al. 2000). A similar defect was found in the CSF-1 receptor null mutant mice (Dai et al. 2002). The continuous requirement for macrophages in mammary development was shown by transgenic add-back experiments where CSF-1 was expressed exclusively in the mammary epithelium on a tetracycline-regulated system without rescue of any systemic phenotypes. In these transgenic

Figure 4. Topography of immune cells in the developing terminal end bud. The diagram shows a schematic of a terminal end bud (TEB) that is bifurcating to give two ductal branches. This TEB is surrounded by a dense fibroblastic stoma and encased by a fibrillar collagen network that is aligned in the direction of the outgrowth through the mammary fat pad that is densely populated by adipocytes. Abundant numbers of innate immune cells are recruited to the TEB and have preferred domains as indicated. Macrophages are enriched around the base and shaft of the TEB but move rapidly up and down the collagen fibers. In addition, they are found in the TEB itself where they phagocytose the apoptotic epithelial cells in the process of lumen formation. Mast cells in contrast, are preferentially located in the stroma in front of the invading TEBs where they provide proteases that enhance TEB invasion. Eosinophils in turn, are found around the bulbous head of the TEB and also are found in the cleft of the bifurcating TEB. Genetic ablation of each of these cell types as described in the text indicates roles for them in the branching morphogenesis of the mammary gland and their combined functions results in a fully branched ductal tree that forms during puberty.
mice ductal development was rescued in the homozygous Csf1op null mice coincident with macrophage recruitment, but only for the duration of the CSF-1 expression (Van Nguyen and Pollard 2002). These data together with expression data showing that the CSF-1 receptor is only expressed in macrophages in the mammary gland, indicates an important role for macrophages in mammary ductal development throughout puberty (Gouon-Evans et al. 2000). This conclusion was reinforced by studies that showed macrophage depletion using a conditional suicide gene approach inhibited mammary epithelial cell proliferation in a model of fibroblast growth factor (FGF) receptor-induced epithelial hyperplasia (Schwertfeger et al. 2006b).

The mammary ductal epithelium can be seeded from a single bipotential stem cell (Shackleton et al. 2006). These stem cells can form an entire mammary epithelial tree on transplantation into a recipient mammary fat pad. The efficiency of this process is dramatically reduced in mice depleted for macrophages either by homozygosity of the CSF-1 null mutation or chemically, using the macrophage-specific liposome-encapsulated clodronate (Gyorki et al. 2009). Thus, macrophages potentiate the stem cell niche that enables engraftment and growth of the stem cells. This data is consistent with effects of macrophage depletion on mammary ductal development because this development is driven by proliferation and differentiation of the stem cells that lie in the TEBs.

Depletion of eosinophils using mice homozygous for a null mutation in the gene encoding the eosinophil chemoattractant, eotaxin, resulted in a decreased numbers of TEBs, and reduced branching although ductal lengths were normal (Gouon-Evans et al. 2000). In contrast, depletion of interleukin (IL)-5, another chemoattractant for eosinophils, did not affect mammary development (Gouon-Evans et al. 2002). However, overrecruitment of eosinophils by transgenic IL-5 expression throughout the mammary gland inhibited TEB formation and ductal branching compared to wild-type mice. This in part, was because of an inhibition of epithelial cell proliferation (Sferruzzi-Perri et al. 2003). The unique position of eosinophils in the normal mammary gland and the preferential effect of their depletion on ductal branching strongly suggest that their role is in regulating branching complexity perhaps by providing inhibitory signals at branch point (Gouon-Evans et al. 2000).

Mast cell depletion in mice carrying a mutation in the W locus (W<sup>ash</sup>) that encodes the c-kit receptor required for the formation of these cells results in a reduced number of TEBs and branches as well as defective TEB outgrowth caused by a reduction of epithelial cell proliferation (Lilla and Werb 2010). These data indicate that hematopoietic cells of the innate immune system play an important role in the branching morphogenesis of the mammary gland. The precise location of the different types of cells suggests they have an important role in patterning as over-abundance can also lead to inhibited development. Indeed the migratory nature of these cells makes them perfectly equipped for precisely delivering growth factors or growth inhibitors as well as proteases in a temporal and spatial manner. However, the mechanisms whereby these innate immune cells potentiate mammary development are largely unknown.

TEB proliferation is accompanied by apoptosis so that the multilaminate epithelium is remodeled to give a duct consisting of a single layer of columnar epithelium overlaying a single layer of myoepithelial cells. Failure of this ductal clearance inhibits proper mammary development (Humphreys 1999). Macrophages are found in the TEBs engulfing apoptotic cells and in their absence, this process may be inefficient (Humphreys et al. 1996; Gouon-Evans et al. 2000). However, even in macrophage-deficient mice, the ductal structure is reduced to a single columnar layer (Gouon-Evans et al. 2000) and thus there are likely to be compensatory mechanisms through the action of nonprofessional phagocytes as is found for development of other organs (Dai et al. 2002).

TEBs are surrounded by a collagenous matrix (Hinck and Silberstein 2005; Schwertfeger et al. 2006b) and these structures grow...
out through a funnel of organized fibers that are composed primarily of fibrillar collagen type 1 (Ingman et al. 2006). Intravital imaging of TEBs using multiphoton microscopy of mice expressing green fluorescent protein (GFP) regulated by a Csf1r-promoter (Sasmono et al. 2003) show that macrophages travel up and down these fibers at a fast rate and also “jump” between them (Ingman et al. 2006). Macrophage depletion using Csf1^op/op mice results in a reduced number of these complex fibers compared to wild-type mice but without any effect on collagen 1 synthesis that is from the fibroblasts and epithelial cells. Furthermore the fibers that remain in the macrophage-depleted mice are also less orientated than the fibers in wild-type mammary glands (Ingman et al. 2006). Multiphoton imaging shows that in the absence of macrophages, the orientation of TEB outgrowth is perturbed with a failure to form the smooth fanlike pattern at the ductal front as observed in normal mice. In addition, macrophage deficiency results in TEBs that are rounder and more distorted than those in wild-type mice (Fig. 3). Correction of the macrophage defect by transgenic expression of CSF-1 in the mammary epithelium resulted in restoration of collagen bundling and a correction of the distortion of the TEBs. These data suggest a primary role for macrophages in bundling of these large collagen fibers and that these fibers in turn are required for the guiding the morphogenesis of the TEBs to generate a properly spaced ductal structure (Ingman et al. 2006). The mechanism for this action on collagen bundling is unknown but macrophages synthesize many proteases including matrix metalloprotease (MMP)-7 an enzyme that remodels collagen that might be necessary for the processing of the extracellular matrix (ECM) and in particular, the collagenous structures. Macrophages may also significantly affect other matrix molecules through expression of different proteases as well as molecules involved in matrix remodeling such as Lysyl oxidase and SPARC (Sangaletti et al. 2008). For example, macrophages are rich sources of MMP9 that is involved in ECM remodeling (Djonov et al. 2001; Egeblad and Werb 2002).

Mast cell degranulation is required for ductal outgrowth indicating a requirement for granule products. These products consist of proteases and growth factors. Indeed, depletion of serine proteases with a mutation in the di-peptidyl peptidase 1 (DPPI; cathepsin C) gene that activates many serine proteases results in inhibited mammary ductal development that includes a reduction in the numbers of TEBs and an inhibition in terminal duct formation (Lilla and Werb 2010). Cathepsin C degrades collagens 1 and IV and this protease is synthesized by macrophages as well as mast cells (Gocheva et al. 2010; Lilla and Werb 2010). Thus, cathepsin C alterations of the ECM might also be part of the mechanism behind the effects of depletion of these two cell types on ductal development. However, cell type-specific ablation studies have not been performed to definitively assign these functions to mast cells or macrophages.

PREGNANCY AND LACTATION

Ductal development is re-initiated on pregnancy under the influence of many systemic and local factors including the hormones progesterone and prolactin. Around mid-pregnancy in mice, there is a dramatic growth of lobuloalveolar structures that decorate these newly expanded branches with the mammary fat pad expanding to accommodate the outgrowths. At the end of pregnancy the adipocytes have largely been replaced by the lobuloalveolar structures and a milk-producing gland is fully formed that is 7–10-times heavier than the virgin gland (Atabai et al. 2007). After parturition with its associated loss of progesterone and on pup suckling, a lactational switch is effected and the alveoli secrete milk and appear as flattened cells surrounding large milk-filled lumens (Neville et al. 2002). There are relatively few studies of the pregnant human breast but it appears as in rodents that there is an expansion of lobular-alveolar structures with commensurate loss of adipocytes. The lactating gland has alveoli full of milk that is followed by involution postweaning with the alveolar cells dying of apoptosis (Howard and Gusterson 2000).
Mast cells are found adjacent to the expanding alveolar structures and their density increases according to lobuloalveolar development, but their density significantly declines on the lactational switch (Szewczyk et al. 2000). Macrophages also accumulate during pregnancy and they are found in close apposition to and aligned alongside the epithelial structures (Gouon-Evans et al. 2002). Although these somewhat diminish in numbers during lactation, macrophages are found stretched along the outside of the alveolar walls (Gouon-Evans et al. 2002). Both macrophages and neutrophils are found abundantly in milk of farm species and humans where they are thought to have an antimicrobial role. In addition, B cells are found in milk and this in turn a rich source of antibodies (Atabai et al. 2007). These innate and acquired immune cells are recruited and undergo a transepithelial migration into the alveolar lumens although the mechanisms behind this biology are poorly understood. Nevertheless, it is clear that the lactating mammary gland is an extension of the mucosal immune system such that the milk confers immune protection to the suckling neonate (Brandtzaeg 2010).

Transcriptome analysis of progesterone responsive genes in mammary organoid cultures has also shown up-regulation of many inflammatory molecules several of which are chemoattractants for innate immune cells including serum amyloid proteins A1, 2, and 3 (Saa1-3), suggesting that these molecules may be involved in leukocyte recruitment (Santos et al. 2009). In progesterone treated mice undergoing alveolar development, there was increased expression of SAA1 that is associated with leukocyte recruitment (Santos et al. 2009). CSF-1 is also synthesized in the epithelia of pregnant and lactating mammary glands and milk is a rich source of CSF-1 (Roth 1991; Sapi et al. 1998; Ryan et al. 2001). This epithelial CSF-1 synthesis is regulated by prolactin and progesterone (Sapi and Kacinski 1999). In the absence of CSF-1 in the Csf1op/op null mutant mouse, there is an almost complete absence of macrophages in the pregnant and lactational glands suggesting that CSF-1 is a major chemoattractant and survival factor for these cells during these phases of development (Gouon-Evans et al. 2002).

Transcriptome analysis of total RNA extracted from mammary gland isolated during pregnancy and lactation also revealed a surprising number of immune mediators expressed at these stages. These include T-cell regulatory cytokines and acute phase proteins (Watson 2009). Some of these molecules (such as IL-4 and IL-13), which under most circumstances act on immune cells, appear to play important roles by directly regulating mammary epithelial function (Khaled et al. 2007) whereas others synthesized by the mammary epithelium undoubtedly influence the immune cell population during pregnancy and lactation (Watson 2009). This plethora of immune mediators remains a fertile area both for discovery and for functional studies.

There are few functional studies on leukocytes during pregnancy and lactation except in their immune roles in response to infection, particularly in farm species (Wheeler et al. 1997a,b; Paape et al. 2002; Marshall et al. 2006; Atabai et al. 2007; Bleck et al. 2009). However, loss of CSF-1 in Csf1op/op mice results in a failure of ductal branching and premature differentiation of the lobuloalveolar structures with earlier expression of milk proteins than detected in wild-type mice (Pollard and Hennighausen 1994). This suggests a tradeoff between growth and differentiation during epithelial development. This effect can be partially rescued by recombinant CSF-1 administered subcutaneously (Pollard and Hennighausen 1994). These data showing that macrophage depletion results in a loss of ductal outgrowth during pregnancy reinforces the concept that macrophages have an important role in branching morphogenesis in the mammary gland. Ablation of immune cell types other than macrophages during pregnancy has not been reported and thus their roles in the processes of lobuloalveolar development and during lactation are unknown.

INVOLUTION

The cessation of suckling triggers involution in the mammary gland. Usually this occurs gradually and thus the process is not synchronized.
However, synchronization can be achieved by abrupt removal of pups. This forced weaning model revealed two stages of involution. The first within 24 hours of pup removal is characterized by epithelial cell apoptosis—a process that can be reversed by re-exposure to pups causing lactation to continue. The second phase of involution at around 48 hr postweaning involves massive apoptosis, matrix modeling and regression of the alveolar structure. With the loss of epithelial structures the gland is repopulated with adipocytes and eventually it returns to a virgin-like state (Atabai et al. 2007).

It was originally thought that the professional phagocytic macrophages would be recruited early in involution to ingest the apoptotic bodies. However, this does not appear to be the case (Monks and Henson 2009). Instead macrophage recruitment is only observed 3–4 days postweaning and instead of professional phagocytes, the removal of the early apoptotic bodies is effected through autophagy by adjacent epithelial cells (Monks and Henson 2009). In mice there is also evidence of neutrophils that are recruited before macrophages and, these cells together with macrophages are scattered through the interstitium and in the alveolar spaces (Monks et al. 2002; Atabai et al. 2007). In the human, cells bearing the pan-leukocytic marker CD45, are also found abundantly in the involuting gland (O’Brien and Schedin 2009). This is preceded by the expression of leukocytic chemoattractants including CCL6, CCL7, CCL8, and CXCL14 that are expressed 24 h postweaning (Clarkson et al. 2004). However, the exact role of these chemoattractive molecules remains to be determined.

The other functions of these immune cells in involution, apart from garbage clearance and immunity, remains elusive because transient depletions of particular populations have not been performed. Interestingly Csf1op/op mice are unable to efficiently undergo a lactational switch and consequently there is a rapid involution in these mice (Pollard and Hennig-hausen 1994). However, this may be due less to the biology of the mammary gland and more to do with the failure of these mice to feed their young perhaps because of problems in olfaction required for pup finding (Erblich et al. unpubl.). Recent studies show that recruited macrophages are polarized to a tissue remodeling state and that their presence, just as in the developing gland, is associated with collagen deposition and matrix remodeling (O’Brien et al. 2010). Macrophages have also recently been shown to be important in adipogenesis at least during obesity, through their secretion of adipocyte growth factors and matrix remodeling molecules (Pollard 2009). However, whether they function as such during involution remains to be determined.

The intense remodeling of the mammary gland during involution associated with abundant macrophages may have negative consequences. This immediate postpartum period is associated with a higher risk of breast cancer that can manifest itself in a very aggressive form (O’Brien and Schedin 2009). Because macrophages play an important role in breast cancer progression and promotion of metastasis (see “Myeloid Cells and Breast Carcinogenesis”) (Qian and Pollard 2010), it has been suggested...
that these cells during involution may encourage the growth of stem cells carrying oncogenic mutations through their effects on matrix remodeling and production of growth and angiogenic factors (Schedin 2006; O’Brien and Schedin 2009). Indeed, the matrix formed during early involution is tumor promoting in models of breast cancer when compared with virgin and late-involutio matrix (McDaniel et al. 2006; O’Brien et al. 2010). Interestingly, this increased risk of breast cancer is transient and in the longer term, parity is protective. This has been suggested to be because of sustained expression of transforming growth factor (TGF-β3 (D’Cruz et al. 2002) that in many contexts is tumor inhibiting in part through effects on stem cell senescence (Boulangé and Smith 2001) and inhibition of mammary epithelial cell proliferation (Ewan et al. 2002). TGFβ3 is one of the earliest signaling molecules starting involution by stimulating apoptosis and it is expressed in two waves through involution (Nguyen and Pollard 2000). Coincidently macrophages are also important TGFβ producing and modifying cells suggesting that they may have dual roles in cancer risk dependent on context.

**IMMUNE CELLS AND BREAST CANCER DEVELOPMENT**

Breast cancer (BrCa) is the most frequent malignant tumor of women in North America (Society 2007). Although genetic and epigenetic changes in genes that regulate mammary epithelial cell (MEC) proliferation, survival, polarity, and/or differentiation are likely “initiators” of breast carcinogenesis, several lines of evidence indicate that stromal cell responses in premalignant mammary tissue may “promote” progression to cancer and/or metastatic capability of malignant MECs. Cellular components of tumor stroma include (myo)fibroblasts, vascular cells, infiltrating leukocytes, and specialized mesenchymal support cells unique to each tissue microenvironment.

Although BrCa has not historically been linked to underlying inflammation or infection, it shows tumor-associated inflammation as defined by infiltration of leukocytes into developing tumors where increases in some immune cell subsets in neoplastic stroma parallels disease progression (de Visser et al. 2006; DeNardo and Coussens 2007; DeNardo et al. 2009). Breast cancer development in woman is characterized by a significant increase in the presence of both innate and adaptive immune cells, with B and T lymphocytes as well as macrophages representing the most abundant leukocytes present in neoplastic stroma (DeNardo and Coussens 2007) (Fig. 5). Retrospective clinical studies examining identity of leukocytes in human breast cancer have revealed that high immunoglobulin (Ig) levels in tumor stoma (and serum), and increased presence of extrafollicular B cells, T regulatory (Treg) cells, high ratios of CD4/CD8, or Th2/Th1 T lymphocytes in primary tumors or in draining lymph nodes correlates with tumor grade, stage, and overall patient survival (Shimokawara et al. 1982; Lee et al. 1985; Chin et al. 1992; Punt et al. 1994; Coronella et al. 2001; Coronella-Wood and Hersh 2003; Fernandez Madrid 2005; Kohrt et al. 2005; Bates et al. 2006); thus, some facets of adaptive immunity may indeed be involved in fostering cancer development in the breast. On the other hand, clinical (Leek and Harris 2002) studies show association with poor prognosis and experimental studies have shown that macrophages in primary mammary adenocarcinomas regulate early tumor development by activating angiogenic programs, as well as late-stage carcinogenesis by virtue of their secretion of paracrine factors required for stimulating proinvasive and prometastatic programs in malignant mammary epithelial cells (MECs) (Lin et al. 2001; Wyckoff et al. 2004, 2007; Goswami et al. 2005; Lin and Pollard 2007; Wang et al. 2007; Philippar et al. 2008; Yang et al. 2008; DeNardo et al. 2009; Gocheva et al. 2010).

**MYELOID CELLS AND BREAST CARCINOGENESIS**

Myeloid-lineage immune cells, such as mast cells, macrophages, and neutrophils, have been shown to promote tumor progression by exerting a number of protumoral activities, e.g., by stimulating angiogenesis (Coussens et al. 1999;
Figure 5. Leukocytes in human breast, ductal carcinoma in situ, and invasive carcinoma. (A) Hematoxylin and Eosin (H&E; top row) staining of normal human breast tissue, ductal carcinoma in situ (DCIS) and invasive breast cancer (invasive), showing ductal epithelial structures (E), collagenous stroma (C), and darkly stained immune cells infiltrating stroma (S). Immunodetection of infiltrating leukocytes by CD45 (leukocyte common antigen) immunoreactivity (brown staining; bottom row) reveals significant leukocyte infiltration in DCIS and invasive cancer, as compared to normal breast tissue. (B) Immunodetection of specific lineages of immune cells in invasive breast cancer, B lymphocytes (CD20+; brown staining), CD4+ T lymphocytes (brown staining), CD8+ T lymphocytes (brown staining), macrophages (CD68+; brown staining), neutrophils (neutrophil elastase+; brown staining), and mast cells (chymase+; blue staining). Original magnifications are shown for each panel.
(Puxeddu et al. 2005), and hematopoietic progenitors (Takakura et al. 2000), also participate in angiogenic processes, but it remains to be established whether they can directly promote tumor angiogenesis, rather than having a broader function in supporting tissue inflammation and remodeling.

The potential of neoplastic cells to spread locally (i.e., progress to malignancy) and systemically (i.e., metastasize) is linked to activation of angiogenic vasculature (Hanahan and Weinberg 2000). Tumor-associated blood vessels generated by angiogenesis support tumor growth and development, as well as provide escape routes for malignant cells to intravasate into the circulation (Hanahan and Weinberg 2000; Wyckoff et al. 2007). Although several studies have found that some highly metastatic breast cancer cell lines have up-regulated expression of genes encoding proangiogenic factors such as vascular endothelial growth factor (VEGF) that favor activation of angiogenic vessels (Lee et al. 2007), tumor angiogenesis in the mammary gland, as well as in other organs, is likely initially activated by activated myeloid cells attracted to neoplastic tissue (Coussens et al. 1999; Bergers et al. 2000; Condeelis and Pollard 2006).

In breast carcinomas, macrophages are one of the most abundant innate immune cell types in which they enhance angiogenic programming by production of proangiogenic factors such as VEGF and proteases, i.e., urokinase-type plasminogen activator (uPA) and MMP9 (Lin and Pollard 2007). In a mouse model of mammary adenocarcinoma development, e.g., MMTV-PyMT mice (Guy et al. 1992), increased macrophage infiltration in premalignant tissue occurs immediately before the angiogenic switch and the onset of malignancy (Lin et al. 2001, 2006). CSF-1 is broadly expressed by tumors of the reproductive system and its expression correlates with the extent of leukocyte infiltration and represent a poor prognostic indicator in these tumors (Lin and Pollard 2007). In a mouse model of mammary adenocarcinoma development, e.g., MMTV-PyMT mice (Guy et al. 1992), increased macrophage infiltration in premalignant tissue occurs immediately before the angiogenic switch and the onset of malignancy (Lin et al. 2001, 2006). CSF-1 is broadly expressed by tumors of the reproductive system and its expression correlates with the extent of leukocyte infiltration and represent a poor prognostic indicator in these tumors (Lin and Pollard 2007). In a mouse model of mammary adenocarcinoma development, e.g., MMTV-PyMT mice (Guy et al. 1992), increased macrophage infiltration in premalignant tissue occurs immediately before the angiogenic switch and the onset of malignancy (Lin et al. 2001, 2006). CSF-1 is broadly expressed by tumors of the reproductive system and its expression correlates with the extent of leukocyte infiltration and represent a poor prognostic indicator in these tumors (Lin and Pollard 2007). In a mouse model of mammary adenocarcinoma development, e.g., MMTV-PyMT mice (Guy et al. 1992), increased macrophage infiltration in premalignant tissue occurs immediately before the angiogenic switch and the onset of malignancy (Lin et al. 2001, 2006). CSF-1 is broadly expressed by tumors of the reproductive system and its expression correlates with the extent of leukocyte infiltration and represent a poor prognostic indicator in these tumors (Lin and Pollard 2007). In a mouse model of mammary adenocarcinoma development, e.g., MMTV-PyMT mice (Guy et al. 1992), increased macrophage infiltration in premalignant tissue occurs immediately before the angiogenic switch and the onset of malignancy (Lin et al. 2001, 2006). CSF-1 is broadly expressed by tumors of the reproductive system and its expression correlates with the extent of leukocyte infiltration and represent a poor prognostic indicator in these tumors (Lin and Pollard 2007).
of chemo-attractant receptors. Once in hypoxic areas, macrophages produce a wide array of proangiogenic molecules and matrix-remodeling factors, including IL-8/CXCL8, VEGF, FGF, platelet-derived growth factor (PDGF), MMPs, and uPA, but it remains to be clarified how crucial these macrophage-secreted factors are in the economy of tumor angiogenesis, because many proangiogenic molecules are also produced by other components of the tumor stroma and by the tumor cells themselves.

In addition to the aforementioned proangiogenic factors, macrophages release other molecules that can influence angiogenesis (Pollard 2004; Ojalvo et al. 2009). Macrophages are key producers of TNF-α, that can up-regulate expression of thymidine phosphorylase and MMP-9. Macrophages also produce IL-1 that may increase VEGF transcription by up-regulating expression of HIF-1α through cyclooxygenase (COX)-2. In addition, macrophages also release nitric oxide (NO), a molecule that provokes vasodilatation and increased vascular flow, through the activity of inducible NO synthase (iNOS). Macrophages can also produce FGF, hepatocyte growth factor (HGF), other EGFR ligands (HB-EGF), as well as platelet-derived growth factor (PDGF) and TGFα (Condeelis and Pollard 2006). In particular, leukocyte-derived tumor necrosis factor (TNF)α has been found to enhance invasive/migratory phenotypes of breast cancer cells in culture (Hagemann et al. 2005). In breast cancer cell lines, TNFα regulates epithelial invasion through activation of downstream signaling cascades including Jun amino-terminal kinase (JNK) and nuclear factor kB (NFkB) transcription factor. Activated JNK and NFkB in turn induce gene expression of proinvasive factors such as EMMPRIN (extracellular matrix metallo-protease inducer) and MIF (migration inhibitory factor), whose expression enhances MMP-2 and -9 secretion and activity (Hagemann et al. 2005). Production of TGFβ by alternatively activated macrophages in mammary tumors, mesenchymal support cells, and immature myeloid cells (IMCs) can also enhance the invasive and metastatic programming of malignant cells (Stover et al. 2007; Bierie et al. 2008; Yang et al. 2008) consistent with existence of a TGFβ-responsive gene signature that a predicts breast cancer pulmonary metastasis (Arribas et al. 1997).

Another myeloid population recently implicated in tumor angiogenesis are the so-called immature myeloid suppressor cells (Gallina et al. 2006; Serafini et al. 2006). Myeloid suppressor cells express low to undetectable levels of MHC-II and co-stimulatory molecules, thus cannot induce antitumor responses. Rather, these cells promote tumor development by exerting a profound inhibitory activity on both tumor-specific and nonspecific T lymphocytes and, as recently described, by providing factors essential for tumor growth and neo-vascularization such as TGFβ (Yang et al. 2004, 2008). The frequency of myeloid suppressor cells is significantly increased in the bone marrow (BM) and spleen of cancer patients and mice carrying large tumors. Moses and colleagues (Yang et al. 2004) found that CD11b+Gr1+ myeloid suppressor cells obtained from spleens of tumor-bearing mice promoted angiogenesis and tumor growth when co-injected with tumor cells, largely in response to their high level secretion of TGFβ (Yang et al. 2008). Myeloid suppressor cells also produce high levels of MMP-9, deletion of which minimizes their tumor-promoting activity. Similar to DC precursors, CD11b+Gr1+ cells have also been found to occasionally incorporate into tumor endothelium as endothelial-like cells (Yang et al. 2004).

Concentration gradients of growth factors established by leukocytes present in neoplastic stroma coordinate tumor cell movements toward, and intravasation into, tumor-associated vasculature. For example, macrophages are the primary source of EGF in breast cancer microenvironments (Leek et al. 2000; Lewis and Pollard 2006). EGF promotes invasion/chemotaxis and intravasation of breast carcinoma cells through its interaction with EGFR as shown using an in vivo needle chemotaxis assay through engagement of cofilin-dependent actin polymerization (Wyckoff et al. 2000; Wang et al. 2007). Paracrine interactions between macrophages and breast carcinoma cells form positive feed-forward loops involving...
macrophage-expressed EGF and CSF-1 expressed by neoplastic cells, that together result in breast carcinoma cells showing “high-velocity” polarized movement (chemotaxis) along collagen fibers toward blood vessels directed by perivascular macrophages (Condeelis and Segall 2003; Condeelis and Pollard 2006). These experimental data combined with the positive correlation between CSF-1 levels, macrophage recruitment and poor prognosis in human cancers (Scholl et al. 1994; Leek and Harris 2002), together support the notion that macrophages and/or their products play a major role in facilitating late-stage metastatic progression of tumors (Lin et al. 2001). Significantly a stromal CSF-1 gene expression signature in breast cancer correlated with poor outcomes and was observed to be similar between primary tumors and lymph node metastases (Webster et al. 2010).

Similar to CSF-1, several CC chemokines, particularly CCL2 and CCL5 (RANTES, or regulated on activation normal T cells expressed and secreted), have been implicated in recruitment of monocytes to tumors (Lin and Pollard 2004). CCL2/MCP-1 overexpression by genetically modified tumor cells implanted in mice promoted monocyte uptake by the tumor mass. In human tumors, CCL2/MCP-1 and CCL5/RANTES are produced predominately by neoplastic cells and fibroblasts correlating with macrophage infiltration. Furthermore, both CCL2/MCP-1 and CCL5/RANTES have been found to stimulate monocyte/macrophage-lineage cells to secrete MMP-9 and urokinase-type plasminogen activator (uPA), which through their ECM-remodeling functions are potent activators of angiogenesis (Murdoch et al. 2004). Thus, thanks to their ability to attract proangiogenic monocytes/macrophages to tumors, both CSF-1 and CCL2/MCP-1 can be regarded as major players in the orchestration of the angiogenic process in tumors.

T LYMPHOCYTES AND BREAST CANCER

Clinical evaluation of human breast adenocarcinomas reveals that presence of CD4+ T cells in primary breast cancers positively correlate with markers of disease progression, including metastatic spread to sentinel lymph nodes and increased primary tumor size (Chin et al. 1992; Kohrt et al. 2005). The extent of T-cell infiltration into invasive breast carcinomas has been reported to range from 1%–45% of the total cellular mass (Chin et al. 1992). In rapidly proliferating tumors, presence of T lymphocytes (by histopathological determination) at tumor sites is a good prognostic indicator when compared to nonimmunogenic tumors, and correlates with auxillary lymph node negativity, smaller tumor diameter, lower histological grade, and recurrence-free survival (Aaltomaa et al. 1992); thus, supporting an overall role for T cells in immune surveillance. However, the exact composition of T-lymphocyte infiltration varies greatly and may profoundly affect disease progression and overall patient survival. Perhaps more significant, the ratio of CD4+ to CD8+ T cells or T_{H2} to T_{H1} cells present in primary tumors, where CD4+ or T_{H2} cells are more frequent than CD8+ or T_{H1} cells, correlates with lymph node metastasis and reduced overall patient survival (Chin et al. 1992; Kohrt et al. 2005). More recently, unsupervised expression profiling from breast cancer-associated stroma revealed a gene signature predictive of good prognostic outcome (>98%, 5-year survival) that was functionally enriched for elements of a T_{H1}-type immune response, including genes suggestive of cytotoxic T lymphocyte (CTL) and natural killer (NK) cell activity (Finak et al. 2008). Conversely, high levels of FOXP3+ T_{reg} cells predict diminished relapse-free and overall patient survival (Bates et al. 2006, 2007). Although it is unclear if presence of CD8+ CTLs alone provides any prognostic information for breast cancer, the presence of high percentages of CD4+ T helper cells at primary tumor sites positively correlates with disease progression, e.g., metastatic spread to sentinel lymph node (LN) and primary tumor size (Chin et al. 1992; Kohrt et al. 2005). The interpretation based on these clinical studies is that the type of CD4+ effector T-cell response elicited in an emergent breast cancer may in

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part determine malignant and metastatic potential. Although presence of metastatic MEGs in sentinel lymph nodes draining the primary tumor represents the strongest prognostic indicator for disease progression and overall patient outcome, combinatorial analysis evaluating presence and composition of leukocytes, together with other clinical markers indicative of stage, may provide utility for predicting outcome.

Why are CD8$^+$ CTL-mediated responses not more effective in eradicating or minimizing cancer occurrence and how might CD4$^+$ T cells be involved in enhancing breast cancer progression? One plausible mechanism may have to do with the “polarity” of the CD4$^+$ T helper cell response at primary tumor sites and/or their distant metastases. We recently reported that interleukin (IL)-4-expressing T$_{h2}$ CD4$^+$ T lymphocytes promote invasion and metastasis of mammary adenocarcinomas by directly regulating T$_{h2}$ activity in macrophages, their bioeffector function and EGF expression, that in turn regulate invasive tumor growth, presence of circulating tumor cells (CTCs), and pulmonary metastasis (DeNardo et al. 2009). Using the PyMT mouse model of mammary carcinogenesis (Guy et al. 1992), we found that whereas primary tumor development was unchanged in PyMT/CD4$^{-/-}$ mice, both showed a significantly attenuated metastatic phenotype (DeNardo et al. 2009) similar to that of tissue macrophage-deficient/PyMT mice (Lin et al. 2001). We found that CD11b$^+$Gr1$^+$ monocytes and macrophages of CD4-deficient/PyMT tumors expressed significantly elevated levels of type 1 cytokines (e.g., TNF$\alpha$, IL-6, IL-12p40, and IL-1$\beta$) andNos2 mRNA, indicative of a prevalent T$_{h1}$ immune microenvironment and M1 phenotype (DeNardo et al. 2009), whereas CD11b$^+$Gr1$^+$ monocytes and macrophages from CD4-proficient/PyMT mice were instead indicative of alternatively activated M2 cells that expressed higher levels of arginase-1 (Arg-1) and Tgf$\beta$, thus characterizing a T$_{h2}$ microenvironment (DeNardo et al. 2009). To determine if CD4$^+$ T-cell regulated myeloid cell phenotypes by a T$_{h2}$ pathway, we evaluated CD4$^+$ T cells from LNs and carcinomas of PyMT mice and found elevated expression of GATA3, IL-4, IL-13, and IL-10 (T$_{h2}$) and to a lesser extent T-bet (T$_{h1}$) and the T$_{h1}$ cytokine IFN$\gamma$, but neither FOXP3 (T$_{reg}$) nor IL-17a were expanded as compared to wild-type littermates indicating presence of both T$_{h1}$1 and T$_{h2}$2 effector lineages.

PyMT/IL4R$\alpha$-deficient mice and PyMT mice treated with neutralizing antibodies to IL-4 phenocopied PyMT/CD4-deficient mice with diminished pulmonary metastasis and presence of M1-CD11b$^+$Gr1$^+$ monocytes and macrophages in carcinomas (DeNardo et al. 2009). Moreover, Joyce and colleagues recently reported that IL-4 activation of tumor-associated macrophages was significant for cathepsin B and S production, that together also enhance invasion and metastatic progression (Gocheva et al. 2010). Together this data indicates that T$_{h2}$-CD4$^+$ T cells promote metastasis by enhancing the protumor bioactivities of macrophages, and engaging intracellular signaling cascades (EGF) required for dissemination and metastasis and indicating that blockade of T$_{h2}$-based and/or IL-4/IL-13-regulated pathways may provide a survival advantage by limiting late-stage disease progression and metastatic spread. By comparison, loss of CD4$^+$ T lymphocytes, similarly to loss of macrophage recruitment to mammary tumors, results in reduced presence of CTCs and diminished development of pulmonary metastasis, but without impacting microvessel density or the character of angiogenic vasculature in mammary carcinomas. These distinctions reflect the fact that CD4$^+$ T lymphocyte-derived factors, including IL-4, partially regulate the protumor properties of macrophages, in particular a unique population that promotes invasion and metastasis (Ojalvo et al. 2010), likely because of their impact on EGF expression. Therefore, the proangiogenic properties of macrophages are regulated independently of their T$_{h1}$- or T$_{h2}$-type cytokine expression characteristics, and instead are more likely dependent on other factors such as hypoxia (Lewis and Murdoch 2005; Zinkernagel et al. 2007).

