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14. ABSTRACT Macrophages are a major inflammatory cell in the asbestos-induced tumor, mesothelioma, and may help maintain tumor resistance to therapy. In this second year, we have completed many tasks that confirm the importance of the macrophage in the inflammatory cell environment of mesothelioma. We can polarize macrophages to either an M1 (anti-tumor) or M2 (pro-tumor) phenotype and that M1-polarized macrophages (derived from a variety of sources including human mesothelioma) increase mesothelioma cell apoptosis and chemosensitivity. In our orthotopic murine mesothelioma model, the depletion of macrophages has increased the mesothelioma response to chemotherapy. We now plan to move to more clinically relevant models, using a denovo mouse mesothelioma model (MexTag) that closely models human mesothelioma, chemotherapy regimens used in patients with mesothelioma and a non-toxic CSF1 receptor inhibitor already in clinical trials to manipulate macrophages. In our final year, we aim to resolve important questions about the role of the macrophage and how polarization or depletion of macrophages within the tumor can sensitize the mesothelioma cell to treatment.						
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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 anti-bacterial 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 second year of our grant-funded research, we have made real progress in these aims. We can present confirmation of our first year findings of the importance of macrophages and extend more into mechanism of the in vitro, ex vivo and in vivo models. We also are poised to reach clinically useful findings in the final year, where we can take these findings into a new in vivo model, the MexTAG mouse [9, 10].

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: We have now analyzed 40 fresh tumors sent from our collaborators at the Brigham & Womens Hospital and from Dr. Jablons by FACS for inflammatory cell populations using high density FACS. The data from the high density (able to detect 14 colors and thus to refine the analysis for many inflammatory subtypes) indicates a predominance of macrophages and other innate immune cells such as neutrophils.

In additional FACS studies, we have continued to disaggregate the tumor and stain cells for mesothelin to determine the percentage of cells within tumors that were mesothelioma cells (mesothelin-positive). These results will be of value for future studies in which mesothelin-positive cells will be sorted and used in hybrid spheroid studies. We continue to find that the actual malignant cells constitute approximately 50% of the tumor, which is consistent with the finding that the inflammatory cell population constitutes approximately 30% of the tumor (as in Task 1a). The additional 20% of the cell population may consist of non-malignant, non-inflammatory tumor-associated cells such as endothelial cells and fibroblasts.

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).

We are continuing to study the profile of the other immune cells by measuring their activation status using the high density FACS, as described above. In our last progress report 2010, we showed evidence of a CD69+ activation marker for T cells that was present on the tumor cells but not in blood. This had now been confirmed in additional tumor: blood pairs. Additional activation markers are being studied as well. This new FACS approach will enable the assessment of activation status within many of the inflammatory cell populations. The CD69 early activation marker has been reported to be activated in lymphocytes within tumors, leading to proliferation but not necessarily to a cytotoxic effector function. Nonetheless, the presence of T cells with early activation markers indicates the potential for such T cells to be stimulated to a cytotoxic effector function. This task is moving ahead well and should be completed in the next year of the grant.

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 are expanding this task to ask whether the percentages are changed after exposure to chemotherapy. For this important and related questions, we are now examining tissue sections from tumors previously exposed to chemotherapy compared to tumors never exposed to treatment.

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. (In contrast, macrophages developed from the THP-leukemia line have not shown good results are not being used.) Thus, we are pursuing the hybrid spheroid studies with the peripheral blood-derived monocyte/macrophages for the following reasons: 1) they are more consistent allowing clearer results, 2) they are more numerous allowing us to speed our studies, and 3) they can be used in other laboratories to confirm our findings.

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)

As described in the 2010 Annual Progress Report, we are testing new staining approaches to identify the macrophage properties in fixed tissue. As previously stated, cytokine staining in tissue has proved to be a challenging technical problem. The fixed tissues are stored and the tumor tissue microarrays generated for these studies so the task is only postponed until the second year of funding. These tumors will serve as a tissue bank for this task and others for the next two years of the project. Staining for key M1 or M2 phenotype cytokines has shown promise in the last few months, suggesting that we are now on track to finish this task in the final year of the grant.

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).

We have now extended our PCR analysis of mesothelioma to 25 tumors and continue to 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 IL10 indicating an M2 phenotype. Nonetheless, this task requires several additional normal pleura tissues as controls before interpreting the results as final. The normal pleura tissues have now been obtained from normal human lungs that are obtained for lung organ donation but are not selected for transplantation. As such, they are available for our use. We have developed a technique for dissecting the pleura, separating it from the underlying lung (and its numerous alveolar macrophages). Normal pleura becomes available intermittently, serving to slow the completion of this task. All is set in place for us to complete the task as planned.

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

As described in the 2010 Annual Progress Report, this task has proved difficult. The cytokines remain too dilute for consistent results. We are currently nearing the use of intracellular cytokines via flow cytometry and, following further standardization in the lab, should be able to apply this to disaggregated tumor specimens.

As mentioned, there are several potential advantages of the intracellular cytokine assay compared to an ELISA which measures secreted, soluble cytokines. For one, the dilution of cytokines is not a factor. For another, we can co-stain the cells with an anti-mesothelin or an anti-CD14 antibody to determine what cytokines are produced specifically by the mesothelioma cells or macrophages, respectively. Thus, we have modified our plan for these studies and we expect them to be complete by the end of the grant period.

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 appears to require 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).

As described below, we have decided to move from additional studies with clodronate to studies using agents that are potential therapeutic agents. The use of liposomal-clodronate to deplete macrophages showed the feasibility of macrophage depletion, confirmed the importance of macrophages in mesothelioma and advanced the mouse studies, however clodronate is too toxic ever to be used in patients. Our studies have moved to a variety of clinically exciting agents, particularly the Plexxikon compound PLX-3397, a blocker of the CSF1 receptor. This compound has already entered clinical trials in some tumors and we hope to find value in mesothelioma that will enable the application of this or other CSF1 receptor inhibitors to this tumor.

We have now begun incubating hybrid spheroids and macrophages alone with the PLX-3397 compound. After 24-48 h of PLX-3397, we have confirmed that macrophages start to round up and float in the wells; macrophages in the hybrid spheroids are reduced in number. PCR studies are ongoing to show if the polarization state of these macrophages are altered by inhibition of their CSF1 receptor, which mediates an important survival pathway for macrophages.

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

These studies are ongoing and will be completed in a few months, using the PLX-3397 compound instead of the more toxic clodronate. We have shown that the PLX-3397 compound does increase the apoptotic response of the tumor fragment spheroids to carboplatin/pemetrexed, an exciting result (see below). Staining of these tumor fragments is ongoing to show whether the population of macrophages has changed in number.

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).

We have continued these studies using the no macrophage and with macrophage hybrids, now with and without the PLX-3397 compound to inhibit macrophages. As shown in the 2010 Annual Progress Report, growing the macrophages with the mesothelioma cells leads to a well formed chimeric structure we call a hybrid spheroid, with the macrophages well distributed throughout the spheroid (**Figure 1**). We have confirmed our preliminary studies that polarization of the macrophages to an M1 phenotype enhances the apoptosis of mesothelioma cells grown with macrophages. The macrophages that are not polarized (called M0) do not have a significant effect in these in vitro experiments. Polarization of the macrophages to an M1 phenotype however induces a robust pro-apoptotic effect. This effect, as shown in **Figure 2** and reproduced in all our experiments, requires the presence of the macrophages; the polarization program has no effect on mesothelioma cells themselves.

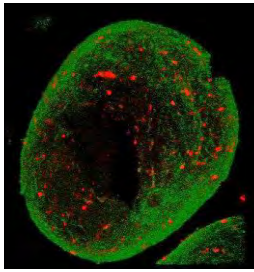


Figure 1. Macrophages and mesothelioma cells form hybrid spheroids. This spheroid contains 10,000 mesothelioma cells (green, anti-cytokeratin) and 500 (5%) macrophages (red, anti-CD69) (see arrows). By fluorescent microscopy, the macrophages are found to be distributed throughout the spheroid. Spheroid is approximately 0.5 mm in diameter.

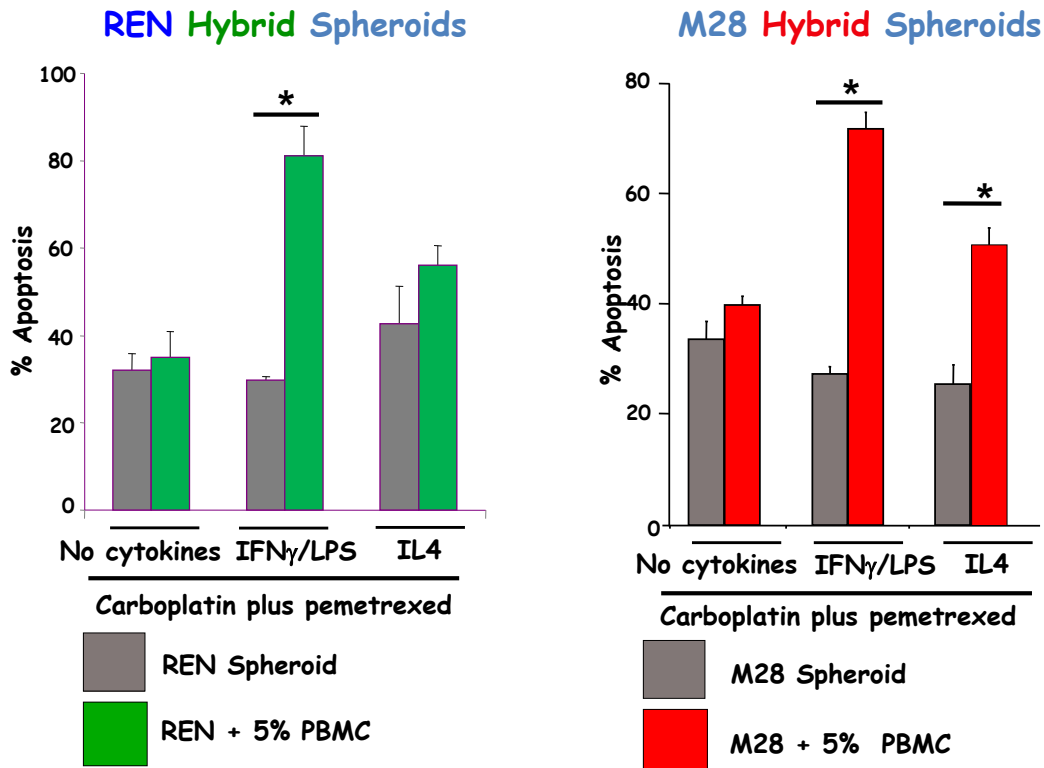


Figure 2. In 5 experiments in each cell line, polarization increases the apoptotic potential of mesothelioma cells, but only when macrophages are present. In these studies, we have used the most reproducible macrophage source, macrophages derived from peripheral blood monocytes, and the most clinically relevant therapeutic regimen, carboplatin plus pemetrexed, the treatment considered the standard for patients and the treatment now being used in the mice studies.

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).

As mentioned above, we have begun these studies, using not clodronate but the PLX-3397 compound. In our first studies since obtaining the PLX compound, we have seen no change in apoptosis of the tumor fragment spheroids exposed to PLX-3397 alone, but have seen a significant increase in apoptosis of mesothelioma cells when the tumor fragment spheroids were exposed to PLX-3397 in addition to the chemotherapy (carboplatin plus pemetrexed). In other words, the PLX compound, and even the chemotherapy alone were not effective in increasing apoptosis; however, the PLX compound given with the chemotherapy was effective. These studies are ongoing in parallel with the mouse mesothelioma studies, where a similar result has been seen. Now that we have the proper PLX compound, we expect these studies to be completed in between 9-12 months. Staining will follow to confirm the effect of the PLX compound

on the macrophage number; PCR studies will follow to confirm the effect on polarization state of the macrophages.

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 extended from the data presented in the 2010 Annual Progress Report (**Figure 3**), we have confirmed that polarization is effective in reprogramming macrophages to an M1 or M2 phenotype. We continue to see no significant difference in the cytokine expression of the different subsets of M2: M2a and M2c.

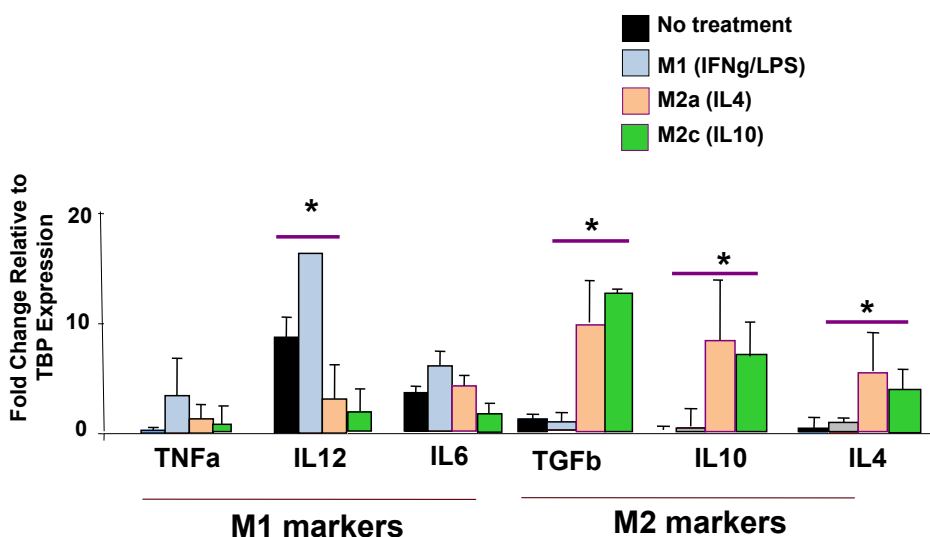


Figure 3. Human macrophages derived from peripheral blood monocytes can be polarized to an M1 or M2 phenotype. Peripheral blood monocytes were matured to macrophages and exposed for 24 h to IFN γ plus LPS (to stimulate toward a TH1 or M1 phenotype), IL-4 (toward TH2 or M2a) or IL-10 (toward TH2 or M2c). 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).

This is underway and results from these experiments are shown in Task 2h below.

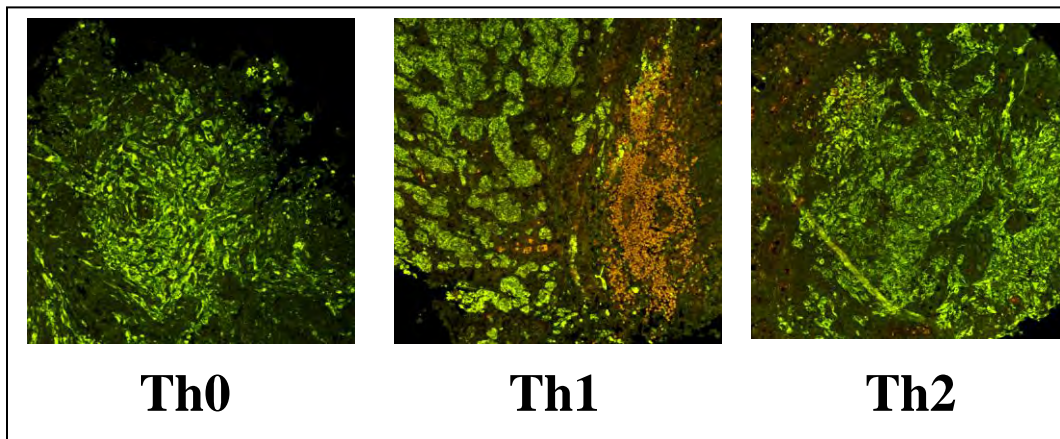
g. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in multicellular spheroids (grown with primary mesothelioma macrophages or THP-1 macrophages) when treated with TRAIL plus gemcitabine (months 12-24).

COMPLETE: We have extensive data on the effect of repolarization on the apoptotic responses of the mesothelioma cells. As shown in Figure 2 and reported in the 2010 Annual Progress Report, 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 phenotype leads to an increased apoptotic response of

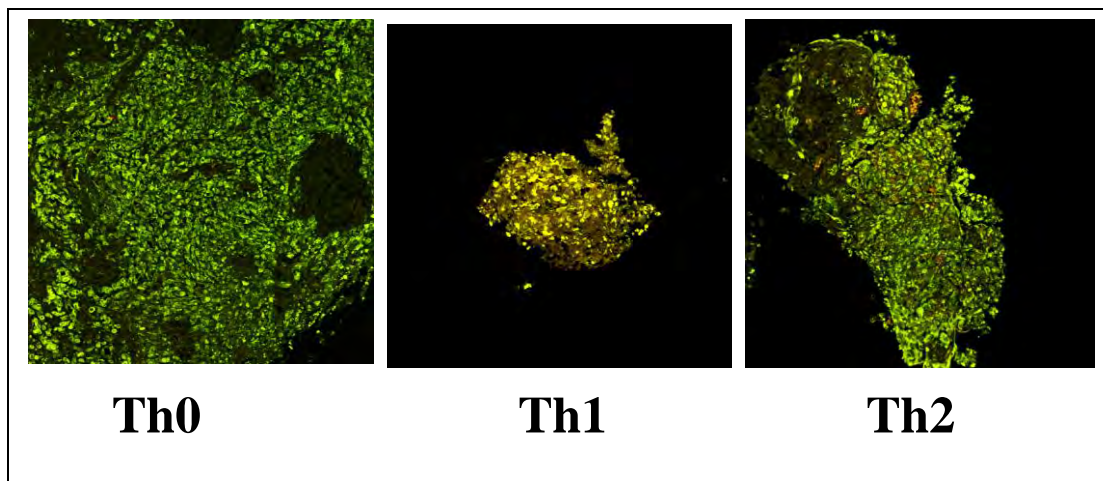
the mesothelioma cells. The mechanism is still under investigation and may depend on the intracellular cytokine assays planned to show the cytokines responsible. Early studies have implicated TNF for part of the pro-apoptotic response, but other cytokines may well be playing a role.

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

We are finding an early striking effect of polarization in the tumor fragment spheroids (**Figure 4A and B**). When the spheroids are polarized toward an M1 or M2 phenotype (or a no polarization, or M0 condition), they 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 are finding significantly increased apoptosis in spheroids polarized to M1. These findings are similar to those in the hybrid spheroids and indicate a pro-apoptotic effect of the M1 polarization state of macrophages. However, in the tumor fragment spheroids, the M1 phenotype itself appears to be associated with increased apoptosis.



A Untreated



B Treated

Figure 4A and B. 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)

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).

After extensive studies in the NF2+/- mice, we have concluded that this de novo model has serious limitations. The two major limitations are 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. In addition, a Shared Instrumentation Grant was submitted to the NIH (S10 Program) that received a top score. If funded, this would support a newer model of the Vevo2100 Small Animal Imaging Ultrasound, with Doppler for detecting tumor vascularity.

The MexTag mice now have been shipped to our animal care facility and should be available for study following quarantine in 4-8 weeks, if all goes well.

Immune studies were begun in the NF2+/- but will be repeated and confirmed in the MexTag mesothelioma mice.

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 wish to pursue clinically-relevant studies of macrophage inhibition using compounds that are nearing clinical use, non-toxic reagents such as the PLX-3397 inhibitor of CSF1 receptor. These studies have begun and show that the PLX compound 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:

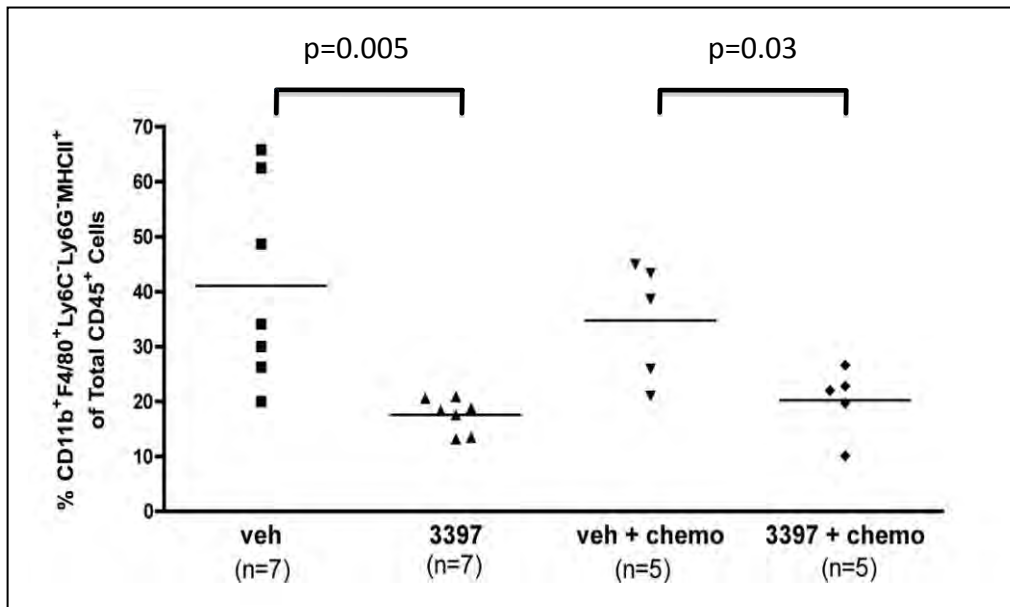
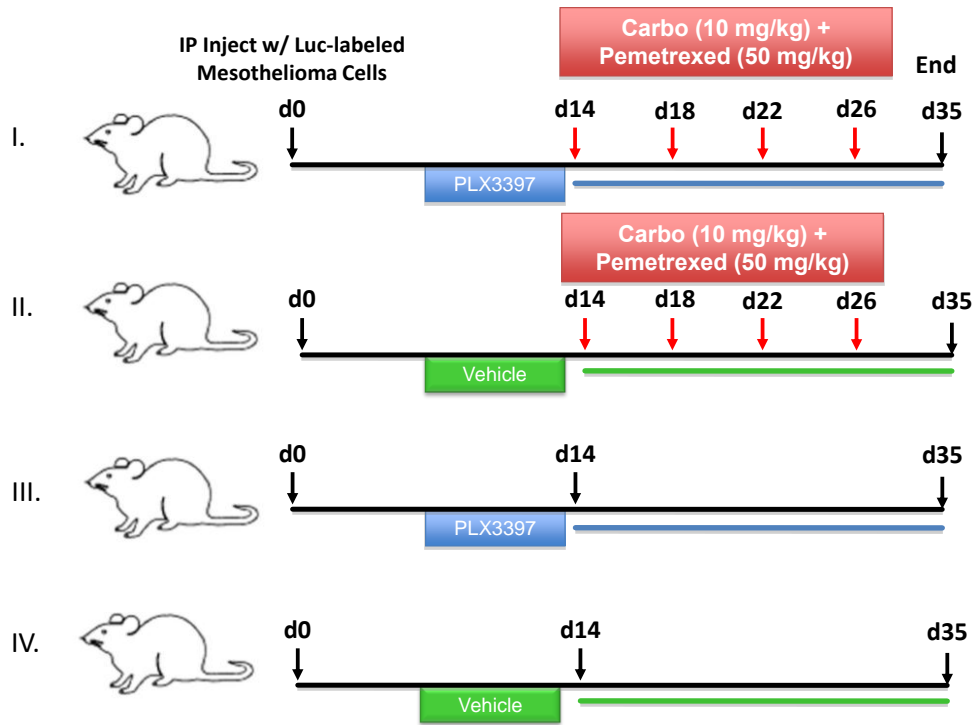


Figure 5. In mice with orthotopic 40L luciferase-labeled intraperitoneal mesothelioma, the PLX3397 compound was injected intraperitoneally with or without chemotherapy (carboplatin (10 mg/kg) plus pemetrexed (50 mg/kg) given in 4 doses over 2 weeks). By flow cytometry, the presence of macrophage population in the tumors was significantly reduced. The reduction in macrophage was seen whether or not the chemotherapy was given.

Thus, in this model, the CSF1 receptor antagonist is able to decrease macrophage populations in the orthotopic 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).

In the orthotopic 40L mesothelioma mice, the PLX 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 6**). Such a result parallels findings in breast cancer and may indicate that CD8 T cells will be shown to mediate cytotoxicity against the tumor.

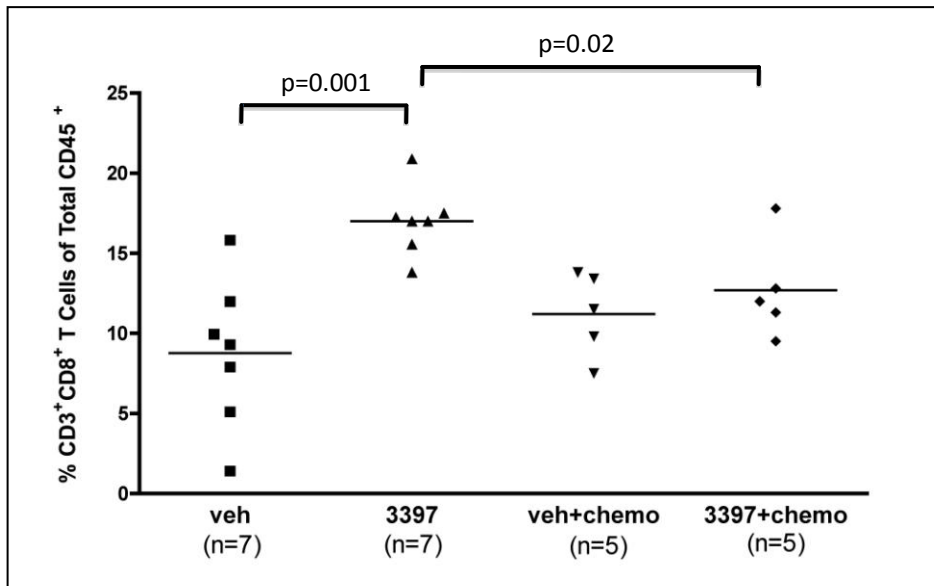


Figure 6. In the same experiment as described in the legend to Figure 5, the PLX3397 compound was associated also with an increase in the CD8 positive T cells. The increase in T cells was most evident in the untreated mice; with treatment, the T cells were suppressed.

In addition, there are complex changes in all the immune cells following PLX3397 (**Figure 7**). Besides increases in CD8+ cells, there are increases in CD4+ T cells.

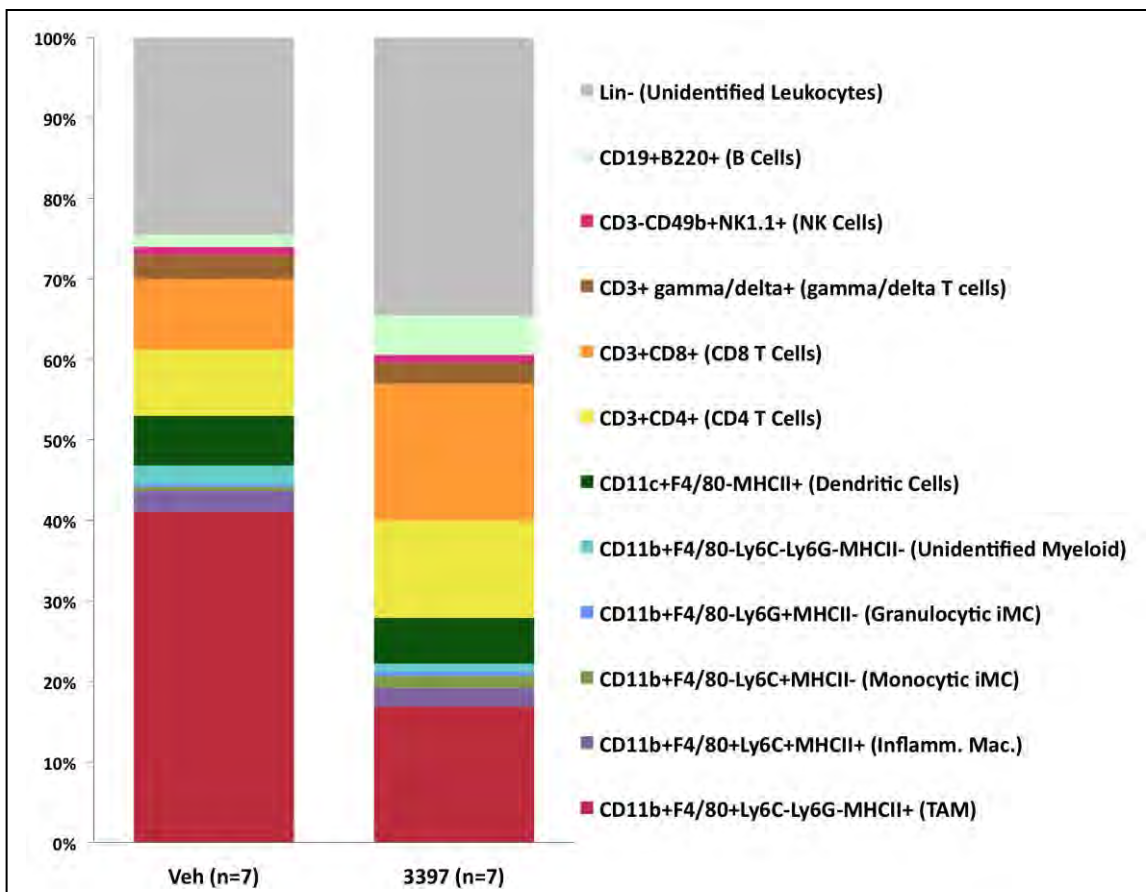


Figure 7. Multicolor flow cytometry of mesothelioma in the mouse following PLX3397 shows many changes in inflammatory cell populations.

