AD________________

Award Number: DAMD17-01-1-0312

TITLE: STAT6 Deletion Enhances Immunity to Mammary Carcinoma

PRINCIPAL INVESTIGATOR: Suzanne Ostrand-Rosenberg, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland Baltimore County
Baltimore, MD 21250

REPORT DATE: June 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20051101 069

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE
01-06-2005

2. REPORT TYPE
Final

3. DATES COVERED
1 Jun 2001 – 31 May 2005

4. TITLE AND SUBTITLE
STAT6 Deletion Enhances Immunity to Mammary Carcinoma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-01-1-0312

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Suzanne Ostrand-Rosenberg, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland Baltimore County
Baltimore, MD 21250

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT – SEE ATTACHED PAGE

15. SUBJECT TERMS
Tumor immunity/metastatic disease/transcription factors

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE		133	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Abstract

The Stat6 (signal transducer activator of transcription) gene is essential for the production of IL-4 and IL-13, two cytokines that govern the activation of $CD4^{+}$ T helper type 2 (Th2) cells. We hypothesized that mice with a deleted Stat6 gene ($Stat6^{-/-}$) would have enhanced tumor immunity because they would preferentially make tumor-reactive Th1 cells, which are thought to facilitate the activation of $CD8^{+}$ cytotoxic T cells (Tc), thereby improving tumor-specific immune responses. Our preliminary results demonstrate that tumor immunity to a metastatic mammary carcinoma is enhanced in the absence of the Stat6 gene. Although additional experiments demonstrated that tumor rejection in $Stat6^{-/-}$ mice is immunologically mediated by $CD8^{+}$ T lymphocytes, this effect is not due to an improved Th1 response. Therefore, elimination of the Stat6 gene is a potent strategy for enhancing rejection of mammary cancer cells; however, the mechanistic explanation for the improved tumor immunity is not clear. The purpose of this project is to determine the potency of the Stat6 effect for enhancing immunity to metastatic mammary carcinoma, and to identify the mechanism underlying the improved immunity. These experiments will not only provide insight into regulation of anti-tumor immunity, but may also suggest novel approaches for enhancing anti-tumor immune responses.

Table of Contents

Cover..

SF 298..

Introduction.. 4

Body.. 4

Key Research Accomplishments.. 9

Reportable Outcomes... 9

Conclusions.. 10

References... 10

Appendices.. 11

INTRODUCTION

The Stat6 (signal transducer activator of transcription 6) gene is essential for the production of IL-4 and IL-13, two cytokines that govern the activation of $CD4^+$ T helper type 2 (Th2) cells. We hypothesized that mice with a deleted Stat6 gene ($Stat6^{-/-}$ mice) would have enhanced tumor immunity because they would preferentially make tumor-reactive Th1 cells, which are thought to facilitate the activation of $CD8^+$ cytotoxic T cells (Tc), thereby improving tumor-specific immune responses. Our preliminary results demonstrate that tumor immunity to a metastatic mammary carcinoma is enhanced in the absence of the Stat6 gene. Although additional experiments demonstrated that tumor rejection in $Stat6^{-/-}$ mice is immunologically mediated by $CD8^+$ T lymphocytes, this effect is not due to an improved Th1 response. Therefore, elimination of the Stat6 gene is a potent strategy for enhancing rejection of mammary cancer cells; however, the mechanistic explanation for the improved tumor immunity is not clear. The purpose of this project is to determine the potency of the Stat6 effect for enhancing immunity to metastatic mammary carcinoma, and to identify the mechanism underlying the improved immunity. These experiments will not only provide insight into regulation of anti-tumor immunity, but may also suggest novel approaches for enhancing anti-tumor immune responses.

BODY

During the final year of this grant we have made the following progress:

Objective #3. Determine if the $STAT6^{-/-}$ effect is the result of a Type 1 vs. Type 2 response. (As noted in the 2004 report, we have extended these studies to include type 1 and type 2 macrophages).

Obective #5: Determine which cells must be $STAT6^{-/-}$ for enhanced anti-tumor immunity.

During the past year, these two objectives have merged. While trying to decipher which cells must be deleted for STAT6, we observed that another knockout strain of mice, CD1d knockouts, are also highly resistant to 4T1 metastatic disease. This observation led us to examine if immunity in $CD1^{-/-}$ mice is regulated by the same mechanisms that regulate immunity in $STAT6^{-/-}$ mice. These studies led us to better understand resistance in $STAT6^{-/-}$ mice and to demonstrate that immunity to metastatic mammary carcinoma requires three cellular events: 1) Down-regulation/elimination of myeloid-derived suppressor cells (MSC); 2) Induction of cytotoxic M1 macrophages; and 3) Induction of tumor-specific $CD8^+$ T cells. The following sections summarizes these studies:

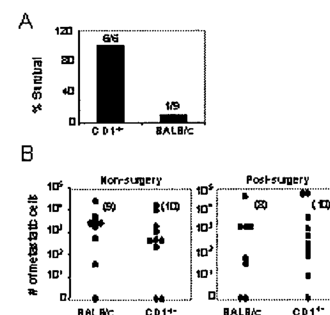


Figure 1. $CD1^{-/-}$ mice are resistant to disseminated metastatic disease. $CD1^{-/-}$ and BALB/c mice were inoculated in the mammary gland on day 0 with 7000 4T1 tumor cells, and primary tumors left in place (non-surgery group) or removed 2-3 weeks later (post-surgery group). **(A)** Post-surgery mice were followed for survival. Numbers indicate the number of mice surviving > 180 days /total mice. Data are from one of two independent experiments. **(B)** Lungs were harvested and the number of metastatic cells quantified by the clonogenic assay. Each symbol represents an individual mouse. Data are pooled from two independent experiments.

<u>CD1-deficient Mice Survive Indefinitely After Surgical Removal of Primary 4T1 Mammary Carcinoma.</u> CD1-deficient and control syngeneic CD1-competent BALB/c mice were injected s.c. in their abdominal mammary gland with 7000 4T1 cells, primary tumors were either left in place (non-surgery group) or surgically removed 2-3 weeks later (post-surgery group), and mice were followed for survival. As shown in **figure 1A**, 100% of post-surgery $CD1^{-/-}$ mice survived >180 days, whereas 89% of the BALB/c mice died with a mean survival time (MST) of 53.4 days. To determine if the differential in survival time between CD1-deficient and BALB/c mice was due to differences in metastatic disease burden, the lungs of non-surgery and post-surgery CD1-deficient and BALB/c mice were removed 30-39 days after 4T1 challenge (9-11 days after surgery for the surgery groups), and tested for metastatic tumor cells. Non-surgery and post-surgery CD1-deficient and BALB/c mice have very similar levels of metastatic cells in their lungs **(figure 1B)**. Therefore, despite the presence of high levels of metastatic tumor, $CD1^{-/-}$ mice whose primary tumors are removed

survive, while BALB/c mice die. To determine if CD1$^{-/-}$ mice survive because they eliminate metastatic cells, lung metastases were quantified by the clonogenic assay in long-term (4-10 month) CD1$^{-/-}$ survivors. These mice had no detectable 4T1 cells and splenic MSC levels were in the normal range (<8%), indicating that post-surgery CD1$^{-/-}$ mice are resistant because they reject 4T1 tumor cells (data not shown).

Myeloid Suppressor Cell Levels Return to Normal in CD1-deficient Mice After Removal of Primary Tumor. Our previous studies demonstrated that regression of 4T1 metastatic disease in STAT6$^{-/-}$ mice was associated with a reduction/elimination of MSC after removal of the primary mammary tumor. In contrast, MSC levels remained elevated after surgery in tumor-susceptible BALB/c mice (1). To determine if the resistance of CD1-deficient mice is related to MSC activity, MSC levels were measured in tumor-bearing CD1-deficient and CD1-competent mice. CD1$^{-/-}$ and wild type BALB/c mice were inoculated with 4T1 tumor cells, and splenocytes were harvested 30-39 days later and analyzed for CD11b^{+} Gr1^{+} cells by flow cytometry. Tumor-free BALB/c and CD1$^{-/-}$ mice have <8% splenic MSC, while tumor-bearing (non-surgery) mice have elevated levels of MSC (**figure 2A**). Therefore, non-surgery CD1-deficient and CD1-competent mice both have elevated levels of MSC relative to tumor-free mice.

To determine if surgical removal of primary tumor differentially affects MSC levels, BALB/c and CD1-deficient mice were inoculated with 4T1 tumor, primary tumors were removed 21-28 days later, and splenocytes were analyzed 9-11 days later (day 30-39 after initial tumor inoculation). After surgery, MSC levels in 90% of post-surgery CD1$^{-/-}$ mice are within the normal range (< 8%), while only 21% of post-surgery BALB/c mice have < 8% MSC (**figure 2A**). Therefore, while MSC levels are high in the spleens of both BALB/c and CD1$^{-/-}$ mice when primary tumor is present, MSC levels drop to the normal level in most post-surgery CD1$^{-/-}$ mice.

The accumuation of MSC is most likely driven by factors secreted by tumor cells (2). Therefore, the dramatic decrease in post-surgery CD1-deficient mice may be because they have less metastatic disease than wild type BALB/c mice. To test this hypothesis, the numbers of metastatic cells in the lungs of the post-surgery mice of figure 1A were graphed with the percent of splenic MSC from the corresponding mice (**figure 2B**). Both wild type BALB/c and CD1-deficient mice have extensive metastatic disease, and there is no correlation between percentage of MSC and the number of metastatic cells. Therefore, the decrease in MSC in CD1-deficient mice after surgery is independent of metastatic tumor burden.

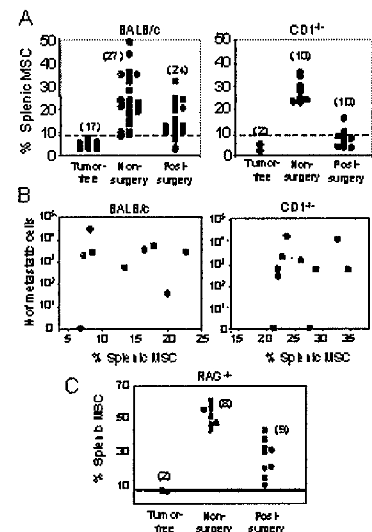


Figure 2. Splenic MSC levels return to baseline after removal of primary tumor from CD1$^{-/-}$ mice. BALB/c or CD1$^{-/-}$ (A and B) or RAG$^{-/-}$ (C) mice were inoculated on day 0 with 4T1 tumor cells and primary tumors left in place (non-surgery groups) or removed on day ~28 (post-surgery groups). Tumor-free groups were not given 4T1. On day ~38 lungs and spleens were harvested, splenocytes were labeled with Gr1-PE and CD11b-FITC to determine percent MSC (**A and C**), and the number of metastatic cells in the lungs quantified by the clonogenic assay (**B**). Each symbol represents an individual mouse. Numbers in ( ) indicate the number of mice per group. Dotted lines indicate the maximum amount of MSC in tumor-free mice (<8%). Data are pooled from two independent experiments. * and ** indicates statistically significantly different values ($p<0.05$)

Although MSC levels are driven by tumor-secreted factors, lymphocytes may also play a role. To determine if the decrease in MSC is lymphocyte-dependent, splenic MSC levels were determined for BALB/c RAG$^{-/-}$ mice inoculated with 4T1 according to the schedule for BALB/c and CD1$^{-/-}$ mice in figure 2A. The baseline level of Gr1^{+}CD11b^{+} splenocytes in tumor-free RAG$^{-/-}$ mice is <8%, while non-surgery RAG$^{-/-}$ mice have significantly ($p < 0.01$) more MSC than BALB/c or CD1$^{-/-}$ mice (**figure 2**). After surgery, MSC in RAG$^{-/-}$ mice remain significantly higher than in BALB/c or CD1$^{-/-}$ mice ($p<0.01$).

To determine if CD4^{+} and/or CD8^{+} T cells are also involved in immune surveillance, post-surgery CD1$^{-/-}$ mice were in vivo depleted for CD4^{+} or CD8^{+} T cells or treated with irrelevant antibodies. Both CD4^{+} and CD8^{+} T cells are required for tumor resistance since 100% of CD4-depleted (3/3) and 80% of CD8-depleted (4/5) post-surgery CD1$^{-/-}$ mice, but none of the irrelevant antibody treateded mice (3/3), die. Therefore, lymphoid cells are essential for tumor rejection and may act by reducing the amounts of MSC in post-surgery BALB/c mice.

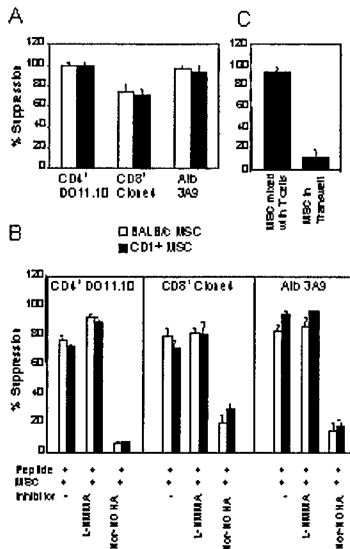


Figure 3. MSC inhibit T cell activation by an arginase-dependent mechanism. **(A)** Syngeneic DO11.10 or clone 4 or allogeneic 3A9 transgenic splenocytes were stimulated with $OVA_{323\text{-}339}$, $HA_{518\text{-}526}$, or HEL protein, respectively, in the presence of non-surgery BALB/c (open bars) or $CD1^{-/-}$ (filled bars) MSC. **(B)** The experiment of part A was repeated with or without the inhibitors for iNOS (L-NMMA) or arginase (nor-NOHA). MSC from 2-3 mice were pooled for each group. Control J774 cells gave no suppression (not shown). **(C)** DO11.10 T cells were stimulated with $OVA_{323\text{-}339}$ peptide and co-cultured in the same well with $CD1^{-/-}$ MSC, or the MSC were contained in a transwell chamber suspended in the well containing the T cells.

MSC Inhibit T Cells by an Arginase-dependent Mechanism. $CD1^{-/-}$ mice may have greater tumor immunity because their MSC are less suppressive than MSC of BALB/c mice. To test this possibility, splenocytes from non-surgery BALB/c and $CD1^{-/-}$ mice were MACS purified for Gr1. The resulting MSC were then co-cultured with antigen-specific $CD4^+$ or $CD8^+$ syngeneic T cells or $CD4^+$ allogeneic T cells plus the appropriate peptide ($H\text{-}2^d$ DO11.10 with OVA-peptide, $H\text{-}2^d$ clone 4 with HA-peptide, or $H\text{-}2^k$ 3A9 with HEL, respectively), and T cell activation measured by ^{3}H-thymidine uptake (**Figure 3A**). On a per cell basis, purified BALB/c and $CD1^{-/-}$ MSC were equally capable of suppressing syngeneic $CD4^+$ or $CD8^+$, or allogeneic $CD4^+$ T cells.

MSC are thought to mediate their effects via the production of arginase and/or inducible nitric oxide synthase (iNOS) (3). To ascertain if $CD1^{-/-}$ MSC mediate suppression by either of these molecules, DO11.10 transgenic T cells were co-cultured with $CD1^{-/-}$ MSC in the presence of OVA-peptide and the arginase inhibitor nor-NOHA, or the iNOS inhibitor L-NMMA, and T cell proliferation measured by ^{3}H-thymidine uptake. The arginase inhibitors, but not the iNOS inhibitor, reverses the suppression (**figure 3B**). Therefore, $CD1^{-/-}$ MSC inhibit T cell activation via arginase production.

To determine if suppression requires direct contact between the MSC and T cells, $CD1^{-/-}$ MSC were suspended in transwell chambers in wells containing OVA-peptide-pulsed DO11.10 T cells (**figure 3C**). Proliferation of DO11.10 cells was not inhibited when the MSC were separated from the T cells by a semi-permeable membrane. Therefore, suppression requires direct contact between the MSC and the affected T cells.

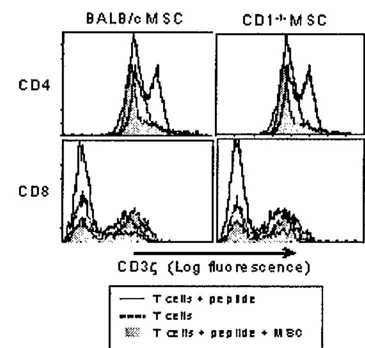


Figure 4. MSC down-regulate CD3ζ chain in $CD4^+$, but not $CD8^+$, T cells. $CD4^+$ DO11.10 or $CD8^+$ clone 4 transgenic splenocytes were stimulated with OVA or HA peptide, respectively, in the presence of non-surgery BALB/c or $CD1^{-/-}$ MSC. Cultures were harvested and cells stained with CD3ζ-FITC, KJ1-26-tricolor, and CD4-PE or CD3ζ-FITC, Vβ8-PE, and CD8-tricolor. CD3ζ expression was determined by gating on double positive cells. Dotted lines are unstimulated T cells (no peptide); solid lines are stimulated T cells (with peptide); filled histograms are stimulated T cells with MSC.

MSC Down-regulate T Cell Receptor-associated Zeta Chain in $CD4^+$, but not $CD8^+$ T Cells. Ochoa and colleagues have demonstrated that T cell dysfunction caused by macrophages or bacteria is associated with the down-regulation of the TCR-associated CD3ζ chain (4, 5). To determine if MSC induce suppression by this mechanism, OVA peptide-pulsed $CD4^+$ DO11.10 T cells were co-cultured with MSC from BALB/c or $CD1^{-/-}$ mice. Following three days of incubation, the cultures were harvested, and the cells triple labeled for CD3ζ, CD4, and the D011.10 clonotype (KJ1-26). The cells were analyzed by flow cytometry by gating on the $DO11.10^+$ $CD4^+$ double positive population and assessing CD3ζ expression. Fifty-three percent of D011.10 transgenic T cells co-cultured with OVA peptide have elevated levels of CD3ζ chain (**figure 4**, top two panels). If BALB/c or $CD1^{-/-}$ MSC are added to the cultures, then only 17% and 15% of the T cells, respectively, have elevated CD3ζ expression. Therefore, BALB/c and $CD1^{-/-}$ MSC reduce CD3ζ chain expression which probably inhibits T cell activation by inhibiting signal transduction.

To determine if MSC also suppress the activation of $CD8^+$ T cells via the down-regulation of CD3ζ, $CD8^+$ clone 4 T cells were cultured as per the $CD4^+$ DO11.10 T cells but with HA peptide. The resulting cells were gated on the $CD8^+$ $V\beta8^+$ double positive population and analyzed for CD3ζ expression (**figure 4,** bottom two panels). Similar to the $CD4^+$ T cells, more than half of the activated $CD8^+$ T cells had elevated

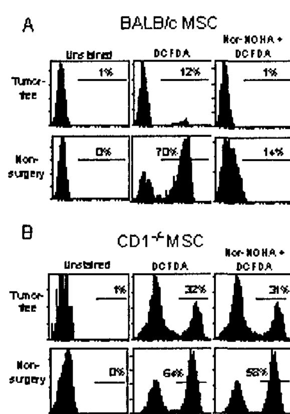


Figure 5. ROS production by BALB/c, but not CD1$^{-/-}$, MSC is arginase-dependent. Purified BALB/c **(A)** and CD1$^{-/-}$ **(B)** MSC from tumor-free and non-surgery mice were unstained, or incubated with DCFDA in the presence or absence of the arginase inhibitor, nor-NOHA.

levels of CD3ζ. However, in contrast to the CD4$^+$ T cells, CD3ζ levels did not decrease following co-culture with either BALB/c or CD1$^{-/-}$ MSC. Therefore, BALB/c and CD1$^{-/-}$ MSC suppress CD4$^+$ T cells by down-regulating CD3ζ chain, but suppress CD8$^+$ T cells via a different mechanism.

BALB/c and CD1$^{-/-}$ MSC Produce Reactive Oxygen Species (ROS). Gabrilovich and colleagues have shown that production of ROS is a characteristic of MSC (6) and we have previously noted that ROS production characterizes different populations of MSC (1). To determine if ROS are differentially expressed in BALB/c vs. CD1$^{-/-}$ MSC, splenic MSC were MACS purified from tumor-free and non-surgery mice and analyzed by flow cytometry for ROS. Staining with DHE, which measures superoxide, was negative (data not shown). Staining with DCFDA, which measures hydrogen peroxide, hydroxyl radical, peroxynitrile, and superoxide, demonstrates that Gr1$^+$CD11b$^+$ splenic cells from non-surgery BALB/c (**figure 5A**) and CD1$^{-/-}$ (**figure 5B**) mice contain more ROS than MSC from the corresponding tumor-free mice. To assess if arginase is involved in ROS production, the arginase inhibitor nor-NOHA was added to the purified Gr1$^+$CD11b$^+$ cells prior to their staining with DCFDA. Although nor-NOHA has no effect on ROS expression in CD1$^{-/-}$ MSC, it inhibits ROS expression in BALB/c MSC. Therefore, MSC from both BALB/c and CD1$^{-/-}$ mice contain ROS; however, ROS expression in the CD1$^{-/-}$ MSC is arginase-independent, while in BALB/c MSC it is arginase-dependent.

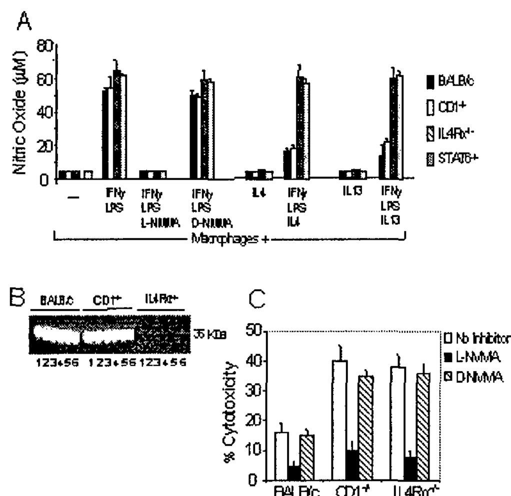


Figure 6. CD1$^{-/-}$ mice have tumoricidal iNOS-producing M1 macrophages. **(A)** Peritoneal macrophages from BALB/c, CD1$^{-/-}$, IL-4Rα$^{-/-}$, or STAT6$^{-/-}$ mice were not activated, activated with LPS and IFNγ, and/or treated with IL-4 or IL-13 in the presence or absence of the iNOS inhibitor, L-NMMA or its inactive enantiomere, D-NMMA. NO was measured using the Griess reagent. **(B)** Peritoneal macrophages were prepared as in 6A, and cell lysates western blotted with mAb to arginase. Lane 1: not-activated; lane 2: activated with LPS and IFNγ; lane 3: pre-treated with IL-4 before activation with LPS and IFNγ; lane 4: pre-treated with IL-13 before activation with LPS and IFNγ; lane 5: pre-treated with IL-4, not activated; lane 6: pre-treated with IL-13, not activated. **(C)** LPS and IFNγ activated BALB/c, CD1$^{-/-}$, or IL-4Rα$^{-/-}$ peritoneal macrophages were co-cultured with 4T1 cells in the presence or absence of the iNOS inhibitor L-NMMA, and percent cytotoxicity measured by LDH release. Activated CD1$^{-/-}$, or IL-4Rα$^{-/-}$ macrophages are significantly more cytotoxic than BALB/c macrophages ($p < 0.05$).

CD1$^{-/-}$ Mice Have Tumoricidal M1 Macrophages. iNOS-producing M1 macrophages are associated with heightened anti-tumor immunity and inhibition of tumor progression (7-9). IL-4 and IL-13 are known to polarize macrophages away from an M1 phenotype and towards an M2 phenotype (8, 9). Since CD1$^{-/-}$ mice lack NKT cells, which are a major source of IL-13 (10-13), they may preferentially generate M1 macrophages, which may contribute to tumor resistance. Since resistance to metastatic disease in STAT6$^{-/-}$ mice is dependent on the presence of M1 macrophages, we were particularly interested in determining if they were also required for resistance of CD1-deficient mice. To test this hypothesis, peritoneal macrophages from BALB/c and CD1$^{-/-}$ mice were activated in vitro with LPS and IFNγ and assayed for iNOS production. LPS and IFNγ activated macrophages from STAT6$^{-/-}$ and IL-4Rα$^{-/-}$ mice were used as controls. The IL-4Rα is a common chain that is shared between the receptors for IL-4 and IL-13 and hence is required for transmitting signals for both of these cytokines (14, 15). Therefore, STAT6$^{-/-}$ and IL-4Rα$^{-/-}$ macrophages should make iNOS regardless of the presence or absence of IL-4 and/or IL-13 (16). Macrophages from all four strains that are activated in vitro with LPS and IFNγ in the absence of IL-4 or IL-13, produce iNOS (**figure 6**). However, if the macrophages are treated with IL-4 or IL-13 prior to activation with LPS and IFNγ, then

BALB/c and $CD1^{-/-}$ macrophages make much less iNOS, whereas iNOS production by $STAT6^{-/-}$ and IL-$4R\alpha^{-/-}$ is unaffected. Since BALB/c mice will produce IL-4 and/or IL-13 in vivo, their macrophages will not make significant levels of iNOS, and hence BALB/c mice will not have M1 macrophages. In contrast, $CD1^{-/-}$ mice will have iNOS-producing M1 macrophages in vivo because they have diminished levels of IL-4 and IL-13 since they lack NKT cells.

The production of arginase has been associated with M2 type macrophages which are thought to promote tumor progression (7-9). To determine if arginase production by macrophages is associated with tumor progression, BALB/c, $CD1^{-/-}$, and IL-$4R\alpha^{-/-}$ peritoneal macrophages were isolated and tested for arginase by western blot (**figure 6B**). Macrophages were either not activated (lane 1), activated with LPS plus IFNγ (lane 2), pretreated with IL-4 before LPS and IFNγ activation (lane 3), pretreated with IL-13 before LPS and IFNγ activation (lane 4), unactivated and treated with IL-4 (lane 5), or not activated and treated with IL-13 (lane 6). BALB/c and $CD1^{-/-}$ macrophages, regardless of treatment, contain arginase, whereas IL-$4R\alpha^{-/-}$ macrophages contain very little, if any arginase.

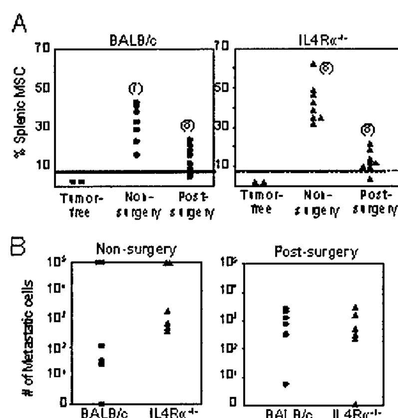


Figure 7. IL-$4R\alpha^{-/-}$ mice are not resistant to 4T1 metastatic disease and retain elevated levels of MSC after surgery. IL-$4R\alpha^{-/-}$ and BALB/c mice were inoculated in the mammary gland on day 0 with 7000 4T1 tumor cells, and primary tumors left in place (non-surgery groups) or removed on day ~28 (post-surgery groups). Tumor-free mice were not given 4T1. **(A)** Lungs and spleens were harvested on day ~38 and splenocytes were labeled with Gr1-PE and CD11b-FITC to determine percent MSC. **(B)** The number of metastatic cells in the lungs was quantified by the clonogenic assay. Data for (A) and (B) are pooled from two independent experiments and include 6 mice/group. Dotted lines indicate the maximum amount of MSC in tumor-free mice (<8%). Each symbol represents an individual mouse.

Macrophage tumoricidal activity is attributed to iNOS production (9), so $CD1^{-/-}$ macrophages may be tumoricidal even though they also contain arginase. To test this hypothesis, BALB/c, $CD1^{-/-}$, and IL-$4R\alpha^{-/-}$ peritoneal macrophages were harvested, activated in vitro with LPS and IFNγ and tested for cytotoxic activity against 4T1 tumor cells. $CD1^{-/-}$ and positive control IL-$4R\alpha^{-/-}$ macrophages are significantly more cytotoxic than BALB/c macrophages (**figure 6C**) ($p<0.05$). The cytotoxicity is due to iNOS, since addition of the iNOS inhibitor, L-NMMA, eliminates the cytotoxic effect, whereas the inactive inhibitor D-NMMA has no effect. Therefore, although $CD1^{-/-}$ macrophages contain both iNOS and arginase, they have strong tumoricidal activity indicating they are polarized towards the M1 phenotype. To confirm the role of M1 macrophages in tumor resistance, macrophages were depleted from post-surgery $CD1^{-/-}$ mice by treatment with liposomes loaded with clodronate. Macrophage-depleted (3/3) mice were dead by 42 days after injection of primary tumor, whereas, mice treated with PBS loaded liposomes survived (MST > 83 days). Therefore, NO-producing M1 macrophages are essential for the survival of post-surgery $CD1^{-/-}$ mice, as they are for resistance of $STAT6^{-/-}$ mice.

IL-$4R\alpha^{-/-}$ Mice are Tumor Susceptible and Maintain Elevated Levels of MSC After Surgery. If the presence of M1 macrophages is sufficient for tumor resistance, then IL-$4R\alpha^{-/-}$ mice, which have tumoricidal M1 macrophages, may survive after removal of primary tumor. To test this possibility, BALB/c and IL-$4R\alpha^{-/-}$ mice were inoculated with 4T1, primary tumors surgically removed 2-3 weeks later, and the mice followed for survival. IL-$4R\alpha^{-/-}$ mice are just as susceptible as BALB/c mice (5/6 IL-$4R\alpha^{-/-}$ vs. 7/8 BALB/c mice die), indicating that despite the presence of M1 macrophages, IL-$4R\alpha^{-/-}$ mice do not have heightened tumor immunity. Because tumor immunity in $CD1^{-/-}$ (see figure 2) and $STAT6^{-/-}$ (1) mice is associated with a rapid decrease to baseline in MSC, we assessed MSC levels in tumor-bearing non-surgery and post-surgery IL-$4R\alpha^{-/-}$ mice. Non-surgery IL-$4R\alpha^{-/-}$ mice have elevated levels of MSC (**figure 7A**), and MSC remain elevated after surgery similar to BALB/c ($p \geq 0.05$), with only 14% of IL-$4R\alpha^{-/-}$ mice having normal levels (<8% MSC). Likewise, post-surgery IL-$4R\alpha^{-/-}$ mice contain high levels of metastatic cells (**figure 7B**). Therefore, although IL-$4R\alpha^{-/-}$ mice generate tumoricidal M1 macrophages, they are not tumor resistant and they have elevated levels of MSC, even after removal of primary tumor.