In addition to indirectly potentiating cancer development by regulating protumor properties...
of myeloid cells, work from several groups have reported that IL-4 and IL-13 also regulate tumor growth directly through activation of IL-4/IL-13 receptors on epithelial cells. In some human breast carcinoma cell lines, particularly those that express the estrogen receptor α, IL-4, and IL-13 inhibit both basal and estrogen-induced cell proliferation in vitro and in xenograph tumor models in vivo (Toi et al. 1992; Gingras et al. 2000; Nagai and Toi 2000; Gooch et al. 2002). However, in other breast carcinoma cell lines, IL-4 regulates tumor cell survival by conferring resistance to apoptosis (in vitro) that translates to resistance to chemotherapy in xenograph models (Todaro et al. 2008).

Palucka and colleagues reported that CD4+ T cells directly enhance early breast tumor development in xenograph tumors using humanized NOD/SCID mice, and reported that the ability of CD4+ T cells to produce Th2 cytokines including IL-4 and IL-13 was key (Aspord et al. 2007). Taken together these data indicate that the effects of CD4-derived Th2 cytokines on tumor development and progression is likely regulated by the organ microenvironment or IL-4/IL-13 receptor status of malignant cells, and may also be subject to tumor cell etiology and the specific genetic programs altered during the initiation phase of cancer development. This realization, however, has profound implications for development of future therapeutics targeting CD4+ T cells to either bolster antitumor immunity or neutralize protumor immunity by revealing the complexities of their bioeffector functions that are regulated by multiple factors not common to all tumor models.

**B LYMPHOCYTES, HUMORAL IMMUNITY, AND BREAST CANCER**

During breast carcinogenesis, mature B cells (including naive and activated) can be found in secondary lymphoid tissues as well as in tumor-associated stroma. As compared to healthy patients without evidence of cancer, the sentinel (draining) LNs of breast cancer patients contain enriched populations of proliferating and affinity matured (IgG+) B lymphocytes. Moreover, data from retrospective studies examining percentages of B cells present in sentinel and auxiliary LNs of breast cancer patients reveals that their presence and/or maturation (IgG+) correlates with increases in disease stage (stage I versus stage II) and total tumor burden (Wernicke 1975). Urdiales-Viedma and colleagues used immunohistochemical detection of IgA, IgG, and IgM in axillary LNs from 50 unselected ductal breast carcinomas and found that LNs with IgG+ lymphoid follicles and/or metastatic LNs with IgM+ lymphoid cells were statistically related to breast tumors of high histologic grade and more than three LN metastases (Urdiales-Viedma et al. 1986). These data correlate the presence of various populations of B cells in lymph nodes with malignancy.

During breast cancer development, Ig deposition in neoplastic mammary stroma is known to increase the bioavailability of VEGF by Ig binding to FcRs on tumor-associated macrophages and thus triggering release VEGF into the interstitium (Barbera-Guillem et al. 2002). Moreover, in breast carcinoma tissues, presence of endocytosed Ig in macrophages corresponds with local extracellular VEGF protein levels and local angiogenic vascular buds (Barbera-Guillem et al. 2002).

How might B lymphocytes regulate carcinoma development? A vast literature exists describing the occurrence of (auto)antibodies in either the serum of cancer patients, or interstitial antibody deposition in tumors (Tomer et al. 1998). Early presence of autoantibodies (in particular antinuclear and smooth muscle antibodies) in serum of cancer patients is well known to correlate with unfavorable prognosis (Tan and Shi 2003). Approximately 50% of breast cancer patients contain circulating IgGs that specifically react with tumor-derived antigens—autoantibodies against ErbB2/HER2/neu are present in 20% of patients with ErbB2-positive breast cancer, making it the most common breast cancer “auto-antigen” (Disis et al. 1994). Paradoxically, presence of specific autoantibodies in serum and/or at tumor sites correlates with poor patient survival (Tomer et al. 1998; Tan and Shi 2003; Fernandez Madrid...
2005); thus, perhaps indicating that Igs resulting from chronic B cell activation in response to tumor-specific antigens might promote disease progression. Despite the presence of antitumor antibodies in greater than half of all breast cancer patients, there are only few reports of spontaneous tumor regression (presumed to be immunologic) in the absence of therapy (Sheikh et al. 1979; Lee et al. 1985). Several factors may influence efficiency of antitumor antibodies in inducing tumor regression/destruction, including Ig concentration, HLA expression, tumor tolerance/immune suppression, and impaired cytotoxic T-cell activity. Thus, whether individuals with progressing tumors harbor a higher antigen load that thus triggers enhanced Ig production, or whether increased presence of serum or interstitial Igs predisposes patients to development of more advanced or recurrent cancers, requires further study. That said, B-cell-depletion in MMTV-PyMT mice was shown to be without consequence in regulating early or late-stage mammary carcinogenesis (DeNardo et al. 2009).

CONCLUSIONS

During the last decade, insights have been gained regarding mechanisms underlying the dynamic roles of immune cells as mediators of developmental processes, tissue homeostasis in adult tissues, and tumorigenesis. During the various phase of mammary development the immune system is engaged at every stage. This response appears to be restricted to the innate immune system except during lactation. Although these cells of the innate system are called immune cells this attribution appears to be a misnomer in these developmental contexts as there are no pathogens present. Instead these cells are trophic to developing tissue and act to enhance rates of epithelial growth and invasion through the fat pad and influence complexity of the ductal structures (Pollard 2009). During tumorigenesis a similar cast of innate immune cells are recruited. However in contrast to the developmental context there is a much greater engagement of the acquired immune response. This presence of many acquired immune cells in tumors suggests recognition of new “foreign” tumor antigens or of the extensive tissue damage caused by tumor growth. Indeed in some cases a tumor-directed immune response involving cytolytic CD8+ T cells, Treg cells, and NK cells appears to protect against tumor development and progression. However, if the immune response involves B cells and activation of humoral immunity, and infiltration of Treg cells as well as innate inflammatory cells into an organ harboring initiated tumor cells, the likely outcome is promotion of tumor development and progression. Thus innate and acquired immune cells are in an evolving dynamic that result in the trophic functions of the innate system being conferred on the epithelial tumors in a fashion that mimics development. In mammary cancers of both mice and women macrophages appear to be the dominant innate immune cell type. Differing populations of macrophages confer on the tumor an ability to outgrow, invade, and become vascularized. Unfortunately unlike the developing mammary gland that has intrinsic programs to suppress the external trophic support once it is no longer required, the oncogenic and tumor suppressor mutations in cancer cells result in the loss of these “off” switches. Consequently the tumors obtain continuing support from these recruited immune cells and malignancy is enhanced. However, the balance between a protective cytotoxic response and a harmful humoral or Treg response can be regulated systemically by the general immune status of the individual, as well as locally by myeloid suppressor cells and Treg cells, and thus offers clinicians with attractive targets for anticancer immune-based therapies.

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APPENDIX B

Leukocytes in Mammary Development and Cancer


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Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy


ABSTRACT

Immune-regulated pathways influence multiple aspects of cancer development. In this article we demonstrate that both macrophage abundance and T-cell abundance in breast cancer represent prognostic indicators for recurrence-free and overall survival. We provide evidence that response to chemotherapy is in part regulated by these leukocytes; cytotoxic therapies induce mammary epithelial cells to produce monocyte/macrophage recruitment factors, including colony stimulating factor 1 (CSF1) and interleukin-34, which together enhance CSF1 receptor (CSF1R)-dependent macrophage infiltration. Blockade of macrophage recruitment with CSF1R-signaling antagonists, in combination with paclitaxel, improved survival of mammary tumor-bearing mice by slowing primary tumor development and reducing pulmonary metastasis. These improved aspects of mammary carcinogenesis were accompanied by decreased vessel density and appearance of antitumor immune programs fostering tumor suppression in a CD8+ T-cell-dependent manner. These data provide a rationale for targeting macrophage recruitment/response pathways, notably CSF1R, in combination with cytotoxic therapy, and identification of a breast cancer population likely to benefit from this novel therapeutic approach.

SIGNIFICANCE: These findings reveal that response to chemotherapy is in part regulated by the tumor immune microenvironment and that common cytotoxic drugs induce neoplastic cells to produce monocyte/macrophage recruitment factors, which in turn enhance macrophage infiltration into mammary adenocarcinomas. Blockade of pathways mediating macrophage recruitment, in combination with chemotherapy, significantly decreases primary tumor progression, reduces metastasis, and improves survival by CD8+ T-cell-dependent mechanisms, thus indicating that the immune microenvironment of tumors can be reprogrammed to instead foster antitumor immunity and improve response to cytotoxic therapy. Cancer Discovery; 1(1); 54–67. ©2011 AACR.

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INTRODUCTION

Tumor-associated macrophages (TAM) have been identified as regulators of solid tumor development based on their capacity to enhance angiogenic, invasive, and metastatic programming of neoplastic tissue (1, 2). Colony stimulating factor-1 (CSF1) is a key cytokine involved in recruitment and activation of tissue macrophages, exerting these effects through binding to a high-affinity receptor tyrosine kinase, the cFMS/CSF1 receptor (CSF1R; ref. 3). A second CSF1R ligand, interleukin 34 (IL-34), possesses similar binding affinities and regulates macrophage recruitment to tissues, but exhibits distinct tissue distribution characteristics (4).

Macrophage presence in several types of human cancer, including breast, ovarian, non–small cell lung cancer, and Hodgkin’s lymphoma, correlates not only with increased vascular density but also a worse clinical outcome (1, 5, 6). Accordingly, a CSF1-response gene signature was identified in human breast cancer that predicts risk of recurrence and metastasis, and is similarly predictive for clinical outcome in colon cancer and leiomyosarcoma (7–9).

On the basis of these findings, it seems reasonable to postulate that blockade of the molecular programs enhancing macrophage recruitment or protumor bioactivity in tumors may represent tractable targets for anticancer therapy. Accordingly, genetic or pharmacologic blockade of CSF1 or its receptor has been reported to decrease macrophage presence in tissues and in some experimental solid tumors, correlating with diminished tumor angiogenesis, reduced primary tumor growth, and pulmonary metastasis (1, 2).

Experimental studies have recently revealed that B and T lymphocytes can exert protumor activity indirectly by regulating the bioactivity of myeloid cells, including macrophages, monocytes, and mast cells, resulting in resistance to endocrine therapies and development of metastasis (10–13). We reported that in the absence of a significant CD8+ CTL response, CD4+ T-effector lymphocytes potentiate mammary adenocarcinoma metastasis by directly enhancing the protumor bioactivity of TAMs (11). On the basis of these results, we hypothesized that human breast cancers containing leukocytic infiltrates dominated by CD4+ T lymphocytes and CD68+ macrophages, without significant CD8+ T-cell infiltration, would have a higher relative risk for metastasis and therefore reduced overall survival (OS) of patients. In this article, we report on an immune signature consisting of CD68+high/CD4+low/CD8+low that significantly correlates with reduced OS for patients with breast cancer. Moreover, to demonstrate the biologic significance of this signature, we investigated a combination of standard-of-care chemotherapy and agents that block TAM infiltration of mammary tumors in an aggressive transgenic mouse model of mammary adenocarcinoma development [MMTV-polyoma middle T (PyMT) mice; ref. 14], in which late-stage carcinogenesis and pulmonary metastasis are regulated by CSF1 and tissue macrophages (15). We found that when TAM presence in mammary adenocarcinomas was minimized, antitumor immunity and CD8+ CTL infiltration were enhanced; together, this improved chemosensitivity and resulted in reduced primary tumor development, significant decrease in pulmonary metastases, and improved OS, when compared with treatment using standard chemotherapy alone.

RESULTS

Single Immune Marker Analysis of Leukocyte Infiltration Predicts Breast Cancer Survival

In previous studies, we demonstrated that in the absence of a significant CD8+ CTL response, CD4+ T lymphocytes indirectly promoted invasion and metastasis of mammary adenocarcinomas by directly regulating the protumor bioactivities of TAMs (11). From these data, we predicted that infiltration of primary human breast cancer by CD4+ and CD8+ T lymphocytes and CD68+ macrophages would correlate with aspects of breast cancer regulating OS. To address this issue, we analyzed CD4+, CD8+, and CD68+ leukocyte density in tissue microarrays consisting of tumor tissue obtained at the time of primary surgery from 179 treatment-naïve breast cancer patients (Fig. 1A). We employed a fully automated nuclear algorithm to quantify CD4+, CD8+, and CD68+ cells following immunohistochemical (IHC) detection. For survival analyses, high and low thresholds for each marker were established using a classification and regression decision tree analysis with 10-fold cross-validation (16). Kaplan-Meier analyses indicated that, as single variables, “high” CD4+ T-cell density (P = 0.032) and “low” CD8+ T-cell density (P = 0.012) correlated with reduced OS, whereas CD68+ cell density alone showed no statistical significance (Fig. 1B). However, analysis of CD68+ and CD8+ immune cell infiltration demonstrated an inverse association between stromal infiltration by CD68+ macrophages and CD8+ T lymphocytes in human breast cancer tissues (Spearman’s rho, −0.38; P < 0.001; Supplementary Table S1).

Three-Marker Immune Signature Is Prognostic for Breast Cancer Survival

Heterotypic interactions between diverse leukocyte populations often determine the outcome of immune responses in tissues (17). As such, we proposed that combined analysis of CD4, CD8, and CD68 would allow for improved prognostic stratification of breast cancer patients by assessing both antitumor immunity (i.e., CD8+ density) and protumor immunity (i.e., high CD4+ and CD68+ leukocyte density). Thus, we predicted that an immune profile characterized by CD68+high/CD4+low/CD8+low would represent primary breast cancer controlled by local resection, with improved OS and relapse-free survival (RFS). In contrast, an immune response characterized by CD68+low/CD4+high/CD8+low would instead represent a population of patients at risk for distant metastasis and thus reduced OS. A classification and regression tree algorithm was used to define the signature in the screening cohort (Cohort 1, n = 179; Supplementary Fig. S1). High and low thresholds for each marker were established by decision tree analysis with 10-fold cross-validation.
Figure 1. CD68/CD4/CD8 immune signature is an independent prognostic indicator of breast cancer survival. A, high-magnification images (40×; 80× for inlays) of human breast cancer tissue sections showing immunoreactivity for representative CD68⁺, CD4⁺, and CD8⁺ leukocyte infiltration. B, automated analysis of CD68⁺, CD4⁺, and CD8⁺ immune cell detection, revealing relationship between leukocyte density and OS. Kaplan-Meier estimate of OS comparing auto-score leukocyte-high- and low-infiltration groups is shown; 179 samples from Cohort I were used for analyses, and log-rank (Mantel-Cox) P values are denoted for difference in OS. C and D, Kaplan-Meier estimate of RFS, comparing CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ and CD68⁺⁻/CD4⁺⁻/CD8⁺⁺ immune profiles as assigned by random forest clustering to identify optimal thresholds using Cohort I (C). Identified CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ and CD68⁺⁻/CD4⁺⁻/CD8⁺⁺ immune profiles were used to stratify a second independent cohort, Cohort II (D). Cohort I (n = 179) and Cohort II (n = 498) samples were assessed, and the log-rank (Mantel-Cox) P value is denoted for difference in RFS. E and F, results from multivariate Cox regression analysis of RFS for the CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ signature in Cohort I (E) and Cohort II (F). Hazard ratios (HR) and P values are shown for all characteristics. G and H, RFS in node-positive breast cancer predicted by CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ immune signature, and the same thresholds were then applied to a validation cohort (Cohort II, n = 498 patients, primary tumor samples). Kaplan-Meier analysis in the 2 independent cohorts (totaling 677 patients) showed significantly reduced OS and RFS in patients whose tumors harbored the CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ signature (Fig. 1C and D; Supplementary Fig. S2A and B). Multivariate Cox regression analysis revealed that the CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ signature was an independent predictor of decreased OS and RFS after controlling for grade, nodal status, tumor size, estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki-67 status in both cohorts (Fig. 1E and F; Supplementary Tables S2–S5), indicating that the immune signature predicted OS independently of established histopathologic parameters.

Three-Marker Immune Signature Is an Independent Predictor of RFS in Node-Positive Patients

The OS of breast cancer patients is greatly reduced if metastasis to regional or draining lymph nodes is present at the time of primary tumor detection. Therefore, node-positive patients require aggressive treatment with neoadjuvant or adjuvant systemic chemotherapy, or targeted therapies such as anti-estrogens or trastuzumab. To assess whether immune infiltration by macrophages and T lymphocytes affected the survival of this high-risk group, we examined the impact of the CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ signature following stratification for nodal status. Whereas the CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ signature was not predictive in node-negative patients (Fig. 1G), Kaplan-Meier analysis of Cohort II demonstrated significantly reduced RFS in node-positive patients whose tumors harbored the CD68⁺⁺/CD4⁺⁺/CD8⁺⁻ signature.
(Fig. 1H). Multivariate Cox regression analysis revealed that the CD68hi/CD45mi/CD8lo signature was an independent predictor of decreased RFS after controlling for grade, tumor size, ER, PR, HER2, and Ki-67 status (Supplementary Table S6), indicating that the immune signature predicts RFS independently of established histopathologic parameters. Thus, tumor infiltration by macrophages and T lymphocytes may influence breast cancer recurrence in lymph node–positive patients, a group often aggressively treated with neoadjuvant and adjuvant chemotherapy.

### Cytotoxic Therapies Induce TAM Recruitment and CSF1 and IL-34 Cytokine Expression

These findings led us to hypothesize that blocking TAM infiltration in breast cancer patients bearing the CD68hi/CD45mi/CD8lo signature might enhance antitumor T-cell responses and facilitate CD8+ CTL infiltration and/or repolarization of CD4+ T-cell responses toward T-helper 1 cell (T(H)1). Consistent with this hypothesis, we found an inverse association between stromal infiltration by CD68+ macrophages and CD8+ T lymphocytes in human breast cancer tissues (Spearman’s rho, −0.38; P < 0.001; Supplementary Table S1). On the basis of this observation, we postulated that chemosen-sitivity might in part be regulated by TAM and/or CD8+ T-cell presence in breast cancer tissue. To address this, we initially evaluated leukocyte infiltration in a small cohort of freshly isolated breast tumors from women who had received neoadjuvant chemotherapy, compared with those undergoing primary surgery without preoperative treatment (Supplementary Table S7). We found a higher percentage of CD45+CD11b+CD14+ macrophages in breast cancer from women who had received neoadjuvant chemotherapy than in tumors from women treated with surgery alone (Fig. 2A). In contrast, we observed no difference in tumor-infiltrating CD45+CD3+CD8+ T lymphocytes between the 2 groups (Supplementary Fig. S3A).

To determine whether TAM presence in breast cancers was directly enhanced by chemotherapy, we evaluated leukocyte responses in MMTV-PyMT mice, a transgenic mouse model of mammary carcinogenesis, following treatment with paclitaxel (PTX; Supplementary Fig. S4). We found that infiltration of mammary tumors by CD45+CD11b+Ly6C+Ly6G+F4/80+ TAMs was significantly increased following PTX treatment, with no significant change in the presence of CD3+CD8+ T lymphocytes (Fig. 2B; Supplementary Fig. S3B). Similar PTX-induced TAM recruitment was observed in syngeneic orthotopic PyMT-derived tumors (Supplementary Fig. S3C). Consistent with our hypothesis, PTX treatment of MMTV-PyMT mice only modestly slowed primary tumor growth (Fig. 2B). For studies herein, we defined TAMs as CD45+CD11b+Ly6G+Ly6C+Ly6Chi/F4/80hi, monocytic immature myeloid cells (iMCs) as CD45+CD11b+Ly6G+Ly6Chi/Lo, and granulocytic iMCs/neutrophils as CD45+CD11b+Ly6Ghi/Ly6Chi (Supplementary Fig. S5A and B), in agreement with previously published studies (18, 19).

To reveal molecular mediators involved in regulating PTX-induced TAM recruitment, we examined mRNA expression of several monocye/macrophage cytokines and chemokines in murine mammary epithelial carcinoma cells (MEC) following exposure to several forms of cytotoxic therapy in vitro. CSF1, CCL8/MCP2, and IL34 mRNAs were increased in MMTV-PyMT-derived MECs following exposure to PTX (Fig. 2C). Of these, CSF1 and IL34 mRNAs were also increased following exposure to either CDDP or ionizing radiation (Fig. 2D; Supplementary Fig. S3D). Increased mRNA expression was not merely a response of malignant PyMT-derived MECs (pMEC), because a similar induction was also observed in nontransgenic MECs exposed to PTX in culture (Fig. S3E). Similarly, CSF1 mRNA expression was also induced by PTX and CDDP in 5 of 6 human breast cancer cell lines reflecting the major subtypes of breast cancer (Fig. 2E). In vivo, CSF1 mRNA was increased in mammary tissue of MMTV-PyMT mice following treatment with either PTX or ionizing radiation (Fig. 2F; Supplementary Fig. S3F). Together, these data indicate that induction of CSF1 (and IL34) mRNA and subsequent TAM recruitment into mammary tissue represent a common response of MECs to cytotoxic agents.

### Blockade of Chemotherapy-Induced TAM Recruitment

To determine whether tumor-infiltrating TAMs also regulate sensitivity of MECs to cytotoxic therapy, we blocked TAM infiltration in vivo with immunologic and pharmacologic agents (Supplementary Fig. S4) and evaluated myeloid cell infiltration of tumors from treated mice (Supplementary Fig. S5). Mice bearing orthotopic mammary tumors were treated with neutralizing monoclonal antibodies (mAb) CSF1 (clone 5S1) or CD11b (clone M1/70), or a competitive ATP inhibitor with potent (nM) specificity for CSF1 and cKIT receptor tyrosine kinases (PLX3397), either as a monotherapy or in combination with PTX. CD11b is an integrin cell adhesion molecule expressed on granulocytes, macrophages, monocytes, dendritic cells (DC), and natural killer cells that in part regulates transendothelial migration of cells into tissue and tumor parenchyma. PLX3397 has 10- to 100-fold selectivity for cKIT and CSF1R, as opposed to other related kinases, such as KDR (see Supplementary Fig. S6A and Methods; ref. 20).

Fluorescence-activated cell sorting analysis of the predominant myeloid subtypes infiltrating mammary tumors revealed that either as monotherapy, or in combination with PTX, CD45+CD11b+Ly6C+Ly6G+F4/80+ TAM recruitment was significantly diminished following treatment with either aCSF1 mAb or PLX3397, with no effect on infiltration of CD45+CD11b+Ly6G+ iMCs or CD45+CD11b+Ly6C+CD22+Ly6G+CD11c+MHCIi+ DCs (Fig. 3A and B; Supplementary Fig. S5A and B). Treatment with aCD11b mAb decreased both TAM and iMC infiltration (Fig. 3A). Analysis of the maturation and differentiation status of TAMs remaining in mammary tumor tissue following aCSF1 or PLX3397 treatment revealed no significant change in CD11b+CD11c+ F4/80, CD45, or MHCIi expression (Supplementary Fig. S5B). However, examination of mammary tumor sections revealed a population of perivascular CSF1-independent F4/80+ TAMs remaining (Fig. 3C). Blockade of TAM recruitment was a direct effect of CSF1/CSF1R blockade: In vitro CSF1R inhibition efficiently blocked CD11b+ monocyte chemotaxis in response to control or PTX-treated pMEC-conditioned medium, with no effect on chemotaxis of CD3+ T lymphocytes (Fig. 3D; Supplementary Fig. S5G). These results were mirrored in vivo; treatment of late-stage MMTV-PyMT mice with PLX3397 significantly inhibited both steady-state and PTX-induced tumor infiltration by CD45+CD11b+Ly6C+Ly6G+F4/80+ TAMs (Fig. 3E; Supplementary Fig. S5) without altering TAM maturation/differentiation (Supplementary Fig. S6B).
We next treated 80-day-old MMTV-PyMT mice, or mice bearing syngeneic orthotopic PyMT-derived tumors (~1.0 cm) with cCSF1, cCD11b, or PLX3397 (vs controls) for 5 days, followed by 4 cycles of PTX (10 mg/kg, i.v.; Supplementary Fig. S4). Primary tumor burden at study endpoints (2.0 cm primary tumors or 100 days of age) was significantly reduced in mice treated with combined cCSF1/PTX, cCD11b/PTX, or PLX3397/PTX therapy, compared to mice treated with these as single agents (Fig. 4A and B; Supplementary Fig. S7A). Similar results were observed in syngeneic mice bearing orthotopic PyMT-derived mammary tumors receiving combined PLX3397/carboplatin (CBDCA) therapy (Fig. 4B).

Mammary tumors in MMTV-PyMT mice progress through well-defined stages of cancer development, similar to progression of breast cancer in women, including tissue with florid ductal hyperplasia, ductal carcinoma in situ with early stromal invasion, and poorly differentiated invasive ductal carcinoma (15, 21). Using this staging criterion, we observed that mammary tumors arising in MMTV-PyMT mice treated with combined PLX3397/PTX therapy exhibited decreased development of late-stage carcinoma, compared with tumors in age-matched mice treated with either PTX or PLX3397 as monotherapy (Fig. 4C; Supplementary Fig. S7B). Moreover, the late-stage carcinomas that did develop in PLX3397/PTX-treated mice contained large areas of necrosis (Supplementary Fig. S7C) characterized by increased presence of apoptotic cells, as measured by cleaved caspase 3-positivity (Fig. 4D) with no accompanying change in epithelial proliferation (Supplementary Fig. S7D).

**Decreased Vascular Density Accompanies Improved Chemosensitivity**

It is known that TAMs provide VEGF to developing mammary tumors and thereby regulate angiogenic programming of tissue (22–24). Chemosensitivity to CDDP in MMTV-PyMT mice is in part regulated by myeloid-derived VEGF (25); thus, we sought to determine if TAM depletion altered VEGF expression and/
or density of CD31+ vessels in MMTV-PyMT mice treated with PTX. Whereas total VEGF mRNA expression was significantly reduced by PLX3397 (Fig. 4E), this 70% reduction did not correlate with a change in vascular density (Fig. 4F). In contrast, combined PLX3397/PTX therapy resulted in a significant reduction in CD31+ vessel density within mammary tumors, paralleling induction of apoptosis and necrosis (Fig. 4F).

**CSF1-Signaling Blockade Enhances Antitumor Immunity and CTL Infiltration in Response to Chemotherapy**

Because analysis of human breast cancer tissues revealed that high stromal TAM density inversely correlated with CD8+ T-cell infiltration (Supplementary Table S1), we predicted that depletion of TAMs would enhance CD8+ CTL infiltration and thereby foster an antitumor immune microenvironment. Analyses of tumor-infiltrating T lymphocytes in mice treated with αCSF1/PTX or PLX3397/PTX by flow cytometry or IHC revealed significantly increased presence of CD4+ and CD8+ T cells in mammary tumors (Fig. 5A and B; Supplementary Fig. S8A). Consistent with these findings, cytokine mRNA expression in mammary tissue derived from PLX3397/PTX-treated MMTV-PyMT mice revealed increased mRNA expression of cytotoxic effector molecules, including IFN-γ, granzyme A, granzyme B, perforin-1, and the type 1 DC effector molecules IL12p35 and IFN-α (Fig. 5C). In contrast, expression of the immunosuppressive molecule arginase-1 was
decreased by PLX3397/PTX therapy (Fig. 5C). This reprogramming of the immune microenvironment was accompanied by increased tumor infiltration of CD45+ CD11b−CD19− Ly6G− Ly6C+CD11c+ MHCIId+ DCs (Fig. 5D), indicating that combined treatment of MMTV-PyMT mice with PLX3397/PTX fostered an antitumor immune response by T lymphocytes expressing high levels of cytotoxic effector molecules.

Given these findings, we assessed the capacity of TAMs (isolated from mammary tumors of MMTV-PyMT mice) to directly repress CD8+ T-cell activation in vitro. Using carboxyfluorescein succinimidyl ester (CFSE) dilution as a marker for T-cell proliferation, we found that CD45+ CD11b+/− F4/80+ Ly6G+ Ly6C+ TAMs significantly repressed CD8+ T-cell activation and proliferation in a dose-dependent manner (Fig. 5E) that was reflective of the altered ratio of TAMs to CD8+ T cells in mammary tumors of untreated versus PLX3397/PTX-treated MMTV-PyMT mice (Fig. 5F).

One mechanism by which TAMs may suppress CD8+ T cells involves expression of inhibitory B7 family members that interact with “checkpoint” receptors expressed on infiltrating CD8+ T cells. In particular, growing interest has been expressed in the PD1–PD-L1 ligand system, in which PD-L1/B7-H1 expression by TAMs represents a major source of the inhibitory PD1 ligand. Thus, we evaluated TAMs isolated from mammary tumors for expression of PD-L1/B7-H1 and PD-L2/B7-DC, as well as co-stimulatory molecules CD80 and CD86, and MHCIId. TAMs expressed high levels of MHCIId and B7-H1, but relatively lower levels of CD80 and CD86, indicating a possible role in inducing tolerance/anergy in tumor antigen–specific CD4+ and CD8+ T lymphocytes (Supplementary Fig. S5C).

### Macrophage Depletion Enhances Chemotherapeutic Response in a CD8+ CTL-Dependent Manner

To determine whether increased chemosensitivity of mammary tumors in PLX3397/PTX-treated mice was dependent on enhanced CD8+ T-cell response, we depleted CD8+ T cells from late-stage MMTV-PyMT mice treated with PTX or PLX3397 or both. Findings from this study revealed that the improved outcome with enhanced chemosensitivity resulting...
Figure 5. PTX in combination with PLX397 induces antitumor T-cell response. A and B, tumor infiltration by T lymphocytes enhanced by combined PTX and CSF1 or CSF1R blockade. Flow cytometric analyses of tumor infiltrating CD3+CD8+ and CD3+CD4+ T lymphocytes depicted as the mean number of positive cells, assessed as a percentage of total cells following treatment of MMTV-PyMT mice with PTX or PLX3397 or both (A), or of treatment of mice having orthotopic PyMT-derived tumors with combined PTX/αCSF1 or PTX/PLX3397 (B), compared with controls. Mean values ± SEM are depicted. C, cytokine mRNA expression assessed in orthotopic PyMT-derived tumors from mice treated with PTX alone or in combination with PLX3397. Graph depicts mean fold change in mRNA expression compared with PTX treatment group (5 animals/group). SEM is depicted. D, tumor infiltration by DCs depicted as mean number of total cells from MMTV-PyMT mice treated with PTX or PLX3397 or both (versus controls). E, CD8+ T-lymphocyte activation repressed by TAMs. Purified T cells were loaded with CFSE and activated in vitro by plate-bound CD3/28 and cocultured with the indicated ratio of CD45+Ly6G−Ly6C+CD11b+F4/80+ TAMs to CD3+CD8+ T lymphocytes exhibiting CFSE dilution after 60 hours. Data are representative of 2 independent experiments run in triplicate. Error bars represent SEM. F, analysis of the ratio of tumor-infiltrating CD45+Ly6G−Ly6C+CD11b+F4/80+ TAMs to CD3+CD8+ T lymphocytes depicted as mean ratio (TAM/CD8 CTL) ± SEM from MMTV-PyMT mice treated with vehicle or with PTX and/or PLX3397. *, statistically significant differences (P < 0.05; Mann-Whitney) in A–F; **, statistically significant differences (P < 0.05; Mann-Whitney) between PLX3397-treated groups in F.

from combined PLX3397/PTX therapy was indeed a CD8+ T-cell–dependent response (Fig. 6A and B; Supplementary Fig. S8B). We found that CD8 depletion also resulted in increased tumor grade and decreased presence of cleaved caspase-3–positive cells in mice that had received combined PLX3397/PTX therapy (Fig. 6C and D). Taken together, these data indicate that the enhanced cytotoxic response elicited by CSF1R-signaling blockade was CD8+ T-cell–dependent.

Combined Macrophage Depletion and Chemotherapy Blocks Metastasis in a CD8-Dependent Manner

Long-term survival of breast cancer patients is often limited by disseminated metastases following surgical resection of primary tumors. Analysis of leukocyte profiles in human breast cancers demonstrated that OS, and thus presumably metastatic spread, were regulated by the spectrum of tumor-infiltrating T lymphocytes and macrophages present. In MMTV-PyMT mice, although neither CSF1R-signaling blockade nor PTX therapy alone inhibited development of pulmonary metastasis, mice receiving combined PLX3397/PTX exhibited >85% reduction in pulmonary metastases that was in part CD8+ T-cell–dependent (Fig. 6E).

Macrophages and CD8 Infiltration Predicts Survival and Chemotherapeutic Response

Overall, our data indicated that in the absence of TAMs, antitumor CD8+ CTLs bolster response to chemotherapy and thereby influence outcome; thus, we predicted that TAM and CD8 T-cell ratios would correlate with pathologic responses in patients with breast cancer. Accordingly, we analyzed CD68 and CD8a mRNA expression in a cohort of 311 patients constructed from 2 independent datasets (26, 27). All patients provided fine-needle aspirates (FNA) prior to neoadjuvant chemotherapy, and pathologic response was assessed at the time of definitive surgery. CD68 mRNA expression in FNA samples correlated with pathologic complete response (pCR; R = 0.216; P < 0.001); however, CD68 did not. With median expression as a threshold, examination of both CD8 and CD68 mRNA revealed 3 groups—CD68 > CD8, CD68 < CD8, and CD8 = CD68 (denoted CD68high/CD8low, CD68low/CD8high, and CD68/
Figure 6. Combined PLX3397 and PTX treatment inhibits metastasis in a CD8-dependent manner. A and B, improved outcome following PLX3397/PTX treatment dependent on CD8+ T cells. A, 85-day-old MMTV-PyMT mice were treated with PTX or PLX3397 or both, as well as anti-CD8 IgG. Total tumor burden per animal was assessed every 5 days. B, orthotopic PyMT-derived tumors were grown to a median diameter of 1.0 cm, at which time mice were treated with PTX or PLX3397 or both in combination with anti-CD8 or control IgG for 21 days, and total tumor burden per animal was assessed every 2 to 3 days. Treatment regimens are depicted along with SEM; *, statistically significant differences between mice treated with PTX alone and those treated with PLX3397/PTX. **, significant differences between mice treated with PLX3397/PTX and those treated with anti-CD8 and PTX/PLX3397/control IgG.