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

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).

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 2×10^6 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 8**).

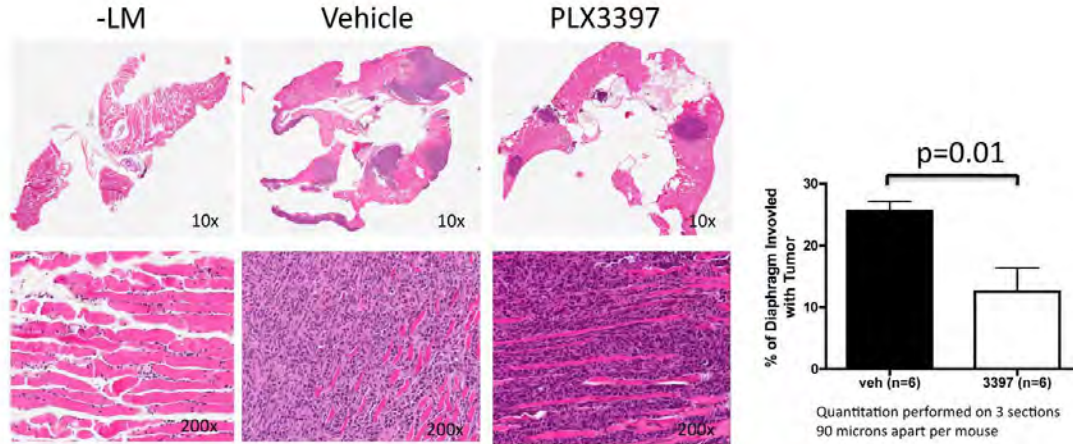


Figure 8. Monotherapy with PLX3397 reduces the burden of mesothelioma in an orthotopic mouse mesothelioma model.

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 (**Figure 9**).

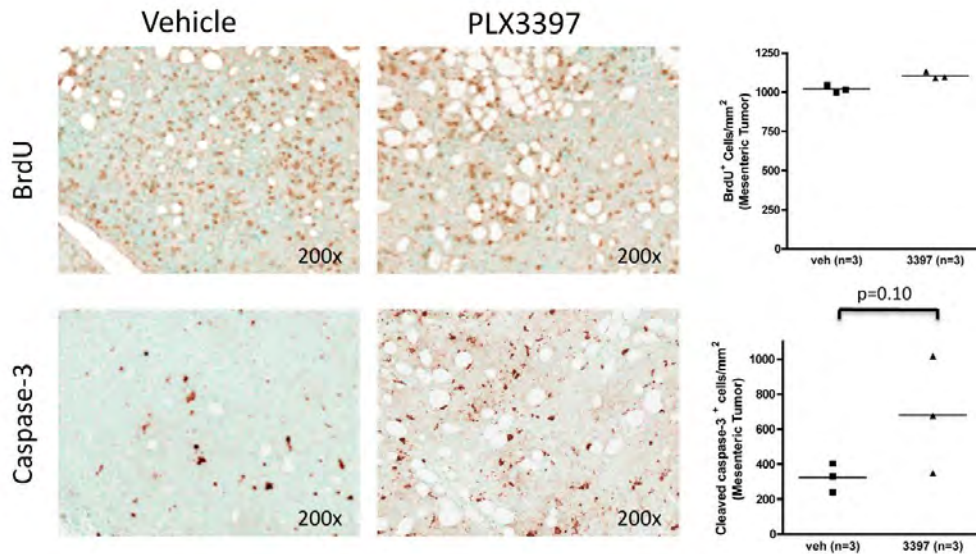


Figure 9. Monotherapy with PLX3397 led to an increase in apoptosis of the mesothelioma cells, as shown by an increase in cleaved caspase-3. There was no change in the proliferation of the tumor cells, as shown by a constant level of BrdU staining.

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).

We are on target to accomplish this task by the third funding cycle.

f. Determine whether 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).

We are on target to accomplish this task in the third funding cycle.

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).

We are on target to accomplish this task in the third funding cycle.

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
- Established 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

Months 13-24

- Determined that macrophages from human mesothelioma acted the same as the peripheral blood monocyctic 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)

Months 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

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).

Months 1-12

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

Months 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

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

Months 1-24

- Determined that cytokines were secreted by tumor fragment spheroids, but were too dilute for accuracy
- Initiated testing of an intracellular cytokine assay

- Developed mesothelin staining and gating by flow cytometry for use in this intracellular and other flow cytometry studies to identify mesothelioma cell population
- Testing and standardizing the intracellular cytokine assay for use in these studies

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

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

- Have begun using PLX3397 with tumor fragment spheroids showing an enhancement of apoptosis (staining of macrophages pending)

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
- Macrophage depletion studies await the studies with PLX3379

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

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 classic 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 1-12

- 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

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 which will soon arrive and go under quarantine

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

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

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 13-24

- 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 1-24 due to focus on the clinically relevant approach of CSF1 receptor antagonism

f. Determine whether 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

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).

- Planned for the coming year.

IV. REPORTABLE OUTCOMES

A. MANUSCRIPTS

Months 1-12

- DeNardo DG, Andreu P, Coussens LM. (2010). Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev*, 29(2):309-316, PMID: 20405169

Months 13-24 (Provided as Appendix Material)

- Barbone D, Ryan J, Kolhatkar N, Chacko A, Jablons DM, Sugarbaker DJ, Bueno R, Letai i AG, Coussens LM, Fennel DA, Broaddus VC. (2011) The Bcl-2

repertoire of mesothelioma spheroids underlies acquired apoptotic multicellular resistance. *Cell Death & Disease* [Epub ahead of print] PMID: 21697949

- Broaddus VC, Everitt JI, Black B, Kane AB. Non-neoplastic and neoplastic pleural endpoints following fiber exposure. *J Toxicol Environ Health* (2011) 14:153-178.
- Coussens LM, Pollard JW. (2011) Leukocytes in mammary development and cancer. *Cold Spring Harbor Perspectives in Biology*, 3(3) PMID: 21123394
- DeNardo DG, Brennan DJ, Rexhapaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhvani N, Keil SD, Junaid SA, Rugo HS, Hwang ES, Jirstrom K, West BL, Coussens LM. (2011) Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discovery*, 1(1): 54-67.
 - Featured in:
 - *Nature* (2011), 472 303-304. 'Macrophages Limit Chemotherapy' by, M. De Palma and C.E.Lewis
 - Nature Reviews Cancer (2011) 11:3056. 'Bad company', by N. McCarthy
- Erez M. Coussens LM. Leukocytes as paracrine regulators of metastasis and determinants of organ-specific colonization. *Int J of Cancer* (2011) 128:2536-2544.

B. ABSTRACTS

Months 13-24 (Provided as Appendix Material 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 Jablons, David M. *Exploiting emerging biology for the treatment of malignant mesothelioma*. In Symposium entitled: Translational Initiatives in Mesothelioma. American Association of 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. A role for macrophages in a recalcitrant tumor, mesothelioma. In: Cancer, Immunity and Microenvironment Program, UCSF.
- 2011 Broaddus VC. Malignant mesothelioma: what is the role of radiotherapy? Radiation Oncology Grand Rounds, UCSF.

Invited Lectures/Seminars: International

- 2010 Broaddus VC. Invited speaker, International Mesothelioma Interest Group, Kyoto, JAPAN.
- 2010 Coussens LM. Invited speaker. Nature CNIO Cancer Symposium "Frontiers in Tumour Progression", Madrid, Spain.
- 2011 Broaddus, VC. Invited Expert. 14th World Conference on Lung Cancer (WCLC), International Association for the Study of Lung Cancer, Amsterdam, The Netherlands.

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
- 2010 Coussens LM. *Inflammation and cancer*. MRS-AACR Joint Conference on Metastasis and the Tumor Microenvironment. Philadelphia, PA

Presentations by Coussens or Broaddus Lab Members:

Nikita Kolhatkar (Coussens and Broaddus' lab, pre-doctoral student)

- 2009 Kohatkar, Nikita. *Macrophages contribute to mesothelioma chemoresistance*. Poster presentation. Annual UCSF Pulmonary Research Retreat, San Francisco, CA.
- 2010 Kohatkar, Nikita. Targeting macrophages as a novel therapeutic approach for malignant pleural mesothelioma. Invited presentation. American Association for Cancer Research. Washington, DC.
- 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

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, Dario. Mesothelioma Applied Research Foundation. 2011
- Barbone D. SPORE Mechanism Career Development Award. 2011
- Broaddus VC & Coussens LM. Small animal imaging system, the Vevo 2100. S10 Shared Instrumentation Grant, 2011

- Coussens LM, Blakely CM. PAR 10-003 (P50) (Carbone)
NIH / NCI
Mesothelioma SPORE
Reprogramming Mesothelioma Immune Microenvironments to Enhance Chemosensitivity.

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.

In our third and final funding cycle, we will aim to complete the important studies to resolve these key outstanding issues about the role of the macrophage in mesothelioma and about our ability to block it or manipulate it to improve chemoresponsiveness. We will focus on the CSF1 receptor blockade, using our *in vitro* hybrid and *ex vivo* tumor fragment models to identify the molecular mechanisms of the macrophage-mesothelioma interaction and on our two *in vivo* models of mesothelioma (40L syngeneic orthotopic and now MexTag) to alter macrophage function. The MexTag model represents a distinct improvement in speed and reliability over the previous NF2^{+/-} model and should allow us to move *in vitro* studies quickly to the *in vivo* clinically relevant studies. The macrophage, the predominant inflammatory cell in all the subtypes of mesothelioma, is already infiltrated throughout the tumor mass. Manipulation of this powerful cell type has the potential to transform mesothelioma from a chemoresistant, intractable and incurable tumor to one more sensitive to current or novel therapies.

VI. BIBLIOGRAPHY

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5. Dave, S.S., et al., *Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells.* . *N. Engl. J. Med.*, 2004. **351**: p. 2159-2169.
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8. Montovani, A., A. Sica, and M. Locati, *New vistas on macrophage differentiation and activation*. *Eur. J. Immunol.* , 2007. **37**: p. 14-16.
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11. Miselis, N.R., et al., *Targeting tumor-associated macrophages in an orthotopic murine model of diffuse malignant mesothelioma*. *Mol. Cancer Ther.*, 2008. **7**(4): p. 788-799.

VII. APPENDICES

- A. Complete academic curriculum vitae for Dr. V. Courtney Broaddus and Dr. Lisa Coussens
- B. Publications for months 1-12
- C. Abstracts for months 1-12

University of California San Francisco

CURRICULUM VITAE

Name: V. Courtney Broaddus

Position: Professor of Medicine, Step 3
Department of Medicine
School 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
- 2005-2011 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. Stulberg Outstanding Teaching Award, UCSF Pulmonary
- 2010 Pioneer Award, The Mesothelioma Applied Research Foundation.

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
1989-1991	ALA/American Thoracic Society	Manpower Review Committee
1991-1992	American Thoracic Society	Program Committee, Respiratory Structure and Function
1993-1994	American Thoracic Society	Nominating Committee, Respiratory Cell and Molecular Biology
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
1995-2001	American Thoracic Society	Long Range Planning Committee, Respiratory Cell and Molecular Biology
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
2000-2002	American Thoracic Society	Planning on ATS Research Agenda
2000-2002	American Thoracic Society	Assembly Structure Task Force
2003-2004	American Thoracic Society	Education Committee
2003-2007	American Thoracic Society	Scientific Advisory Committee
2005-now	International 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
2007-2008	American Thoracic Society	Member, Search Committee for Editor, Am. Journal of Respiratory Cell Mol. Biol.
2008-2011	Mesothelioma Applied Research Foundation	Member, Scientific Advisory Board

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)
2002-2006	Editor, <u>Murray and Nadel's Textbook of Respiratory Medicine</u> 4 th Edition
2007-2010	Editor, <u>Murray and Nadel's Textbook of Respiratory Medicine</u> 5 th Edition
2005-now	Principal Editor, Website for <u>Murray and Nadel's Textbook of Respiratory Medicine</u> www.expertconsult.com
1996-1998	Associate Editor, <u>American Journal of Respiratory Cell and Molecular Biology</u>
2000-2007	Editorial Board, <u>American Journal of Physiology:</u> <u>Lung Cellular and Molecular Physiology</u>

INVITED PRESENTATIONS

INTERNATIONAL

International Chemokine Symposium, Bath, England, 1995 (Invited speaker)
International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles, Lake Placid, NY, 1996 (Platform)
International Meeting of the Formosan Medical Association, Taiwan, 1999 (Platform)
Centre for Cancer Research and Cell Biology, Queen's University, Belfast, No. Ireland 2008 (Invited Speaker)
International Mesothelioma Interest Group, Kyoto Japan, 2010 (Invited Speaker)
IASLC/World Conference on Lung Cancer, 2011 (Invited Speaker)

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)

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, Speaker, Chair Symposia)

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
1996 Co-Moderator, Pleural Disease Minisymposium. American College of Chest Physicians, San Francisco, CA
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.

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
- 2011 Member, U.S. EPA Science Advisory Board Committee, Libby Asbestos Review Panel (Nominated, Approval Pending)

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, Search 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
- 2008-9 Fundraiser, Annual Fund, International High School, San Francisco.
- 2009-11 Class Captain, Annual Fund, International High School, San Francisco.

TEACHING AND MENTORINGOther 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

<i>Dates</i>	<i>Name</i>	<i>Position while supervised</i>	<i>Current position</i>
1995-1997	Sudha Rani Narasimhan	Medical Student	Medical Resident, UCLA
1997-1999	Jack Wu	Undergraduate	Medical resident Cook County, Chicago, IL
2002-2004	Kevin Lee	Undergraduate	Medical student
2009- 2010	Nikita Kolhatkar	Graduate	current

Graduate Students Supervised

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

Postdoctoral Fellows Supervised

<i>Dates</i>	<i>Name</i>	<i>Position while supervised</i>	<i>Current position</i>
1989-1991	Alice M. Boylan, MD	Pulmonary research fellow	Associate Professor of Medicine Medical Univ of South Carolina
1991-1993	Rex Yung, MD	Pulmonary research fellow	Associate Professor of Medicine Johns Hopkins Univ.
1993-1995	Hans Folkesson, PhD	Postdoctoral research fellow	Associate Professor of Physiology Northeastern Ohio University, OH
1995-1996	Jamie Bigelow, MD	Pulmonary research fellow	Pulmonologist St. Francis Hospital, SF
1996-1997	Evaldo Marchi, MD	Visiting research fellow	Professor of Surgery Sao Paulo, Brazil
1998-2000	Tom Geiser, MD	Postdoctoral research fellow	Associate Professor of Medicine University Hospital, Bern, SW

2000-2001	Masa Ishigaki, MD PhD	Visiting research fellow	Associate Professor, Japan
2000-2003	Claire Vivo, PhD	Postdoctoral research fellow	Research Scientist Ordway Research Institute, NY
2003-2004	Ki Up Kim, MD	Visiting research fellow	Associate Professor of Medicine Soonchunhyang University Hospital South Korea
2004-2005	Lorriana Leard, MD	Pulmonary research fellow	Assistant Prof of Medicine, UCSF
2003-2006	Keith Abayasiriwardana, PhD	Postdoctoral research fellow	Research Scientist, Merck Laboratories, London, UK
2006- 2008	Tsung-Ming Yang, MD	Postdoctoral research fellow	Assistant Professor, Chiayi Chung-Gung Memorial Hosp Chiayi, Taiwan
2008-on	Eunice Kim, MD	Pulmonary Fellow	Pulmonary Research Fellow
2008-on	Denitza Blagev, MD	Pulmonary Fellow	Pulmonary Research Fellow
2008-on	Joyce Lee, MD	Pulmonary Fellow	Pulmonary Research Fellow
2009-on	Joshua Galanter, MD	Pulmonary Fellow	Pulmonary Research Fellow
2006-now	Dario Barbone	Postdoctoral Research Fellow	Postdoctoral Research Fellow

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)

1999-2002 Attending Rounds, Medicine Service, SFGH

FACULTY MENTORING

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

<i>Dates</i>	<i>Name</i>	<i>Position While Mentoring</i>	<i>Role</i>	<i>Current Position</i>
2000-2002	David Morris, MD	Division chief	Informal advisor	Head Respiratory Research Roche, Palo Alto, CA
2000-2005	Robert Jasmer, MD	Division chief	Advisor	Physician, private practice
2004-now	Payam Nahid, MD	Division chief	Advisor, Reviewed grants	Assoc Prof, UCSF
2004-now	Mary Gray, MD	LBC Assoc Director	Recruited into LBC, collaborator/advisor	Assoc Prof, UCSF
2002-now	Laura Koth, MD	LBC Assoc. Dir.	Career advisor	Asst Prof, UCSF
2004-now	Lorriana Leard, MD	Lab head/Div.chief	Advisor, formal mentor	Asst Prof, UCSF
2005-2007	Dana McClintock, MD	Career committee	Selected formal mentor	Physician, academic practice
2005-now	Harold Collard, MD	Division chief	Advisor, reviewed grants	Asst Prof, UCSF
2006-2010	Janet Diaz, MD	Division chief	Advisor	Asst Prof, UCSF
2010-now	Antonio Gomez, MD	Division chief	Advisor	Asst Prof, UCSF

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: 04/01/2009 – 3/31/2012

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: Ireland- NCI Cancer Consortium

Total Funding Period: 1/1/2009 – 12/31/2011

Grant Title: *Role of the Bcl-2 Family in Apoptotic Resistance of Mesothelioma to Bortezomib.*

Role on project: Co-Principal Investigator

Time Commitment to Project: No time

Program Overlap: None

Goals: To support a post-doctoral fellow to determine the mechanism of apoptotic resistance in 3 dimensional models of mesothelioma.

Specific Aims: 1) To study the mitochondrial anti-apoptotic repertoire that determines resistance of mesothelioma to bortezomib. 2) To uncover the role of the mitochondrial anti-apoptotic repertoire that contributes to the acquired apoptotic resistance in 3D models to bortezomib.

Supporting Agency: NCI

Grant mechanism: RO1 CA135358-01

Total Funding Period: 8/11/2008 – 5/31/2012

Grant Title: *Targeted Liposomal Radiotherapy of Malignant Mesothelioma*

Role on project: Co-Investigator

Time Commitment to Project: 5%

Program Overlap: None

Goals: To engineer nanoliposomes as the dose delivery media to carry high payload of radionuclides for specific targeting of the mesothelioma tumor cells.

Specific Aims: 1) To identify optimal scFv antibodies targeting to malignant mesothelioma from a panel of 15 antibodies we have recently discovered. The native antibodies will be labeled with 99mTc for in vitro and in vivo. 2) To focus on the 177Lu labeling of the nanosized liposome conjugated with optimal antibody (177Lu-LP-scFv) to achieve high specific activity and characterize the targeting capability, stability and radiolysis in vitro 3) To determine the biodistribution and treatment efficacy of the best 177Lu-LP-scFv in mice with malignant peritoneal mesothelioma xenografts.

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

PEER REVIEWED PUBLICATIONS:

1. BROADDUS C, Dake M, Stulberg MS, Blumenfeld W, Hadley K, Golden JA, Hopewell PC. Bronchoalveolar lavage and transbronchial biopsy for the diagnosis of pulmonary infections in patients with the acquired immunodeficiency syndrome. Ann Intern Med 102:747-752, 1985.
2. Wiener-Kronish JP, Goldstein R, Matthay RA, Biondi JW, BROADDUS VC, Chatterjee K, Matthay MA. Lack of association of pleural effusion with chronic pulmonary arterial and right atrial hypertension. Chest 92:967-970, 1987.
3. BROADDUS VC, Wiener-Kronish JP, Berthiaume Y, Staub NC. Removal of pleural liquid and protein by lymphatics in awake sheep. J Appl Physiol 64:384-390, 1988.
4. Berthiaume Y, BROADDUS VC, Gropper MA, Tanita T, Matthay MA. Alveolar liquid and protein clearance from normal dog lungs. J Appl Physiol 65:585-593, 1988.
5. Wiener-Kronish JP, BROADDUS VC, Albertine KH, Gropper MA, Matthay MA, Staub NC. Relationship of pleural effusions to increased permeability pulmonary edema in anesthetized sheep. J Clin Invest 82:1422-1429, 1988.
6. BROADDUS VC, Wiener-Kronish JP, Staub NC. Clearance of lung edema into the pleural space of volume-loaded anesthetized sheep. J Appl Physiol 68:2623-2630, 1990.
7. BROADDUS VC, Araya M, Carlton DP, Bland RD. Developmental changes in pleural liquid protein concentration in sheep. Am Rev Resp Dis 143:38-41, 1991.
8. Jacobson MA, Mills J, Rush J, Peiperl L, Seru V, Mohanty PK, Hopewell PC, Hadley WK, BROADDUS VC, Leoung G, Feigal DW. Morbidity and mortality of patients with AIDS and first-episode *Pneumocystis carinii* pneumonia unaffected by concomitant pulmonary cytomegalovirus infection. Am Rev Respir Dis 144:6-9, 1991.
9. BROADDUS VC, Araya M. Liquid and protein dynamics using a new, minimally invasive pleural catheter in rabbits. J Appl Physiol 72:851-857, 1992.
10. Boylan AM, Rüegg C, Hoeffel J, Kim KJ, Hébert CA, Pytela R, Sheppard D, Goldstein IM, BROADDUS VC. Evidence of a role for mesothelial cell-derived interleukin-8 in the pathogenesis of asbestos-induced pleurisy in rabbits. J Clin Invest 89:1257-1267, 1992.
11. BROADDUS VC, Hébert CA, Vitangcol RV, Hoeffel JM, Bernstein MS, Boylan AM. Interleukin-8 is a major neutrophil chemotactic factor in pleural liquid of patients with empyema. Am Rev Respir Dis 146:825-830, 1992.
12. BROADDUS VC, Feigal DW Jr. Starting an academic career: a survey of junior academic pulmonary physicians. Chest 105:1858-1863, 1994.
13. BROADDUS VC, Hoeffel JM, Boylan AM, Sadick M, Chuntharapai A, Kim KJ, Hébert CA. Neutralization of interleukin-8 inhibits neutrophil influx in a rabbit model of endotoxin-induced pleurisy. J Immunol 152:2960-2967, 1994.
14. Boylan AM, Hébert CA, Sadick M, Wong WL, Hoeffel JM, Hartiala KT, BROADDUS VC. Interleukin-8 is a major component of pleural liquid chemotactic activity in a rabbit model of endotoxin pleurisy. Am J Physiol Lung Cell Mol Physiol 267(11):L137-L144, 1994.

15. Folkesson HG, Matthay MA, Hébert CA, BROADDUS VC. Acid aspiration lung injury in rabbits is mediated by interleukin-8 dependent mechanisms. J Clin Invest 96:107-116, 1995.
16. Boylan AM, Sanan DA, Sheppard D, BROADDUS VC. Vitronectin enhances internalization of crocidolite asbestos by rabbit pleural mesothelial cells via the integrin $\alpha v \beta 5$. J Clin Invest 96:1987-2001, 1995.
17. BROADDUS VC, Yang L, Scavo LM, Ernst JD, Boylan AM. Asbestos induces apoptosis of human and rabbit pleural mesothelial cells via reactive oxygen species. J Clin Invest 98:2050-2059, 1996. (* identified by the Editors as being of broad interest)
18. BROADDUS VC, Yang L, Scavo LM, Ernst JD, Boylan AM. Crocidolite asbestos induces apoptosis of pleural mesothelial cells: Role of reactive oxygen species and poly (ADP-ribose) polymerase. Environ Health Perspect 105 (Suppl 5):1147-1152, 1997.
19. Narasimhan SR, Yang L, Gerwin BI, BROADDUS VC. Resistance of pleural mesothelioma cell lines to apoptosis: relation to expression of Bcl-2 and Bax. Am J Physiol (Lung Cell Mol Physiol) 275(19): L165-L171, 1998.
20. Ernst JD, Yang L, BROADDUS VC. Preparation and characterization of an endogenously fluorescent annexin for detection of apoptotic cells. Anal Biochem 260:18-23, 1998.
21. Perkins RC, BROADDUS VC, Shetty S, Hamilton S, Idell S. Asbestos upregulates expression of the urokinase-type plasminogen activator receptor on mesothelial cells. Am J Respir Cell Mol Biol 21:637-646, 1999.
22. Miyazaki H, BROADDUS VC, Wiener-Kronish JP, Sawa T, Pittet J-F, Kravchenko V, Mathison JC, Nishizawa H, Hattori S, Yamakawa T, Yamada H, Kudoh I. The effects of two anti-inflammatory pretreatments on bacterial-induced lung injury. Anesthesiology 90:1650-1662, 1999.
23. Modelska K, Pittet J-F, Folkesson HG, BROADDUS VC, and Matthay MA. Acid-induced lung injury: protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits. Am J Respir Crit Care Med 160:1450-1456, 1999.
24. Marchi E, Liu W, BROADDUS VC. Mesothelial cell apoptosis is confirmed in vivo by morphologic change in cytokeratin distribution. Am J Physiol Lung Cell Mol Physiol 278: L528-L535, 2000.
25. Levresse V, Renier A, Levy F, BROADDUS VC, Jaurand M-C. DNA breakage in asbestos-treated normal and transformed (TSV40) rat pleural mesothelial cells. Mutagenesis 15(3): 239-244, 2000.
26. Liu W, Ernst JD, BROADDUS VC. Phagocytosis of crocidolite asbestos induces oxidative stress, DNA damage and apoptosis in mesothelial cells. Am J Respir Cell Mol Biol 23(3): 371-378, 2000.
27. Wu J, Liu W, Koenig K, Idell SI, BROADDUS VC. Vitronectin adsorption to chrysotile asbestos increases phagocytosis and toxicity for mesothelial cells. Am J Physiol Lung Cell Mol Physiol 279:L916-L923, 2000.
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- (* equal contributors as senior author)
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46. Xiang X, Phung Y, Feng M, Nagashima K, Zhang J, BROADDUS VC, Hassan R, FitzGerald D, Ho M. The development and characterization of a human mesothelioma in vitro 3D model to investigate immunotoxin therapy. PLoS ONE 6(1): e14640, 2011.
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NON-PEER REVIEWED PUBLICATIONS AND OTHER CREATIVE ACTIVITIES:

EDITORIALS

1. BROADDUS VC, Light RW. What is the origin of pleural transudates and exudates? Chest 102:658-659, 1992.
2. BROADDUS VC. Asbestos, the mesothelial cell and malignancy: a matter of life or death. Am J Respir Cell Mol Biol 17:657-659, 1997.
3. BROADDUS VC. Apoptosis and asbestos-induced disease – is there a connection? J Lab Clin Med 137(5):314-5, 2001.
4. BROADDUS VC. Diuresis and transudative effusions-changing the rules of the game. Am J Med 110(9):732-5, 2001.