The studies with CD1-deficient mice strongly support our findings in $STAT6^{-/-}$ mice that resistance to metastatic 4T1 mammary carcinoma in post-surgery mice requires a reduction in MSC, the generation

of M1 macrophages, and tumor-specific $CD8^+$ T cells. Since these characteristics are needed in both mouse strains, it is likely that these findings are generally applicable.

KEY ACCOMPLISHMENTS

- Demonstrated that CD1-deficient mice reject metastatic 4T1 mammary carcinoma and survive indefinitely if their primary tumors are surgically removed.

- Demonstrated that MSC levels are elevated in CD1-deficient mice with primary tumors, but regress to normal levels after surgery.

- Demonstrated that $CD4^+$ and $CD8^+$ T cells are required for resistance to metastatic disease.

- Demonstrated that MSC inhibit T cell activation by the production of arginase and that they down-regulate CD3-zeta chain in $CD4^+$ T cells, but not in $CD8^+$ T cells.

- Demonstrated that CD1-deficient mice have M1 macrophages that mediate tumor cell killing by the production of iNOS.

- Demonstrated that the polarization of macrophages towards an M2, non-tumoricidal
- phenotype is regulated by IL-13 and/or IL-4 via the IL-4Ralpha.

- Concluded that the combination of M1 macrophages, regression of MSC, and induction of tumor-specific $CD8^+$ T cells is required for rejection of established metastatic mammary carcinoma and survival.

REPORTABLE OUTCOMES

- The following peer-reviewed article was published: *Sinha, P., V. K. Clements, and S. Ostrand-Rosenberg, 2005. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease.* ***J. Immunol.*** *174:636-645.*

- The following review article was published: *Ostrand-Rosenberg, S., P. Sinha, E. Danna, S. Miller, C. Davis, and S. Dissanayake, 2005. Antagonists of tumor-specific immunity: tumor induced immune suppression and host genes that co-opt the anti-tumor immune response.* ***In: Breast Diseases 20:****127-135.*

- The following review article is in press: *Ostrand-Rosenberg, S., 2005.* *$CD4^+$ T lymphocytes: Critical components of anti-tumor immunity.* ***Cancer Invest., in press.***

- The following review article is in press: *Sinha, P., V. Clements, and S. Miller, 2005. Tumor immunity: A balancing act between T cell activation, macrophage activation and tumor-induced immune suppression.* ***Cancer Immunol. Immunother., in press.***

- The following manuscript is in revision: *Sinha, P., V. Clements, and S. Ostrand-Rosenberg, 2005. M2 macrophages and myeloid suppressor cells block immune surveillance against metastasis via an IL-13-dependent pathway. (In revision, Cancer Res.)*

- Short talk on the reported results presented at the *Basic Aspects of Tumor Immunology II Conference* in Keystone, CO, March 19-24, 2005.

- Talk on the reported results presented at the *Tumor Escape and Its Determinants Conference* in Salzburg, Austria, October 10-13, 2004.

- Talk on the reported results presented at the *International Workshop IT-2004 Immunotherapy for the New Century,* Havana, Cuba, November 15-19, 2004.

- Poster presentation of the reported results was presented at the *Basic Aspects of Tumor Immunology II Conference* in Keystone, CO, March 19-24, 2005.

CONCLUSIONS/SIGNIFICANCE

This project is aimed at determining the potency of the STAT6 gene effect in reducing metastatic mammary cancer and at understanding the mechanisms responsible for the increased resistance. During the 4 year period of support (3 years plus a one year no-cost extension), we have made significant progress in both of these areas. Our fundamental observation is that $STAT6^{-/-}$ mice reject established metastatic mammary carcinoma if the primary mammary tumor is surgically removed. Rejection is mediated by a variety of effector mechanisms including polarized M1 macrophages and tumor-specific $CD8^+$ T cells. Since 4T1 (similar to many human mammary tumors) induces a profound immune suppression, resistance to metastatic disease also requires a drastic reduction in myeloid-derived suppressor cells. These same three characteristics also govern rejection of 4T1 mammary carcinoma in CD1-deficient mice, suggesting that our findings may be universally applicable. The results of this last year are particularly informative because they identify IL-4 and IL-13 as the molecules that govern M2 macrophage differentiation. These studies are significant because 1) They demonstrate that multiple immune effector mechanisms are necessary for rejection of established metastatic disease, suggesting that the most effective immunotherapies will activate the host's immune system via multiple pathways; and 2) Since M1 type macrophages are essential for tumor rejection and most patients have M2 macrophages, immunotherapies must be developed that polarize macrophages towards an M1 phenotype.

REFERENCES

1. Sinha, P., Clements, V. K., and Ostrand-Rosenberg, S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. J. Immunol. *174*:636-645. , 2005.
2. Kusmartsev, S. and Gabrilovich, D. I. Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother, *51:* 293-298, 2002.
3. Bronte, V., Serafini, P., De Santo, C., Marigo, I., Tosello, V., Mazzoni, A., Segal, D. M., Staib, C., Lowel, M., Sutter, G., Colombo, M. P., and Zanovello, P. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J Immunol, *170:* 270-278, 2003.
4. Rodriguez, P. C., Zea, A. H., DeSalvo, J., Culotta, K. S., Zabaleta, J., Quiceno, D. G., Ochoa, J. B., and Ochoa, A. C. L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. J Immunol, *171:* 1232-1239, 2003.
5. Zabaleta, J., McGee, D. J., Zea, A. H., Hernandez, C. P., Rodriguez, P. C., Sierra, R. A., Correa, P., and Ochoa, A. C. Helicobacter pylori arginase inhibits T cell proliferation and reduces the expression of the TCR zeta-chain (CD3zeta). J Immunol, *173:* 586-593, 2004.
6. Kusmartsev, S., Nefedova, Y., Yoder, D., and Gabrilovich, D. I. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J Immunol, *172:* 989-999, 2004.
7. Mills, C. D. Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. Crit Rev Immunol, *21:* 399-425, 2001.
8. Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., and Hill, A. M. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol, *164:* 6166-6173, 2000.

9. Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol, *23:* 549-555, 2002.
10. Smiley, S. T., Kaplan, M. H., and Grusby, M. J. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. Science, *275:* 977-979, 1997.
11. Chen, Y. H., Chiu, N. M., Mandal, M., Wang, N., and Wang, C. R. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. Immunity, *6:* 459-467, 1997.
12. Mendiratta, S. K., Martin, W. D., Hong, S., Boesteanu, A., Joyce, S., and Van Kaer, L. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. Immunity, *6:* 469-477, 1997.
13. Yoshimoto, T., Bendelac, A., Watson, C., Hu-Li, J., and Paul, W. E. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. Science, *270:* 1845-1847, 1995.
14. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K., and Sugamura, K. Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. Science, *262:* 1874-1877, 1993.
15. Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E., and et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. Science, *262:* 1880-1883, 1993.
16. Takeda, K., Kamanaka, M., Tanaka, T., Kishimoto, T., and Akira, S. Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. J Immunol, *157:* 3220-3222, 1996.

APPENDICES

Manuscripts published as a result of this project from 2001-2005. (All are attached):

1. Sinha, P., V. K. Clements, and S. Ostrand-Rosenberg, 2005. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. ***J. Immunol. 174***:636-645.

2. Ostrand-Rosenberg, S., P. Sinha, E. Danna, S. Miller, C. Davis, and S. Dissanayake, 2004. Antagonists of tumor-specific immunity: tumor induced immune suppression and host genes that co-opt the anti-tumor immune response. ***In: Breast Diseases 20:***127-135.

3. Danna, E., Sinha, P., and Clements, V., 2004. Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease , **Cancer Research 64:2205-2211**.

4. Ostrand-Rosenberg, S., 2004, Animal models of tumor immunity, immunotherapy and cancer vaccines, **Curr. Opin. Immunol. 16:143-150.**

5. Pulaski, B., M. Smyth, and S. Ostrand-Rosenberg, 2002. IFN-gamma-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma. ***Cancer Res. 62:***4406-4412.

6. Ostrand-Rosenberg, S., V. Clements, M. Terabe, J. Park, J. Berzofsky, and S. Dissanayake, 2002. Hematopoietic and non-hematopoietic cells facilitate survivial of Stat6-deficient mice with metastatic breast cancer. ***J. Immunol. 169:***5796-5804.

7. Ostrand-Rosenberg, S., Sinha, P., Clements, V., Dissanayake, S. I., Miller, S., Davis, C., and Danna, E. 2004. Signal transducer and activator of transcription 6 (Stat6) and CD1: inhibitors of immunosurveillance against primary tumors and metastatic disease, **Cancer Immunol. Immunother 53:86-91**.

8. Sinha, P., V. Clements, and S. Miller, 2005. Tumor immunity: A balancing act between T cell activation, macrophage activation and tumor-induced immune suppression. ***Cancer Immunol. Immunother., in press.***

Manuscripts currently in revision:

9. Sinha, P., V. Clements, and S. Ostrand-Rosenberg, 2005. M2 macrophages and myeloid suppressor cells block immune surveillance against metastasis via an IL-13-dependent pathway. (In revision)

Reduction of Myeloid-Derived Suppressor Cells and Induction of M1 Macrophages Facilitate the Rejection of Established Metastatic Disease[1]

Pratima Sinha, Virginia K. Clements, and Suzanne Ostrand-Rosenberg[2]

More than 60% of STAT6$^{-/-}$ mice immunologically reject spontaneous metastatic mammary carcinoma and survive indefinitely if their primary tumors are removed, whereas 95% of STAT6-competent BALB/c mice succumb to metastatic disease. BALB/c and STAT6-deficient mice with primary tumors have elevated levels of Gr1^{+}CD11b^{+} myeloid suppressor cells (MSCs), which inhibit T cell activation. After removal of primary tumor, MSC levels revert to baseline in STAT6-deficient mice, but remain elevated in BALB/c mice. The decrease is IFN-γ dependent, as is the reduction in metastatic disease. Neither BALB/c nor STAT6-deficient MSCs produce inducible NO synthase; however, both produce arginase and reactive oxygen species. STAT6-deficient mice produce M1 macrophages, which contain high levels of NO and are tumoricidal, whereas BALB/c mice produce M2 macrophages, which make arginase and are not tumoricidal. Immunity in STAT6-deficient mice requires the activation of NO-producing M1 macrophages that are tumoricidal, the reduction in MSC levels to baseline after surgical removal of primary tumor, and the activation of tumor-specific T cells. These mechanisms occur in STAT6$^{-/-}$ mice because STAT6 deficiency prevents signaling through the type 2 IL-4Rα, thereby blocking the production of arginase and promoting the synthesis of NO. ***The Journal of Immunology,*** **2005, 174: 636–645.**

Recent laboratory and clinical studies suggest that immunosurveillence and immunotherapy may be effective mechanisms for preventing tumor onset and/or limiting the growth and progression of established tumors (1, 2). However, effective anti-tumor immunity is frequently impeded by complicating factors such as 1) host tolerance to tumor Ags (3), 2) down-regulation of MHC molecules on tumor cells, rendering them resistant to cell-mediated immunity (4), 3) tumor cell expression of ligands that mediate T cell destruction or dysfunction (5), 4) production of CD4^{+}CD25^{+} T regulatory cells that block activation of effector T cells (6), and 5) tumor-induced immune suppression via the production of myeloid-derived suppressor cells (MSCs)[3] (7). The balance between these factors is critical for determining whether the host's immune system rejects tumor or if it is inhibited and is ineffective in controlling tumor growth.

We and others have recently identified a gene whose deletion favors the development of anti-tumor immunity, thereby tipping the balance away from inhibitory processes and toward a productive tumor-specific immune response. Mice that are deficient for the STAT6 gene are resistant to the metastatic 4T1 mammary carcinoma (8, 9), to recurrence of the 15-12RM fibrosarcoma (10), and to growth of the P815 mastocytoma (11). Tumor resistance in STAT6-deficient mice is T cell mediated and CD8 dependent. Because STAT6 is essential for activation of Type 2 CD4^{+} T cells, it was hypothesized that STAT6-deficient mice have a polarized Type 1 CD4^{+} T cell response and hence are better able to generate tumor-specific CD8^{+} T cells than are STAT6$^{+/+}$ mice. Surprisingly, additional studies with the mammary carcinoma and fibrosarcoma tumors did not confirm a role for Type 1 vs Type 2 CD4^{+} T cell responses, but instead indicated that deletion of the STAT6 gene removed an inhibitor that interfered with the generation of tumor-specific CD8^{+} T cells. In the fibrosarcoma system, inhibition is IL-13 dependent and involves NKT cells that activate Gr1^{+}CD11b^{+} MSCs, which in turn produce immunosuppressive TGFβ (12). In contrast, inhibition in the mammary carcinoma system does not involve IL-13, suggesting that STAT6 deficiency can mediate tumor regression via more than one mechanism (9).

Because a better understanding of the pathways by which STAT6 deficiency leads to tumor regression may suggest novel immunotherapy strategies, we are studying the mechanisms that underlie immunity to the BALB/c-derived 4T1 mammary carcinoma. This tumor shares many characteristics with human breast cancer, particularly its ability to spontaneously metastasize to the lungs, brain, liver, blood, lymph nodes, and bone marrow (13, 14). Although >95% of wild-type BALB/c (STAT6$^{+/+}$) mice die from 4T1 metastatic disease even if the primary mammary gland tumor is surgically removed, >60% of STAT6-deficient BALB/c mice survive indefinitely with the same treatment (9). In the present study we find that immunity in postsurgery STAT6-deficient mice is associated with a rapid decrease in a novel MSC population and with the activation of Type 1 tumoricidal macrophages that produce NO. Combined with our earlier studies, these results indicate that immunity to the 4T1 tumor in wild-type mice is blocked by two inhibitors: 1) a novel MSC population that interferes with

Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD 21250

Received for publication August 20, 2004. Accepted for publication October 18, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Institutes of Health (Grants R01CA52527 and R01CA84232) and the U.S. Army Breast Cancer Program (Grant DAMD-17-01-1-0312).

[2] Address correspondence and reprint requests to Dr. Suzanne Ostrand-Rosenberg, Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250. E-mail address: srosenbe@umbc.edu

[3] Abbreviations used in this paper: MSC, myeloid-derived suppressor cell; HA, influenza hemagglutinin; HEL, hen eggwhite lysozyme; DCFDA, dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; LCCM, L-cell conditioned medium; BMDM, bone marrow-derived macrophage; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; nor-NOHA, N^{W}-hydroxyl-nor-L-arginine; L-NMMA, N^{G}-monomethyl-L-arginine; LDH, lactate dehydrogenase; ATRA, all-trans-retinoic acid; TD, tumor diameter; iNOS, inducible NO synthase.

Copyright © 2005 by The American Association of Immunologists, Inc.

0022-1767/05/$02.00

CD4$^+$ T cell activation, and 2) macrophages that preferentially produce arginase instead of NO. These studies also demonstrate that suppressor cell characteristics vary between different tumors and hosts, suggesting that multiple subpopulations of MSC exist.

Materials and Methods

Mice

Mice were maintained and/or bred in the University of Maryland Baltimore County (UMBC) animal facility according to the National Institutes of Health guidelines for the humane treatment of laboratory animals. All animal procedures have been approved by the UMBC Institutional Animal Care and Use Committee. BALB/c, STAT6-deficient BALB/c (STAT6$^{-/-}$) and double-deficient STAT6$^{-/-}$IFN-$\gamma^{-/-}$ BALB/c mice were obtained and/or generated as described (8, 9). BALB/c DO11.10 TCR-transgenic mice expressing an $\alpha\beta$-TCR restricted to chicken OVA peptide 323–339 restricted by I-A^d (15) were obtained from The Jackson Laboratory. BALB/c clone 4 TCR-transgenic mice expressing an $\alpha\beta$-TCR specific for amino acids 518–526 of influenza hemagglutinin (HA) restricted to H-2K^d (16) and 3A9 TCR-transgenic mice expressing an $\alpha\beta$-TCR specific for hen eggwhite lysozyme (HEL) peptide 46–61 restricted to I-A^k (17) were kindly provided by Drs. E. Fuchs (Johns Hopkins, Baltimore, MD) and B. Wade (Dartmouth, Hannover, NH), respectively.

Reagents and Abs

Dichlorodihydrofluorescein diacetate (DCFDA) and dihydroethidium (DHE) were from Molecular Probes; recombinant mouse IFN-γ and LPS were from Pierce-Endogen and Difco, respectively. OVA$_{323-339}$ peptide was synthesized in the Biopolymer Core Facility at the University of Maryland, Baltimore. Diff-Quik stain set was from Dade Behring.

Fluorescently labeled anti-mouse Abs Gr1-PE, CD3-FITC, CD4-PE, CD8-FITC, B220-PE, CD11c-PE, I-A^d/I-E^d, D^d-FITC, CD86-PE, CD80-FITC, CD40-PE, CD44-FITC, CD14-FITC, CD23-FITC, CD31-FITC, CD34-FITC, CD16/CD32-FITC, rat IgG2a-PE isotype control, and rat IgG2a-FITC isotype control were from BD Pharmingen. CD11b-FITC, F4/80-FITC, and KJ1-26, an anti-clonotypic mAb that recognizes the DO11.10 TCR (18), were from Caltag; PDL2-PE was from eBioscience; CD33-FITC was from Biocarta; DEC205-FITC was from Cedarlane; and rat anti-mouse Gr-1 Ab for MACS sorting (clone RB6-8C5) was from BD Pharmingen.

Cell lines

The J774 macrophage and L929 fibroblast cell lines were obtained from the American Type Culture Collection and maintained in DMEM (Biofluids) supplemented with 10% FBS (HyClone), 1% penicillin, 1% streptomycin (Biofluids), and 1% Glutamax (Invitrogen Life Technologies). The 4T1 mammary carcinoma was maintained as described (8).

L-cell conditioned medium (LCCM)

L929 cells were grown in 75-cm^2 T flasks in bone marrow-derived macrophage (BMDM) medium (DMEM, 10% FBS, 1% penicillin, 1% streptomycin, and 1% Glutamax) at 37°C in 5% CO_2. One confluent T flask was split into five 75-cm^2 T flasks and the cells were cultured for 48 h or until confluent. Culture supernatants were collected, filtered through 0.22-μm filters, and diluted 5-fold with BMDM medium. Resulting LCCM was stored frozen until used.

BMDMs

BMDMs were prepared as described (19). Briefly, femurs were removed from euthanized mice and flushed with DMEM. The resulting cells were pelleted at 290 × *g* and incubated in BMDM medium at 37°C in 5% CO_2. Twenty-four hours later the adherent cells, containing mostly fibroblasts and stromal cells, were discarded, and the nonadherent cells were replated in 10-cm dishes in 10 ml of LCCM. Four days later, another 5 ml of LCCM was added to each dish. Cultures were maintained for 10–20 days. Resulting cells were assayed by flow cytometry and were >90% CD11b$^+$ or F4/80$^+$.

Splenic MSCs

Splenocytes were depleted of RBCs (20) and washed twice with degassed, cold MACS buffer (0.5% BSA in PBS with 2 mM EDTA). Washed cells were resuspended at 1–2 × 10^9 cells in 2 ml of MACS buffer, incubated with 100 μl of rat anti-mouse Gr1 Ab for 30 min at 4°C, and then washed twice with MACS buffer. Gr1-labeled splenocytes (in 400 μl of MACS buffer in a 50-ml tube) were then incubated at 4°C with 100 μl of goat anti-rat IgG microbeads (Miltenyi Biotec) for 15 min. The tube was then filled with 45 ml of MACS buffer and centrifuged for 10 min at 290 × *g*. Pelleted cells were resuspended in 5 ml of MACS buffer and sequentially applied to two MACS-LS columns for positive selection according to the manufacturer's instructions (Miltenyi Biotec). The resulting cells were assayed by flow cytometry and were >90% Gr1$^+$CD11b$^+$.

Tumor inoculation, surgery, metastasis (clonogenic) assay, and carrageenan treatment

Female BALB/c mice were inoculated in the abdominal mammary gland with 7 × 10^3 4T1 tumor cells in 50 μl of serum-free IMDM (Biofluids) as described (13). Surgical resection of primary tumors, measurement of primary tumor diameters, and quantification of metastatic disease using the clonogenic assay were performed as previously described (13). Day of tumor inoculation is day 0. Carrageenan-treated (Sigma-Aldrich) mice were inoculated with 1 mg/mouse i.p. on days −6 and −4 and every 14 days thereafter and were followed for survival for 73 days. Treated mice were assayed for depletion of phagocytic cells by measuring reduced susceptibility to LPS-induced toxic shock as described (21). Rapidly progressing primary tumors are defined as those that are >4 mm in diameter by day 25–30.

Cytokine assays

4T1 cells at 5 × 10^5 cells/well/4 ml growth medium (IMDM, 10% Fetal Clone I, 1% penicillin, 1% streptomycin, and 1% Glutamax) were cultured in six-well plates. Supernatants were collected after 48 h and assayed in triplicate by the Cytokine Core Facility at the University of Maryland, Baltimore (IL-6, IL-10, GM-CSF, and activated TGFβ) or by using an ELISA kit from R&D Systems according to the manufacturer's guidelines vascular endothelial growth factor (VEGF).

Flow cytometry

Cells were labeled for direct immunofluorescence as described (13). Abs were diluted in HEPES buffer (0.01 M, pH 7.35) with 2% FCS (HyClone). Samples were analyzed on an Epics XL flow cytometer (Beckman Coulter) and analyzed using Expo32 ADC software (Beckman Coulter).

Reactive oxygen species (ROS)

ROS production was measured by DCFDA and DHE as described (22). Briefly, 10^6 MSCs were incubated at 37°C in serum-free DMEM containing 2 μM DCFDA for 20 min to measure ROS or with 2 μM DHE for 60 min to measure superoxide. To block ROS production, MSCs were incubated at 37°C with the arginase inhibitor N^W-hydroxyl-nor-L-arginine (nor-NOHA; 500 mM) for 10 min, followed by a 20-min incubation with DCFDA (2 μM). Treated cells were washed twice with excess cold PBS and analyzed by flow cytometry.

NO and arginase assays

BMDMs or MACS-sorted MSCs were activated by culturing 2 × 10^5 cells/200-μl well in DMEM containing 5% FBS and IFN-γ and LPS at final concentrations of 2 and 100 ng/ml, respectively, in 96-well flat-bottom plates for 18 h. The supernatants of individual wells were then removed for the NO assay, and the remaining attached cells were used for the arginase assay.

NO assay

NO was measured using Griess reagents (23). Briefly, 100 μl of culture supernatant was incubated for 10 min at room temperature with 50 μl of Griess reagent A (1% sulfanilamide in 2.5% H_3PO_4) plus 50 μl of Griess reagent B (0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) per well in 96-well flat-bottom plates. Absorbance at 540 nm was measured using a Biotek 311 microplate reader. Data are the mean ± SD of triplicate wells.

Arginase assay

Arginase was quantified by measuring the production of urea as described (24, 25) with the following modifications. BMDMs were washed twice with 0.5% BSA in PBS, resuspended in 50 μl of lysis buffer (10 ml of 0.1% Triton X-100 in water with one tablet of protease inhibitor mixture; Roche), and incubated at 37°C for 30 min. Lysates were transferred to 1.5-ml microfuge tubes and arginase was activated by adding 50 μl of 25 mM Tris-HCl and 10 μl of 2 mM $MnCl_2$ per tube and heating the mixture at 56°C for 10 min. One hundred microliters of 500 mM L-arginine (pH 9.7) was added per tube, and the tubes were incubated at 37°C for 30 min

to hydrolyze the L-arginine. L-Arginine hydrolysis was stopped by adding 800 μl of acid solution (H_2SO_4 (96%):H_3PO_4 (85%):H_2O (1:3:7)) per tube. To measure the degradation of L-arginine to urea, 40 μl of α-isonitrosopropiophenone (dissolved in 100% ethanol) was added to each tube and the resulting precipitate was dissolved by heating the tubes at 100°C for 15 min. A calibration curve was run in parallel with the experimental samples and consisted of 50 μl of serial dilutions of urea dissolved in lysis buffer. Two hundred microliters from each tube was transferred to wells of a 96-well flat-bottom plate, and the urea concentration was measured at 540 nm using a Biotek microplate reader. Nor-NOHA, L-norvalin, N^G-monomethy-L-arginine (L-NMMA; Calbiochem) were used as described (26). The inactive enantiomer D-NMMA served as a negative control for L-NMMA. Data are the mean ± SD from triplicate wells.

T cell proliferation assay

Splenocytes from DO11.10, clone 4, or 3A9 mice were depleted of RBCs and cocultured in 96-well plates at 37°C in proliferation medium (HL1 medium (BioWhittaker), 1% penicillin, 1% streptomycin, 1% Glutamax, and 5×10^{-5} M 2-ME) at 10^5 cells/well with 2500 Rad irradiated MACS-sorted MSCs and 14 μM OVA peptide, 28 μM HA peptide, or 5 μg of HEL protein in a total volume of 200 μl/well in 5% CO_2. Cells were pulsed with 1 μCi [^{3}H]thymidine/well (ICN Biochemicals) on day 3, and 18 h later the cells were harvested onto glass fiber filter mats using a Packard Micromate 196 cell harvester. Filter mats were sealed in plastic bags with 4 ml of Betaplate scintillation fluid (PerkinElmer) and [^{3}H]thymidine incorporation was measured using a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer). Data are expressed as cpm (mean ± SD) of triplicate cultures. Transwell chambers (0.02-μm pores) were from Nunc.

percent suppression = 100% [1 − (cpm of spleen + peptide + MSC/ cpm of spleen + peptide)].

Cytotoxicity assay

Cytotoxicity was measured as described (27). IFN-γ- and LPS-activated BMDMs (10^5/well) were washed twice with cytotoxicity medium (IMDM, 3% FBS) and cocultured with 4T1 target cells (10^4/well) in 200 μl/well for 24 h at 37°C in 5% CO_2. Lactate dehydrogenase (LDH) activity in the culture supernatants was determined using a Cytotoxicity Detection kit (Roche) per the manufacturer's guidelines. The absorbance at 490 nm was measured using a Biotek 311 microplate reader. Spontaneous LDH release was obtained from nonactivated BMDMs incubated with 4T1 target cells. Experimental LDH release was obtained from activated BMDMs incubated with 4T1 target cells. Maximum release was obtained by adding 100 μl of 2% Triton X-100 (in cytotoxicity medium) to wells of 4T1 target cells alone. Values are the average of triplicates ± SD. Background values for media were subtracted from each point. Activated and nonactivated BMDMs without 4T1 were routinely run and gave no LDH release.

$$\text{Percent specific lysis} = 100\% \times [(A_{490}\ \text{Experimental} - A_{490}\ \text{Spontaneous})/(A_{490}\ \text{Maximum})]$$

All-trans-retinoic acid (ATRA) treatment

Pellets with or without ATRA (Innovative Research of America) were implanted in the neck on the day of removal of primary tumor as described (28). Lung metastases were quantified on day 38 after 4T1 inoculation.

Statistical analysis

Student's one-tailed (see Fig. 2, *A* and *B*) or two-tailed (see all other figures) *t* test for unequal variance was performed using Microsoft Excel 2000.

Results

MSC levels return to normal in STAT6-deficient mice, but not in BALB/c mice, after surgical removal of primary tumor

Immature myeloid cells or MSCs frequently accumulate in mice and patients as tumor burden increases, resulting in immune suppression. To determine whether deletion of the STAT6 gene affects MSC levels in 4T1 tumor-bearing mice, wild-type BALB/c and STAT6$^{-/-}$ mice were inoculated in the mammary gland with 4T1 tumor cells, and splenocytes were harvested 30–39 days later, stained, and analyzed by flow cytometry for Gr1$^+$CD11b$^+$ MSCs (nonsurgery group). Because immune suppression has been shown to decrease if primary tumor is removed (29), we also assessed MSC levels in mice whose primary tumors were surgically resected (postsurgery group). For the postsurgery group, primary tumors were resected on day 21–28 after initial tumor inoculation, and splenic MSC levels were assessed 9–11 days later. The nonsurgery BALB/c and STAT6$^{-/-}$ groups were matched for primary tumor diameter (TD) (6.05 ± 0.75 mm and 5.4 ± 0.97 mm, respectively) as were the BALB/c and STAT6$^{-/-}$ surgery groups at the time of surgery (TD: 6.11 ± 0.81 mm and 5.56 ± 0.95 mm, respectively) to minimize differences due to primary tumor burden. Tumor-free BALB/c (Fig. 1*A*) and STAT6$^{-/-}$ (Fig. 1*B*) mice have <8% splenic MSCs, whereas BALB/c and STAT6$^{-/-}$ nonsurgery mice have similarly high levels of splenic MSCs. Although both postsurgery groups showed decreases in splenic MSCs, the decrease in STAT6$^{-/-}$ mice is significantly greater than in BALB/c mice in that 67% of STAT6$^{-/-}$ mice vs 21% of BALB/c mice have <8% splenic MSCs 11 days after surgery ($p < 0.036$). The average MSC levels between the two postsurgery groups are also statistically significantly different (BALB/c: 12% ± 2.5; STAT6$^{-/-}$: 7% ± 1.9; $p < 0.01$). Therefore, although BALB/c and STAT6-deficient mice have comparably high levels of splenic MSCs while primary tumor is present, postsurgery STAT6$^{-/-}$ mice have fewer MSCs.