C, histologic stage analysis of MMTV-PyMT tumors. Tumors from 100-day-old MMTV-PyMT mice treated with anti-CD8 IgG or with PTX and/or PLX3397 were assessed for presence of premalignant tissue and early- and late-stage carcinoma; data expressed as mean percentage of total gland area ± SEM. D, quantification of cleaved caspase-3-positive cells in mammary tumors of MMTV-PyMt mice treated with anti-CD8 IgG or with PTX and/or PLX3397 versus control (vehicle). Graph depicts mean positive cells per μm² of tumor tissue. E, quantification of metastatic foci per lung section per mouse from 100-day-old MMTV-PyMt mice treated with PTX and/or PLX3397 and/or anti-CD8 IgG, versus controls. Each lung was serially sectioned, 6 sections 100 μm apart were stained with hematoxylin and eosin (H&E), and the total number of metastatic foci (>8 cells) was quantified per mouse (n ≥ 10 mice per cohort). SEM is depicted. *, Statistically significant differences (P < 0.05, Mann-Whitney). Representative photomicrographs of lung tissue sections reveal metastatic foci from 100-day-old MMTV-PyMt mice treated with vehicle or with PTX and/or PLX3397. Scale bar, 500 μm.
DISCUSSION

The immune microenvironment in which a tumor evolves influences multiple parameters of the tumorigenic process. In this article we demonstrate that the immune microenvironment in breast cancer is a predictor of RFS and OS. Moreover, we provide evidence that response to chemotherapy is in part regulated by the immune microenvironment and that cytotoxic therapies induce neoplastic cells to produce monocyte/macrophage recruitment factors, including CSF1 and IL-34, which in turn enhance CSF1R-dependent macrophage infiltration into mammary adenocarcinomas. This is significant in light of our finding that blockade of the CSF1-signaling pathway mediating TAM recruitment, in combination with chemotherapy, decreases primary tumor progression, reduces metastasis, and improves survival—a CD8+ T-cell–dependent outcome resulting from a reprogrammed immune microenvironment that fosters antitumor immunity. These data provide a compelling rationale for combinatorial therapies targeting TAM recruitment, notably CSF1R-mediated signaling pathways, in combination with cytotoxic therapy for breast cancer.

The Immune Microenvironment in Breast Cancer Predicts Outcome

In a previous study, we reported that T-helper 2 (Th2)–CD4+ T-effector cells regulate TAM bioactivity and thereby promote late-stage mammary carcinogenesis and development of pulmonary metastasis (11). We now extend these findings and demonstrate that the complexity of CD8+ T lymphocytes, CD4+ T lymphocytes (presumably Th2 or T regulatory cells or both), and CD68+ TAMs is a predictive biomarker for OS and RFS in node-positive breast cancer (Fig. 1). Retrospective clinical studies have previously revealed that the ratio of CD4+ to CD8+ T lymphocytes, or Th2 to Th1 CD4+ T cells, infiltrating breast cancer correlates with increased tumor grade, lymph node metastasis, and reduced OS (31). Unsupervised expression profiling of breast cancer-associated stroma revealed a gene signature predictive of good prognostic outcome (>98% 5-year survival) that was functionally enriched for genes suggestive of CTL and natural killer cell activity (32). Moreover, elucidation of a CSF1-response gene expression signature in breast cancer demonstrated that CSF1 signaling correlates with response to therapy and OS (7–9). In this article, we revealed that not only does increased macrophage density correlate with poor outcome (Fig. 1), as reported by others, as well (5, 31, 33), but also the ratio of macrophages/CD68 to CD8+ T lymphocytes/CD8a in breast cancer is inversely correlated, an important finding when considering that TAMs can also suppress antitumor immunity. These findings indicate that our immune-based signature may be a useful predictor of recurrence and poor OS for multiple breast cancer subtypes, and, as such, may improve existing gene expression-based prognostic profiling to evaluate risk.

Breast Cancer CSF1 and CSF1R Expression

Our findings demonstrate that macrophage CSF1R signaling is necessary for their recruitment following induction of CSF1 mRNA and interaction with ligand in carcinoma cells treated with chemotherapy (Fig. 3). Recent studies by Patsialou and colleagues (34) demonstrated that in some human breast carcinoma cell lines, specifically MDA-MB231, steady-state CSF1R mRNA is expressed at high levels and autocrine CSF1-CSF1R signaling enhances invasion (44). Notably, MDA-MB231 cells in our studies did not respond to CTX with increased CSF1 mRNA expression (Fig. 3), likely because these cells already express 10- to 50-fold higher levels of CSF1 mRNA than do other breast cancer cell lines evaluated (data not shown). Carcinoma cells isolated from MMTV-PyMT mice do not express significant levels of CSF1R mRNA (15, 22), indicating that CSF1R blockade in MMTV-PyMT mice influences myeloid biology, as opposed to MECs.

Figure 7. Ratio of CD68 to CD8 predicts patient survival and response to neoadjuvant chemotherapy. **A**, frequency of pCR in a cohort of 311 patients constructed from 2 independent datasets. All patients received FNAs prior to neoadjuvant chemotherapy and pathologic response was assessed at definitive surgery. With median expression as a threshold, examination of CD8a and CD68 mRNA in FNA samples revealed 3 separate groups: CD68 > CD8, CD68 < CD8, and CD68 = CD8 (denoted CD68+, CD8+, and CD68/CD8+, respectively). Analysis of the rate of pCR in the groups is shown **B**, Kaplan-Meier estimate of survival, comparing CD68+/CD8+ and CD68+/CD8+ immune profiles as assessed by mRNA expression from 3,872 patient samples assembled from 14 different platforms. Median expression for both CD8 and CD68 was used to determine high and low groups within each of the 22 individual datasets. Once a sample was assigned to a particular group, the 22 datasets were combined and a global survival analysis was performed. The log-rank (Mantel-Cox) P value is shown for difference in survival. **C**, Kaplan-Meier estimate of survival, comparing CD68+/CD8+ and CD68+/CD8+ immune profiles as assessed by mRNA expression from 3,872 patient samples grouped into basal and HER2+ breast cancer. The log-rank (Mantel-Cox) P value is shown for difference in survival.
Immunosuppressive Macrophages and Chemosensitivity

Development and progression of pulmonary metastases in mammary carcinoma is impaired in mice containing a recessive null mutation in the CSF1 gene (15, 35). Similarly, blockade of CSF1R signaling impairs aspects of mammary carcinogenesis (36) and metastases (37). We used αCSF1 mAb and PLX3397, a novel small-molecular-weight tyrosine kinase inhibitor, to efficiently deplete CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>dim</sup>F4/80<sup>+</sup> TAMs (70%) without altering the presence of CD11b<sup>+</sup>Ly6C<sup>dim</sup>F4/80<sup>+</sup> iMCs or perivascular F4/80<sup>+</sup> macrophages in mammary tumor stroma (Fig. 3).

Malignant mammary epithelial cells from MMTV-PyMT mice express high levels of CSF1, which directly regulates TAM recruitment (and EGF expression) and induction of macrophage HIF1α (11, 38, 39). We previously reported that IFN-γ<sup>+</sup> CD8<sup>+</sup> CTL activity is impaired by myeloid-derived ARG1 and nitric oxide synthase that is HIF1α-dependent (38). Thus, on the basis of inverse correlation between TAMs and CD8<sup>+</sup> T cells in human breast cancer, and the fact that TAMs infiltrating mammary carcinomas directly suppress CTL activity in a HIF1α-dependent manner (38), we postulated that TAM depletion would foster antitumor immunity by relieving TAM-mediated CTL suppression and thereby enhance response to cytotoxic therapy. Accordingly, combined treatment of MMTV-PyMT mice with PTX, and either αCSF1 mAb or PLX3397, slowed primary tumor development, reduced development of high-grade carcinomas (Fig. 4), and decreased pulmonary metastasis by 85% (Fig. 6), features of mammary carcinogenesis accompanied by decreased vascular density (Fig. 4), and increased CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltration in primary tumors (Fig. 5). Increased presence of CD8<sup>+</sup> T cells was significant in this regard, as when specifically depleted, the added benefit of combined PLX3397/PTX therapy was lost (Fig. 6).

Immunosuppressive myeloid cells encompass a diverse population of CD11b<sup>+</sup>Gr1<sup>+</sup>Ly6G<sup>−</sup> cells, including myeloid-derived suppressor cells, inflammatory monocytes, neutrophils, and iMCs. Human equivalents have been identified as LIN<sup>−</sup>Lys<sup>−</sup> human leukocyte antigen (HLA)−DR−CD33<sup>+</sup>CD11b<sup>+</sup> and CD14<sup>+</sup>HLA-DR<sup>−</sup>/low cells (40). CSF1R blockade by PLX3397 depleted CD11b<sup>+</sup>Ly6G<sup>−</sup>Lys<sup>−</sup>CD4<sup>+</sup>/80 TAMs, but not CD11b<sup>+</sup>Ly6G<sup>−</sup> cells (Fig. 3C), which are 12-fold less abundant in MMTV-PyMT carcinomas. In contrast, immunosuppressive CD11b<sup>+</sup>Ly6G<sup>−</sup> cells are more abundant in other mammary tumor models, such as 4T1 (41). This may be an important distinction between tumor types considering the fact that monoocyte mobilization from bone marrow is impaired by genetic loss of CSF1, but unaltered following pharmacologic or immunologic inhibition of CSF1R. Gr1<sup>+</sup>Ccr2<sup>−</sup>Cx3cr1<sup>−/−</sup> iMCs are highly responsive to CCL2 (42), and CCL2 (MCP1) is expressed at high levels in MMTV-PyMT mammary tumors (Fig. 2C). Therefore, in extrapolating to the clinical scenario, it will be important to stratify human breast cancers containing predominantly high levels of mature tissue TAMs, compared with those containing LIN<sup>−</sup>Lys<sup>−</sup>HLA-DR<sup>−</sup>CD33<sup>−</sup>CD11b<sup>+</sup> or CD14<sup>+</sup>HLA-DR<sup>−</sup>Cx3cr1<sup>−/−</sup> iMCs, because these breast cancers would likely be less responsive to combinatorial therapy involving CSF1R-targeted agents.

Tissue Specificity and Clinical Implications

Stromal infiltration of TAMs is a poor prognostic indicator for some solid tumor types (43); however, infiltration of TAMs inside tumor nests, particularly when CD8<sup>+</sup> CTLs are also present, can correlate with improved survival outcome (44). These differences might be explained in part by the fact that TAMs produce either protumor or antitumor bioactivities depending on the types of cytokines to which they are exposed (43). TAMs regulated by T<sub>h</sub>1 cytokines including IFN-γ, TNF-α, and granulocyte macrophage colony stimulating factor enhance TAM cytotoxic activity, production of proinflammatory cytokines, and antigen presentation (45). In contrast, tissue macrophages exposed to T<sub>h</sub>2 cytokines, immune complexes, or immunosuppressive cytokines instead block CTL activity and promote angiogenesis and tissue remodeling (43, 45). In non–small cell lung cancer, TAMs that localize to tumor nests and correlate with favorable clinical outcomes exhibit an M1/T<sub>h</sub>1 cytokine profile and express high levels of HLA-DR (46, 47). In contrast, CD163 and CD204 expressing TAMs (M2/T<sub>h</sub>2 markers) correlate with poor clinical outcomes in melanoma, non–small cell lung cancer, and pancreatic cancer (48, 49). We found that tumor tissue from PLX3397/PTX-treated mice had increased IL12p35 and IFNα1 mRNA expression, indicative of bolstered antitumor immunity, indicating that PLX3397/PTX therapy fostered a general reprogramming of the immune microenvironment, in addition to blocking TAM infiltration that together favored CD8<sup>+</sup> T-cell–mediated tumor suppression. Data presented herein do not reveal whether the improved outcome for tumor-bearing mice or the antitumor immune microenvironment fostered under these conditions resulted directly from reduced presence of alternatively activated TAMs, decreased vessel density, or a combination of the two. Given the fact that PLX3397 as monotherapy efficiently reduced TAM presence but had no effect on vessel density, primary carcinoma (Fig. 4), or pulmonary metastasis development (Fig. 6), it seems reasonable to speculate that TAM depletion resulted in loss of an important epithelial cell survival pathway (possibly mediated by EGF) that resists chemotherapy-induced cell death; certainly, however, effects on vascular pathways may also play a role, as has been recently reported by Rolny and colleagues (50). It will be interesting to determine whether directly reprogramming TAMs—for example, by neutralization of IL-4—to favor the presence of classically activated (M1) TAMs, as we have previously reported (11), also similarly enhances antitumor immune programs and chemosensitivity and, if so, whether those responses are also CD8<sup>+</sup> T-cell–dependent.

Microtubule inhibitors constitute one of the most effective classes of cytotoxic agents available for treating both early- and late-stage breast cancer, and are considered the standard of care for treatment of metastatic disease. Several agents that affect microtubule dynamics are active antitumor agents and induce polymerization or cause nonfunctional tubulin aggregates. These compounds block cell division by interfering with function of the mitotic spindle and consequently result in cell-cycle arrest and cell death. PTX is among the most widely used agents in this class. Despite the clinical activity of taxanes, median time to progression in patients treated with PTX is only 6 to 9 months in the first- and second-line setting, and 3 to 4 months in patients with previous exposure to taxanes (51). Although addition of the antiangiogenic agent bevacizumab to PTX improved response and time to progression, it was without impact on OS (52). Glucocorticoid premedication is required for PTX to prevent increased bone marrow and peripheral nerve toxicity as well as allergic reactions and anaphylaxis due to the Cremaphor solvent base. For other cytotoxic agents, glucocorticoids are also commonly used to prevent
toxicities such as nausea, vomiting, and fluid retention. Although these agents are standard additions to many chemotherapies, they suppress production of proinflammatory cytokines and chemokines, severely impair differentiation of antigen presentation by DCs, suppress development of $T_H^1$ cells, and bias immune responses toward $T_H^2$ cell types (53). Our neoadjuvant studies in MMTV-PyMT and orthotopic tumor–bearing mice were performed without dexamethasone, an $H_2$ antagonist, or diphenhydramine. Thus, it is possible that the CD8$^+$ T-cell–dependent antitumor program fostered by combined PLX3397/PTX would have been dampened in the presence of dexamethasone. An understanding of the mechanisms that lead to inadequate or poor response to taxanes is urgently needed, as are prognostic biomarkers that predict which patients will respond favorably. That said, it seems reasonable that administration of cytotoxic agents not requiring steroids, in combination with novel strategies such as TAM ablation (or TAM reprogramming), which together bolster natural antitumor immunity, would improve outcomes and extend long-term survival for patients with breast cancer, as well as other cancer types. The clinical outcome of pharmacologically (or immunologically) targeting TAMs directly or the pathways that regulate their recruitment must be considered carefully because all cancer types may not respond in a similar fashion. This study provides a compelling rationale for clinical evaluation of combinatorial therapies inhibiting TAM recruitment, in combination with standard-of-care chemotherapy for treatment of breast cancer, and underscores the importance of identifying a population of patients who, by virtue of their immune profile and CSF1R status, may benefit most from such therapies.

**METHODS**

**Patients and Tumor Samples**

Tissue microarray studies were conducted on 2 separate patient cohorts. The screening cohort, Cohort I, described elsewhere in detail (54), was constructed from 179 cases of invasive breast cancer diagnosed at the Department of Pathology, Malmö University Hospital, Malmö, Sweden, between 2001 and 2002. The median age at diagnosis was 65 years of age, and the median follow-up time for OS was 52 months. Patients had not received neoadjuvant therapy and were treated with either modified radical mastectomy or wide local excision. The median tumor size was 2.2 cm; 62% of the tumors were PR-positive and 72% were ER-positive. Complete endocrine treatment data were available for 143 patients, 67 of whom received adjuvant tamoxifen, 3 an aromatase inhibitor, and 25 a combination of tamoxifen and an aromatase inhibitor. Information on adjuvant chemotherapy was available for 143 patients, of whom 30 received treatment. The second (validation) cohort, Cohort II, included 498 patients with primary invasive breast cancer diagnosed at the Malmö University Hospital between 1988 and 1992. These cases belonged to an original cohort of 512 patients, as previously described in detail (55). The median age at diagnosis was 65 years, and the median follow-up time to first breast cancer event was 128 months. Information regarding the date of death was obtained from regional cause-of-death registries for all patients in both cohorts. Complete endocrine treatment data were available for 379 patients, 160 of whom received adjuvant tamoxifen. Information on adjuvant chemotherapy was available for 382 patients, of whom 23 received treatment. To assemble tissue microarrays, clearly defined areas of tumor tissue were indicated on a slide with a fresh tissue section from the paraffin block. Two biopsy samples, 1.0 mm in diameter, were taken from each donor paraffin block corresponding to the marked area. Recipient blocks were limited to ~200 cores each. In general, cores were taken from the peripheral aspect of the tumor. Necrotic tissue was avoided. For IHC analyses, 4-μm paraffin sections were used. The ethical committee at Lund University (Malmö, Sweden) approved this study.

**Automated Image Acquisition, Management, and Analysis**

Fully automated image acquisition was used for the results presented in this article. The Aperio ScanScope XT Slide Scanner (Aperio Technologies) was used to capture whole-slide digital images with a 20× objective. Slides were dearrayed to visualize individual cores, using Spectrum software (Aperio). A tumor-specific nuclear algorithm (IHC-MARK) developed in house was modified to quantify CD4, CD8, and CD68 expression. IHC-MARK was initially designed to identify tumor cells on the basis of nuclear morphologic features (56); however, this was modified for evaluating leukocyte infiltration based on specific nuclear morphologic features.

**Image and Statistical Analysis**

A decision tree–supervised algorithm was used to group patients based on immune cell IHC density. For decision tree analysis, all patients were randomly divided into 10 subsets. A decision tree model was selected using a 10-fold cross-validation approach (16). Ten consecutive decision tree models were independently constructed with the immune cell infiltration density continuous output from 9 subsets. Survival outcome predictive power of each decision tree model was tested on the remaining set of patients, and the model with the higher accuracy was selected as optimal for the dataset. Kaplan-Meier analysis and the log-rank test were used to illustrate differences between OS and RFS according to individual CD68, CD4, and CD8 expression. A Cox regression proportional hazards model was used to estimate the relationship to OS of the CD68/CD4/CD8 immune profile; lymph node status; tumor grade; and HER2, PR, and ER status in the patient cohorts. A P value of <0.05 was considered statistically significant, and calculations were assessed using Statistical Package for the Social Sciences (SPSS, Inc.).

**Neoadjuvant Cohort**

The neoadjuvant cohort consisted of 2 gene expression cohorts representing 311 patients treated with neoadjuvant chemotherapy (26, 27), with complete pathologic response in 60 (19%) patients. The majority of patients received PTX and fluorouracil-doxorubicin-cyclophosphamide. Both datasets were examined on the same array platform (Affymetrix U133A), using a standard operating procedure and normalization method (dCHIP) as previously reported (57, 58). Data were downloaded from the Gene Expression Omnibus (59) and an institutional website (60). Normalized expression values for both CD8 and CD68 were established as previously described (54).

**Preclinical Mouse Models and Animal Husbandry**

Mice harboring the PyMT transgene under the control of the MMTV promoter in the FVB/n strain were obtained from Dr. Zena Werb [University of California, San Francisco (UCSF), San Francisco, California] and have been previously described (14). Two murine models of mammary tumor development were used to analyze response to chemotherapy (Supplementary Fig. S3). The first model used MMTV-PyMT mice (Supplementary Fig. S3A). The 80-day-old MMTV-PyMT female littermates were randomized by initial tumor volume and fed either PLX3397 (20, 61, 62) formulated in mouse chow or control chow (provided by Plexxikon Inc.). PLX3397 was formulated in mouse chow so that the average dose per animal per day was 40 mg/kg. When PLX3397-treated MMTV-PyMT mice reached 85 days of age, they were then administered PTX (Hospira) every 5 days by i.v. injection into the retroorbital plexus. PTX was given at 10 mg/kg of the animal per injection, diluted in PBS. Tumor burden was evaluated by caliper measurement every 5 days following the start of PLX3397 treatment. Prior to tissue collection, mice werecardiac-perfused with PBS to clear peripheral blood. Mammary tumor tissue from PBS-perfused MMTV-PyMT mice was analyzed by flow cytometry and qRT-PCR 2 days after the second dose of PTX, when metastatic burden and tumor grade were determined. Primary tumor burden was determined by caliper measurements on live sedated mice. Metastatic burden was assessed by serial sectioning of formalin-fixed paraffin-embedded lung tissue whereby the entire lung was sectioned and the number of metastatic foci (>5 cells) was determined on 6 sections taken every 100 μm following H&E staining. Lungs from >10 mice/group were analyzed.
To assess tumor grade, the stage characterization technique classified tumor tissue into 3 levels of histologic progression by quantifying the area of transformed glands occupied by each stage (15, 38). Progression follows from a “precancerous stage” characterized by premalignant hyperplasia and adenoma/mouse intestinal epithelium but with the retention of some normal ductal and acinar mammary gland morphology, to a more epithelial cell–dense “early carcinoma” with some stromal invasion, and finally to an invasive, high–mitotic index “late-stage carcinoma.”

The IHC analysis was conducted on tissue sections following the end of studies on 100-day-old MMTV-PyMT mice (detailed in Supplementary Fig. S4A). Vehicle-treated mice received PBS-only injections. We also used a syngeneic orthotopic implantable tumor model (referred to as PyMT-implantable in all figures and detailed in Supplementary Fig. S4B). For this model, single-cell suspensions of tumor cell pools isolated from mammary tumors of 3 or 4 100-day-old MMTV-PyMT mice were generated following collagenase A digestion (see discussion of flow cytometry analysis earlier). A total of 1.0 million tumor cells from pools were diluted in medium and basement membrane extract (Matrigel, BD Pharmingen) and injected orthotopically into uncleared mammary fat pads (4th gland) of 10-week-old virgin FVB/n female mice. Following implantation, tumors were allowed to grow to a mean diameter of 1.0 cm before enrollment into studies. Mice were randomized into treatment groups based on tumor size and treated with PLX3397 and PTX, as described above. For some studies, CBDDCA (Hospira) was used and administered at 10 mg/kg of mouse per injection, in a similar manner to administration of PTX (see above). For mice with implantable tumors, tumor burden was evaluated by caliper measurement every 2 to 3 days following the start of PLX3397 treatment, and mammary tissue was analyzed by flow cytometry, IHC, and qRT-PCR at the end of the study (Supplementary Fig. S3B). Immune-depleted mice were injected i.p. every 5 days with either 1.0 mg anti-CD8 immunoglobulin G (YTS169.4) or isotype control rat immunoglobulin on day 1 followed by 500 μg every 5 days. All mice were maintained within the UCSF Laboratory for Animal Care barrier facility, and the UCSF Institutional Animal Care and Use Committee approved all experiments involving animals.

Additional information on methods and cohorts is available in the Supplementary Data.

Disclosure of Potential Conflicts of Interest

E. Rexhepaj, D.J. Brennan, and W.M. Gallagher are inventors of a pending patent application in relation to the development of novel automated image analysis approaches in histopathology, and D.G. DeNardo, D.J. Brennan and L.M. Coussens are inventors of a pending patent application in relation to immune-based signatures for predicting breast cancer risk. B.L. West is an employee of Plexxikon Inc. but had no involvement in data collection, analysis, or interpretation.

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Leukocytes as paracrine regulators of metastasis and determinants of organ-specific colonization

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It is now well recognized that tumor cell–host interactions regulate all aspects of cancer development. Amongst the various host response programs that facilitate primary cancer development, an emerging body of literature points to a critical role for leukocytes and their soluble mediators as regulating discrete events during primary tumor development and metastasis. This review focuses on the multiple aspects of leukocytes and their effector molecules as regulators of the metastatic process.

Dissemination of malignant cancer cells to distant organs is a multistage process requiring detachment and escape from primary tumor sites, extravasation through multiple basement membranes and matrix, survival in peripheral blood or lymphatics and ability to survive and proliferate in foreign tissue locales. As, for many tumor types, there is a temporal lag (months to decades) between when malignant cells arrive in ectopic locations and when proliferative capabilities are acquired,\textsuperscript{1,2} this implies that in addition to activation of survival programs at the metastatic site, disseminated malignant cells must acquire additional capabilities enabling their survival that likely rely on harnessing embedded regulatory programs at secondary sites. Thus, although cell-intrinsic programs are necessary for successful progression through each of these hurdles,\textsuperscript{2,3} cell-extrinsic programs regulated by non-neoplastic cells of mesenchymal, vascular and immune origins are also critical determinants for successful metastatic progression.\textsuperscript{4}

Chronic infiltration of tissue by leukocytes, \textit{i.e.}, chronic inflammation, is associated with predisposition to cancer.\textsuperscript{5} Chronic inflammation triggered by bacterial and viral infections or by autoimmune disease is estimated to be linked with 20\% of all deaths from cancer worldwide.\textsuperscript{6} Indeed, epidemiological studies reporting that nonsteroidal anti-inflammatory drugs reduce the risk of some cancers provide evidence for a causal link between inflammation and cancer.\textsuperscript{7–9} Thus, chronic inflammation that precedes neoplasia provides a fertile microenvironment whereby secreted growth factors, reactive oxygen species and cytokines support epithelial proliferation and create a permissive microenvironment to foster ongoing genetic instability and accumulation of genetic alterations that predispose to malignancy.\textsuperscript{5,6} Alternatively, inflammation can also be a physiological response to aberrant proliferation and tissue remodeling initiated by mutational activation of intrinsic programs that sustain proliferation and/or block cell death, and thus represent a secondary event enabling progression of neoplastic cells.\textsuperscript{10} In any event, hallmarks of inflammation such as the infiltration of neoplastic tissue by innate and adaptive leukocytes, activated angiogenic vasculature, tissue remodeling, and high levels of chemokines and cytokines that regulate these processes typify most solid tumors.\textsuperscript{5,6,11} This review discusses how key components and pathways of the immune microenvironment are associated with adult solid tumors and thereby promote the multistep cascade of tumor metastasis to distant organs.

Leukocytes Implicated in Mediating Solid Tumor Metastasis

It has been generally accepted that the chronic presence of activated leukocytes in primary tumors is a “hallmark” of the tumorigenic process and also represents a predictor of aggressive disease. Tumor-associated macrophages (TAMs) are one of the most abundant innate immune cells present in several types of human cancer (DeNardo \textit{et al.}, manuscript submitted),\textsuperscript{12–15} regulated in part by colony-stimulating factor (CSF)-1, a key cytokine involved in macrophage maturation, tissue recruitment and activation mediated by the CSF-1 receptor (CSF-1R/cFMS).\textsuperscript{16} A second CSF-1R ligand, interleukin (IL)-34, possesses similar binding affinities and also regulates TAM recruitment to tissues, but exhibits distinct tissue...
distribution. TAM presence in several types of human cancer correlates with increased vascular density and worse clinical outcomes. Studies in transgenic mouse models of mammary carcinogenesis revealed that TAMs promote tumor growth and enhance pulmonary metastasis by high-level expression of epidermal growth factor (EGF) and by activation of EGF-regulated signaling in mammary epithelial cells critical for invasive tumor growth and metastatic dissemination. In mouse models of mammary carcinogenesis, TAMs activated by IL-4 and CSF-1 have been identified as essential determinants of pulmonary metastasis because of the prometastatic mediators they secrete. The transcription factor Ets2 was recently implicated in regulating some aspects of these activities as selective deletion of Ets2 in TAMs decreased the frequency and size of pulmonary metastases in mouse models.

T lymphocytes were classically studied in the context of their tumor surveillance and antitumor capabilities. However, recent investigations have revealed that CD4⁺ T cells and T-regulatory cells instead promote pulmonary metastasis in part by regulating protumor versus antitumor bioactivity of innate leukocytes. We reported that IL-4-expressing CD4⁺ T cells promote invasion and metastasis of mammary adenocarcinomas by directly regulating TAM phenotype, biofactor function and EGF expression, which in turn regulates invasion, presence of circulating tumor cells (CTCs) and pulmonary metastasis. Other mediators found to be significant with regards to T-cell enhancement of pulmonary metastasis mammary carcinomas are S100A4 and receptor activator of nuclear factor-B ligand (RANKL). S100A4 protein mediates T cell attraction to developing neoplasms and permissive lungs of tumor-bearing mice, and in turn, it stimulates T-cell production of cytokines, particularly granulocyte CSF and eotaxin-2. RANTES stimulates externalization of S100A4 via microparticle shedding from plasma membranes and induces upregulation of fibronectin (FN) from fibroblasts and a number of other cytokines, including RANTES in tumor cells that together enhance tumor cell motility.

During prostate carcinogenesis, T lymphocyte and macrophage-derived RANKL induces metastasis through activation and nuclear localization of inhibitor of nuclear factor kappa-B kinase subunit alpha leading to repression of maspin, a critical suppressor of metastasis. Lung metastasis of mammary carcinomas is also regulated by CCR4⁺ T regulatory cells that can directly kill natural killer (NK) cells.

Other myeloid cell types implicated in regulating metastasis include neutrophils, mast cells and monocytes harboring T-cell suppressive activity that are potent suppressors of antitumor adaptive immunity and directly facilitate metastasis by regulating angiogenic programs via enhanced metalloproteinase (MMP) activity. Cancer-associated fibroblasts (CAFs) are implicated in regulating the activities of these myeloid cell types through their secretion of proinflammatory chemokines that recruit immune cells to sites of developing neoplasms.
In particular, murine macrophages are known to express elevated levels of the cysteine protease cathepsin B following exposure to IL-4.67 Macrophages at the invasive edge of pancreatic islet cancers express cathepsin B, and this is associated with loss of epithelial E-cadherin on neighboring malignant cells.4 Secretion of proteases by cells within the tumor microenvironment may not only foster metastatic activity and motility of neoplastic cells through matrix and into vasculature but also enhance and/or regulate the presence and activity of leukocytes: overexpression of cathepsin B in a transgenic mouse model of mammary carcinoma regulates pulmonary metastases, accompanied by increased numbers of B cells, Ig deposition and degradation of mast cells in the primary tumor site.68

Protease secretion by TAMs is in part regulated by IL-6 emanating from neoplastic cells. Tumor cell-derived IL-6 induces secretion and activation of the cysteine protease cathepsin B and secretion of matrix MMPs by monocytes.69 Similarly, neoplastic cell and T-cell-derived IL-4 induces cathepsin expression and activity in TAMs in several cancer types.28 TAMs in turn regulate neoplastic cell motility by secreting factors such as matrix-stimulating factor (MSF).70 MSF is an oncofetal isoform of FN and is induced in TAMs by macrophage-CSF, IL-4 and transforming growth factor beta (TGF-β). Notably, some immune cell-derived proteases also harbor tumor-suppressive activity: the aspartic proteinase cathepsin E, expressed predominantly by immune cells, including lymphocytes, macrophages and dendritic cells, mediates neoplastic cell apoptosis by catalyzing the proteolytic release of soluble TNF-related apoptosis-inducing ligand from the cell surface. Tumor growth is subsequently enhanced by this cascade, and survival in tumor-bearing mice is impaired.71

**Leukocytes and Survival of CTCs**

Before productive metastatic colonization is possible, CTCs must develop mechanisms enabling their survival within the circulation. Mechanical shear stress, detachment-induced cell death (anoikis) and cell-mediated cytotoxicity within the microcirculation effectively clear most CTCs.72 It has been estimated that only 0.01% of CTCs survive and eventually extravasate at distal locales.4 Mechanisms that enhance the probability for CTC survival rely on physical interaction with leukocytes. Activated platelets aggregate around CTC and thereby protect them from NK cell-mediated lysis72 by both thrombin-dependent and -independent mechanisms.73

Adhesion to capillary walls is largely regulated by the availability of adhesion molecules on CTCs, the endothelium, and the composition of the underlying extracellular matrix (ECM). Platelets and neutrophils facilitate these interactions via their production of matrix attachment molecules such as beta(2)-integrin/intracellular adhesion molecule-1 (Refs. 74 and 75) and selectins.76 Once CTCs attach to capillary lumens, another obstacle to surmount is inhibition of detachment-induced cell death, or anoikis, which is thought to be a major impediment for productive metastatic spread. Chemo-kine receptors CXCR4 and CCR7 and their ligands reduce the sensitivity of CTCs to not only arrest on capillary lumens but also on CTC anoikis by selective regulation of proapoptotic Bmf and antiapoptotic Bcl-xl proteins; thus, in the absence of appropriate cell–ECM interactions, selectins and chemokine receptors regulate CTC survival by mediating attachment and blocking cell death.76,77

**Site-specific colonization**

**Organ-specific migration.** The development of productive metastasis is a highly regulated process that is also subject to organ-specific mechanisms. Some solid tumors metastasize to preferred organ sites, for example, breast cancers metastasize to lung, liver, bone and brain; melanoma to liver, brain and skin; prostate cancer to bone; colorectal cancer to liver and lung; and lung adenocarcinoma metastasizes to bone, liver and brain.2,4 Several studies have reported that tropism of CTCs to specific organ locales is regulated by the complexity of genetic alterations intrinsic to neoplastic cells,2 while also recognizing that altered expression of important genes can also regulate tropism. Cyclooxygenase-2 (also known as PTGS2), the EGF receptor (EGFR) ligand heparin-bound EGF and the α-2,6-sialyltransferase (ST6GALNAC5) all act as mediators of malignant cell passage through the blood–brain barrier when breast cancers metastasize to brain.78,79 In contrast, when breast cancer metastasizes to bone, IL-11 and connective tissue growth factor regulated by TGF-β are important.80

However, a growing body of literature has also identified cell-extrinsic mechanisms, in addition to intrinsic, that dictate organ specificity of metastases, including differential expression of chemokines and their receptors. Chemokines expressed by specific organs promote tumor cell adhesion to microvessel walls, facilitate extravasation into target tissues and induce tumor cell migration. CXCL12-CXCR4, CCR7 and its corresponding chemokine ligands, CCL21 and CCL19, significantly regulate lymph node metastasis, whereas CCR10-CCL27 and CCR4-CCL22 regulate melanoma metastasis.81,82 Many malignant cells upregulate expression of chemokine receptors during premalignancy, partially as a result of autocrine and paracrine signaling mediated by TNF-α, IL-1 and IL-6 at the primary tumor site and subsequent chemokine gradients and then in part regulate migration toward specific organs.70 One such functional chemokine-signaling axis involves CXCR4 and its ligand CXCL12. Expression of CXCL12 by mesenchymal bone marrow-derived cells directs migration of metastatic breast cancer cells to bone. These cells constitutively secrete the chemokine SDF-1 (SDF-1/CXCL12) and thereby attract CXCR4+ malignant cells. Activation of CXCR4 promotes tumor progression by enabling survival and growth programs in malignant cells in ectopic tissues, regulates survival and growth of neoplastic cells in a paracrine manner and promotes tumor angiogenesis by attracting endothelial cells.83 This important axis has been implicated for metastasis of multiple solid tumors including...
pancreatic, hepatocellular, melanoma, lung and renal cancers.84–91 Other functional chemokine receptors include CCR7, implicated in lymphatic metastasis, and CCR9 that is associated with metastasis to small intestine where its ligand is expressed.92 Other chemokine receptors including CCR10, CXCR1, CXCR2, CXCR3, CXCR5 and CXCR7 expressed by malignant cells by a variety of solid tumors have also been implicated in organ-specific metastasis.11

Chemokine signaling is also an important feature of site-directed metastasis where neoplastic cell-secreted cytokines and chemokines signal to receptors expressed by various subtypes of myeloid cells, particularly significant with regards to colon carcinoma metastasis to liver.93 Both murine and human colon cancer cells secrete the CC-chemokine ligands CCL9 and CCL15, and thereby induce recruitment of CD34+/Gr1+ immature myeloid cells that express the CCL9/15 receptor CCR1, activation of which directly induces MMP-2 and MMP-9 expression. Lack of the Ccr1, Mmp2 or Mmp9 genes in myeloid cells suppresses disseminated tumor growth in the liver and significantly prolongs the survival of tumor-bearing mice.93

Autocrine signaling loops by malignant cells have also been implicated in organ-specific metastasis. Malignant cellderived TGF-β induces expression of the cytokine angiopoietin-like 4 (ANGPTL4) in mammary carcinoma cells, which is critical for carcinoma dissemination and colonization in lungs. TGF-β induces expression of ANGPTL4 through Smad-signaling cascades in carcinoma cells just prior to their entry into circulation. This subsequently enhances their retention in lungs, but not in bone, by disruption of vascular endothelial cell–cell junctions, which increases permeability of lung capillaries and facilitates transendothelial passage of tumor cells.94 These results indicate that a cytokine in the primary tumor microenvironment can induce expression of another cytokine in exiting tumor cells, thus enabling those cells to disrupt lung capillary walls and seed pulmonary metastases.