WORKSHOP OR MEETING SUMMARIES

1. Crapo JD, BROADDUS VC, Brody AR, Malindzak G, Samet J, Wright JR; American Thoracic Society. ATS-NIEHS Workshop on lung disease and the environment; Where do we go from here? Am J Respir Crit Care Med 168(2):250-4, 2003.
2. BROADDUS VC. Advances in Mesothelioma Research. Report of the IMIG meeting. International Pleural Newsletter 2 (3): 10-11, 2004.
3. Carbone M, Albelda SM, BROADDUS VC, Flores RM, Hillerdal G, Jaurand, M-C, Kjaerheim K, Pass HI, Robinson B, Tsao A. Meeting Review: 8th International Mesothelioma Interest Group Oncogene 26 (49): 6959-6967, 2007.
4. Boylan A, Broaddus VC. Pleural Disease. In: Respiratory Disease in America: An ATS Fact Book. Schraufnagel, DE Editor 2010.
5. BROADDUS VC, Everitt JI, Black B, Kane AB. Non-neoplastic and neoplastic pleural endpoints following fiber exposure. J Toxicol Environ Health (Critical Reviews Part B): 14:153-178, 2011.

REVIEWS

1. Staub NC, BROADDUS VC, Zylak C, Lai-Fook SJ, Light RW, Vinegar R, Gaensler EA, Sahn SA. Pathophysiology of the pleural space. Am Rev Respir Dis 134:820-821, 1986.
2. BROADDUS VC, Berthiaume Y, Biondi JW, Matthay MA. Hemodynamic management of the adult respiratory distress syndrome. J Intensive Care Med 2:190-213, 1987.
3. BROADDUS C and Staub NC. Pleural liquid & protein turnover in health & disease. Sem in Respir Med 9:7-12, 1987.
4. Wiener-Kronish JP, BROADDUS VC. Interrelationship of pleural and pulmonary interstitial liquid. Ann Rev Physiol 55:209-226, 1993.
5. Matthay MA, BROADDUS VC. Fluid and hemodynamic management in acute lung injury. Sem in Respir Med 15:271-288, 1994.
6. BROADDUS, V.C. Infections in the pleural space: An update on pathogenesis and management. Sem in Respir Crit Care Med 16:303-314, 1995.
7. Marchi E, BROADDUS VC. Mechanisms of pleural liquid formation in pleural inflammation. Curr Opinion in Pulmonary Med 3:305-309, 1997.
8. Nishimura SL, BROADDUS VC. Asbestos-induced pleural disease. Clinics in Chest Medicine 19 (2): 311-329, 1998.
9. Leard LE, BROADDUS VC. Mesothelial cell proliferation and apoptosis. Respirology 9: 292-299, 2004.
10. Mutti L, BROADDUS VC. Malignant mesothelioma as both a challenge and an opportunity. Oncogene 23:9155-9161, 2004.

CHAPTERS

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2. BROADDUS VC, Wiener-Kronish JP. Pleural diseases. In: Annual Review of Pulmonary and Critical Care Medicine, 1987-1988. Philadelphia: Hanley & Belfus, 1987, 197-219.
3. Wiener-Kronish JP, BROADDUS VC. Pleural diseases. In: Annual Review of Pulmonary and Critical Care Medicine, 1988-1989. Philadelphia: Hanley & Belfus, 1989, 85-102.
4. Wiener-Kronish JP, Boylan AM, BROADDUS VC. Pleural diseases. In: Annual Review of Pulmonary and Critical Care Medicine, 1991-1992. Philadelphia: Hanley & Belfus, 1991, 93-106.
5. BROADDUS VC. Cardiac diseases. In: Pulmonary Manifestations of Systemic Disease. JF Murray, ed. New York: Marcel Dekker, Inc., 1991; 59:149-190.
6. Light RW, BROADDUS VC. Disorders of the pleura: general principles and diagnostic approach. In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, eds. 2nd edition. Philadelphia: WB Saunders Co., 1994, 2145-2163.
7. BROADDUS VC. Mechanisms of Pleural Liquid Turnover in the Normal State. UptoDate in Pulmonary and Critical Care Medicine (CDROM), S.E. Weinberger, Editor, American Thoracic Society, 1996-2004.
8. BROADDUS VC. Mechanisms of Pleural Liquid Accumulation in Disease. UptoDate in Pulmonary and Critical Care Medicine (CDROM), S.E. Weinberger, Editor, American Thoracic Society, 1996-2004.
9. BROADDUS VC, Hébert, CA. The Role of IL-8 in Inflammatory Diseases. In: Chemoattractant Ligands and Their Receptors. R. Horuk, Editor. CRC Press: New York. 1996, pp. 1-28.
10. Bigelow JM, BROADDUS VC. Empyema and Lung Abscess. In: Pulmonary/Respiratory Therapy Secrets. P.E. Parsons, J.E. Heffner, Editors. Hanley & Belfus, Inc. Medical Publishers, Phila, PA. 1996, pp. 179-185.
11. BROADDUS VC, Hébert CA. Neutralization of IL-8 in *in Vivo* models of lung and pleural injury. In: Methods in Enzymology. Chemokines and Chemokine Receptors. R. Horuk, Editor. Academic Press. Orlando, FL. 1997; 288: 161-181.
12. BROADDUS VC, Light RW. Disorders of the Pleura: General Principles and Diagnostic Approach (Chapter 73). In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, RJ Mason, HA Boushey, eds. 3rd edition. Philadelphia: WB Saunders Co., 2000; pp. 1995-2012.
13. Light RW, BROADDUS VC. Pleural Effusion. (Chapter 74) In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, RJ Mason, HA Boushey, eds. 3rd edition. Philadelphia: WB Saunders Co., 2000; pp. 2013-2041.
14. Light RW, BROADDUS VC. Pneumothorax, Chylothorax, Hemothorax and Fibrothorax. (Chapter 75) In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, RJ Mason, HA Boushey, eds. 3rd edition. Philadelphia: WB Saunders Co., 2000; pp. 2043-2066.

15. Light RW, BROADDUS VC. Tumors of the Pleura. (Chapter 76) In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, RJ Mason, HA Boushey, eds. 3rd edition. Philadelphia: WB Saunders Co., 2000; pp. 2067-2078.
16. BROADDUS VC, Jaurand MC. Asbestos Fibers and the Biology of Mesothelial Cells. In: Mesothelioma. P Chahinian, BWS Robinson, ed. Gordon & Breach Science Publishers, Harwood Academic Publishers 2004.
17. Bigelow JM, BROADDUS VC. Empyema and Lung Abscess. In: Pulmonary/Respiratory Therapy Secrets. 2nd edition. P.E. Parsons, J.E. Heffner, Editors. Hanley & Belfus, Inc. Medical Publishers, Phila, PA. 2001.
18. BROADDUS VC. Transudative pleural effusions. In: Pleural Diseases. Loddenkemper R, Antony V, Editors. European Respiratory Society, Sheffield, UK. Eur Respir Monograph 2002: 22, pp. 157-176.
19. Nahid P, BROADDUS VC. Liquid and Protein Exchange. (Chapter 3) In: Pleural Diseases: an International Textbook. Light RW and Lee G.Y.C. Editors Hodder Arnold, London, England 2003; pp. 35-44.
20. BROADDUS VC. Physiology. (Chapter 6) and Transudates (Chapter 27). In: Derrame Pleural (Translation: Pleural Effusion). Vargas FS, Teixeira LR, and Marchi E, Editors. Roca Publishers, Brazil, 2003; pp. 15-24 and 233-248.
21. BROADDUS VC, Light RW. Pleural effusion (Chapter 68). In: Textbook of Respiratory Disease. RJ Mason, VC Broaddus, JF Murray, JA Nadel, eds. 4th edition. Philadelphia: Elsevier 2005; pp.1913-1960.
22. Boylan AM, BROADDUS VC. Tumors of the pleura (Chapter 70). In: Textbook of Respiratory Disease. RJ Mason, VC Broaddus, JF Murray, JA Nadel, eds. 4th edition. Philadelphia: Elsevier 2005; pp. 1989-2009.
23. BROADDUS VC. Fluid and solute exchange in normal physiological states. In: Textbook of Pleural Diseases, 2nd Edition. Light RW, Lee YGC (eds) London: Hodder, Arnold, 2008:43-48.
24. BROADDUS VC, Light RW. Pleural effusion. In: Murray & Nadel's Textbook of Respiratory Medicine. RJ Mason, VC Broaddus, Martin TR, King TE, Schraufnagel DE, JF Murray, JA Nadel, eds. 5th edition. Philadelphia: Elsevier, 2010: 1719-1763.
25. BROADDUS VC, Robinson BWS. Tumors of the pleura. In: Murray & Nadel's Textbook of Respiratory Medicine. RJ Mason, VC Broaddus, Martin TR, King TE, Schraufnagel DE, JF Murray, JA Nadel, eds. 5th edition. Philadelphia: Elsevier, 2010: 1792-1813.

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 the 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, we are now exploring the mitochondria as a central integrator of apoptotic signaling.

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
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I. EDUCATION:

1976 - 1980	San Francisco State University	B.A.	Biology
1988 - 1993	University of California, Los Angeles	Ph.D.	Biological Chemistry
1993 - 1997	University of California, San Francisco	Post-Doctoral Fellow	Cancer Biology

II. PRINCIPAL POSITIONS HELD:

1981 - 1988	Genentech, Inc., South San Francisco	Research Associate	Molec. & Devel. Biology
1997 - 1999	Univ. of California, San Francisco	Assistant Research Biochemist	Hormone Research Inst.
1999 - 2004	Univ. of California, San Francisco	Assistant Professor, In Residence	Cancer Research Inst. & Dept of Pathology
2004 - 2006	Univ. of California, San Francisco	Associate Professor, In Residence	Cancer Research Inst. & Dept. of Pathology
2006 - 2007	Univ. of California, San Francisco	Associate Professor	Dept. of Pathology & Cancer Research Inst.
2007 - present	Univ. of California, San Francisco	Professor	Dept. of Pathology & Cancer Research Inst.

OTHER POSITIONS HELD CONCURRENTLY:

1989 - 1992	Whittier College, Whittier, CA	Lecturer	Biology Dept
1992	Genentech, Inc., South San Francisco	Scientific Consultant	Dept. of Legal Affairs
2000-present	Helen Diller Family Comprehensive Cancer Center, UCSF	Co-Director	Mouse Pathology Core
2007-2009	<i>CANCER RESEARCH</i>	Senior and Deputy Editor	<i>Tumor Microenvironment Section</i>
2009-present	Helen Diller Family Comprehensive Cancer Center, UCSF	Co-Leader	<i>Program in Cancer Immunity & Microenvironment</i>
2009-present	<i>CANCER RESEARCH</i>	Deputy Editor	<i>Breaking Advances</i>

III. HONORS AND AWARDS:

1985	Recognition Award	Genentech, Inc.,
1986	Recognition Award	Genentech, Inc.,
1988	Recognition Award	Genentech, Inc.
2000 - 02	Hellman Family Award For Early Career Faculty	Univ. of Calif., San Francisco
2000 - 01	V Foundation Scholar	The V Foundation for Cancer Research
2000 - 03	Edward Mallinckrodt, Jr. Fndt. Award for Medical Research	Edward Mallinckrodt, Jr. Fndt.
2002	Gertrude B. Elion Cancer Research Award	Am. Assoc. for Cancer Research
2006 - 11	Era of Hope Scholar Award	Dept. of Defense, Breast Cancer Research Program
2011 - 16	Era of Hope Scholar Expansion Award	Dept. of Defense, Breast Cancer Research Program
2011 - 16	KOMEN Promise Award	Susan G Komen For the Cure Foundation

IV. PROFESSIONAL ACTIVITIES

1999 - present	Member	Graduate Program in BioMedical Sciences (BMS)	Univ. of Calif., San Francisco
1999 - present	Member	Helen Diller Family Comprehensive Cancer Center	Univ. of Calif., San Francisco
2000 - present	Member	Graduate Program in Biological Sciences (PIBS)	Univ. of Calif., San Francisco
2001 - present	Co-Director	Mouse Pathology Core	Helen Diller Family Comprehensive Cancer Center, Univ. of Calif., San Francisco
2004 – present	Member	Program in Immunology	Univ. of Calif., San Francisco
2004 – 2007	Senior Editor	Cancer Research (<i>Cell, Tumor and Stem Cell Biology</i> Section)	American Association Cancer Research
2007 – 2009	Deputy Editor	Cancer Research (<i>general</i>)	American Association Cancer Research
2007 – 2009	Senior Editor	Cancer Research (<i>Tumor Microenvironment</i> Section)	American Association Cancer Research
2007 - 2010	Member	External Scientific Advisory Board, University of Minnesota Cancer Center	University of Minnesota Cancer Center
2007 – 2011	Member	External Scientific Advisory Board, CA U54: <i>Aging, Tumor Microenvironment and Prostate Cancer</i> , P.I. Steve Plymate, Univ. of Washington, HMC.	University of Washington
2008 – 2011	Member	Board of Directors (<i>elected</i>)	American Association of Cancer Research
2009 - present	Member	External Scientific Advisory Board; Neuroblastoma Program Project Grant	Children's Hospital Los Angeles, Univ. of Southern California
2009 – 2011	Deputy Editor	Cancer Research (<i>Breaking Advances section</i>)	American Association Cancer Research
2009 - present	Co-Director	Program in <i>Cancer Immunity and Microenvironment</i>	Helen Diller Family Comprehensive Cancer Center, Univ. of Calif., San Francisco
2010-2012	Council Member	<i>Women in Cancer Research</i> , Council (<i>elected</i>)	American Association of Cancer Research
2009 - present	Member	External Advisory Committee, P01, Program in <i>Motility and Invasion</i> , John Condeelis, Ph.D., PI, Program Director	Albert Einstein College of Medicine
2011	Member	Scientific Review Board	Starr Cancer Consortium

Local, National and International Meetings Organized:

- 2005 Keystone Symposia, *Inflammation and Cancer*, Co-organizer with Dr. Ray DuBois, Vanderbilt Univ, TN), Breckenridge, CO, USA
- 2006 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
- 2006 Co-Organizer (with Dr. Lewis Lanier), UCSF HDFCCC Annual Symposium, '*Inflammation & Cancer: Bench to Bedside*'.
- 2007 Keystone Symposia, *Inflammation and Cancer*, Co-Organizer with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA); Santa Fe, New Mexico, USA
- 2008 AACR Special Conference: *Inflammation and Cancer*, Co-organizer with Drs. Michael Karin and

- Larry Marnett. Oahu, Hawaii, USA.
- 2008 International Society for Biological Therapy of Cancer (ISBTc), *2008 Workshop on Inflammation in Cancer Development*, Co-Organizer with Drs. Michael Karin, (UCSD), Steven Dubinett (UCLA), and George Weiner (WU); San Diego CA USA
- 2010 Co-Organizer (with Dr. Lewis Lanier), UCSF HDFCCC Program in *Cancer Immunity and Microenvironment* Symposium
- 2011 AACR *Special Conference: Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy*, Co-Organizer with Drs. Yves DeClerck (USC, Children's Hospital) and Melody Swartz (EPFL)

V. PROFESSIONAL ORGANIZATIONS

Memberships

- | | |
|----------------|----------------------------------------------|
| 2000 - 2009 | American Society for Matrix Biology |
| 2000 – present | American Association for Cancer Research |
| 2001 – 2008 | American Society for Cell Biology |
| 2004 – present | American Society for Investigative Pathology |
| 2004 - 2009 | International Protease Society |

Service to Professional Organizations

American Association for Cancer Research

- | | |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2003 | Subsection Co-chair (Tumor Progression, Invasion and Metastasis) Cellular, Molecular and Tumor Biology Subcommittee, AACR Program Committee for <i>94th Annual Meeting</i> . |
| 2003 | Chair and organizer, Educational Session (Proteases: Successes and Failures): <i>94th Annual Meeting</i> , Washington D.C., USA |
| 2003 | Minisymposium Co-chair (Inflammatory Mediators & Cancer): <i>94th Annual Meeting</i> , Washington D.C., USA |
| 2004 - 2006 | Member, Grants Committee |
| 2005 | Minisymposium Co-Chair (Inflammation, Microenvironment and Tumor Progression): <i>96th Annual Meeting</i> , Anaheim, CA USA |
| 2005 | Session Chair (Inflammation): <i>AACR Special Conference: Cancer, Proteases and the Microenvironment</i> , Bonita Springs, Florida. USA |
| 2006 | Subsection Co-chair (Tumor Progression, Invasion and Metastasis) of the Tumor Biology Subcommittee, AACR Program Committee for <i>97th Annual meeting</i> |
| 2006 | Minisymposium Co-Chair (Inflammation and Cancer): <i>97th Annual Meeting</i> , Washington DC, USA |
| 2006 | Co-Chairperson, Program Committee: <i>6th Annual Frontiers in Cancer Prevention Research Conference</i> , December 5-8, 2007, Philadelphia, PA USA. |
| 2006 - 2010 | Steering Committee Member: AACR Tumor Microenvironment Working Group (TME/AACR). |
| 2007 | Organizer, Education session (Inflammation and Cancer), <i>98th Annual Meeting</i> , Los Angeles, USA |
| 2007 | Minisymposium Co-Chair (Tumor Microenvironment): <i>98th Annual Meeting</i> , Los Angeles, CA USA |
| 2007 | Co-Chairperson, Program Committee: <i>2008 99th Annual Meeting of the AACR</i> . April 12-16, 2008, San Diego, CA. USA |
| 2008 | Program Committee Member, Tumor Microenvironment Subcommittee for <i>99th Annual Meeting of the AACR</i> . April 12-16, 2008, San Diego, CA. USA |
| 2007 - 2010 | Member, AACR Special Conferences Committee |
| 2008 | Co-Organizer Special Conference: <i>Inflammation and Cancer</i> , with Drs. Michael Karin and Larry Marnett. Oahu, Hawaii, USA. |
| 2008 - 2011 | Member, Board of Directors (elected) |
| 2009 | Member, 2009 Education Committee, 2009 100 th AACR Annual Meeting, Denver, CO. USA |
| 2009 | Organizer and Chair: <i>Inflammation and Cancer: Novel Mechanisms Regulating Protumor Immunity</i> Major Symposium, 2009 100 th AACR Annual Meeting, Denver, CO. USA |
| 2009 | Organizer and Chair: Education Session, <i>Aspects of the Tumor Microenvironment that Regulate Solid Tumor Development</i> , 2009 100 th 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 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 - 2013 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 102st 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, 2010, 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.
- 2002 Poster Judge (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 - present Editorial Board, ***Cancer Microenvironment***
- 2009 - 2011 Deputy Editor for Breaking Advances, ***Cancer Research***

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
 2006 Nature Reviews Cancer; Nature; Nature Medicine; Cell; Cancer Research; Clinical Cancer Research; J Exp Med; Cancer Cell: Am J Pathology; J Cell Biology
 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
 2010 Nature, J Exp Med, Nature Medicine, J Invest Dermatology, Cell, Cancer Cell, J Clinical Onc; PNAS; J Clin Invest; Cancer Research; Dis Mech Models; Cancer Immuno Immunother;
 2011 Nature; Nature Medicine; Cancer Research; Cancer Cell; J Clin Invest; Breast Cancer Research; PlosOne, PNAS;

VI. INVITED PRESENTATIONS**Symposia and Workshops: International**

- 1996 *Human Tumor Heterogeneity II: Cytometric Measurement of Growth Regulation and Genetic Alterations: International Society of Analytical Cytometry.* Kananaskas, Alberta, Canada.
 1997 *GeneMedicine-Boehringer Mannheim Cancer Alliance: Technology Workshop.* Cancún Mexico.
 2001 *2nd Annual International Protease Society.* Freising, Germany.
 2002 *6th International Symposium on Predictive Oncology & Intervention Strategies,* Pasteur Institute, Paris, France
 2002 **KEYNOTE LECTURE,** *Dutch Cancer Society Annual Symposium,* Luntern, The Netherlands
 2002 **KEYNOTE LECTURE,** *Cancer: Genome, Signal & Environment, Takeda Genome Urology International,* Kyoto, Japan
 2003 *2nd Annual International Symposium on Epithelial Biology,* Timberline, Oregon USA
 2004 *10th International Congress of the Metastasis Research Society, 'Progress Against Tumor Progression',* Genoa Italy
 2005 *2005 International Consortium Meeting of the Children's Tumor Foundation: Molecular Biology of NF1, NF2 and Schwannomatosis,* Aspen, CO, USA
 2005 *International Symposium on Systems Genome Medicine - Bench to Bedside,* Institute of Medical Sciences University of Tokyo, Tokyo, Japan
 2005 *Immunotherapy of Cancer, XI Annual Symposium of the Danish Cancer Society,* Copenhagen, Denmark
 2005 *4th General Meeting of the International Proteolysis Society,* Quebec City, Canada
 2006 *Centro Nacional de Investigaciones Oncológicas (CNIO) Cancer Conference: Inflammation and Cancer,* Madrid SPAIN
 2006 *18th Annual Pezcoller Symposium 'Tumor Microenvironment: Heterotypic Interactions',* Trento ITALY
 2006 *European Association for Cancer Research (EACR) 1st Annual Meeting,* Budapest HUNGARY
 2006 *XXXIVth Meeting of the International Society for Oncodevelopmental Biology and Medicine (ISOBM): Tumor Biology, Detection and Therapy,* Pasadena, CA, USA

- 2006 37th International Symposium of the Princess Takamatsu Cancer Research Fund '*Cancer Cells and Their Microenvironment*', Tokyo, JAPAN
- 2007 4th International Conference on Tumor Microenvironment, Florence, ITALY
- 2007 2nd International Symposium on Cancer Metastasis and the Lymphovascular System: Basis for Rational Therapy, San Francisco CA USA
- 2007 CNIO – Nature Symposium on "Oncogenes and Human Cancer". The Next 25 Years", Madrid SPAIN
- 2007 **KEYNOTE LECTURE**, 7th International Symposium on Hodgkin Lymphoma, Cologne, GERMANY
- 2007 **CANDLELIGHT LECTURE**, *Inflammation and Cancer: From molecular links to bed side*; Inaugural meeting for the *Istituto Clinico Humanitas*, Milan ITALY
- 2008 7th Annual International Congress on the Future of Breast Cancer, Kauai, Hawaii USA
- 2008 Cancer Research UK Cambridge Research Institute (CRI) Inaugural Annual Symposium, '*Unanswered Questions in the Tumour Microenvironment*', Homerton College, Cambridge UK
- 2008 5th International Kloster Seeon Meeting, *Angiogenesis: Molecular Mechanisms and Functional Interactions*. Kloster Seeon, GERMANY
- 2008 **CANCER RESEARCH UK LECTURE**, NCRI Cancer Conference, Birmingham UNITED KINGDOM
- 2009 21ST Lorne Cancer Conference, Lorne AUSTRALIA
- 2009 6th International Symposium on the Intraductal Approach to Breast Cancer, Santa Monica CA USA
- 2009 **STATE-OF-THE-ART LECTURE**, International Cancer Conference, *CANCER 2009*, Dublin IRELAND
- 2009 19th Annual BioCity Symposium, 'Tumor Microenvironment in Cancer Progression', Tirku FINLAND
- 2009 **KEYNOTE LECTURE**, European Association of Cancer Research, Special Conference on *Inflammation and Cancer*, Berlin GERMANY
- 2009 7th International Symposium on Minimal Residual Cancer, Athens, GREECE
- 2009 Tri-Society Annual Conference of the Society for Leukocyte Biology, International Cytokine Society, and the International Society for Interferon and Cytokine Research, Lisbon, Portugal
- 2009 5th International Conference on Tumor Microenvironment, Versailles, FRANCE
- 2009 **PRESIDENT'S PLENARY LECTURE**: Italian Cancer Society Annual Meeting, Milano ITALY
- 2010 **PLENARY LECTURE**, CHUV Research Day, University hospital (CHUV) and the Faculty of Biology and 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

UPCOMING INVITATIONS

- 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 8th Ipsen Foundation meeting on *Mouse Models of Human Cancers*, Ouro Preto, BRAZIL
- 2012 Keystone Symposium on *The Role of Inflammation During Carcinogenesis*, Dublin IRELAND
- 2012 25th International IGB Workshop, CNR, Capri Island, Naples, ITALY

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 *Pathobiochemistry 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.
- 2004 19th Aspen Cancer Conference: *Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy*. Aspen, CO, 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, *Mouse Models of Cancer*, Cambridge, MA, USA
- 2006 AACR Special Conference, *Tumor Immunology: An Integrated Perspective*. Miami, FL, USA
- 2007 7th AACR-Japanese Cancer Association Joint Conference: *In the Forefront of Basic and Translational Cancer Research*, Waikoloa, Hawaii, USA
- 2007 Keystone Symposium, *'Mouse Models at the Frontiers of Cancer Discovery'*, Whistler, British Columbia, CANADA
- 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 American Thoracic Society 2007 International Conference, *San Francisco Science: Inflammation, Immunity and Signaling*. San Francisco, 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 AACR, *Frontiers in Cancer Prevention Research Conference*, Philadelphia, PA, 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 DOD BCRP Era of Hope Meeting 2008, Symposium Session: *Immune and Inflammatory Contributions to Breast Cancer*, AND Era of Hope Spotlight Session, Baltimore MD, 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 2009 Geoffrey Beane Cancer Research Symposium: *Inflammation and Cancer*, Memorial-Sloane Kettering Cancer Center, New York NY 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

UPCOMING INVITATIONS

- 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

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, UK
- 2004 Cancer Research UK, London Research Institute, Lincoln's Inn Fields Laboratories, London, UK
- 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 UK
- 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 GB

UPCOMING INVITATIONS

- 2012 Excellence in Genetics and Immunology Lecture Series, Complex Traits Group at McGill University, Montreal, Qc, Canada

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 McArdle 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

UPCOMING INVITATIONS

- 2011 Abbott Biotherapeutics, Redwood City CA USA
- 2012 University of Rochester, Department of Microbiology and Immunology, Rochester, NY, USA
- 2012 Brown Foundation Institute of Medicine, Univ. of Texas, Health Science Center at Houston, Houston TX USA
- 2012 Harvard Medical School's (HMS) Committee on Immunology Seminar Series
- 2012 Massachusetts General Hospital's (MGH) Seminar Series
- 2012 **VISITING PROFESSOR**, Frontiers in Oncology Program, University of Maryland Marlene and Stewart Greenebaum Cancer Center, Univ. of Maryland, Baltimore MD USA

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
- 2009 Helen Diller Family Comprehensive Cancer Center *Research Symposium*; UCSF
- 2010 Bay Area Workshop on Lung Development, Physiology and Cancer, San Francisco CA USA
- 2010 UCSF-GIVI Center for AIDS Research (CFAR) Scientific Symposium for 2010: *HIV Infection, Inflammation, and Premature Aging*, San Francisco, CA USA
- 2010 Breast Oncology Program Seminar, Helen Diller Family Comprehensive Cancer Center, UCSF
- 2011 Breast Oncology Program Annual Retreat, Helen Diller Family Comprehensive Cancer Center, UCSF

UPCOMING INVITATIONS

- 2011 Helen Diller Family Comprehensive Cancer Center Symposium, San Francisco CA, USA
 2012 BMS Faculty Lecture Series, UCSF