STAT6-deficient mice have less metastatic disease than do BALB/c mice

Tumor cells are known to secrete cytokines that stimulate the accumulation of MSCs (30). Therefore, the different levels of MSCs in postsurgery BALB/c vs STAT6-deficient mice could be due to differences in cytokine production by 4T1 tumor cells as they grow in the two mouse strains. To test this hypothesis, supernatants from in vitro-cultured 4T1 cells were compared with supernatants from ex vivo-cultured 4T1 cells harvested from primary tumor or from lungs of nonsurgery mice. As measured by ELISA, 24-h supernatants from in vitro-cultured 4T1 cells contained IL-6 (265 ± 16 pg/ml), GM-CSF (177 ± 9 pg/ml), VEGF (88 ± 14 pg/ml), and activated TGFβ (2410 ± 27 pg/ml). Although supernatants from ex vivo-cultured primary and metastatic 4T1 cells contained variable levels of IL-6, GM-CSF, VEGF, and activated TGFβ, there

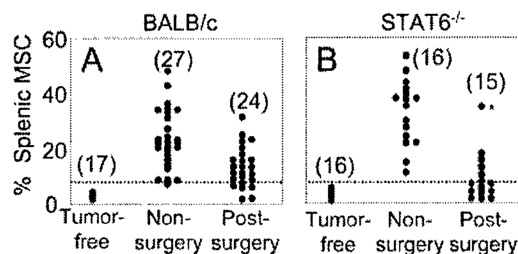


FIGURE 1. Gr1$^+$CD11b$^+$ splenic MSC levels are elevated in nonsurgery BALB/c and STAT6$^{-/-}$ mice, but return to normal in STAT6$^{-/-}$ mice after removal of primary tumor. BALB/c (*A*) or STAT6-deficient (*B*) mice were inoculated on day 0 with 4T1 tumor cells in the mammary gland, and primary tumors either were left in place (nonsurgery) or were surgically resected on approximately day 28 (postsurgery). Tumor-free groups were not inoculated with 4T1. Percent splenic MSCs is the percent of splenocytes that were CD11b$^+$Gr1$^+$ on approximately day 38. Postsurgery groups have statistically significantly fewer MSCs than do their corresponding nonsurgery groups ($p < 0.05$). Postsurgery STAT6-deficient mice have statistically fewer MSCs than do postsurgery BALB/c mice ($p < 0.036$). (Outlier value denoted with an asterisk was omitted for the *t* test). Each symbol represents an individual mouse. Numbers indicate the number of mice in each group. Data are pooled from four independent experiments. Dotted lines indicate the level of MSCs in tumor-free mice.

were no significant differences between cytokine levels from BALB/c and STAT6$^{-/-}$ mice (data not shown). Therefore, 4T1 tumor cells secrete several cytokines known to induce MSCs; however, the difference in MSC levels in postsurgery BALB/c and STAT6$^{-/-}$ mice is not due to differential secretion of these cytokines.

Because tumor load can affect MSC levels, we have used the clonogenic assay to quantify lung metastatic disease in the nonsurgery and postsurgery BALB/c and STAT6-deficient mice of Fig. 1. Although STAT6$^{-/-}$ mice have significantly ($p < 0.01$) reduced metastatic disease relative to BALB/c mice, there is no direct correlation between number of metastatic cells and percent MSCs (Fig. 2).

MSCs from STAT6$^{-/-}$ and BALB/c mice are phenotypically and functionally equivalent and their suppressive activity is due to arginase production

Qualitative differences between MSCs of BALB/c and STAT6$^{-/-}$ mice may also contribute to the increased anti-tumor immunity of STAT6-deficient animals. To test this hypothesis, BALB/c and STAT6$^{-/-}$ mice were inoculated in the mammary gland with 4T1 tumor cells; 21–28 days later splenocytes were removed and MSCs were purified by MACS sorting and phenotyped by Ab staining. To minimize differences due to primary tumor load, BALB/c and STAT6$^{-/-}$ donors were matched for primary 4T1 TD at the time of sacrifice (8.96 ± 0.35 mm and 9.01 ± 0.33 mm, respectively). MACS-sorted MSCs from both BALB/c and STAT6$^{-/-}$ mice were ≥95% pure (Gr1$^+$CD11b$^+$) (Fig. 3*A*) and had the morphology of immature myeloid cells (Fig. 3*B*). Although the MSCs from both strains are phenotypically similar, MSCs from STAT6-deficient mice express more CD16/CD32 and CD80, whereas MSCs from BALB/c mice express more CD11c, DEC205, and CD8 (Fig. 3*C*). Therefore, there are subtle differences between the MSCs of tumor-bearing BALB/c and STAT6-deficient mice.

To determine whether the MSCs of BALB/c and STAT6-deficient mice have similar immunosuppressive activity, equal numbers of MACS-sorted Gr1$^+$CD11b$^+$ cells from tumor-free, nonsurgery, or postsurgery mice were cocultured with OVA peptide-pulsed splenocytes from DO11.10 transgenic mice, and T cell proliferation was measured by [^{3}H]thymidine incorporation. Gr1$^+$CD11b$^+$ cells from all three groups and from both strains are highly suppressive, whereas control J774 cells do not suppress (Fig. 4*A*). Purified MSCs similarly inhibit HA-specific CD8$^+$ T cells and HEL-specific allogeneic CD4$^+$ T cells (Fig. 4*B*). Culture supernatants were also assayed for IL-2 activity. In the presence of MSCs, IL-2 levels were reduced ~50%; however, IL-2 levels rebounded completely when the arginase inhibitor nor-NOHA was added (data not shown). Therefore, the suppressive capacity of Gr1$^+$CD11b$^+$ cells on a per cell basis is similar in BALB/c and STAT6-deficient mice, is independent of whether tumor is present, involves a reduction in IL-2 production, and is not MHC-, CD4-, or CD8-restricted.

To determine whether differences in anti-tumor immunity between BALB/c and STAT6$^{-/-}$ mice could be due to differential expression of ROS, as suggested by Kusmartsev et al. (31), MACS-purified Gr1$^+$CD11b$^+$ MSCs from tumor-free and nonsurgery mice were treated with DCFDA or DHE and were analyzed by flow cytometry. DCFDA is oxidized by hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), peroxynitrile ($ONOO^-$), or superoxide to yield a fluorescent compound, and thus measures ROS.

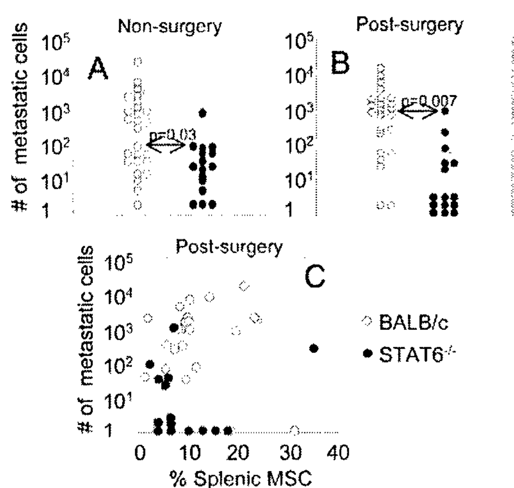


FIGURE 2. STAT6-deficient mice have less metastatic disease than do BALB/c mice. Mice were inoculated with 4T1 tumor as indicated in Fig. 1. Lungs were harvested from the nonsurgery (*A*) or postsurgery (*B* and *C*) groups, and the number of metastatic cells was quantified using the clonogenic assay. Each symbol represents the number of metastatic tumor cells in the lungs of an individual mouse. *C*, MSC levels do not directly correlate with number of metastatic cells. Data are pooled from four independent experiments.

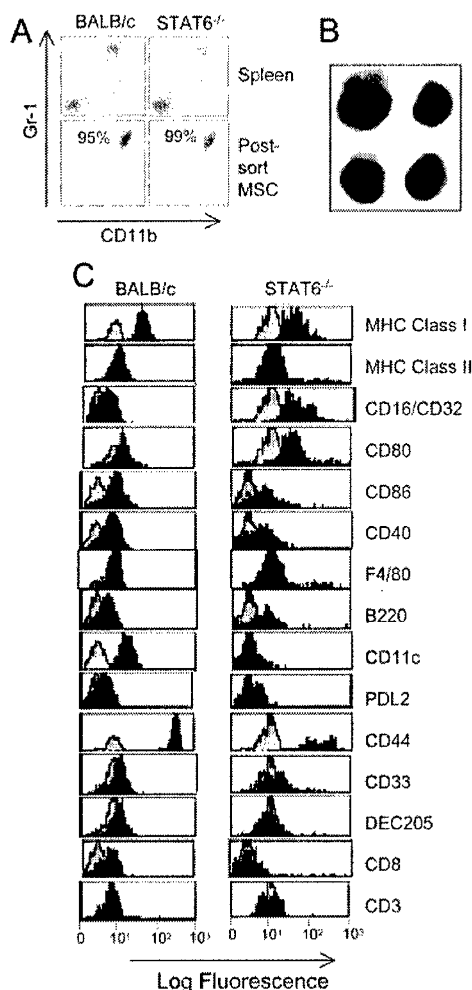


FIGURE 3. MSCs from STAT6-deficient and BALB/c mice have different phenotypes. *A*, Spleen cells from BALB/c and STAT6$^{-/-}$ nonsurgery mice stained with Gr1 and CD11b mAbs before and after MACS sorting. *B*, Wright-Giemsa-stained, MACS-purified Gr1$^+$CD11b$^+$ cells (magnification ×630). *C*, MACS-sorted MSCs stained with the indicated mAbs. CD3 represents the staining by CD4, CD14, CD23, CD31, and CD34 mAbs. Gray and black peaks are the isotype control and Ag-specific staining, respectively. Data are from one of two independent experiments.

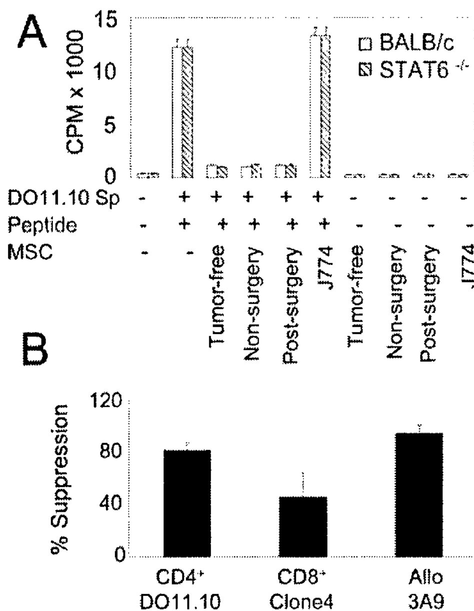


FIGURE 4. MSCs suppress Ag-specific $CD4^+$ and $CD8^+$ T cell proliferation and are not MHC restricted. *A*, DO11.10 transgenic splenocytes were stimulated with $OVA_{323-339}$ peptide in the presence or absence of MACS-sorted $Gr1^+CD11b^+$ MSCs from tumor-free, nonsurgery, or postsurgery STAT6-deficient or BALB/c mice, or with control J774 cells. *B*, DO11.10, clone 4, or 3A9 transgenic splenocytes were stimulated with $OVA_{323-339}$ or $HA_{518-526}$ peptide or HEL protein, respectively, in the presence of nonsurgery BALB/c MSCs. MSCs from two to three mice were pooled for each group. Data are from one of two independent experiments.

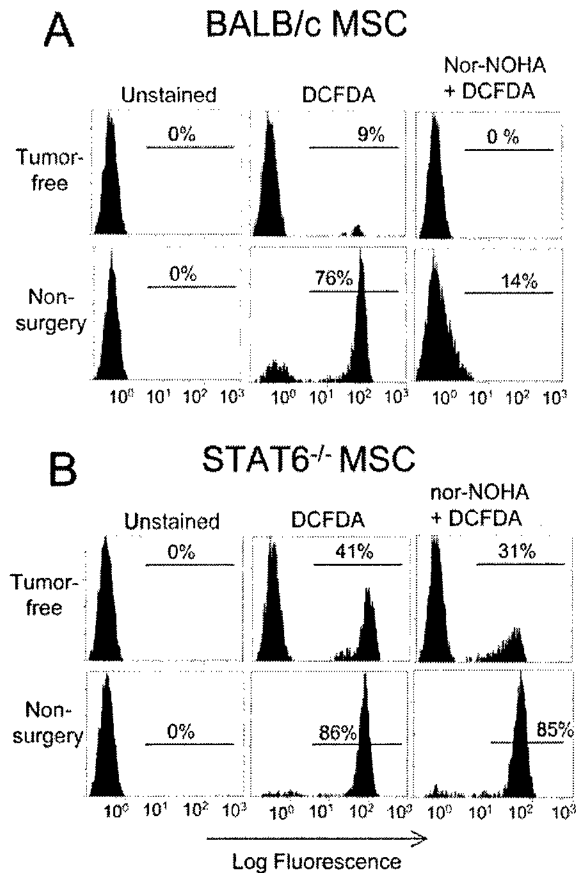


FIGURE 5. MSCs from tumor-bearing BALB/c and STAT6-deficient mice produce ROS; however, ROS production is arginase dependent in BALB/c mice and arginase independent in STAT6-deficient mice. MACS-purified MSCs from tumor-free or nonsurgery BALB/c (*A*) or STAT6-deficient (*B*) mice were incubated with DCFDA in the presence or absence of the arginase inhibitor nor-NOHA. Data are from one of two independent experiments.

Likewise, DHE is oxidized by superoxide to a fluorescent species. MSCs from nonsurgery BALB/c (Fig. 5*A*) and STAT6-deficient (Fig. 5*B*) mice contain more ROS than do MSCs from tumor-free mice. Neither MSC population stains with DHE (data not shown), indicating that the MSCs do not make superoxide. To determine whether arginase is required for ROS production, MSCs from nonsurgery mice were treated with the arginase inhibitor nor-NOHA before staining with DCFDA. Nor-NOHA blocks the production of ROS from MSCs of BALB/c nonsurgery mice, but has no effect on ROS expression by MSCs from nonsurgery $STAT6^{-/-}$ mice. In addition, $STAT6^{-/-}$ MSCs have a high baseline level of ROS in tumor-free mice, so there is only a 2-fold increase in ROS in MSCs from tumor-free vs nonsurgery $STAT6^{-/-}$ MSCs, whereas there is an 8-fold increase in BALB/c MSCs. Therefore, MSCs from tumor-free BALB/c and STAT6-deficient mice contain different baseline levels of ROS, whereas ROS levels are comparable in nonsurgery mice; however, the ROS produced by BALB/c mice are arginase dependent, whereas the ROS produced by STAT6-deficient MSCs are arginase independent.

Bronte et al. (26, 32) have shown that MSC activity is dependent on arginase and/or inducible NO synthase (iNOS). To determine whether MSCs from nonsurgery mice differ because of selective expression of arginase and/or iNOS, OVA peptide-pulsed splenocytes from DO11.10 transgenic mice were cocultured with MSCs in the presence or absence of inhibitors of arginase or iNOS. BALB/c and $STAT6^{-/-}$ MSCs inhibit DO11.10 proliferation, and this inhibition is reversed by the arginase inhibitors norvalin and nor-NOHA, but not by the iNOS inhibitor L-NMMA (Fig. 6*A*). Similar inhibition was seen for clone 4 $CD8^+$ T cells and for allogeneic 3A9 $CD4^+$ T cells (data not shown). To determine whether suppression requires cell contact, peptide-pulsed DO11.10 cells were cocultured with MSCs contained in transwell chambers. Proliferation of DO11.10 cells separated from MSCs by a semipermeable membrane was not inhibited, indicating that MSCs must directly contact the target cells they are suppressing (Fig. 6*B*). Therefore, in agreement with earlier studies with other tumors (33), 4T1-induced MSCs suppress via a contact-dependent mechanism involving arginase

Reduction of MSC levels and decrease in metastatic disease in $STAT6^{-/-}$ mice is IFN-γ dependent

Previous studies established that resistance to the 4T1 tumor in STAT6-deficient mice requires IFN-γ because $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice were just as susceptible to metastatic disease as were BALB/c mice (9). If tumor resistance in postsurgery $STAT6^{-/-}$ mice is dependent on the rapid decrease in MSCs and if IFN-γ is involved in that decrease, then postsurgery $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice should have relatively high levels of MSCs. To test this hypothesis, $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice were inoculated with 4T1 on day 1, and tumors were either left in place (nonsurgery group) or they were removed 21–28 days later (postsurgery group). Nine to 11 days after the surgery date, all mice were sacrificed, their splenocytes were stained for Gr1 and CD11b, and their lungs were assayed by the clonogenic assay for metastatic 4T1 cells. The nonsurgery and postsurgery groups were matched for primary TD on the day of surgery (nonsurgery: 6 ± 2.02 mm; postsurgery: 6.22 ± 1.74 mm). There is a modest decrease in MSCs in postsurgery $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice (Fig. 7*A*); however, the decrease is

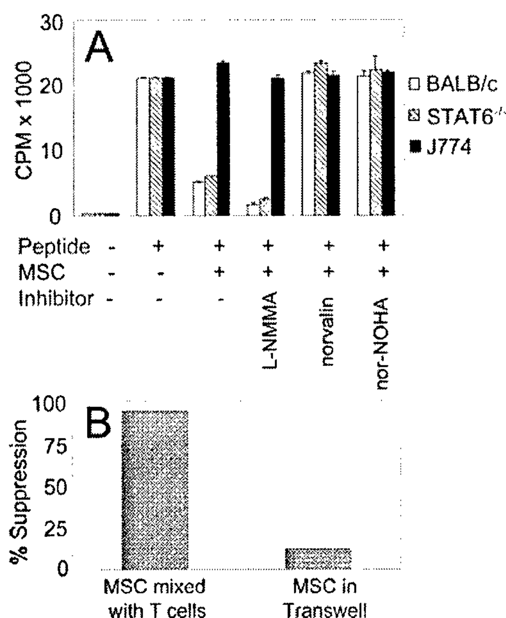


FIGURE 6. MSC-induced suppression of Ag-specific T cells is arginase dependent and cell contact dependent. *A*, DO11.10 transgenic splenocytes were cocultured with $OVA_{323-339}$ peptide in the presence or absence of MSCs from nonsurgery mice or with control J774 cells in the presence or absence of the iNOS inhibitor L-NMMA, or with the arginase inhibitors nor-NOHA or norvalin. *B*, MSCs were contained in a transwell chamber or directly mixed with DO11.10 T cells plus peptide. Data are from one of three to five independent experiments.

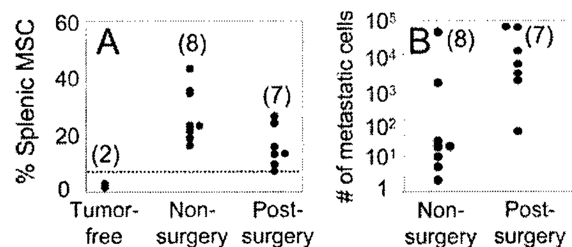


FIGURE 7. Regression of MSCs and metastatic disease in STAT6-deficient mice is IFN-γ dependent. $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice were inoculated with 4T1 tumor as described in Fig. 1. *A*, Percent splenic MSCs is the percent of splenocytes that stained $Gr1^{+}CD11b^{+}$ on approximately day 38 after tumor inoculation. Dotted line indicates the level of MSCs in tumor-free mice. MSCs in the postsurgery $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ group are significantly higher than in the postsurgery $STAT6^{-/-}$ group ($p \leq 0.01$), but they are not significantly different from the postsurgery BALB/c group (see Fig. 1*A* for BALB/c data). *B*, Lungs from the mice in *A* were harvested on approximately day 38 and were assayed by the clonogenic assay for the number of metastatic tumor cells. Each symbol represents an individual mouse. Numbers in parentheses indicate the number of mice in each group. Data are pooled from two independent experiments.

comparable to that seen in tumor-susceptible BALB/c mice (14% and 21% have <8% MSCs, respectively; see Fig. 1*A* for BALB/c data) and does not approach the larger decrease seen in $STAT6^{-/-}$ mice, in which 67% of mice have <8% MSCs (see Fig. 1*B* for $STAT6^{-/-}$ data). The average MSC levels between the $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ and $STAT6^{-/-}$ postsurgery groups are also statistically significantly different ($STAT6^{-/-}IFN\text{-}\gamma^{-/-}$: 14.8 ± 5.6; $STAT6^{-/-}$: 7 ± 1.9; $p < 0.01$). In contrast, the average MSC levels between postsurgery $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ and BALB/c mice are not significantly different ($STAT6^{-/-}IFN\text{-}\gamma^{-/-}$: 14.8 ± 5.6; BALB/c: 12 ± 2.5). In agreement with the high MSC count, the number of metastatic cells in the $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice is also significantly ($p = 0.05$) elevated (Fig. 7*B*) compared with $STAT6^{-/-}$ postsurgery mice. However, there is no direct correlation between number of metastatic cells and percent MSCs (data not shown). Therefore, resistance to metastatic disease in $STAT6^{-/-}$ mice requires IFN-γ, and IFN-γ is essential for the rapid decrease in MSCs after surgery.

$STAT6^{-/-}$ macrophages produce high levels of iNOS, are cytotoxic for 4T1 tumor cells, and do not produce arginase

In addition to cytotoxic $CD8^{+}$ T cells, macrophages can have tumoricidal activity. Type 1 macrophages (M1 macrophages), which make iNOS and do not make arginase, are particularly tumoricidal, whereas M2 macrophages, which make arginase and do not make iNOS, are not tumoricidal (34–36). Previous studies indicated that phagocytic cells are an important component of immune surveillance against the 4T1 tumor (21), suggesting that macrophages may be involved in the enhanced immunity of STAT6-deficient mice. To determine whether macrophages are involved in the heightened immunity in STAT6-deficient mice, BMDMs were prepared from tumor-free, nonsurgery, and postsurgery BALB/c and $STAT6^{-/-}$ mice and were tested in vitro for arginase and iNOS production and for cytotoxicity against 4T1 tumor cells. The nonsurgery and postsurgery groups were matched for primary TD at the time of surgery (BALB/c nonsurgery, 7.75 ± 0.8 mm vs postsurgery, 8.08 ± 0.8 mm; $STAT6^{-/-}$ nonsurgery, 7.46 ± 0.59 mm vs postsurgery, 7.6 ± 0.41 mm). Both activated and nonactivated BALB/c macrophages from nonsurgery and postsurgery mice synthesize arginase, as measured by urea production, whereas $STAT6^{-/-}$ macrophages, regardless of activation state, do not produce arginase (Fig. 8*A*). Nonactivated macrophages from both strains do not produce iNOS, as measured by NO production. iNOS production by BALB/c macrophages decreases with tumor progression, whereas STAT6-deficient macrophages maintain high iNOS production regardless of the presence of primary tumor or

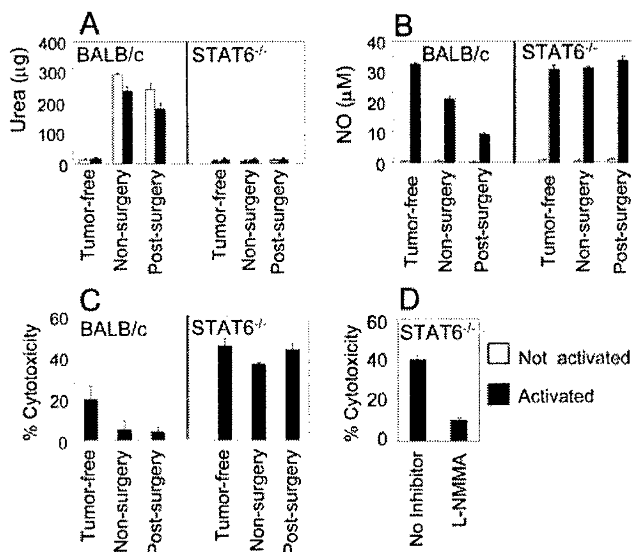


FIGURE 8. STAT6-deficient mice make M1 BMDMs, which produce NO and are tumoricidal, whereas BALB/c mice make M2 BMDMs, which produce arginase and are not tumoricidal. BMDMs from tumor-free, nonsurgery, or postsurgery mice were not activated or activated with IFN-γ and LPS and were assayed for urea production (*A*) as a measure of arginase content or for NO production (*B*) as a measure of iNOS. *C* and *D*, BMDMs were cocultured with 4T1 target cells and percent cytotoxicity was determined. Data are from one of two independent experiments.

metastatic disease (Fig. 8*B*). Activated STAT6$^{-/-}$ macrophages are also more tumoricidal for 4T1 cells compared with activated BALB/c macrophages (Fig. 8*C*), and the cytotoxic activity is iNOS dependent (Fig. 8*D*). Collectively, these data indicate that BALB/c mice make an M2 macrophage response that is ineffective in destroying 4T1 tumor, whereas STAT6-deficient mice make an M1 macrophage response that kills 4T1 tumor cells.

Depletion of macrophages reduces survival

To determine whether macrophages are critical for increased survival of STAT6-deficient mice, STAT6$^{-/-}$ mice were either untreated or depleted of phagocytic cells by carrageenan treatment, inoculated with 4T1 tumor, had primary tumors removed, and were followed for survival. As shown in Table I, only 45% of carrageenan-treated mice survived, whereas 75% of nontreated mice survived. The macrophage-depleted mice that died also died more rapidly (37 ± 14 vs 47 ± 21 days for the carrageenan-treated vs nontreated groups, respectively); however, these values were not statistically signficantly different. Before surgery, the two groups were also followed for primary tumor growth. Sixty-three percent and 29% of carrageenan-treated and untreated mice, respectively, had rapidly progressing primary tumors. Therefore, macrophages are essential for survival and for enhanced immunity to primary and metastatic disease in STAT6-deficient mice.

To determine whether reduction in MSCs is sufficient for tumor rejection, postsurgery BALB/c mice were implanted with pellets containing ATRA, which has been shown to reduce MSC levels (28). Although MSC levels were reduced to baseline in 50% of the postsurgery BALB/c mice implanted with the ATRA pellets, metastatic tumor levels in these mice remained high (data not shown). Therefore, reduction in MSC levels in the absence of cytotoxic macrophages is not sufficient for metastasis rejection.

Discussion

Global deletion of the STAT6 gene provides potent immunity/immunosurveillance to a variety of tumors (8, 10, 11). The enhanced immunity is particularly impressive against metastatic disease in that 60–80% of mice whose primary mammary tumors are removed reject their established metastases and survive indefinitely. In contrast, <5% of STAT6-competent BALB/c mice survive (9). Previous studies established that CD8^{+} T cells are critical for immunity (8). The current study identifies two additional cell populations that are also involved: 1) immunosuppressive MSCs, which rapidly and irreversibly decrease to background levels after surgical removal of primary tumor, and 2) tumoricidal M1-type macrophages that produce NO and do not produce arginase.

Fig. 9 shows a model of how these effectors and inhibitors may interact to mediate tumor regression vs tumor progression. In both STAT6-competent and STAT6-deficient mice, 4T1 cells of the primary tumor secrete cytokines (IL-6, TGFβ, GM-CSF, and VEGF) that stimulate the accumulation of MSCs. MSCs are thought to inhibit T cell function by secreting arginase, which depletes L-arginine, causing a loss of CD3ζ chain expression (37–39). Therefore, the high levels of arginase-producing MSCs block activation of tumor-reactive CD4^{+} and CD8^{+} T cells, which would normally be activated to tumor Ags by cross-presentation by professional APCs. In the absence of functional CD4^{+} T cells, potent tumor-specific CD8^{+} T cells are not generated and tumor growth progresses. However, after removal of 4T1 primary tumor from STAT6-deficient mice, MSC levels decrease to baseline, allowing tumor-specific CD4^{+} and CD8^{+} T cells to be activated. In contrast, MSC levels in postsurgery STAT6-competent mice do not return to baseline, so T cells remain suppressed. In addition, macrophages of STAT6-deficient mice are tumoricidal because they make NO, whereas BALB/c macrophages are not cytotoxic and make arginase, which supports tumor growth (35). Therefore, complete rejection of metastatic disease and survival of STAT6-deficient mice requires the reduction in MSC levels, coupled with the presence of NO-secreting macrophages and tumor-specific T cells. This model is consistent with the results presented in this report, as well as with earlier studies demonstrating a requirement for CD8^{+} T cells (8) and phagocytic cells (21).

Previous studies in patients have demonstrated that MSCs decrease after surgical removal of tumor, presumably due to the reduction of tumor cells secreting factors that mediate MSC accumulation (reviewed in Refs. 7 and 30). The rapid decrease of MSCs in postsurgery STAT6-deficient mice identified in the current study demonstrates that MSC levels after surgery are not exclusively regulated by tumor burden. In agreement with others, our working hypothesis is that MSC regression is regulated by a ligand that binds to a receptor on the MSCs and causes them to accumulate or regress. Because STAT6 deficiency favors rapid regression to baseline levels, the relevant ligand must signal through STAT6. Although others have proposed that the IL-4Rα regulates MSC levels (10, 12, 26), this receptor is not relevant in the 4T1 system because IL-4Rα knockout mice have the same levels of MSCs as do wild-type mice (P. Sinha and S. Ostrand-Rosenberg, unpublished observations). Therefore, an alternative receptor on 4T1-induced MSCs that signals through STAT6 must mediate the regression to baseline in postsurgery mice.

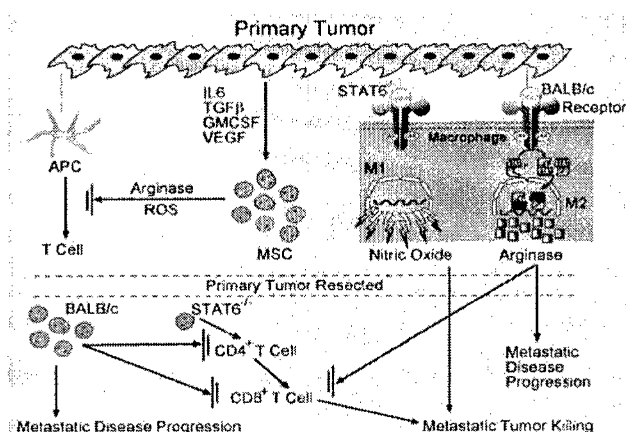


FIGURE 9. Proposed model for tumor resistance of STAT6-deficient mice. Resistance to metastatic disease in postsurgery STAT6-deficient mice requires the regression of MSCs and the presence of M1 macrophages and activated T cells. See details for a *Discussion* of the roles of NO, arginase, MSCs, macrophages, and T cells.

Table I. *Macrophage-depleted STAT6-deficient mice have decreased survival*[a]

Treatment	Percent Survival	Percentage of Mice with Rapid Primary Tumor Growth
None	75% (9/12)	29% (4/14)
Carrageenan	45% (5/11)	63% (10/16)

[a] STAT6-deficient mice were inoculated in the abdominal mammary gland with 7000 4T1 cells on day 0, primary tumors were removed 3–4 wk later, and mice were followed for 73 days. Macrophages were depleted by inoculation of 1 mg/mouse carrageenan on days −6, −4, and every 14 days thereafter for the duration of the experiment. Rapid primary tumor growth is defined as tumors that are >4 mm in diameter by days 25–30.