Immune cell support for colonization and organ-specific metastasis is also mediated by nonchemokine mechanisms: The two NF-κB targets, S100A8 and S100A9, are inflammatory mediators with chemotactic activity expressed and secreted by neoplastic cells, as well as by tumor-associated myeloid cells, and are associated with metastasis and a poor outcome in a variety of human tumors.95 S100A8 and S100A9 act through the RAGE receptor (receptor for advanced glycation end products). They induce migration of myeloid cells with T-cell suppressive activity into tumors, in murine models of mammary and squamous carcinogenesis. Recruited suppressive myeloid cells facilitate tumor progression by inhibiting T cell and NK cell activation and by polarizing immunity toward a tumor-promoting type 2 phenotype.96,97 Recently, S100A8/S100A9 were also implicated in site-specific colonization of melanoma to lungs: lack of the endogenous anti-inflammatory protein urotoglobin in mice leads to overexpression of S100A8/S100A9. Overexpression results in induction of MMP expression by neoplastic cells, and chemoattraction of melanoma cells according to the S100A8/S100A9 gradient, thus enhancing colonization of B16 melanoma in lungs.98 Another mechanism by which tumor cell-secreted S100A8/S100A9 facilitate metastasis is through serum amyloid A-3 (SAA). Secretion of S100A8/S100A9 into premetastatic lungs induces local expression of SAA, that acts through Toll-like receptor (TLR) 4 to recruit additional myeloid cells, thus creating an inflammatory environment that accelerates migration of primary tumor cells to lung parenchyma.99

Leukocyte support for tumor cell survival and colonization at the metastatic organ. The temporal gap between infiltration to distant organs and the ability to colonize and form macro-metastases, a process sometimes requiring decades, depending on tumor type, suggests that to grow at the metastatic site, disseminated tumor cells must acquire an ability to “educate” their new microenvironment to support their own survival. Although changes in the metastatic microenvironment that enable growth of disseminated cells are poorly defined, emerging data indicate that immune-mediated signaling plays an important role. During the earliest stages of liver metastasis, microvascular arrest of neoplastic cells triggers a local inflammatory response: tumor-secreted vascular endothelial growth factor (VEGF) induces expression of proinflammatory cytokines by sinusoidal endothelial cells resulting in upregulation of adhesion molecules such as VCAM-1, allowing arrest of metastatic melanoma cells.100 Another prometastatic mechanism in liver supporting survival of CTCs is mediated by tumor-activated proinflammatory cytokine signaling by liver stellate cells, hepatocytes and myofibroblasts. These are recruited into sites of avascular micrometastases and create a microenvironment that supports metastatic growth through specific release of both proangiogenic factors and tumor cell invasion- and proliferation-stimulating factors provided by tumor-activated hepatocytes and myofibroblasts.101

An intriguing mechanism by which tumor cells take advantage of immune pathways to increase their metastatic potential is the ectopic expression of FcγRIIB by metastatic melanoma cells. FcγRIIB is an inhibitory low-affinity receptor for IgG that terminates activation signals initiated by antigen crosslinking of the B-cell receptor through its inhibitory immunoreceptor tyrosine-based inhibiting motif.102 Forty percent of human metastatic melanomas gain expression of FcγRIIB, in particular, in liver metastases (69%), suggesting that gain of expression supports their survival in liver by escaping humoral immunity.103,104 Experimental studies with B16 melanoma cells in immunocompetent mice indicate that tumor-expressed FcγRIIB operates as a decoy receptor inhibiting antibody-dependent cell cytotoxicity mediated by tissue macrophages, neutrophils and NK cells, which are abundant in liver: antitumor antibodies bind tumor cells via Fab domains, whereas the Fc portion is “caught” by the tumor FcγRIIB and cannot be recognized by FcγR of the effector
cells. Thus, liver offers a prometastatic microenvironment that supports metastasis of cancer cells able to resist antitumor hepatic defenses and takes advantage of hepatic cell-derived factors that are key phenotypic properties of liver-metastasizing cancer cells.

The bone and bone marrow are also among the most frequent sites of cancer metastasis. During bone metastasis, breast carcinoma cells, through secretion of IL-6, IL-11, TNF-α and parathyroid hormone-related peptide, are able to activate osteoclasts through RANKL, which is critical for the formation of osteolytic metastases. In other cancer types, such as neuroblastomas, IL-6 is secreted by bone marrow stromal cells and promotes osteolysis through the induction of RANKL in osteoblasts as well as in tumor cells. NF-κB-regulated signaling in breast carcinoma cells promotes osteolytic bone metastasis by induction of osteoclastogenesis via granulocyte macrophage colony-stimulating factor. Late-stage breast cancers also metastasize to brain, where recruitment of glial cells and a brain inflammatory response correlates with tumor cell proliferation and growth in both experimental metastasis in mice and in human brain metastases (Figure 1).

**A Premetastatic Niche**

Several studies suggest that primary tumors can “prepare” the distant target organ of metastasis by creating a prometastatic niche, whereas other studies indicate that a small number of metastatic cells activate their new microenvironment on arrival. Regardless, neoplastic cells secrete factors that mobilize bone marrow-derived VEGF receptor (VEGFR)-1-expressing hematopoietic progenitor cells to sites of metastasis that induce expression of FN by resident fibroblasts, thus creating favorable conditions for arrival of would-be metastatic cells. Gr1⁺CD11b⁺ myeloid cells have also been identified to play a potential role in mediating changes that activate premetastatic lung into a permissive haven by diminishing immune-protective programs. Mammary tumor cells growing in mammary pads remotely activate expression of TARC/CCL17 and MDC/CCL22 in lungs. These chemokines acting through CCR4 attract both tumor and immune cells. Distant primary tumor-derived factors induce the expression of the inflammatory chemoattractants, S100A8 and S100A9, which in turn attract Mac1⁺ myeloid cells to premetastatic lungs mediated by TLR-4-expressing cells that accelerate migration of primary tumor cells to lung tissues. Lysyl oxidase (LOX) is a tumor cell-derived factor often induced in primary tumors in response to hypoxia. However, systemic secretion of LOX leads to its accumulation in the lung, where it has been found to act on ECM proteins establishing a permissive niche for infiltrating cancer cells by crosslinking collagen IV in basement membranes and by recruiting CD11b+ myeloid cells that adhere to...
crosslinked collagen IV, produce MMP-2 and thereby enhance the invasion and recruitment of BMDCs and metastasizing tumor cells.\textsuperscript{116} These data indicate that, through multiple mechanisms, creation of a proinflammatory microenvironment in metastatic organs, whether that be prior to or at the time of malignant cell arrival, enhances the survival and proliferative possibilities for metastatic cells.

**Implications for Therapy and Perspectives**

Elucidation of the changes in metastatic microenvironments is a significant clinical goal for eradicating cancer-associated death. Immune-based signaling pathways have emerged as central players in facilitating growth of micrometastases into clinically relevant macrometastases. Anticancer therapies that target these programs are gaining attention and in a few cases are being evaluated in clinical trials.\textsuperscript{117} Denosumab, an anti-RANKL antibody, originally developed for treatment of osteoporosis, has been found effective for inhibiting bone metastasis in prostate cancer.\textsuperscript{118} Disruption of tumor cell adhesion to protective stroma by targeting the CXCR4-CXCL12 axis using a small molecule of CXCR4 antagonist, such as Plerixafor (AMD3100), is a novel, attractive therapeutic approach being explored in ongoing clinical trials for metastatic multiple myeloma, leukemia and other types of cancer.\textsuperscript{119,120}

Several therapeutic agents that limit IL-1 activity are approved for treating chronic inflammatory diseases, e.g., recombinant IL-1R\textsuperscript{a} (anakinra), and neutralizing monoclonal antibodies to IL-1\textsuperscript{b} and a soluble receptor to IL-1, which have also been found to exert benefits in animal models of metastasis and tumor-associated angiogenesis. A goal for the future would be to evaluate this activity in clinical trials of IL-1 blockade.\textsuperscript{121} Despite their critical involvement in invasion and metastasis, there has been conflicting results with antiproteases, possibly due to antitumorigenic activity of some enzymes.\textsuperscript{122}

As cancer research and clinical oncology progress increasingly toward a new era of integrative cancer therapy based on combinatorial drug regimens that act synergistically by targeting intrinsic pathways in neoplastic cells, as well as extrinsic pro-oncogenic pathways in the tumor microenvironment, the intensive research in deciphering the role of the metastatic microenvironment and of tumor-promoting inflammation will hopefully result in innovative therapeutic strategies in the future.

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Rapid Generation of In Vitro Multicellular Spheroids for the Study of Monoclonal Antibody Therapy

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Abstract

Tumor microenvironments present significant barriers to penetration by antibodies and immunonoconjugates and are difficult to study in vitro. Cells cultured as monolayers typically exhibit less resistance to therapy than those grown in vivo. Therefore, it is important to develop an alternative research model that better represents in vivo tumors. We have developed a protocol to produce multicellular spheroids, a simple and more relevant model of in vivo tumors that allows for further investigations of the microenvironmental effects on drug penetration and tumor cell killing. The protocol is used to produce in vitro three-dimensional tumor spheroids from established human cancer cell lines and primary cancer cells isolated from patients without the use of any extracellular components. To study the ability of tumor-targeting immunoconjugates to penetrate these tumor spheroids in vitro, we have used an immunotoxin targeting mesothelin, a surface protein expressed in malignant mesotheliomas. This method for producing consistent, reproducible 3D spheroids may allow for improved testing of novel monoclonal antibodies and other agents for their ability to penetrate solid tumors for cancer therapy.

Key words: multicellular spheroids, protocol, monoclonal antibody therapy

INTRODUCTION

Treatment of solid tumors remains a major challenge despite the availability of a vast number of anti-cancer agents. Antibody-based therapies represent a promising new approach for treating solid tumors. However, a major challenge involves delivering sufficient amounts of antibodies and immunoconjugates within tumor masses. For an anti-cancer antibody agent to be successful, it must (a) be effective in the tumor microenvironment, and (b) reach the tumor cells in optimal quantities to exert a therapeutic effect. Tumors are more resistant to therapy than cancer cells cultured as monolayers. This can be explained by “multicellular resistance,” a mechanism for drug resistance attributed to cell-cell contacts, cell-matrix contacts, and the three-dimensional (3D) shape found in tissue. The majority of the ~10^{13} cells in the human body are only within a few cell diameters of a blood vessel. This feature facilitates the delivery of oxygen and nutrients to the cells that form the tissues of the body and also enables the efficient delivery of most medicines.
In light of this, the solid tumor microenvironment has several major characteristics, including hypoxia, large distances between blood vessels, high interstitial fluid pressure, structure and composition of the extracellular matrix, and cell-cell adhesion. Overall, these features of the tumor microenvironment limit the delivery of anticancer drugs to cancer cells that are situated far from blood vessels. Multicellular resistance acquired by tumor cells may contribute to difficulties in translating promising findings from in vitro studies into clinical therapy.

Here, we describe a detailed protocol to establish an in vitro 3D tumor spheroid model. This model can be used to identify potential new therapeutic targets that are highly expressed in mesothelioma cells in 3D spheroids, but not in monolayers, and therefore be relevant in the 3D tumor. Furthermore, this protocol may be easily applied to in vitro studies of other tumor-targeting antibodies and immunoconjugates in vitro. It may also be a useful system for screening tumor penetrating monoclonal antibodies by antibody engineering technologies such as phage display.

**Development of the protocol**

Most studies of anticancer drugs consider only cellular and/or genetic mechanisms at the level of the single cell. However, drug penetration is a highly important additional mechanism and requires a more complex cellular environment to study. Indeed, in vitro spheroid models have become the most commonly used tools to assess drug penetration. Although animal studies, when feasible, hold the advantage of mimicking the clinical environment most closely, in vitro spheroids offer the benefit of being straightforward and relatively easy to establish.
able to examine the distribution of drugs in the absence of complicating factors such as pharmacokinetics, which often differ between mice and humans. Not only are tumor spheroids an excellent model to evaluate drug penetration, they play an increasingly meaningful role in drug discovery and development.

In 2006, Ivascu and Kubbies at Roche Pharmacutical Research Oncology in Germany first reported a simple method to generate tumor spheroids for potential high-throughput functionality and toxicity analysis. Briefly, a defined number of cancer cells ranging from 1,000 to 20,000 were seeded into wells of poly(2-hydroxyethylmethacrylate)—coated, 96-well, round- or conical-bottom plates in standard growth medium and centrifuged for 10 minutes at 1000 x g. Within 24 hours of culturing, this procedure generated individual spheroids in each well with homogeneous sizes, morphologies, and stratification of proliferating cells found in the rim that also include dying cells in the core region. In addition, by adding basement membrane extract Matrigel to some cell lines, they were able to improve the structure from an aggregate to spheroid morphology. In 2008, after evaluating several techniques, V. Courtney Broaddus’ group at the University of California San Francisco (USA) first established mesothelioma spheroids for the study of apoptotic resistance using multicellular spheroids, modifying the method originally reported by Ivascu and Kubbies. Interestingly, although Broaddus’ study did not use any basement membrane extract, they found the formation of spheroids to be stably intact.

Our laboratory at the National Cancer Institute (NCI) focuses on producing human monoclonal antibodies (mAbs) for the development of cancer therapy. Although leukemia treatments involving mAbs have been in clinical use for years, this approach has not been as successful for solid tumors. The proliferation of tumor cells forces blood vessels apart, reducing vascular density and creating a population of cells distant (>100μm) from vessels. Drugs generally do not penetrate further than three to five cell diameters from blood vessels, thereby depriving more distantly located tumor cells of any drugs. Penetrating antibody technology is increasingly seen by many to be the holy grail of antibody therapy. A limitation in our ability to identify and evaluate effective penetrating antibody reagents has been the lack of an in vitro model that recapitulates the features of a solid tumor. In the last two years, in collaboration with our group, the Taka- yama laboratory at the University of Michigan has produced two publications related to 3D spheroid cultures. One study established co-cultures by introducing different types of cells on the membrane or in the bottom chamber of commercially available Transwells. They found that cancer cells formed 3D spheroids while maintaining their viability and used this procedure to evaluate mouse embryonic stem (mES) cell differentiation based on heterogeneous cell-cell interactions. Co-culture of mES cells and HepG2 human hepatocellular carcinoma (HCC) cells decreased SOX17 expression of mES cells, and direct cell-cell contact further decreased SOX17 expression, indicating that co-culture with HepG2 cells inhibits endoderm differentiation. Another study described high-throughput 3D spheroid culture and drug testing using a 384-well format hanging drop culture plate.

In collaboration with V. Courtney Broaddus, we made a 3D spheroid model culture using a human mesothelioma cell line and primary cell lines isolated from the ascites of malignant mesothelioma patients. Using this model, we treated spheroids with SS1P, a recombinant immunotoxin targeting mesothelin, and evaluated how the microenvironment affected the ability of the drug to penetrate a 3D cancer cell mass and kill cancer cells. Mesothelin is highly expressed in mesothelioma and several other human cancers, including pancreatic adenocarcinomas, ovarian cancers, lung adenocarcinomas and cholangiocarcinoma. Although mesothelioma cells grown as monolayers or as spheroids express comparable levels of mesothelin, we observed that spheroids were at least 100 times less sensitive to SS1P and that penetration within spheroids was limited after four hours. The core area of spheroids revealed a substantial number of tight junctions, along with a significant increase in expression of E-cadherin, a protein involved in the assembly and sealing of tight junctions and highly expressed in malignant mesothelioma. Moreover, siRNA silencing and antibody inhibition targeting E-cadherin enhanced SS1P immunotoxin therapy in vitro.

Advantages and limitations of the present protocol

3D spheroid culture is often complicated to follow and varies frequently according to individual practices. It can be cumbersome for labs with limited resources and expensive to manage. However, it is worthwhile to mention that although not all features of solid cancers, such as the influence of stroma and immune cells, are modeled by multicellular spheroids, spheroids do reflect many important properties of solid tumors, including the development of an extracellular matrix, tight junctions between epithelial cells, and gradients of nutrient concentration and cell proliferation from the exterior to the center. Spheroids...
can develop central necrosis and regions of hypoxia. By incubating spheroids with an anticancer drug, it then becomes possible to examine the kinetics of drug penetration. Unlike a previous method, the protocol described here is relatively simple and straightforward to learn, rapid in producing samples, and economically feasible.

**Experimental Design**

NCI-H226 (American Type Culture Collection or ATCC, Rockville, MD) is a human lung squamous carcinoma cell line. HepG2 and Hep3B (ATCC) are two human liver cancer (HCC) cell lines. NCI-M-03 and NCI-M-13 (Raffit Hassan, NCI, Bethesda, MD) are two human mesothelioma primary cell lines isolated from the ascites of malignant mesothelioma patients treated at the NCI.

**MATERIALS**

**REAGENTS FOR FORMATION OF SPHEROIDS**

- Poly-HEMA (Sigma; Cat. #P3932)
- 95% ethanol (in milliQ water)
- U-bottomed 96-well plates (Greiner; Cat. #650185)
- SS1P (Ira Pastan, Laboratory of Molecular Biology, NCI, Bethesda, MD)
- RPMI 1640 Media (Invitrogen; Cat. #21870-076) containing 10% fetal bovine serum (FBS), 1% L-glutamine (Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA)
- FreeStyle 293 Media (Invitrogen; Cat. #12338-018)
- Accutase (BD Biosciences; Cat. #561527)
- T-75cm² tissue culture flasks (Sarstedt; Cat. #83.1813.002)

**ADDITIONAL REAGENTS FOR CHARACTERIZATION OF SPHEROIDS**

- Coomassie Plus Protein Assay Reagent (Thermo Scientific; Cat. #1856210)
- RNeasy Mini Kit (Qiagen; Cat. #74104)
- Cell Counting Kit-8 (Dojindo Laboratories; Cat. #CK04)
- Costar white opaque-walled 96-well plates adequate for cell culture (Corning; Cat. #3922)
- CellTiter-Glo Luminescent Cell Viability Assay (Promega; Cat. #G7572)

**REAGENT SETUP**

**Lysis buffer**

To make a stock solution of lysis buffer, combine one “Complete Mini-EDTA free” protease inhibitor tablet (Roche; Cat. #1873580), 10 mL of RIPA buffer, and 1 mL of 20% SDS.

**Poly-HEMA**

Prepare a 120 mg/mL stock solution of poly-HEMA in 95% ethanol. Incubate while stirring with a sterile magnetic bar at room temperature (15-20°C) overnight. To make a working solution of Poly-HEMA, pipette 1 mL of Poly-HEMA stock solution into 23 mL of 95% ethanol to obtain a final concentration of 5 mg/mL (this volume is enough to coat four plates). Vortex solution and leave at room temperature (15-20°C) until ready to coat. Prepare fresh working solution every time new plates are made.

**EQUIPMENT**

Device (plate shaker) for mixing multiwell plates
Luminometer or charge-coupled device (CCD) camera imaging device capable of reading multiwell plates

**PROCEDURE**

**Preparation of non-adsorbent poly-HEMA plates**

1. Pipette 60 μL of poly-HEMA stock solution into each well of a 96-well U-bottomed plate.
2. Evaporate with lids on at room temperature (15-20°C) inside a sterile hood for 72 hours.
3. After plates are completely dried, seal with parafilm, and store at 4°C.
4. Before use, bring to room temperature (15-20°C) and sterilize in the hood with lids off for 30 minutes.

**Preparation of Spheroids**

1. Maintain cell line as adherent monolayer cultures in RPMI 1640 medium supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin.
2. Incubate at 5% CO₂ with balance of air at 37°C.
3. Seed at 2x10⁶ cells/mL in T-75cm² tissue culture flasks.
4. Change cell media twice a week.
5. Rinse the cells with PBS and add 5 mL of Accutase to detach cells and incubate at 37°C for 3-5 minutes.
Neutralize with 5 mL of growth media and perform a cell count.

Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes.

Dilute cell concentration to 5x10⁴ cells/mL with growth media in order to form 10,000-cells per well spheroids (approximately 1 million cells are needed per plate).

Pipette 200 µL of the cell suspension into each well of the 96-well plate pre-coated with poly-HEMA.

Centrifuge the plate at 216 x g at room temperature (15–20°C) for 10 minutes.

Incubate for 24-48 hours at 37°C, 5% CO₂.

**Preparation of Cell Lysate from Spheroids and Monolayers**

**Spheroids**

Pipette growth media and spheroid completely from each well and collect in a 50 mL tube.

Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes, aspirate the supernatant, and resuspend the cells with 1.5 mL of PBS.

Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes, aspirate the supernatant, and resuspend the cells in 50 µL of lysis buffer (for approximately 1 million cells).

Vortex thoroughly.

Repeat four cycles of freezing at 80ºC for approximately 10 minutes and thawing at 37ºC for approximately 20 minutes.

Centrifuge at 216 x g at room temperature (15–20°C) for 1 minute, and collect all of the supernatant.

Measure concentration by Coomassie stain, a reliable standard for protein quantification. Load approximately 50-100 µg of cell protein lysate per lane for western blot analysis.

**Monolayers**

Aspirate growth media of monolayer cells and rinse three times with PBS.

Δ CRITICAL STEP Allow monolayer cells to grow for two to three days after seeding until 50-60% confluent.

Add 2 mL of PBS and scrape monolayer cells on ice, and collect in a 15 mL tube.

Add another 2 mL of PBS to the plate, scrape, and collect the remaining cells.

Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes, aspirate the supernatant, and resuspend the cells in 50 µL of lysis buffer (for approximately 1 million cells)

**Purification of RNA for Microarray Analysis**

Collect monolayer and spheroid cell lysate on three separate days.

Δ CRITICAL STEP Save a sufficient amount of cell lysate for RNA quality validation.

Follow manufacturer’s kit instructions to purify RNA.

**WST Assay**

Form 3D spheroids on a 96-well poly-HEMA-coated plate.

Allow monolayers and spheroids to grow for 24 hours.

Add 20 µL of SS1P/BL22 (negative control)/Cycloheximide (CMH) (positive control) to 200 µL of RPMI growth media per well for a 1:10 dilution.

Prepare a sufficient volume of WST reagent in serum-free FreeStyle media (e.g. 1 mL of reagent in 9 mL of media).

Remove growth media and replace with 100 µL/well of a working solution of WST reagent-FreeStyle media mentioned in the previous step.

Transfer all 100 µL containing each spheroid from each well to a flat-bottom 96-well plate.

Allow to incubate for 2-4 hours at 37ºC until brown-yellow color develops.

Measure absorbance at 450 nm with reference wavelength of 650 nm.

**ATP Assay**

Form 3D spheroids on a 96-well poly-HEMA coated plate.

Allow monolayers and spheroids to grow for 24 hours.

Seed 10,000 cells/well for monolayer cells and one 10,000-cell spheroid/well containing 200 µL of RPMI growth media in a 96-well flat bottom plate.

Add 20 µL of SS1P and BL22 (negative control) from 1000 ng/mL to 0.0001 ng/mL to each well.
46 Incubate for 72 hours at 37°C.
47 Transfer monolayer cells and spheroids via a pipette to opaque-walled multiwell plates in culture medium (100 µL/well for 96-well plates).
48 Prepare control wells containing medium without cells to obtain background luminescence value.
49 Equilibrate the plate and its contents at room temperature (15-20°C) for approximately 30 minutes.
50 Add a volume of CellTiter-Glo Reagent equal to the volume of cell culture medium present in each well (e.g., add 100 µL of reagent to 100 µL of growth media containing cells for a 96-well plate).
51 Mix contents for 2 minutes on an orbital shaker to induce cell lysis.
52 Allow plate to incubate at room temperature (15-20°C) for 10 minutes to stabilize luminescent signal.
53 Measure luminescence.

TROUBLESHOOTING

1. For proper storage, the stock solution of poly-HEMA can be stored at room temperature (15-20°C) indefinitely.
2. It is optional to evaporate with the lids on at room temperature (15-20°C) on the bench for 1 to 2 weeks for slower drying, or to evaporate at 37°C inside a humidity-free bacterial incubator for 48 hours.
3. Within six hours, mesothelioma cells begin to form as an aggregate and should be left alone as they may be easily disturbed by pipetting. By 24 hours, most mesothelioma cells form into a disk-like shape called a multicellular spheroid and can be transferred gently by a pipette without dissociating.
4. For proteins that may be more difficult to visualize or detect (e.g. E-Cadherin), use at least 100 µg of cell protein lysate per lane for western blot analysis.
5. Selected GeneChip Human Genome U133 Plus 2.0 Array was performed by NCI Affymetrix Group (Frederick, MD) for reliability. For optimal results, the recommended final concentration of RNA for array analysis is 100 ng/mL in a 6 µL volume per sample.
6. To properly prepare SS1P at the original concentration (0.25 mg/mL): For 100 ng/mL final concentration, add 20 µL of 10 µg/ml SS1P into 200 µL growth media per well. For 1000 ng/mL final concentration, add 20 µL of 1 µg/ml SS1P into 200 µL growth media per well. Original concentration of BL22 (1 µg/mL): For 100 ng/mL final concentration, add 20 µL of 1 µg/ml BL22 into 200 µL growth media per well. Original concentration of CHM (100 µg/mL): For working solution, dilute 1 µL in 99 µL of growth media. For 1:1000 final concentration, add 20 µL of CHM working solution into 200 µL growth media per well.
7. To properly prepare SS1P at the original concentration (0.25 mg/mL): Dilute to 10,000 ng/mL working solution (adding 20 µL of this in 200 µL of cell suspension results in a final concentration of 1000 ng/mL). For a 1000 ng/mL working solution, add 100 µL of 10,000 ng/mL SS1P in 900 µL of growth media (adding 20 µL of this in 200 µL of cell suspension results in a final concentration of 100 ng/mL). Continue to serially dilute 100 µL of SS1P in 900 µL of growth media. Original concentration of BL22 (684 µg/mL): Dilute to 10,000 ng/mL working solution (adding 20 µL of this in 200 µL of cell suspension results in a final concentration of 1000 ng/mL). For a 1000 ng/mL working solution, add 100 µL of 10,000 ng/mL BL22 in 900 µL of growth media (adding 20 µL of this in 200 µL of cell suspension results in a final concentration of 100 ng/mL). Continue to serially dilute 100 µL of BL22 in 900 µL of growth media.
8. It is highly recommended to do more than one type of cell proliferation or viability assay (e.g. WST and ATP).

TIMING

Step 1-4, Preparation of non-adsorbent Poly-HEMA plates: 3 days
Step 5-15, Preparation of Spheroids: 2 days
Step 16-30, Preparation of Cell Lysate: 3 hours
Step 31-32, Purification of RNA for Microarray Analysis: 1 day
Step 33-41, WST Assay for SS1P-Treated Monolayers and Spheroids: 4 days
Step 42-53, ATP Assay for SS1P-Treated Monolayers and Spheroids: 4 days

ANTICIPATED RESULTS

Using the experimental conditions described in this protocol, almost all of the established cancer cell lines and primary cells isolated from patients are able to form 3D spheroids in vitro (Fig. 2). We found that the mesothelioma and HCC cells we used formed spheroids well.
Figure 2. Establishment of in vitro tumor spheroids. Microscopic images of monolayers and spheroids of human cancer cell lines, NCI-H226 (mesothelioma), HepG2 (hepatocellular carcinoma or HCC), Hep3B (HCC), and primary mesothelioma lines, NCI-M-03 and NCI-M-13, isolated from patients taken after 24 hours. Scale bars, 400 µm.
Within only 2 days after seeding cells, spheroids are ready for tumor penetration studies of antibodies or immunoconjugates, RNA extraction for microarray analysis, protein lysis for proteomics analysis or discovery of tumor penetration antibodies by phage display and other antibody technologies.

To investigate how tumor microenvironments affect the killing activity and penetration of an antibody agent, monolayers and spheroids were treated with SS1P and a negative control. Cell growth inhibition (WST) and cell viability (ATP) assays showed that the IC$_{50}$ of SS1P for spheroids was $>$1000 ng/mL, at least 100 times the IC$_{50}$ for monolayers, ~10 ng/mL, after 72 hours. Both assays revealed that greater than 50% of the cancer cells from spheroids could not be killed by SS1P concentrations as high as 1,000 ng/mL. Finally, we tested SS1P on primary lines isolated from malignant mesothelioma patients and confirmed that SS1P was far less effective on spheroids.

We explored two different experimental approaches to examine the roles of protein regulation in regard to immunotoxin penetration. The first involved silencing E-Cadherin using siRNA. A reduction in protein expression (greater than 80%) was observed followed by an increased sensitization to SS1P therapy. Finally, an inhibitory antibody against E-Cadherin also demonstrated enhanced anti-tumor activity only if added before, but not after, spheroid formation.

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Author Contributions

MH conceived and designed the experiments. YP performed the experiments. MH, VCB and DB revised the manuscript. YP and MH wrote the manuscript. YP, MH, VCB and DB analyzed the data. Conflict of Interest

There is no conflict of interest and the financial disclosure has been described in the Acknowledgements.

References
MRI of Tumor-Associated Macrophages with Clinically Applicable Iron Oxide Nanoparticles

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Abstract

**Purpose:** The presence of tumor-associated macrophages (TAM) in breast cancer correlates strongly with poor outcome. The purpose of this study was to develop a clinically applicable, noninvasive diagnostic assay for selective targeting and visualization of TAMs in breast cancer, based on magnetic resonance and clinically applicable iron oxide nanoparticles.

**Experimental Design:** F4/80-negative mammary carcinoma cells and F4/80-positive TAMs were incubated with iron oxide nanoparticles and were compared with respect to magnetic resonance signal changes and iron uptake. MMTV-PyMT transgenic mice harboring mammary carcinomas underwent nanoparticle-enhanced magnetic resonance imaging (MRI) up to 1 hour and 24 hours after injection. The tumor enhancement on MRIs was correlated with the presence and location of TAMs and nanoparticles by confocal microscopy.

**Results:** In vitro studies revealed that iron oxide nanoparticles are preferentially phagocytosed by TAMs but not by malignant tumor cells. In vivo, all tumors showed an initial contrast agent perfusion on immediate postcontrast MRIs with gradual transendothelial leakage into the tumor interstitium. Twenty-four hours after injection, all tumors showed a persistent signal decline on MRIs. TAM depletion via CSF1 monoclonal antibodies led to significant inhibition of tumor nanoparticle enhancement. Detection of iron using 3,3'-diaminobenzidine-enhanced Prussian Blue staining, combined with immunodetection of CD68, localized iron oxide nanoparticles to TAMs, showing that the signal effects on delayed MRIs were largely due to TAM-mediated uptake of contrast agent.

**Conclusion:** These data indicate that tumor enhancement with clinically applicable iron oxide nanoparticles may serve as a new biomarker for long-term prognosis, related treatment decisions, and the evaluation of new immune-targeted therapies.

Introduction

Although breast cancer has not historically been linked to underlying inflammation or infection, it exhibits tumor-associated inflammation marked by infiltration of leukocytes into developing tumors where increases in some leukocyte subsets parallels disease progression (1–3). In the majority of cases, however, the natural immunity to cancer that is present is not protective, but instead fosters progression. Studies in transgenic mouse models of mammary carcinogenesis revealed that tumor-associated macrophages (TAM) promote tumor growth and enhance pulmonary metastasis by high-level expression of epidermal growth factor (EGF) and activation of EGF-regulated signaling in mammary epithelial cells (MEC) critical for invasive tumor growth and metastatic dissemination (4). Histopathologic and flow cytometric evaluations have revealed that TAMs are the most abundant innate immune cell present in murine mammary carcinomas and in human breast cancers (2, 5). TAM presence in several types of human cancer, including breast, correlates with increased vascular density and worse clinical outcome (6–11). A clinically reliable noninvasive in vivo imaging test that could reliably detect and quantify TAMs could be employed as a novel, widely applicable prognostic assay for stratifying individual patients to more aggressive and/or TAM-targeted therapies.