VII. GOVERNMENT AND OTHER PROFESSIONAL SERVICE:**GOVERNMENT SERVICE**

- | | | |
|----------------|--------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2003 -
2006 | National Institutes of Health, Center for Scientific Review | Ad hoc reviewer (10/2003; 02/2005; 10/2005; 06/2006), Tumor Progression & Metastasis (TPM) Study Section, Oncological Sciences Review group |
| 2003 | Division of Cancer Biology, National Cancer Institute: <i>Microenvironment Think Tank</i> | Participant and <i>Reporter</i> |
| 2003 | Division Cancer Etiology, National Cancer Institute: <i>Validation of A Causal Relationship: Criteria to Establish Etiology Think Tank</i> | 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) |
| 2010 -
2011 | Department of Defense (DOD), Breast Cancer Research Program (BCRP) | 6 th Era of Hope conference Technical Planning Committee (TPC) |
| 2011 | National Institutes of Health, Center for Scientific Review | 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

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|------|--------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| 1999 | Arkansas Science & Technology Authority | Ad hoc Grant Review |
| 2000 | McGraw-Hill, ' <i>Biology</i> ' 6 th edition, Ed. P.H. Raven and G.B. Johnson | Ad hoc Review, Chapters 17 and 18 |
| 2000 | Division of Cancer Biology, NCI: <i>Epithelial-Stromal Interactions & Tumor Progression Workshop</i> | 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 | ' <i>H. pylori-induced Inflammation and Gastric Adenocarcinoma</i> , PO1 External Advisory Panel |
| 2004 | Cancer Research Ireland, Irish Cancer Society | Ad hoc grant review |
| 2004 | Dutch Cancer Society | Ad hoc grant review |
| 2004 | Vanderbilt University, Nashville TN, USA; | Ad hoc reviewer for SPORE Developmental |

	SPORE in GI Cancer	Research Program
2005	Keystone Symposia, <i>Inflammation and Cancer</i>	Co-organizer (with Dr. Ray DuBois, Vanderbilt Univ, TN), Breckinridge, CO, USA
2006	5 th Annual Timberline Symp. on Epithelial Cell Biology, <i>'Intrinsic and Microenvironmental Regulation of Epithelial Cancer'</i>	Co-Organizer (with Dr. Harold Moses, Vanderbilt University, TN, USA), Timberline, OR, USA
2006	Keystone Symposia Cancer Study Group for 2009 programming	Study group member
2007	Keystone Symposia, <i>Inflammation and Cancer</i>	Organizer (with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA) Santa Fe, New Mexico, USA
2008	AACR Special Conference on <i>'Inflammation and Cancer'</i>	Co-Organizer (with Drs. Michael Karin and Larry Marnett)
2007 - 2010	University of Minnesota Cancer Center; Douglas Yee, M.D., Director	External Scientific Advisory Board Member
2007 - 2011	University of Washington, Seattle WA, USA	Member, External Scientific Advisory Board, CA U54 TMEN: <i>Significance of Microenvironment for Prostate Cancer Initiation and Progression</i> ; P.I. Stephen R Plymate, Univ. of Washington School of Medicine.
2007 - 2011	Albert Einstein College of Medicine of Yeshiva University, New York, NY USA	Member, External Scientific Advisory Board, CA U54 TMEN: <i>Novel Methods for Detection Cell Interactions in the Tumor Microenvironment</i> ; P.I. John S. Condeelis, Albert Einstein College of Medicine.
2008	International Society for Biological Therapy of Cancer (iSBTc), <i>2008 Workshop on Inflammation in Cancer Development</i>	Co-Organizer (with Drs. Michael Karin, Steven Dubinett, George Weiner)
2009	GlaxoSmith Kline	Member, Tykerb Post-ASCO KOL Advisory Board
2009	University of Southern California, Children's Hospital	Member, External Scientific Advisory Board, Neuroblastoma, Program Project grant (P01), PI: Robert Seeger, M.D.,
2009 - 2010	Cancer Prevention and Research Institute of Texas (CPRIT)	Member, Scientific Review Committee; Basic Cancer Biology Review Committee
2011	Starr Cancer Consortium Scientific Review Board	Member, Scientific Review Committee
2011	AACR Special Conference on <i>'Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy'</i>	Co-Organizer (with Drs. Yves DeClerck and Melodie Shwartz)

VIII. UNIVERSITY AND PUBLIC SERVICE

UNIVERSITY SERVICE

System wide

1992-1993	Graduate Student Representative, Dept. of Biological Chemistry Faculty Council, UCLA
2004	<i>ad hoc</i> 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 <i>Inflammation and Disease</i> . 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: open.
2009	Member, Committee to choose 1 st Bonnie J. and Anthony Addario Endowed Chair in Thoracic Oncology, School of Medicine, UCSF
2010	Member, 2010 Selection Committee for the Hellman Family Early-Career Faculty Award.

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, <i>'Inflammation & Cancer: Bench to Bedside'</i> .
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
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University of California, San Francisco, Department of Pathology

2003	Member, Committee to recommend faculty for the <i>Robert E. Smith Endowed Chair in Experimental Pathology</i>
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-present 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
- 1991 Organizer and 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
- 2002 Photo credits and interviewed for '*Misdiagnosis: Failure of Promising Cancer Treatment Starts Soul Searching by Researchers & Drug Companies*', in: *San Francisco Chronicle*, May 12, 2002.
- 2003 Interviewed for article '*Body's First Defense May Be Root of Diseases*', in: *The Washington Post*, February 20, 2003
- 2003 Interviewed for article '*The Body on Fire*', in: U.S. News & World Report, October 20, 2003
- 2004 Interviewed for comments in: *Science News*, '*Early Warming: Inflammatory protein tied to colon cancer risk*' February 7, 2004, Vol 165.
- 2004 Interviewed for article '*The Fires Within*', in: *TIME Magazine*, February 23, 2004
- 2004 Interviewed for comments on AACR Annual Meeting in: *Oncology Times*, '*Exercise Reduces Inflammatory Response, May also Reduce Cancer Risk*', Robert H Carlson, 26(11):33-34, June 10, 2004
- 2004 Interviewed for article '*Inflammation and Cancer: The Link Grows Stronger*', in: *Science*, 306, 966-968 (2004)
- 2005 Interviewed for article '*Quieting a Body's Defenses*', in: *Newsweek*, Special Edition, Summer 2005
- 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 Event for Journalists, September 27, 2007, UCSF Mission Bay Campus
- 2007 On-Air radio interview by Dave Iversen, KQED *FORUM*, September 28, 2007 San Francisco CA USA

IX. TEACHING AND MENTORING

Formal Scheduled Classes for UCSF Students:

Qtr	Academic Yr	Course No. & Title	Teaching Contribution	Units	Class Size
W	1997/98	IDS 100; Histology Laboratory	<i>Neoplastic Skin Histopathology</i> ; Laboratory lecture & instruction	10	150
W	1998/99	IDS 100; Histology Laboratory	<i>Neoplastic Skin Histopathology</i> ; Laboratory lecture & instruction	10	150
W	1999/00	IDS 100; Histology	<i>Neoplastic Skin Histopathology</i> ; Laboratory	10	150

		Laboratory	lecture & instruction		
S	1999/00	BMS 297A; Molecular Biology & Pathology of Neoplasia	<i>Animal Models of Cancer Laboratory</i> ; Laboratory lecture & instruction	3	15
S	2000/01	BMS 297A; Molecular Biology & Pathology of Neoplasia	<i>Animal Models of Cancer Laboratory</i> ; Laboratory lecture & instruction	3	15
W	2000/01	BMS 225; Tissue and Organ Biology	Lecture and laboratory instruction	3	15
S	2000/01	BMS 260; Cell Biology	Discussion group leader	1	6
F/W	2001/02	IDS 101; Prologue	Laboratory Instructor	9	30
W	2001/02	BMS 225; Tissue and Organ Biology	Lecture and laboratory instruction	3	15
W	2001/02	IDS 103; Cancer Block	<i>Invasion & Metastasis</i> ; Lecturer	7	150
S	2001/02	BMS 260; Cell Biology	Discussion group leader	1	7
F	2002/03	BMS 260; Cell Biology	Discussion group leader	1	6
W	2002/03	IDS 103; Cancer Block	<i>Invasion & Metastasis</i> ; Lecturer	7	150
F/W	2002/03	IDS 101; Prologue	Laboratory Instructor	9	30
F	2003/04	BMS 260; Cell Biology	Discussion group leader	1	6
S	2003/04	BMS 225B, Tissue and Organ Biology	Lecturer and Laboratory Instructor	1.5 - 5	tbd
W	2003/04	Biochem 297; Molecular Biology & Pathology of Neoplasia	<i>Angiogenesis</i> : Lecturer	3	30
W	2003/04	BMS 297A Molecular Biology & Pathology of Neoplasia Laboratory	Lecturer and Laboratory Instructor, <i>Animal Models of Neoplasia</i>	1	10
S	2003/04	BMS 225B; Tissue & Organ Biology	Lecturer: Cancer I & Cancer II	1.5 - 5	16
F	2004/05	BMS 260; Cell Biology	Discussion group leader	1	6
F	2005/06	BMS 260; Cell Biology	Discussion group leader	1	7
W	2006/07	Biochem 297; Molecular Biology & Pathology of Neoplasia	<i>Inflammation and Cancer</i> : Lecturer	3	30
W	2008/09	BMS230; Cellular & Molecular Biology of Cancer	Course Co-Director	3.5	22
W	2008/09	BMS230; Cellular & Molecular Biology of Cancer	Lecturer: <i>Cancer Microenvironments; Inflammation and Cancer</i>	3.5	22
W	2010/11	BMS230; Cellular & Molecular Biology of Cancer	Course Co-Director	3.5	22
W	2010/11	BMS230; Cellular & Molecular Biology of Cancer	Lecturer: <i>Tumor cell heterogeneity; Cancer Microenvironments; Inflammation and Cancer</i>	3.5	22

Postgraduate and Other Courses:

1989	M204, <i>Biochemistry Lab</i> Univ. of Calif., Los Angeles	Student Teaching Assistant for quarter long course (100 medical students)
1989	Biology 250, <i>Human Heredity</i> , Dept. of Biology Whittier College, Whittier CA	Organized and taught entire lecture-based course (30 undergraduate students)
1990	Biology 350 & 350L, <i>Molecular</i>	Organized and taught entire lecture and laboratory course (16

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

High School and Undergraduate Students Supervised or Mentored:

Dates	Name	Program or School	Faculty Role	Current position
1998	Christopher Tinkle	Undergraduate, Univ. of Texas, Austin, TX, USA	Summer Research Training Program Supervisor	Ph.D. awarded 2008; M.D. awarded 2010; Resident, Rad/Onc, UCSF
2000	Adam Zucker	Undergraduate, Oberlin College, Ohio USA	Supervised Summer work	unknown
2000	Ashkan Hirari	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA, USA	Supervised Summer work	unknown
2001	Jason Reuter	Undergraduate, Univ. of Calif., Berkeley,	Supervised Summer work	unknown

		Berkeley CA USA		
2002	Destinee Cooper	Undergraduate, Univ. of Calif., Davis USA	Summer Research Training Supervisor	unknown
2006	Sunum Mobin	UCSF Science & Health Education Partnership: High School Intern Program	Summer Research Training Supervisor	unknown
2008-2009	Julia Lam	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA	Independent study (199), Mentor	B.S. awarded 2009
2010	Scott Keil	Undergraduate, The University of Glasgow, Scotland	Summer Research Training Supervisor	The University of Glasgow, Scotland
2010-2011	Heather Chen	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA	Summer Research Training Supervisor	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA
2010	Amy Desalazar	Cupertino High School Cupertino, CA USA	Summer Research Training Supervisor	Cupertino High School
2010-2011	Nikhil Wadhvani	Undergraduate, Sarah Lawrence College, Bronxville, NY USA	Summer Research Training Supervisor	Sarah Lawrence College, Bronxville, NY
2010	Sharfa Junaid	Undergraduate, Univ. of Calif., San Diego, San Diego CA USA	Summer Research Training Supervisor	Undergraduate, Univ. of Calif., San Diego, San Diego CA USA
2010-2011	Jon Lau	University of Nevada, Reno, Reno, Nevada USA	Summer Research Training Supervisor	University of Nevada, Reno, Reno, Nevada USA
2011	Kara Wang	Undergraduate, Pomona College, Claremont, CA USA	Summer Research Training Supervisor	Undergraduate, Pomona College, Claremont, CA USA
2011	Graham Litchman	Undergraduate, San Francisco State Univ, San Francisco CA USA	Summer Research Training Supervisor	Masters student, Univ. of Nevada, Reno
2011	Jessica Wignall	Smith College, Northampton, MA USA	Summer Research Training Supervisor	Smith College, Northampton, MA USA

Predocctoral Students Supervised or Mentored:

Dates	Name	Program or School	Faculty Role	Current position
2000	Jin-Sae Rhee	UCSF MSTP/BMS, graduate student	Rotation Supervisor	PhD awarded 2003, M.D. awarded 2005
2000 - 2003	Jin-Sae Rhee	UCSF M.D., Ph.D.,	Ph.D. supervisor	Pediatrician, Private Sector
2000	Maria Christophorou	UCSF BMS, graduate student	Faculty coach, BMS 297	Ph.D. awarded 2006
2001	Leslie Chu	UCSF BMS, graduate student	Rotation Supervisor	Ph.D. awarded 2005
2001	Rayna Takaki	UCSF BMS, graduate student	Rotation Supervisor	Ph.D. awarded 2006
2001 – 2002	Sophia Bruggerman	University of Nijmegen, The	Masters Thesis Supervisor	Ph.D. awarded 2007

		Netherlands		
2002	Lucy Lebedeva	UCSF PIBS, graduate student	Faculty coach, BMS 297	Ph.D. awarded 2005
2002	Leslie Chu	UCSF BMS, graduate student	Ph.D. Orals committee	Ph.D. awarded 2005
2002	Andre Whitkin	MSTP student, Cornell University USA	Supervised Summer work	unknown
2002	Karin deVisser	The Netherlands Cancer Institute, The Netherlands	Ph.D. Thesis Reading Committee	Ph.D. awarded 2002
2003	Cathy Collins	UCSF MSTP student	MSTP Advisor	PhD awarded 2009 M.D. awarded 2011
2004	Eric Tamm	University of British Columbia, Canada	Doctoral Dissertation External Examiner	Ph.D. awarded 2004
2004	Annie Hsieh	University of Södertörn, Sweden	Masters Thesis Supervisor	unknown
2005	Geoff Benton	UCSF TETRAD/PIBS, graduate student	Ph.D. Orals committee	Ph.D. awarded 2011
2006	Morgan Truitt	UCSF BMS, graduate student	Rotation Supervisor	UCSF BMS PhD graduate student
2006	Danielle Shin	UCSF MSTP student	Rotation Supervisor	Ph.D. awarded 2011
2006-2008	Celeste Rivera	SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student	M.S. research advisor	M.S. awarded 2010; Research Technician, EPFL Lausanne Switzerland
2007-2009	Leslie Vasquez	SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student	M.S. research advisor	M.S. awarded 2009; PharmD student, UCSF
2008	Ashley Martin	UCSF BMS, graduate student	Rotation Supervisor	UCSF BMS PhD graduate student
2009	Kay Wiebrands	Master's Student Utrecht University, the Netherlands	Masters Thesis Internship Supervisor	M.S. awarded 2009
2009-2010	David Tawfik	Medical Student III, UCSF	MSIII break year. Dean's Quarterly Research Fellowship; PACCTR Fall Quarter Fellowship;	M.D., awarded 2011; Resident in Internal Medicine, UCLA Harbor Hospital
2009-present	Renee Vanderlaan	UCSF BMS, graduate student	Chair: Thesis Committee	UCSF BMS graduate student, Lab of Matthias Hebrook, Ph.D.
2009-2011	A. Preethi Ganessan, M.D., PhD.	Ph.D. Graduate Student (Cancer Research UK, Univ of Southampton)	Ph.D. supervisor	Pediatric Hematology Resident, Univ of Southampton, UK

2010	Lucia Cottone	Visiting Ph.D. graduate student (9-mo) (San Raffaele Institute, Milan Italy)	Ph.D. supervisor	Ph.D. student, San Raffaele Institute, Milan Italy
2011 - present	Conny Hainer	Master's student, Technical University of Berlin, Berlin, Germany	MS supervisor	Master's student, Technical University of Berlin, Berlin, Germany
2011 - present	Melissa Wheeler	PharmD student, UCSF	PharmD research mentor	PharmD student, UCSF
2011 - present	Paul Huynh	PharmD student, UCSF	PharmD research mentor	PharmD student, UCSF

Postdoctoral Fellows and Residents Directly Supervised or Mentored

Dates	Name	Position & Funding	Faculty Role	Current Position
2000 - 2001	Ernst Lengyel, M.D., Ph.D.	Post-Doc Researcher, Senior Clinical Fellow	Research Supervisor	Assoc. Adj. Prof., Dept. Gyn. & Oncology, UCSF
2000 -2002	Leon Van Kempen, Ph.D.	Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship	Research Supervisor	Assoc. Prof., Univ. of Nijmegen, Dept. of Pathology, The Netherlands
2002 – 2005	Robert Diaz, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Scientist, Roche Pharmaceuticals
2002 – 2005	Karin deVisser, Ph.D.	Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship	Research Supervisor	Research Scientist, The Netherlands Cancer Inst, Amsterdam, The Netherlands
2003 – 2007	Alexandra Eichten, Ph.D.	Post-Doc Researcher, Serono Fndt for the Advancement of Medical Science (2003-2005);	Research Supervisor	Scientist, Regeneron Corp., New York USA
2003 - 2005	Stephen Robinson, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Private sector, United Kingdom
2003 - 2004	H. Jennifer Shen, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	unknown
2005 -2010	David DeNardo, Ph.D.	Post-Doc Researcher; 1) NGA: 5T32CA09043 PI: BISHOP; <i>Molec. Analysis of Tumor Viruses</i> ; 2) Am Cancer Society Fellowship 2007-2010	Research Supervisor	Assistant Professor, Molecular Oncology and Immunology, Washington University, St Louis, St Louis MS USA
2005 – 2007	Nor Eddine Sounni, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Research Scientist, Univ. of Liege, Belgium
2006 – 2007	Tingting Tan, M.D., Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Hem/Onc Fellow, Fox Chase Cancer Center, Phil., PA
2006 -2010	Magnus Johansson, Ph.D.	Post-Doc Researcher; Swedish Cancer Society Fellowship 2006-08	Research Supervisor	MBA candidate, Stanford Univ., Palo Alto CA
2006- present	Nessrine Affara, Ph.D.	Post-Doc Researcher; AACR-Astellas USA Fndt in Basic Cancer	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF

		Research 2009-2010		
2007 -2010	Pauline Andreu, Ph.D.	Post-Doc Researcher; Cancer Research Institute Postdoctoral Fellowship 2008-2011	Research Supervisor	Research Funding Agency in Private Sector, France
2008-present	Brian Ruffell, Ph.D.	Post-Doc Researcher; Dept of Defense Postdoctoral fellowship 2009-2012	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF
2009-2011	Stephen Shiao, M.D., Ph.D.	UCSF Radiation Oncology Resident	Research Supervisor	UCSF Radiation Oncology, Chief Resident
2010-present	Collin Blakeley, M.D., Ph.D.	UCSF Hematology-Oncology Fellow	Research Supervisor	UCSF Hematology-Oncology Fellowship
2010-present	Anna Wasiuk, Ph.D	Post-Doc Researcher; Coussens R01	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF

FACULTY MENTORING

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

Sabbatical Visitors:

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

SUMMARY OF TEACHING HOURS

Academic Year	Teaching/Mentoring Summary	Hours
1997/98	<u>Total hours of teaching /mentoring:</u> Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	<u>27</u> 2 1 24
1998/99	<u>Total hours of teaching /mentoring:</u> Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	<u>71</u> 2 1 68
1999/00	<u>Total hours of teaching /mentoring:</u> Formal class or course teaching hours: Informal teaching hours including prep time:	<u>108</u> 4 2

	Mentoring hours:	102
2000/01	<u>Total hours of teaching /mentoring:</u>	<u>130</u>
	Formal class or course teaching hours:	16
	Informal teaching hours including prep time:	9
	Mentoring hours:	105
2001/02	<u>Total hours of teaching /mentoring:</u>	<u>201</u>
	Formal class or course teaching hours:	18
	Informal teaching hours including prep time:	19
	Mentoring hours:	164
2002/03	<u>Total hours of teaching /mentoring:</u>	<u>314.5</u>
	Formal class or course teaching hours:	15.5
	Informal teaching hours including prep time:	17
	Mentoring hours:	282
2003/04	<u>Total hours of teaching /mentoring:</u>	<u>402</u>
	Formal class or course teaching hours:	20
	Informal teaching hours including prep time:	28
	Mentoring hours:	354
2004/05	<u>Total hours of teaching /mentoring:</u>	<u>395</u>
	Formal class or course teaching hours:	17
	Informal teaching hours including prep time:	28
	Mentoring hours:	350
2005/06	<u>Total hours of teaching /mentoring:</u>	<u>395</u>
	Formal class or course teaching hours:	17
	Informal teaching hours including prep time:	28
	Mentoring hours:	350
2006/2007	<u>Total hours of teaching /mentoring:</u>	<u>473</u>
	Formal class or course teaching hours:	45
	Informal teaching hours including prep time:	28
	Mentoring hours:	400
2008/2009	<u>Total hours of teaching /mentoring:</u>	<u>499</u>
	Formal class or course teaching hours:	51
	Informal teaching hours including prep time:	48
	Mentoring hours:	400
2009/2010	<u>Total hours of teaching /mentoring:</u>	<u>499</u>
	Formal class or course teaching hours:	51
	Informal teaching hours including prep time:	48
	Mentoring hours:	400
2010/2011	<u>Total hours of teaching /mentoring:</u>	<u>499</u>
	Formal class or course teaching hours:	51
	Informal teaching hours including prep time:	48
	Mentoring hours:	400
2011/2012 (future)	<u>Total hours of teaching /mentoring:</u>	<u>499</u>
	Formal class or course teaching hours:	51
	Informal teaching hours including prep time:	48
	Mentoring hours:	400

X. RESEARCH AND CREATIVE ACTIVITIES

RESEARCH AWARDS AND GRANTS:

CURRENT

W81XWH-10-BCRP-EOHS-EXP (PI: Coussens, LM; UCSF)

09/30/11 – 09/29/16

Era of Hope Scholar Expansion Award

Source: DOD/U.S. Army Medical Research & Materiel Command

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.

KG111084 (PI: Coussens, LM; Hwang S, Rugo HUCSF) 07/01/11 – 06/31/16

Source: Komen Foundation**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 (PI: Hwang S; Coussens, LM; UCSF) 07/1/11 – 06/31/14
Source: Komen Foundation**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.

R01 CA155331 (PI: Coussens, LM; UCSF) 05/01/11 – 03/31/16
Source: NIH/NCI**Title:** *Regulating the Immune Microenvironment in Breast Cancer*

The major goal of this study is to evaluate the efficacy of T_H2-blockade as a therapy for reprogramming pro-tumor immune cells in mouse models of mammary carcinogenesis.

Role: P.I.

R01 CA140655 (PI: Boudreau, N; UCSF) 05/07/10 – 02/28/15
Source: NIH/NCI**Title:** *Regulation of Tumor Progression by Endothelial HoxA5*

The major goal of this project is to establish the role of endothelial HoxA5 in regulating angiogenesis and vascular permeability in response to acute angiogenic stimuli in vivo; to evaluate the role of endothelial HoxA5 in regulating angiogenesis during de novo carcinogenesis; and to evaluate efficacy of hoxA5 as an anti-cancer therapeutic

Role: co-investigator

R01CA140943 (multi-PI: Coussens, Boudreau, Daldrup-Link; UCSF) 07/01/09 –06/30/13
Source: NIH/NCI**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; UCSF) 07/01/08-06/30/13
Source: NIH/NCI**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 (multi PI: Coussens, LM; Jablons DM; UCSF) 05/01/08-04/30/13
Source: NIH/NCI**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.

W81XWH-08-PRMRP-IIRA (multi-PI: Broaddus, C; Coussens, LM; UCSF) 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.

P30 CA82103 (PI: McCormick, F; UCSF) 08/5/99-05/31/12

Source: NIH/NCI

Title: *Cancer Center Support Grant*

Role: Co-Director, Mouse Pathology Shared Resource

The Cancer Center Support Grant provides support for administration and infrastructure for the UCSF Comprehensive Cancer Center. Dr. Coussens is the Core Co-Director of the Mouse Pathology Shared Resource that provides routine hematologic and histopathologic processing of tissue and blood samples to members of the UCSF community.

PREVIOUS

USPHS 5 T32 CA09056 (PI: Fox, F, UCLA) 07/01/89 –06/30/92

Source: NIH/UCLA

Title: Regulation of *junB* Gene Expression by TGF-Beta

Competitive Pre-Doctoral award to study transcription factor *junB*.

Univ. of Calif., Dissertation Year Fellowship (PI: Coussens, LM, UCLA) 10/1/92 – 09/31/93

Source: University of California, Office of the President

Title: *Effects of E1A on TGF-Beta-inducible junB Expression*

Competitive Pre-Doctoral award to study transcription factor *junB*.

USPHS 5 T32 CA09043 (PI: Bishop, KM, UCSF) 10/01/93-06/31/96

Source: NIH/UCSF

Title: *Molecular Analysis of Tumor Viruses*

Post-Doctoral fellowship to study mouse model of epithelial carcinogenesis.

American Social Health Association/Pfizer Post-Doctoral Research Fellowship in Sexually Transmitted Diseases (PI: Coussens, LM, UCSF) 10/01/96 – 9/30/98

Source: Private Foundation

Title: *Metalloproteinases and Malignant Progression of Squamous Epithelium*

in K14-HPV16 Transgenic Mice

Role: Principal Investigator

Competitive Post-Doctoral fellowship to study proteases and tumor development

P01 CA072006 (PI: Shuman M, UCSF) 06/10/97 – 06/30/03

Source: NIH/NCI

Title: *Proteases in Cancer Biology and Drug Development*

Project 3 – Proteases in Models of Tumor Initiation/Progression

Role: Co-Investigator, Project 3

The major goal of this project is to study the role of proteases in cancer biology.

Core C – Transgenic Animal Models

Role: Director (year 4 and 5)

The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.

UCSF IRG-97-150-01 (PI: Coussens LM, UCSF) 07/01/99-06/30/00

Source: American Cancer Society

Title: *Proteases and Genomics in a Mouse Model of Epithelial Cancer*

Role: Principal Investigator

Pilot project tested role of proteinases as effectors of genomic instability.

UCSF Cell Cycle and Dysregulation Program (PI: Coussens LM, UCSF) 02/01/00-01/31/01

Source: UCSF Comprehensive Cancer Center, Intramural

Title: *Epithelial Neoplastic Progression and Degradation of Type I Collagen*

Role: Principal Investigator

Pilot project assessed functional significance of type I collagen metabolism during epithelial carcinogenesis.

Research Evaluation & Allocation Committee (PI: Coussens LM, UCSF)

07/01/00-06/30/01

Source: UCSF Academic Senate

Title: *Role of Gelatinase B in Maintenance of Genomic Instability*

Role: Principal Investigator

Pilot project tested the role of MMP9 as an indirect regulator of genomic instability.

UCSF IRG AC-04-02 (PI: Coussens LM, UCSF)

10/01/00-09/30/01

Source: American Cancer Society

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.

The V Foundation for Cancer Research (PI: Coussens LM, UCSF)

06/02/00-05/31/02

Source: Private Foundation

Title: *Gelatinase B and Epithelial Cancer Development*

Role: Principal Investigator

Pilot project to study role of MMP9 during epithelial carcinogenesis.

Gertrude B. Elion Cancer Research Award (PI: Coussens LM, UCSF)

07/1/01 – 06/30/02

Source: American Association of Cancer Research

Title: *Functional Role of MMP-2 During Epithelial Carcinogenesis*

Role: Principal Investigator

Pilot project to study role of MMP-2 during epithelial carcinogenesis.