Macrophages are a heterogenous population of cells whose phenotype, characteristics, and functions are determined by the cytokine milieu in which they reside. M1 macrophages produce NO and reduce tumor growth, whereas M2 macrophages produce arginase and facilitate tumor progression (34–36). In activated macrophages, iNOS converts arginine and oxygen to citrulline and NO. If arginine is not available, NO is not produced. In M2 macrophages, where arginase production is high, the arginine pool is depleted, resulting in low levels of iNOS and minimal NO production (32, 40). Because the production of arginase requires IL-4 and IL-13 signaling through the JAK3/STAT6 pathway (19, 41), STAT6-deficient mice should not produce arginase and hence should not make M2 macrophages. Our finding that macrophages from STAT6-deficient mice are strongly polarized toward an M1 phenotype is consistent with the role of STAT6 in regulating arginase production and suggests that, in the absence of arginase, activated macrophages default toward a tumoricidal M1 phenotype. Because signaling by IL-4, presumably through the IL-4Rα and the JAK-STAT pathway, is thought to be essential for the production of arginase (35, 42), the preferential generation of M1 macrophages by STAT6-deficient mice is entirely consistent with the deletion of the STAT6 gene.

In addition to its ability to mediate cytotoxicity, NO is also important in CD8$^+$ T cell differentiation. Decreased NO levels due to depletion of arginine by arginase have been associated with CD3ζ chain deficiencies and defects in CD8$^+$ T lymphocytes (39, 43). Because NO preferentially induces type 1 T cell differentiation (44, 45), CD8$^+$ T cells of STAT6-deficient mice may be more efficacious because STAT6$^{-/-}$ macrophages are polarized toward NO production. This scenario is consistent with earlier observations that STAT6-deficient mice have 4T1-specific cytotoxic CD8$^+$ T cells, whereas STAT6-competent mice do not (8). Given the multiple inhibitory effects of arginase, strategies that specifically block or degrade arginase in macrophages may be novel and useful cancer immunotherapy approaches.

NO production by activated macrophages has also been shown to stimulate differentiation of MSCs to normal myeloid-derived cells (46, 47). We find a direct correlation between elevated levels of NO-producing macrophages and decreased MSC levels in STAT6-deficient mice, suggesting that NO-producing macrophages may also enhance immunity by reducing MSC levels.

Arginase production by MSCs is thought to be triggered by IL-4 and/or IL-13 binding to the cell's IL-4Rα, followed by signaling through the JAK3/STAT6 pathway (19, 26, 32). Our finding that MSCs from STAT6-deficient and STAT6-competent mice make comparable levels of arginase suggests that there is an additional mechanism for induction of arginase by MSCs that is independent of the IL-4Rα JAK3/STAT6 pathway. This hypothesis is supported by our observation that MSCs from IL-4R-deficient mice also suppress via the production of arginase (P.S. and S.O.-R., unpublished observations).

Myeloid-derived cells with suppressor activity have been reported in many tumor systems in both experimental animals and patients (7, 30, 48). Although these cells have the common feature of suppressing T cells, there are significant differences in their phenotype and function. Bronte et al. (26) describe MSCs that express MHC class II, B220, F4/80, CD86, CD16/32, and DEC205, whereas Gabrilovich et al. (33) report the expression of MHC class I and the absence of MHC class II and costimulatory molecules. Not only do the MSCs described in this report differ between BALB/c and STAT6-deficient mice, but they also differ from these previously described MSCs with respect to their cell surface markers. The 4T1-induced STAT6-deficient and BALB/c MSCs also differ in the mechanism by which they generate ROS. Although ROS production by 4T1-induced MSCs from BALB/c mice is arginase dependent in agreement with Kusmartsev et al. (31), ROS production by STAT6$^{-/-}$ MSCs is arginase independent, suggesting that there are multiple pathways for stimulating ROS biosynthesis in MSCs.

MSCs are also heterogeneous with respect to function. Some MSCs inhibit MHC class I-restricted CD8$^+$ T cells and have no effect on CD4$^+$ T cells (33, 49, 50), whereas others inhibit CD4$^+$ T cells (51). The MSCs reported here inhibit both CD4$^+$ and CD8$^+$ T cells. Kusmartsev et al. (31) report that Gr1$^+$CD11b$^+$ splenic cells from tumor-free mice are not suppressive, whereas the splenic Gr1$^+$CD11b$^+$ cells from tumor-free mice in this report are as suppressive on a per cell basis as are Gr1$^+$CD11b$^+$ cells from tumor-bearing mice.

The role of IFN-γ in suppressor cell development/activity is also variable. In the MSCs described by Mazzoni et al. (51), IFN-γ enhances MSC suppressive activity by inducing production of NO from MSCs. Kusmartsev and Gabrilovich (30) find that IFN-γ increases the production of reactive oxygen intermediates by MSCs, which in turn decreases the net amount of IFN-γ by down-regulating IFN-γ production by CD4$^+$ and CD8$^+$ T cells. Because IFN-γ is critical for effective anti-tumor immunity (52), these authors conclude that this net decrease in IFN-γ is one of the mechanisms by which MSCs mediate immune suppression. In the 4T1 system described in this report, IFN-γ is essential for the postsurgery decrease in MSCs in STAT6$^{-/-}$ mice, supporting the concept that MSC function is IFN-γ dependent.

Different mechanisms of suppression have also been postulated. Suppression is most frequently attributed to overexpression of arginase and the resulting loss of NO production (26, 31, 51), although it has also been ascribed to TGFβ production (12). The MSCs of this report do not produce TGFβ (data not shown) and their suppressive activity is blocked by inhibitors of arginase. 4T1-induced MSCs also differ from other described suppressor cell populations in that they express B220 in addition to Gr-1 and CD11b. This phenotype is characteristic of plasmacytoid dendritic cells, which are also immunosuppressive (53). These variations in Gr1$^+$CD11b$^+$ MSCs suggest that there are multiple subpopulations of MSCs. Not only do the various subpopulations differ in their phenotype and characteristics, but they also suppress different target cells. Although it is unclear why there is such variation, it is likely that the variation is due to differential cytokine production by the various tumors and the interaction of these cytokines with the host.

Jensen et al. (54) have suggested that enhanced immunity in STAT6$^{-/-}$ mice is due to CD8$^+$ T cell reactivity against tumor-expressed STAT6 protein, and they report rejection of primary 4T1 tumors by STAT6-deficient mice. In contrast, we find that, although 4T1 primary tumor growth is slightly delayed in STAT6-deficient mice, all mice die (8), indicating that immunity in the presence of primary tumor is only modestly effective, presumably due to the presence of high levels of MSCs. We also find heightened immunity in STAT6-deficient mice against STAT6-negative tumors because STAT6$^{-/-}$NeuT$^{+/-}$ mice, which spontaneously develop STAT6$^{-/-}$ mammary tumors, have significantly extended survival times relative to STAT6$^{+/+}$NeuT$^{+/-}$ mice (55). We also do not find evidence for CD8$^+$ T cell reactivity against STAT6 protein because CTLs from 4T1 immune STAT6$^{-/-}$ mice are not cytotoxic for $H\text{-}2^d$ STAT6$^+$ tumors other than 4T1 (V.K.C. and S.O.-R., unpublished observations). Therefore, heightened immunity in STAT6-deficient mice is not due to tumor-expressed STAT6 protein.

Anti-tumor immunity is a careful balance between the presence of anti-tumor effector mechanisms such as $CD4^+$ and $CD8^+$ T cells, NK cells, and tumoricidal macrophages and counterproductive inhibitory phenomena such as MSCs and $CD4^+CD25^+$ T regulatory cells. Ultimately, the ability to exploit the immune system to control tumor growth depends on tipping the balance toward the effector mechanisms and away from the inhibitory mechanisms, and deletion of the STAT6 gene achieves this polarization.

Acknowledgments

We thank Drs. Ephraim Fuchs and Bill Wade for kindly providing clone 4 and 3A9 mice, respectively; Dr. B. Pulaski for performing the carrageenan experiment; Drs. Dmitry Gabrilovich, Enzo Bronte, and Peter Murray for providing the ROS, arginase, and BMDM protocols, respectively; Dr. Jeff Leips for statistical consultation; and Sandy Mason for her excellent care of our mice.

References

1. Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber. 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat. Immunol. 3:991.*
2. Finn, O. J. 2003. Cancer vaccines: between the idea and the reality. *Nat. Rev. Immunol. 3:630.*
3. Pardoll, D. 2003. Does the immune system see tumors as foreign or self? *Annu. Rev. Immunol. 21:807.*
4. Marincola, F. M., E. Wang, M. Herlyn, B. Seliger, and S. Ferrone. 2003. Tumors as elusive targets of T-cell-based active immunotherapy. *Trends Immunol. 24:335.*
5. Iwai, Y., M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato. 2002. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. USA 99:12293.*
6. Shevach, E. M. 2002. $CD4^+CD25^+$ suppressor T cells: more questions than answers. *Nat. Rev. Immunol. 2:389.*
7. Serafini, P., C. De Santo, I. Marigo, S. Cingarlini, L. Dolcetti, G. Gallina, P. Zanovello, and V. Bronte. 2004. Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol. Immunother. 53:64.*
8. Ostrand-Rosenberg, S., M. J. Grusby, and V. K. Clements. 2000. Cutting edge: STAT6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. *J. Immunol. 165:6015.*
9. Ostrand-Rosenberg, S., V. K. Clements, M. Terabe, J. M. Park, J. A. Berzofsky, and S. K. Dissanayake. 2002. Resistance to metastatic disease in STAT6-deficient mice requires hemopoietic and nonhemopoietic cells and is IFN-γ dependent. *J. Immunol. 169:5796.*
10. Terabe, M., S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D. D. Donaldson, D. P. Carbone, W. E. Paul, and J. A. Berzofsky. 2000. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat. Immunol. 1:515.*
11. Kacha, A. K., F. Fallarino, M. A. Markiewicz, and T. F. Gajewski. 2000. Cutting edge: spontaneous rejection of poorly immunogenic P1.HTR tumors by Stat6-deficient mice. *J. Immunol. 165:6024.*
12. Terabe, M., S. Matsui, J. M. Park, M. Mamura, N. Noben-Trauth, D. D. Donaldson, W. Chen, S. M. Wahl, S. Ledbetter, B. Pratt, et al. 2003. Transforming growth factor-β production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J. Exp. Med. 198:1741.*
13. Pulaski, B. A., and S. Ostrand-Rosenberg. 1998. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. *Cancer Res. 58:1486.*
14. Lelekakis, M., J. Moseley, T. Martin, D. Hards, E. Williams, P. Ho, D. Lowen, J. Javni, F. Miller, J. Slavin, and R. Anderson. 1999. A novel orthotopic model of breast cancer metastasis to bone. *Clin. Exp. Metastasis 17:163.*
15. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes in vivo. *Science 250:1720.*
16. Morgan, D. J., R. Liblau, B. Scott, S. Fleck, H. O. McDevitt, N. Sarvetnick, D. Lo, and L. A. Sherman. 1996. $CD8^+$ T cell-mediated spontaneous diabetes in neonatal mice. *J. Immunol. 157:978.*
17. Ho, W. Y., M. P. Cooke, C. C. Goodnow, and M. M. Davis. 1994. Resting and anergic B cells are defective in CD28-dependent costimulation of naive $CD4^+$ T cells. *J. Exp. Med. 179:1539.*
18. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med. 157:1149.*
19. Rutschman, R., R. Lang, M. Hesse, J. N. Ihle, T. A. Wynn, and P. J. Murray. 2001. Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. *J. Immunol. 166:2173.*
20. Lamouse-Smith, E., V. K. Clements, and S. Ostrand-Rosenberg. 1993. $\beta_2m^{-/-}$ knockout mice contain low levels of $CD8^+$ cytotoxic T lymphocyte that mediate specific tumor rejection. *J. Immunol. 151:6283.*
21. Pulaski, B. A., M. J. Smyth, and S. Ostrand-Rosenberg. 2002. Interferon-γ-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma. *Cancer Res. 62:4406.*
22. Kusmartsev, S., and D. I. Gabrilovich. 2003. Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J. Leukocyte Biol. 74:186.*
23. Green, S. J., J. Aniagolu, and J. J. Raney. 2003. Oxidative metabolism of murine macrophages. In *Current Protocols In Immunology*, Vol. 3. J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds. Wiley, Hoboken, NJ, p. 14.5.1.
24. Munder, M., K. Eichmann, J. M. Moran, F. Centeno, G. Soler, and M. Modolell. 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J. Immunol. 163:3771.*
25. Corraliza, I. M., M. L. Campo, G. Soler, and M. Modolell. 1994. Determination of arginase activity in macrophages: a micromethod. *J. Immunol. Methods 174:231.*
26. Bronte, V., P. Serafini, C. De Santo, I. Marigo, V. Tosello, A. Mazzoni, D. M. Segal, C. Staib, M. Lowel, G. Sutter, M. P. Colombo, and P. Zanovello. 2003. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. *J. Immunol. 170:270.*
27. Decker, T., and M. L. Lohmann-Matthes. 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods 115:61.*
28. Kusmartsev, S., F. Cheng, B. Yu, Y. Nefedova, E. Sotomayor, R. Lush, and D. Gabrilovich. 2003. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res. 63:4441.*
29. Danna, E. A., P. Sinha, M. Gilbert, V. K. Clements, B. A. Pulaski, and S. Ostrand-Rosenberg. 2004. Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease. *Cancer Res. 64:2205.*
30. Kusmartsev, S., and D. I. Gabrilovich. 2002. Immature myeloid cells and cancer-associated immune suppression. *Cancer Immunol. Immunother. 51:293.*
31. Kusmartsev, S., Y. Nefedova, D. Yoder, and D. I. Gabrilovich. 2004. Antigen-specific inhibition of $CD8^+$ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J. Immunol. 172:989.*
32. Bronte, V., P. Serafini, A. Mazzoni, D. M. Segal, and P. Zanovello. 2003. L-Arginine metabolism in myeloid cells controls T-lymphocyte functions. *Trends Immunol. 24:302.*
33. Gabrilovich, D. I., M. P. Velders, E. M. Sotomayor, and W. M. Kast. 2001. Mechanism of immune dysfunction in cancer mediated by immature $Gr-1^+$ myeloid cells. *J. Immunol. 166:5398.*
34. Mills, C. D., K. Kincaid, J. M. Alt, M. J. Heilman, and A. M. Hill. 2000. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J. Immunol. 164:6166.*
35. Mills, C. D. 2001. Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. *Crit. Rev. Immunol. 21:399.*
36. Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol. 23:549.*
37. Kiessling, R., K. Wasserman, S. Horiguchi, K. Kono, J. Sjoberg, P. Pisa, and M. Petersson. 1999. Tumor-induced immune dysfunction. *Cancer Immunol. Immunother. 48:353.*
38. Rodriguez, P. C., A. H. Zea, K. S. Culotta, J. Zabaleta, J. B. Ochoa, and A. C. Ochoa. 2002. Regulation of T cell receptor CD3ζ chain expression by L-arginine. *J. Biol. Chem. 277:21123.*
39. Rodriguez, P. C., D. G. Quiceno, J. Zabaleta, B. Ortiz, A. H. Zea, M. B. Piazuelo, A. Delgado, P. Correa, J. Brayer, E. M. Sotomayor, et al. 2004. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res. 64:5839.*
40. El-Gayar, S., H. Thuring-Nahler, J. Pfeilschifter, M. Rollinghoff, and C. Bogdan. 2003. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. *J. Immunol. 171:4561.*
41. Wei, L. H., A. T. Jacobs, S. M. Morris, Jr., and L. J. Ignarro. 2000. IL-4 and IL-13 upregulate arginase I expression by cAMP and JAK/STAT6 pathways in vascular smooth muscle cells. *Am. J. Physiol. Cell Physiol. 279:C248.*
42. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol. 3:23.*
43. Rodriguez, P. C., A. H. Zea, J. DeSalvo, K. S. Culotta, J. Zabaleta, D. G. Quiceno, J. B. Ochoa, and A. C. Ochoa. 2003. L-Arginine consumption by macrophages modulates the expression of CD3ζ chain in T lymphocytes. *J. Immunol. 171:1232.*
44. Niedbala, W., X. Q. Wei, D. Piedrafita, D. Xu, and F. Y. Liew. 1999. Effects of nitric oxide on the induction and differentiation of Th1 cells. *Eur. J. Immunol. 29:2498.*
45. Niedbala, W., X. Q. Wei, C. Campbell, D. Thomson, M. Komai-Koma, and F. Y. Liew. 2002. Nitric oxide preferentially induces type 1 T cell differentiation by selectively up-regulating IL-12 receptor $\beta 2$ expression via cGMP. *Proc. Natl. Acad. Sci. USA 99:16186.*
46. Kim, S. J., O. S. Bang, Y. S. Lee, and S. S. Kang. 1998. Production of inducible nitric oxide is required for monocytic differentiation of U937 cells induced by vitamin E-succinate. *J. Cell Sci. 111:435.*

47. Phung, Y. T., J. M. Bekker, O. G. Hallmark, and S. M. Black. 1999. Both neuronal NO synthase and nitric oxide are required for PC12 cell differentiation: a cGMP independent pathway. *Brain Res. Mol. Brain Res. 64:165.*

48. Almand, B., J. I. Clark, E. Nikitina, J. van Beynen, N. R. English, S. C. Knight, D. P. Carbone, and D. I. Gabrilovich. 2001. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J. Immunol. 166:678.*

49. Bronte, V., E. Apolloni, A. Cabrelle, R. Ronca, P. Serafini, P. Zamboni, N. P. Restifo, and P. Zanovello. 2000. Identification of a $CD11b^+/Gr\text{-}1^+/CD31^+$ myeloid progenitor capable of activating or suppressing $CD8^+$ T cells. *Blood 96:3838.*

50. Liu, Y., J. A. Van Ginderachter, L. Brys, P. De Baetselier, G. Raes, and A. B. Geldhof. 2003. Nitric oxide-independent CTL suppression during tumor progression: association with arginase-producing (M2) myeloid cells. *J. Immunol. 170:5064.*

51. Mazzoni, A., V. Bronte, A. Visintin, J. H. Spitzer, E. Apolloni, P. Serafini, P. Zanovello, and D. M. Segal. 2002. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J. Immunol. 168:689.*

52. Shankaran, V., H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old, and R. D. Schreiber. 2001. IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature 410:1107.*

53. Munn, D. H., M. D. Sharma, D. Hou, B. Baban, J. R. Lee, S. J. Antonia, J. L. Messina, P. Chandler, P. A. Koni, and A. L. Mellor. 2004. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J. Clin. Invest. 114:280.*

54. Jensen, S. M., S. L. Meijer, R. A. Kurt, W. J. Urba, H. M. Hu, and B. A. Fox. 2003. Regression of a mammary adenocarcinoma in $STAT6^{-/-}$ mice is dependent on the presence of STAT6-reactive T cells. *J. Immunol. 170:2014.*

55. Ostrand-Rosenberg, S., P. Sinha, V. Clements, S. I. Dissanayake, S. Miller, C. Davis, and E. Danna. 2004. Signal transducer and activator of transcription 6 (Stat6) and CD1: inhibitors of immunosurveillance against primary tumors and metastatic disease. *Cancer Immunol. Immunother. 53:86.*

Breast Disease 20 (2004) 127–135
IOS Press

Antagonists of Tumor-Specific Immunity: Tumor-Induced Immune Suppression and Host Genes that Co-opt the Anti-Tumor Immune Response

Suzanne Ostrand-Rosenberg*, Pratima Sinha, Erika A. Danna, Seth Miller, Cordula Davis and Samudra K. Dissanayake
University of Maryland Baltimore County, Baltimore, MD 21250, USA

Abstract. Metastatic disease is the principle cause of death for most patients with breast cancer. Conventional therapies including radiation therapy and chemotherapy are largely uneffective against metastatic disease. It is now generally appreciated that the immune system can destroy tumor cells, and numerous novel immunotherapies are currently under development. Many of these immunotherapies are dependent on activation of the host's immune system so the success of a cancer vaccine will depend on the immune status of the patient. Tolerance to tumor antigens, tumor-induced immune suppression, and the presence of immunomodulatory genes that block the development of tumor-specific immunity can potentially interfere with the therapeutic efficacy of immune-based therapies. Studies from the authors' laboratory demonstrate that although mice with bulky primary mammary tumors are immunosuppressed for T cell and antibody-mediated immunity, surgical removal of the primary tumor reverses the suppression, even when disseminated metastatic disease is present. The post-surgical reversal is associated with a large decrease in myeloid suppressor cells. In addition to tumor-induced suppression, two genes, the Stat6 and CD1 genes, are also associated with inhibiting tumor-specific immunity, since mice deficient for these genes have dramatically enhanced resistance to metastatic mammary carcinoma. Therefore, optimal delivery of immunotherapy should be coordinated with methodology that decreases immune suppression and eliminates or blocks inhibitory factors.

Abbreviations: DC: Dendritic cells; HEL: hen eggwhite lysozyme; MSC: Myeloid suppressor cells (immature dendritic cells); VEGF: Vascular endothelial growth factor; TGF-β: Transforming growth factor beta; 6-TG: 6-thioguanine (4T1 cells are 6-TG-resistant, and hence selectively grow in medium supplemented with 6-TG)

INTRODUCTION

Most deaths from breast cancer are due to metastatic disease. Immunotherapy is a promising, novel treatment that may be therapeutically effective

Of the approximately 41,000 women per year who die of breast cancer, most die from metastatic disease [1]. Although improved early detection may increase survival because primary tumors are detected before they metastasize, more effective therapies are required for women who have metastatic disease at the time of diagnosis. In addition, establishing a state of "immunosurveillence" in patients with latent metastatic breast cancer may be an effective method for preventing metastatic disease progression years after removal of a primary tumor. Although cancer immunotherapy has gone through multiple cycles of respectability during the past 100 years, it is now generally appreciated that the immune system can destroy

*Corresponding author: Dr. S. Ostrand-Rosenberg, Dept. Biological Sciences, U. of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250, USA. Tel.: +1 410 455 2237; Fax: +1 410 455 3875; E-mail: srosenbe@umbc.edu.

0888-6008/04/$17.00 © 2004 – IOS Press and the authors. All rights reserved

endogenous tumor cells [2,3], and a number of promising immunotherapies are currently in clinical trials (http://clinicaltrials.gov/). Indeed, many investigators now feel that the immune system is particularly well suited to destroy disseminated metastatic tumor cells, and that it will be possible to harness the immune system as an effective immunotherapy/immunoprevention agent [4].

Before immunotherapy can be used as a therapeutic and/or prophylactic strategy, there are several issues that must be addressed. A critical factor is the immune status of the tumor-bearing individual. There is some evidence demonstrating that tumor-bearing individuals may be immune suppressed. Obviously, immune suppression could severely compromise the therapeutic and/or prophylactic success of immunotherapy. This article will briefly review some of the mechanisms that lead to immune suppression in tumor-bearing individuals, and then review some of the studies in the authors' laboratory that address these issues in experimental animals with metastatic mammary carcinoma.

The immune status of the tumor-bearing host is pivotal for effective immunotherapy

There are several central problems that impede the clinical development of immunotherapy. A likely use of immunotherapy will be in patients whose primary tumor has been surgically removed, but who are at high risk for metastatic disease. In this setting, a cancer vaccine or other immunotherapy may eliminate proliferating tumor cells, as well as destroy latent, nonproliferating metastatic cells. Since immunotherapies work by activating the host's immune response, a critical issue is the immune status of the tumor-bearing individual during the administration of immunotherapy. For example, if post-surgery patients are immunosuppressed, then it is unlikely that immunotherapy in the post-surgery setting will be productive.

Aside from the immune suppression that can be induced by some chemotherapies (reviewed by [5]), there are three general phenomena that account for much of the decreased immune competence in tumor-bearing individuals: 1) Tolerance to antigens expressed by tumor cells; 2) Immunosuppression via myeloid suppressor cells or immature dendritic cells; and 3) Skewing of immunity towards a non-productive immune response by $CD4^{+}CD25^{+}$ T regulatory cells.

Immunocompetence in tumor-bearing individuals is modulated by tumor-induced tolerance, T regulatory cells, and/or immunosuppressive cells

Although some investigators argue that the immune system of tumor-bearing individuals is "ignorant" of resident tumors [6,7], most investigators concur that tumor-bearing individuals are actively tolerized or anergized to their tumor antigens [8–12]. Tolerance typically occurs when tumor antigens are over-expressed "self" antigens that are present throughout the life of the individual. However, constitutive expression of a tumor antigen, even before tumor onset, does not ensure full tolerance. For example, transgenic mice that spontaneously develop breast cancer because they contain the ErbB-2 (her2/neu) gene under the control of a constitutive viral promoter display varying levels of tolerance to her2/neu protein [13–18]. Despite this tolerance, experimental immunotherapies have been developed that effectively target tolerized tumor antigens. For example, the her2/neu transgenics can be induced to develop immunity to her2/neu protein which results in significant delay of tumor onset [17,19]. Therefore, although individuals with cancer may be tolerant to the antigens of their tumors, some immunotherapies overcome this tolerance and result in effective anti-tumor immunity.

Modulation of the immune response is also mediated by $CD4^{+}CD25^{+}$ T regulatory cells. $CD4^{+}CD25^{+}$ T regulatory cells suppress the activation of $CD8^{+}$ T cells by blocking the production of IL-2, although the precise mechanism by which they block is unknown [20]. These cells are critical for preventing autoimmunity [21,22]; however, they also inhibit antitumor immunity [23]. They mediate their effects at the time of immunization, so if $CD4^{+}CD25^{+}$ T cells are present at the onset of tumor growth, they may block activation of $CD8^{+}$ T cells [20,23]. Numerous studies have demonstrated that depletion of $CD4^{+}CD25^{+}$ T cells results in enhanced $CD8^{+}$-mediated immunity against primary, solid, s.c. tumors. Unfortunately, immunotherapy involving elimination of $CD4^{+}CD25^{+}$ T regulatory cells can be accompanied by significant increases in autoimmunity [24]. Therefore, although anti-tumor immunity can be enhanced by deletion of $CD4^{+}CD25^{+}$ T regulatory cells, the onset of autoimmunity is a significant side-effect that must be considered.

Another mechanism by which tumors down-regulate anti-tumor immunity is via the induction of myeloid suppressor cells (MSC) or immature myeloid cells

which inhibit the activation of $CD4^+$ and $CD8^+$ T cells. MSC were originally identified in patients with head and neck cancer who had excessively high levels of immature $CD34^+$ dendritic cells (DC) and low levels of mature DC [25]. MSC have subsequently been found in patients with many other types of cancers (including breast cancer) and in tumor-bearing experimental animals [26,27]. These immature DC express the surface markers Gr1 and CD11b [28,29], and are induced by vascular endothelial cell growth factor (VEGF) and transforming growth factor beta (TGFβ) that are produced by many tumor cells [30,31]. They suppress activation of both $CD4^+$ and $CD8^+$ T cells [28,29,32]. There is a strong correlation between MSC and immune suppression in both patients and mice with tumors, and investigators have concluded that MSC may interfere with active immunotherapy strategies [33].

Tumor-induced immune suppression is reversed following removal of primary tumors, despite the presence of metastatic disease

Since immunotherapy is likely to be used in a post-surgery setting, it is important to assess if removal of primary tumor restores the tumor-bearing individual's ability to respond to immunological stimuli. Since many breast cancer patients may have established or latent metastatic disease after resection of their primary tumors, we have determined if the presence of metastatic lesions affects tumor-induced immune suppression. In the following sections we describe our studies examining this question using the 4T1 mouse mammary carcinoma model.

Extensive studies in this laboratory during the past eight years have demonstrated that the BALB/c-derived 4T1 mammary tumor, originally derived by F. Miller [34,35], is an excellent mouse model for human breast cancer because it shares many common features with the human disease [36–38]. For example, 4T1 is virtually non-immunogenic and highly malignant. As few as 7000 cells inoculated into the abdominal mammary gland give rise to mammary carcinomas in >95% of inoculated mice [36]. Since 4T1 cells are resistant to 6-thioguanine (6-TG), body organs can be explanted into medium supplemented with 6-TG and the number of clonogenic tumor cells quantified by counting 6-TG-resistant colonies (clonogenic assay) [34,36]. Inoculation of 7000 4T1cells into the abdominal mammary gland results in spontaneous metastasis to the lungs, liver, blood, lymph nodes, brain, and bone marrow [36, 39] Clements and Ostrand-Rosenberg, unpublished].

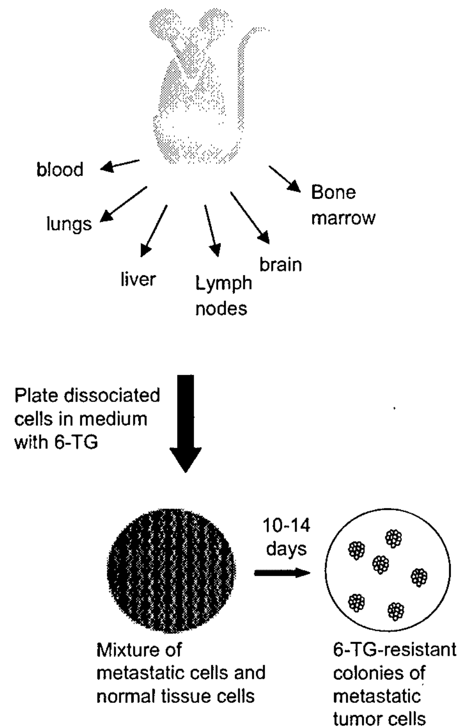


Fig. 1. The clonogenic assay for quantifying the number of metastatic mammary tumor cells in distant organs. Target organs are removed from tumor-bearing (non-surgery) or post-surgery mice, dissociated into single cell suspensions, and cultured in growth medium containing 6- thioguanine. Ten to 14 days later, culture plates are stained and the number of colonies is counted. Since 4T1 tumor cells are 6-TG resistant and normal tissue cells are not, the number of colonies is equal to the number of metastatic cells in the organ.

Furthermore, metastatic cells proliferate in distant organs while the primary tumor is in place, and they continue to proliferate when the primary tumor is surgically removed, resulting in death of >95% of inoculated mice [37,40–42]. Figure 1 is a schematic diagram showing how the clonogenic assay is used to quantify the number of metastatic tumor cells in distant organs. Figure 2 shows a time line for the onset of metastatic disease, surgery, and death, based on experiments with >200 BALB/c mice [38,40–42]. These data identify a well-defined 2–3.5 week "window" after surgery and before death during which experiments can be performed assessing the immune status of individual mice.