Intravenously injected superparamagnetic iron oxide (SPIO) nanoparticles are effective contrast agents for magnetic resonance imaging (MRI). SPIO are phagocyted by macrophages in various target tissues depending on their particle size and composition. Relatively large SPIO with
Translational Relevance

The presence of tumor-associated macrophages (TAM) in adenocarcinomas correlates strongly with poor outcome in patients with breast cancer. Our data indicate that the Food and Drug Administration (FDA)-approved iron oxide nanoparticle compound ferumoxytol (Feraheme) is preferentially phagocytosed by TAMs, but not by neoplastic tumor cells. In vivo, ferumoxytol administration was associated with an initial tumor perfusion, followed by tumor retention and persistent magnetic resonance-enhancement at 24 hours after intravenous administration, which correlated with phagocytosed nanoparticles in TAMs. Together, these data indicate that ferumoxytol-enhancement may serve as a new biomarker for long-term prognosis and related treatment decisions that will support ongoing development of new immune-targeted therapies. Since ferumoxytol is FDA-approved as an iron supplement, this application is immediately clinically applicable as an imaging approach via an “off label” use.

hydrodynamic diameters in the order of 80 to 150 nm are rapidly phagocytosed by macrophages of the reticuloendothelial system (RES), such as liver, spleen, and bone marrow, whereas ultra small SPIO (USPIO) with diameters of less than 50 nm escape RES phagocytosis to some extent, leading to a prolonged blood pool circulation and accumulation in inflamed tissues and tumors due to transendothelial leak and macrophage phagocytosis (12–14).

The goal of this study was to utilize novel USPIO to develop an immediately clinically applicable molecular imaging approach for enhanced imaging of TAMs in breast cancer. Our imaging technique relies on the iron supplement ferumoxytol (Feraheme), recently Food and Drug Administration–approved for intravenous treatment of iron deficiency in patients (15–17). Ferumoxytol is also a USPIO compound, providing a strong signal effect on MRIs and thus exerting properties of an magnetic resonance contrast agent (18–21). On the basis of these properties, we postulated that ferumoxytol would be phagocytosed by TAMs in breast cancer, thereby enabling selective detection of TAMs on delayed, postperfusion MRIs.

Materials and Methods

Contrast agents

Three USPIO nanoparticle compounds were investigated: (i) Ferumoxytol (Feraheme, AMAG Pharmaceuticals Inc.) is a USPIO nanoparticle applied for intravenous treatment of iron deficiency in patients with impaired renal function (15, 16, 20, 22, 23). Ferumoxytol consists of an iron oxide core and a carboxydextran coating. Ferumoxytol has a mean hydrodynamic diameter of 30 nm, an $r_1$ relaxivity of $38 \text{s}^{-1}\text{mM}^{-1}$, and an $r_2$ relaxivity of $83 \text{s}^{-1}\text{mM}^{-1}$ at $0.94\text{T}$ and at $37^\circ\text{C}$. Ferumoxytol was conjugated to fluorescein isothiocyanate (FITC; Ferumoxytol-FITC) for detection by immunofluorescent microscopy. (ii) P904 (Guerbet Group, Paris, France) is a USPIO compound currently in phase I clinical trials in Europe with plans for global distribution (24–27). P904 consists of an iron oxide core and a hydrophilic coating by a monomeric organic molecule with 20 hydroxyl groups per nanoparticle in its coating, added via an amino PEG derivative of folic acid coupled on its g-carboxyl moiety to the carboxylate-bearing iron core. P1133 has a mean hydrodynamic diameter of 26 nm, an $r_1$ relaxivity of $12 \text{s}^{-1}\text{mM}^{-1}$, and an $r_2$ relaxivity of $95 \text{s}^{-1}\text{mM}^{-1}$ at $1.5\text{T}$ and $37^\circ\text{C}$. (iii) P1133 (Guerbet) is a preclinical USPIO with potential future clinical development (24). P1133 is based on P904 but also incorporates 8 to 10 folate moieties per nanoparticle in its coating, added via an amino PEG derivative of folate acid coupled on its g-carboxyl moiety to the carboxylate-bearing iron core. P1133 has a mean hydrodynamic diameter of 26 nm, an $r_1$ relaxivity of $12 \text{s}^{-1}\text{mM}^{-1}$, and an $r_2$ relaxivity of $95 \text{s}^{-1}\text{mM}^{-1}$ at $1.5\text{T}$ and $37^\circ\text{C}$. Animal model

This study was approved by the animal care and use committees at the respective institutions. MMTV-PyMT mice that spontaneously develop multifocal, multiclonal mammary adenocarcinomas were used at 12 to 14 weeks of life (28). Seven animals each received intravenous injections of ferumoxytol, P904, or P1133. Six additional animals received injections of P1133 + free folic acid. Animal age, weight, and tumor size were not significantly different between experimental groups that received different contrast agents ($P > 0.05$). Additional experiments were carried out in 7 postpubertal female FVB/n mice (10–12 weeks), which received injections of 50,000 PyMT-derived tumor cells into the right lower mammary fat pad for induction of orthotopic tumors. Three of these animals were treated with anti- colony stimulating factor (CSF)-1 monoclonal antibody (mAb), clone 5A1, purified by the UCSF Hybridoma core using the ATCC hybridoma (#CRL-2702). The animals received an intraperitoneal injection of 2 mg of anti-CSF1 mAb, consisting of 1 mg starting dose followed by 0.5 mg chaser doses on day 5 and 8, and ferumoxytol-enhanced MRI on day 9. Three additional animals served as controls and received intraperitoneal injections of PBS at the corresponding time points above, followed by ferumoxytol-enhanced MRI. One additional mouse received 3 subsequent MRIs at 0, 1, and 24 hours without any contrast agent injection to confirm that tumors did not show any changes in magnetic resonance signal over a 2 day observation period. For all animals, MRI experiments were carried out when mammary tumors reached an approximate size of 1.0 cm.

Macrophage isolation and in vitro labeling

Tumors from MMTV-PyMT mice at day 90 to 95, or PyMT-orthotopic tumors, were isolated and digested in collagenase and DNAse (Roche Applied Sciences), stained over a cell strainer (BD Falcon, BD Biosciences), and incubated with phycoerythrin (PE)-conjugated rat anti-mouse F4/80 antibody (clone CI:A3-1, Caltag). Cells were then incubated with anti-PE magnetic beads and isolated...
over a magnetic column to provide F4/80⁺ cells (macrophages and monocytes) and F4/80⁻ cell fractions (malignant mammary epithelial cells and other stromal populations). In a previous study, we reported that F4/80⁺ cells represent Ly6C⁻Ly6C⁺CD11b⁺F4/80⁺ TAMs (2). A total of 4 x 10⁶ of both F4/80⁺ and F4/80⁻ cells were plated onto cell culture dishes in DMEM supplemented with 10% fetal calf serum. A total of 200 μg [Fe]/mL of either Ferumoxytol, P1133, or P904 were added to the cell culture medium. Additional samples were incubated with P1133 + 1.67 μmol/mL of free folic acid, a dose that corresponds to 10 times the dose of folic acid engrafted onto P1133. Cells were incubated overnight at standard cell culture conditions (37°C, 5% CO₂). The next day, nonadherent cells were discarded and adherent cells were removed via a cell lifter. Removed cells were washed 3 times in PBS and resuspended in 400 μL of ficoll at a density of 1.07 g/mL and placed into 2.0 mL conical tubes for imaging. Experiments were done in duplicates.

**In vitro cell imaging and data analysis**

For in vitro imaging of nanoparticle-loaded cells, a clinical 3T scanner was used (Signa Excite HD, GE Medical Systems) with a standard wrist coil (USA Instruments). Test tubes were immersed in a water bath and a multiecho spin echo sequence was obtained with the following parameters: TE 15, 30, 45, 60 ms, TR 2000 ms, FOV 8 x 8 cm, matrix 256 x 196 pixels, slice thickness 2 mm, and 2 acquisitions. Image processing was done by using MRVision software (MR Vision Co.). T2 relaxation times were calculated assuming a monoexponential signal decay and using nonlinear least square curve fitting on a pixel by pixel basis.

**Determination of cell iron content**

After imaging, cell samples were digested overnight in trypsin and placed in 10% HNO₃. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was done to quantify the iron content per sample (Perkin-Elmer).

**In vivo imaging**

Animals were randomly assigned to MRI when their tumor reached a size of approximately 1.0 cm. Imaging of MMTV-PyMT mice before and after injection of different nanoparticles was done with a 2 T Omega CSI-II magnetic resonance scanner (Bruker Instruments) and imaging of mice before and after anti-CSF1 mAb treatment was done with a 1 T desktop magnetic resonance scanner (Aspect M2 Compact High Performance MR System). Animals were anesthetized with isoflurane and placed on a recirculating water warming pad in a dedicated radiofrequency coil for high resolution MRI. A butterfly cannula filled with heparinized saline solution was introduced into the tail vein and left in place. T1, T2, and T2* weighted imaging sequences were obtained with the following parameters: T1 Spin echo (SE): TR 500 ms, TE 12 ms; T2 SE: TR 2000-2500 ms, TE 15, 30, 45, 60 ms (2T), and TE 20, 40, 60, 80 μs (1T); T2* Gradient echo (GE): TR 240 ms, TE 10 ms, flip angle 30 degrees (2T). MRIs were obtained with a field of view (FOV) of 3 x 3 cm (2T) or 6 x 6 cm (1T), a matrix of 128 x 128 or 200 x 200 pixels, and a slice thickness of 1 to 2 mm.

Following precontrast T1, T2, and T2* weighted imaging, 24 PyMT animals received intravenous injections of 0.5 mmol [Fe]/kg ferumoxytol (n = 7), P904 (n = 7), P1133 (n = 7), P1133 + 2.35 mmol/kg free folic acid (100 times the dose of folate engrafted onto P1133; n = 3), or P1133 + 0.235 mmol/kg free folic acid (10 times the dose of folate engrafted onto P1133; n = 3). Additional tumor-bearing mice after anti-CSF1 mAb treatment (n = 3) or controls (n = 3) were injected with 0.5 mmol [Fe]/kg ferumoxytol. After contrast media injection, without repositioning the mouse, 6 subsequent multiecho T2 SE sequences were obtained over the course of an hour, followed by T1- and T2*-weighted images. Mice were removed from the scanner, allowed to wake up, and imaged 24 hours later with T1, T2, and T2* weighted sequences. T2-relaxation times of the tumor were calculated based on multiecho SE sequences and converted to R2-relaxation rates (R2 = 1/T2), which is proportional to contrast agent concentration. The relative change in R2 data between pre- and postcontrast MRIs, ΔR2 (%), was determined as a quantitative measure of tumor contrast enhancement.

**Histology**

After the last MRI, at 24 hours postcontrast media injection, mice were sacrificed, and mammary tumors explanted and placed in optimal cutting temperature (OCT) compound on dry ice for histologic processing. Samples were cut onto slides and warmed to room temperature, followed by fixation in 100% ice-cold acetone. Some samples were then washed in H₂O, and iron deposits in the tissue were detected using the Accustain Iron Stain Kit (Sigma-Aldrich) according to the manufacturer’s instructions, followed by signal enhancement with Fast 3,3'-diaminobenzidine (DAB, Vector Laboratories) for 2 minutes. After blocking sections with PBS containing 5% goat serum and 2.5% bovine serum albumin (blocking buffer), sections were incubated overnight at 4°C with 0.5× blocking buffer containing either rabbit anti-mouse folate receptor α (1:100, Abcam) or rat anti-mouse folate receptor β (1:8, kind gift from Prof. Matsuyama, Kagoshima University, Japan; ref. 29). Staining for folate receptor β was enhanced using a biotinylated anti-rat secondary antibody (1:200, Vector Laboratories) and the Tyramide Signal Amplification kit (Perkin-Elmer). After extensive washing, sections were incubated overnight with FITC-conjugated rat anti-mouse CD68 (1:50, Serotec), followed by Alexa 488 conjugated goat anti-FITC and either Alexa 546-conjugated donkey anti-rabbit or Alexa 546-conjugated Streptavidin (1:500, Invitrogen). For detection of Ferumoxytol-FITC, sections were stained with rat anti-mouse CD68 (1:100, Serotec), washed, and then incubated with a combination of Alexa 546-conjugated donkey anti-rat and Alexa 488-conjugated goat anti-FITC. All slides were mounted using ProLong Gold with DAPI (Invitrogen) and analyzed using an LSM510 confocal microscope (Zeiss).
Statistics
Statistical analysis comparing the differences of tumor relaxation rates between mice receiving different contrast agents was done with a Wilcoxon rank sum test. A t test was used to determine the significance of differences between different cell samples and differences between age and tumor size of mice. A P value of less than 0.05 was considered significant.

Results

F4/80-positive TAMs phagocytose USPIO in vitro

Following incubation with the iron oxide nanoparticle ferumoxytol, F4/80+ TAMs showed a markedly decreased signal on T2-weighted MRIs, whereas F4/80− cells showed minimal signal changes compared with untreated controls (Fig. 1A). Calculation of changes in relaxation rates (ΔR) as quantitative measures of the magnetic resonance signal enhancement (Fig. 1B) corroborated the qualitative findings with significantly higher ΔR2 data for ferumoxytol-exposed F4/80+ TAMs compared with ferumoxytol-exposed F4/80− cells consisting primarily of carcinoma cells (P > 0.05). Determination of iron content in the samples revealed that increased iron uptake was responsible for the observed relaxation rate changes (Fig. 1C).

Since both TAMs and malignant epithelial cells highly express the folate receptor, folate-linked USPIO have been recently developed for “tumor-targeted imaging” (24, 30, 31). F4/80+ TAMs incubated with folate-engrafted P1133 nanoparticles showed significantly stronger visual and quantitative magnetic resonance signal enhancement as compared with ferumoxytol and P904 (Fig. 1). However, folate-engraftment also leads to significantly increased nanoparticle uptake and magnetic resonance enhancement of F4/80− populations. The P1133-induced signal effect was inhibited by coincubation with free folic acid to P904 levels (Fig. 1), thus indicating that folate-targeting mediates increased USPIO uptake in vitro.

Ferumoxytol leads to persistent tumor enhancement on delayed, postperfusion MRIs and corresponds to specific nanoparticle retention in TAMs

We investigated 90-day-old MMTV-PyMT mice bearing late-stage mammary adenocarcinomas before and after intravenous injection of ferumoxytol, as well as syngeneic mice with PyMT-derived orthotopic mammary tumors. All tumors showed an initial negative (dark) enhancement on immediate postcontrast T2-weighted MRIs, which was most pronounced in the tumor periphery and increased slowly and gradually up to 1.0-hour postinjection (p.i.). This corresponds to an initial blood pool perfusion of USPIO with slow, gradual transendothelial leakage of the nanoparticles into the tumor interstitium (32–35). At 24-hour p.i. of ferumoxytol, all tumors showed a persistent signal decline, which was most pronounced in tumor centers (Fig. 2). We used DAB-enhanced Prussian Blue staining for detection of iron, and immunodetection of CD68+ TAMs in tissue sections of mammary tumors localized ferumoxytol to CD68+ TAMs (Fig. 3A). As it was difficult to show selective uptake using DAB-generated contrast due to high background, we also generated ferumoxytol-FITC to show colocalization by immunofluorescence using an Alexa 488-conjugated anti-FITC antibody. As shown in Figure 3B, ferumoxytol was specifically found within all TAMs, these results indicate that the magnetic resonance signal effects on delayed MRIs were largely due to TAM-mediated uptake of contrast agent.

USPIO-mediated TAM enhancement on delayed MRIs can be increased by folate receptor targeting of nanoparticles

To determine whether folate receptor targeting could enhance the MRIs, we obtained additional MRIs of
MMTV-PyMT mice with late-stage mammary adenocarcinomas injected with the folate-engrafted USPIO P1133, the nontargeted analogue P904, or P1133 plus free folic acid. P1133 and P904 caused a nonspecific tumor-perfusion effect on T2-weighted images during the first hour p.i., which was not significantly different as compared with tumor-bearing mice injected with ferumoxytol (Fig. 2).

Delayed MRIs showed a significantly stronger persistent tumor signal decline at 24-hour p.i. of P1133 compared with ferumoxytol ($P < 0.05$).

*In vivo* inhibition experiments with free folic acid are limited due to rapid liver uptake and renal elimination of free folic acid (36). Inhibition experiments with free folic acid at a 10 times increased dose as compared with the folate dose delivered with P1133 resulted in a minor, albeit not significant inhibition of the P1133-induced tumor enhancement ($P > 0.05$). Inhibition experiments with higher folic acid doses proved toxic in tumor-bearing mice, similar to previous reports (37). However, delayed MRIs showed significantly less tumor enhancement at 24-hour p.i. of P1133.
p.i. of folate-free P904 compared with folate-linked P1133 (P < 0.05; Fig. 2). Because P1133 and P904 are chemically identical except for folate engraftment on P1133, this data indicates increased nanoparticle uptake via folate receptor targeting.

We next evaluated TAMs versus epithelial cells for expression of α and β folate receptor in mammary tumors and revealed folate receptor α staining throughout epithelium, with no expression detectable on CD68⁺ TAMs (Fig. 4A). In contrast, expression of folate receptor β was observed exclusively on CD68⁺ cells, although these represented only a portion of the total CD68⁺ TAMs found within tumors (Fig. 4B) and seemed to be primarily associated with vascular and peripheral regions of mammary tumors. Consistent with the MRIs, Prussian Blue staining for iron was more prominent within tumors from mice injected with P1133 compared with P904 or ferumoxytol (Fig. 4C). Furthermore, although some iron staining was observed in areas that did not seem occupied by TAMs (blue arrow), this was minor compared with staining within stromal areas likely enriched with TAMs (red arrow). Thus, while folate engraftment did increase uptake of USPIOs by cells...
other than TAMs, these results indicate that folate modification of USPIOs may still improve their clinical use as evaluators of TAM presence within tumors.

**Ferumoxytol-enhanced MRI detects TAM-depletion after anti-CSF1-mAb treatment**

Imaging data from a control mouse that underwent 3 subsequent MRIs at 0, 1, and 24 hours without any contrast agent injection confirmed that MMTV-PyMT tumors do not show any intrinsic changes in magnetic resonance signal within a 2-day observation period. Mice treated with anti-CSF1 mAb showed a similar ferumoxytol-tumor perfusion effect compared with untreated controls during the first hour after intravenous ferumoxytol-injection. However, at 24-hour p.i., anti-CSF1 mAb-treated tumors showed less magnetic resonance contrast effects and significantly smaller ΔR2 enhancement data compared with untreated controls (Fig. 5A). Corresponding confocal microscopy evaluations confirmed TAM-depletion of anti-CSF1 mAb-treated tumors (Fig. 5B), indicating that ferumoxytol-enhanced MRI is related to TAM density.

**Discussion**

Results from this study show that ferumoxytol can be used as a reliable tool to quantitatively monitor macrophage presence in tumors, suggesting that this imaging technique can be readily investigated as a surrogate measure to predict outcomes for patients with breast cancer, and applied to monitor TAM-targeted therapies now in clinical trials.

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**Figure 4. Folate receptor expression and folate-targeted uptake of nanoparticles.**

A, staining for folate receptor α (FRα; red) and CD68⁺ macrophages (green) shows that expression of FRα is localized to carcinoma cells within mammary tumors. B, a subpopulation of CD68⁺ macrophages (green) expresses folate receptor β (FRβ; red staining). C, Prussian Blue staining for iron with DAB enhancement within mammary tumors from mice injected with ferumoxytol, P904, or P1133. Scale bars are shown in images.
displayed as mean ± SD of 3 mice treated with anti-CSF1 mAb or PBS control. An additional control mouse underwent serial magnetic resonance without any contrast agent injection to confirm that MMTV-PyMT tumors do not show any intrinsic changes in magnetic resonance signal within a 2-day observation period. Note that mice treated with anti-CSF1 mAb showed significantly smaller ΔR2 enhancement data compared with untreated controls. B, corresponding confocal microscopy evaluations confirmed TAM-depletion within anti-CSF1 mAb-treated tumors.

Figure 5. Ferumoxytol-enhanced MRI detects TAM-depletion noninvasively in vivo. A, quantitative magnetic resonance signal enhancement (delta R2 measurement) of MMTV-PyMT mammary tumors before and after iron oxide-nanoparticle administration, displayed as mean ± SD of 3 mice treated with anti-CSF1 mAb or PBS control. An additional control mouse underwent serial magnetic resonance without any contrast agent injection to confirm that MMTV-PyMT tumors do not show any intrinsic changes in magnetic resonance signal within a 2-day observation period. Note that mice treated with anti-CSF1 mAb showed significantly smaller ΔR2 enhancement data compared with untreated controls. B, corresponding confocal microscopy evaluations confirmed TAM-depletion within anti-CSF1 mAb-treated tumors.

To the best of our knowledge, this is the first report of utilizing a clinically applicable nanoparticle for TAM-detection by MRI. Other investigators have reported TAM-detection with nanoparticles that are not clinically applicable, for either MRI (38) or optical imaging (39). In addition, there have been reports of radiotracers-based approaches for TAM detection by positron emission tomography (PET; ref. 39). The latter is associated with radiation exposure and therefore not utilized for breast imaging. MRI, on the other hand, is radiation free, established for breast cancer detection, and integrates near-microscopic anatomic resolution, high sensitivity, and excellent soft tissue contrast. Although histologic methods for quantifying TAMs are more precise, they are invasive, limited to one or few observations, and not representative of the whole tumor in the case of biopsies.

Preclinical and clinical evidence indicates that chronic presence of diverse leukocyte subsets within the stroma of breast cancers promotes tumor growth and metastasis (3, 40, 41). TAMs play a significant protumorigenic role in this context by augmenting neoplastic cell survival and motility via elaboration of cytokines, chemokines, proteases, and reactive oxygen species (3, 4, 42, 43). TAMs also potentiate pulmonary metastasis of mammary adenocarcinomas through enhanced angiogenesis via regulation of VEGF bioavailability and supplying epidermal growth factor (EGF) to mammary epithelium (5, 44), in addition to suppression of protective adaptive immune responses (3, 42, 43, 45, 46). Exuberant macrophage recruitment to breast cancer has been reported to be strongly associated with poor prognosis, both in animal models and in patients (2, 4, 42–44). Although phagocytotic capacity can be altered by in vitro polarization of macrophages, we have no evidence that ferumoxytol uptake corresponds to a particular TAM phenotype. Regardless, aggressive human breast cancers have been reported to contain few (if any) TH1-polarized macrophages (47), and in the MMTV-PyMT transgenic model in particular, TAMs are strongly TH2-polarized by interleukin 4 (2).

Ferumoxytol enhancement is a new, noninvasive indicator for TAM-tumor infiltration, which may serve as a novel biomarker for breast cancers with poor outcome and may be utilized to stratify tumors with high TAM infiltration for immune-targeted therapies. There have been multiple approaches for specific targeting and/or blockade of TAMs for therapeutic purposes (29, 48, 49), some of which are currently in clinical trials based on experimental data showing that genetic, immunologic, or pharmacologic blockade of CSF1, or its receptor (CSF1R), decreases TAM presence in tissues and in experimental solid tumors, correlating with diminished tumor angiogenesis, and reduced primary tumor growth and pulmonary metastasis (50–54). Because these therapies are not cytotoxic, biomarkers of their efficiency at inducing macrophage depletion would be of great clinical benefit. Moreover, since clinical trials of new therapeutic drugs and new combination therapies are expensive and take years to complete, the immediate value and impact of imaging TAMs and/or TAM-depletion via MRI would be immense.

We recognize several limitations with this approach. Studies reported herein were done with ferumoxytol doses of 0.5 mmol/kg. Previously described ferumoxytol doses in humans were in the order of 0.035–0.072 mmol/kg (18, 19). Iron oxide nanoparticles are generally applied in higher doses in rodents as opposed to humans to compensate for the relatively shorter blood half life in rodents. However, future clinical applications must show if the currently applied dose in patients is sufficient for TAM detection, or if the dose can be safely increased. Of note, ferumoxytol showed an excellent safety profile in more than 700 patients (17). Larger superparamagnetic iron oxide nanoparticles (SPIO, diameter >50 nm) provide higher cellular uptake via ex vivo labeling. However, SPIOs are rapidly phagocytosed by macrophages in liver, spleen, and bone marrow and do not reach TAMs in vivo. USPIOs, on the other hand, are not as quickly recognized by the RES and have a longer circulation time, and can therefore leak into tumor interstitium, where they can be phagocytosed by TAMs. Thus, for “in vivo” TAM labeling, USPIOs are advantageous (32, 33).
As shown by our data, an alternative approach to increase the sensitivity of MRI would be to utilize folate-engrafted nanoparticles. Although such particles are currently not available for clinical use, precursors of such compounds are currently entering clinical trials and thus folate-engrafted derivatives may become clinically available in the future. Folate-encraftment enhances nanoparticle uptake via the folate receptor, which is highly expressed on TAMs (31). Several investigators including us have reported uptake of USPIO and folate-engrafted USPIO by neoplastic cells, which may be a confounding variable when aiming for TAM detection (24, 55, 56). However, data presented here reveal that the ferumoxytol and P1133 uptake in malignant epithelial cells is significantly lower as compared with macrophage uptake, leading to negligible interferences of our imaging approach.

In conclusion, we have shown that iron oxide nanoparticle-enhanced MRI can be utilized to detect TAMs in a mouse model of mammary carcinogenesis. Ferumoxytol is a clinically available nanoparticle that can be readily applied for TAM imaging in patients with breast cancer via an "off label" use. Macrophage detection may be enhanced by using folate-engrafted nanoparticles that may become available for clinical use in the near future. Clinical studies are underway to evaluate these findings in patients.

Disclosure of Potential Conflicts of Interest

C. Corot works for Guerbet Research and provided contrast agents for this study. She was not involved in data analyses. The other authors disclosed no potential conflicts of interest.

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Immune microenvironments in solid tumors: new targets for therapy


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Immune microenvironments in solid tumors: new targets for therapy

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Leukocytes and their soluble mediators play important regulatory roles in all aspects of solid tumor development. While immunotherapeutic strategies have conceptually held clinical promise, with the exception of a small percentage of patients, they have failed to demonstrate effective, consistent, and durable anti-cancer responses. Several subtypes of leukocytes that commonly infiltrate solid tumors harbor immunosuppressive activity and undoubtedly restrict the effectiveness of these strategies. Several of these same immune cells also foster tumor development by expression of potent protumor mediators. Given recent evidence revealing that immune-based mechanisms regulate the response to conventional cytotoxic therapy, it seems reasonable to speculate that tumor progression could be effectively diminished by combining cytotoxic strategies with therapies that blunt protumor immune-based effectors and/or neutralize those that instead impede development of desired anti-tumor immunity, thus providing synergistic effects between traditional cytotoxic and immune-modulatory approaches.

Despite expanded appreciation for the diversity of cellular mechanisms fostering solid tumor development, anti-cancer therapy remains heavily reliant on cytotoxic modalities—including chemotheraphy (CTX) and radiation therapy (RT)—that kill rapidly proliferating (neoplastic) cells within tumors. Emerging clinical and experimental data indicate that clinical responses to cytotoxic therapy can be improved if immunogenic cell death pathways are also concurrently activated (Ma et al. 2010). Evidence for simultaneous engagement of immunogenic cell death programs has been provided for some tumors following conventional cytotoxic therapy, based on the increased presence of molecules released by dying cells thought to be “sensed” by leukocytes [Kepp et al. 2011], the result of which leads to enhanced “killing” of target cells. While an obvious clinical strategy has been to bolster these anti-tumor mechanisms, achieving clinical success has been limited. Possible mechanisms underlying these clinical failures include the underappreciated properties of some immune cell types that can harbor both immunosuppressive activity—e.g., blunting malignant cell killing by CD8+ cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells—simultaneously with protumor activities that promote survival, invasion, and dissemination of malignant cells [Ruffell et al. 2010]. Experimental studies in immunocompetent murine models of human cancer have provided support for this concept by revealing that blockade of some protumor immune-based pathways effectively bolsters antitumor immunity (neoplastic cell killing) when combined with cytotoxic therapy [DeNardo et al. 2011].

Cancer and chronic inflammation

In homeostatic tissue, resident immune cells serve as sentinels that safeguard tissue and organ integrity. Following acute damage [e.g., infiltration/infection by pathogens or physical trauma], one activity of resident leukocytes is to limit tissue damage while engaging tissue repair programs [e.g., activation of stromal fibroblasts and vasculature for matrix resynthesis and angiogenesis, respectively, and recruitment of leukocytes from peripheral blood to remove damaged cells and debris] and facilitate re-epithelialization, all without inducing autoimmunity. Following resolution of wound responses, tissue damage is [hopefully] minimal and homeostatic maintenance programs return such that organ physiology is unperturbed.

In cancer, immune cells play dual roles with potential to either eliminate or promote malignancy. Premalignant tissues contain proliferating cells harboring genomic damage [e.g., “initiated” cells] that typically activate critical proliferation/survival pathways. In these tissues, chronic engagement/activation of immune cells, stromal fibroblasts, and vascular and mesenchymal support cells together fosters survival of “initiated” cells, culminating in tissue expansion and development of premalignant lesions via a process reminiscent of typical “inflammatory-type” responses observed in tissues responding to acute damage/truma (Coussens and Werb 2002). When these chronic inflammatory-type events are sustained, neoplastic progression can ensue. Unresolved chronic immune responses thus resemble the resolution phase of wound healing, where the tumor microenvironment contains...
significant infiltrations of cells with immunosuppressive activity akin to a wound failing to heal (Coussens and Werb 2002). Consistent with this, studies evaluating leukocyte complexity by flow cytometry in human (and murine) tumors have identified multiple immune cell types that variably contain immunosuppressive activity (e.g., block anti-tumor CTL- or NK T-cell-mediated killing of malignant cells)—including regulatory T cells \( (T_{reg}) \), immature monocytes \( (iMCs) \), alternatively activated macrophages \( (AAMs) \), mast cells, neutrophils, Tie2\(^{+}\) monocytes, dendritic cells \( (DCs) \), and T helper 2 \( (T_{H2}) \)-CD4\(^{+}\) effector T cells \( (DeNardo et al. 2011; Rolny et al. 2011; Ruffell et al. 2011) \)—and thus afford developing malignancies a mechanism to escape killing by T cells. Mouse modeling studies indicate that the net effect of these assemblages results in favoring tumor expansion \( (Fig. 1; DeNardo et al. 2010; Grivennikov et al. 2010; Qian and Pollard 2010; Ruffell et al. 2010) \). Three types of leukocytes in particular have emerged as playing significant roles in suppressing anti-tumor immune responses: \( T_{reg} \) cells, iMCs, and AAMs.

**Immune-based programs that blunt anti-tumor immunity**

\[ T_{reg} \] cells, a subset of the CD4\(^{+}\) T-cell population, constitutively express the high-affinity interleukin-2 \( (IL-2) \) receptor \( (CD25, CTLA-4) \), glucocorticoid-induced tumor necrosis factor receptor \( (GITR) \), and the lineage-specific transcription factor Foxp3 and play an important physiological role in suppressing responses to self-antigens, thereby preventing autoimmunity \( (Hori et al. 2003) \). As many malignant cell types express self-antigens \( (Kawakami and Rosenberg 1997) \), it follows that \( T_{reg} \) cells in their physiologic capacity...
hamper anti-tumor immunity and that tumors may evade immune detection by engaging or activating T\textsubscript{reg} cell-based pathways. This notion has been borne out by studies evaluating peripheral blood, tumor-draining lymph nodes, and tumors—e.g., breast (Bates et al. 2006) and gastrointestinal (Sasada et al. 2003)—where increased presence of T\textsubscript{reg} is prominent. Importantly, increased frequency of T\textsubscript{reg} also correlates with poor outcome for several cancer types (Sasada et al. 2003; Curiel et al. 2004; Bates et al. 2006). Further support for the notion of tumors activating development of T\textsubscript{reg} comes from studies showing that stromal cells produce chemokines such as CCL22 (Curiel et al. 2004) and cytokines such as transforming growth factor-\(\beta\) (TGF\(\beta\)) (Ghiringhelli et al. 2005) that enhance T\textsubscript{reg} infiltration.

The ability of T\textsubscript{reg} to block anti-tumor immunity has been confirmed in vivo, where adoptive transfer of CD3\^-CD25\^- T cells from patients into NOD/SCID mice was found to retard tumor growth, while simultaneous transfer of T\textsubscript{reg} abrogated the protective effect (Curiel et al. 2004). Mechanistically, in vitro studies have revealed that leukocytes isolated from melanoma and ovarian cancer patients depleted of T\textsubscript{reg} cells ex vivo enabled remaining leukocytes to respond to selective tumor antigens (Nishikawara et al. 2005). Also significant is the observation that T\textsubscript{reg} directly promote malignant cell proliferation and dissemination via soluble mediators they express (Tan et al. 2011). Given evidence demonstrating that T\textsubscript{reg} block anti-tumor immunity (Dunn et al. 2004), it stands to reason that, in order to augment anti-tumor immunity therapeutically, neutralizing pathways that bolster the presence or activity of T\textsubscript{reg} would likely provide a survival advantage.

**AAMs**

Unlike T\textsubscript{reg}, macrophages derived from circulating immature myeloid precursors play a more complex role in regulating immune responses owing to their ability to possess both pro- and anti-tumor bioactivity, depending on the cytokine milieu they encounter once within tissue (Qian and Pollard 2010). Classically activated macrophages (CAMs) regulated by \(T_{H}1\) cytokines—e.g., interferon \(\gamma\) (IFN\(\gamma\)), tumor necrosis factor \(\alpha\) (TNF\(\alpha\)), or granulocyte/monocyte colony-stimulating factor (GM-CSF)—possess enhanced cytotoxic activity, produce proinflammatory \(T_{H}1\) cytokines, and have antigen presentation capability (Mosser and Edwards 2008). In contrast, macrophages exposed to \(T_{H}2\) cytokines (IL-4, IL-13, etc.), immune complexes, or immunosuppressive cytokines become alternatively activated (AAMs) (Qian and Pollard 2010) and instead typically lack cytotoxic activity, block CD8\^+ T-cell proliferation or infiltration, and express a diverse assortment of proliferative, proangiogenic, and tissue remodeling mediators (DeNardo et al. 2009, 2011; Andreu et al. 2010; Qian and Pollard 2010; Ruffell et al. 2010). Experimental data from murine models indicate that AAMs become \(T_{H}2\)-skewed due to high levels of type 2 cytokines (IL-4 and IL-13) released by infiltrating CD4\^+ T cells and neoplastic epithelial cells (DeNardo et al. 2009; Gocheva et al. 2010) or TSLP (thymic stromal lymphopoietin) also produced by neoplastic epithelial cells (Pedroza-Gonzalez et al. 2011). While AAMs are typical constituents of tissue repair processes, in solid tumors, rather than aiding in “healing,” they instead foster neoplasia (Qian and Pollard 2010). AAMs produce a multitude of factors—including epidermal growth factor (EGF), TGF\(\beta\), and cathepsin proteases—that together provide a survival advantage to malignant epithelia and regulate their response to cytotoxic therapies (DeNardo et al. 2011; Shree et al. 2011). Data from human tumors support this hypothesis, since the presence of AAMs that are CD163^+CD204^+ correlate with reduced survival for patients with breast cancer, non-small-cell lung cancer, and Hodgkin’s lymphoma (Kawai et al. 2008; Steidl et al. 2010; DeNardo et al. 2011). Owing to lack of specificity for CD68 as a macrophage-specific marker, however, some of these findings may need to be revisited (Ruffell et al. 2011).