Univ. of Calif., Cancer Research Coordinating Committee (PI: Coussens LM, UCSF)

07/01/01 – 06/30/02

Source: University of California

Title: *Gelatinase A/MMP-2 and Epithelial Cancer Development*

Role: Principal Investigator

Pilot project to study role of MMP-2 as a potentiator of tumor development.

Hellman Family Award For Early Career Faculty (PI: Coussens LM, UCSF)

11/01/00-09/30/02

Source: UCSF Intramural

Title: *Paracrine Regulation of Epithelial Carcinogenesis by MMP-9*

Role: Principal Investigator

Pilot project to identify matrix molecules regulated by MMP-9.

Edward Mallinckrodt, Jr. Foundation (PI: Coussens LM, UCSF)

10/01/00-09/30/03

Source: Private Foundation

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.

P50 CA58207 (PI: Gray, J: UCSF)

03/01/03-02/28/05

Source: NIH/NCI

Bay Area Breast Cancer Translational Research Program (SPORE)

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.

DE-FG02-05ER6401 (PI: Franc, B; UCSF)

03/01/05 – 01/16/06

Source: DOE Medical Applications Grant

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

Title: *Proteases in Prostate Cancer Bone Metastasis*

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

Title: *B Lymphocytes as Targets for Cancer Prevention*

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

Breast Cancer Center of Excellence

Title: *Validation of Proteases as Therapeutic Targets in Breast Cancer Functional Imaging of Protease Expression, Activity and Inhibition*

Role: Subcontract Principal Investigator

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

Title: *Regulation of Epithelial Cancer by MMP-9/gelatinase B*

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

Title: Center on Proteolytic Pathways

Role: Principal Investigator (Driving Biological Problem #1)

DBP#1 *Proteolytic Pathways in Acute Vascular Response*

P01 CA72006 (PI: Werb, Z; UCSF)

07/07/03 – 06/30/08

Source: NIH/NCI

Title: *Proteases in Cancer Biology and Drug Development*

Project 3 - *Proteases in Models of Tumor Initiation/Progression*

Role: Co-Investigator, Project 3

The major goal of this project is to study the role of proteases in cancer biology.

Core C - *Transgenic Animal Models*

Role: Director

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

Title: *Microenvironmental Regulation of Tumor Progression*

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
(5/1/10-04/30/11)

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

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.

XI. PEER-REVIEWED PUBLICATIONS

1. Francke U, de Martinville B, **Coussens L**, Ullrich A. (1983) The human gene for the Beta subunit of nerve growth factor is located on the proximal short arm of chromosome 1. *Science* 222:1248-1251.
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5. Lauffer L, Garcia P, Harkins R, **Coussens L**, Ullrich A, Walter P. (1985) Topology of the signal recognition particle receptor in the endoplasmic reticulum membrane. *Nature* 318:334-338.
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7. **Coussens L**, Yang-Feng T, Liao T-C, Chen E, Gray A, McGrath J, Seeburg P, Libermann T, Schlessinger J, Francke U, Levinson A, Ullrich A. (1985) Tyrosine kinase receptor with extensive homology to the EGF receptor shares chromosomal location with *neu* oncogene. *Science* 230:1132-1139.
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11. **Coussens L**, Rhee L, Parker P, Ullrich A. (1987) Alternative splicing increases the diversity of the human protein kinase C family. *DNA* 6:389-394.
12. Yarden Y, Kuang W-J, Yang-Feng T, **Coussens L**, Munemitsu S, Dull T, Schlessinger J, Francke U, Ullrich A. (1987) Human proto-oncogene *c-kit*: A new cell surface receptor-tyrosine kinase for an unidentified ligand. *EMBO J.* 6:3341-3351.
13. MacDonald R, Pfeffer S, **Coussens L**, Tepper M, Brocklebank C, Mole J, Anderson J, Chen E, Czech M, Ullrich A. (1988) A single receptor binds both insulin-like growth factor II and mannose-6-phosphate. *Science* 239:1134-1137.
14. Formby B, Ullrich A, **Coussens L**, Walker L, Peterson C. (1988) Growth hormone stimulates insulin gene expression in cultured human fetal pancreatic islets. *J. Clin. Endo. Metab.* 66:1075-1079.
15. Mosthaf L, Grako D, Dull T, **Coussens L**, Ullrich A, McClain D. (1990) Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *EMBO J.* 9:2409-2413.
16. **Coussens LM**, Yokoyama K, Chiu R. (1994) Transforming Growth Factor β -mediated induction of *junB* is selectively inhibited by expression of Ad.12-E1A. *J. Cell. Physio.* 160:435-444.
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 - *Cancer Cell* (2009), 16:81-82. 'Metastasis-Promoting Immunity: When T cells turn to the dark side' by D. Pardoll
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- *Nature Reviews Immunology* (2010) 10:158 'Antibodies lend support to tumors', by, L. Byrd
 - *Faculty of 1000 – Exceptional (Factor 6.4)*. Authoritative evaluations by X. Ma (Weill Medical College of Cornell) and F. Kiefer (Max-Planck-Institute, Germany). <http://f1000biology.com/article/id/2467956>
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 - *Nature Reviews Cancer* (2011) 11:3056. 'Bad company', by N. McCarthy
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- Johansson M, Affara NI, Junankar S, Korets L, Peters C, Sloane BF, McKerrow J, **Coussens LM**. Cathepsin B-deficiency does not alter parameters of neoplastic progression in the K14-HPV16 transgenic mouse model of epithelial carcinogenesis. *Manuscript submitted*.
- Shiao S, Ganesan A.P, Rugo HS, **Coussens LM**. Immune microenvironments in solid tumors: New targets for therapy. Commissioned review, *Genes and Development*, submitted.
- Ruffell B, Cottone L, DeNardo DG, Affara NI, Vasquez L, Deu E, Barreto J, Bogyo M, **Coussens LM**. Mammary carcinogenesis is cathepsin C-independent. *Manuscript submitted*

XII. PATENTS

1. U.S. Patent Application Serial No. 10/567,873
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
International filing date: August 9, 2004. Application Filing Number: 60/493,643; Docket Number: 23540-09361/PCT; International Publication Number WO 2005/013915 A2; International Application Number: PCT/US2004/025902
2. DeNardo D, Brennan D, **Coussens LM**. *Phenotyping Tumor-Infiltrating Leukocytes*, Provisional patent application number: 61/227,035

XIII. NON-PEER REVIEWED PUBLICATIONS AND EDITORIALS:

Review Articles, Symposium Proceedings and Editorials

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Published Abstracts:

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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 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. 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

1. de Visser KE, Korets LV, **Coussens LM.** (2005) De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell*, 7:411-423.

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. de Visser wrote drafts of the manuscript under Dr. Coussens' direct supervision.

2. DeNardo DG, Baretto JB, Andreu P, Vasquez L, Kolhatkar N, **Coussens LM.** (2009) CD4⁺ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell*, 16:91-102.

Role: Infiltration of T lymphocytes in human breast cancers has been recognized by pathologist for decades, however their functional role has been undetermined. We utilized a transgenic mouse model of mammary carcinogenesis and demonstrated a tumor-promoting role for T_H2-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.

3. Andreu P, Johansson M, Affara NI, Tan TT, Junankar S, Korets L, Lam J, Tawfik D, Pucci F, De Palma M, DeNardo D, de Visser KE, **Coussens LM**. (2010) FcR γ activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell*, 17(2):121-134.

Role: This work presented novel findings demonstrating functional significance of Fc γ 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 Fc γ 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 FcR γ 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.

4. Sounni NE, Dehne K, vanKempen LCL, Egeblad M, Affara NI, Cuevas I, Wiesen J, Junankar S, Korets L, Lee J, Shen J, Morrison C, Overall CM, Krane SM, Werb Z, Boudreau N, **Coussens LM**. (2010) Stromal regulation of vessel stability by MMP14 and TGF β . *Disease Model. Mech.* 3:317-332.

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 (TGF β) 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 TGF β . In wild-type mice, inhibitors of either MMP14 or TGF β signaling induce blood vessel permeability. Conversely, enhanced MMP14 or TGF β activity in the mouse epidermis decreases leakage across cutaneous vessels. This pathway remains functional during tumor progression, as acute blockade of either MMP14 or TGF β 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 TGF β 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.

5. DeNardo DG, Brennan DJ, Rexhapaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhvani N, Keil SD, Junaid SA, Rugo HS, Hwang ES, Jirstrom K, West BL, **Coussens LM**. (2011) Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discovery*, 1(1): 54-67.

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.

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

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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.^{1,2} 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.^{3–5}

Understanding multicellular resistance may provide key insights into effective therapies for recalcitrant solid tumors such as human malignant mesothelioma,⁶ which usually presents at a late stage as a thick tumor mass.⁷ At this time, few effective therapeutic options are available for mesothelioma patients and survival remains poor. Our group⁸ and others⁹ have found that mesothelioma spheroids acquire multicellular resistance to a variety of treatments.^{8,9} We decided to focus on resistance to bortezomib (PS-341, Velcade), an agent that has shown promise in pre-clinical studies of mesothelioma^{10,11} 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,¹² has been a common problem in solid tumors.¹³

Bortezomib, a proteasome inhibitor, is thought to induce apoptosis via the alteration of expression of Bcl-2 proteins.¹⁴ 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.¹⁵ 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/X_L/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 apoptosis¹⁰ and known to form spheroids.⁸ 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).

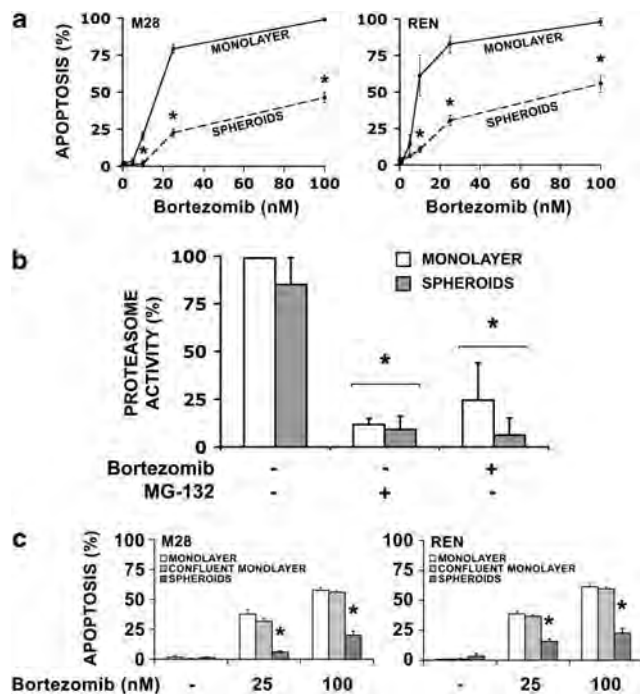


Figure 1 Spheroids grown from mesothelioma cell lines acquire multicellular resistance to bortezomib. (a) M28 and REN monolayers and spheroids were treated with bortezomib 1, 5, 10, 25 or 100 nM for 48 h. Apoptosis was measured in Hoechst-stained cells; cells with signs of nuclear condensation were considered apoptotic. Spheroids grown from both cell lines acquired a marked multicellular resistance to bortezomib even at the highest concentrations. (* $P < 0.05$ spheroids compared with monolayers, $n = 3$). (b) REN monolayers and spheroids were treated with bortezomib (100 nM) or MG-132 (10 μ M), a potent proteasome inhibitor, for 4 h before cells were lysed and analyzed for proteasomal activity. Monolayers and spheroids showed similar proteasomal activity at baseline and were both equally inhibited by bortezomib or MG-132 after 4 h. (* $P < 0.05$ compared with untreated control, $n = 3$). (c) M28 and REN monolayers at standard confluence (70%), completely confluent monolayers (obtained by plating twice the number of cells to achieve at least 100% confluence) and spheroids were treated with bortezomib (25 or 100 nM). Completely confluent monolayers showed an apoptotic response comparable to monolayers and did not acquire multicellular resistance. (* $P < 0.05$ spheroids compared with monolayers and confluent monolayers, $n = 3$)

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.¹⁶ 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 cells¹⁷ 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.^{14,18–20} 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 Noxa^{BH3} peptide bound to 8 arginine residues (R8-Noxa^{BH3}), a modification that aids diffusion of the peptide across cell membranes.^{21,22} Although an R8 control peptide had no activity, R8-Noxa^{BH3} enhanced the apoptotic response of the spheroids, thereby significantly reducing the multicellular resistance of spheroids to bortezomib (Figure 3b). In addition, R8-Noxa^{BH3} alone induced apoptosis in REN spheroids. To determine whether R8-Noxa^{BH3} peptide had off-target effects, we ablated Mcl-1 by siRNA. Because Noxa activity requires Mcl-1, we would expect R8-Noxa^{BH3} to have no activity in the absence of Mcl-1. Without Mcl-1, R8-Noxa^{BH3} lost all apoptotic activity indicating that, at

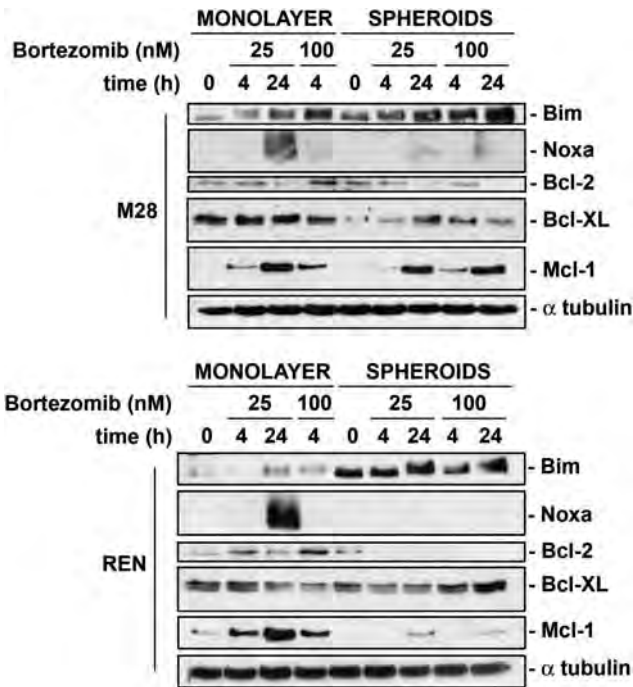


Figure 2 Spheroids fail to upregulate Noxa after bortezomib but have a high expression of Bim. M28 and REN monolayers and spheroids were treated with bortezomib (25 or 100 nM) for 4 or 24 h. Whole cell lysates (50 μ g) were resolved on SDS-PAGE and immunoblotted for a panel of pro-/anti-apoptotic Bcl-2 proteins. Both M28 and REN monolayers upregulated Noxa after bortezomib (25 nM) at 24 h. (Owing to the high degree of apoptosis, monolayers treated with 100 nM bortezomib at 24 h were not collected.) Spheroids, however, failed to upregulate Noxa, even after bortezomib 100 nM at 24 h. Interestingly, at baseline and during exposure to bortezomib, spheroids expressed more Bim protein than monolayers. (Representative of three experiments)

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/ X_L / w , would be effective in spheroids by unmasking the pro-apoptotic potential revealed by the R8-Noxa^{BH3} peptide. Indeed ABT-737 (1 μ M) 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'¹⁵ 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

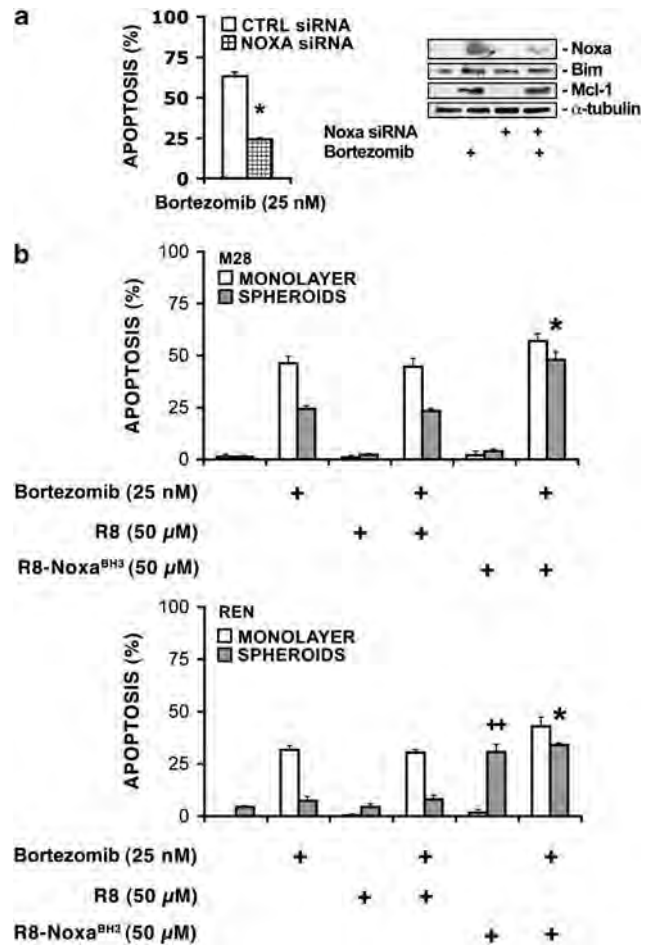


Figure 3 A Noxa peptide sensitizes spheroids to bortezomib-induced apoptosis. (a) Noxa was silenced in REN cells and, 24 h later, cells were plated as monolayers and treated with bortezomib (25 nM). After 4 h, cells were collected and lysed to verify the efficacy of Noxa siRNA (see immunoblot). No changes in other Bcl-2 proteins, Mcl-1 and Bim, were detected upon Noxa silencing, with or without bortezomib. After 24 h, Hoechst-stained cells were counted for the presence of apoptosis. Noxa siRNA significantly reduced the apoptosis induced by bortezomib. (* $P < 0.05$, $n = 3$). (b) M28 and REN monolayers and spheroids were treated with bortezomib (25 nM), a cell-permeable R8-Noxa^{BH3} peptide (50 μ M) or a control R8 peptide (50 μ M), either alone or in combination, for 24 h. R8-Noxa^{BH3}, but not the R8 peptide, eliminated the acquired multicellular resistance of spheroids to bortezomib without affecting monolayers. Interestingly, REN spheroids, but not M28, were sensitive to R8-Noxa^{BH3} alone. (* $P < 0.05$ compared with untreated and R8 control; + + $P < 0.05$ spheroids versus monolayers, $n = 3$)

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/ X_L / w , Mcl-1 is not inhibited by ABT-737 and is known to mediate resistance to ABT-737.^{23,24} 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/ X_L / 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.

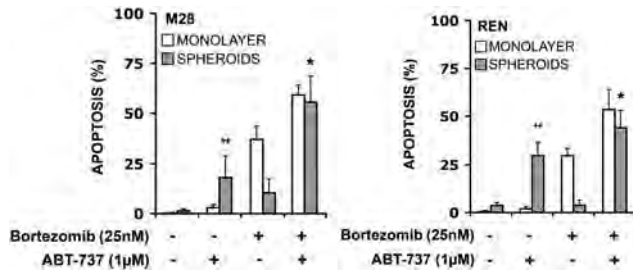


Figure 4 ABT-737 eliminates multicellular resistance to bortezomib. M28 and REN monolayers and spheroids were treated with bortezomib (25 nM) and ABT-737 (1 μ M), either alone or in combination, for 24 h. ABT-737 eliminated the acquired multicellular resistance of spheroids to bortezomib. Interestingly, 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)

ABT-737 has been shown in some settings to alter levels of Bcl-2 proteins,^{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 6 h. 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 *in vivo* exist as single cell monolayer 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 bortezomib treatment for 6 h (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, SARC 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 for the loss of mitochondrial potential indicative of apoptosis.¹⁵

Cells derived from monolayers and spheroids were equally sensitive to the Bim BH3 peptide, confirming an equal ability to activate the apoptotic program (Figure 6). However, compared with cells from monolayers, cells from spheroids were more sensitive to sensitizer BH3-only molecules, Bad and Hrk, targeting Bcl-2 and Bcl-X_L, respectively. In addition, cells from spheroids were confirmed to be more sensitive to ABT-737, which had no effect on the cells from monolayers.

Because cells from spheroids of the two additional mesothelioma cell lines, VAMT and SARC, were found to be sensitive to pro-apoptotic sensitizers by BH3-profiling, we

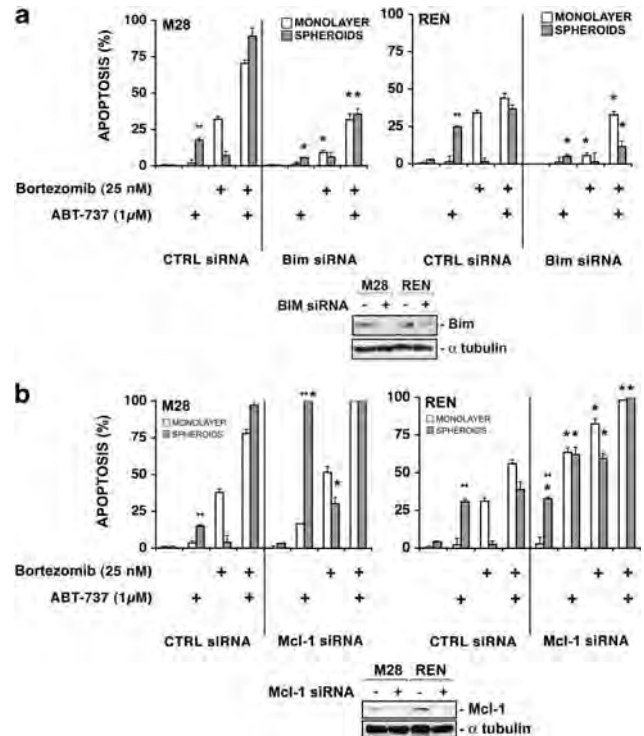


Figure 5 ABT-737 sensitization of spheroids to bortezomib is dependent on Bim and aided by ablation of Mcl-1. (a) Bim was ablated in M28 and REN cells and, 24 h later, cells were plated to generate monolayers and spheroids, exposed to bortezomib 25 nM and/or ABT-737 1 μ M for 24 h and lysed and probed for Bim at 72 h after siRNA (see immunoblot insert), the time when cells were counted for the presence of apoptosis. The absence of Bim in spheroids completely abolished the apoptotic effect of ABT-737 when given alone and significantly reduced the effect when given with bortezomib. (* P <0.05 compared with control siRNA, ++ P <0.05 spheroids versus monolayers, n = 3). (b) Mcl-1 was ablated, as performed for Bim above (see immunoblot insert). The absence of Mcl-1 reduced the acquired multicellular resistance of spheroids to bortezomib. The absence of Mcl-1 also increased the apoptotic response to ABT-737 when given alone or in combination with bortezomib. (* P <0.05 compared with control siRNA, ++ P <0.05 spheroids versus monolayers, n = 3)

anticipated that they would also show sensitivity to BH3-only sensitizers when tested in cell culture. Indeed, spheroids of both cell lines were sensitive to ABT-737- and R8-Noxa^{BH3}-induced apoptosis, even in the absence of bortezomib (Figure 7a). When spheroids from all four cell lines were compared, the M28 spheroids appeared to have less sensitivity, with no response to R8-Noxa^{BH3} given alone (see also Figure 3b). We then confirmed that the expression levels of Bim increased in each cell line when grown as spheroids. Of interest, the increase in Bim levels as estimated by the ratio of densitometry values was less in M28 (1.9-fold increase) when compared with the more sensitive lines (3.2–4.5-fold increase) (Figure 7b and Supplementary Figure 4)

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

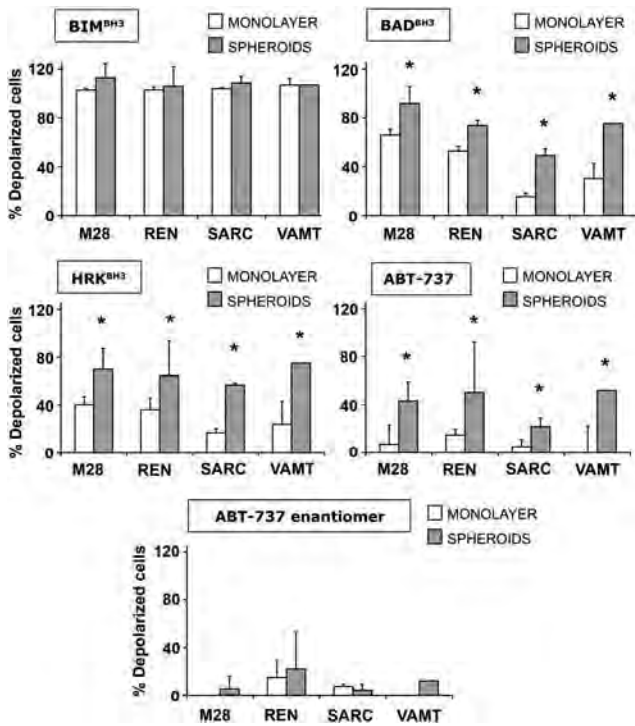


Figure 6 Spheroids are 'primed for death'. BH3-profiling was performed on cells disaggregated from monolayers and spheroids grown from M28, REN, SARC and VAMT cells. The cells were gently permeabilized as described³⁹ and exposed to BH3 peptides (100 μ M) for 90 min before JC1 was added for 30 min and depolarization measured as a percentage of the total. Cells from spheroids and monolayers were equally depolarized by Bim^{BH3}, the positive control, confirming a functioning apoptotic apparatus. However, cells disaggregated from spheroids were more sensitive to Bad^{BH3}, Hrk^{BH3} and ABT-737 than cells from monolayers, confirming that cells in 3D acquired apoptotic priming. Cells showed no response to an ABT-737 inactive enantiomer, used as negative control. (* P <0.05 compared with monolayers, $n=3$)

fragment model we previously developed for *ex vivo* studies,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 (<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

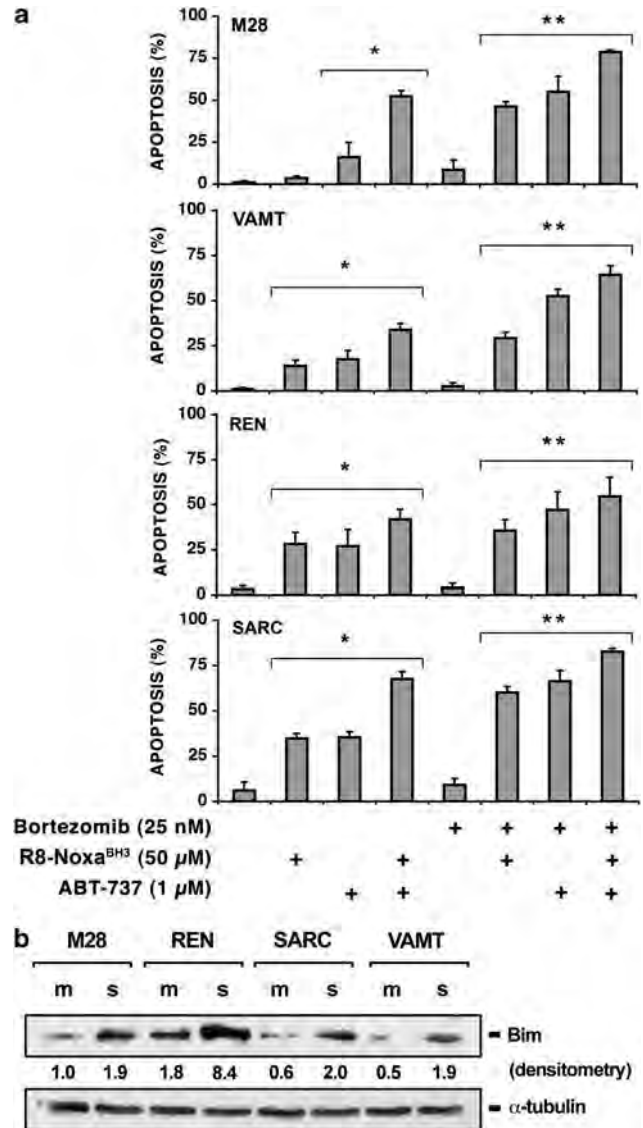


Figure 7 Apoptotic priming of spheroids increases with elevated Bim expression. (a) Spheroids grown from M28, VAMT, REN and SARC cell lines were treated with R8-Noxa^{BH3} (50 μ M), ABT-737 (1 μ M), bortezomib (25 nM) or their combination for 24 h. Whereas R8-Noxa^{BH3} alone failed to induce apoptosis in M28 spheroids, ABT-737 alone induced apoptosis in the spheroids generated from all four cell lines. Nevertheless, when given with bortezomib, either R8-Noxa^{BH3} or ABT-737 was effective in increasing apoptosis of spheroids. (* P <0.05 compared with untreated spheroids, ** P <0.05 compared with bortezomib alone, $n=3$). (b) Immunoblot analysis of Bim expression in M28, REN, SARC and VAMT monolayers and spheroids with densitometry shown for Bim, standardized for α -tubulin expression. Immunoblot and densitometry values are representative of three separate experiments. In all four cell lines, spheroids expressed increased Bim levels. Compared with monolayers, spheroids of M28 displayed a 1.9-fold increase in Bim levels whereas spheroids of REN, SARC and VAMT showed a 4.5-, 3.2- and 4.0-fold, increase, respectively

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).