Immunosuppression is frequently mediated by factors secreted by tumor cells [27,43], and tolerance can be regulated by quantity of tolerizing antigen [44,45]. Therefore, after removal of primary tumor, if a patient

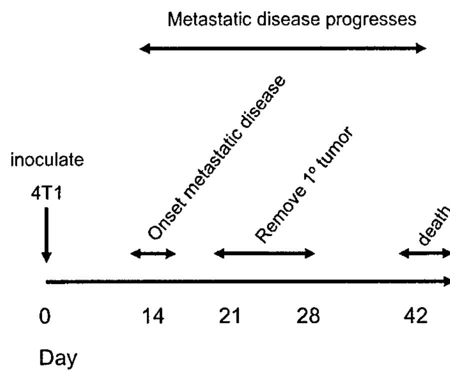


Fig. 2. Timeline for the onset and progression of spontaneous metastatic disease in mice inoculated with the transplantable 4T1 mammary carcinoma.

Table 1
Antibody production and T cell responses are suppressed in tumor-bearing mice, but revert to normal levels after surgical removal of primary tumor

Mice	Antibody response (ng/ml)[1]	T cell response (cpm)[2]
Tumor-free	47.8 ± 20.6	12,859 ± 3,330
Non-surgery	21.3 ± 13.2	7490 ± 1944
Post-surgery	37.6 ± 7.7	11,469 ± 2,374

[1]Tumor-free, non-surgery, or post-surgery mice were immunized and boosted with hen eggwhite lysozyme (HEL) on days 0 and 7, and serum collected on day 14 and assayed by ELISA for total (IgG plus IgM) anti-HEL antibodies.
[2]Tumor-free, non-surgery, or post-surgery mice were immunized with HEL in adjuvant on day 0 and splenocytes harvested on day 9 and co-cultured for 6 days with HEL. Cultures were pulsed for the last 18 hours with ^{3}H-thymidine.

has active or latent metastatic disease, there is the potential for immune suppression. Since immunotherapy is a potential treatment for the approximately 30% percent of breast cancer patients who will ultimately die from metastatic disease after their primary tumors are resected, it is important to determine if disseminated metastatic tumor induces immune suppression and/or tolerance.

To assess immunosuppression in individuals with metastatic disease, we have compared antibody, T cell, macrophage and dendritic cell (DC) function in mice without tumor ("tumor-free"), with a primary tumor in place ("non-surgery"), or with a primary tumor surgically removed but with established metastatic disease ("post-surgery"). 4T1 tumor is inoculated and surgery was performed according to the time line of Fig. 1. For antibody responses, nonsurgery and post-surgery mice were challenged with a nominal antigen on day 2–3 after surgery and bled on day 16–18 after surgery for primary responses, and boosted on day 9 after surgery for secondary antibody responses. Specific antibody production was uniformly suppressed in the non-surgery group, but post-surgery mice made specific antibodies at the same level as tumorfree mice (Table 1). Assays quantifying the number of metastatic tumor cells in the lungs demonstrated that the post-surgery mice had established metastatic disease. Similar results were seen for antigen-specific T cell responses in which tumor-free, non-surgery, and post-surgery mice were inoculated with a nominal antigen and T cell responses assessed five days later (Table 1). Therefore, antigen-specific T can B cell responses are dysfunctional in tumor-bearing individuals; however, immunocompetence recovers after resection of the primary tumor [46].

In contrast, macrophage activity as measured by lipopolysaccharide activation, and dendritic cell antigen presentation as measured by allogeneic and MHC class II-restricted antigen presentation, were not suppressed in 4T1 non-surgery or 4T1 post-surgery mice. This latter result was somewhat surprising since two previous studies have described suppressed DC function in tumor-bearing individuals [47,48]. Collectively, these results demonstrate that although immune responses of tumor-bearing individuals are globally suppressed, ability to respond to antigen is restored by removal of primary tumor, even if disseminated metastatic disease is present [46]. Therefore, post-surgery patients with or without metastatic disease are potential candidates for immunotherapy.

Although there is a significant reduction in myeloid suppressor cells after surgical removal of primary tumor, mice still die from metastatic mammary carcinoma

Because MSC effectively inhibit both CD4^{+} and CD8^{+} T lymphocytes, reduction in immunocompetence in tumor-bearing individuals is often attributed to increasing levels of MSC. To determine if MSC levels are altered in individuals with or without primary tumor and metastatic disease, we monitored splenic MSC in tumor-free, non-surgery tumor-bearing, and post-surgery BALB/c mice carrying the 4T1 mammary carcinoma. Mice were inoculated with 4T1 in the abdominal mammary gland on day 0 and primary tumors were either left in place (non-surgery group), or removed approximately 21–28 days later (post-surgery group). MSC levels were measured by immunofluorescence staining of splenocytes for Gr1^{+}CD11b^{+} cells on day 38–40. As seen in Fig. 3, the non-surgery group

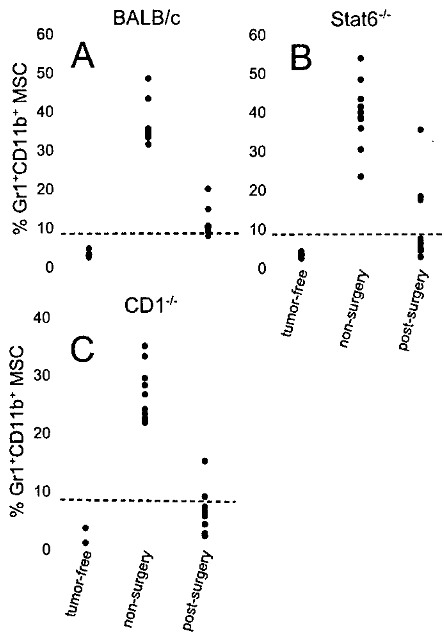


Fig. 3. Myeloid suppressor cell levels are elevated in tumor-bearing mice and decrease after removal of primary tumor, particularly in Stat6-deficient and CD1-deficient mice. For the nonsurgery and post-surgery groups, mice were inoculated with 4T1 tumor cells on day 0. Primary mammary tumors were surgically removed from the post-surgery group on day 28. Splenocytes were stained on day 38–42 with Gr1-PE and CD11b-FITC, and assessed by flow cytometry. Each point represents an individual mouse.

has elevated levels of MSC as compared to tumor-free mice, while the post-surgery group has fewer MSC than the non-surgery group, but more than the tumor-free group. Functional assays demonstrated that MSC from both the nonsurgery and post-surgery groups equally suppress T cell activation. However, despite the decrease in MSC after surgery, >95% of the post-surgery mice die from metastatic disease with the same kinetics as the non-surgery mice [40]. Therefore, although surgery produces a decrease in MSC levels, BALB/c mice still die from metastatic disease, suggesting that either the MSC decrease is not sufficient to eliminate suppression, or additional factors beyond MSC are involved in depressing anti-tumor immunity.

The Stat6 and CD1 genes inhibit the development of anti-tumor immunity and lead to immune suppression

Type 1 $CD4^+$ T helper lymphocytes are considered by many tumor immunologists to facilitate tumor rejection by providing "help" to activate tumor-specific $CD8^+$ T cells [49,50]. This hypothesis has led to the assumption that if $CD4^+$ T cell responses could be skewed towards a Type 1 phenotype and away from a Type 2 phenotype, then tumor-specific immunity would be enhanced. We have tested this hypothesis using knockout mice that are unable to make a Type 2 $CD4^+$ T cell response, and hence default to a Type 1 response. The Signal Transducer and Activator of Transcription 6 (Stat6) gene is essential for transmitting signals by IL-4 and IL-13. Since these cytokines are critical for development of Type 2 $CD4^+$ T cells, Stat6-deficient ($Stat6^{-/-}$) mice do not produce Type 2 T cells [51,52].

To determine if Stat6-deficient BALB/c mice have enhanced anti-tumor immunity, they were inoculated with the 4T1 mammary carcinoma and followed for primary tumor progression and metastatic disease. Not surprisingly, primary mammary tumors grew slightly more slowly in Stat6-deficient vs. Stat6-competent BALB/c mice. Metastatic disease, as measured by metastatic tumor cells in the lungs, liver, bone marrow, and brain was significantly reduced in the Stat6-deficient mice relative to Stat6-competent animals. T cell depletion studies demonstrated that the decrease in tumor progression was T-cell mediated [53]. Other investigators have found a similar enhancement of tumor-specific immunity in Stat6-deficient mice for a primary fibrosarcoma [54] and a mastocytoma [55]. Thus, elimination of the Stat6 gene allows for the development of effective tumor-specific immunity.

Subsequent studies with the 4T1 mammary carcinoma demonstrated that Stat6-deficient mice were highly resistant to metastatic disease provided the primary tumor in the mammary gland was surgically removed [42] (see Table 3). Because anti-tumor immunity is improved by the *deletion* of the Stat6 gene, these studies have collectively led to the concept that Stat6 regulates an *inhibitor* that blocks or suppresses the induction of tumor-specific immunity. Some investigators have argued that the inhibitors are IL-4 and IL-13 and that they mediate their effect by skewing the response away from a Type 1 $CD4^+$ T cell response [54, 56].

In addition to being resistant to the transplanted 4T1 tumor, mice deficient for the Stat6 gene are also resistant to spontaneous mammary carcinoma. BALB/c $neuT^{+/-}$ mice are transgenic mice that contain the transforming her2/neu (ErbB-2) gene under the control of the mouse mammary tumor virus LTR. These mice spontaneously develop multi-focal mammary neoplasia starting at approximately 10 weeks of age [13]. To determine if Stat6-deficiency affects spontaneous mam-

Table 2
Mice with deleted Stat6 or CD1 genes have increased survival and decreased metastatic mammary cancer

Number survivors/total mice[1]		
BALB/c	$Stat6^{-/-}$	$CD1^{-/-}$
2/45 (4%)	30/44 (68.2%)	8/10 (80%)

[1]Mice were inoculated in the abdominal mammary gland with 7000 4T1 mammary carcinoma cells and primary tumors were surgically removed 2.5–3.5 weeks later. Mice were followed for survival. Survivors are defined as mice that remain alive for >280 days after initial 4T1 inoculation.

Table 3
Mice with a deleted Stat6 gene have decreased metastatic mammary cancer

Site of metastasis	Percent mice with metastatic mammary carcinoma[1]	
	BALB/c	$Stat6^{-/-}$
Lungs	92.5	40
Bone marrow	54	0
Liver	92	15

[1]Mice were inoculated with 4T1 mammary carcinoma on day 0, primary tumors were surgically removed 2–3 weeks later, and organs explanted on day 42–25 and assayed by the clonogenic assay for metastatic tumor cells.

mary cancer development and/or progression, neuT mice were crossed with Stat6-deficient mice and the offspring backcrossed to Stat6-deficient mice to obtain $Stat6^{-/-}neuT^{+/-}$ mice. The $Stat6^{-/-}neuT^{+/-}$ mice developed mammary tumors more slowly, had fewer tumors, and survived longer than $neuT^{+/-}$ mice, demonstrating that Stat6-deficiency also reduces spontaneous mammary cancer (S. Miller, C. Davis, S. Dissanayake, and S. Ostrand-Rosenberg, unpublished results).

CD1-deficient mice also have greatly increased tumor-specific immunity and survive 4T1 metastatic disease (Table 2) [42] and are also resistant to recurrence of the 15–12RM fibrosarcoma [54]. CD1-deficient mice lack NKT cells which are a principle source of IL-13, and therefore, it has been hypothesized that IL-13 is the inhibitor that blocks tumor-specific immunity in CD1-deficient mice [54]. Blocking IL-13 and/or IL-4 either via *in vivo* administered inhibitors or through the use of receptor-deficient mice does not yield enhanced tumor immunity to the 4T1 mammary carcinoma. Therefore, we have concluded that IL-13 and IL-4 are not the exclusive inhibitors that prevent the induction of immunity to 4T1 mammary carcinoma [42].

Further studies have shown that $CD4^{+}CD25^{+}$ T regulatory cells are not responsible for preventing the induction of tumor-specific immunity against the 4T1 mammary tumor because depletion of these cells does not affect progression of either primary tumor or metastatic disease. Therefore, the Stat6 and CD1 genes may regulate as yet unidentified molecules that inhibit $CD8^{+}$ T-cell-mediated tumor-specific immunity [42].

Stat6-deficient and CD1-deficient mice rapidly loose MSC after surgical removal of primary mammary tumors

Similar to many tumors, 4T1 secretes growth factors such as VEGF and TGF that stimulate MSC and block differentiation of DC. As described above and shown in Table 2, Stat6-deficient and CD1-deficient mice have very effective immunity against metastatic disease, provided the primary mammary tumor is surgically removed. To determine if the enhanced immunity is associated with reduced MSC levels, we have compared MSC levels in tumor-free, non-surgery (primary tumor in place), and post-surgery mice (with metastatic disease). Splenic MSC levels in tumor-free mice are typically below 8%, while MSC levels in tumor-bearing mice (equivalent to our non-surgery group) can be as high as 60%. As shown in Fig. 3, a high percentage of post-surgery Stat6-deficient and CD1-deficient (panels B and C, respectively) have splenic MSC levels below 8%. In contrast, most post-surgery BALB/c mice have MSC levels above 10% (panel A). Therefore, mice deleted for the Stat6 or CD1 gene display a rapid reversal in MSC levels following surgical removal of primary tumor. Collectively, these results suggest that the deleted inhibitor in Stat6-deficient and CD1-deficient mice may act by either directly or indirectly regulating MSC levels.

CONCLUSIONS

Most deaths from breast cancer are the result of metastatic disease that progresses after surgical removal of primary tumor. Immunotherapy has been proposed as a potentially efficacious treatment to destroy micro (and perhaps macro) metastasis in post-surgery patients. The potency of immunotherapy will directly depend on the immune status (competence) of the host. Since tumor burden profoundly influences immune status, it is critical to understand the impact of latent and/or active metastatic disease on immunocompetence. In this report we have reviewed the mechanisms known to decrease immune responsiveness in mice with primary and metastastic mammary carcinoma, and we have presented data demonstrating that surgical removal of primary tumor reverses this

immune suppression. These studies demonstrate that there is a "window" of time after surgical removal of primary tumor during which tumor-bearing individuals may be candidates for immune-based therapies. This "window" is present even if the post-surgery individual has active metastatic disease. However, as metastatic disease and metastatic tumor burden increase, immune suppression may recur, making immunotherapy less efficacious. In addition to complications arising from tumor-induced immune suppression, we have also described two genes, the Stat6 and CD1 genes, that inhibit the development of effective tumor-specific immunity. These genes antagonize the development of tumor-specific immunity regardless of the presence of tumor. If the Stat6 and CD1 genes have comparable inhibitory function in humans, then therapies based on their selective deletion may be possible. Given the dramatic affects of both tumor-induced immune suppression and inhibitory genes on the downregulation of tumor-specific immunity, the design of any immune strategy for the treatment of metastatic cancers must factor in these variables.

ACKNOWLEDGEMENTS

We appreciate Ms. Virginia K. Clements for providing outstanding technical support and Ms. Sandra Mason for her excellent care of our mouse colony. These studies were supported by NIH R01CA52527 and R0184232, DOD US Army Research and Development Command DAMD17-01-0312. E.A.D. is a BCURE fellow and was partially supported by US Army Research and Development Command DAMD17-01-0313.

REFERENCES

[1] E. Winer, M. Morrow, C. Osborne and J. Harris, Malignant tumors of the breast, in: *Cancer, Principles & Practice of Oncology*, (Vol. 2), J. De Vita, V., S. Hellman and S. Rosenberg, eds, Lippincott Williams & Wilkins, Philadelphia, 2001, pp. 1651–1717.

[2] C.R. Parish, Cancer immunotherapy: The past, the present and the future, *Immunol Cell Biol* **81** (2003), 106–113.

[3] G.P. Dunn, A.T. Bruce, H. Ikeda, L.J. Old and R.D. Schreiber, Cancer immunoediting: from immunosurveillance to tumor escape, *Nat Immunol* **3** (2002), 991–998.

[4] M. Whelan, J. Whelan, N. Russell and A. Dalgleish, Cancer immunotherapy: an embarrassment of riches?, *Drug Discov Today* **8** (2003), 253–258.

[5] A. Terando and J.J. Mule, On combining antineoplastic drugs with tumor vaccines, *Cancer Immunol Immunother* **52** (2003), 680–685.

[6] A.F. Ochsenbein, S. Sierro, B. Odermatt M. Pericin, U. Karrer, J. Hermans, S. Hemmi, H. Hengartner and R.M. Zinkernagel, Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction, *Nature* **411** (2001), 1058–1064.

[7] M.T. Spiotto, P. Yu, D.A. Rowley, M.I. Nishimura, S.C. Meredith, T.F. Gajewski, Y.X. Fu and H. Schreiber, Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells, *Immunity* **17** (2002), 737–747.

[8] K. Staveley-O'Carroll, E. Sotomayor, J. Montgomery, I. Borrello, L. Hwang, S. Fein, D. Pardoll and H. Levitsky, Induction of antigen-specific T cell anergy: An early event in the course of tumor progression, *Proc Natl Acad Sci USA* **95** (1998), 1178–1183.

[9] E.M. Sotomayor, I. Borrello, F.M. Rattis, A.G. Cuenca, J. Abrams, K. Staveley-O'Carroll and H.I. Levitsky, Cross-presentation of tumor antigens by bone marrow-derived antigenpresenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression, *Blood* **98** (2001), 1070–1077.

[10] P. Shrikant, A. Khoruts and M.F. Mescher, CTLA-4 blockade reverses $CD8^+$ T cell tolerance to tumor by a $CD4^+$ T cell- and IL-2-dependent mechanism, *Immunity* **11** (1999), 483–493.

[11] E.M. Sotomayor, I. Borrello, E. Tubb, F.M. Rattis, H. Bien, Z. Lu, S. Fein, S. Schoenberger and H.I. Levitsky, Conversion of tumor-specific $CD4^+$ T-cell tolerance to T-cell priming through in vivo ligation of CD40, *Nat Med* **5** (1999), 780–787.

[12] D. Pardoll, Does the immune system see tumors as foreign or self?, *Annu. Rev. Immunol.* **21** (2003), 807–839.

[13] K. Boggio, G. Nicoletti, E. Di Carlo, F. Cavallo, L. Landuzzi, C. Melani, M. Giovarelli, I. Rossi, P. Nanni, C. De Giovanni, P. Bouchard, S. Wolf, A. Modesti, P. Musiani, P.L. Lollini, M.P. Colombo and G. Forni, Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice, *J Exp Med* **188** (1998), 589–596.

[14] S. Rovero, A. Amici, E.D. Carlo, R. Bei, P. Nanni, E. Quaglino, P. Porcedda, K. Boggio, A. Smorlesi, P.L. Lollini, L. Landuzzi, M.P. Colombo, M. Giovarelli, P. Musiani and G. Forni, DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice, *J Immunol* **165** (2000), 5133–5142.

[15] A.M. Ercolini, J.P. Machiels, Y.C. Chen, J.E. Slansky, M. Giedlen, R.T. Reilly and E.M. Jaffee, Identification and characterization of the immunodominant rat HER-2/neu MHC class I epitope presented by spontaneous mammary tumors from HER-2/neu-transgenic mice, *J Immunol* **170** (2003), 4273–4280.

[16] C.T. Guy, R.D. Cardiff and W.J. Muller, Activated neu induces rapid tumor progression, *J Biol Chem* **271** (1996), 7673–7678.

[17] R.T. Reilly, M.B. Gottlieb, A.M. Ercolini, J.P. Machiels, C.E. Kane, F.I. Okoye, W.J. Muller, K.H. Dixon and E.M. Jaffee, HER-2/neu is a tumor rejection target in tolerized HER- 2/neu transgenic mice, *Cancer Res* **60** (2000), 3569–3576.

[18] M.P. Piechocki, Y.S. Ho, S. Pilon and W.Z. Wei, Human ErbB-2 (Her-2) Transgenic Mice: A Model System for Testing Her-2 Based Vaccines, *J Immunol* **171** (2003), 5787–5794.

[19] E. Di Carlo, S. Rovero, K. Boggio, E. Quaglino, A. Amici, A. Smorlesi, G. Forni and P. Musiani, Inhibition of mammary carcinogenesis by systemic interleukin 12 or p185neu DNA vaccination in Her-2/neu transgenic BALB/c mice, *Clin Cancer Res* **7** (2001), 830s–837s.

[20] E.M. Shevach, $CD4^+$ $CD25^+$ suppressor T cells: more questions than answers, *Nat Rev Immunol* **2** (2002), 389–400.

[21] C.A. Piccirillo and E.M. Shevach, Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells, *J Immunol* **167** (2001), 1137–1140.

[22] S. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh and M. Toda, Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25); Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases, *J Immunol* **155** (1995), 1151–1164.

[23] R.P. Sutmuller, L.M. van Duivenvoorde, A. van Elsas, T.N. Schumacher, M.E. Wildenberg, J.P. Allison, R.E. Toes, R. Offringa and C.J. Melief, Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses, *J Exp Med* **194** (2001), 823–832.

[24] W.Z. Wei, G.P. Morris and Y.C. Kong, Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells, *Cancer Immunol Immunother* (2003).

[25] T. Garrity, R. Pandit, M.A. Wright, J. Benefield, S. Keni and M.R. Young, Increased presence of CD34+ cells in the peripheral blood of head and neck cancer patients and their differentiation into dendritic cells, *Int J Cancer* **73** (1997), 663–669.

[26] V. Bronte, P. Serafini, E. Apolloni and P. Zanovello, Tumor-induced immune dysfunctions caused by myeloid suppressor cells, *J Immunother* **24** (2001), 431–446.

[27] S. Kusmartsev and D.I. Gabrilovich, Immature myeloid cells and cancer-associated immune suppression, *Cancer Immunol. Immunother.* **51** (2002), 293–298.

[28] S. Kusmartsev, Y. Li and S. Chen, Gr-1+ myeloid cell derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/Cd28 costimulation, *J. Immunol.* **165** (2000), 779–785.

[29] V. Bronte, E. Apolloni, A. Cabrelle, R. Ronca, P. Serafini, P. Zamboni, N.P. Restifo and P. Zanovello, Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells, *Blood* **96** (2000), 3838–3846.

[30] R. Young, G. Petruzzelli, K. Kolesiak, N. Achille, M. Lathers and D.I. Gabrilovich, Human squamous cell carcinomas of the head and neck chemoattract immune suppressive CD34+ progenitor cells, *Human Immunol.* **62** (2001), 332–341.

[31] D.I. Gabrilovich, T. Ishida, T. Oyama, S. Ran, V. Kravtsov, S. Nadaf and D.P. Carbone, Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineasges in vivo, *Blood* **92** (1998), 4150–4166.

[32] D.I. Gabrilovich, M.P. Velders, E.M. Sotomayor and W.M. Kast, Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells, *J Immunol* **166** (2001), 5398–5406.

[33] B. Almand, J.R. Resser, B. Lindman, S. Nadaf, J.I. Clark, E.D. Kwon, D.P. Carbone and D.I. Gabrilovich, Clinical significance of defective dendritic cell differentiation in cancer, *Clin Cancer Res* **6** (2000), 1755–1766.

[34] F. Miller, B. Miller and G. Heppner, Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability, *Invasion Metastasis* **3** (1983), 22–31.

[35] C. Aslakson and F. Miller, Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor, *Cancer Res.* **52** (1992), 1399–1405.

[36] B. Pulaski and S. Ostrand-Rosenberg, MHC class II and B7.1 immunotherapeutic cell-based vaccine reduces spontaneous mammary carcinoma metastases without affecting primary tumor growth, *Cancer Res* **58** (1998), 1486–1493.

[37] B.A. Pulaski, V.K. Clements, M.R. Pipeling and S. Ostrand-Rosenberg, Immunotherapy with vaccines combining MHC class II/CD80+ tumor cells with interleukin-12 reduces established metastatic disease and stimulates immune effectors and monokine induced by interferon gamma, *ancer Immunol Immunother* **49** (2000), 34–45.

[38] B. Pulaski and S. Ostrand-Rosenberg, Mouse 4T1 breast tumor model, in: *Current Protocols in Immunology*, J. Coligan, D. Margulies, E. Shevach, W. Strober and A. Kruisbeek, eds, John Wiley & Sons, Inc., 2000, pp. 20.22.21–20.22.11.

[39] M. Lelekakis, J. Moseley, T. Martin, D. Hards, E. Williams, P. Ho, D. Lowen, J. Javni, F. Miller, J. Slavin and R. Anderson, A novel orthotopic model of breast cancer metastasis to bone, *Clin. Exp. Metastasis* **17** (1999), 163–170.

[40] B.A. Pulaski, D.S. Terman, S. Khan, E. Muller and S. Ostrand-Rosenberg, Cooperativity of Staphylococcal aureus enterotoxin B superantigen, major histocompatibility complex class II, and CD80 for immunotherapy of advanced spontaneous metastases in a clinically relevant postoperative mouse breast cancer model, *Cancer Res* **60** (2000), 2710–2715.

[41] B.A. Pulaski, M.J. Smyth and S. Ostrand-Rosenberg, Interferon-gamma-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma, *Cancer Res* **62** (2002), 4406–4412.

[42] S. Ostrand-Rosenberg, V.K. Clements, M. Terabe, J.M. Park, J.A. Berzofsky and S.K. Dissanayake, Resistance to metastatic disease in STAT6-deficient mice requires hemopoietic and nonhemopoietic cells and is IFN-gamma dependent, *J Immunol* **169** (2002), 5796–5804.

[43] S. Salvadori, G. Martinelli and K. Zier, Resection of solid tumors reverses T cell defects and restores protective immunity, *J Immunol* **164** (2000), 2214–2220.

[44] D.A. Nemazee and K. Burki, Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes, *Nature* **337** (1989), 562–566.

[45] C.C. Goodnow, Transgenic mice and analysis of B-cell tolerance, *Annu Rev Immunol* **10** (1992), 489–518.

[46] E. Danna, P. Sinha, M. Gilbert, V.K. Clements, B. Pulaski and S. Ostrand-Rosenberg, Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease, *submitted* (2004),

[47] E. Orsini, A. Guarini, S. Chiaretti, F.R. Mauro and R. Foa, The circulating dendritic cell compartment in patients with chronic lymphocytic leukemia is severely defective and unable to stimulate an effective T-cell response, *Cancer Res* **63** (2003), 4497–4506.

[48] A.S. Yang and E.C. Lattime, Tumor-induced interleukin 10 suppresses the ability of splenic dendritic cells to stimulate CD4 and CD8 T-cell responses, *Cancer Res* **63** (2003), 2150–2157.

[49] H.M. Hu, W.J. Urba and B.A. Fox, Gene-modified tumor vaccine with therapeutic potential shifts tumor-specific T cell response from a type 2 to a type 1 cytokine profile, *J Immunol* **161** (1998), 3033–3041.

[50] M. Shurin, L. Lu, P. Kalinski, A. Stewart-Akers and M. Lotze, Th1/Th2 balance in cancer, transplantation and pregnancy, Springer Semin, *Immunopathol* **21** (1999), 339–359.

[51] M. Kaplan and M. Grusby, Regulation of T helper cell differentiation by STAT molecules, *J. Leukoc. Biol.* **64** (1998), 2–5.

[52] K. Shimoda, J. van Deursen, M. Sangster, S. Sarawar, R. Carson, R. Tripp, C. Chu, F. Quelle, T. Nosaka, D. Vagnali,

P. Doherty, G. Grosveld, W. Paul and J. Ihle, Lack of IL-4-induced Th2 response and IgE class switching in mice with distrupted STAT6 gene, *Nature* **380** (1996), 630–633.
[53] S. Ostrand-Rosenberg, M.J. Grusby and V.K. Clements, Cutting edge: STAT6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma, *J Immunol* **165** (2000), 6015–6019.
[54] M. Terabe, S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D.D. Donaldson, D.P. Carbone, W.E. Paul and J.A. Berzofsky, NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway, *Nat Immunol* **1** (2000), 515–520.
[55] A.K. Kacha, F. Fallarino, M.A. Markiewicz and T.F. Gajewski, Cutting edge: spontaneous rejection of poorly immunogenic P1.HTR tumors by Stat6-deficient mice, *J Immunol* **165** (2000), 6024–6028.
[56] S.M. Jensen, S.L. Meijer, R.A. Kurt, W.J. Urba, H.M. Hu and B.A. Fox, Regression of a mammary adenocarcinoma in STAT6-/- mice is dependent on the presence of STAT6-reactive T cells, *J Immunol* **170** (2003), 2014–2021.

[CANCER RESEARCH 64, 2205–2211, March 15, 2004]

Surgical Removal of Primary Tumor Reverses Tumor-Induced Immunosuppression Despite the Presence of Metastatic Disease

Erika A. Danna, Pratima Sinha, Mileka Gilbert, Virginia K. Clements, Beth A. Pulaski, and Suzanne Ostrand-Rosenberg

Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, Maryland

ABSTRACT

Immunotherapy is a promising approach for the management of malignancies. It may be particularly useful for tumors that do not respond to conventional therapies, such as many metastatic cancers. The efficacy of immunotherapy will depend on many factors, one of which is the immunocompetence of the host. Patients with large primary tumors frequently are immunosuppressed, making them poor candidates for immunotherapy. Although a few studies have reported that surgical removal of primary tumor reverses immunosuppression, it is not known whether metastatic disease in postsurgery patients inhibits this recovery. To determine the role of metastatic disease, we examined tumor-free mice *versus* mice with primary tumor and metastatic disease *versus* mice whose primary tumors were removed surgically but who had metastatic disease. We have used the mouse 4T1 mammary carcinoma, a BALB/c-derived transplantable tumor that shares many characteristics with human breast cancer and is an established model for spontaneous, metastatic cancer. Cell-mediated and humoral adaptive immunity, as measured by rejection of allogeneic tumor, antigen-specific T-cell proliferation, and antigen-specific antibody responses, was suppressed in 4T1-bearing nonsurgery mice relative to tumor-free mice. Surgical removal of primary tumor resulted in rebounding of antibody and cell-mediated responses, even in mice with metastatic disease. Macrophage activity, as measured by lipopolysaccharide responsiveness, and dendritic cell function, as measured by nominal and alloantigen presentation, were not suppressed in tumor-bearing mice. Therefore, the presence of primary tumor suppresses T-cell and antibody responses; however, surgical removal of primary tumor restores immunocompetence even when disseminated metastatic disease is present.