The importance of macrophages in tumor progression is further underscored by mouse modeling data revealing that genetic loss of CSF1/CSF1 receptors (Lin et al. 2001) or blockade of M-CSF-induced signaling cascades (DeNardo et al. 2011) reduces macrophage presence in tumors and correlates with reduced mammary tumor metastasis. Thus, AAMs, through their ability to differentially regulate immunity and express molecules that support angiogenesis/tissue remodeling and proliferation, profoundly affect the development, maintenance, and dissemination of malignant tumors.

**Immunosuppressive monocytes**

Sharing the same common myeloid progenitor as macrophages, immunosuppressive monocytes in rodent tumor models encompass a diverse population of cells characterized by expression of surface markers, including CD11b and Gr1 (Ostrand-Rosenberg 2008; Gabrilovich and Nagaraj 2009), and include monocytes variably referred to as myeloid-derived suppressor cells (MDSCs), iMCs, inflammatory monocytes, and neutrophils (Ostrand-Rosenberg 2008). Human equivalents have been identified as LIN^-/Lo^ human leukocyte antigen (HLA)-DR CD33^+ CD11b^+ and CD14^+HLA-DR^-/Lo^ cells (Serafini et al. 2006); however, as with mice, these share markers with multiple mature granulocytic subtypes and thus likely represent a mixed population in which some cells contain immunosuppressive properties. MDSCs and iMCs are functionally characterized by their T-cell-suppressive activity; e.g., the ability to suppress T- and NK cell proliferation via arginase I, inducible nitric oxide synthase expression, and peroxynitrite, and, at the same time, promote generation of T\textsubscript{reg} cells (Mazzoni et al. 2002; Gabrilovich and Nagaraj 2009; Doedens et al. 2010; Lu et al. 2011). In mice, systemic increases in the presence of MDSCs and iMCs have been observed when syngeneic mice are transplanted with or develop spontaneous tumors (Ostrand-Rosenberg 2008). Significant increases in MDSCs in peripheral blood are also a common feature for patients with several types of cancer (Almand et al. 2001). Moreover, in murine models of cancer, MDSCs/iMCs have also been found to mediate resistance to some forms of anti-angiogenic
Cytotoxic therapy and immune cells

Cytotoxic therapy and immunogenic cell death

Cytotoxic therapy (CTX and RT), in combination with surgery, forms the cornerstone of systemic treatment for most clinically detectable solid tumors. Significantly, most cytotoxic therapies result in immune suppression due to a higher sensitivity of bone marrow-derived stem cells and many leukocyte subsets, especially lymphocytes, to their cytotoxic effects. Through specialized cell death pathways, including Fas–FasL, lymphocytes respond to DNA damage induced by CTX and RT by undergoing early apoptosis at doses significantly lower than other cell types, especially epithelial or neural cell types. Bone marrow-derived stem cells are also uniquely sensitive to CTX and RT [Apetoh et al. 2007; Ghiringhelli et al. 2009], and their early destruction is likely a dose-limiting toxicity for many of these modalities; thus, administration of cytotoxic agents can lead to systemic immune suppression. That said, there is increasing evidence that within tumors, cell death generated by these agents also triggers activation of other immune response pathways that serendipitously also regulate therapeutic efficacy of the particular cytotoxic agent/modality (Table 1).

Whereas neoplastic cells have long been thought to undergo an “immunologically silent” demise following cytotoxic therapy, whereby apoptotic machinery eliminates them [Albert et al. 1998], recent studies have challenged this notion [Ma et al. 2011] and revealed that nonapoptotic and biochemically distinct cell death pathways are also activated following RT and some forms of CTX [e.g., anthracyclines and oxaliplatin] (Fig. 1). Mechanistically, leukocytes detect cell death through immune-based receptors selective for molecules released by dying cells (often termed “danger signals”), including Toll-like receptor-4 (TLR-4) and its ligand, the high-mobility group box protein 1 (HMGB1) [Apetoh et al. 2007]. Detection of danger signals by resident leukocytes results in subsequent activation of both innate [myeloid and NK cells] and adaptive (T and B) cell lineages. Molecular mechanisms underlying immunogenic cell death following cytotoxic therapy involve activation of several critical sequential checkpoints. These include [1] exposure of endoplasmic reticulum (ER)-resident protein complexes, comprised of calreticulin/ERp57 on plasma membranes of neoplastic cells that serve as “eat me” signals for DCs; [2] release of the chromatin-binding HMGB1 protein, which by a TLR4/MyD88-dependent mechanism inhibits degradation of DC phagosomes, thereby facilitating antigen presentation [Apetoh et al. 2007]; [3] ATP release from dying neoplastic cells and subsequent engagement of DC P2RX7 purinergic receptors, leading to IL-1β release [Ghiringhelli et al. 2009]; and [4] effective antigen cross-presentation by DCs with increased production of IFNγ/IFNγ receptors and CD8+ CTL-dependent killing responses. Experimental evidence supporting these pathways emanates from in vivo evaluation in murine tumor models where the immune response induced by CTX or RT efficiently prevents tumor growth dependent on activation of these pathways [Apetoh et al. 2007; Ghiringhelli et al. 2009]. Clinical evidence for the importance of these mechanisms is provided by human breast cancer patients harboring Asp299Gly TLR4 polymorphisms or loss-of-function mutations in the P2RX7 gene, both of which disrupt DC–T-cell functional interactions by impairing DCs’ ability to sense HMGB1 and ATP release by dying cells, and correlates clinically with resistance to CTX (anthracyclines) and RT [Ghiringhelli et al. 2009].

Recognition that immune-based mechanisms modulate the response to cytotoxic therapy implies that the ultimate effectiveness of cytotoxic modalities could be improved by combinatorial approaches that also engage immunogenic death programs. Thus, strategies improving antigen presentation [to T cells] and/or increasing macrophage cytolytic activity would theoretically impede tumorigenesis if the protumorigenic properties of those leukocytes following cytotoxic therapy could also be effectively blunted. Requisite for success of this scenario is that T11-based immune programs would be fostered, and dominant T12-type programming would be blunted. T11 programming in response to increased expression of type 1 cytokines [TNFs, IFNγ, and IL-2] activates cell-mediated responses that are “anti-tumor” in nature. T12 programming, on the other hand, is mediated by expression of type 2 cytokines (IL-4, IL-10, and TGFβ) that instead initiate tissue remodeling, angiogenesis, and [sometimes] humoral immunity, and together foster a protumorigenic state [Yang et al. 2008; DeNardo et al. 2010; Ruffell et al. 2010].

Evidence that forced T11 polarization of tumor microenvironments can improve response to cytotoxic therapy has been observed. For example, immunization with plasmacytoma supernatant plus IL-1 resulted in decreased tissue/tumor levels of IL-10 and TGFβ and increased levels of IFNγ and IL-2, thus favoring T11 immunity and tumor regression [Li et al. 1998]. Other studies reported that combined CTX or RT with DC vaccination, which augments the initial T11 response through enhanced antigen presentation, also resulted in tumor regression [Koike et al. 2008; Matsumura et al. 2008]. A general conclusion from these studies is that cytotoxic therapy indeed fosters an anti-tumor immune microenvironment; however, this response tends not to be durable, likely due to protumor, immunosuppressive programs that become dominant, thereby fueling tumor recurrence and subsequent resistance to therapy.

Cytotoxic therapy and cancer immunotherapy—a different approach

Harnessing the body’s own immune system to fight cancer has long been considered the ultimate treatment for cancer because of its potential to specifically and durably target antigen-positive neoplastic cells while limiting damage to
Table 1. **Immune effects of cytotoxic agents**

<table>
<thead>
<tr>
<th>Cytotoxic agent</th>
<th>Example</th>
<th>Effect on tumor immune response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating agents</td>
<td>Cyclophosphamide, ifosfamide, busulfan, melphalan, dacarbazine</td>
<td></td>
<td>Bracci et al. 2007; Robert et al. 2011</td>
</tr>
<tr>
<td>Anthracyclines</td>
<td>Daunorubicin, doxorubicin</td>
<td>●</td>
<td>Ma et al. 2010; Ramakrishnan et al. 2010</td>
</tr>
<tr>
<td>Anti-metabolites</td>
<td>Methotrexate, 5-fluorouracil, fludarabine, gemcitabine</td>
<td></td>
<td>Suzuki et al. 2005; Ma et al. 2010</td>
</tr>
<tr>
<td>DNA methytransferase</td>
<td>2′-Deoxy-5-azacytidine [decitabine or DAC]</td>
<td>●</td>
<td>Ma et al. 2010</td>
</tr>
<tr>
<td>Platinum agents</td>
<td>Carboplatin, cisplatin, oxaliplatin</td>
<td>●</td>
<td>Ghiringhelli et al. 2009; Ma et al. 2010;</td>
</tr>
<tr>
<td>Spindle poisons</td>
<td>Paclitaxel, docetaxel, vincristine, vinblastine [Vinca alkaloids]</td>
<td>●</td>
<td>Ramakrishnan et al. 2010; Apetoh et al. 2007;</td>
</tr>
<tr>
<td>Topoisomerase inhibitors</td>
<td>Etoposide, irinotecan</td>
<td></td>
<td>Ma et al. 2010; Ramakrishnan et al. 2010</td>
</tr>
<tr>
<td>Radiation</td>
<td>γ/X-rays, electrons, Protons, α particles</td>
<td>●</td>
<td>Matsumura et al. 2008; Ahn et al. 2010;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>●</td>
<td>Meng et al. 2010; DeNardo et al. 2011</td>
</tr>
</tbody>
</table>

Common classes of cytotoxic agents are listed, with their known effects on immune responses indicated.
Cancer vaccines

Inspired by success with vaccination against bacterial and some viral pathogens, a variety of approaches have been explored in an attempt to immunize patients against their own cancers, some of which include use of whole [killed] tumor cells, proteins, peptides, or DNA vaccines [Giaccione et al. 2005; Testori et al. 2008; Doughan and Dranoff 2009; Amato et al. 2010]. In spite of limited success with these, there is renewed interest following recent positive clinical results in prostate cancer and lymphoma. Sipuleucel-T (Provenge), a cellular vaccine comprising autologous antigen-presenting cells (APCs) cultured with a fusion protein of prostatic acid phosphatase with GM-CSF, extended median survival in two independent phase III trials, leading to FDA approval for treatment of advanced prostate cancer [Small et al. 2006; Kantoff et al. 2010]. Other encouraging results have been reported, most notably idiotypic vaccination for follicular lymphoma in a phase III trial that demonstrated a prolonged period of CTX-induced remission [Neelapu et al. 2005].

DCs link innate and adaptive immunity and can induce contrasting states, including immunity and tolerance to self. Multiple populations of DCs are recognized in vivo in both human and murine tumors, each with distinct properties that vary according to the type of DC and its environment [Hashimoto et al. 2011]. While antibody responses are preferentially mediated by CD11c+ dermal DCs, CTL responses are instead preferentially mediated by Langerhans cells [Hashimoto et al. 2011], thus indicating that DC-mediated mechanisms inducing humoral and/or cellular immunity are fundamentally distinct. Early clinical trials testing vaccination with ex vivo generated DCs pulsed with tumor antigens provided proof-of-principle evidence that therapeutic immunity could be elicited, however, clinical benefit measured by regression of established tumors in patients with stage IV cancer was observed in only a small percentage of patients [Palucka et al. 2008]. Patients with soft tissue sarcoma who received fractionated external beam radiation in combination with administration of intratumoral DCs demonstrated an increased T-cell infiltration, with tumor-specific CD4+ T cells positively correlating with tumor-specific immune responses [Finkelstein et al. 2011]. Thus, new-generation DC vaccines are needed that generate large numbers of high avidity effector anti-tumor T cells able to overcome suppressive mechanisms in the tumor microenvironment. These, combined with therapies blunting T112-based pro-tumor immunity and CTX or RT, would thus be anticipated to provide much more durable tumor repression.

Cancer immunotherapy II: targeting immunosuppressive pathways and cells

Treg cells Evidence for the role of Treg cells in anti-tumor immunity was first provided by Sakaguchi and colleagues (Shimizu et al. 1999; Sakaguchi 2005) using a syngeneic murine heterotopic transplant model. This was later reproduced in several murine tumor models, all of which demonstrated that depletion of Treg cells via anti-CD25 mAb prior to tumor inoculation led to syngeneic tumor rejection [Casares et al. 2003]. In conjunction with cytotoxic therapy, strategies targeting CD25, such as depleting mAbs or an IL-2-diphtheria toxin fusion protein, enhanced...
<table>
<thead>
<tr>
<th>Immunotherapy type</th>
<th>Species</th>
<th>Cancer type</th>
<th>Agent</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>Mouse</td>
<td>Colon, breast, lymphoma</td>
<td>CTL + paclitaxel or cisplatin or doxorubicin</td>
<td>Significant tumor growth delay seen with combination therapy due to increased sensitivity of tumor cells, Granzyme B via mannose-6 phosphate receptor</td>
<td>Ramakrishnan et al. 2010</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Melanoma</td>
<td>Ex vivo expanded tumor-infiltrating lymphocytes + IL-2; cyclophosphamide + fludarabine + total body irradiation</td>
<td>Objective response rate of 50%–70% in patients with combined regimen vs. 15% with historical standard of IL-2 + dacarbazine</td>
<td>Dudley et al. 2008</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Melanoma</td>
<td>Ex vivo expanded CD8+ T cells against melanoma antigen Melan-A</td>
<td>Tumor-specific immune response detected in 27.2% [three of 11 patients]</td>
<td>Mackensen et al. 2006</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Melanoma</td>
<td>Peptide vaccine [Hsp96]</td>
<td>No improvement in overall survival (P = 0.32)</td>
<td>Testori et al. 2008</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Kidney</td>
<td>Poxvirus encoding tumor antigen ST4</td>
<td>Specific antibody responses detected, but no improvement in overall survival (P = 0.55)</td>
<td>Amato et al. 2010</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Lung</td>
<td>Antibody that mimics ganglioside GD3</td>
<td>Specific antibody responses detected, but no improvement in overall survival (P = 0.28)</td>
<td>van Meerheek et al. 2005</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Follicular lymphoma</td>
<td>Idiotype vaccination</td>
<td>Induction of prolonged remission</td>
<td>Neclapu et al. 2005</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Sarcoma</td>
<td>Intratumoral DCs + RT</td>
<td>Tumor-specific immune responses detected in 52.9% of patients [nine of 17 patients]</td>
<td>Finkelstein et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Prostate</td>
<td>Tumor antigen [prostatic acid phosphatase] coupled to GM-CSF [Sipuleucel-T]</td>
<td>4-mo improvement in median survival compared with no treatment (P = 0.02)</td>
<td>Small et al. 2006, Kantoff et al. 2010</td>
</tr>
<tr>
<td>Inhibition or depletion of (T_{reg}) cells</td>
<td>Mouse</td>
<td>Lymphoma, colon</td>
<td>Anti-CD25 depleting mAb + attenuated Poxvirus + RT</td>
<td>Increased antigen-specific immune responses with combined modalities</td>
<td>Kudo-Saito et al. 2005</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Melanoma</td>
<td>Agonistic anti-OX40 mAb + cyclophosphamide</td>
<td>Improved survival and significantly delayed tumor growth secondary to intratumoral (T_{reg})-specific hyperactivation and apoptosis</td>
<td>Hirschhorn-Cymerman et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Melanoma</td>
<td>IL-2 fusion protein with diphtheria toxin [denileukin difitox] + DC vaccine</td>
<td>Increased T-cell and antibody response to tumor antigen CEA</td>
<td>Morse et al. 2008</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Melanoma</td>
<td>Anti-CTLA4 [ipilimumab] mAbs + dacarbazine</td>
<td>2-mo improvement in median survival versus dacarbazine alone (P &lt; 0.001)</td>
<td>Hodi et al. 2010, Robert et al. 2011</td>
</tr>
</tbody>
</table>
Table 2. continued

<table>
<thead>
<tr>
<th>Immunotherapy type</th>
<th>Species</th>
<th>Cancer type</th>
<th>Agent</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depletion or repolarization of macrophages</td>
<td>Mouse</td>
<td>Melanoma</td>
<td>Clodronate liposomes + RT</td>
<td>Delayed tumor growth following RT only when macrophages were depleted</td>
<td>Meng et al. 2010</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Breast</td>
<td>CSF-1 receptor [CSF-1R] inhibitor + paclitaxel</td>
<td>Delayed tumor growth with combination of paclitaxel and macrophage deletion but not with macrophage depletion alone</td>
<td>DeNardo et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Human, mouse</td>
<td>Pancreas</td>
<td>Agonistic CD40 mAb + gemcitabine</td>
<td>Tumor response rate of 30% [vs. 5.4% historically for gemcitabine alone] in both humans and mice; mechanism depends on repolarization of tumor-associated macrophages</td>
<td>Beatty et al. 2011</td>
</tr>
<tr>
<td>Depletion of iMCs/MDSCs</td>
<td>Human, mouse</td>
<td>Lung [non-small cell]</td>
<td>ATRA + paclitaxel/cisplatin</td>
<td>2.9 mo increase in progression-free survival (P = 0.008); mouse model indicates that ATRA mechanism may be combination of differentiation of iMC into macrophages and increased sensitivity of tumor cells to CTX</td>
<td>Arrieta et al. 2010</td>
</tr>
<tr>
<td></td>
<td>Human, mouse</td>
<td>Multiple</td>
<td>Sunitinib [tyrosine kinase inhibitor] + RT</td>
<td>59% [21 of 36] of lesions had complete or partial response; mouse model shows decreased circulating (T_{reg}) and iMC/MDSC and suppressive function with sunitinib</td>
<td>Kao et al. 2009; Ozao-Choy et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Head and neck, breast</td>
<td>Anti-CD11b mAb</td>
<td>Delayed tumor growth with combination of mAb and RT or paclitaxel</td>
<td>Ahn et al. 2010; DeNardo et al. 2011</td>
</tr>
</tbody>
</table>

Results from selected immunotherapy studies in murine models of human cancer and in patients with various types of solid cancers.
immune cells as targets for anti-cancer therapy

anti-tumor immune responses in both murine models and humans [Kudo-Saito et al. 2005; Mackensen et al. 2006; Morse et al. 2008]. However, strategies targeting CD25 lack specificity, in that activated T cells also express CD25; thus, these agents may also blunt formation of robust anti-tumor T-cell responses while also depleting Treg cells [Curtin et al. 2008]. Therefore, other strategies targeting the Treg—such as the agonistic antibody against OX40, a TNR receptor (TNFR) family costimulatory molecule expressed on T cells and DCs—in combination with cyclophosphamide minimize this paradox by inducing Treg-specific apoptosis [Hirschhorn-Cymerman et al. 2009].

Targeting Treg with CTLA-4 antagonists has been perhaps the most successful of the strategies targeting an immunosuppressive pathway, although others such as B7-H3, PD-L1, and CD73 are currently under investigation [Yi and Chen 2009; Ascierto et al. 2010; Jin et al. 2011]. CTLA-4 is a negative costimulatory molecule expressed on both activated T cells and Treg cells that helps dampen ongoing immune response, is frequently up-regulated on chronically activated and exhausted T cells [Engelhardt et al. 2006; Wherry et al. 2007], and not only inhibits T-cell activation, but also promotes Treg function [Teft et al. 2006]. Results from a phase III trial evaluating the CTLA-4-blocking mAb ipilimumab, recently approved for the treatment of advanced malignant melanoma by the FDA, revealed extended overall survival of previously treated melanoma patients, correlating with increased CD8+ T-cell activation and Treg inhibition [Hodi et al. 2010]. A subsequent landmark study demonstrated improved survival in patients with untreated advanced melanoma who received ipilimumab combined with the CTX agent dacarbazine, as compared with those receiving CTX alone [Robert et al. 2011]. This study supports the hypothesis that the combination of CTX with a reduction in the suppressive environment—in this case, elimination of Treg—is a strategy that can lead to more effective anti-tumor immunity.

AAMs CTX and RT stimulate recruitment of macrophages (and monocytes) into tissues through direct induction of myeloid cell chemoattractant molecules. Epithelial cells rapidly respond to CTX (paclitaxel, cisplatin, and carboplatin) and RT by direct mRNA induction of monocyte-promoting chemokines such as csf-1, IL-34, ccl2/MCP-1, ccl5, cxcl10, cxcl11, cx3c11, and HIF1 [Kioi et al. 2010; DeNardo et al. 2011; Rufell et al. 2011]. Cyclophosphamide, oxaliplatin, and RT induce Tg1 (Bracci et al. 2007; Ghiringhelli et al. 2009) as well as Tg12 cytokines [Gremy et al. 2008], indicating that CTX and RT have the potential to skew macrophage phenotypes to either anti- or protumorigenic states. Thus, while CTX and RT may initially mediate a cytotoxic/cytolytic macrophage response [Lambert and Paulinock 1987], enhanced presence of Tg12 cytokines may contribute to ongoing skewing and maintenance of AAMs in tumors and subsequent repulsion of CD8+ T-cell-mediated antitumor immunity [Doedens et al. 2010].

The duality of macrophages as mediators of cytotoxic therapy responses has been demonstrated in experimental murine models showing that macrophage depletion significantly slows tumor growth, but only when provided in combination with either CTX or RT. Selective depletion of macrophages using clodronate liposomes in an orthotopic murine melanoma model given before RT increased latency and slowed tumor regrowth, whereas coimplantation of malignant cells along with bone marrow-derived macrophages increased tumor radioresistance mediated by TNFα signaling pathways [Meng et al. 2010]. Macrophage depletion strategies in combination with CTX or RT slow tumor development in murine models of sarcoma and melanoma in part by altering, or perhaps “normalizing,” tumor vascular [Meng et al. 2010; Rolny et al. 2011]. Vascular normalization in this context likely improves tumor hemodynamics, thereby increasing delivery of chemotherapeutic agents and oxygenation of tumor parenchyma, and thereby reducing hypoxia.

Given the evidence that cytotoxic agents are also potent immune adjuvants, it would not be surprising that strategies abolishing or reprogramming AAMs would enhance both cell killing by cytotoxics and immunogenic cell death. Thus, in order to overcome the immunosuppressive barriers established by tumors, it may be important to not only provide antigenic stimulus in the form of cytotoxic therapy, but also neutralize myeloid-based pathways established in the tumor that blunt effective anti-tumor immune responses. To address this possibility, we recently used a mouse model of mammary carcinogenesis [MMTV-PyMT mice] and reported that CSF1R blockade depleted CD11b+Ly6G+Ly6C+ F4/80+ macrophages, and the less abundant population of granulocytic CD11b+Ly6G−-expressing myeloid cells, and resulted in slowed primary tumor growth and diminished metastasis, but only when given in combination with CTX, by CD8+ T-cell-dependent mechanisms [DeNardo et al. 2011]. Cathepsin protease-expressing macrophages have been found to mediate many of these effects, and cathepsin B and S protect malignant mammary epithelial cells from Taxol-induced [as well as etoposide and doxorubicin] tumor cell death in coculture [Shree et al. 2011]. Combining Taxol with cathepsin inhibition in vivo significantly enhanced efficacy against primary and metastatic mammary tumors, supporting the therapeutic relevance for this effect [Shree et al. 2011]. These experimental studies provide a compelling rationale for clinical evaluation of combinatorial approaches inhibiting macrophage recruitment or altering macrophage response pathways and mediator expression/activity in combination with “standard of care” CTX for treatment of some solid tumors in order to overcome inherent resistance to CTX. These strategies are an active area of clinical research in the phase I and II setting, testing a variety of agents designed to either block macrophage recruitment or stimulate alternative macrophage programming [Anthony et al. 2011] with the hope that combinations will provide improved clinical outcomes.

In addition to targeting macrophage recruitment, it is also possible to target macrophage polarization in an attempt to elicit the presence of more favorable Tg1,1-polarized cytotoxic macrophages in tumors. One such strategy currently being explored is via targeting CD40, a member of the TNFR
superfamily and a costimulatory molecule expressed on a diverse assortment of cells, including DCs, B cells, and macrophages, as well as endothelial, mesenchymal, and epithelial cells. Binding of the CD40 ligand (CD40L) to CD40 mediates distinct effects on cells, depending on cell type and the tissue and microenvironment in which they reside. On immune cells, CD40 regulates humoral and cellular immunity, while apoptotic and anti-proliferative pathways are regulated by CD40 on some neoplastic cells [Fonsatti et al. 2010]. Activation of APCS requires binding of CD40L on T\textsubscript{H} cells to CD40, whereas macrophage activation requires IFN\textgamma produced by T\textsubscript{H}1-CD4\textsuperscript{+} T cells in addition to CD40L–CD40 interaction. This results in macrophage up-regulation of CD40 and TNFR and induction of cytotoxic activity, including increased expression of nitric oxide and reactive oxygen species [Fonsatti et al. 2010]. To investigate whether agonist CD40 mAbs would thwart tumor-induced immune suppression and instead invoke productive T-cell-dependent anti-tumor immunity, Beatty et al. (2011) treated 21 patients with pancreatic ducal adenocarcinoma (PDAC) with a fully humanized agonistic CD40 mAb in combination with gemcitabine and reported tumor regression in some patients. Using a mouse model of PDAC to reveal the molecular/cellular mechanisms underlying the improved response, tumor regression was found to be dependent on CD40-activated MHC-II\textsuperscript{+}CD8\textsuperscript{+} tumoricidal macrophages as opposed to CD8\textsuperscript{+} T cells [Beatty et al. 2011]. In addition to these approaches, others have investigated the efficacy of CD47 blockade to foster macrophage and DC phagocytic activity [Jaiswal et al. 2010]. CD47, also known as integrin-associated protein (IAP), encodes a membrane protein mediating intracellular calcium levels following cell adhesion to extracellular matrix. CD47 binds to the SIRP\alpha inhibitor receptor on macrophages and DCs and thereby inhibits phagocytosis; in autoimmune processes, these interactions limit tissue damage [Jaiswal et al. 2010]. Expression of CD47 has been found to be significantly increased on some malignant tumor cells, especially in non-Hodgkin’s lymphoma, thus rendering malignant cells resistant to macrophage and DC phagocytosis [Chao et al. 2010]. Since agonistic CD40 mAb in combination with gemcitabine provides a survival advantage for PDAC dependent on tumoricidal macrophages, it seems reasonable to speculate that combining similar approaches with therapies blocking CD47 may be efficacious in solid tumors where CD47 is up-regulated. Taken together, the experience with immunotherapy makes a compelling case for integrating strategies that restrain and/or reprogram tumor immune microenvironments, resulting in bolstering of diverse anti-tumor pathways to achieve meaningful therapeutic gains.

**Immunosuppressive myeloid cells** Minimizing suppressive iMCs/MDSCs in tumors has been investigated using all-trans retinoic acid (ATRA), which induces differentiation of iMCs into macrophages and correlates with enhanced anti-tumor immunity in murine models [Kusmartsev and Gabrilovich 2003]. In human clinical trials, addition of retinoic acid to standard CTX improved outcome for patients with advanced non-small-cell lung cancer [Arrieta et al. 2010]. While ATRA decreased accumulation of iMCs in both tumor-bearing mice and humans, exposure to this agent also increased sensitivity of malignant cells to CTX, likely accounting for at least some of its anti-tumor efficacy [Arrieta et al. 2010]. Other strategies to eliminate iMCs have used c-KIT antagonists that decrease accumulation of iMCs in murine and human tumors but have only improved anti-tumor immunity when given in the presence of tumor vaccines [Ozao-Choy et al. 2009]. A phase I/II clinical study revealed that concurrent administration of sunitinib—an oral, small-molecule, multi-targeted receptor tyrosine kinase inhibitor of the vascular endothelial growth factor receptor (VEGFR), c-KIT, and platelet-derived growth factor receptor approved by the FDA for treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor—with RT in patients with one to five distant oligometastases improved progression-free survival; responses correlated with decreased peripheral blood monocyte levels 7 d following start of therapy [Kao et al. 2009]. While evidence supporting the use of c-KIT antagonists and cytotoxic therapy is encouraging, agents that target c-KIT can also have effects on many other cell types, including hematopoietic stem cells, mast cells, and melanocytes, due to activity also against other related kinases, thus posing a significant challenge for interpreting data in terms of effects on iMC subtypes. Although many of the agents used for targeting iMCs lack specificity, data from agents such ATRA and c-KIT antagonists provide suggestive evidence that immature myeloid populations may have important roles in regulating anti-tumor immune responses.

Another approach for depletion of immunosuppressive myeloid cells has been treatment of tumor-bearing mice with cCD11b mAbs. CD11b is an integrin cell adhesion molecule involved in transendothelial migration expressed predominantly by myeloid lineage cells, including neutrophils, macrophages, monocytes, and DCs. Bone marrow-derived CD11b\textsuperscript{+} myeloid cells are recruited to tumors following RT, where they restore vascular programming via VEGF secretion, thus aiding subsequent tumor regrowth. Neutralizing CD11b mAbs inhibit recruitment of CD11b\textsuperscript{+} myeloid cells into RT-treated tumors, slowing tumor regrowth and thus improving RT response [Ahn et al. 2010]. Similarly, mice bearing syngeneic 4T1 mammary tumors treated with CTX and cCD11b mAbs demonstrated significantly slowed primary tumor growth as well as reduced pulmonary metastases [DeNardo et al. 2011]. Gr1\textsuperscript{+}CCR2\textsuperscript{+}CX3CR1\textsuperscript{+} iMCs are highly responsive to CCL2 [Zhang et al. 2010], and CCL2/MCP1 is expressed at high levels in mammary tumors and is now mechanistically demonstrated to potentiate pulmonary metastasis [Qian et al. 2011].

A neutralizing antibody specific for human CD11b–CD18 integrin heterodimers, rovelizumab (LeukArrest), has previously been investigated and was found to have an excellent safety profile, but lacked therapeutic efficacy in inflammatory diseases such as multiple sclerosis. However, based on murine studies, it seems reasonable to speculate that a drug like rovelizumab could be
administered safely for transient blockade of myeloid cell infiltration following local RT or systemic CTX and thereby provide a window of opportunity when tumors could be prevented from efficient revascularization and anti-tumor immunity could be bolstered. Thus, extrapolating to the clinical scenario, it will be important to stratify human tumors containing predominately high levels of mature tissue macrophages, as compared with those containing iMCs/MDSCs, as these tumors would likely be less responsive to therapy directed at CSF1R-positive macrophages, but might be expected to instead respond to drugs like rovelizumab.

Conclusions
The relatively modest gains provided by immunotherapy despite intense investigation can be in part attributed to the presence of pathways that suppress anti-tumor immunity. These mechanisms likely evolved as part of tumor development where the local microenvironment contains an immune set point skewed favoring Th2-type pathways, an immune set point skewed favoring Th12-type pathways, as compared with homeostatic tissue. Cell types including Tregs, AAMs, and iMCs form an inhibitory network that suppresses local immunity, thereby limiting the efficacy of many forms of anti-cancer therapy reliant on formation of productive anti-tumor immune responses.

Studies investigating the mechanism of action for CTX and RT have historically focused on cell-intrinsic molecules regulated by these cytotoxic agents; however, recent evidence indicates the importance of cell-extrinsic factors, particularly for induction of anti-tumor immunity. Given this, inhibitory mechanisms that stymied development of effective immunotherapy may also play an important role in regulating response to cytotoxic agents. Emerging data indicate that targeting immune inhibitory/stimulatory pathways, in conjunction with conventional cytotoxic therapy and current immunotherapy, significantly enhances the effectiveness of cytotoxic therapy by augmenting anti-tumor immunity and preventing its suppression. Further exploration to better characterize and understand inhibitory immune pathways will aid in identification of new targets that redefine our understanding of the anti-tumor mechanism of traditional cytotoxic therapies and direct us to new strategies that improve the efficacy of standard therapy.

Acknowledgments
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References


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Leukocyte composition of human breast cancer

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Retrospective clinical studies have used immune-based biomarkers, alone or in combination, to predict survival outcomes for women with breast cancer (BC); however, the limitations inherent to immunohistochemical analyses prevent comprehensive descriptions of leukocytic infiltrates, as well as evaluation of the functional state of leukocytes in BC stroma. To more fully evaluate this complexity, and to gain insight into immune responses after chemotherapy (CTX), we prospectively evaluated tumor and nonadjacent normal breast tissue from women with BC, who either had or had not received neoadjuvant CTX before surgery. Tissues were evaluated by polychromatic flow cytometry in combination with confocal immunofluorescence and immunohistochemical analysis of tissue sections. These studies revealed that activated T lymphocytes predominate in tumor tissue, whereas myeloid lineage cells are more prominent in “normal” breast tissue. Notably, residual tumors from an unselected group of BC patients treated with neoadjuvant CTX contained increased percentages of infiltrating myeloid cells, accompanied by an increased CD8/CD4 T-cell ratio and higher numbers of granzyme B-expressing cells, compared with tumors removed from patients treated primarily by surgery alone. These data provide an initial evaluation of differences in the immune microenvironment of BC compared with nonadjacent normal tissue and reveal the degree to which CTX may alter the complexity and presence of selective subsets of immune cells in tumors previously treated in the neoadjuvant setting.

**Results**

**Increased Presence of T Cells in Tumor Tissue.** To evaluate the composition of tumor-infiltrating leukocytes in human BC, tumors from 20 patients were evaluated by polychromatic flow cytometry and IHC detection of leukocyte lineages in tissue sections as described in Materials and Methods. Nine invasive ductal carcinomas (IDC) and five invasive lobular carcinomas (ILC)—mostly histological grade two or three—were obtained from patients with no prior exposure to CTX (CTX-naïve) at the time of primary surgery for early stage BC, although one patient had received neoadjuvant tamoxifen. Six tumor samples were obtained from patients previously treated with neoadjuvant CTX before resection (CTX-treated), consisting entirely of grade two or three IDC. Notably, three of six CTX-treated tumors were HER2/neu-positive, compared with only 1 of 14 CTX-naïve tumors, whereas both groups contained roughly equal nadir serum chemotherapy (CTX) expression of TAM presence and bioactivity within mammary tumors correspond to their clinical activity, further indicating the importance of TAMS, not only in promoting tumor development, but also in suppression of anti-tumor immunity.