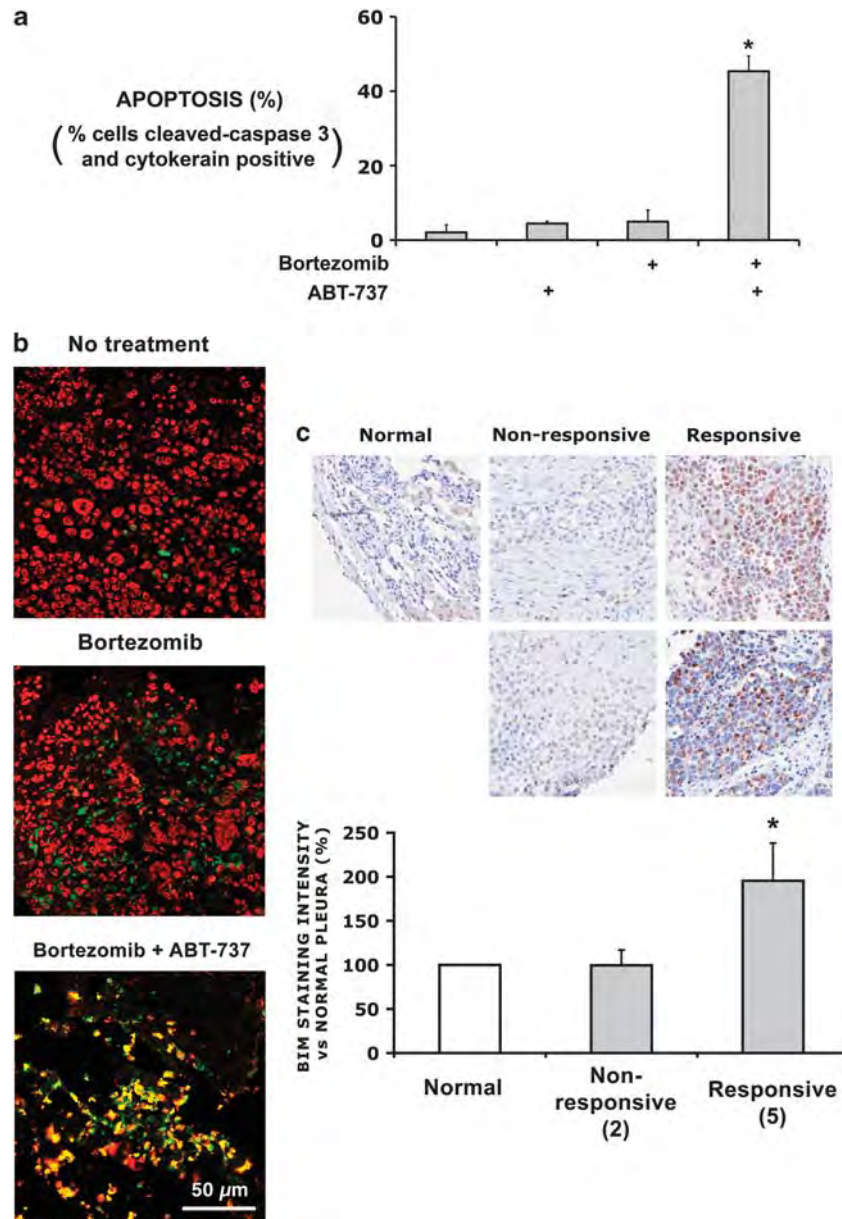


Figure 8 ABT-737 increases activity of bortezomib against mesothelioma cells in spheroids derived from tumors with high expression of Bim. (a) TFS generated from seven tumors were treated with bortezomib (50 nM), ABT-737 (2.5 μ M) or their combination for 24 h. In the responsive tumors ($n = 5$) (shown), ABT-737 increased apoptosis when given together with bortezomib. In unresponsive tumors ($n = 2$) (not shown), ABT-737 with or without bortezomib had no effect (apoptosis < 5%), ($*P < 0.05$ compared with no treatment, bortezomib alone or ABT-737 alone, $n = 5$). (b) Confocal images representative of the responsive tumor fragment spheroids treated with bortezomib (50 nM) with or without ABT-737 (2.5 μ M) for 24 h. Pan-cytokeratin (red) was used to identify mesothelioma cells within the spheroids and cleaved caspase-3 (green) to identify apoptosis. The merged color (yellow) identifies apoptotic mesothelioma cells. The mesothelioma cells are resistant to ABT-737 alone (not shown) and to bortezomib alone. The addition of ABT-737 to bortezomib, however, effectively increased apoptosis of the human mesothelioma cells. (c) Using a TMA generated with tissue from the original seven tumors tested for apoptosis along with normal pleural tissue as control, Bim expression was determined by immunohistochemistry and quantified using an imaging analysis system (ScanscopeXT, Aperio). Every sample was represented three times on the slide. Normal pleura and the two tumors not responsive to ABT-737 and bortezomib showed comparable, very low amounts of Bim. Conversely, the responsive tumors showed strong Bim staining. Intensity of staining was expressed as percentage compared with normal pleura. ($*P < 0.05$ compared with normal and non-responsive)

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,²⁹ 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.³⁰ In many systems, Noxa has been shown to mediate bortezomib activity,^{31–33} a role we confirmed in the mesothelioma monolayers. In spheroids, the addition of a R8-Noxa^{BH3} 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/X_L/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-X_L. 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.³⁴

Mcl-1 can confer resistance to ABT-737 as well as to bortezomib,^{31–33} and can be a major resistance factor in 3D spheroids, as we have previously shown in lung cancer.³⁵ 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-Noxa^{BH3} or by Mcl-1 siRNA) did increase the response to ABT-737, as has been previously shown.²³ 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/X_L/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-Noxa^{BH3} 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/X_L/w, as has been described,³⁶ 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.^{23,25,26}

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.⁵ 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,³⁷ 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.³⁸ 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.

Materials and Methods

Cell cultures and reagents. The human mesothelioma cell lines M28, REN, VAMT 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 described²⁸. Tumor samples were obtained from extrapleural pneumonectomy (EPP) or pleurectomy procedures performed by DJS and RB at Brigham and Women's Hospital in Boston, MA, USA.

R8-Noxa^{BH3} (RRRRRRRR-EVECATQLRRFGDKLNFQRQL) 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-adsorbent round-bottomed 96-well plates. The 96-well plates were coated with a 1:24 dilution of polyHEMA (120 mg/ml) (#P3932, 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 for 5 min at 800 r.p.m. 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 grown 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 immunostaining.

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, 3% glycerol, 0.01% bromophenol blue and 200 mM DTT). 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-X_L (#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 (#559685) was from BD Pharmingen (San Jose, CA, USA), and the Noxa antibody was from Calbiochem (#OP180, San Diego, CA, USA). The α-tubulin antibody (#T-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 chymotryptic 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 (4×10^6) were pelleted and resuspended in 100 µl of nucleofection buffer (solution T, Amaxa 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 T-20 on a Nucleofector II device (Amaxa 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'-ACUUACAUCAGAAG GUUGCt-3'), Mcl-1 (5'-CCAGUAUACUUCUAGAAAt-3') and Noxa (5'-GAAAU GUGUCAAAUUAUCt-3'), non-targeting control (5'-GCAACCUUCUGAUGUAA GUtt-3').

BH3 profiling. Experiments were performed at the Dana Farber Cancer Institute (Boston, MA, USA) by JA Ryan and AG Letai as previously published.³⁹ Monolayers and spheroids formed from equal numbers of cells were disaggregated in an identical fashion to single cells. The cells were gently permeabilized as described³⁹ and exposed to BH3 peptides (100 µM) 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% EtOH (2 × 2 min), 95% EtOH (2 × 2 min), 70% EtOH (2 × 2 min), 50% EtOH (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; #31821) and anti-guinea pig AlexaFluor 633 (Invitrogen; #A-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 ± 1 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, 2008). This space (10–20 μ m 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 et al., 1988).

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

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#This author has disclosed a potential conflict of interest as described by one or more of the following: He/She may have also received (and may also apply in future for) competitive-funding research grants from U.S. publicly financed, peer-reviewed grant approval process agencies concerning asbestos exposure and disease, including topics covered in all aspects of the workshop, including but not limited to research support from NIEHS.

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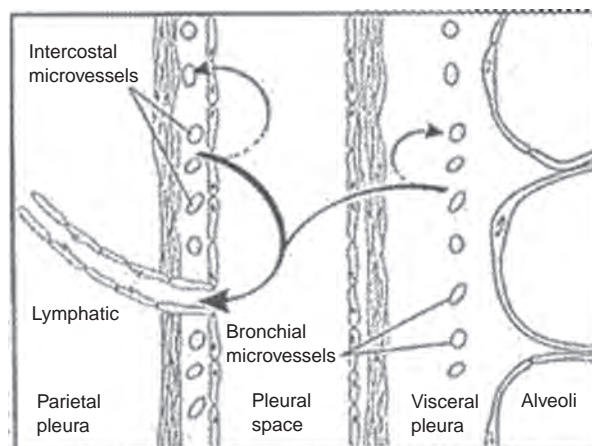


FIGURE 1. Fluid turnover and lymphatic drainage from the pleural space. In the normal pleural space (shown here), as in other interstitial spaces of the body, liquid slowly filters from systemic capillaries and is absorbed via lymphatics (solid arrows). In the pleural space, the capillary filtrate from systemic capillaries moves across a permeable pleural membrane toward the lower pressure pleural space and is absorbed via the parietal pleural lymphatics. From there, liquid moves via lymphatic propulsion to the central veins. When interstitial edema forms in the adjacent lung, some of that excess liquid moves across the visceral pleura into the pleural space. Asbestos fibers may follow similar routes from the lung to the pleura and are thought to lodge in the parietal pleura preferentially at sites of lymphatic drainage.

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 pre-neoplastic biomarkers (Greillier et al., 2008) that might lead to intervention and prevention of 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 μm ; however, the majority of asbestos fibers in human tissue samples are shorter than 5 μm (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

Fiber type	Fiber number/g wet lung
Chrysotile asbestos fibers	130.0×10^3
Antigorite	2.5×10^3
Noncommercial amphiboles:	
Tremolite	15.0×10^3
Actinolite	5.1×10^3
Anthophyllite	3.7×10^3
Commercial amphiboles:	
Amosite and crocidolite	1.1×10^3

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

Fiber type	Percent of patients with fibers	Percent of controls with fibers
Chrysotile	80	67
Tremolite	20	11
Crocidolite	59	16
Amosite	81	40
Other:		
Mullite	98	98
Iron	88	65
Rutile	83	79
Muscovite	61	65
Silica	55	65

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 or ashing of 25- μm tissue sections using high-resolution analytical electron microscopy. The majority of fibers were $\leq 5 \mu\text{m}$ long and 92.7% were $\leq 0.25 \mu\text{m}$ 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 $\geq 5 \mu\text{m}$ long. The mean asbestos fiber concentration in the 8 exposed cases was $12.4 \pm 9.8 \times 10^6$ fibers/g dry lung tissue, $4.1 \pm 1.9 \times 10^6$ fibers/g black spots on the parietal pleura, and $0.5 \pm 0.2 \times 10^6$ 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 $>1000/\text{g}$ 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 distribution 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 predominantly 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 ($\geq 20 \mu\text{m}$) and thin ($\leq 0.4 \mu\text{m}$) 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)- β 1 (Decolonne 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.

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

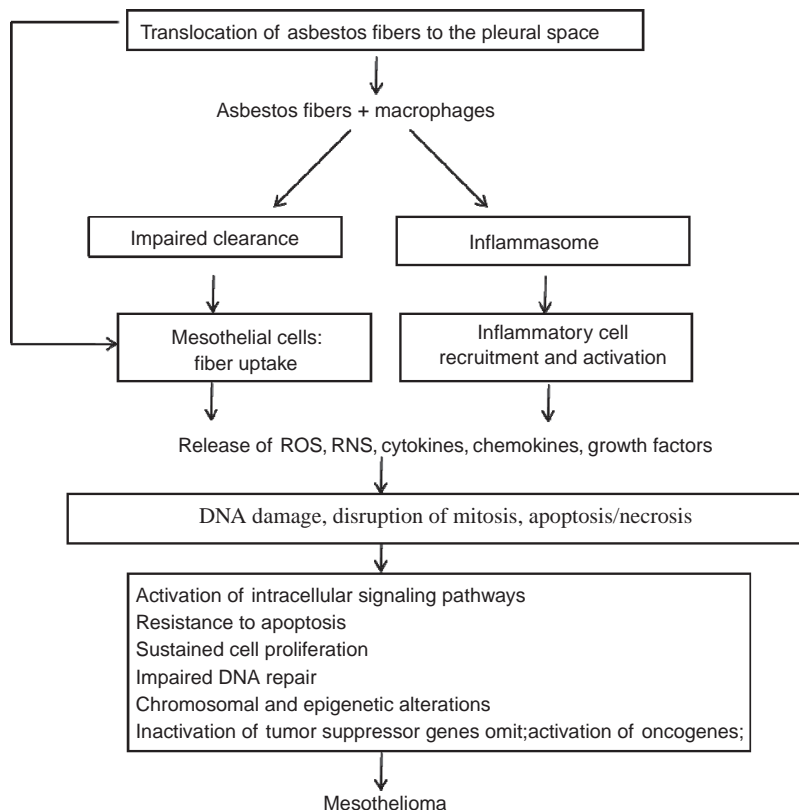


FIGURE 2. Proposed mechanisms for asbestos-induced mesothelioma. Asbestos fibers are thought to lead to mesothelioma via mechanisms as outlined in this algorithm. Asbestos fibers enter the pleural space, where they interact with pleural macrophages and mesothelial cells and induce an influx of inflammatory 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-1 β (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)- κ B 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; Daubriac 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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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-17 β (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 multilaminar 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

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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 multilaminar 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; Szweczyk 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 chemoattractant 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^{op}*) have shown that many tissues but not all are severely depleted of macrophages. The mammary gland is one of these severely affected tissues indicating

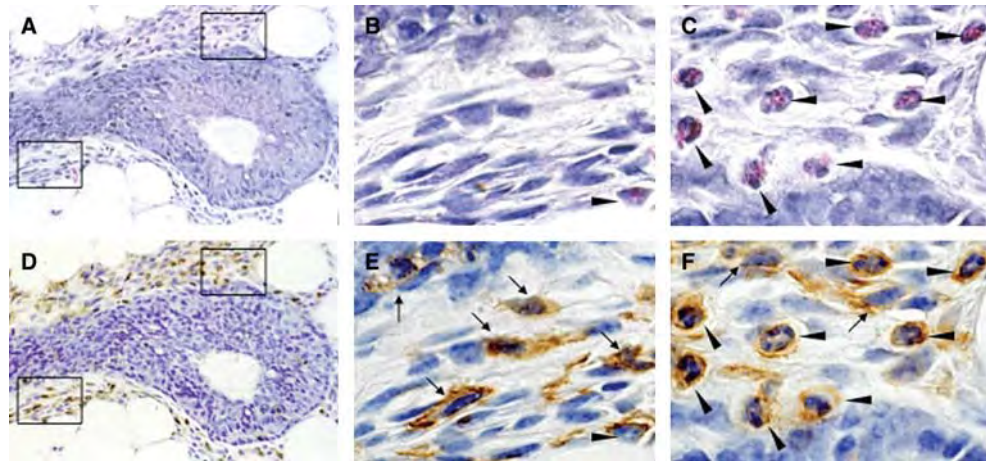


Figure 1. Macrophage and eosinophil recruitment to the terminal end buds of mice. H&E longitudinal sections of terminal end buds at 5 wk of age. Sections were first stained with H&E (A–C) and then destained and immunostained using anti-F4/80 antibody followed by a peroxidase detection system for positive 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.)

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

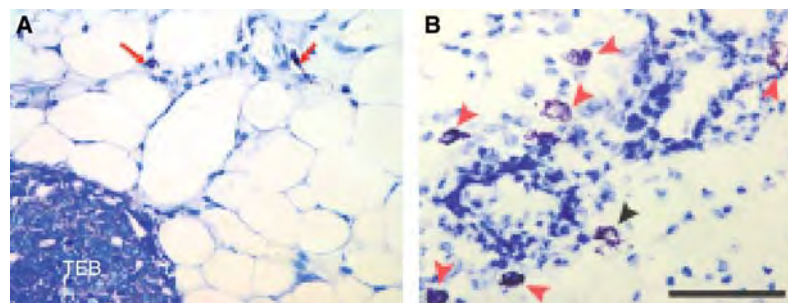


Figure 2. Distribution of mast cells around the terminal end bud of mice. Shown are formalin fixed, paraffin embedded sections stained with toluidine blue in which mast cells are identified by an enzymatic reaction to detect chymase a defining enzyme of these cells. (A) Mast cells (red arrows) shown 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 μ m.

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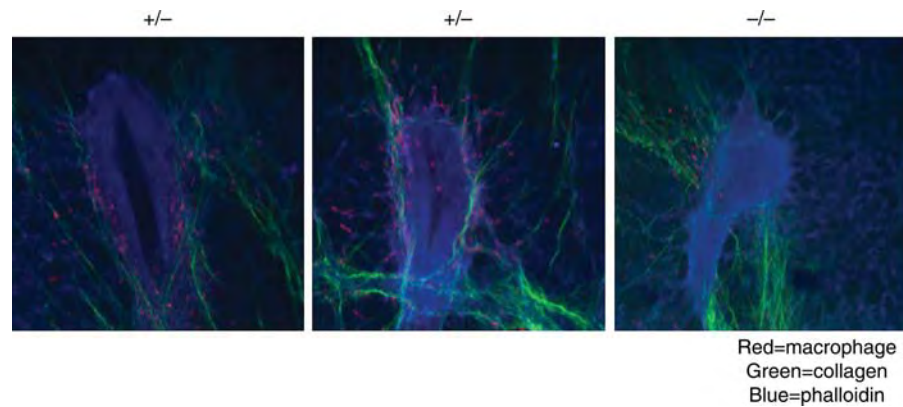


Figure 3. Association of macrophages and collagen fibers with the terminal end bud. Multiphoton imaging of frozen sections of terminal end buds with nuclei stained with DAPI in which the collagen fibers are shown by second harmonic resonance and pseudo-colored in green while macrophages are shown by expression of GFP from the *Cs1r*-promoter (data from Sasmono et al. 2003) pseudocolored to red. In *A* and *B*, TEBs from mice heterozygous ($^{+/-}$) for the *Csfl*^{OP} 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).

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 a cross-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. Extramedullary 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

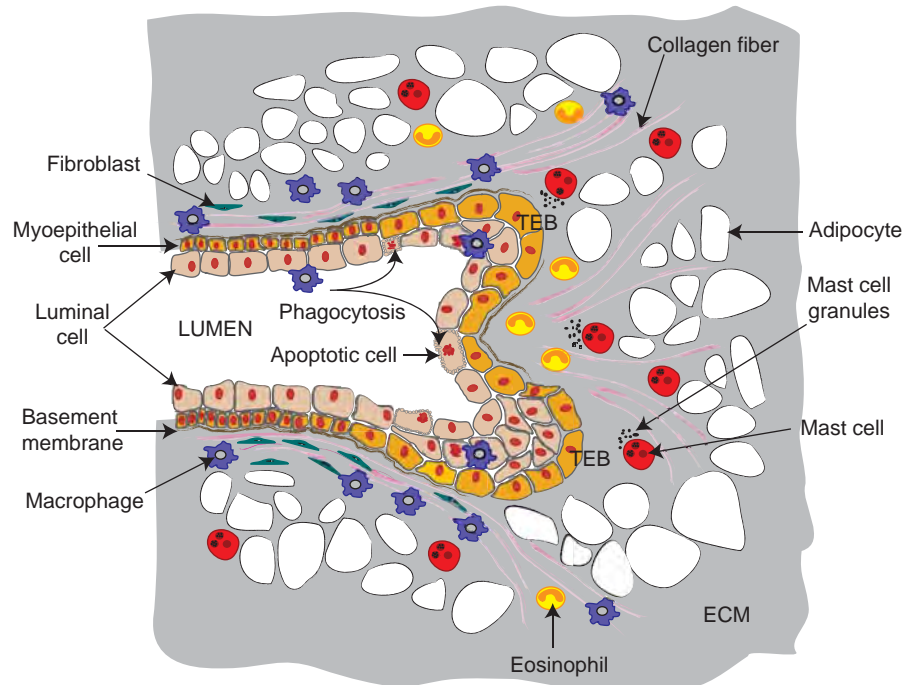


Figure 4. Topography of immune cells in the developing terminal end bud. The diagram shows a schematic of a terminal end bud (TEB) that is bifurcating to give two ductal branches. This TEB is surrounded by a dense fibroblastic stroma and encased by a fibrillar collagen network that is aligned in the 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.

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^{op}*) 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

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mice ductal development was rescued in the homozygous *Csf1^{op}* 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^{ash1}*) 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 multilaminar 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).

Leukocytes in Mammary Development and Cancer

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).

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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 is 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 *Csf1^{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 *Csf1^{op/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). Similarly in farm species where it has been studied extensively, macrophages, neutrophils, and lymphocytes are found in the secretions from involuting glands. It is extremely likely that these macrophages scavenge debris and apoptotic cells (Atabai et al. 2007; Monks and Henson 2009) as well as bacteria (Tatarczuch et al. 2000, 2002). They may therefore be important in the prevention of mastitis in the vulnerable period of milk stasis (Atabai et al. 2007).

This sequential recruitment of neutrophils, macrophages, and lymphocytes at day 3–4 of involution is supported by the transcriptome analysis of involuting mammary glands. These data show expression of a wide range of inflammatory and acute phase molecules in the involuting mammary gland (Stein et al. 2004;

Watson 2009). At 3–4 d postweaning these include markers for monocytes and macrophages such as F4/80, Ly6c, CD11b CSF-1R, and CD14, as well as markers for alternatively activated tissue-remodeling macrophages such as Arginase 1 (Stein et al. 2004; Monks and Henson 2009; 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 *Csf1^{op/op}* mice are unable to efficiently undergo a lactational switch and consequently there is a rapid involution in these mice (Pollard and Hennighausen 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

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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-involution 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)- β 3 (D'Cruz et al. 2002) that in many contexts is tumor inhibiting in part through effects on stem cell senescence (Boulanger and Smith 2001) and inhibition of mammary epithelial cell proliferation (Ewan et al. 2002). TGF β 3 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). Coincidentally macrophages are also important TGF β 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 stroma (and serum), and increased presence of extrafollicular 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 (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;

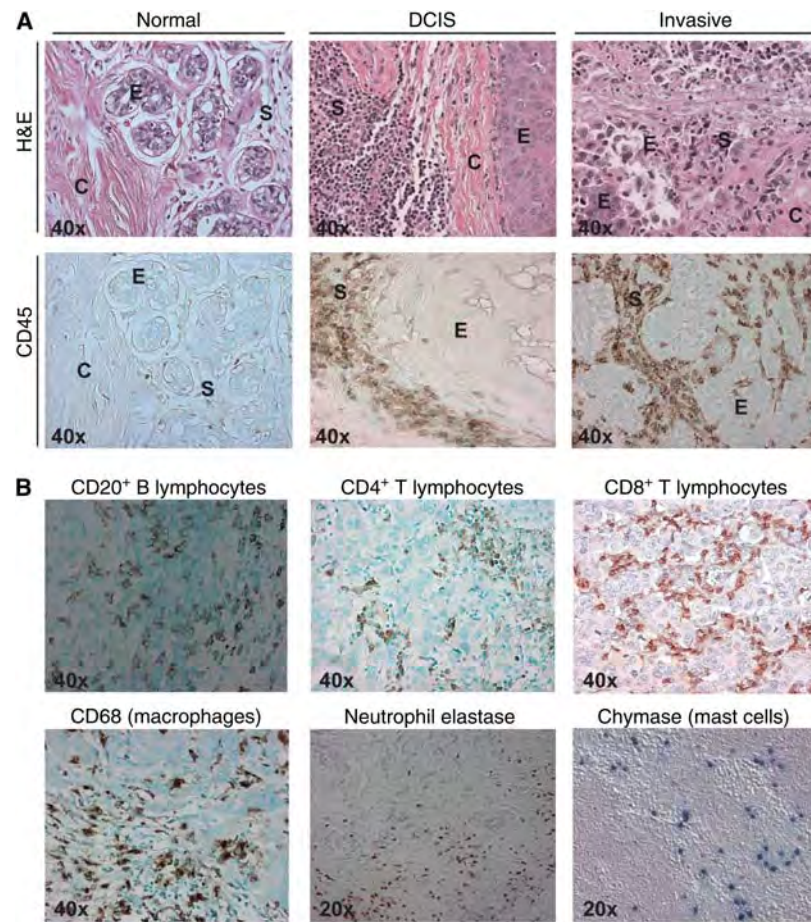


Figure 5. Leukocytes in human breast, ductal carcinoma in situ, and invasive carcinoma. (A) Hematoxylin and Eosin (H&E; top row) staining of *normal* human breast tissue, ductal carcinoma in situ (DCIS) and invasive breast cancer (invasive), showing ductal epithelial structures (E), collagenous stroma (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 (neutrophils elastase⁺; brown staining), and mast cells (chymase⁺; blue staining). Original magnifications are shown for each panel.