INTRODUCTION

For >20 years immunologists have noted that tumor-bearing patients often are immunosuppressed and unable to respond to their tumors (1). For some patients, the suppression is limited to responses to their resident tumor cells (2), whereas others are unable to respond to a variety of tumors (3), and still others are suppressed globally and unable to respond to nominal antigens (4). In many of the earlier studies, cells were identified as the "suppressor" elements (5–7). In more recent studies, a range of additional mechanisms has been identified that decrease tumor immunity in tumor-bearing persons. These mechanisms include (*a*) immune tolerance of the host to tumor antigens (8); (*b*) genetic changes in tumor cells that render the tumor cells "immune" to the host's immune system (9–11); (*c*) "ignorance" or lack of activation to tumor antigens (12); (*d*) dysfunction of potentially tumor-reactive lymphocytes rendering them unresponsive to antigen (13); and (*e*) immune suppression mediated by tumor cell secretion of inhibitor factors and/or activation of systemically immunosuppressive cells (14, 15). Many of these mechanisms have been documented in a variety of animal models of cancer and in cancer patients, and T-cell (13), B-cell (16), and antigen-presenting cell (14, 17, 18) deficits have been reported.

Immunotherapy has been proposed as a novel therapy for cancers that do not respond to conventional therapies. However, if cancer patients are immunosuppressed, then immunotherapy may be less effective. Studies with experimental animals have led to the conclusion that cancer immunotherapy efficacy is inversely proportional to tumor burden. This conclusion is supported by the paucity of studies in the literature demonstrating effective immunotherapy against large, established tumors (1). The relationship between tumor burden and immunosuppression raises the important question of whether tumor-induced immunosuppression is reversible by surgical removal of the primary tumor. Only a few studies have assessed immunosuppression after primary tumor removal in either mice (19, 20) or humans (21, 22). Although these authors observed different levels of immunosuppression, most report at least partial recovery of immune function following tumor resection.

Although these immunosuppression studies are important to understand the role of primary tumor in inducing immune suppression, they do not address the important question of immune suppression in postsurgery patients with metastatic disease. Surgical removal of primary, solid tumors can be curative. However, if metastatic disease is present at surgery and if the metastases do not respond to conventional therapies, then the cancer can be lethal. Therefore, metastatic cancer is a major target for immunotherapy, and immunotherapy is likely to be used in a postsurgery setting. Because immunotherapy will be most effective for patients who are maximally immunocompetent, it is important to determine whether patients whose primary tumors have been removed, but who have established metastatic disease, are immunosuppressed.

To address this question, we used the 4T1 mouse mammary carcinoma. This poorly immunogenic, BALB/c-derived transplantable tumor shares many characteristics with human breast tumors and is an established model for metastatic cancer (23–25). Using this model, we compared immune responses in mice without tumor ("tumor-free") *versus* mice with intact primary tumors ("nonsurgery") *versus* mice whose primary tumors have been removed but who have established, spontaneous metastatic disease ("postsurgery"). Our studies demonstrate that although tumor-bearing animals have reduced B- and T-cell responses, the immunosuppression is reversed following primary tumor removal even when metastatic disease is present. Therefore, immunotherapy may be useful for postsurgery patients with metastatic disease and for whom conventional therapies are not effective.

MATERIALS AND METHODS

Mice

Female BALB/c, C57BL/6, and C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred at the University of Maryland Baltimore County animal facility. All of the mice used were between 6 weeks and 6 months of age. Mice were housed and bred according to the NIH guidelines for the humane treatment of laboratory animals, and the University

Received 8/24/03; revised 12/4/03; accepted 1/13/04.

Grant support: NIH grants (R01CA52527, R01CA84232) and the United States Army Research and Development Command (DAMD17-01-0312). E. A. Danna is a BCURE fellow and was partially supported by a grant from the United States Army Research and Development Command (DAMD17-01-0313).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Suzanne Ostrand-Rosenberg, Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250. Phone: (410) 455-2237; Fax: (410) 455-3875; E-mail: srosenbe@umbc.edu.

of Maryland Baltimore County Institutional Animal Care and Use Committee approved all of the procedures.

Tumor Cell Lines, 4T1 Inoculations, and Metastasis Assays

4T1 and B16 melF10 cells were grown *in vitro* as described previously (23, 26). BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 4T1 tumor cells/50 μl serum-free Iscove's modified Dulbecco's medium or RPMI. Primary tumor growth and spontaneous lung metastases were measured as described previously (23). Briefly, mean primary tumor diameter (TD) was calculated as the square root of the product of two perpendicular diameters. Lung metastases were quantified using the clonogenic assay by plating dissociated lung cells in medium containing 6-thioguanine and counting foci of 6-thioguanine-resistant 4T1 tumor cells (23, 24).

Surgery

Mice were anesthetized, and tumors were resected as described previously (24, 27). Wounds were closed with Nexaband liquid (Henry Schein, Melville, NY). Mice underwent autopsy at the time of death to confirm the presence of lung metastases and to check for recurrence of the primary tumor.

Immunizations and Bleeds for Antibody Studies

Mice were injected i.p. with 200 μg hen egg white lysozyme (HEL; Sigma-Aldrich, St. Louis, MO) in 100 μl RIBI adjuvant (monophosphoryl lipid A, synthetic trehalose dicorynomycolate, and mycobacteria cell wall skeleton; Sigma-Aldrich) prepared according to the manufacturer's instructions. Briefly, RIBI adjuvant was prepared by warming the vial to 45°C, injecting 1 ml PBS into the vial, and vortexing vigorously for 3 min. Equal volumes of RIBI adjuvant and 4 mg/ml HEL in PBS were mixed by vortexing. Following immunization, mice were bled from the tail vein or heart at selected intervals. For studies of primary antibody responses, results of two experiments were pooled. In the first primary response experiment, 4T1 was inoculated on day −21; primary tumors were surgically removed on day 0; and mice were immunized with HEL on day 2 and bled on days 2 (prebleed) and 17–18 (final bleed). In the second experiment, 4T1 was inoculated on day −26; primary tumors were removed on day 0; and mice were immunized with HEL on day 3 and bled on days 3 (prebleed) and 16 (final bleed). Lung metastases were quantified on day 16.

ELISA for Anti-HEL Antibody

Anti-HEL antibody (total immunoglobulin) in serum was quantified by ELISA. Flat-bottomed 96-well plates (Nalge Nunc International, Rochester, NY) were coated overnight with 5 μg/ml HEL in PBS or 5 μg/ml BSA (Sigma-Aldrich) in PBS. Excess protein was removed by washing with PBS, 0.2% Tween, and 0.05 M Tris using an ELISA plate washer (Tecan, Research Triangle Park, NC) set for six passes of 300 μl/well/cycle. All of the subsequent washes used the same solution and same number of wash cycles. The wells were blocked with 0.02% horse hemoglobin containing 0.01% thimerosal in PBS for 1 h and then washed. Diluted serum samples were added, and after an overnight incubation, the plates were washed. One hundred μl of affinity-purified biotinylated antimouse IgG (whole molecule H and L chains; Cappel/ICN, Irvine, CA; in PBS, 0.02% hemoglobin, and 0.01% thimerosal) were added to each well, and after a 1-h incubation, excess antibody was removed by washing. One hundred μl of a 0.156 μg/ml solution of streptavidin-horseradish peroxidase (Zymed, San Francisco, CA; in PBS, 0.02% hemoglobin, and 0.01% thimerosal) then were added to each well and incubated for 30 min, followed by washing. Tetramethylbenzidine substrate then was added (100 μl/well for 5–15 min; Dako, Carpinteria, CA), and by adding 100 μl 0.18 M H_2SO_4 per well, the enzymatic reaction was stopped. Plates were read at 450 nm using a Microplate 311 Autoreader (Bio-Tek Instruments, Winooski, VT). To assay levels of anti-HEL IgG or IgM in serum, the aforementioned procedure was followed, substituting affinity-purified biotinylated monoclonal antibody to mouse IgG (γ chain; 0.6 μg/ml; Zymed) or mouse IgM (μ chain; Cappel/ICN), respectively. The positive control HyHEL7 antibody (28) was prepared as described previously (29). Positive control purified mouse IgM was obtained from Zymed.

Absorbance values were converted to μg/ml of anti-HEL antibody using a standard curve. Final values for anti-HEL antibody concentrations were calculated as follows:

$$Anti\text{-}HEL\ Ab = (dilution\ factor) \times \{[(\mu g/ml\ HEL\ Ab\ on\ Day\ x) - (\mu g/ml\ BSA\ control\ for\ Day\ x)] - [(\mu g/ml\ HEL\ Ab\ on\ Day\ 0) - (\mu g/ml\ BSA\ control\ for\ Day\ 0)]\}$$

B16 Inoculations

B16 melF10 cells (1×10^6 or 5×10^5/100 μl serum-free Iscove's modified Dulbecco's medium or RPMI) were inoculated s.c. in the flank of BALB/c, C57BL/6, or 4T1 tumor-bearing BALB/c mice. Tumor growth was assessed as described previously (23). Mice were followed for B16 growth until 4T1 tumor-bearing mice died. Because BALB/c mice die ~42 days after 4T1 inoculation (23), mice that had 4-week-old 4T1 tumors at the time of B16 inoculation died relatively soon after B16 was administered compared with mice with 3-week-old 4T1 tumors. As a result, B16 TDs for mice in the 4-week group were smaller than for mice in the 3-week group.

T-Cell Proliferation Assay and Immunizations

Mice were immunized with HEL in PBS (1:1 v/v emulsion with complete Freund's adjuvant; Sigma) s.c. at the base of the tail (25 μg/50 μl) and in each hind footpad (12.5 μg/25 μl per footpad). Nine days later, spleens were removed; splenocytes were depleted for RBC as described previously, washed twice with serum-free Iscove's modified Dulbecco's medium, and cultured in flat-bottomed 96-well plates at 5×10^5 cells/210 μl/well in serum-free HL1 medium (Bio-Whittaker, Walkersville, MD) containing 1% penicillin, 1% streptomycin, 1% Glutamax (Life Technologies, Rockville, MD) 5×10^{-5} M β-mercaptoethanol, and 10 μg HEL/well (30). Each splenocyte sample also was cocultured with concanavalin A (0.2 μg/ml; Sigma) to ensure cell viability. Each well was pulsed with 1 μCi [^{3}H]thymidine (ICN Biochemicals, Costa Mesa, CA) on day 4, and cells were harvested 16 h later onto glass fiber filter mats using a Packard Micromate 196 cell harvester (Packard, Downers Grove, IL). Filter mats were sealed into plastic bags with 4 ml of Betaplate scintillation fluid (Perkin-Elmer, Gaitherburg, MD), and radioactivity was assessed using a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer). The HEL-specific T-cell proliferative response is reported as:

$$\Delta CPM = (CPM\ for\ HEL\text{-}stimulated\ splenocytes) - (CPM\ for\ splenocytes\ without\ HEL).$$

Values are the average of five replicate wells per point.

Lipopolysaccharide Injections

Mice were injected i.p. with 50 or 100 μg of lipopolysaccharide (LPS; Sigma)/200 μl PBS and weighed daily. Percent weight change was calculated as follows:

$$\%\ weight\ change = 100\% \times [(weight\ on\ day\ x\ after\ injection)/(weight\ before\ injection)].$$

Dendritic Cell (DC) Assays

DC Isolation. Splenic DCs were isolated by a modification of the procedure described previously (31). Briefly, spleens were injected with 0.5 ml of a 1-mg/ml solution of collagenase D (Boehringer Mannheim, Indianapolis, IN) in HEPES Hanks' solution, chopped into small pieces, incubated at 37°C for 45–60 min, and filtered through a 70-μM cell strainer (Falcon, BD, Franklin Lakes, NJ). Resulting cells were depleted for RBC, washed twice with DC wash buffer (PBS containing 0.5% BSA and 2 mM EDTA), and resuspended to 400 μl in DC wash buffer. One hundred μl of CD11c magnetic microbeads (Miltenyi Biotech, Auburn, CA) were added per up to 10^8 splenocytes; the mixture was incubated on ice for 15 min and washed once with DC wash buffer; and the resulting cells were resuspended to 3 ml. Beaded cells were applied to LS columns (Miltenyi Biotech), eluted in 5 ml of DC wash buffer, reapplied to a second LS column, and eluted with 2.5 ml of wash buffer.

Resulting cells were >60% CD11c positive as measured by flow cytometry using a CD11c-FITC monoclonal antibody (PharMingen, San Diego, CA). DC enrichment produced ~2.9–5.2 × 10^6 cells per spleen.

DC Presentation of Ovalbumin. To isolate DO11.10 T cells, up to 4 × 10^8 splenocytes from D011.10 transgenic mice were depleted of RBC, resuspended in wash buffer (HEPES Hanks' solution and 2% calf serum), and adhered to plastic to remove macrophages and DCs (1 spleen, 10 ml wash buffer, and T75 flask; 37°C for 90 min; Ref. 32). Cells then were washed once, resuspended in 2 ml of a 1:20 dilution of B220 culture supernatant, incubated at 4°C for 1 h, washed once, resuspended in 2–4 ml of Lowtox M rabbit complement (Accurate, Westbury, NY), and incubated at 37°C for 30 min. Resulting DO11.10 T cells were >46% $CD4^+DO11.10^+$, <4% $CD8^+DO11.10^+$, and <4% $B220^+$ as measured by flow cytometry. Purified D011.10 T cells were washed once and resuspended in assay medium (RPMI, 10% FCS, 1% penicillin/streptomycin, 1% gentamicin sulfate, 1% Glutamax, and 5 × 10^{-5} M β-mercaptoethanol). Antigen presentation assays were performed in 96-well flat-bottomed plates with 5 × 10^4 DO11.10 T cells, the indicated number of DCs, and 10 μg ovalbumin (Sigma) or 1 μg ova peptide 323–339 (synthesized in the University of Maryland Biopolymer facility) per 200 μl assay medium per well. Cells were incubated at 37°C for 48–72 h, and the supernatants were harvested and assayed by ELISA for IFN-γ using matched pairs of antibodies according to the manufacturer's directions (Pierce-Endogen, Rockford, IL). Values are the average of triplicates.

Allogeneic DC Assay. Splenocytes from C3H/J mice were harvested, depleted of RBC, mixed with 1500 rad irradiated (Gammator B; Kewaunee Scientific, Statesville, NC) BALB/c DCs (2 × 10^5 responders plus 1.4 × 10^5 DCs in 200 μl assay medium/well in 96-well round-bottomed plates), and incubated at 37°C for 5 days. Cultures were pulsed with 1 μCi/well [^{3}H]thymidine and harvested and counted as per the T-cell activation assays. Values are the average of triplicates.

Statistical Analyses

Student's *t* test for unequal variances was performed using Microsoft Excel 2000 (Redmond, WA). Statistical analyses were performed for all of the experiments for which there were sufficient data points.

RESULTS

Antigen-Specific Antibody Responses Are Suppressed in Tumor-Bearing Mice but Return to Normal Following Primary Tumor Removal Despite the Presence of Metastatic Disease. Antibody production in response to immunization is a fundamental element of adaptive immunity. Thus, we examined B-cell activity in tumor-bearing mice by assaying antibody production in response to the foreign antigen HEL. To compare primary antibody responses in mice without tumors ("tumor-free"), mice with intact primary tumors ("nonsurgery"), and mice whose primary tumors have been surgically removed but who retain established, spontaneous metastatic disease ("postsurgery"), BALB/c mice were inoculated with 4T1 tumor cells (nonsurgery and postsurgery groups only), and primary tumors were removed surgically from mice in the postsurgery group after 21 or 26 days (day 0 is the day of surgery; see the timeline in Fig. 1*A*). Mice in the nonsurgery and postsurgery groups were matched for primary TD on the day of surgery (nonsurgery TD, 5.59 ± 1.93 mm; postsurgery TD, 5.52 ± 1.82 mm). On day 2 or 3 after surgery, all of the mice were bled (prebleed) and then immunized with HEL in RIBI adjuvant. On days 16–18, mice were bled again, and serum samples were assayed using ELISA for primary antibody responses to HEL.

To assay total HEL-specific immunoglobulin by ELISA, a biotinylated antibody to whole-molecule mouse IgG was used. Because this antibody reacts with light chains and heavy chains, it detects all of the isotypes of HEL-specific antibody. To control for the binding of non-HEL-specific serum immunoglobulin to ELISA plates, each sample was tested in wells coated with HEL and in wells coated with BSA. After converting absorbance values to g/ml of antibody, antibody levels from BSA wells were subtracted from the HEL antibody value for each sample, and HEL antibody levels from day 0 prebleeds were subtracted from postimmunization values. Because the immunization time course varied slightly between experiments, HEL-specific antibody values from each experiment were normalized by dividing the antibody concentration for each mouse by the antibody concentration of the highest responder in the experiment.

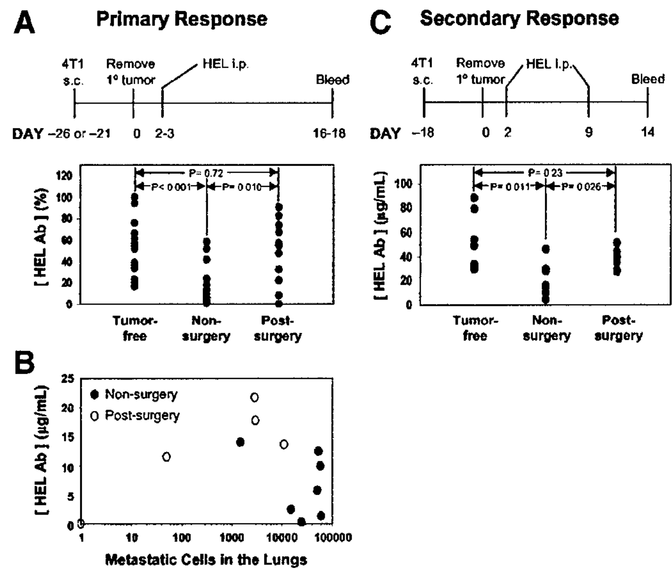


Fig. 1. Antibody production is reduced in 4T1 tumor-bearing mice but recovers following primary tumor removal despite the presence of metastatic disease. BALB/c mice without tumors ("tumor-free"), with a primary tumor in place ("nonsurgery"), or with their primary tumors surgically removed ("postsurgery") were immunized with hen egg white lysozyme (*HEL*) and bled as indicated in the timelines. Sera were tested using ELISA for total HEL-specific antibodies. *A*, primary antibody responses; pooled results of two independent experiments. HEL-specific antibody concentrations were normalized as described in the text. *B*, lungs of some of the mice from *A* were harvested, and metastases were quantified using the clonogenic assay. *C*, secondary antibody responses. Each dot represents antibody levels or number of metastatic cells in an individual mouse.

As shown in Fig. 1*A*, following HEL immunization, serum levels of total HEL-specific antibody were significantly lower in nonsurgery mice than in tumor-free mice. However, postsurgery mice had HEL-specific antibody levels that were significantly higher than nonsurgery mice and comparable with tumor-free mice. Interestingly, serum levels of HEL-specific IgM were not significantly different between tumor-free, nonsurgery, and postsurgery mice (data not shown). Therefore, production of total antibody, but not of IgM, is suppressed in mice with 4T1 tumors but returns to normal following primary tumor removal.

We have established previously that mice inoculated with 4T1 develop disseminated metastases within 10–21 days of 4T1 inoculation (23). To confirm that mice in the present experiment had metastatic disease, lung metastases were quantified using the clonogenic assay. As shown in Fig. 1*B*, mice in the nonsurgery and postsurgery groups developed metastatic disease. However, there appears to be no relationship between the number of lung metastases and the ability to mount a primary antibody response. Thus, the observed recovery of primary antibody responses in postsurgery mice occurred despite the presence of extensive, established metastatic disease.

To determine whether secondary B-cell responses also recover following primary tumor removal, we compared secondary antibody responses in tumor-free, nonsurgery, and postsurgery mice. BALB/c mice were inoculated with 4T1 tumor cells (nonsurgery and postsurgery groups only), and primary tumors were removed surgically from mice in the postsurgery group after 18 days (day 0 is the day of surgery; see the timeline in Fig. 1*C*). Mice in the nonsurgery and

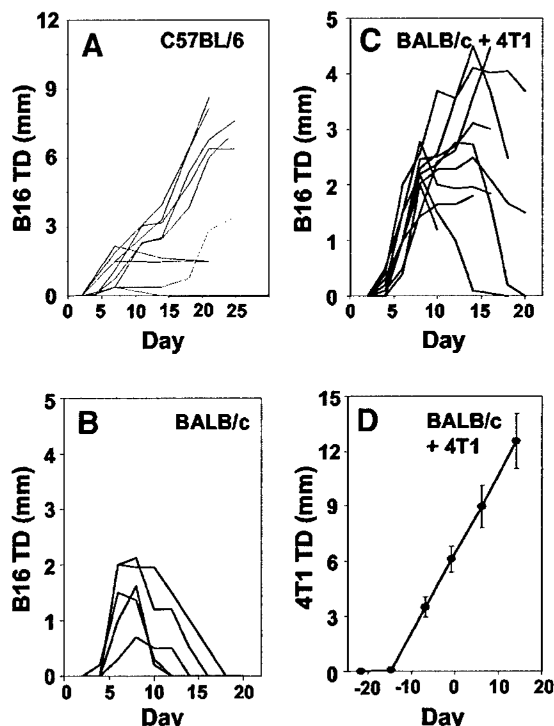


Fig. 2. 4T1 tumor-bearing mice do not efficiently reject allogeneic tumor. B16 tumor growth in C57BL/6 (*A*) or BALB/c (*B*) mice inoculated s.c. in the flank on day 0 with 1×10^6 B16 tumor cells (*H-2^b^*). *C*, B16 tumor growth in BALB/c mice inoculated s.c. in the mammary gland on day −22 with 7×10^3 4T1 cells and additionally inoculated on day 0 with 1×10^6 B16 tumor cells. Each line represents B16 tumor growth in an individual mouse. Termination of a line indicates mouse death. *D*, average growth of 4T1 tumor in the 10 mice shown in *C*. Data are from one of two independent experiments, except for *A*, which is pooled from two separate experiments.

postsurgery groups were matched for primary tumor diameter on the day of surgery (nonsurgery TD, 3.82 ± 0.68 mm; postsurgery TD, 4.05 ± 1.13 mm). All of the mice were bled on day 2 after surgery and immunized with HEL in RIBI adjuvant on days 2 and 9. On day 14, mice were bled, and serum samples were assayed using ELISA for secondary antibody responses to HEL.

As shown in Fig. 1*C*, in response to two immunizations with HEL, serum levels of HEL-specific antibody were lower in nonsurgery, tumor-bearing mice than in tumor-free mice. However, HEL-specific antibody levels in postsurgery mice were significantly higher than in nonsurgery mice and comparable with tumor-free mice. Therefore, like primary responses, secondary antibody responses are reduced in mice with 4T1 tumors but recover following primary tumor removal despite the presence of established metastatic disease.

Rejection of Allogeneic Tumor Is Impaired in Mice with 4T1 Tumors. Many experimental cancer immunotherapeutic strategies focus on the activation of T lymphocytes. Because rejection of allogeneic tumor is mediated by T cells, we used the growth of the C57BL/6-derived (*H-2^b^*) B16 melF10 melanoma to measure T-cell activity in tumor-free *versus* nonsurgery BALB/c (*H-2^d^*) mice (33). The numbers of B16 cells inoculated were based on previous studies in which B16 melF10 tumors grew progressively in syngeneic C57BL/6 mice.[1] Tumor-free BALB/c or BALB/c mice that had been inoculated 22 days earlier with 4T1 cells were inoculated with 1×10^6 B16 cells (contralateral side for 4T1-bearing mice). C57BL/6 mice also were inoculated with 1×10^6 B16 cells to monitor tumor progression in the syngeneic host. Growth of B16 tumors was tracked for 20 days or until mice became moribund or died. As expected, B16 tumors grew progressively in most C57BL/6 mice (Fig. 2*A*) but were rejected by BALB/c mice within 20 days of B16 inoculation (Fig. 2*B*). In contrast, B16 tumors were not rejected by 60% of 4T1 tumor-bearing BALB/c mice within the same period (Fig. 2*C*). 4T1 tumors in these mice (Fig. 2*D*) grew at a rate comparable with growth in mice without B16 tumors (data not shown). Therefore, allogeneic tumor rejection is impaired in mice carrying 4T1 tumors.

In a second experiment, C57BL/6, tumor-free BALB/c, or BALB/c mice that had been inoculated 3 weeks earlier with 4T1 cells (4T1 TD, 5.21 ± 1.61 mm) were inoculated with 5×10^5 B16 cells (contralateral side for 4T1-bearing mice). Growth of B16 tumors was tracked for 26 days or until mice became moribund or died. As expected, palpable B16 tumors were present in 100% of C57BL/6 mice (Fig. 3*A*). When compared with BALB/c mice without 4T1, mice with 3-week established 4T1 tumors were more likely to develop palpable B16 tumors (Fig. 3*A*), and the tumors that developed grew to a significantly larger maximum TD before regression or mouse moribundity (Fig. 3*B*). In a similar experiment, B16 growth was tracked in C57BL/6, tumor-free BALB/c, and BALB/c mice that were inoculated with 5×10^5 B16 cells 4 weeks after 4T1 inoculation (4T1 TD, 8.41 ± 1.85 mm). Tumor growth was tracked until 16 days post-B16 inoculation or until mice became moribund or died. In this experiment, palpable B16 tumors developed in 100% of C57BL/6 mice. B16 tumor incidence and maximum TD were higher in mice with 4-week established 4T1 tumors than in mice without 4T1. Therefore, allogeneic B16 tumors grew larger and were more likely to develop in 4T1 tumor-bearing BALB/c mice than in mice without 4T1 tumors. These results collectively demonstrate that mice with bulky, primary tumor are less able to immunologically reject allogeneic tumor, suggesting that their cellular immunity is compromised.

T-Cell Responses to HEL Are Impaired in Mice with 4T1 Tumors but Recover Following Surgery Despite the Presence of Disseminated Metastatic Disease. To assess antigen-specific T-cell-mediated immunity, tumor-free, nonsurgery, and 9-day postsurgery mice with metastatic disease were immunized with HEL. Mice in the nonsurgery and postsurgery groups were matched for primary TD on the day of surgery (4.93 ± 0.83 mm and 4.71 ± 1.2 mm, respectively). Splenocytes were harvested 9 days after immunization, restimulated in culture with HEL, and T-cell proliferation was measured

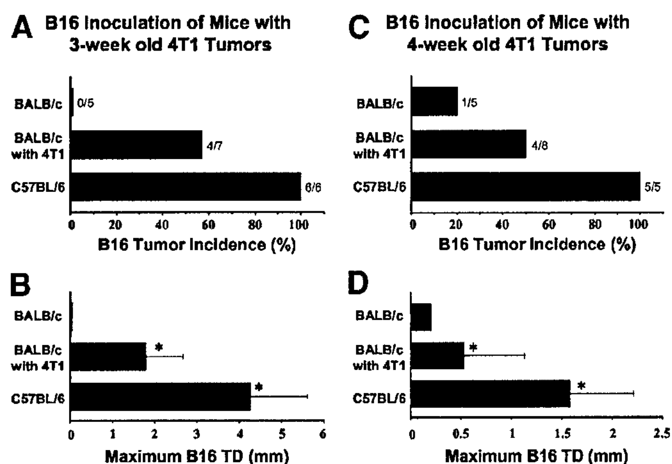


Fig. 3. Incidence and growth of allogeneic tumor are greater in 4T1 tumor-bearing mice than in tumor-free mice. BALB/c, C57BL/6, or 4T1 tumor-bearing nonsurgery BALB/c mice were inoculated s.c. with 5×10^5 B16 cells and followed for incidence of B16 tumor growth (*A* and *C*) and maximum B16 tumor diameter (*B* and *D*). BALB/c mice were inoculated s.c. with 7×10^3 4T1 cells 3 weeks (*A* and *B*) or 4 weeks (*C* and *D*) before B16 inoculation. Fractions indicate number of mice that developed palpable B16 tumors/number of mice inoculated with B16. *, A significant difference between groups ($P < 0.05$). Data are from one of three independent experiments.

[1] Unpublished observations.

by [^{3}H]thymidine incorporation. As shown in Fig. 4*A*, HEL-specific proliferative responses were reduced significantly in nonsurgery mice compared with tumor-free mice. However, surgical removal of primary tumor returned HEL-proliferative responses to levels comparable with that of tumor-free mice.

To ascertain that the HEL-immunized mice had metastatic disease, the lungs of the nonsurgery and postsurgery groups were removed at the time of splenocyte removal and assayed using the clonogenic assay for metastatic tumor cells. As shown in Fig. 4*B*, both groups have metastatic cells in their lungs. Therefore, although the presence of bulky primary tumor significantly inhibits antigen-specific T-cell responses, surgical resection of primary tumor reverses this inhibition even when metastatic disease is present.

Macrophage Activity Is Unimpaired in Mice with 4T1 Tumors. As professional antigen-presenting cells, macrophages play an important role in adaptive immune responses. The endotoxin LPS induces toxic shock and cachexia in mice via a macrophage-dependent mechanism, leading to severe weight loss (34). Thus, we have examined responses of mice to LPS as a measure of macrophage function, with the degree of weight loss corresponding to macrophage activity. To compare responsiveness to LPS in tumor-free mice and nonsurgery mice, BALB/c mice were inoculated with 4T1 cells (tumor-bearing group only) and 2 or 4 weeks later inoculated with LPS. 4T1 TDs for mice with 2-week and 4-week established 4T1 tumors were 2.68 ± 1.47 mm and 7.87 ± 1.81 mm, respectively. Weight change was tracked for 3 days or until mice became moribund or died. Tumor-free (Fig. 5*A*) and nonsurgery (Fig. 5*B*) mice experienced significant weight loss within 1 day of LPS inoculation. Percent weight change did not vary significantly between tumor-free and nonsurgery mice.