**CD4**+ T cells isolated from human BC produce high levels of type II helper (Th2) cytokines including IL-4 and IL-13 (25, 26), which are significant in light of studies demonstrating that several protumor activities of TAMS are regulated by IL-4 derived from CD4+ T cells (1, 27). Based on these findings, we recently reported that infiltration by CD68+, CD4+, and CD8+ immune cells in human BC is predictive of overall survival, and that the ratio of CD68 to CD8α mRNA in tumor tissue correlates with complete pathologic response (pCR) in patients undergoing neoadjuvant chemotherapy (CTX) for early stage BC (6). Herein, we evaluated leukocytic infiltrates in breast tissue from predominantly hormone receptor positive patients who had, or had not, received CTX before definitive surgery. In CTX-naïve patients, we found that activated T lymphocytes comprised the majority of immune cells within tumors, whereas myeloid-lineage cells predominate in nonadjacent normal breast tissue. In contrast, tumors from patients with residual disease after neoadjuvant CTX contained higher levels of infiltrating myeloid cells, with a simultaneous shift away from a Th1-dominated lymphocyte response.

**Inflammation | Macrophage**

Several subtypes of CD45-expressing leukocytes infiltrate breast cancer (BC), including CD4+ and CD8+ T cells, CD20+ B cells, and multiple myeloid-lineage cells including tumor-associated macrophages (TAMS) that are often identified by immunohistochemical (IHC) detection of CD68 (1). High lymphocyte infiltration is associated with increased survival in patients <40 y of age (2) and with a favorable prognosis in subsets of patients whose tumors are also heavily infiltrated by TAMS (3). More specifically, large cohort studies of patients with BC have revealed that the presence of CD68+ cells in tumor tissue correlates with poor prognostic features (4–6), higher tumor grade (7–9), increased angiogenesis (10–13), decreased disease-free survival (6, 11, 14, 15), and increased risk for systemic metastasis when assessed in conjunction with endothelial and carcinoma cell markers (16).

The functional significance of specific leukocytes in BC development has been implied based on experimental studies using murine models of mammary carcinogenesis where mice harboring homozygous null mutations in genes specifying leukocyte development or recruitment have been evaluated. In transgenic mice expressing the polyoma virus middle T antigen regulated by the mouse mammary tumor virus promoter (MMTV-PyMT mice), progression of mammary carcinomas and metastases to lungs are reduced in mice lacking the colony-stimulating factor-1 (cstf1) gene, a cytokine critical for macrophage maturation and recruitment (17, 18). TAMs in mammary tumor tissue are often associated with vasculature (19), where their production of VEGFA fosters angiogenic programming of tissue (20, 21), and their production of EGF promotes invasive tumor growth and subsequent metastases (22, 23). Moreover, TAMs regulated by epithelial CSF1 express higher levels of several hypoxia-induced genes (iNOS and arginase-1) that, in turn, mediate suppression of anti-tumor immunity by blocking cytotoxic T-cell proliferation and activation (6, 24). Thus, TAM presence and bioactivity within mammary tumors correspond to their clinical activity, further indicating the importance of TAMS, not only in promoting tumor development, but also in suppression of anti-tumor immunity.

The authors declare no conflict of interest.

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for use as “normal” tissue, in addition to tissues from two contralateral prophylactic mastectomies from patients with ipsilateral ductal carcinoma in situ (DCIS).

Immune infiltrates detected with the pan-leukocyte marker CD45 were present in both normal and tumor tissue, but with substantially increased density in BC (Fig. 1A). Leukocyte subsets were evaluated by using a combination of lineage markers to identify specific subpopulations (Figs. S1 and S2), with the complexity of these populations shown in Fig. 1B as a percentage of the total number of CD45+ cells in each sample. BC tissues from CTX-naïve patients contained infiltrates dominated by T lymphocytes (CD3ε+), with minor populations of natural killer cells (CD3ε−CD56−NK2G2−) and B lymphocytes (CD19/20+HLA-DR−CD3ε−). In comparison, myeloid-lineage cells including macrophages (CD14hiCD11b+HLA-DR−), mast cells (FcerIα+CD117+CD11b−CD49d+) and neutrophils (CD15+CD11b+CD49d+) were more evident in the normal tissue from these patients. A similar immune profile was observed in breast tissues obtained from the two prophylactic mastectomies (Fig. 1B).

**Increased Presence of Myeloid-Lineage Cells in Residual Tumors from Patients Exposed to Neoadjuvant CTX.** Comparative analysis of residual BC tissue removed from patients after neoadjuvant CTX revealed an obvious difference in the percentages of myeloid-lineage cells compared with the CTX-naïve group. With some exceptions, this difference included an increased presence of macrophages as a percent of total leukocytes (Fig. 2A), as well as by density evaluation of CSF1R receptor (CSF1R)-positive cells in tissue by IHC (Fig. 2B). Increased percentages of mast cells (Fig. 2C) and neutrophils (Fig. 2D) were also evident in most CTX-treated patients, with an >14-fold increase in CTX-treated versus CTX-naïve groups. Basophils (FcerIα+CD117+CD11b−CD49d+; Fig. 2E) were highly increased in only one of six CTX-treated samples, whereas the percentage of myeloid dendritic cells (CD11c+HLA-DR+CD14hi; Fig. 2F) was unchanged. Evaluation of plasmacytoid dendritic cells expressing CD85g/ILT7 detected an insufficient number of events for analysis. Thus, with the exception of basophils, dendritic cells, and CD15+CD11b+CD49d+ eosinophils—which were present just at a detectable level in the tissues examined—increased presence of myeloid-lineage cells typified residual tumors of women treated with neoadjuvant CTX.

**CD68 Is Not a Macrophage-Specific Marker in Human BC.** Macrophages are well established as regulators of murine mammary tumorigenesis (30), where they can represent up to 80% of leukocytes present within late stage mammary carcinomas (1). In human BC, immunoreactivity for CD68 has been used extensively for identification of macrophages, with CD68+ cell density associated with reduced overall survival (6, 11, 14, 15).

The high number of CD68+ cells reported in the literature, and shown in Fig. 3A, was in contrast to the limited number of CD14hiCD11b+HLA-DR+ macrophages observed by flow cytometry in the BC suspensions examined (Figs. 1B and 2A). To understand this discrepancy, we first evaluated CD68 expression in BC tissue sections, compared with CD163 (a hemoglobin scavenger receptor also commonly used as a marker for macrophages) and CSF1R (Fig. 3A). This comparative analysis revealed a lack of correlation in cell density among the three markers. We next evaluated frozen BC tissue sections by confocal microscopy after immunofluorescent detection of CD68 in combination with CSF1R or CD45 (Fig. 3B). Although all cells expressing high levels of the CSF1R also expressed CD68, there was a distinct population of CD68+ cells that expressed neither CSF1R nor CD45. CD68 did not significantly colocalize with keratin+ epithelial cells, CD31+ endothelial cells, or smooth muscle actin α-expressing mural cells surrounding vasculature (Fig. 3C). This expression contrasted with murine mammary tumors isolated from MMTV-PyMT transgenic mice (17), where CD68+ cells coexpressed both CSF1R and the murine macrophage marker F4/80 (Fig. S3). In agreement with historic literature (31, 32), these results thus indicate that CD68 is not a macrophage-specific marker in human BC.

**Tumor-Infiltrating T Cells Display an Activated Phenotype.** To reveal the phenotype of T cells infiltrating BCs, we examined surface marker and chemokine receptor expression of tissue-infiltrating CD4+ and CD8+ T cells (Fig. 4A and B). Specifically, both CD4+ and CD8+ T cells displayed increased expression of activation markers CD69 and HLA-DR compared with peripheral
blood T cells, with a corresponding loss of markers for naïve T cells, CD45RA, and CCR7. Furthermore, although all T cells constitutively expressed the costimulatory receptor CD28 (Fig. S4 A), expression of CD27, another costimulatory receptor, was reduced in a large proportion of tissue-infiltrating cells, indicative of shedding after interaction with its ligand CD70 (33) and potential acquisition of effector functions (34, 35). CD4+ and CD8+ T cells also displayed substantially up-regulated expression of chemokine receptors CCR4 and CCR5 (Fig. 4 B), and although CD8+ T cells constitutively expressed CXCR3, tissue-infiltrating CD4+ T cells exhibited higher CXCR3 expression than their counterparts in peripheral blood. Surface marker expression by tissue-infiltrating CD4+ and CD8+ T cells was subtly different between tumor and benign tissue in some samples; however, these changes were not consistent across patients, or between CTX-naïve and CTX-treated groups (Fig. S4 B).

Altered Lymphocyte Balance in Residual Tumors After Neoadjuvant CTX. Although we observed no difference in the percent of CD3ε−CD56+NKG2D+ natural killer (NK) cells (Fig. S4), higher levels of CD19/CD20+HLA-DR+ B cells were evident in several CTX-naïve tumors compared with both normal tissue and CTX-treated tumors. As has been reported (36), B cells were clustered together in association with T cells (Fig. S4A). Notably, CD4+ T cells as a percent of the total CD45+ population were also increased in CTX-naïve tumors compared with both normal tissue and residual postneoadjuvant tumors (Fig. 5D). As the percent of CD8+ T cells was unchanged (Fig. 5E), the lower percentage of CD4+ T cells within the CTX-treated group resulted in an increased CD8 to CD4 ratio (Fig. 5F). Although it was unclear whether the density of CD8+ cells in CTX-treated residual tumors was increased (Fig. 5G), the number of cells expressing granzyme B was strikingly evident in two of six CTX-treated tumors (Fig. 5H), whereas minimal granzyme B staining was observed in CTX-naïve tumors, even in areas with high numbers of CD8+ T cells (Fig. 5F).

Despite the reduced percentage of CD4+ T cells in tumors from CTX-treated patients, there was no change in the density of IHC detected regulatory T cells expressing FoxP3 (Fig. S5A), which was specifically expressed by CD3ε+CD4+ cells in the tumor (Fig. S5B). Gating on CD25hi cells, consisting of >80% FoxP3+ cells in all samples tested, also revealed that the relative percentage of these cells was invariant between groups (Fig. S5C). Phenotypically, CD4εFoxP3ε cells displayed an activated phenotype with equivalent surface levels of CD45RO and CD69.
to CD4+FoxP3− cells and, as has been reported for cells in peripheral blood (37), expressed lower levels of CD127 (Fig. S5D).

Interestingly, although not all FoxP3+ cells expressed HLA-DR, they did comprise the majority of HLA-DR−expressing CD4+ T cells, in addition to coexpressing high levels of CD25.

These data collectively reveal a shift within tumors toward a TH2-type response in BC characterized by increased presence of B cells and CD4+ T cells, in comparison with nonadjacent normal breast tissue. This shift is reversed in tumors obtained from CTX-treated patients, with additional evidence of a cytotoxic T-cell response through a more favorable CD8/CD4 T-cell ratio and increased presence of granzyme B-expressing lymphocytes; thus, even residual tumors from patients with a poor response to CTX may contain immune microenvironments that are more favorably skewed towards an anti-tumor, TH1-type immune response.

Discussion

Herein, we present a detailed description of leukocyte complexity in BC as evaluated in a cohort of CTX-naïve patients with stage 2/3 tumors, compared with patients with significant residual disease after neoadjuvant CTX. T lymphocytes were the major population within both CTX-naive and CTX-treated tumors, found almost exclusively in an activated state as determined by increased expression of CD69 and chemokine receptors, with simultaneous loss of naïve markers CCR7 and CD45RA. The presence of activation markers, however, does not definitively demonstrate that intratumoral T cells are functionally active. In fact, granzyme B expression was minimal within tumors from CTX-naive patients, suggesting negligible cytotoxic activity by infiltrating CD8+ T cells. In comparison, granzyme B was highly expressed in one-third of the CTX-treated tumors, suggestive of a more cytotoxic T-cell response within some tumors after exposure to CTX.

Importantly, residual tumors from CTX-treated patients also contained reduced percentages of B cells and CD4+ T cells. Tumor-infiltrating CD4+ T cells in BC are known to express the T H2 cytokines IL-4 and IL-13 concomitantly with the production of IFN-γ (25, 26), consistent with coexpression of CXCR3 and CCR4 (38, 39) as we observed herein. It remains to be determined whether cytokine production by CD4+ T cells is altered by neoadjuvant CTX; however, the combined reduction in both CD4+ T cells and B cells is indicative of a favorable shift away from a TH2 microenvironment. This shift could be relevant for TAM function, as has been described in the MMTV-PyMT model where TAMs are programmed by IL-4 toward a TH2 phenotype (1), and more recently in pancreatic ductal adenocarcinoma during treatment where an agonist CD40 monoclonal antibody fostered cytolytic macrophage activities (40).

Although the extent of lymphocyte infiltration has been associated with improved prognosis in subsets of patients (2, 3), and with pCR after CTX (41, 42), information regarding the relationship between individual lymphocyte subsets to survival is limited. High FoxP3 counts correlate with reduced overall and relapse-free survival in estrogen receptor (ER)-positive tumors (43), and pCR to neoadjuvant CTX is associated with reduced FoxP3 grading (44, 45). Although two studies examining T-cell infiltration by flow cytometry found conflicting results regarding the CD8:CD4 ratio and lymph node metastasis (46, 47), the number of CD8+ T cells within tissue has been associated with improved patient survival (48). We have also reported a CD68/CD4/CD8 immune signature predicting overall and relapse-free survival, with inverse correlations evident for CD4 when used in conjunction with other markers (6). There is thus an urgent need for additional prospective investigations where multiple parameters of lymphocytic infiltration and functionality are evaluated to determine the most significant biomarker comparisons that predict outcome and guide specific therapy.
Although used successfully in multiple studies to relate TAM infiltration with clinically relevant outcomes, our results indicate that CD68 alone cannot accurately evaluate macrophage presence in human breast tissue given that multiple stromal cells express it and that a subset of these are CSF1R- and CD45-negative. We observed that the nonleukocytic CD68+ cells were predominantly located within tumor stroma and, thus, based on this localization and morphology, we speculate that CSF1R−CD68+ cells likely reflect tumor-associated fibroblasts or monocyte-derived fibroblasts in agreement with other reports (31, 32, 49–52). Our findings do not invalidate CD68 as a clinically relevant marker and, importantly, CSF1-response gene signatures have been identified in breast adenocarcinomas that are predictive of recurrence risk and metastasis (53, 54). However, given the important role that fibroblasts (and perhaps fibrocytes) play in fostering aspects of tumorigenesis (55–57), differentiating among macrophages, fibroblasts, and other stromal populations within tumors has the potential to improve diagnostic information currently generated by immunodetection of CD68.

As we have reported for expression of csf1 mRNA (6), multiple genes encoding myeloid cell chemoattractants are differentially expressed by human BC cell lines, with variable induction of these genes in response to CTX (Fig. S6). Although differential expression between cell lines corresponding to particular subtypes of BC is evident, it is doubtful these cell lines accurately represent the response of BC tumor tissue; thus, we are investigating whether differences in myeloid cell infiltrates reflect distinct molecular subtypes of BC and to what extent these differ in residual tumors from CTX-treated patients.

It is important to acknowledge that leukocyte composition within tumors responding to CTX likely differs substantially from residual or recurrent tumors from patients that have received CTX, given what is known regarding immune responses to CTX-induced cell death (28). However, we recently reported that in mammary carcinomas of MMTV-PyMT mice, blockade of the CSF1-CSF1R pathway critical for TAM recruitment improved response to CTX through a CD8+ T-cell–dependent effect (6). Thus, even though the findings presented herein are based on a small dataset of heterogeneous tumor subtypes, and our results may be biased because of sample selection favoring large and/or less CTX-responsive tumors among the CTX-treated group, the clear distinctions in the myeloid profiles between CTX-naive and CTX-treated tumors is provocative and indicates that a CSF1-targeted strategy may be a promising approach to enhance therapeutic efficacy of cytotoxic CTX, particularly for treatment of refractory BC. Moreover, given the increase in granulocytic populations within tumors resistant to CTX, and the involvement of these cells in regulating immune responses in chronic inflammatory diseases (58–62), these populations may also be functionally relevant, and targeting common pathways of immune suppression within the tumor microenvironment may provide additional therapeutic opportunities to increase efficacy of neoadjuvant CTX.
Materials and Methods

Tissues were collected at the time of surgery from consenting patients at the University of California, San Francisco under approval from the institutional review board. Tumor and ipsilaterial nonadjacent normal tissues were collected by a certifi- cation board. Tumor and ipsilaterial nonadjacent normal tissues were collected for a subset of the patients described here (6). Flow cytometry, immunohisto- chemistry, and immunofluorescence were performed as described (6), with detailed methods contained in SI Materials and Methods, and a list of anti- bodies available in Tables S2 and S3. Statistical differences between two in- dependent groups were determined by using Student’s t test via Prism 4.0 software (GraphPad Software).

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Clinical and experimental studies have established that chronic infiltration of neoplastic tissue by leukocytes, i.e., chronic inflammation, promotes development and/or progression of various solid tumors\(^1\), however, the organ-specific cellular and molecular programs that favor pro-tumor, as opposed to anti-tumor immunity by leukocytes are incompletely understood. While some leukocytes certainly exhibit anti-tumor activity, i.e., cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells\(^3\), other leukocytes, most notably mast cells, CD4\(^+\) T cells, B lymphocytes, dendritic cells (DCs), granulocytes, immature monocytes and macrophages exhibit more bipolar roles, by virtue of their capacity to either hinder or potentiate tumor progression\(^1\),\(^2\). A major question regarding these disparate leukocytes bioactivities is the degree to which their various pro- or anti-cancer activities are regulated by tissue-specificity and/or are responsive to individual oncogenic or tumor suppressive gene programming in early neoplastic tissue.

**Leukocytes and Breast Carcinogenesis:** In the breast, cancer development is in part characterized by a significant increase in both innate and adaptive immune cells, with B and T lymphocytes and macrophages representing the most abundant leukocytes present in neoplastic stroma\(^4\). Retrospective clinical studies examining identity of leukocytes in human breast cancer have revealed that high immunoglobulin (Ig) levels in tumor stroma (and serum), and increased presence of extra follicular B cells, T regulatory (T\(_{reg}\)) cells, high ratios of CD4/CD8 or T\(_{h2}/T_{h1}\) T lymphocytes in primary tumors or in draining lymph nodes correlates with tumor grade, stage and overall patient survival\(^3\). On the other hand, experimental studies have demonstrated that macrophages in primary mammary adenocarcinomas regulate late-stage carcinogenesis by virtue of their pro-angiogenic properties\(^5\),\(^6\), as well as fostering pulmonary metastasis by providing epidermal growth factor (EGF) and cathepsin proteases\(^7\) to malignant mammary epithelial cells (MECs) and thereby enhancing their invasive (and metastatic) behavior\(^8\),\(^9\). Based on these seemingly disparate observations, we sought to determine if adaptive immunity also fostered malignancy in the breast by regulating the phenotype or effector functions of tumor-associated macrophages (TAMs) and either activated their pro-tumor properties or alternatively by suppressing their anti-tumor capabilities.

Utilizing the MMTV-PyMT mouse model of mammary carcinogenesis\(^10\), we revealed a provocative role for CD4\(^+\) T cells as potentiators of peripheral blood dissemination and pulmonary metastasis of malignant mammary adenocarcinomas through their ability to regulate pro-tumor properties of TAMs\(^11\). Specifically, T\(_{h2}\)-polarized CD4\(^+\) T cells secrete high levels of interleukin (IL)-4 and thereby regulate M1 and M2-type TAM bioactivity by activation of IL4R\(\alpha\)-signaling cascades. M2-TAMs in turn promote invasive behavior of malignant MECs by high level production of cathepsin protease activity\(^7\) and EGF that subsequently activates MEC invasion and EGF receptor signaling programs, activities that are essential for entry into peripheral blood, dissemination and outgrowth in the lung. These findings indicate that when CD4\(^+\) T lymphocytes are present in a T\(_{h2}\)-type tumor microenvironment, they promote metastasis by regulating the pro-tumor properties of TAMs mediated by IL4R\(\alpha\)-signaling, as opposed to limiting or eradicating malignant cells by engaging cytotoxic mechanisms. These realizations provide rational for development of anti-cancer therapeutics that neutralize pro-tumor properties of IL4R\(\alpha\)-based signaling in both adaptive and innate immune cells in the tumor microenvironment and periphery, that when delivered in combination with cytotoxic drugs or therapeutics bolstering anti-tumor immunity, may extend survival of patients with advanced disease.

**Leukocytes and Squamous Carcinogenesis:** B lymphocytes constitute a central component of adaptive immunity and not only serve in antibody production but also as antigen-presenting cell; thus, B lymphocyte expression of major histocompatibility complex (MHC) and co-stimulatory molecules as well as secretion of pro-inflammatory cytokines induces optimal CD4\(^+\) and CD8\(^+\) T cells activation,
expansion, memory T lymphocyte formation and antigenic spreading. As such, B cells have been historically associated with anti-tumor immunity. More recently, the heterogeneity of B lymphocyte responses has been recognized and diverse B cell subtypes with either pro-immune or regulatory properties have been identified in vivo. In particular, regulatory B lymphocytes, which include various flavors of IL-10 producing cells, have been identified in the context of autoimmune diseases that exert anti-inflammatory activities. However, the role of these individual B lymphocyte subpopulations in malignant disease has yet to be fully elucidated.

Using a transgenic mouse model of multi-stage epithelial carcinogenesis, i.e., K14-HPV16 mice, we previously revealed that adaptive immunity is an important regulator of inflammation-associated cancer development. Combined B and T lymphocyte-deficiency in HPV16 mice, e.g. HPV16/RAG1-/- mice, resulted in a failure to initiate and/or sustain leukocyte infiltration during premalignancy. As a consequence, tissue remodeling, angiogenesis and epithelial hyperproliferation were significantly reduced, culminating in attenuated premalignant progression and a 43% reduction in carcinoma incidence. Importantly, adoptive transfer of B lymphocytes or serum from HPV16 mice into HPV16/RAG1-/- mice reinstated chronic inflammation in premalignant tissues, indicating that B cell-derived soluble mediators were necessary to potentiate malignant progression. More recently, we revealed that B cell-derived IgGs regulate neoplastic progression and subsequent carcinoma development by engagement of Fcγ receptors (FcγR) expressed on resident and recruited immune cells. Specifically, we found that immune complex (IC)-stimulation of leukocyte FcγR is critical for establishing a pro-tumor microenvironment in premalignant tissue that directs not only recruitment of leukocytes from peripheral blood, but also leukocyte composition, phenotype and bioeffector functions once within neoplastic tissue. As such, proangiogenic and protumorigenic functions of mast cells and macrophages are differentially regulated by humoral immunity and functionally contribute to squamous carcinogenesis. These findings have broad clinical implications as they reveal critical signaling pathways regulated by humoral immunity and FcγR to target therapeutically in patients suffering from chronic inflammatory diseases at risk for cancer, as well as individuals harboring premalignant lesions where chronic inflammation compromises tissue integrity and enhances risk of malignancy.

**Summary:** While many factors regulate leukocyte propensity to either promote or repress tumor development, in tissue- and/or oncogene-specific manners, polarized T H2-type adaptive immune responses foster pro- as opposed to anti- tumor programming of myeloid cells that in turn directly regulate many of the “hallmarks” of solid tumor development. Neutralizing these various “pro-tumor” regulatory pathways may provide relief for some aspects of late-stage cancer development as monotherapy, but more likely when combined with cytotoxic-, targeted- and/or immuno-therapy will provide a survival advantage by bolstering induction of anti-tumor bioactivities of tumor-associated leukocytes that extend efficacy of therapy.

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*LMC acknowledges support from the NIH/NCI, and Dept of Defense Era of Hope Scholar Award (W81XWH-06-1-0416) and Investigator-Initiated Research Award in Mesothelioma (PR080717).*
Clinical and experimental studies have established that chronic infiltration of neoplastic tissue by leukocytes, i.e., chronic inflammation, promotes development and/or progression of various solid tumors [1, 2]; however, the organ-specific cellular and molecular programs that favor protumor, as opposed to anti-tumor immunity by leukocytes are incompletely understood. While some leukocytes certainly exhibit anti-tumor activity, i.e., cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells [3-5], other leukocytes, most notably mast cells, CD4+ T cells, B lymphocytes, dendritic cells (DCs), granulocytes, immature monocytes and macrophages exhibit more bipolar roles, by virtue of their capacity to either hinder or potentiate tumor progression [1, 2]. A major question regarding these disparate leukocytes bioactivities is the degree to which their various pro- or anti-cancer activities are regulated by tissue-specificity and/or are responsive to individual oncogenic or tumor suppressive gene programming in early neoplastic tissue.

Leukocytes and Breast Carcinogenesis: In the breast, cancer development is in part characterized by a significant increase in the presence of both innate and adaptive immune cells, with B and T lymphocytes as well as macrophages representing the most abundant leukocytes present in neoplastic stroma [6]. Retrospective clinical studies examining identity of leukocytes in human breast cancer have revealed that high immunoglobulin (Ig) levels in tumor stoma (and serum), and increased presence of extra follicular B cells, T regulatory (Treg) cells, high ratios of CD4/CD8 or Th2/Th1 T lymphocytes in primary tumors or in draining lymph nodes correlates with tumor grade, stage and overall patient survival [7-11]; thus, some facets of adaptive immunity may indeed be involved in fostering cancer development.

On the other hand, experimental studies have demonstrated that macrophages in primary mammary adenocarcinomas regulate late-stage carcinogenesis by virtue of their pro-angiogenic properties [12, 13], as well as fostering pulmonary metastasis by providing epidermal growth factor (EGF) and cathepsin proteases [14] to malignant mammary epithelial cells (MECs) and thereby enhancing their invasive (and metastatic) behavior [15, 16]. Based on these seemingly disparate observations, we sought to determine if adaptive immunity also fostered malignancy in the breast by regulating the phenotype or effector functions of tumor-associated macrophages (TAMs) and either activated their pro-tumor properties or alternatively by suppressing their anti-tumor capabilities.

Utilizing the MMTV-PyMT mouse model of mammary carcinogenesis [17], we revealed a provocative and functional role for CD4+ T effector cells as potentiators of peripheral blood dissemination and pulmonary metastasis of malignant mammary adenocarcinomas through their ability to regulate the pro-tumor properties of TAMs [18]. Specifically, Th2-polarized CD4+ T lymphocytes secrete high levels of interleukin (IL)-4 and thereby regulate M1 and M2-type TAM bioactivity by activation of IL4R –signaling cascades. M2-TAMs in turn promote invasive behavior of malignant MECs by high level production of cathepsin protease activity [14] and
EGF that subsequently activates MEC invasion and EGF receptor signaling programs, activities that are essential for entry into peripheral blood, dissemination and outgrowth in the lung. These findings indicate that when CD4⁺ T lymphocytes are present in a TH2-type tumor microenvironment, they promote metastasis by regulating the pro-tumor properties of TAMs mediated by IL4R- -signaling, as opposed to limiting or eradicating malignant cells by engaging cytotoxic mechanisms. These provocative realizations provide rational for development of anti-cancer therapeutics that neutralize the pro-tumor properties of IL4R -based signaling in both adaptive and innate immune cells in the tumor microenvironment and periphery, that when delivered in combination with cytotoxic drugs or therapeutics bolstering anti-tumor immunity, may thereby extend survival of cancer patients with advanced disease.

Leukocytes and Squamous Carcinogenesis: B lymphocytes constitute a central component of adaptive immunity and not only serve in antibody production but also as antigen-presenting cell; thus, B lymphocyte expression of major histocompatibility complex (MHC) and co-stimulatory molecules as well as secretion of pro-inflammatory cytokines induces optimal CD4⁺ and CD8⁺ T cells activation, expansion, memory T lymphocyte formation and antigenic spreading. As such, B cells have been historically associated with anti-tumor immunity. More recently, the heterogeneity of B lymphocyte responses has been recognized and diverse B cell subtypes with either pro-immune or regulatory properties have been identified in vivo. In particular, regulatory B lymphocytes, which include various flavors of IL-10 producing cells, have been identified in the context of autoimmune diseases that exert anti-inflammatory activities [19, 20]. However, the role of these individual B lymphocyte subpopulations in malignant disease has yet to be fully elucidated.

Using a transgenic mouse model of multi-stage epithelial carcinogenesis, i.e., K14-HPV16 mice [21], we previously revealed that adaptive immunity is an important regulator of inflammation-associated cancer development [22]. Combined B and T lymphocyte-deficiency in HPV16 mice, e.g. HPV16/RAG1⁻ mice, resulted in a failure to initiate and/or sustain leukocyte infiltration during premalignancy [22]. As a consequence, tissue remodeling, angiogenesis and epithelial hyperproliferation were significantly reduced, culminating in attenuated premalignant progression and a 43% reduction in carcinoma incidence [22]. Importantly, adoptive transfer of B lymphocytes or serum from HPV16 mice into HPV16/RAG1⁻ mice reinstated chronic inflammation in premalignant tissues, indicating that B cell-derived soluble mediators were necessary to potentiate malignant progression. More recently, we revealed that B cell-derived IgGs regulate neoplastic progression and subsequent carcinoma development by engagement of Fc receptors (Fc R) expressed on resident and recruited immune cells [23].

Specifically, we found that immune complex (IC)-stimulation of leukocyte FcR is critical for establishing a pro-tumor microenvironment in premalignant tissue that directs not only recruitment of leukocytes from peripheral blood, but also leukocyte composition, phenotype and bioeffector functions once within neoplastic tissue. As such, proangiogenic and protumorigenic functions of mast cells and macrophages are differentially regulated by humoral immunity and functionally contribute to squamous carcinogenesis. These findings have broad clinical implications as they reveal critical signaling pathways regulated by humoral immunity and FcR to target therapeutically in patients at risk for cancer development, e.g., patients suffering from chronic inflammatory diseases, as well as individuals harboring premalignant lesions where chronic inflammation compromises tissue integrity and enhances risk of malignancy.
Summary: While many factors regulate the propensity of leukocytes to promote or repress primary tumor development and metastasis, some of which may be tissue- and/or oncogene-specific, polarized Th2-type adaptive immune responses by B and T lymphocytes foster pro- as opposed to anti-tumor programming of myeloid cells that in turn directly regulate many of the “hallmarks” of solid tumor development [24]. Neutralizing these various “pro-tumor” regulatory pathways may provide relief for some aspects of late-stage cancer development as monotherapy, but more likely when combined with cytotoxic-, targeted- and/or immunotherapy will provide a survival advantage by bolstering induction of anti-tumor bioactivities of tumor-associated leukocytes that extend efficacy of therapy.

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis is B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, Th2-polarized CD4⁺ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of M2-polarized macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

Funding from the NIH/NCI and Department of Defense Era of Hope Scholar Award and Program in Mesothelioma
Oral Presentation at the 2010 Annual Meeting of the American Association for Cancer Research (AACR), Washington DC, USA.

Targeting Macrophages as a Novel Therapeutic Approach for Malignant Pleural Mesothelioma

Nikita Kolhatkar¹,³, Magnus Johansson¹, Dario Barbone³, Adam Yagui-Beltrán², Raphael Bueno⁴, David J. Sugarbaker⁴, David M Jablons²,⁵, V. Courtney Broaddus³, Lisa M Coussens¹,⁵

Departments of ¹Pathology, ²Surgery, ³Lung Biology Center, and ⁵Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco. ⁴Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Mesothelioma is a life-threatening tumor, induced by inhalation of asbestos fibers, which is largely resistant to most chemotherapeutic approaches. One feasible approach could be to harness the power of the immune system to increase the chemosensitivity of mesotheliomas. Using a combination of immunohistochemistry and flow cytometry to analyze the leukocyte compositions of human mesotheliomas, we have found that 1) epithelioid and mixed mesothelioma tumor subtypes have a higher degree of immune cell infiltration, when compared to sarcomatous tumors, and 2) mesothelioma tumors have large infiltrations of macrophages (31 ± 4.6% of the inflammatory cell population [CD45⁺]). Indeed, the percentage of macrophages in mesotheliomas exceeded that found in other thoracic malignancies thus far evaluated (NSCLC cancer, 9%; esophageal, 4%). In view of recent data indicating that macrophages can be targeted therapeutically to minimize some aspects of cancer development, we investigated whether macrophages could be targeted to enhance chemosensitivity of human mesotheliomas. To address this question, we adapted a 3-dimensional spheroid growth model, enabling heterotypic culture of mesothelioma cells with macrophages. We found that mesothelioma chemoresistance can be lowered by co-incubation with macrophages. However, the magnitude of the response was dictated by specific macrophage phenotype. Macrophage phenotype and bioactivity is modulated by Th1 versus Th2 cytokine exposure that in turn regulate either an M1 (IFN-γ & LPS) or M2 (IL-4) phenotype. M1-polarized macrophages increased the response of malignant mesothelioma spheroids to pro-apoptotic chemotherapeutic agents, such as TRAIL and gemcitabine. Furthermore, our preliminary data indicate that primary human tumor-associated macrophages, isolated from untreated malignant mesotheliomas, have similar pro-apoptotic effects when polarized with M1 cytokines, suggesting that cytokine re-polarization of macrophages in mesothelioma tumors to an M1 phenotype could augment therapeutic efficacy.