De Palma et al. 2005; Okamoto et al. 2005; Lin et al. 2006; Nozawa et al. 2006; Takakura 2006; Lin and Pollard 2007), suppressing antitumor immunity (Blankenstein 2005; Zou 2005; Bronte et al. 2006), and enhancing tumor cell migration and metastasis (Wyckoff et al. 2004; Condeelis and Pollard 2006; DeNardo et al. 2009; Gocheva et al. 2010). Nucleated hematopoietic cells that have been directly implicated in tumor angiogenesis include mast cells

(Coussens et al. 1999; Soucek et al. 2007), tumor-associated macrophages (Pollard 2004; Balkwill et al. 2005; Lewis and Pollard 2006), Tie2-expressing monocytes (De Palma et al. 2005; De Palma and Naldini 2006), neutrophils (Nozawa et al. 2006), dendritic cell precursors (Coukos et al. 2005), and myeloid immune suppressor cells (Yang et al. 2004; Serafini et al. 2006). Other hematopoietic cell types, such as platelets (Kisucka et al. 2006), eosinophils

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(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 2004). By using PyMT mice carrying a *Csf1* null mutation (*Csf1^{op/op}*), it was further shown that depletion of CSF-1 markedly decreased infiltration of macrophages at tumor sites, inhibited

the angiogenic switch and significantly delayed tumor progression. The knockdown of CSF-1 in transplanted tumor cells (by using antisense oligonucleotides) also resulted in inhibition of tumor growth, with tumors showing extensive necrosis and poor vascularization, phenotypes that could be reversed by treatment of mice with CSF-1. The premature macrophage infiltration in the mammary gland of MMTV-LTR-CSF-1 transgenic mice induced robust angiogenesis even at early pre-malignant stages, providing evidence for a direct link between macrophage infiltration and angiogenesis, independent of tumor stage (Lin et al. 2006). These studies have provided evidence that CSF-1 is a major regulator of macrophage recruitment to tumors and shed light on the important roles of macrophages in tumor progression, and in particular with tumor-associated angiogenesis. Myeloid cell-delivery of VEGF to mammary tumors is clearly significant because myeloid-specific deletion of VEGF inhibited the angiogenic switch but surprisingly accelerated tumor development (Stockmann et al. 2008). In this regard, myeloid VEGF regulates formation of high-density vessel networks, attenuation of which blocks angiogenic programming of tumors while also inducing normalization of vasculature that thus results in accelerated tumor progression by decreasing tumor cell death and tumor hypoxia, together indicating that myeloid-derived VEGF-A is essential for tumor-associated alterations of vasculature. It is known that macrophages heavily infiltrate necrotic areas in tumors where they scavenge cellular debris and cooperate with tumor cells to promote angiogenesis (Murdoch et al. 2004; Lewis and Murdoch 2005). In fact, hypoxia stimulates expression of several proangiogenic molecules by activating hypoxia-inducible factors (HIFs) in macrophages. Expression of the monocyte chemoattractant VEGF, endothelin 2, and endothelial monocyte-activating polypeptide II (EMAP II) by hypoxic tumor cells can attract macrophages into hypoxic areas within tumors. It is believed that macrophages are then retained in hypoxic tumor areas because of abrogation of chemotactic signal transduction and the down-regulation

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-1 α through cyclooxygenase (COX)-2. In addition, macrophages also release nitric oxide (NO), a molecule that provokes vasodilation 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 TGF α (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, TNF α regulates epithelial invasion through activation of downstream signaling cascades including Jun amino-terminal kinase (JNK) and nuclear factor κ B (NF κ B) transcription factor. Activated JNK and NF κ B 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 TGF β 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 TGF β -responsive gene signature that 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 costimulatory 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 neovascularization such as TGF β (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 TGF β (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

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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_H2 T cells and T_{reg} cells increases with disease progression.

High percentages 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 axillary 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

part determine malignant and metastatic potential. Although presence of metastatic MECs 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 α , IL-6, IL-12p40, and IL-1 β) and *Nos2* 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 β* , 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 γ , but neither FOXP3 (T_{reg}) nor IL-17a were expanded as compared to wild-type littermates indicating presence of both T_{H1} and T_{H2} effector lineages.

PyMT/IL4R α -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

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of myeloid cells, work from several groups have reported that IL-4 and IL-13 also regulate tumor growth directly through activation of IL-4/13 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 T_H2 cytokines including IL-4 and IL-13 was key (Aspord et al. 2007). Taken together these data indicate that the effects of CD4-derived T_H2 cytokines on tumor development and progression is likely regulated by the organ microenvironment or IL-4/13 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 contains 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 Igs 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 anti-tumor 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

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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, T_H1 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 T_H2 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 T_H2 response can be regulated systemically by the general immune status of the individual, as well as locally by myeloid suppressor cells and T_{reg} cells, and thus offers clinicians with attractive targets for anticancer immune-based therapies.

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

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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^{high}/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

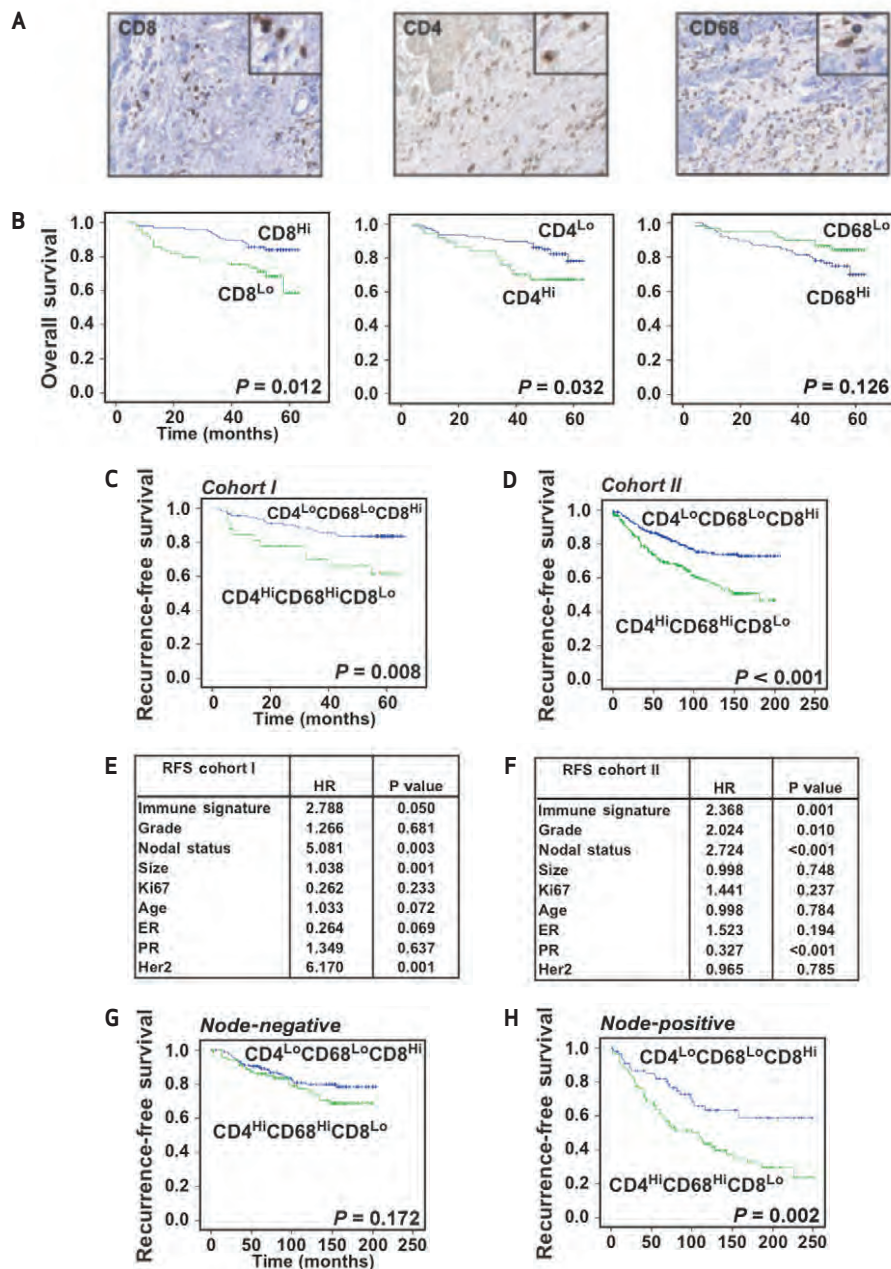
In previous studies, we demonstrated that in the absence of a significant CD8⁺ CTL response, CD4⁺ T lymphocytes indirectly promoted invasion and metastasis of mammary adenocarcinomas by directly regulating the protumor bioactivities of TAMs (11). From these data, we predicted that infiltration of primary 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^{low}/CD4^{low}/CD8^{high} 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^{high}/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 I, $n = 179$; 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 autosome 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^{high}/CD4^{high}/CD8^{low} and CD68^{low}/CD4^{low}/CD8^{high} immune profiles as assigned by random forest clustering to identify optimal thresholds using Cohort I (**C**). Identified CD68^{high}/CD4^{high}/CD8^{low} and CD68^{low}/CD4^{low}/CD8^{high} 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. Kaplan-Meier estimates of RFS comparing CD68^{high}/CD4^{high}/CD8^{low} and CD68^{low}/CD4^{low}/CD8^{high} immune profiles as assigned by random forest clustering of breast cancer tissues. breast cancers were stratified into node-negative (**G**) and node-positive (**H**) patients and analyzed for RFS. The log-rank (Mantel-Cox) *P* value is denoted for difference in RFS.



of each tree model (16). All patients were categorized as having either a CD68^{high}/CD4^{high}/CD8^{low} or a CD68^{low}/CD4^{low}/CD8^{high} 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^{high}/CD4^{high}/CD8^{low} signature (Fig. 1C and D; Supplementary Fig. S2A and B). Multivariate Cox regression analysis revealed that the CD68^{high}/CD4^{high}/CD8^{low} 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^{high}/CD4^{high}/CD8^{low} signature

(Fig. 1H). Multivariate Cox regression analysis revealed that the CD68^{high}/CD4^{high}/CD8^{low} 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 CD68^{high}/CD4^{high}/CD8^{low} 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_H1). 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 chemosensitivity 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^{low}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^{low}F4/80⁺, monocytic immature myeloid cells (iMC) as CD45⁺CD11b⁺Ly6G⁻Ly6C^{high}, and granulocytic iMCs/neutrophils as CD45⁺CD11b⁺Ly6G^{high}Ly6C⁺ (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 monocyte/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 αCSF1 mAb or PLX3397, with no effect on infiltration of CD45⁺CD11b⁺Ly6G^{high} iMCs or CD45⁺CD11b^{low}/Ly6C⁻CD22⁻Ly6G⁻CD11c^{high}MHCII^{high} DCs (Fig. 3A and B; Supplementary Fig. S5A and B). Treatment with αCD11b mAb decreased both TAM and iMC infiltration (Fig. 3A). Analysis of the maturation and differentiation status of TAMs remaining in mammary tumor tissue following αCSF1 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. S3G). 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).

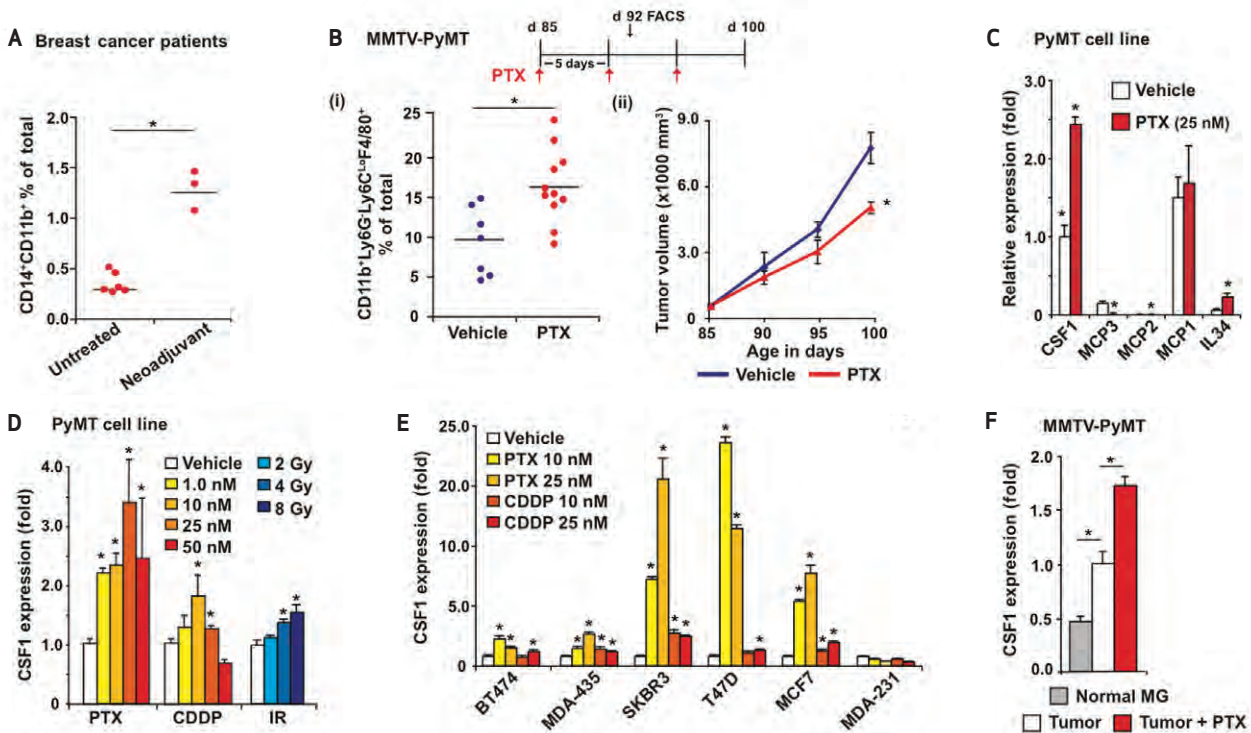


Figure 2. Cytotoxic therapy induces macrophage recruitment, as well as *CSF1* and *IL-34* mRNA expression. **A**, macrophage percentage in fresh human primary breast cancer tissues, depicted as mean of CD45⁺CD11b⁺CD14⁺ macrophages as a percentage of total cells (analyzed by flow cytometry). “Neo-adjuvant” denotes patients who received chemotherapy prior to surgical resection of their primary breast cancer, as opposed to those who did not, denoted as “untreated”; *, statistically significant differences ($P = 0.004$) between the 2 groups. **B**, PTX-induced *CSF1* mRNA expression regulates tumor infiltration of macrophages and limits PTX response. (i), TAM percentage in mammary tumors of MMTV-PyMT mice following PTX treatment with mean number of CD45⁺Ly6G⁺Ly6C⁺CD11b⁺F4/80⁺ TAMs as a percentage of total cells shown (analyzed by flow cytometry); (ii), primary tumor growth reduced by treatment with PTX. The 85-day-old MMTV-PyMT mice were treated with PTX and total tumor burden per animal was assessed every 5 days until endpoint. Treatment schematic is depicted at top, and data are displayed as mean tumor burden \pm SEM; *, statistically significant differences between controls and PTX-treated mice (>8 mice/group). **C**, expression of monocyte/macrophage chemoattractants following chemotherapy. Quantitative reverse transcriptase PCR (qRT-PCR) analyses of *CSF1*, *MCP1*, *MCP2*, *MCP3*, and *IL34* expression in MMTV-pMECs-derived MECs treated with PTX for 24 hours ex vivo, expressed as mean fold change, compared with controls. Samples were assayed in triplicate for each tested condition; *, statistically significant differences between control and PTX-treated groups. **D**, Dose-dependent expression of *CSF1* following chemotherapy or radiation therapy. qRT-PCR analysis of mRNA expression in MMTV-PyMT-derived pMECs 24 hours after treatment with either cisplatin (CDDP), PTX, or a single dose of ionizing radiation, expressed as mean fold change, compared with control \pm SEM. Drug and radiation doses are shown. Samples were assayed in triplicate for each tested condition; *, statistically significant differences between control and the indicated treatment. **E**, *CSF1* expression induced by chemotherapy in human breast carcinoma cell lines. qRT-PCR analysis of mRNA expression in BT474, MDA-MB-435, SKBR3, T47D, MCF7, and MDA-MB-231 at 24 hours after treatment with either CDDP or PTX, expressed as mean fold change, compared with vehicle-treated cells \pm SEM. Chemotherapeutic doses are denoted. Samples were assayed in triplicate for each condition; *, statistically significant differences between control and indicated treatment. **F**, *CSF1* expression induced by cytotoxic therapy in MMTV-PyMT mammary tumors. qRT-PCR analysis of mRNA expression isolated from normal mammary tissue or MMTV-PyMT mammary tumors from mice treated with either PTX (10 mg/kg) every 5 days, or ionizing radiation (single dose of 8 Gy), expressed as mean fold change, compared with vehicle-treated tumors (4 mice/group). SE is depicted; *, statistically significant differences ($P < 0.05$, Mann-Whitney) for all gene expression analyses (**C-F**).

We next treated 80-day-old MMTV-PyMT mice, or mice bearing syngeneic orthotopic PyMT-derived tumors (~ 1.0 cm) with α CSF1, α CD11b, 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 α CSF1/PTX, α CD11b/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 α CSF1/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

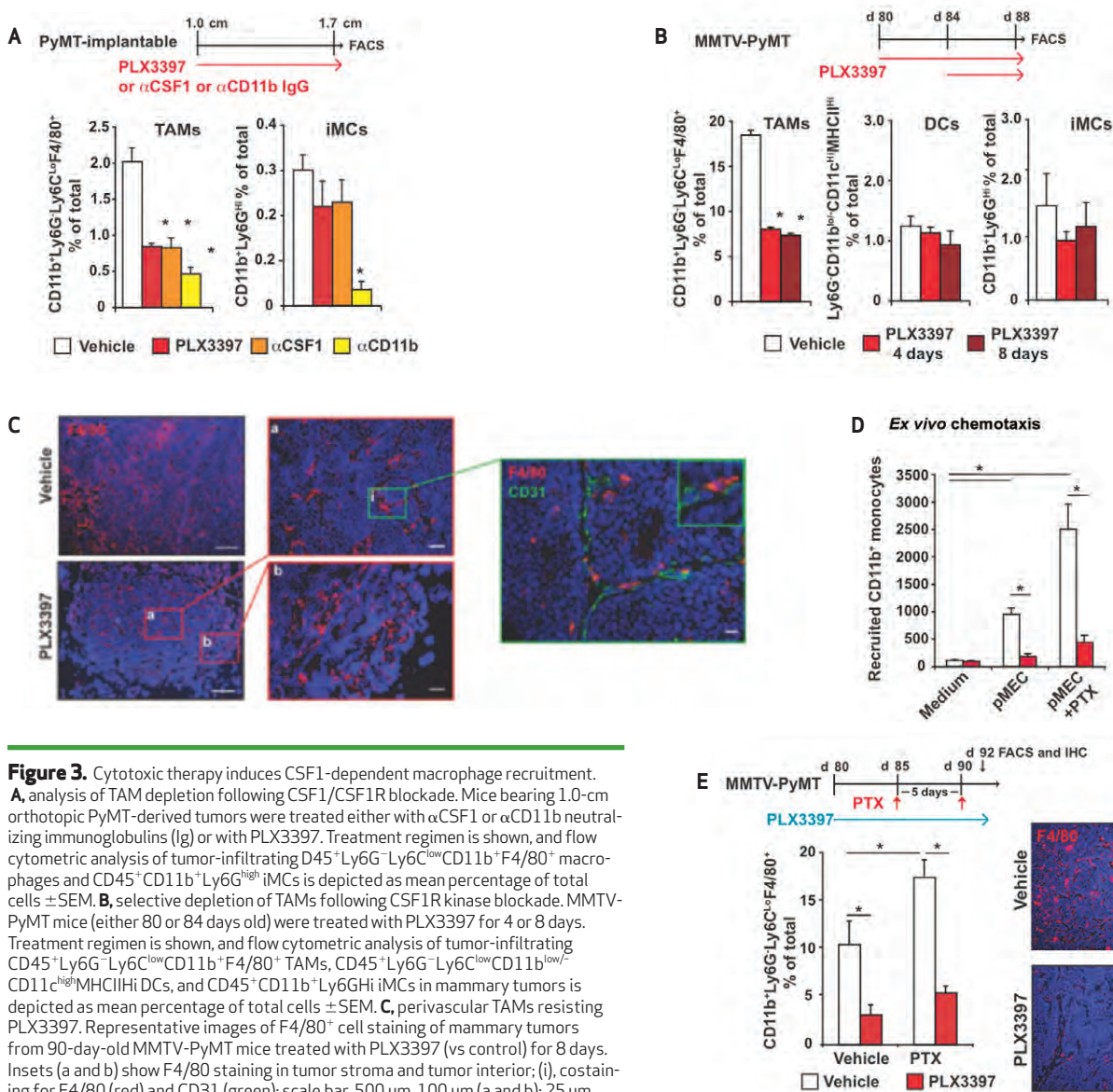


Figure 3. Cytotoxic therapy induces CSF1-dependent macrophage recruitment. **A**, analysis of TAM depletion following CSF1/CSF1R blockade. Mice bearing 1.0-cm or thoptopic PyMT-derived tumors were treated either with α CSF1 or α CD11b neutralizing immunoglobulins (Ig) or with PLX3397. Treatment regimen is shown, and flow cytometric analysis of tumor-infiltrating CD45⁺Ly6G⁻Ly6C^{low}CD11b⁺F4/80⁺ macrophages and CD45⁺CD11b⁺Ly6G^{high}iMCS is depicted as mean percentage of total cells \pm SEM. **B**, selective depletion of TAMs following CSF1R kinase blockade. MMTV-PyMT mice (either 80 or 84 days old) were treated with PLX3397 for 4 or 8 days. Treatment regimen is shown, and flow cytometric analysis of tumor-infiltrating CD45⁺Ly6G⁻Ly6C^{low}CD11b⁺F4/80⁺ TAMs, CD45⁺Ly6G⁻Ly6C^{low}CD11b^{low}/CD11c^{high}MHCII^{hi}DCs, and CD45⁺CD11b⁺Ly6G^{hi}iMCS in mammary tumors is depicted as mean percentage of total cells \pm SEM. **C**, perivascular TAMs resisting PLX3397. Representative images of F4/80⁺ cell staining of mammary tumors from 90-day-old MMTV-PyMT mice treated with PLX3397 (vs control) for 8 days. Insets (a and b) show F4/80 staining in tumor stroma and tumor interior; (i), costaining for F4/80 (red) and CD31 (green); scale bar, 500 μ m, 100 μ m (a and b); 25 μ m (i). **D**, peripheral blood lymphocyte (PBL) migration in response to conditioned medium from MMTV-PyMT MECs treated with either vehicle or PTX (25 nM for 24 hours), evaluated by Boyden chamber assay. CD45⁺CD11b⁺ peripheral blood monocytes after migration to the lower chamber, in the presence or absence of PLX3397 (50 nM), as quantified by flow cytometry. Data are depicted as mean cell number assayed in triplicate. **E**, PTX-induced TAM recruitment inhibited by PLX3397. TAM density in mammary tumors removed from MMTV-PyMT mice following treatment with PTX \pm PLX3397. Treatment regimen is shown with mouse age, and data are depicted as mean number of CD45⁺Ly6G⁻Ly6C^{low}CD11b⁺F4/80⁺ as a percentage of total cells \pm SEM (analyzed by flow cytometry, >5 mice/group). Representative IHC staining for F4/80 (red) and nuclear DNA (blue) from the same cohort of animals is shown. Scale bar, 500 μ m; *, statistically significant differences ($P < 0.05$, Mann-Whitney) in **A–E**.

decreased by PLX3397/PTX therapy (Fig. 5C). This reprogramming of the immune microenvironment was accompanied by increased tumor infiltration of CD45⁺CD11b^{low/-}CD19⁻Ly6G⁻Ly6C^{low}CD11c^{high}MHCII^{high} 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^{low} 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- PDL1 ligand system, in which PDL1/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 PDL1/B7-H1 and PDL2/B7-DC, as well as costimulatory molecules CD80 and CD86, and MHCII. TAMs expressed high levels of MHCII 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

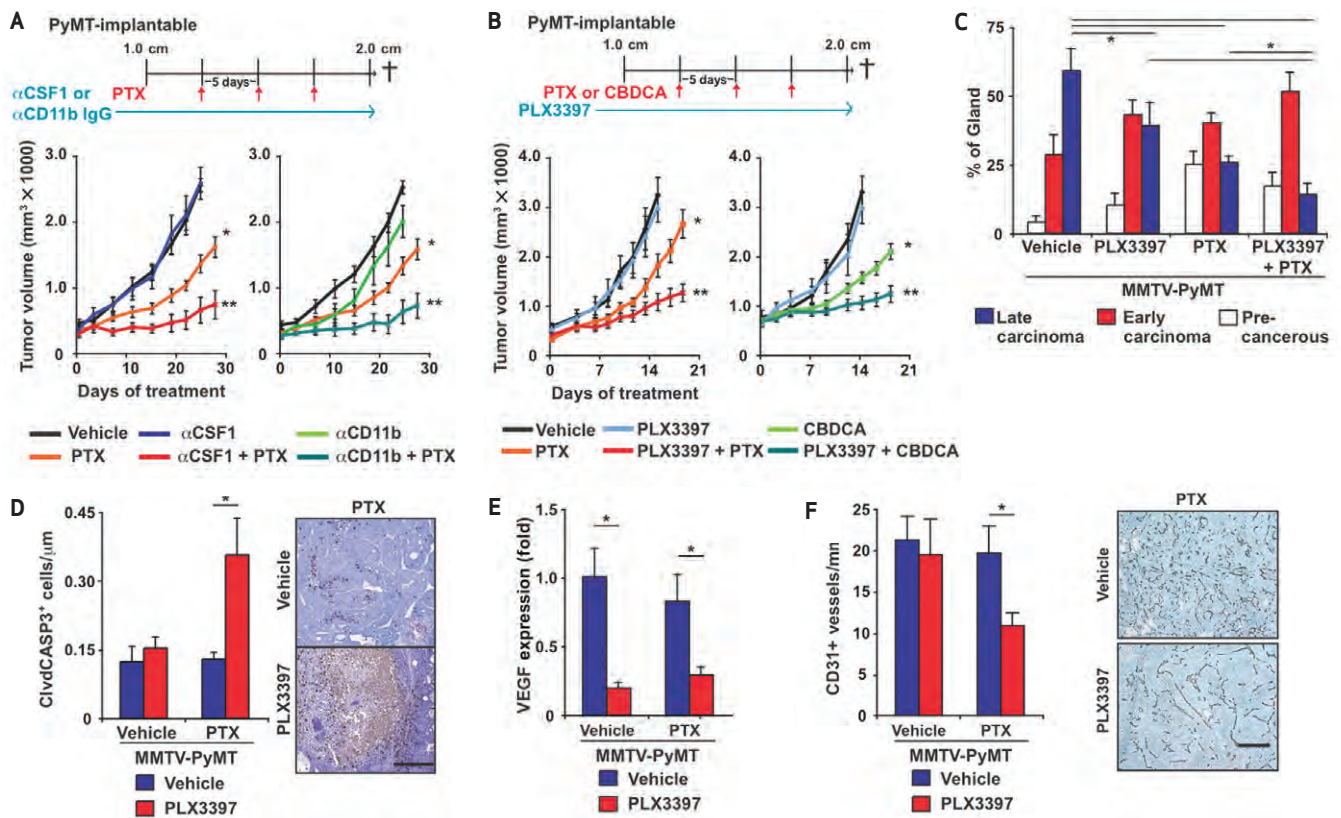
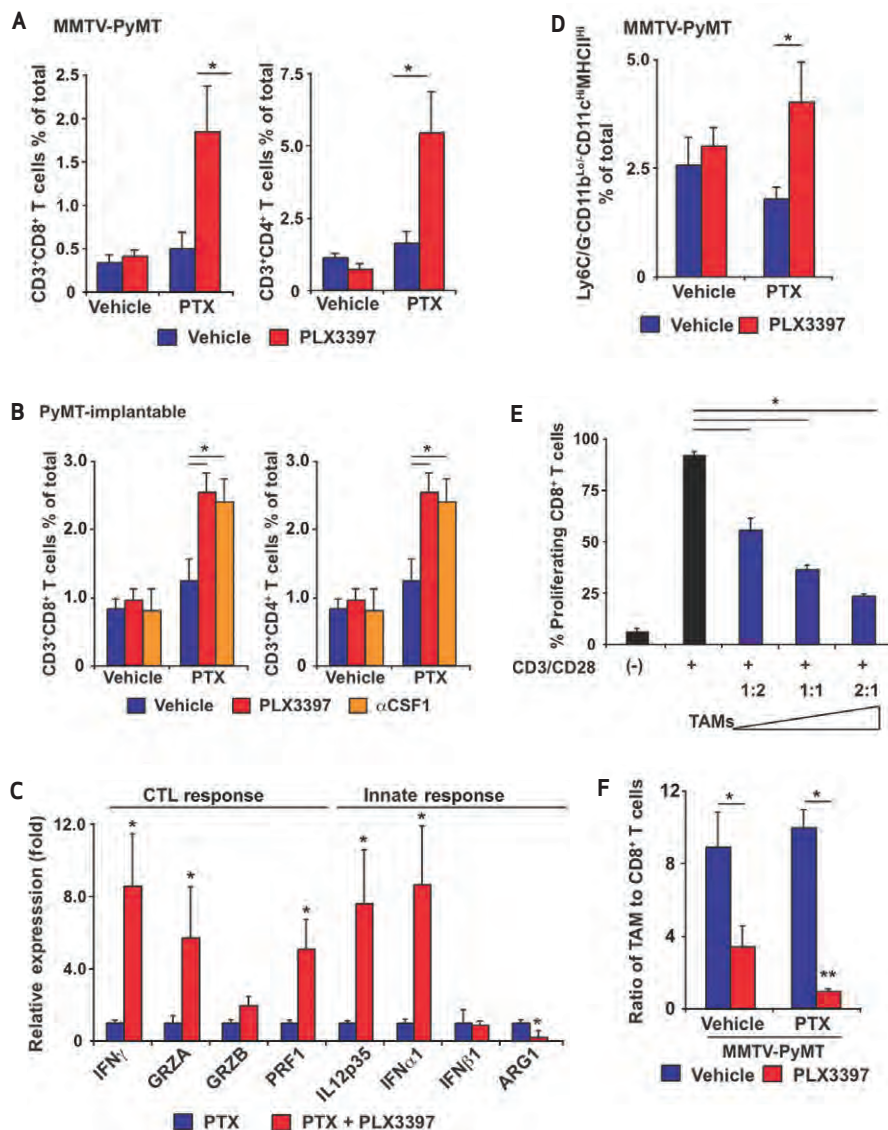


Figure 4. Macrophage depletion improves response to chemotherapy. **A** and **B**, primary tumor growth reduced by treatment with macrophage-depleting agents in combination with chemotherapy. Orthotopic PyMT-derived tumors were grown to a median diameter of 1.0 cm, and mice were then treated with PTX, CBDCA, and/or αCSF1, αCD11b neutralizing Igs ± PLX3397 for 21 or 28 days, with total tumor burden per animal assessed every 2 to 3 days. Treatment regimens are depicted for all cohorts, and data displayed as mean tumor burden ± SEM; *, statistically significant differences between vehicle- and PTX-treated mice. **, significant differences between mice treated with PTX alone and mice treated with PTX/PLX3397 or αCSF1 or αCD11b in combination. **C**, histologic stage analysis of MMTV-PyMT tumors. Tumors from 100-day-old MMTV-PyMT mice treated with PTX or PLX3397 or both were assessed for the 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 PTX or PLX3397 or both versus control (vehicle). Graph depicts mean positive cells per μm² of tumor tissue. Representative images show cleaved caspase-3-positive cells (brown staining) in tumors of MMTV-PyMT mice; scale bar, 500 μm. **E**, VEGF mRNA expression assessed by qRT-PCR of tumor tissue from MMTV-PyMT mice treated with vehicle or PTX or PLX3397 or both. Graph depicts mean fold change in gene expression compared with vehicle-treated control group. **F**, quantification of CD31⁺ positive vessels in mammary tumors from MMTV-PyMT mice treated with PTX or PLX3397 or both, versus control (vehicle). Data represent the mean number of CD31⁺ positive vessels per mm² of carcinoma tissue. Representative photomicrographs show CD31⁺ positive vessels (brown staining); scale bar, 400 μm; *, statistically significant differences ($P < 0.05$, Mann-Whitney) in **C-F**.