To determine whether 4T1 tumor burden affected LPS-induced weight loss, tumor-free and nonsurgery mice with either 2-week or

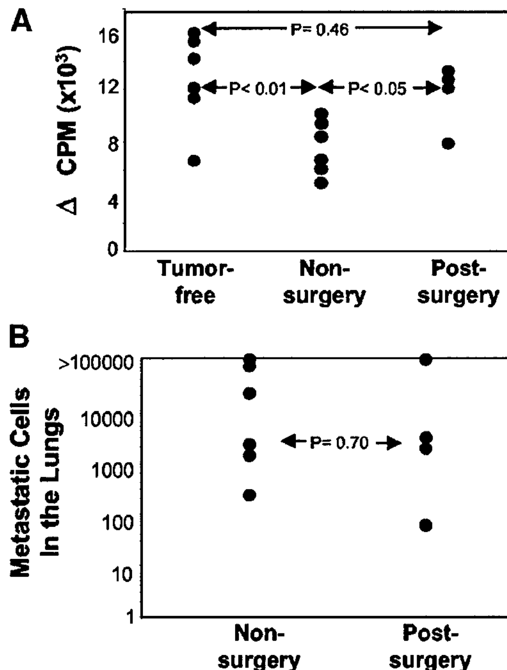


Fig. 4. Hen egg white lysozyme (HEL)-specific T-cell responses are reduced in 4T1 tumor-bearing mice but recover following primary tumor removal despite the presence of metastatic disease. *A*, tumor-free, nonsurgery, and postsurgery BALB/c mice were immunized with HEL. Nine days later, their spleens were removed and boosted *in vitro* with HEL, and T-cell proliferation was measured by incorporation of [^{3}H]thymidine. *B*, lungs of some of the mice from *A* were harvested, and metastases were quantified using the clonogenic assay. Each symbol represents the number of metastatic tumor cells in the lungs of an individual mouse. Data are pooled from three independent experiments.

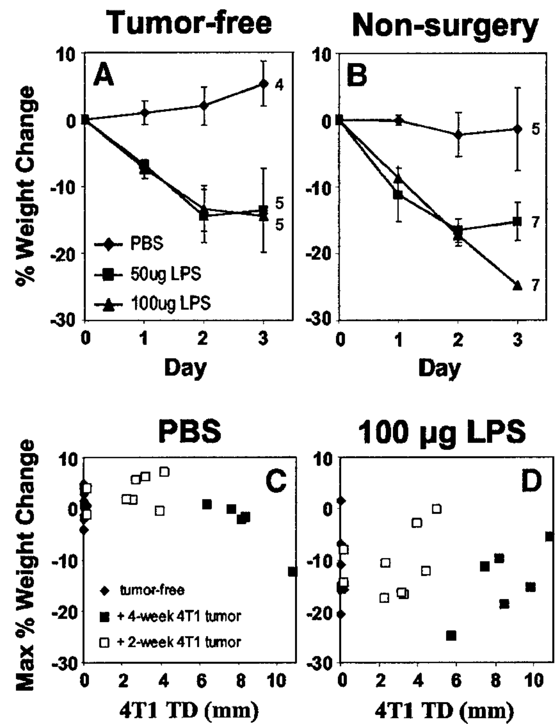


Fig. 5. 4T1 tumor burden does not affect macrophage activity. *A* and *B*, tumor-free BALB/c mice (*A*) and BALB/c mice inoculated s.c. with 4T1 on day −28 (nonsurgery; *B*) were inoculated with lipopolysaccharide (*LPS*) or with PBS on day 0. Weight loss was tracked daily or until mice died. At day 1, there were statistically significant differences between PBS-injected and LPS-inoculated tumor-free mice (50 μg LPS, $P = 0.001$; 100 μg LPS, $P < 0.001$) and between PBS-injected and LPS-inoculated tumor-bearing mice (50 μg LPS, $P = 0.012$; 100 μg LPS, $P < 0.001$). The number of mice in each group is indicated at the end of each line. Nine nonsurgery mice and five tumor-free mice died within 3 days of LPS inoculation. Data are from one of three independent experiments, in which mice with 2-week or 4-week 4T1 tumors were used. *C* and *D*, tumor-free BALB/c mice and BALB/c mice with 2-week or 4-week established 4T1 tumors were inoculated with PBS (*C*) or LPS (*D*) on day 0 and weighed daily for 3 days or until they became moribund or died. 4T1 tumor diameter (*TD*) was measured on the day of LPS inoculation. Each symbol represents the weight of an individual mouse. Five tumor-free mice, five mice with 4-week 4T1 tumors, and six mice with 2-week 4T1 tumors died within 3 days of LPS inoculation. Data are pooled from three independent experiments.

4-week established 4T1 tumors were inoculated with PBS (Fig. 5*C*) or LPS (Fig. 5*D*) and followed for weight changes. TD does not impact percent weight change. Therefore, macrophage activity is not altered in mice carrying 4T1 tumors, suggesting that macrophage function is not suppressed by the presence of bulky, primary tumor.

DC Activity Is Not Suppressed in Tumor-Bearing or Postsurgery Mice. Impaired DC activity has been reported in patients with bulky, primary tumors (31, 35). To determine whether DC activity is decreased in 4T1 tumor-bearing mice and/or is affected by surgery, splenic DCs were purified from tumor-free, nonsurgery, and 10–15-day postsurgery mice with metastatic disease using Miltenyi magnetic bead sorting for CD11c$^+$ cells. Mice in the nonsurgery and postsurgery groups were matched for primary TD on the day of surgery (7.7 ± 1.6 mm and 7.6 ± 1.6 mm, respectively). Recovery of splenic CD11c$^+$ cells from the three groups ranged from 3–5.2 × 10^6 per spleen, and there were no significant differences in yield between the treatment groups. Purified cells were double stained for CD11c plus CD40, CD80, or I-A^d to ascertain phenotype. More than 60% of the recovered cells were CD11c$^+$, and the CD11c$^+$ cells from the three groups did not differ in cell surface expression of MHC class II, CD40, or CD80 as measured by immunofluorescence and flow cytometry (data not shown). Functional activity of the CD11c$^+$ cells from the three treatment groups was measured by (*a*) activation of allogeneic (C3H/HeJ) T cells, (*b*) presentation of exogenous ovalbu-

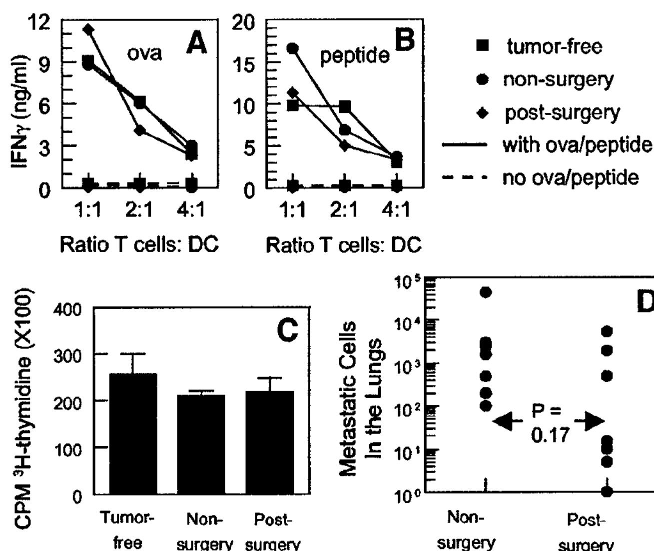


Fig. 6. Dendritic cell (*DC*) activity is not suppressed in tumor-bearing mice. Splenic DCs were purified from tumor-free, nonsurgery, and 10-day postsurgery BALB/c mice. DCs were pulsed with ovalbumin protein (*A*) or ovalbumin peptide 323–339 (*B*) and cocultured at varying ratios with I-A^d-restricted $ova_{323-339}$-specific DO11.10 T cells, and supernatants were assayed for IFN-γ. *C,* irradiated DCs were cocultured with allogeneic C3H splenocytes, and T-cell proliferation was measured by [^{3}H]thymidine uptake. The cpm for DC and C3H splenocytes cultured separately were <8% of the allo response. Each graph in *A* through *C* represents splenocytes from one or two mice per treatment group and is representative of three to five independent experiments. *D,* lungs of mice from the same inoculation cohort of *A* and *B* were harvested, and metastases were quantified using the clonogenic assay. Each symbol represents the number of metastatic tumor cells in the lungs of an individual mouse. Data are pooled from two independent experiments.

min to I-A^d-restricted, $ovalbumin_{323-339}$-specific CD4$^+$ DO11.10 transgenic T cells (32), and (*c*) presentation of ovalbumin peptide 323–339 to DO11.10 T cells. CD11c$^+$ cells from tumor-free, nonsurgery, and postsurgery mice are approximately equivalent in their ability to present ovalbumin protein (Fig. 6*A*) and ovalbumin peptide (Fig. 6*B*). Similarly, CD11c$^+$ cells from all of the three treatment groups are equal in their ability to activate allogeneic T cells (Fig. 6*C*). To confirm that mice in the nonsurgery and postsurgery groups have metastatic disease, lungs were harvested and assayed by the clonogenic assay for metastatic tumor cells (Fig. 6*D*). These results collectively demonstrate that splenic DCs from 4T1 tumor-bearing mice and from postsurgery mice are not impaired in their ability to process and present antigen and to activate T cells.

DISCUSSION

Immunotherapy offers a promising approach for the management of metastatic cancers; however, the development of effective strategies is complicated by the ability of tumors to evade host immunity. Although there are a few studies in which tumor-induced immunosuppression has not been noted (36), most investigators have reported reduced immune functions in tumor-bearing individuals (1), establishing tumor-induced immunosuppression as a fundamental mechanism allowing tumors to escape immune destruction. Because immunotherapy becomes less effective as tumor mass increases, it is thought that immunosuppression intensifies with increasing tumor burden (1). Despite the apparent critical role of tumor-induced immune suppression, few studies have evaluated immunocompetence following the reduction of tumor burden via primary tumor resection (19–22). There is a particular shortage of information regarding the clinically relevant question of whether tumor-induced immunosuppression can be reversed by primary tumor resection even when metastatic disease is present. Because many metastatic cancers are not responsive to conventional therapies, postsurgery patients with established metastatic disease may benefit from novel treatments such as immunotherapy. Therefore, a better understanding of the immunocompetence of patients whose primary tumors have been removed surgically, but who have metastatic disease, is essential to evaluate whether immunotherapy will be a useful treatment strategy.

The studies reported here indicate deficits in cell-mediated and humoral immune responses in mice with bulky primary tumors, relative to tumor-free mice. CD8$^+$ and CD4$^+$ T-cell responses are suppressed based on inadequate rejection of allogeneic tumor, inability to switch from antigen-specific IgM to IgG isotypes following immunization, and reduced antigen-specific T-cell activation following immunization. Whether the decreased antigen-specific IgG responses of tumor-bearing mice demonstrate direct suppression of B lymphocytes or are the result of T-cell dysfunction is unclear. Because immunized tumor-bearing mice make normal levels of antigen-specific IgM but produce significantly reduced levels of total antibody, the tumor-induced deficit in antibody production may reflect a problem with CD4$^+$ T-helper cell-mediated immunoglobulin class switching rather than an inherent B-cell defect. Regardless of the mechanisms responsible for the reduced immunocompetence, our studies agree with previous reports documenting T-cell (13) and B-cell deficiencies (16, 37) in tumor-bearing patients. Surprisingly, we do not find deficiencies in macrophage or DC activity, although previous reports have documented such defects (31, 35, 38).

Tumor cells are known to synthesize and secrete several immunosuppressive factors. For example, transforming growth factor β inhibits CD8$^+$ effector T cells and Th1 CD4$^+$ T cells, thereby suppressing T-cell-mediated antitumor immunity (39). Vascular endothelial growth factor also is an effective immunosuppressive agent. It blocks normal myeloid cell differentiation and causes a buildup of immature myeloid cells, known as myeloid suppressor cells, that inhibit the activity of CD4$^+$ and CD8$^+$ T cells (14, 17, 21, 40). Similar to many tumors, the 4T1 mammary carcinoma used in this study produces both of these cytokines.[1] Other immunosuppressive factors secreted by tumor cells include interleukin 10, which when present in high levels is hypothesized to skew the immune response toward a type 2 response, thereby minimizing an effective Th1 response (41). The activity of these cytokines is thought to be roughly proportional to their *in vivo* level, and this level correlates directly with tumor burden because the cytokines are synthesized and secreted by the tumor cells. Therefore, it is likely that surgery reverses immune suppression because it reduces the quantity of immunosuppressive factors, thereby allowing the immune response to recover in the absence of the inhibitory cytokines. If this is the case, then immunosuppression may recur as metastatic lesions grow and inhibitory cytokine levels increase, becoming more severe as metastatic tumor burden increases. However, after surgery there clearly is a "window" during which relatively large quantities of metastatic cells are present, but immune suppression is not active. This window of immunocompetence may be the result of less efficient cytokine production by metastatic tumor cells *versus* primary tumor cells, or alternatively, there may be qualitative differences in cytokine production by primary tumor cells *versus* metastatic tumor cells. Such differences could result from distinct cytokine secretion profiles for primary tumor *versus* metastatic tumor cells or from a requirement for a large focus of tumor cells in a common location to induce immunosuppression. Either of these mechanisms would result in the recurrence of immune suppression as metastatic tumor burden increases.

The reversal of tumor-induced immunosuppression in patients with metastatic disease following primary tumor resection has important implications for cancer immunotherapy. Because patients will be most

responsive to immunotherapy when they are maximally immunocompetent, it is imperative that tumor-induced immune suppression is considered when planning immunotherapy regimens. Our studies indicate that although patients with bulky primary tumors are profoundly immunosuppressed, primary tumor removal reverses immune suppression even in the presence of extensive metastatic disease. Thus, for maximal efficacy, immunotherapy should be administered only after tumor burden is reduced, either by surgery or by other conventional therapies. Because conventional treatments such as radiation therapy, chemotherapy, and surgery also can reduce host immunocompetence, the ultimate timing of an immunotherapy regimen must consider all of these conditions (42).

ACKNOWLEDGMENTS

We thank Mr. Charles Anderson and Dr. Tariq Akbar for their preliminary work on the HEL antibody assay. We also thank Ms. Sandy Mason for providing excellent animal care.

REFERENCES

1. Schreiber H. Tumor immunology. In: Paul WE, editor. Fundamental immunology, ed. 5. Baltimore: Lippincott Williams & Wilkins, 2003. p. 1557–91.
2. Berendt M, North RJ. The cell mediated suppression of anti-tumor immunity. An explanation for progressive growth of an immunogenic tumor. J Exp Med 1980;151: 69–80.
3. Naor D. Suppressor cells: permitters and promoters of malignancy? Adv Cancer Res 1979;29:45–125.
4. North RJ. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J Exp Med 1982;155:1063–74.
5. North RJ, Digiacom A, Dye E. Suppression of antitumor immunity. In: Ruitenberg WDOaE, editor. Tumor immunology: mechanisms, diagnosis, therapy, vol. 8. Amsterdam: Elsevier, 1987. p. 125.
6. North RJ, Awwad M, Dunn P. The immune response to tumors. Transplant Proc 1989;21:575.
7. North RJ. Down-regulation of the antitumor immune response. Adv Cancer Res 1985;45:1–43.
8. Pardoll D. Does the immune system see tumors as foreign or self? Annu Rev Immunol 2003;21:807–39.
9. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Immunol Today 2000;21:455–64.
10. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Adv Immunol 2000;74:181–273.
11. Seliger B, Cabrera T, Garrido F, Ferrone S. HLA class I antigen abnormalities and immune escape by malignant cells. Semin Cancer Biol 2002;12:3–13.
12. Ochsenbein AF, Sierro S, Odermatt B, et al. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. Nature 2001;411:1058–64.
13. Finke J, Ferrone S, Frey A, Mufson A, Ochoa A. Where have all the T cells gone? Mechanisms of immune evasion by tumors. Immunol Today 1999;20:158–60.
14. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother 2002;51:293–8.
15. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2002;2:389–400.
16. Pericle F, Kirken RA, Bronte V, Sconocchia G, DaSilva L, Segal DM. Immunocompromised tumor-bearing mice show a selective loss of STAT5a/b expression in T and B lymphocytes. J Immunol 1997;159:2580–5.
17. Bronte V, Serafini P, Apolloni E, Zanovello P. Tumor-induced immune dysfunctions caused by myeloid suppressor cells. J Immunother 2001;24:431–46.
18. Ting C, Rodrigues D. Switching on the macrophage-mediated suppressor mechanism by tumor cells to evade host immune surveillance. Proc Natl Acad Sci USA 1980; 77:4265–9.
19. Salvadori S, Martinelli G, Zier K. Resection of solid tumors reverses T cell defects and restores protective immunity. J Immunol 2000;164:2214–20.
20. Mullen CA, Rowley DA, Schreiber H. Highly immunogenic regressor tumor cells can prevent development of postsurgical tumor immunity. Cell Immunol 1989;119: 101–13.
21. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol 2001; 166:678–89.
22. Barbieri C, Fujisawa MM, Yasuda CL, et al. Effect of surgical treatment on the cellular immune response of gastric cancer patients. Braz J Med Biol Res 2003;36: 339–45.
23. Pulaski BA, Ostrand-Rosenberg S. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. Cancer Res 1998;58:1486–93.
24. Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology, Vol. 4. New York: John Wiley; 2001. p. 20.2.1.
25. Miller F, Miller B, Heppner G. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. Invasion Metastasis 1983;3:22–31.
26. Ostrand-Rosenberg S, Baskar S, Patterson N, Clements V. Expression of MHC class II and B7-1 and B7-2 costimulatory molecules accompanies tumor rejection and reduces the metastatic potential of tumor cells. Tissue Antigens 1996;47:414–21.
27. Pulaski BA, Clements VK, Pipeling MR, Ostrand-Rosenberg S. Immunotherapy with vaccines combining MHC class II/CD80+ tumor cells with interleukin-12 reduces established metastatic disease and stimulates immune effectors and monokine induced by interferon-ã. Cancer Immunol Immunother 2000;49:34–45.
28. Smith-Gill SJ, Lavoie TB, Mainhart CR. Antigenic regions defined by monoclonal antibodies correspond to structural domains of avian lysozyme. J Immunol 1984;133: 384–93.
29. Armstrong TD, Clements VK, Martin BK, Ting JP, Ostrand-Rosenberg S. Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. Proc Natl Acad Sci USA 1997;94:6886–91.
30. Lamouse-Smith E, Clements VK, Ostrand-Rosenberg S. â2M-/- knockout mice contain low levels of CD8+ cytotoxic T lymphocyte that mediate specific tumor rejection. J Immunol 1993;151:6283–90.
31. Yang AS, Lattime EC. Tumor-induced interleukin 10 suppresses the ability of splenic dendritic cells to stimulate CD4 and CD8 T-cell responses. Cancer Res 2003;63: 2150–7.
32. Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes *in vivo*. Science 1990;250:1720–3.
33. Fidler IJ, Hart IR. Biological diversity in metastatic neoplasms: origins and implications. Science 1982;217:998–1003.
34. Beutler B, Rietschel E. Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol 2003;3:169–76.
35. Orsini E, Guarini A, Chiaretti S, Mauro FR, Foa R. The circulating dendritic cell compartment in patients with chronic lymphocytic leukemia is severely defective and unable to stimulate an effective T-cell response. Cancer Res 2003;63:4497–506.
36. Radoja S, Rao TD, Hillman D, Frey AB. Mice bearing late-stage tumors have normal functional systemic T cell responses *in vitro* and *in vivo*. J Immunol 2000;164: 2619–28.
37. Pilarski LM, Andrews EJ, Mant MJ, Ruether BA. Humoral immune deficiency in multiple myeloma patients due to compromised B-cell function. J Clin Immunol 1986;6:491–501.
38. Watson GA, Lopez DM. Aberrant antigen presentation by macrophages from tumor-bearing mice is involved in the down-regulation of their T cell responses. J Immunol 1995;155:3124–34.
39. Tada T, Ohzeki S, Utsumi K, et al. Transforming growth factor-â-induced inhibition of T cell function. Susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. J Immunol 1991;146:1077–82.
40. Serafini P, De Santo C, Marigo I, et al. Derangement of immune responses by myeloid suppressor cells. Cancer Immunol Immunother 2004;53:64–72.
41. Halak BK, Maguire HC Jr, Lattime EC. Tumor-induced interleukin-10 inhibits type 1 immune responses directed at a tumor antigen as well as a non-tumor antigen present at the tumor site. Cancer Res 1999;59:911–7.
42. Carter JJ, Whelan RL. The immunologic consequences of laparoscopy in oncology. Surg Oncol Clin N Am 2001;10:655–77.

ELSEVIER

Full text provided by www.sciencedirect.com

SCIENCE DIRECT®

Animal models of tumor immunity, immunotherapy and cancer vaccines

Suzanne Ostrand-Rosenberg

Reliable animal models are critical for evaluating immunotherapies and for defining tumor immunology paradigms. Tumor immunologists are moving away from traditional transplantation tumor systems because they do not adequately model human malignancies. Transgenic mouse models in which tumors arise spontaneously have been developed for most cancers. The models use one of three technologies: tissue-specific promoters to drive expression of SV40 large T antigen or tissue-specific oncogenes; deletion of tumor suppressor genes by gene targeting; or, conditional deletion of tumor suppressor genes or activation of oncogenes via Cre-lox technology. Knockin mice expressing human tumor antigens and gene-targeted mice with deletions for immunologically relevant molecules have been integral to advancing knowledge of the tumor–host relationship. Although animal models are becoming more sophisticated, additional improvements are needed so that more realistic models can be developed.

Addresses
Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle Baltimore, MD 21250, USA
e-mail: srosenbe@umbc.edu

Current Opinion in Immunology 2004, **16**:143–150

This review comes from a themed issue on
Tumor immunology
Edited by Olivera J Finn

0952-7915/$ – see front matter
© 2004 Elsevier Ltd. All rights reserved.

DOI 10.1016/j.coi.2004.01.003

Abbreviations

Apc	adenomatosis polyposis coli
ARR_2	androgen-receptor-regulated promoter region
MMTV	mouse mammary tumor virus
Py	polyoma virus
Rb	retinoblastoma
T ag	SV40 large T antigen
t ag	SV40 small t antigen

Introduction

Animal models have played a critical role in establishing basic paradigms of tumor immunology because they provide an *in vivo* milieu that cannot be reproduced *in vitro*. As novel immunotherapies and cancer vaccines have been developed, animal models have also played an important role in pre-clinical testing for therapeutic efficacy. Historically, investigators have used transplantable tumor models, in which inbred animals are inoculated with tumor cells derived from the same genetic strain. The tumors were initially derived from spontaneously occurring malignancies or induced by chemicals or irradiation, and maintained either by *in vivo* or *in vitro* passage. As the tumor immunology field has moved towards developing cancer vaccines and other novel cancer immunotherapies, the same transplantable tumor models have been used to test therapeutic efficacy. Unfortunately, many of these tumor models are not good predictors for human clinical trials, as numerous therapies that look promising in experimental animals have turned out to be ineffective in patients. Although immunotherapy and cancer vaccine studies are moving away from using transplantable tumor models, they remain a mainstay for immunologists examining issues of basic tumor immunology. This review will briefly describe the pros and cons of transplantable tumor models and then focus on the recently developed transgenic mouse models in which tumors develop spontaneously. A brief overview of other mouse models that have been useful in defining basic principles of tumor immunology will also be discussed.

Transplantable tumor models

Although transplantable tumors have long been integral to tumor immunology research, they have several characteristics that limit their applicability to human disease and make them less than optimal for predicting immunotherapy efficacy in patients. First, most transplantable tumors were derived many years ago, and today's 'syngeneic' mouse strains may no longer be fully syngeneic with these tumors. In addition, some transplantable tumors have picked up endogenous viruses and express viral antigens not expressed by their mouse hosts. Therefore, many transplantable tumors may be partially histoincompatible with their 'syngeneic' mouse host and/or contain viral epitopes that make them significantly more immunogenic than naturally arising human tumors. Second, transplanted tumors are typically inoculated subcutaneously or intravenously and therefore do not grow in the anatomically appropriate site. As a result, the animal model does not mimic the organ-specific physiology characteristic of the tumor and the immune system is not exposed to the tumor in a manner comparable to that of naturally occurring malignancies in patients. Third, transplantable tumors generally progress very rapidly following inoculation, whereas spontaneous human tumors usually develop more slowly through a gradual series of cellular changes from pre-malignant to malignant pathologies. Therefore, the immune system of patients is slowly acclimated to tumors, whereas the immune system

of experimental animals with transplanted tumors is abruptly exposed. These kinetic variations may lead to different immunological outcomes, such as tolerance versus activation. Fourth, for patients with solid tumors, disseminated metastatic disease is frequently the predominant cause of death, and many cancer vaccines and immunotherapies are aimed at reducing and/or preventing metastasis. Most transplantable mouse tumors, however, are not spontaneously metastatic, so vaccine efficacy studies using these models are not particularly relevant for human metastatic disease.

Despite these obvious limitations, some transplantable tumors have distinct experimental advantages. For example, when inoculated in the mammary fat pad of syngeneic mice, the mouse 4T1 mammary carcinoma is spontaneously metastatic to the same sites as human mammary adenocarcinoma. If the primary tumor in the mammary gland is removed, then this transplantable tumor serves as an excellent model for the treatment of established, disseminated metastatic disease in a post-surgery setting [1–3].

Transplantable tumors have also been derived from spontaneous tumors that arise in genetically engineered mice. Because these recently derived tumors are syngeneic with their spontaneous tumor counterparts, they have been used in conjunction with the spontaneous models. For example, experiments with such transplantable tumors have demonstrated that older mice are significantly less responsive to cancer vaccines than younger mice [4•], and that combination immunotherapy consisting of passive administration of tumor-antigen-specific antibodies plus a cell-based vaccine provides more effective immunity than either therapy alone [5•].

Models for testing immunotherapy and cancer vaccines

In developing better animal models for both immunotherapy and cancer vaccine studies, investigators have tried to address the problems associated with transplantable tumors and to develop experimental systems that more closely mimic human malignancy. Efforts have been directed towards developing transgenic mouse models in which tumors develop spontaneously and progress through the known pre-malignant and malignant stages; defined human tumor antigens are expressed so that the host is tolerized to tumor-encoded molecules; and, the timing of tumor onset can be controlled so that tumors arise when the host has a mature immune system, as they do in humans.

SV40-driven transgenic models

Numerous transgenic mice have been generated by placing the transforming genes of the SV40 or polyoma virus early regions under the control of a tissue or cell-specific promoter. These mice spontaneously develop tumors in the targeted tissue. Table 1 includes some of these models and summarizes their characteristics by target organ. These models are useful because the mice develop organ-localized tumors, and, in some cases, also develop metastatic lesions. Most of these transgenic mice develop prostate cancer [6,7] or mammary carcinoma [8–10]; however, pancreatic [11,12], ovarian [13] and melanoma [14] models have also been reported.

The SV40 early region contains both large T and small t antigens (SV40 T ag and SV40 t ag, respectively). SV40 T ag inactivates the p53 and retinoblastoma (Rb) tumor-suppressor genes and the t ag activates cyclin Dp, which alters the mitogen-activated protein kinase (MAPK) and stress-activated protein kinase (SAPK) pathways. The original prostate cancer model, called the transgenic adenocarcinoma mouse prostate (TRAMP) mouse, was generated using the entire SV40 early region [7]. However, there has been concern that the multiple perturbations induced by the SV40 early region are not consistent with human prostate cancer, so another model called the 'LADY' mouse, containing only the T ag was developed [6].

A limitation of the SV40-driven prostate models is that the resulting tumors do not morphologically or phenotypically resemble human prostate tumors. For example, TRAMP mice develop seminal vesicle and stromal tumors, and LADY mice develop neuroendocrine tumors, whereas most human prostate cancers (adenocarcinoma) are of epithelial origin. In addition, tumor progression in many of the SV40 models is very rapid and therefore differs from development of human tumors, which typically progress more gradually. These characteristics have led some investigators to question the physiological relevance of SV40-driven transgenic models [15].

Organ-specific oncogene-driven transgenic models

Because of the desire to generate animal models in which the mechanism of tumor induction more closely parallels that of human disease, transgenic models using tissue or cell-specific promoters driving tumor-specific oncogenes have been developed. These models utilize a cell or tissue-specific promoter driving an oncogene that is thought to be causative of tumorigenesis. Table 1 includes some of these models and gives their characteristics. Most of these models involve oncogenes such as Her2/neu (ErbB2), which is driven by mammary tissue-specific promoters such as the Her2/neu endogenous promoter or mouse mammary tumor virus (MMTV) promoter [5•,16–21,22•,23]; however, prostate [24] and intestinal models [25] have also been reported. Several characteristics of these tumors demonstrate their similarity to human malignancies. Tumors in these models progress as they do in humans from pre-malignant lesions to invasive tumors and in some cases metastatic disease. Tumor progression in one of the Her2/neu models

Table 1

Selected transgenic mouse models of spontaneous malignancies.