This work was supported by grants from the NCI and a DoD Mesothelioma Program grant PR080717 to Broaddus and Coussens
Targeting macrophages as a novel therapeutic approach for malignant pleural mesothelioma

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This work was supported by a DoD Mesothelioma Program grant PR080717 (to Broaddus and Coussens).
Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis is B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, TH2-polarized CD4+ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of M2-polarized macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

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Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

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LMC acknowledges generous support from the NIH and NCI, and Department of Defense Era of Hope Scholar Award (W81XWH-06-1-0416) and Investigator-Initiated Research Award in Mesothelioma (PR080717).
Targeting Macrophages in a Preclinical Model of Mesothelioma

Collin M. Blakely¹, Nikita Kolhatkar²,⁴, Magnus Johansson², Dario Barbone⁴, Adam Yagui-Beltrán⁵, Raphael Bueno⁶, David J. Sugarbaker⁵, Thierry M. Jahan¹,⁶, David M. Jablons³,⁶, V. Courtney Broaddus⁴, and Lisa M. Coussens²,⁶

Departments of ¹Medicine, Division of Hematology/Oncology, ²Pathology, ³Surgery, ⁴Lung Biology Center, and ⁵Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Background: Malignant mesothelioma is a debilitating, incurable cancer that exhibits a high degree of resistance to standard chemotherapy. Novel therapeutic approaches to treat this disease are desperately needed. We have found that mesothelioma tumors resected from patients exhibit a high rate of infiltration by macrophages. Increasing evidence suggests that tumor-associated macrophages (TAMs) secrete proangiogenic, prosurvival, and proinvasive factors that act to promote tumor formation and progression. Novel approaches designed to target TAMs have been developed. These include depletion of macrophages with the bisphosphonate clodronate or targeting the Colony Stimulating Factor-1 Receptor (CSF-1R), which is important for monocyte/macrophage-lineage recruitment to tumors.

Objective/Hypothesis: We hypothesize that mesothelioma-associated macrophages promote resistance to the cytotoxic effects of chemotherapy and that inhibition of macrophage infiltration of tumors, or their protumor bioactivity, will improve the efficacy of chemotherapy.


Study Design: To assess the effects of macrophage depletion on mesothelioma responsiveness to chemotherapy we will conduct three independent preclinical trials. In the first experiment we will assess the effects of depleting macrophages with clodronate on mesothelioma responsiveness to chemotherapy. Mice will be injected intraperitoneally with a syngeneic mesothelioma cell line to establish tumors. Tumor-bearing mice will be randomized into 1 of 4 treatment groups: 1) clodronate liposomes alone; 2) control liposomes alone; 3) clodronate liposomes + carboplatin/pemetrexed chemotherapy; 4) control liposomes + carboplatin/pemetrexed. In the second experiment we will assess the effects of CSF-1R inhibition on mesothelioma responsiveness to chemotherapy by using the novel, orally bioavailable, small molecule CSF-1R kinase inhibitor, PLX3397 (Plexxikon Inc.). Mice with orthotopic mesothelioma tumors will be randomized into 1 of 4 treatment groups: 1) PLX3397 chow alone; 2) control chow alone; 3) PLX3397 chow + carboplatin/pemetrexed; 4) control chow + carboplatin/pemetrexed. Finally, to assess the effects of CSF-1R inhibition in a de novo model of mesothelioma, we will treat NF2⁺KO mice with asbestos to induce mesothelioma tumor formation. Mice with mesothelioma will be randomized into 1 of 4 treatment groups to receive chemotherapy with or without PLX3397 as above. In each experiment, mice will be followed for tumor progression by optical imaging and/or ultrasound as well as for overall survival. Tumors will be analyzed histopathologically, and by flow cytometry (FACS) analysis for changes in vasculature, proliferation, apoptosis, and immune cell infiltration. Statistical analysis will be performed to identify significant changes in any of these parameters between treatment groups.

Cancer Relevance: This study will provide a thorough preclinical assessment of the potential benefits of targeting macrophages in mesothelioma. We predict that these results will be readily translated into novel therapies for mesothelioma patients. Many other cancers, including lung and breast cancer, are also infiltrated with inflammatory cells. As such, the potential benefit of studying this new method of targeting cancer is enormous.

This work was supported by an NIH T32 training grant to Dr. Blakely, and by a DoD Mesothelioma Program grant (PR080717) to Drs. Broaddus and Coussens.

MR Imaging of Tumor Associated Macrophages with Ferumoxytol

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Purpose: The presence of tumor-associated macrophages (TAM) in breast cancer correlates strongly with poor outcome. The purpose of this study was to develop a clinically applicable, non-invasive diagnostic assay for selective targeting and visualization of TAMs in breast cancer, based on magnetic resonance (MR) imaging and the clinically applicable iron oxide nanoparticle compound ferumoxytol.

Methods: F4/80-negative cancer cells and F4/80-positive TAM were incubated with ferumoxytol and were compared regarding MR signal changes and iron uptake. Mice with MMTV PyMT breast cancers underwent nanoparticle-enhanced MR up to 1 hour (h) and at 24 h post injection (p.i.). The tumor enhancement on MR images was correlated with the presence of TAMs on histopathology.

Results: In vitro studies revealed that the clinically applicable iron oxide nanoparticle compound ferumoxytol is preferentially phagocytosed by TAMs, but not by tumor cells. In vivo, all tumors demonstrated an initial contrast agent perfusion on immediate postcontrast T2-weighted MR images with gradual transendothelial leakage into the tumor interstitium. At 24 h p.i., all tumors demonstrated a persistent signal decline on MR scans. Detection of iron using DAB-enhanced Prussian Blue staining, and immunodetection of CD68+ TAMs in tumor tissue sections localized iron oxide nanoparticles to CD68+ TAMs, indicating that the MR signal effects on delayed MR images were largely due to TAM-mediated uptake of contrast agent.

Conclusion: Ferumoxytol nanoparticles can selectively target and visualize of TAMs in breast cancer on MR images.

Clinical Implication: These data indicate that tumor-enhancement with clinically applicable iron oxide nanoparticles may serve as a new biomarker for long-term prognosis, related treatment decisions and the development of new immune-targeted therapies.

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A Role for Macrophages in a Recalcitrant Tumor, Mesothelioma
V. Courtney Broaddus, MD
Invited Speaker
March 1, 2011

Mesothelioma is highly resistant to treatment, a problem thought to be due to the resistance to apoptosis. In our collaboration with Dr. Lisa Coussens, we are now investigating the underpinning of this resistance and have focused on the role of the tumor-associated macrophages. We have now shown that mesothelioma contains a predominance of macrophages, more evident in mesothelioma than in other thoracic malignancies such as lung cancer or esophageal cancer. Perhaps the significance of macrophages may stem from their initial involvement in the response to asbestos, the only known etiology of mesothelioma. In hybrid spheroids that we are constructing to study the interaction of mesothelioma cells and macrophages, we have learned that macrophages can be reprogrammed by cytokines to a pro-apoptotic phenotype (M1) which enhances the chemosensitivity of mesothelioma cells to therapy. Most recently, we have shown that this repolarization can be accomplished in tumor fragment spheroids, leading to clear increases in the apoptotic response of the mesothelioma cells ex vivo to standard chemotherapeutic agents (cisplatin or carboplatin plus pemetrexed).

This work was supported by the Simmons Mesothelioma Foundation and a DoD Mesothelioma Program grant PR080717.
Macrophage-depletion as a Novel Therapeutic Approach for Malignant Pleural Mesothelioma

Collin M. Blakely¹, Nikita Kolhatkar², Magnus Johansson², Dario Barbone⁴, Adam Yagui-Beltrán³, Raphael Bueno⁵, David J. Sugarbaker⁵, Thierry M. Jahan¹,⁶, David M. Jablons³,⁶, V. Courtney Broaddus⁴, and Lisa M. Coussens²,⁶

Departments of ¹Medicine, Division of Hematology/Oncology, ²Pathology, ³Surgery, ⁴Lung Biology Center, and ⁶Helen Diller Family Comprehensive Cancer Center, University of California San Francisco. ⁵Division of Thoracic Surgery, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts.

Malignant pleural mesothelioma (MPM) is a debilitating incurable cancer that exhibits a high degree of resistance to standard chemotherapy; thus, we sought to identify novel therapeutic targets for treatment. In the vast majority of cases, MPM is associated with exposure to asbestos fibers, which result in a chronic pro-inflammatory state. As such, we hypothesized that MPMs were infiltrated by leukocytes possessing tumor-potentiating activities. To address this, we evaluated MPMs, resected from 26 patients, by flow cytometry and found that tumors were highly infiltrated with macrophages (31 ± 4.6% of total CD45+), a percentage that is significantly higher that that observed in other thoracic malignancies (NSCLC cancer, 9%; esophageal, 4%). Since recent experimental data has revealed that tumor-associated macrophages (TAMs) secrete proangiogenic, prosurvival, and proinvasive factors that foster tumor progression, we evaluated macrophage-depletion in MPM as a novel therapeutic strategy. Using a syngeneic murine transplantation model and liposomal-encapsulated clodronate that efficiently deplete phagocytic macrophages, as monotherapy, and in combination with chemotherapy, we revealed a significant decrease in tumor growth and a decrease in tumor-burden in tumor-bearing mice depleted of macrophages. However, when macrophage-depletion was combined with the cytotoxic agents gemcitabine and TRAIL, an even greater reduction in tumor burden was observed as compared to mice treated with either agent alone. These studies indicate that: 1) mesothelioma-associated macrophages provide a protumor function, and 2) depletion of mesothelioma-associated macrophages may improve efficacy of cytotoxic chemotherapy. Ongoing studies to reveal the effect of macrophage-depletion plus chemotherapy in limiting MPM development in NF2ko/+ mice exposed to asbestos are underway.

This work was supported by an NIH T32 training grant to Dr. Blakely, and by a DoD Mesothelioma Program grant (PR080717) to Drs. Broaddus and Coussens.
Malignant Mesothelioma: What is the role of radiotherapy?

V. Courtney Broaddus, MD  
Invited Speaker  
April 15, 2011

In this Grand Rounds, I will cover the background of the etiology of mesothelioma, updates in diagnosis and staging, a current assessment of treatment options including radiation therapy, and insights from our laboratory about novel approaches to treatment of mesothelioma. Mesothelioma is induced by asbestos probably as it lodges in the parietal pleural lymphatics, most likely explaining why mesothelioma originates in the parietal pleura. As it develops over decades, it becomes apparent in imaging (plain films, CT scans, PET scans and MRI) at a point when it is incurable by surgical resection. Diagnosis is still difficult although using panels of immunohistochemical markers has made a positive identification of mesothelioma more accurate. Staging criteria are not universally accepted and different systems are used; a new system is under evaluation by the IASLC together with iMig. Therapy used today is generally a multimodality therapy, including surgery, chemotherapy and radiation. Radiation therapy has a long history but not a clear evidence of effectiveness. In our 3D models, radiation of spheroids has encountered resistance due to acquired multicellular resistance of cells in 3D. Our effort to undermine this resistance, by blocking the Bcl-2 anti-apoptotic repertoire or by blocking or repolarizing the macrophages within the tumors, may improve the response of mesothelioma to therapy, including radiation therapy.

This work was supported by the Simmons Mesothelioma Foundation and a DoD Mesothelioma Program grant PR080717.
Tumor-associated Macrophages can be Polarized to Reduce the Chemoresistance of Malignant Mesothelioma

V. Courtney Broaddus, Sailaja Battula, Dario Barbone, Collin Blakely, Raphael Bueno, Nikita Kolhatkar, David J. Sugarbaker, David M Jablons, Lisa M Coussens

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Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Background: Mesothelioma is highly resistant to chemotherapy. One feasible approach could be to harness the immune system to undermine the chemoresistance of mesothelioma. We studied the chemoresistance of mesothelioma cells in a spheroid model wherein mesothelioma cells acquire multicellular apoptotic resistance that resembles that of the solid tumor.

Methods: Immunohistochemistry and flow cytometry were used to analyze the leukocyte compositions of human mesotheliomas. Macrophages, either derived from human mesothelioma, THP-1-derived or matured from peripheral blood monocytes, were polarized to a Th1 or Th2 phenotype by exposure for 48 h to cytokines (IFN-gamma & LPS or IL-4 respectively). Polarized macrophages were then grown with mesothelioma cells either together in heterotypic 3-dimensional spheroids or in a Transwell system in which macrophages were grown separately from mesothelioma spheroids by a 3 micron filter. After 24 h, mesothelioma spheroids with and without macrophages were treated with TRAIL plus gemcitabine or carboplatin plus pemetrexed for 48 h and studied for apoptosis by analysis of Hoescht-stained nuclear morphology.

Results: We found that all subtypes of mesothelioma harbor a large macrophage population (31±4.6% of the inflammatory cell population [CD45⁺]). Indeed, the percentage of macrophages in mesotheliomas exceeded that found in other thoracic malignancies thus far evaluated (NSCLC cancer, 9%; esophageal, 4%). We found that Th1-polarized macrophages increased the response of mesothelioma spheroids to TRAIL plus gemcitabine by 155% and to carboplatin plus pemetrexed by 47.6% respectively, compared to Th2 polarization. The pro-apoptotic effect was similar whether macrophages were grown together with or separately from the mesothelioma cells, suggesting the mediation of macrophage-derived cytokines. The pro-apoptotic effect of Th1 polarization depended on the presence of macrophages: mesothelioma spheroids without macrophages exposed to Th1 cytokines showed no increase in apoptosis.

Conclusion: Polarization of macrophages within mesothelioma tumors to an Th1 phenotype could enhance the efficacy of standard and novel therapies.

This work was supported by grants from the NCI and a DoD Mesothelioma Program grant PR080717 to Broaddus and Coussens
Targeting Macrophages in a Preclinical Model of Malignant Mesothelioma

Collin M. Blakely¹, Nikita Kolhatkar², Magnus Johansson², Dario Barbone⁴, Adam Yagui-Beltrán³, Raphael Bueno⁵, David J. Sugarbaker⁵, Thierry M. Jahan¹, Brian L. West⁷, David M. Jablons³, V. Courtney Broaddus⁴, and Lisa M. Coussens²

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Malignant pleural mesothelioma (MPM) is a debilitating, incurable cancer that exhibits a high degree of resistance to standard chemo-therapy; thus, we sought to identify novel therapeutic targets for treatment. In the vast majority of cases, MPM is associated with prior exposure to asbestos fibers, resulting in a chronic pro-inflammatory state in pleura. As such, we hypothesized that MPMs were infiltrated by leukocytes possessing tumor-potentiating activities. To address this, we evaluated MPMs, resected from 24 patients, by flow cytometry and found that tumors were highly infiltrated by macrophages (31 ± 4.6% of total CD45+), a percentage significantly higher than that observed in other thoracic malignancies (NSCLC cancer, 9%; esophageal, 4%). Since recent experimental data has revealed that tumor-associated macrophages (TAMs) secrete proangiogenic, prosurvival, and pro-invasive factors that foster tumor progression, we evaluated macrophage depletion in MPM as a novel therapeutic strategy. Using a syngeneic murine transplantation model, and liposomal-encapsulated clodronate that efficiently depletes phagocytic macrophages. These studies revealed a significant decrease in tumor growth and a decrease in tumor-burden in mice depleted of macrophages. A similar effect was observed when MPM-bearing mice were treated with an anti-CD11b neutralizing mAB in combination with cytotoxic chemotherapy. Finally, we revealed that an orally bioavailable tyrosine kinase inhibitor of CSF-1R (PLX3397) that decreases macrophage infiltration, also reduces MPM as mono-therapy. These studies indicate that: 1) macrophages potentiate mesothelioma development, and 2) depletion of mesothelioma-associated macrophages improve efficacy of cytotoxic chemotherapy and may provide a survival advantage.

This work was supported by an NIH T32 training grant to Dr. Blakely, and by a DoD Mesothelioma Program grant (PR080717) to Drs. Broaddus and Coussens.

Presented at the UCSF Pulmonary Research Retreat  
University of California San Francisco  
San Francisco, CA  
November 14, 2011

Th1-Polarized Macrophages Enhance the Apoptotic Response To Chemotherapy In Mesothelioma

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The tumor microenvironment (TME) may contribute to tumor chemoresistance. Manipulating the TME may be one of the strategies to target mesothelioma, a highly chemoresistant tumor. We found that macrophages constitute a high percentage of inflammatory cells in mesothelioma. Our aim is to understand whether macrophages influence the tumor cells and whether alteration of macrophage phenotype could enhance the chemoresponsiveness of the tumor.

Two 3D models of mesothelioma were used: 1) the multicellular spheroid, in which mesothelioma cells are grown alone or co-cultured with macrophages derived from peripheral blood monocytes and 2) the tumor fragment spheroid (TFS) where the small fragments of fresh tumor from mesothelioma patients are grown in culture. The macrophages were polarized to Th0 (no cytokines), Th1 (by exposure to LPS & IFNγ) or Th2 (by exposure to IL-4 &IL-13) phenotype and subjected to various chemotherapeutic agents.

Polarization of the macrophages to their respective phenotype was confirmed by qPCR showing an increase in M1 phenotype markers (TNF, IL-12, IFNγ) and M2 phenotype markers (CD206, IL10) in presence of their respective cytokines. We found that, compared to Th0- or Th2-polarized macrophages, Th1-polarized macrophages increased the apoptotic response of multicellular spheroids to carboplatin and pemetrexed by 81%. The presence of macrophages is essential to elicit the pro-apoptotic effect because the cytokines have no effect on the chemoresponsiveness of the mesothelioma cells alone. Moreover, Th1-polarized macrophages enhanced chemoresponsiveness even when separated from the target mesothelioma cells by a filter, suggesting that the effect is mediated by soluble factors.

Furthermore, in TFS from patients, Th1-polarization increased mesothelioma cell apoptosis alone and after chemotherapy. Therefore, our results demonstrate that manipulating the TME may be an effective therapeutic approach to mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and the T32 Training Grant to Battula.
Colony-Stimulating Factor-1 Receptor Blockade by the Tyrosine Kinase Inhibitor PLX3397 Reprograms Malignant Mesothelioma Tumor Microenvironments and Decreases Tumor Growth

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Malignant mesothelioma (MM) is a debilitating, frequently incurable cancer that exhibits a high degree of resistance to standard cytotoxic chemotherapy (CTX). Novel therapeutic approaches to treat this disease are desperately needed. In the vast majority of cases, MM is associated with prior exposure to asbestos fibers, resulting in a chronic pro-inflammatory state in pleura. As such, we hypothesized that MMs are infiltrated by leukocytes possessing tumor-potentiating activities. To address this, we evaluated MMs resected from patients (n=16) and compared the complexity of immune cells infiltrating MM to those found in normal pleura (n=4). Using polychromatic fluorescent-activated cell sorting (FACS) on freshly-resected whole tissues, we found a significant increased presence of CD11b+CD14+HLA-DR+ monocytes/macrophages in MM (37.3 ± 4.4% of total CD45+ cells) as compared to normal pleural tissue (13.8 ± 6.5% of total CD45+ cells), and an even more significant increase in MM resected from patients treated with CTX (48.8 ± 5.5% of CD45+ cells). To determine if increased presence of CD11b+CD14+HLA-DR+ cells was associated with varied expression of cytokine signaling genes, we examined mRNA expression of tissues/tumors. We found increased expression of Colony Stimulating Factor 1 (CSF1), and Colony-Stimulating Factor-1 Receptor (CSF-1R) mRNA, a critical cytokine-signaling axis regulating monocyte/macrophage differentiation and recruitment into tumors, in MM tumors compared to normal pleura, and even higher levels in tumors resected from patients treated with CTX. Since recent experimental data has revealed that tumor-associated macrophages (TAMs) secrete proangiogenic, prosurvival, and pro-invasive factors that foster tumor progression, we evaluated macrophage depletion in MM as a novel therapeutic strategy. We conducted studies evaluating PLX3397 (Plexxikon Inc., Berkeley, CA), a novel, orally bioavailable, small molecule tyrosine kinase inhibitor of CSF-1R. Using a syngeneic orthotopic murine model of MM, we found that treatment of mice with PLX3397 alters the tumor immune microenvironment by decreasing TAM infiltration and increasing the proportion of CD8+ cytotoxic T lymphocytes within tumors. This reprogramming of the tumor immune microenvironment was associated with alterations in the tumor microvasculature as evidenced by a decrease in CD31+ structures, as well as a decrease in VEGFA mRNA expression. Ultimately, these changes resulted in an increase in tumor cell apoptosis and a decrease in tumor burden. These studies indicate that: 1) macrophages potentiate mesothelioma development, and 2) depletion of mesothelioma-associated macrophages may improve the efficacy of cytotoxic chemotherapy and provide a survival advantage.

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MODULATING IMMUNE RESPONSE TO IMPROVE THERAPY FOR BREAST CANCER

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While BC has not historically been linked to underlying inflammation or infection, it exhibits tumor-associated inflammation marked by infiltration of innate and adaptive immune cells into developing tumors. In BC, macrophages are one of the most abundant innate immune cells present. BC-associated macrophages are regulated in part by colony stimulating factor 1 (CSF1), a key cytokine involved in monocyte/macrophage maturation, recruitment and activation, and its cognate receptor CSF1R. Macrophage presence in BC correlates with increased CSF1, increased vascular density, and worse clinical outcome. We reported that CD4+ T cells promote invasion and metastasis of mammary adenocarcinomas by directly regulating macrophage phenotype that in turn fosters invasive tumor growth, presence of circulating tumor cells and pulmonary metastasis. This preclinical data implied that women with BC heavily infiltrated by macrophages would have a worse clinical outcome as compared to tumors not heavily infiltrated with macrophages. We evaluated survival outcomes in 698 women with invasive BC treated with surgery alone and found that recurrence-free survival could be stratified based upon macrophage and T cell infiltration. Thus, we investigated CSF1 and CSF1R antagonists, in combination with standard-of-care chemotherapy (CTX) in mouse models of mammary carcinogenesis. We found that when macrophage infiltration in mammary adenocarcinomas was blocked, paclitaxel (PTX) chemosensitivity was increased, accompanied by development of productive anti-tumor immune responses and CD8+ cytotoxic T cell (CTL) infiltration. The combined effects of these changes were reduced primary tumor growth, 85% reduction in metastases and increased survival. In collaboration with clinical colleagues, we are currently evaluating the clinical benefit of macrophage modulation in preclinical models of BC to facilitate biomarker identification, and inform clinical trials of CTX in combination with macrophage-antagonists. Based on our preliminary data, we hypothesize that components of macrophage responses in BC can be identified to serve as biomarkers for risk stratification. And, that these components can be effectively targeted for therapeutic intervention, resulting in reduced late-stage BC development and metastasis when combined with CTX.

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis are B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, T_{H}2-CD4^{+} T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and new studies evaluating how neutralizing selective aspects of pro-tumor immunity can be exploited to enhance therapeutic responses to cytotoxic therapy and in general bolster tumor-suppressive anti-tumor immune responses.

LMC acknowledges generous support from the NIH/NCI (R01CA130980, R01CA13256, R01CA140943, R01CA15531), the Department of Defense (W81XWH-09-1-0342, W81XWH-10-BCRP-EOHS-EXP) and the Susan G Komen Foundation (KG111084)
Abstract for
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Manipulation of Macrophage Phenotype Enhances the Apoptotic Response To Chemotherapy In Mesothelioma
V. Courtney Broaddus¹,5, Sailaja Battula¹, Dario Barbone¹, Collin Blakely⁴, David J. Sugarbaker⁶, Raphael Bueno⁶, Lisa M. Coussens²,5,7.
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Macrophages within the tumor microenvironment (TME) may contribute to tumor chemoresistance. We have found that macrophages constitute a significant percentage of the infiltrating leukocytes in mesothelioma, a highly chemoresistant tumor. We asked whether reprogramming of macrophage phenotype from a Th2 (pro-tumor) to a Th1 (anti-tumor) phenotype would alter the mesothelioma chemoresponsiveness. To address this, we used 3D organotypic models of mesothelioma growth: 1) multicellular spheroids, with mesothelioma cells grown alone or co-cultured with macrophages derived from peripheral blood monocytes and, 2) primary tumor fragment spheroid (TFS) derived from small fragments of freshly isolated human mesothelioma. Macrophages were incubated with Th1 (LPS & IFNγ) or Th2 (IL-4 &IL-13) cytokines prior to co-culture with mesothelioma spheroids, followed by exposure to standard-of-care chemotherapy, cisplatin plus pemetrexed. Gene expression was evaluated in macrophages to affirm Th1-type (TNF, IL-12, IFNα) versus Th2-type (CD206, IL10) programming. In both spheroid approaches, Th1-type macrophage programming significantly increased the apoptotic response of mesothelioma cells to chemotherapy. Moreover, when CSF1R signaling in macrophages was inhibited by incubation with a small molecular weight kinase antagonist, GW2850, chemoresponsiveness was significantly increased as evidenced by increased presence of apoptotic tumor cells in spheroids. Enhanced chemoresponsiveness of mesothelioma tumor cells was dependent on presence of macrophages because incubation of tumor cells with Th1-type cytokines or GW2850 were without effect. We conclude that manipulating the TME may be a promising therapeutic approach in mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.
Invited Presentation
2nd International Symposium on Lung-sparing Therapies for Malignant pleural Mesothelioma
May 12, 2012
Santa Monica, CA

MANIPULATING THE CORE APOPTOTIC MACHINERY IN MESOTHELIOMA

V. Courtney Broaddus

Thank you for this opportunity to present our research and to describe how it may improve the therapeutic options in mesothelioma. Along with members of my laboratory, Drs. Dario Barbone and Sailaja Battula, and our major collaborators, Dr. Lisa Coussens, we have made promising strides in understanding the core apoptotic resistance in mesothelioma and how the resident macrophages can be manipulated to tilt the balance from chemoresistance to chemosensitivity.

For many years now, our major effort has been in uncovering the apoptotic resistance of malignant pleural mesothelioma, a particularly recalcitrant tumor. Our studies in 3D mesothelioma spheroids have indicated roles for certain pathways and proteins that would not have been recognized by studies in 2D monolayers. Some of these findings are the role played by mTOR and by the Bcl-2 family of pro- and anti-apoptotic proteins. Our current studies using macrophages in co-culture with mesothelioma cells have illustrated the potential role of macrophages in enhancing apoptosis. In fact, these studies have helped advance clinical trials planned to use inhibitors to a key macrophage receptor, the CSF1R, in patients with mesothelioma.

In this talk, I will present findings in the 3D spheroids and in the hybrid spheroids co-cultured with mesothelioma cells plus macrophages. This will then lead to parallel studies in the mouse mesothelioma model and the use of this anti-CSF1R therapeutic agent to enhance chemosensitivity. We hope that these pre-clinical studies will speed clinical trials in which this non-toxic approach can be tested in patients with mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.
Cancer Immunotherapy Consortium's 2012 Colloquium, April 2012

**Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy**

**Lisa M. Coussens.** Department of Cell & Developmental Biology, Knight Cancer Institute, Oregon Health & Sciences University, 3181 SW Sam Jackson Park Road, Portland OR 97239-3098. Email: coussenl@ohsu.edu

The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis are B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, Th2-CD4+ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung, as well as responses to cytotoxic therapies. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and new studies evaluating how attenuating protumor properties of myeloid cells can be exploited to enhance therapeutic responses to cytotoxic therapy.

LMC acknowledges generous support from the NIH / NCI, and Department of Defense Era of Hope Scholar Award Expansion Award, Susan G. Komen Foundation for a Komen Promise award, and an Investigator-Initiated Research Award in Mesothelioma from the DoD.
Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis are B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, Th2-CD4+ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung, as well as responses to cytotoxic therapies. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and new studies evaluating how attenuating protumor properties of myeloid cells can be exploited to enhance therapeutic responses to cytotoxic therapy.

LMC acknowledges generous support from the NIH / NCI, and Department of Defense Era of Hope Scholar Award Expansion Award, Susan G. Komen Foundation for a Komen Promise award, and an Investigator-Initiated Research Award in Mesothelioma from the DoD.
Th1-Polarized Macrophages Enhance The Apoptotic Response To Chemotherapy In Mesothelioma

S. Battula¹, D. Barbone¹, C. Blakely¹, R. Bueno², D. Sugarbaker², D. Jablons³, L.M. Coussens¹, V.C. Broaddus¹
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RATIONALE The tumor microenvironment (TME) may contribute to tumor chemoresistance. As a result, manipulating the TME may be a means of targeting mesothelioma, a highly chemoresistant tumor. We found that macrophages constitute a high percentage of inflammatory cells in mesothelioma. Our aim is to understand whether macrophages influence the tumor cells and whether alteration of the macrophage phenotype could enhance the chemoresponsiveness of the tumor.

METHODS Two 3D models of mesothelioma were used: 1) the multicellular spheroid, in which mesothelioma cells are grown alone or co-cultured with macrophages derived from peripheral blood monocytes and 2) the tumor fragment spheroid (TFS) in which the small fragments of fresh tumor from mesothelioma patients are grown ex vivo in culture. The macrophages were polarized to Th0 (no cytokines), Th1 (by exposure to LPS & IFNγ) or Th2 (by exposure to IL-4
& IL-13) phenotypes and subjected to various chemotherapeutic agents.

RESULTS Polarization of the macrophages to their respective phenotype was confirmed by qPCR showing an increase in Th1 phenotype markers (TNF, IL-12, IFNγ) and Th2 phenotype markers (CD206, IL10) in presence of their respective cytokines. We found that, compared to Th0- or Th2-polarized macrophages, Th1-polarized macrophages increased the apoptotic response of multicellular spheroids to carboplatin and pemetrexed by 80%. The presence of macrophages was essential for the pro-apoptotic effect because the cytokines had no effect on the chemoresponsiveness of the mesothelioma cells alone. Th1-polarized macrophages enhanced chemoresponsiveness even when separated from the target mesothelioma cells by a filter, suggesting that the effect was mediated by soluble factors. Most interestingly, in TFS grown from patient tumors, Th1-polarization significantly increased mesothelioma cell apoptosis to chemotherapy, suggesting that macrophages in the actual tumors can be manipulated to enhance tumor chemoresponsiveness.

CONCLUSION Therefore, our results demonstrate that manipulating the tumor microenvironment by altering the macrophage to a Th1 phenotype may be an effective therapeutic approach to mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.
Asbestos and its toxic relationship with the pleura: update and future concerns.

V. Courtney Broaddus, MD

In this presentation, I will describe current understanding of the interaction of asbestos fibers with the parietal pleura ultimately leading to pleural disease, especially pleural mesothelioma. Initially, asbestos fibers are inhaled into the airways, reaching the airspaces due to their unique shape. There they attract macrophages which enhance an innate inflammatory response to the fibers. However, many fibers translocate to the pleura by moving directly across the visceral pleura, due to their migration toward the negative pressure of the pleural space, and the ‘milking’ motion of the lung in ventilation. In animal studies, asbestos fibers can be shown to reach the pleural space within hours to days. There, the fibers likely move to the parietal pleura along with the absorption of pleural liquid. In early studies, I have shown that the pleural liquid is absorbed by bulk flow, into lymphatic stomata of the parietal pleura. Later studies have shown that carbon also collects at these locations and, in a study by Boutin using VATS biopsies, asbestos fibers were also found to accumulate at these discrete locations. There, the inflammatory stimulus of the asbestos fibers continues over decades. The fibers cannot negotiate the lymphatic openings and remain stuck in the parietal pleura, macrophages are recruited but cannot digest or remove the fibers, and thus inflammation is unchecked. Presumably, the inflammation along with the oxygen radicals and chromosomal damage induced by the asbestos fibers leads to malignant change. Such insights have led to current efforts to inhibit macrophage function and clinical trials that are being developed to block the macrophage receptor, CSF1R. Early studies using co-culture of macrophages with mesothelioma cells and animal studies with asbestos-induced and orthotopic mesothelioma have shown promising results suggesting that targeting the macrophage and its inflammatory responses may be an effective strategy for fighting mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.
Invited Presentation
Boston, MA

Spheroids and 3D Insights into Apoptotic Resistance: Mesothelioma and Lung Cancer

V. Courtney Broaddus, MD

In this presentation, I will describe our laboratory’s evolution into the study of tumor biology using 3D spheroids. Our major effort has been in uncovering the apoptotic resistance of malignant pleural mesothelioma, a particularly recalcitrant tumor, but we have also found application of our findings and approaches to lung cancer. Our studies in 3D mesothelioma spheroids have indicated roles for certain pathways and proteins that would not have been recognized by studies in 2D monolayers. Some of these findings are the role played by mTOR and by the Bcl-2 family of pro- and anti-apoptotic proteins. Our current studies using macrophages in co-culture with mesothelioma cells have illustrated the potential role of macrophages in enhancing apoptosis. In fact, these studies have helped advance clinical trials planned to use inhibitors to a key macrophage receptor, the CSF1R, in patients with mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.
Macrophages can be Manipulated to Enhance the Apoptotic Response To Chemotherapy In Mesothelioma

Sailaja Battula¹, Collin Blakely⁴, Dario Barbone¹, David J. Sugarbaker⁶, Raphael Bueno⁶, Lisa M. Coussens²,⁵,⁷, V. Courtney Broaddus¹,⁵.

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Background: Macrophages within the tumor microenvironment (TME) may contribute to tumor chemoresistance. We have found that macrophages constitute a significant percentage of the infiltrating leukocytes in mesothelioma, a highly chemoresistant tumor. We asked whether reprogramming of macrophage phenotype from a Th2 (pro-tumor) to a Th1 (anti-tumor) phenotype or blockade of macrophages by inhibition of a key macrophage receptor, CSF1R, would alter the chemoresponsiveness of mesothelioma.

Methods: To address this, we used 3D organotypic spheroid models of mesothelioma growth: 1) multicellular spheroids, with mesothelioma cells grown alone or co-cultured with macrophages derived from peripheral blood monocytes and, 2) primary tumor fragment spheroid (TFS) derived from small fragments of resected viable human mesothelioma. Macrophages were incubated with Th1 (LPS & IFNγ) or Th2 (IL-4 & IL-13) cytokines prior to co-culture with mesothelioma spheroids, followed by exposure to standard-of-care chemotherapy, carboplatin plus pemetrexed. Gene expression was evaluated in macrophages to confirm Th1-type (TNF, IL-12, IFNα) versus Th2-type (CD206, IL10) programming. Mice with syngeneic orthotopic mesothelioma were treated with carboplatin plus pemetrexed with and without GW2850, a small molecule inhibitor of CSF1R.

Results: In both spheroid models, Th1-type macrophage programming significantly increased the apoptotic response of mesothelioma cells to chemotherapy. Moreover, when CSF1R signaling in macrophages was inhibited by incubation with GW2850, chemoresponsiveness was significantly increased as evidenced by increased presence of apoptotic tumor cells in spheroids. Enhanced chemoresponsiveness of mesothelioma tumor cells was dependent on presence of macrophages because incubation of tumor cells with Th1-type cytokines or GW2850 was without effect. We extended these findings using a syngeneic orthotopic murine model of malignant mesothelioma. Treatment of tumor-bearing mice with GW2580 in combination with carboplatin plus pemetrexed chemotherapy resulted in a significant decrease in tumor burden and an increase in tumor cell apoptosis compared to treatment of mice with chemotherapy or GW2580 alone.

Conclusion: We conclude that manipulating the macrophage within the TME may be a promising therapeutic approach in mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.