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 treatment of mice having orthotopic PyMT-derived tumors with combined PTX/ α CSF1 or PTX/PLX3397 (**B**), compared with controls. Mean values \pm 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 enhanced by combined PTX/PLX3397. Flow cytometric analysis of tumor-infiltrating CD45⁺Ly6G⁻Ly6C^{low}CD11b^{low}-CD11c^{high}MHCII^{high} DCs depicted as mean percentage of positive cells as a percentage 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^{low}CD11b⁺F4/80⁺ TAMs isolated from late-stage mammary tumors of MMTV-PyMT mice. Data are depicted as the percentage of live 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^{low}CD11b⁺F4/80⁺ TAMs to CD3⁺CD8⁺ T lymphocytes depicted as mean ratio (TAM/CD8 CTL) \pm 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. *CD8* 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 *CD68* = *CD8* (denoted *CD68*^{high}/*CD8*^{low}, *CD68*^{low}/*CD8*^{high}, and *CD68*/*CD8*).

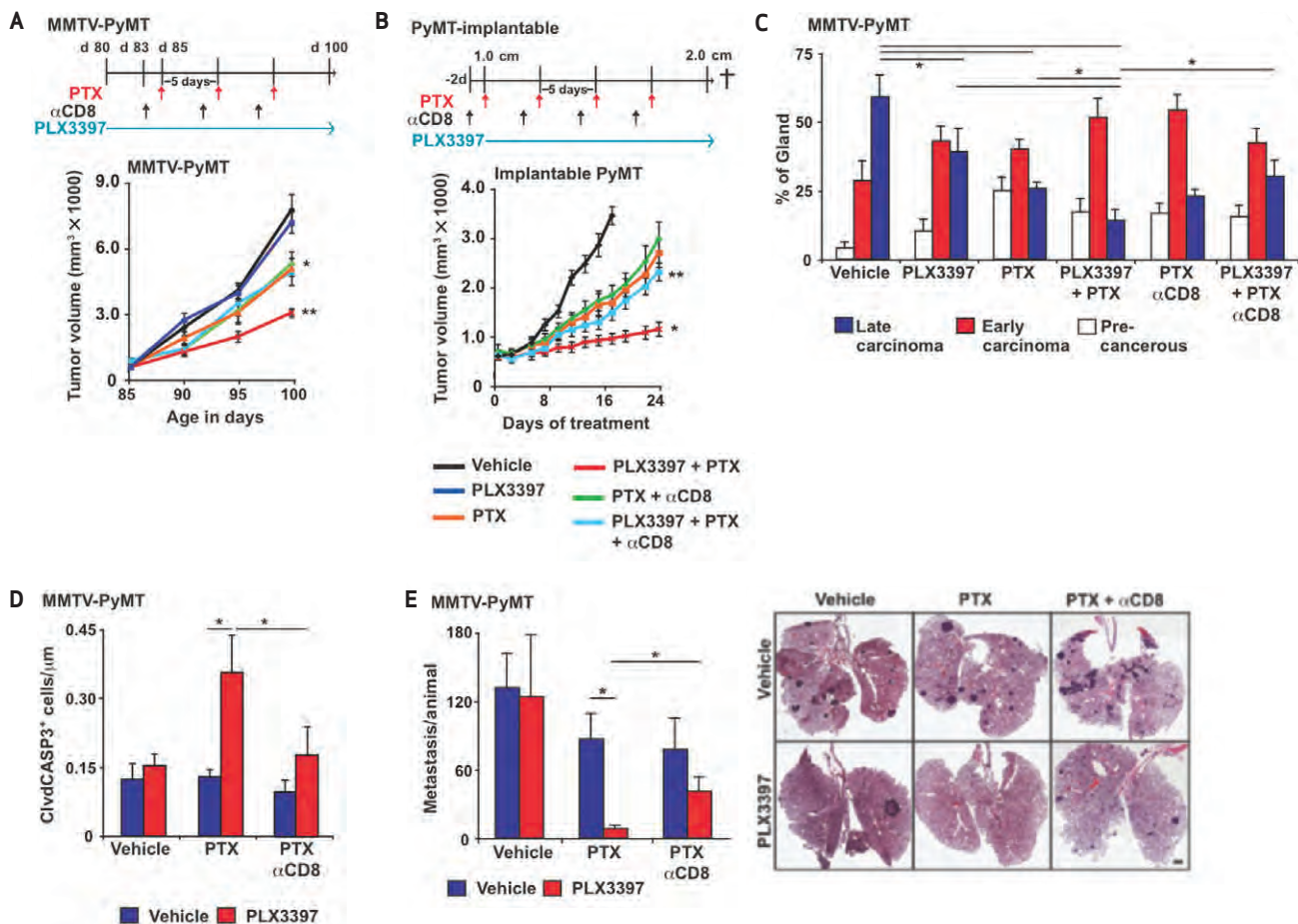


Figure 6. Combined PLX3397 and PTX treatment inhibits metastasis in a CD8-dependent manner. **A** and **B**, improved outcome following PLX3397/PTX treatment dependent on CD8⁺ T cells. **A**, 85-day-old MMTV-PyMT mice were treated with PTX or PLX3397 or both, as well as anti-CD8 IgG. Total tumor burden 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 \pm 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 μ m² 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 μ m apart were stained with hematoxylin and eosin (H&E), and the total number of metastatic foci (>8 cells) was quantified per mouse ($n \geq 10$ 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 μ m.

CD8^{equal}, respectively)—with the CD68^{high}/CD8^{low} group correlating with a significantly lower rate of pCR (7%) and the CD68^{low}/CD8^{high} exhibiting the highest rate of pCR at 27% (Fig. 7A). Thus, the ratio of CD68/CD8a expression represents a predictive response biomarker for neoadjuvant chemotherapy.

We next evaluated CD68 and CD8a mRNA expression in breast cancers representing ~4000 patients assembled from 22 retrospective gene expression datasets (Supplementary Table S8). Median expression for both CD8 and CD68 was used to determine high and low groups. All patients were categorized as either CD68^{high}/CD8^{low} or CD68^{low}/CD8^{high}. Kaplan-Meier estimates of survival demonstrated significantly reduced OS in the CD68^{high}/CD8^{low} group (Fig. 7B). Not surprisingly, these

gene expression results were validated using Kaplan-Meier analysis of OS on IHC data from Cohort I and II stratified for CD68^{high}/CD8^{low} or CD68^{low}/CD8^{high} (Supplementary Fig. S9A and B).

Because breast cancer represents a spectrum of distinct molecular subtypes (luminal A, luminal B, HER2-positive, basal type/triple negative), possessing distinct histopathologic and molecular features and correlating with differential responses to therapy and outcome (28–30), we evaluated CD68/CD8 expression within breast cancer subtypes. CD68^{high}/CD8^{low} expression correlated with reduced OS for breast cancer patients whose tumors were classified as either basal or HER2-positive (Fig. 7C; Supplementary Fig. S10).

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

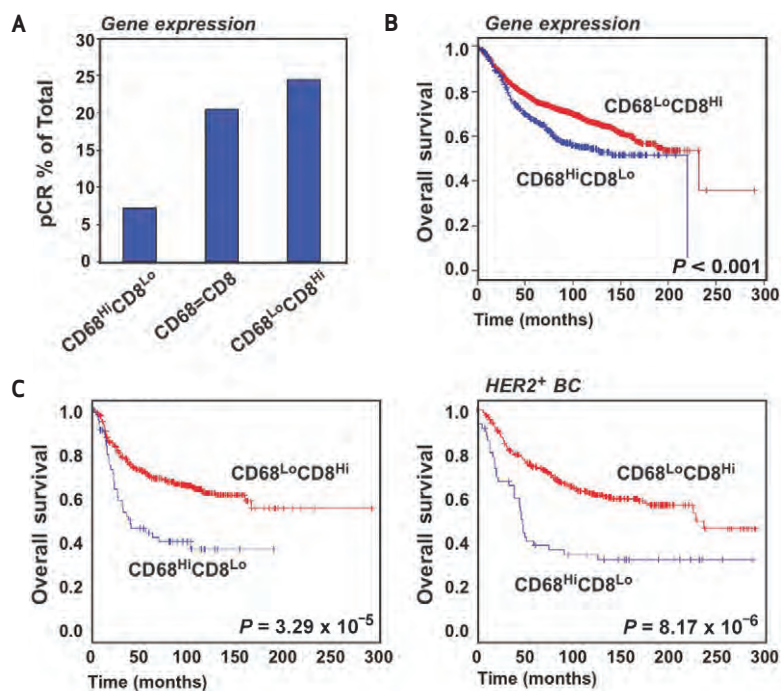
In a previous study, we reported that T-helper 2 (T_H2)-CD4⁺ T-effector cells regulate TAM bioactivity and thereby promote late-stage mammary carcinogenesis and development of pulmonary metastasis (11). We now extend these findings and demonstrate that the complexity of CD8⁺ T lymphocytes, CD4⁺ T lymphocytes (presumably T_H2 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 T_H2 to T_H1 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/CD8 α 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 CD8 α and CD68 mRNA in FNA samples revealed 3 separate groups: CD68 > CD8, CD68 < CD8, and CD68 = CD8 (denoted CD68^{high}/CD8^{low}, CD68^{low}/CD8^{high}, and CD68/CD8^{equal}, respectively). Analysis of the rate of pCR in the groups is shown. **B**, Kaplan-Meier estimate of survival, comparing CD68^{high}/CD8^{low} and CD68^{low}/CD8^{high} 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^{high}/CD8^{low} and CD68^{low}/CD8^{high} immune profiles as assessed by mRNA expression from 3872 patient samples for tumors stratified 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 α CSF1 mAb and PLX3397, a novel small-molecular-weight tyrosine kinase inhibitor, to efficiently deplete CD11b⁺Ly6G⁻Ly6C^{low}F4/80⁺ TAMs (70%) without altering the presence of CD11b⁺Ly6G^{high}F4/80⁻ iMCs or perivascular F4/80⁺ 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 HIF1 α (11, 38, 39). We previously reported that IFN- γ ⁺ CD8⁺ CTL activity is impaired by myeloid-derived ARG1 and nitric oxide synthase that is HIF1 α -dependent (38). Thus, on the basis of inverse correlation between TAMs and CD8⁺ T cells in human breast cancer, and the fact that TAMs infiltrating mammary carcinomas directly suppress CTL activity in a HIF1 α -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 α CSF1 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⁺ and CD8⁺ T-cell infiltration in primary tumors (Fig. 5). Increased presence of CD8⁺ 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⁺Gr1⁺Ly6G⁺ cells, including myeloid-derived suppressor cells, inflammatory monocytes, neutrophils, and iMCs. Human equivalents have been identified as LIN^{low} human leukocyte antigen (HLA)-DR-CD33⁺CD11b⁺ and CD14⁺HLA-DR^{low} cells (40). CSF1R blockade by PLX3397 depleted CD11b⁺Ly6G⁻Ly6C^{low}F4/80⁺ TAMs, but not CD11b⁺Ly6G⁺ cells (Fig. 3C), which are 12-fold less abundant in MMTV-PyMT carcinomas. In contrast, immunosuppressive CD11b⁺Ly6G⁺ 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 monocyte mobilization from bone marrow is impaired by genetic loss of CSF1, but unaltered following pharmacologic or immunologic inhibition of CSF1R. Gr1⁺CCR2⁺CX3CR1^{low} 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^{low}HLA-DR⁻CD33⁺CD11b⁺ or CD14⁺HLA-DR^{low} 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⁺ 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_H1 cytokines including IFN- γ , TNF- α , and granulocyte monocyte colony stimulating factor enhance TAM cytotoxic activity, production of proinflammatory cytokines, and antigen presentation (45). In contrast, tissue macrophages exposed to T_H2 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_H1 cytokine profile and express high levels of HLA-DR (46, 47). In contrast, CD163 and CD204 expressing TAMs (M2/T_H2 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 *IFN α 1* 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⁺ 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⁺ 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_H1 cells, and bias immune responses toward T_H2 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 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, 4.0- μ m 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 \times 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 Plexikon 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 were cardiac-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 μ m 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, CBDCA (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 Plexikon 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,^{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,^{2,3} cell-extrinsic programs regulated by non-neoplastic cells of mesenchymal, vascular and immune origins are also critical determinants for successful metastatic progression.⁴

Chronic infiltration of tissue by leukocytes, *i.e.*, chronic inflammation, is associated with predisposition to cancer.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.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.^{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.^{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.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.^{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 *et al.*, manuscript submitted),^{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).¹⁶ A second CSF-1R ligand, interleukin (IL)-34, possesses similar binding affinities and also regulates TAM recruitment to tissues, but exhibits distinct tissue

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distribution.^{17–19} TAM presence in several types of human cancer correlates with increased vascular density and worse clinical outcomes.^{20–25} 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.¹⁴ 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.^{26–29} 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.³⁰

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, bioeffector function and EGF expression, which in turn regulates invasion, presence of circulating tumor cells (CTCs) and pulmonary metastasis.²⁸ 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- κ B ligand (RANKL).^{31,32} S100A4 protein mediates T cell attraction to developing neoplasms and premetastatic lungs of tumor-bearing mice, and in turn, it stimulates T-cell production of cytokines, particularly granulocyte CSF and eotaxin-2.³² 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.³¹ During prostate carcinogenesis, T lymphocyte and macrophage-derived RANKL induces metastasis through activation and nuclear localization of inhibitor of nuclear factor κ -B kinase subunit alpha leading to repression of *maspin*, a critical suppressor of metastasis.^{33,34} Lung metastasis of mammary carcinomas is also regulated by CCR4⁺ T regulatory cells that can directly kill natural killer (NK) cells.³⁵

Other myeloid cell types implicated in regulating metastasis include neutrophils, mast cells and monocytes harboring T-cell suppressive activity^{36–39} that are potent suppressors of antitumor adaptive immunity and directly facilitate metastasis by regulating angiogenic programs via enhanced metalloproteinase (MMP) activity.^{11,40–42} 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.^{32,43,44}

Proinflammatory Signals that Impact Exit from Primary Tumor Sites

Regulators of the invasive phenotype

For malignant cells to detach from primary tumors and move through their substratum basement membrane, they must transiently acquire a motile and migratory phenotype, sometimes also referred to as epithelial to mesenchymal transition (EMT).^{45,46} This motile state is characterized by loss of homotypic cellular adhesions and apical–basal polarity and increased migratory capabilities. At the molecular level, this transition is largely driven by intrinsic alterations in gene expression, including suppression of *E-Cadherin*, mediated by activation of transcriptional repressors Snail, Slug, Twist and Zeb.⁴⁷

Extrinsic regulation is also important. Specifically, the chronic inflammatory microenvironment, provided by leukocytes and CAFs, plays an important role in regulating the invasive and motile phenotype of potential metastatic cells. Several leukocyte-regulated mediators have been identified as key to these processes. Notably, tumor necrosis factor (TNF)- α secreted by TAMs activates the NF- κ B transcription factor in neoplastic (and other) cells, directly leading to expression of Snail1 and Zeb.^{44,48,49} Other leukocyte-derived cytokines (including IL-6 and IL-23) induce activation of intracellular STAT3 that in turn leads to induced expression of Twist.^{50–52}

Local hypoxia in neoplastic tissue also contributes to induction of motility programs^{53,54} in part by activation of transcription factors hypoxia inducible factor-1 α and NF- κ B, both implicated in EMT via transcription of Snail.⁴⁸ Moreover, the chemokine receptor CXCR4 is upregulated in mammary carcinomas by hypoxia and is associated with invasive behavior in response to its ligand stromal-derived factor-1 (SDF-1/CXCL12).^{55,56} Thus, hypoxic conditions select for a more metastatic phenotype partially through activation of proinflammatory signaling cascades.

Invasion

Movement of malignant cells through basement membrane and stromal matrix requires remodeling of matrix proteins. This process is coordinated by proteolytic enzymes spanning several catalytic classes and includes matrix MMPs, cysteine cathepsins and serine proteases.⁴ Indeed, the increased expression and activity of various proteases has been observed in multiple human and murine tumor types and can be used as a prognostic indicator of shorter survival rates in patients with breast, ovarian, colorectal and head and neck cancers.^{57,58} Although many proteolytic enzymes are produced by motile neoplastic cells, the majority of tumor-promoting proteases are produced by activated stromal cells in the local tumor microenvironment,^{40,59–62} e.g., fibroblasts⁶³ or tumor-associated immune cells. Mast cells, neutrophils and macrophages secrete matrix remodeling proteases^{64–66} implicated in prometastatic activity, as well as serine proteases that are associated with higher tumor grades and lymph node metastasis in breast cancer.³⁷

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 degranulation 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.²⁹ TAMs in turn regulate neoplastic cell motility by secreting factors such as migration-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). Platelets⁷³ 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 cell-derived 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- κ B 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 uteroglobin 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 Fc γ RIIB by metastatic melanoma cells. Fc γ RIIB 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 Fc γ RIIB, 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 Fc γ RIIB 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 Fc γ RIIB and cannot be recognized by Fc γ R of the effector

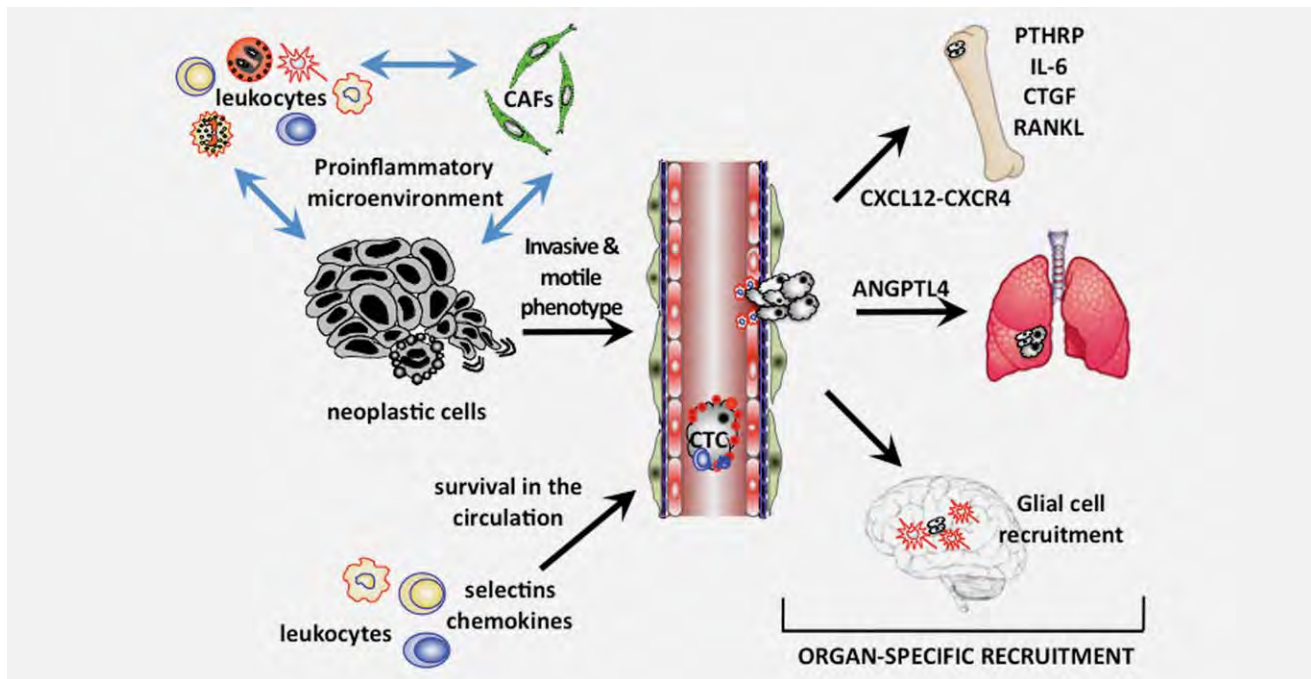


Figure 1. Immune signaling in tumor microenvironments facilitates all stages of tumorigenesis. Soluble mediators secreted by infiltrating and resident leukocytes and by carcinoma-associated fibroblasts (CAFs) within primary tumor sites support signaling programs within neoplastic cells that enable motile and invasive growth. Survival of circulating tumor cells (CTCs) in peripheral blood is facilitated by platelets, neutrophils and production of selectins and chemokine receptors. Organ-specific metastasis is directed by differential expression of chemokines and their receptors that together promote extravasation and retention of CTCs in distal organs. Colonization of distal organs is accomplished by mobilization of leukocytes and other stromal cells in ectopic organs, such as activation of osteoclasts in bone and recruitment of glial cells in brain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells.¹⁰⁵ 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 are critical for the formation of osteolytic metastases.¹⁰⁶ 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.¹⁰⁷ NF- κ B-regulated signaling in breast carcinoma cells promotes osteolytic bone metastasis by induction of osteoclastogenesis via granulocyte macrophage colony-stimulating factor.¹⁰⁸ 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).¹⁰⁹

A Premetastatic Niche

Several studies suggest that primary tumors can “prepare” the distant target organ of metastasis by creating a premetastatic

niche,¹¹⁰ whereas other studies indicate that a small number of metastatic cells activate their new microenvironment on arrival.¹¹¹ 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.¹¹²

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.¹¹³ 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.³⁵ 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.^{99,114} Lysyl oxidase (LOX) is a tumor cell-derived factor often induced in primary tumors in response to hypoxia.¹¹⁵ 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.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.¹¹⁷ Denosumab, an anti-RANKL antibody, originally developed for treatment of osteoporosis, has been found effective for inhibiting bone metastasis in prostate cancer.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.^{119,120}

Several therapeutic agents that limit IL-1 activity are approved for treating chronic inflammatory diseases, *e.g.*, recombinant IL-1R α (anakinra), and neutralizing monoclonal antibodies to IL-1 β 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.¹²¹ Despite their critical involvement in invasion and metastasis, there has been conflicting results with antiproteases, possibly due to antitumorigenic activity of some enzymes.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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Targeting macrophages as a novel therapeutic approach for malignant pleural mesothelioma

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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 mesothelioma 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 macrophage phenotype. Macrophage phenotype and bioactivity is modulated by Th1 versus Th2 cytokine exposure that in turn regulate either an M1 (IFN-gamma & LPS) or M2 (IL-4) phenotype. M1-polarized macrophages increased the response of malignant mesothelioma spheroids to pro-apoptotic agents, such as TRAIL plus anisomycin. Furthermore, our preliminary data indicate that primary human tumor-associated macrophages, isolated from 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 a DoD Mesothelioma Program grant PR080717 (to Broaddus and Coussens).

Abstract for: MRS-AACR Joint Conference on Metastasis and the Tumor Microenvironment

September 7-15, 2010, Philadelphia PA

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 Fc γ R–dependent, during mammary carcinogenesis by contrast, T_H2-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.

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).

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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).

Abstract for: Nature CNIO Cancer Symposium “Frontiers in Tumour Progression”

24th – 27th October 2010

Palacete de los Duques de Pastrana, Madrid, Spain

Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

Lisa M. Coussens. Department of Pathology, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, 513 Parnassus Ave., HSW-450C San Francisco, CA 94143-0502 USA; Phone: 001-415-502-6378; e-mail: Lisa.Coussens@ucsf.edu

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Targeting Macrophages in a Preclinical Model of Mesothelioma

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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.

Specific Aims: (1) Determine the functional significance of macrophage depletion on the effects of chemotherapy in an orthotopic mouse model of mesothelioma. (2) Determine the functional significance of CSF-1R inhibition on the effects of chemotherapy in an orthotopic mouse model of mesothelioma. (3) Determine the functional significance of CSF-1R inhibition on the effects of chemotherapy in a de novo mouse model of mesothelioma.

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.

Abstract for poster presentation: Annual meeting of the Radiological Society of North America (RSNA), Nov. 28- Dec. 3, 2010, Chicago, IL.

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.

The authors acknowledge support from the Department of Defense (W81XWH-06-1-0416, PR080717) to LMC and grants from NIH/NCI R01CA140943 to LMC and HED

Abstract for Invited Talk
Cancer, Immunity, & Microenvironment Program
March 1, 2011
UCSF

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.

Abstract for: American Association of Cancer Research (AACR)
April 2-6, 2011 Orlando, FL

Macrophage-depletion as a Novel Therapeutic Approach for Malignant Pleural Mesothelioma

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Raphael Bueno⁵, David J. Sugarbaker⁵, Thierry M. Jahan^{1,6}, David M. Jablons^{3,6}, V. Courtney
Broaddus⁴, and Lisa M. Coussens^{2,6}

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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 ($31 \pm 4.6\%$ of total CD45⁺), a percentage that is 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, pro-survival, 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 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 NF2^{ko/+} 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**

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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