Target organ	Model name	Promoter/ transgene	Genetic background	Percent mice with tumors	Metastasis	Comments	References
Breast	neuNT	MMTV/rat activated Her-2/neu	FVB	100%		Palpable mammary masses by ~13–14 weeks.	[44]
Breast	BALB/c neuT	MMTV/rat activated Her-2/neu	BALB/c	100%	Lung mets in older mice (~week 33)[a].	Mammary hyperplasia at ~8–13 weeks; DCIS at ~8–17 weeks; 1 palpable mass by ~20 weeks; 10 palpable masses by ~week 30.	[17]
Breast	neuN	MMTV/ unactivated Her-2/neu	FVB	~75%	Lung mets in ~72% of mice >8 months of age.	DCIS at ~37 weeks; 1 palpable mass by ~41–49 weeks; ~2.5 palpable masses thereafter; less disease than BALB/c-neuT mice; tolerant to neu.	[16,17]
Breast	MMT	(MMTV LTR/Py MT) × MUC1 Tg	C57BL/6	100%		Focal hyperplasia at ~4 weeks; palpable mammary tumors by day 65; 50% of mice have tumors by day 80–90; rapid progression.	[8]
Breast	MT	MMTV/PyMT	FVB	100%	Lung mets.	Multifocal mammary adenocarcinoma; rapid progression.	[9]
Breast	neuNT	(MMTV Cre) × loxP activated neu with endogenous promoter	BALB/c	100%		MMTV/Cre transgenics were bred with transgenics containing an inducible activated neu gene under its endogenous promoter; mammary tumors appear by ~8 months.	[18]
Prostate	TRAMP	Truncated rat probasin/SV40 T + t	C57BL/6 and FVB	100%	100% to lymph nodes and/or lungs; less common to kidney, adrenal gland, bone.	Prostate intraepithelial hyperplasia by 10 weeks; invasive neuroendocrine tumors by 20 weeks.	[7,45]
Prostate	LADY (12T-10)	Large probasin/ SV40 Tag	CD-1	100%	88% at 9 months; liver and lung most common; also to lymph nodes, bone.	Low grade prostatic intraepithelial neoplasia (PIN); invasive neuroendocrine tumors by 22 weeks; androgen receptor negative.	[6]
Prostate	$Pten^{-/-}$	Cre-lox conditional knockout	(C57BL/6 × DBA/2)F1 × (129/BALB/c)	100%	~50% with mets to lymph nodes, lungs.	Prostate hyperplasia at 4 weeks; PIN at 6 weeks; invasive prostate adenocarcinoma by 9 weeks; tumors are androgen receptor negative.	[29•]
Prostate	$Nkx3.1^{+/-}$ $Pten^{+/-}$	Double knockout	129/Sv × C57BL/6	84%	25% to lymph nodes after 1 year.	High grade PIN; invasive adenocarcinoma after 1 year; androgen independent; Pten is a tumor suppressor gene; Nkx3.1 is homeobox gene that is prostate-specific.	[30]
Prostate	Lo Myc or Hi Myc	Lo Myc: rat probasin/myc Hi Myc: ARR_2-probasin/myc	FVB	100%		PIN by 2–4 weeks; mice with high levels of myc expression develop invasive prostate adenocarcinoma by 3–6 months; mice with low levels of myc by 10–12 months.	[24]
GI/colorectal	Apc 1638	Truncated Apc gene	B6.129	90%		Colon polyps develop and progress to adenomas and colon carcinoma; 1–7 foci per mouse; mice are heterozygous for the truncated gene product.	[25]

Table 1 Continued

Target organ	Model name	Promoter/ transgene	Genetic background	Percent mice with tumors	Metastasis	Comments	References
GI/colorectal	CEA.Tg/ MIN	CEA.Tg × Apc mutated	C57BL/6	100%		Multifocal; tolerant to CEA.	[34,46]
Pancreas	MET	Rat elastase/ SV40 Tag 1-127 × MUC1.Tg	C57BL/6	50%		Pancreatic dysplasia at birth progressing to microadenomas and acinar cell carcinomas by week 9; by week 12 up to 9 tumor foci per mouse; the shortened SV40 Tag eliminates potential SV40 viral antigens.	[12]
Ovary	Tg MISIIR Tag	Mullerian inhibitory substance type IIR/SV40 Tag	B6C3F1	50%	Ascites	Poorly differentiated ovarian carcinoma.	[13]
Ovary	Ad	Cre-adenovirus/ $p53^{+}$Rb1 floxed recipients		97% by day 227 if both alleles are inactivated.	Ascites; mets to lung and liver.	Cre-adenovirus is inoculated intrabursally in the ovary; 5% of mice get tumors outside of the ovary.	[32•]
Melanocytes	Tyr-SV40E	Mouse tyrosinase/ SV40 T+t	C57BL/6	100%	61% of mice with eye tumors get mets.	Earliest melanomas are in the eye; skin melanomas are later and less frequent.	[14]

CEA, carcinoma embryonic antigen; DCIS, ductal carcinoma *in situ*; GI, gastrointestinal tract; mets, metastases; mo, month; PIN, prostate intraepithelial neoplasia. [a]Piero Musiani and Guido Forni, unpublished.

correlates with increasing tumor-induced immune suppression of the host, a situation that also occurs in patients with malignancies [26••]. Gene expression profiling of mammary tumors regulated by the endogenous Her2/neu promoter shows similarities to human mammary carcinoma [27•].

Although these transgenic models have a high incidence of spontaneous cancer, and are therefore very useful experimentally, investigators have questioned the physiological relevance of those models in which the oncogene is driven by a strong viral promoter such as MMTV [18]. Another limitation of some of these models is that they simultaneously develop multiple primary tumors, unlike their human counterparts in which typically a single primary tumor arises.

Tumor-suppressor-gene knockout models

Many human malignancies are associated with mutations in tumor suppressor genes. Because such mutations are considered causative of malignancy, tumor-suppressor-gene targeted mice ('loss-of-function') have been developed, either with or without co-activation of oncogenes. Table 1 includes some of these transgenic models and gives their characteristics. The most commonly targeted tumor suppressor gene is p53, and these mice typically develop tumors in multiple tissues (e.g. lung, skin, intestine, brain, thymus, lymphocytes and connective tissue). Two prostate cancer transgenic models have also been developed based on loss-of-function of Pten, a tumor suppressor gene that also has anti-apoptotic activity [28,29•,30].

Cre-lox conditional expression models

Traditional knockin and knockout transgenic mouse technology has provided numerous models of spontaneous tumorigenesis; however, these models share a major limitation. Unlike human malignancies, which typically develop after birth, the targeted/transgenes in these mouse models are altered during embryonic development. Therefore, disease onset is much earlier than in humans, and the kinetics of tumor progression do not parallel those of human malignancies. To overcome this problem, mouse models are being developed based on the ability of the bacterial recombinase Cre to activate genes that are flanked by LoxP sites. Typically, one strain of mice will contain a tissue-specific promoter upstream of a floxed oncogene or inactivator of a tumor suppressor gene, and a second strain will contain the Cre recombinase regulated by an inducible promoter. When the two strains are interbred and the F1 mice are given the inducer, then the targeted gene is affected. Using this approach, tumor-inducing genes can be manipulated at any time during the life of the mouse [31].

In an adaptation of the Cre-lox approach, Flesken-Nikitin and colleagues [32•] have devised a novel method for inducing localized ovarian tumors. Instead of mating Cre and floxed mice, they inoculated the ovarian bursa of mice with floxed versions of the *p53* and *Rb1* tumor suppressor genes with adenovirus encoding the bacterial Cre recombinase. The resulting mice developed predominantly ovarian tumors that progressed and metastasized in a similar way to human ovarian carcinomas.

Table 2

Selected transgenic mice expressing human tumor antigens.

Model name	Promoter/tumor antigen	Genetic background	Comments	References
PSA1Tg	Endogenous human/PSA	BALB/c	PSA expressed on prostate ductal epithelium; immune response to immunization with PSA.	[33,47]
Muc1.Tg	Endogenous human/Muc1	C57BL/6	Muc1 tissue distribution similar to human Muc1; no immune response to MUC1-expressing tumor cells or MUC1 protein.	[36]
(CEA Ge)18FJP	Endogenous human/CEA	C57BL/6	CEA expressed in the cecum, colon, gastric foveolar cells, and on 20% of luminal epithelial cells; no circulating CEA; immune response to immunization with CEA.	[46]
hHer-2 Tg	Whey acidic protein/ErbB-2	B6C3 backcrossed to C57BL/6	ErbB-2 expressed constitutively in Bergman glia cells (brain) and in secretory mammary epithelia during pregnancy and lactation.	[48••]

CEA, carcinoma embryonic antigen; PSA, prostate-specific antigen.

Transgenic mice expressing human tumor antigens

Many human tumor antigens are expressed by non-malignant cells, so investigators developing cancer vaccines must study the immunogenicity and host responsiveness to endogenous molecules. Therefore, transgenic mice expressing human tumor antigens have been generated. Some of these models and their characteristics are listed in Table 2. Such models are particularly useful for human tumor antigens, such as prostate-specific antigen (PSA), for which there is no mouse counterpart [33]. In some cases, tumor antigen transgenic mice have been crossed to mice that contain oncogenes, resulting in mice that develop spontaneous tumors expressing relevant tumor antigens (e.g. carcinoma embryonic antigen [CEA]/adenomatous polyposis coli [APC]$^{+/-}$ mice; [34]). In some cases, the tumor antigen itself is an oncogene and causes spontaneous tumor formation. Examples are the neuT and neuN transgenic mice, although both of these models use a rat her-2/neu gene rather than a human gene [16,17,35]. These models have provided valuable information on the challenges of inducing anti-tumor immunity to self antigens for which the host has varying degrees of tolerance [5•,23,36–40].

Gene-targeted (knockout) mice

The availability of knockout mice has allowed investigators to identify many molecules that are pivotal in tumor immunity. Knockout mice have been used in at least two types of scenarios. First, they are inoculated with a transplantable tumor derived from the genetic background of the knockout, and the mice are followed for tumor progression. As most gene-targeted mice are on a C57BL/6 or BALB/c background, experiments are limited to transplantable tumors derived from these strains (for an example of this approach see [41] and [42]). In an alternative experimental design, mice that have increased tumor resistance have been bred with knockout mice and the resulting offspring intercrossed or

Table 3

Websites for animal models.

Website URL	Content
http://emice.nci.nih.gov/	Mouse Models of Human Cancer Consortium.
Http://cancermodels.nci.nih.gov/	These National Cancer Institute (NCI) sites include a database of mouse cancer models, relevant publications and a listing of mice available from the NCI. Models are listed by affected organ and there are minireviews for each organ.
Http://www.jax.org/ Http://jaxmice.jax.org/library/models/cancer.pdf	The Jackson Laboratory. This site provides information and availability on the many mouse models distributed and/or developed at The Jackson Laboratory – the world's largest private supplier of inbred strains of mice.
http://ccr.cancer.gov/tech_initiatives/animalmodels/default.asp	NCI-sponsored Animal Models Initiative. (Password needed to access this site).
Http://tbase.jax.org/	The Jackson Laboratory transgenic/targeted mutation database (searchable).
Http://bioscience.org/knockout/alphabet.htm	Alphabetical listing of gene-targeted mice.
Http://research.bmn.com/mkmd	Mouse knockout and mutation database.
http://immunology.tch.harvard.edu/knockouts	Mouse mutants with immunological phenotypes
http://www.mshri.on.ca/nagy/cre.htm	This page contains links to Cre recombinase and floxed gene databases.

backcrossed to obtained homozygous-deficient mice. By following the incidence and kinetics of tumor development, investigators have assessed the role of the deleted gene in tumor resistance (see [43] for an example of this strategy). Table 3 lists websites containing databases describing mice deficient for various immunologically relevant genes.

Conclusions

Although most investigators believe that animal models can provide useful pre-clinical information about novel immunotherapies and cancer vaccines, others have argued that animal studies are uninformative because they are not predictive of results with humans. If poor prognostic results from animal studies are due to inadequate models, then better models must be developed. As tumor immunologists select the models they use, they should ensure that they mimic as closely as possible the human cancer for which the therapy or vaccine is designed. Is tumor onset comparable to that in humans? Are tumor progression and staging similar? Is the pathology of the animal tumor similar to that of its human counterpart? Is the extent of tumor burden comparable? Is hormone responsiveness similar? If the therapy being tested is designed for the treatment of metastatic disease in a post-surgery setting, is the animal model appropriate? If the targeted patients have tumor-induced immune suppression, is the animal model comparably immune suppressed? If the targeted patients are immunocompromised because of age, does the animal model show a similar immune deficit? Consideration of these issues when selecting the appropriate animal model may yield pre-clinical results that more closely predict clinical outcomes.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Lelekakis M, Moseley J, Martin T, Hards D, Williams E, Ho P, Lowen D, Javni J, Miller F, Slavin J *et al.*: **A novel orthotopic model of breast cancer metastasis to bone.** *Clin Exp Metastasis* 1999, **17**:163-170.

2. Pulaski B, Ostrand-Rosenberg S: **Mouse 4T1 breast tumor model**. In *Current Protocols in Immunology*. Edited by Coligan J, Margulies D, Shevach E, Strober W, Kruisbeek A. John Wiley & Sons; 2000:20.22.21-20.22.11.

3. Pulaski B, Terman D, Khan S, Muller E, Ostrand-Rosenberg S: **Cooperativity of SEB superantigen, MHC class II, and CD80 in immunotherapy of advanced metastases in a clinically relevant post-operative breast cancer model.** *Cancer Res* 2000, **60**:2710-2715.

4. Provinciali M, Smorlesi A, Donnini A, Bartozzi B, Amici A:
• **Low effectiveness of DNA vaccination against HER-2/neu in ageing.** *Vaccine* 2003, **21**:843-848.
This paper uses a transplantable tumor derived from a spontaneous tumor of BALB/c–neuT mice to demonstrate that aging dramatically effects the induction of cellular and humoral immunity following vaccination with a DNA vaccine. The paper points out that age of the recipient must be considered when selecting animal models for testing therapeutic efficacy.

5. Wolpoe ME, Lutz ER, Ercolini AM, Murata S, Ivie SE, Garrett ES,
• Emens LA, Jaffee EM, Reilly RT: **HER-2/neu-specific monoclonal antibodies collaborate with HER-2/neu-targeted granulocyte macrophage colony-stimulating factor secreting whole cell vaccination to augment CD8+ T cell effector function and tumor-free survival in Her-2/neu-transgenic mice.** *J Immunol* 2003, **171**:2161-2169.
This paper describes a combination immunotherapy for the treatment of transplanted mammary tumors derived from neuN transgenic mice. The administration of a whole cell vaccine (3T3/neu/GM-CSF cells) plus her2/neu-specific antibodies stimulates heightened anti-tumor immunity that results in increased survival, demonstrating the improved therapeutic efficacy of therapies that combine humoral and cellular immunity. These studies are an example of how transplantable tumors derived from spontaneous tumors of transgenic mice are used as therapy models.

6. Masumori N, Thomas TZ, Chaurand P, Case T, Paul M, Kasper S, Caprioli RM, Tsukamoto T, Shappell SB, Matusik RJ: **A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential.** *Cancer Res* 2001, **61**:2239-2249.

7. Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, Rosen JM: **Prostate cancer in a transgenic mouse.** *Proc Natl Acad Sci USA* 1995, **92**:3439-3443.

8. Xia J, Tanaka Y, Koido S, Liu C, Mukherjee P, Gendler SJ, Gong J: **Prevention of spontaneous breast carcinoma by prophylactic vaccination with dendritic/tumor fusion cells.** *J Immunol* 2003, **170**:1980-1986.

9. Guy CT, Cardiff RD, Muller WJ: **Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease.** *Mol Cell Biol* 1992, **12**:954-961.

10. Chen D, Xia J, Tanaka Y, Chen H, Koido S, Wernet O, Mukherjee P, Gendler SJ, Kufe D, Gong J: **Immunotherapy of spontaneous mammary carcinoma with fusions of dendritic cells and mucin 1-positive carcinoma cells.** *Immunology* 2003, **109**:300-307.

11. Mukherjee P, Ginardi A, Madsen C, Tinder T, Jacobs F, Parker J, Agrawal B, Longenecker B, Gendler S: **MUC1-specific CTLs are non-functional within a pancreatic tumor microenvironment.** *Glycoconj J* 2001, **18**:931-942.

12. Mukherjee P, Ginardi AR, Madsen CS, Sterner CJ, Adriance MC, Tevethia MJ, Gendler SJ: **Mice with spontaneous pancreatic cancer naturally develop MUC-1-specific CTLs that eradicate tumors when adoptively transferred.** *J Immunol* 2000, **165**:3451-3460.

13. Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC, Hamilton TC: **Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer.** *Cancer Res* 2003, **63**:1389-1397.

14. Bradl M, Klein-Szanto A, Porter S, Mintz B: **Malignant melanoma in transgenic mice.** *Proc Natl Acad Sci USA* 1991, **88**:164-168.

15. Powell WC, Cardiff RD, Cohen MB, Miller GJ, Roy-Burman P: **Mouse strains for prostate tumorigenesis based on genes altered in human prostate cancer.** *Curr Drug Targets* 2003, **4**:263-279.

16. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ: **Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease.** *Proc Natl Acad Sci USA* 1992, **89**:10578-10582.

17. Boggio K, Nicoletti G, Di Carlo E, Cavallo F, Landuzzi L, Melani C, Giovarelli M, Rossi I, Nanni P, De Giovanni C *et al.*: **Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice.** *J Exp Med* 1998, **188**:589-596.

18. Andrechek ER, Hardy WR, Siegel PM, Rudnicki MA, Cardiff RD, Muller WJ: **Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis.** *Proc Natl Acad Sci USA* 2000, **97**:3444-3449.

19. Nanni P, Nicoletti G, De Giovanni C, Landuzzi L, Di Carlo E, Cavallo F, Pupa SM, Rossi I, Colombo MP, Ricci C *et al.*: **Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER-2/neu transgenic mice.** *J Exp Med* 2001, **194**:1195-1205.

20. Reilly RT, Machiels JP, Emens LA, Ercolini AM, Okoye FI, Lei RY, Weintraub D, Jaffee EM: **The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors.** *Cancer Res* 2001, **61**:880-883.

21. Lollini PL, Forni G: **Antitumor vaccines: is it possible to prevent a tumor?** *Cancer Immunol Immunother* 2002, **51**:409-416.

22. • Cappello P, Triebel F, Iezzi M, Caorsi C, Quaglino E, Lollini PL, Amici A, Di Carlo E, Musiani P, Giovarelli M *et al.*: **LAG-3 enables DNA vaccination to persistently prevent mammary carcinogenesis in HER-2/neu transgenic BALB/c mice.** *Cancer Res* 2003, **63**:2518-2525.

This paper describes a novel cancer vaccine consisting of lymphocyte-activating gene-3/CD223. The prophylactic and therapeutic treatment is highly effective in neuT mice, which die from massive multi-focal breast tumors if untreated.

23. Ercolini AM, Machiels JP, Chen YC, Slansky JE, Giedlen M, Reilly RT, Jaffee EM: **Identification and characterization of the immunodominant rat HER-2/neu MHC class I epitope presented by spontaneous mammary tumors from HER-2/neu-transgenic mice.** *J Immunol* 2003, **170**:4273-4280.

24. Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J, Matusik R, Thomas GV, Sawyers CL: **Myc-driven murine prostate cancer shares molecular features with human prostate tumors.** *Cancer Cell* 2003, **4**:223-238.

25. Fodde R, Edelmann W, Yang K, van Leeuwen C, Carlson C, Renault B, Breukel C, Alt E, Lipkin M, Khan PM *et al.*: **A targeted chain-termination mutation in the mouse Apc gene results in multiple intestinal tumors.** *Proc Natl Acad Sci USA* 1994, **91**:8969-8973.

26. •• Melani C, Chiodoni C, Forni G, Colombo MP: **Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity.** *Blood* 2003, **102**:2138-2145.

This paper demonstrates that spontaneous tumor development in transgenic mice that contain the transforming ErbB-2 gene under the control of the MMTV promoter (BALB/c-neuT mice) is directly correlated with the development of myeloid-induced immune suppression. As many patients with malignancies are also profoundly immune suppressed, this spontaneous tumor model is particularly appropriate for studying tumor-induced immune suppression.

27. • Andrechek ER, Laing MA, Girgis-Gabardo AA, Siegel PM, Cardiff RD, Muller WJ: **Gene expression profiling of neu-induced mammary tumors from transgenic mice reveals genetic and morphological similarities to ErbB2-expressing human breast cancers.** *Cancer Res* 2003, **63**:4920-4926.

This paper reports gene expression profiling of ErbB-2-expressing human mammary carcinomas and two mammary carcinomas from transgenic mice in which the her2/neu gene is regulated by its endogenous promoter or by the MMTV promoter. Although the transgenic tumors have similar histology, they are very different in time of onset and metastatic characteristics. Gene profiling results demonstrate that, although expression of some genes is restricted to each type of tumor, there is considerable similarity between the endogenously regulated transgenic tumor and ErbB-2 positive human mammary cancers.

28. Freeman DJ, Li AG, Wei G, Li HH, Kertesz N, Lesche R, Whale AD, Martinez-Diaz H, Rozengurt N, Cardiff RD *et al.*: **PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms.** *Cancer Cell* 2003, **3**:117-130.

29. • Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, Thomas GV, Li G, Roy-Burman P, Nelson PS *et al.*: **Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer.** *Cancer Cell* 2003, **4**:209-221.

This paper describes a $Pten^{-/-}$ knockout mouse that spontaneously and rapidly develops prostate cancer. Unlike the TRAMP and other prostate cancer models that develop neuroendocrine tumors, homozygous deletion of the Pten gene produces epithelial tumors which are the type of tumors seen in human prostate cancer.

30. Abate-Shen C, Banach-Petrosky WA, Sun X, Economides KD, Desai N, Gregg JP, Borowsky AD, Cardiff RD, Shen MM: **Nkx3.1; Pten mutant mice develop invasive prostate adenocarcinoma and lymph node metastases.** *Cancer Res* 2003, **63**:3886-3890.

31. Nagy A: **Cre recombinase: the universal reagent for genome tailoring.** *Genesis* 2000, **26**:99-109.

32. • Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, Nikitin AY: **Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium.** *Cancer Res* 2003, **63**:3459-3463.

This paper describes a novel approach for initiating ovarian cancer development by inoculating the ovarian bursa of p53 and Rb1 floxed mice with a recombinant adenovirus containing the bacterial recombinase Cre. A high percentage of mice with homozygous inactivation of p53 and Rb1 develop progressive and metastatic ovarian tumors.

33. Wei C, Willis RA, Tilton BR, Looney RJ, Lord EM, Barth RK, Frelinger JG: **Tissue-specific expression of the human prostate-specific antigen gene in transgenic mice: implications for tolerance and immunotherapy.** *Proc Natl Acad Sci USA* 1997, **94**:6369-6374.

34. Greiner JW, Zeytin H, Anver MR, Schlom J: **Vaccine-based therapy directed against carcinoembryonic antigen demonstrates antitumor activity on spontaneous intestinal tumors in the absence of autoimmunity.** *Cancer Res* 2002, **62**:6944-6951.

35. Guy CT, Cardiff RD, Muller WJ: **Activated neu induces rapid tumor progression.** *J Biol Chem* 1996, **271**:7673-7678.

36. Rowse GJ, Tempero RM, VanLith ML, Hollingsworth MA, Gendler SJ: **Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model.** *Cancer Res* 1998, **58**:315-321.

37. Reilly RT, Gottlieb MB, Ercolini AM, Machiels JP, Kane CE, Okoye FI, Muller WJ, Dixon KH, Jaffee EM: **HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice.** *Cancer Res* 2000, **60**:3569-3576.

38. Rovero S, Amici A, Carlo ED, Bei R, Nanni P, Quaglino E, Porcedda P, Boggio K, Smorlesi A, Lollini PL *et al.*: **DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice.** *J Immunol* 2000, **165**:5133-5142.

39. Soares MM, Mehta V, Finn OJ: **Three different vaccines based on the 140-amino acid MUC1 peptide with seven tandemly repeated tumor-specific epitopes elicit distinct immune effector mechanisms in wild-type versus MUC1-transgenic mice with different potential for tumor rejection.** *J Immunol* 2001, **166**:6555-6563.

40. Pupa SM, Invernizzi AM, Forti S, Di Carlo E, Musiani P, Nanni P, Lollini PL, Meazza R, Ferrini S, Menard S: **Prevention of spontaneous neu-expressing mammary tumor development in mice transgenic for rat proto-neu by DNA vaccination.** *Gene Ther* 2001, **8**:75-79.

41. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA: **Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma.** *J Exp Med* 2000, **192**:755-760.

42. Pulaski BA, Smyth MJ, Ostrand-Rosenberg S: **Interferon-gamma-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma.** *Cancer Res* 2002, **62**:4406-4412.

43. Ostrand-Rosenberg S, Sinha P, Clements V, Dissanayake S, Miller S, Davis C, Danna E: **Signal transducer and activator of transcirption 6 (Stat6) and CD1: inhibitors of immunosurveillance against primary tumors and metastatic disease.** *Cancer Immunol Immunother* 2004, **53**:86-91.

44. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P: **Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene.** *Cell* 1988, **54**:105-115.

45. Gingrich JR, Barrios RJ, Morton RA, Boyce BF, DeMayo FJ, Finegold MJ, Angelopoulou R, Rosen JM, Greenberg NM: **Metastatic prostate cancer in a transgenic mouse.** *Cancer Res* 1996, **56**:4096-4102.

46. Clarke P, Mann J, Simpson JF, Rickard-Dickson K, Primus FJ: **Mice transgenic for human carcinoembryonic antigen as a model for immunotherapy**. *Cancer Res* 1998, **58**:1469-1477.

47. Wei C, Callahan BP, Turner MJ, Willis RA, Lord EM, Barth RK, Frelinger JG: **Regulation of human prostate-specific antigen gene expression in transgenic mice: evidence for an enhancer between the PSA and human glandular kallikrein-1 genes.** *Int J Mol Med* 1998, **2**:487-496.

48. Piechocki M, Ho Y, Pilon S, Wei W: **Human ErbB-2 (Her-2)**
•• **transgenic mice: a model system for testing Her-2 based vaccines.** *J Immunol* 2003, **171**:5787-5794.
This paper describes the generation of a transgenic mouse model in which the human ErbB-2 gene is expressed under the control of the whey acidic promoter. This model will be very useful for studying the immunogenicity of mammary cancer vaccines because, unlike other her2/neu transgenic models, it contains the human ErbB-2 gene, which is only overexpressed in the mammary gland during pregnancy and lactation, similar to her2/neu expression in humans.

APPENDIX 5

[CANCER RESEARCH 62, 4406–4412, August 1, 2002]

Interferon-γ-dependent Phagocytic Cells Are a Critical Component of Innate Immunity against Metastatic Mammary Carcinoma[1]

Beth A. Pulaski, Mark J. Smyth, and Suzanne Ostrand-Rosenberg[2]

Department of Biological Sciences, University of Maryland-Baltimore County, Baltimore, Maryland 21250 [B. A. P, S. O-R.], and Cancer Immunology Program and Peter MacCallum Cancer Institute, East Melbourne, Victoria 8006, Australia [M. J. S.]

ABSTRACT

IFN-γ is a pleiotropic cytokine that plays an important role in regulating the growth of primary tumors. Although numerous studies of the effects of IFN-γ on primary-solid-tumor growth have been performed and several potential mechanisms for its efficacy have been proposed, it remains unclear how IFN-γ modulates tumor progression and whether it exerts its effects indirectly via host cells or directly by interacting with tumor cells. Using the well-characterized mouse metastatic mammary carcinoma 4T1 in a postsurgery setting, IFN-γ-deficient mice were found to have significantly shorter survival time relative to wild-type mice, demonstrating that IFN-γ is also a critical component in regulating innate immunity to metastatic disease. Experiments quantifying lung and liver metastasis indicate that decreased survival of IFN-γ-deficient mice is attributable to increased metastatic disease. To determine whether IFN-γ is acting directly on the tumor cells, IFN-γ-nonresponsive 4T1 cells were generated by transfection (4t1/IRt). Metastasis experiments with 4T1/IRt demonstrated that IFN-γ mediates its effects via host-derived cells, rather than by directly affecting tumor growth. To identify the population of cells responsible for IFN-γ efficacy, perforin-deficient, T-cell subset-depleted, natural killer cell-depleted, or carrageenan-treated phagocytic cell-depleted mice were inoculated with 4T1 and assessed for primary tumor growth and metastatic disease. None of the conditions altered primary tumor growth; however, the carrageenan treatment significantly increased metastatic disease in the liver and lungs. Survival experiments in 4T1-inoculated, carrageenan-treated mice confirmed that the elimination of phagocytic cells significantly reduces survival time and yields a survival phenotype comparable with IFN-γ deficiency. Therefore, IFN-γ is a critical component of innate immunity to metastatic mammary carcinoma that probably mediates its effects via host-derived phagocytic cells.

INTRODUCTION

IFN-γ is a pleiotrophic cytokine that regulates hundreds of diverse genes. Many of these genes are involved in responses to pathogens, and their effects are manifested via the immune system (1–3). For example, IFN-γ regulates immune functions such as immunoglobulin heavy-chain class switching, cell-mediated (T_{h1} *versus* T_{h2}) and NK[3] cell immune responses, phagocytic cell clearance of bacteria, antigen processing and presentation by MHC class I and class II molecules, and leukocyte-endothelial interactions. In addition to its role in combating pathogens, IFN-γ has also been implicated in immune responses to tumors, and recent studies demonstrated that the induction of IFN-γ underlies the therapeutic efficacy of IL-12-based tumor immunotherapy (4). Although it is appreciated that IFN-γ plays an important role in regulating tumor growth, the mechanism(s) by which it exerts its effect is unclear.

On the basis of *in vivo* and *in vitro* observations, several antitumor mechanisms have been ascribed to IFN-γ. For example, it induces tumor cell production of antiangiogenic factors, which promote antitumor effects through starvation of tumor cells (5). Similarly, Fas/FasL interactions which are necessary for IFN-γ up-regulation of antiangiogenic factors, promote vascular endothelial apoptosis in the tumor microenvironment, thereby limiting tumor growth (6). Other studies show that tumor-induced antiangiogenesis requires expression of IFN-γ receptor (CD119) on nonhematopoietic cells, which suggests that IFN-γ-dependent host factors are involved (7). Other studies show the effects of IFN-γ on the host's immune system that suggest that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression by IFN-γ-activated NK cells (8) and IgG2a and IgG2b antibody production by B cells (9) are important for the generation of IFN-γ-dependent antitumor responses. Clearly IFN-γ potentially acts through multiple mechanisms and may alter tumor growth either by its direct effects on tumor cells or via its action on host cells and/or factors. Not only is it unclear which of these mechanisms is (are) relevant for tumor rejection, but it is also uncertain whether these mechanisms work cooperatively or synergistically. Likewise, the relative contribution of each mechanism to overall tumor rejection is unknown.

We have undertaken the present study to identify the predominant IFN-γ-mediated mechanism(s) responsible for regulating tumor growth and to clarify whether host and/or tumor cells are the target for IFN-γ activity *in vivo*. Because metastatic cancer is the major cause of death for patients with solid tumors, our studies focus on the role of IFN-γ in metastatic disease. Several types of animal models are available for these studies. Transgenic mice carrying transforming genes under the control of viral promoters provide animals with spontaneously developing primary tumors (9, 10). However, these animals typically die from massive, multifocal primary tumors rather than disseminated metastatic disease and, hence, are not optimal models for the study of metastatic cancer. Transplantable tumors include both immunogenic and nonimmunogenic tumors. Because human tumors are poorly immunogenic, nonimmunogenic or poorly immunogenic mouse tumors are the better models. We have used the poorly immunogenic 4T1 mouse mammary carcinoma. This BALB/c-derived transplantable tumor shares many characteristics with human breast tumors and is an established model for studies of metastatic cancer (11–14). After inoculation of small quantities of 4T1 tumor cells in the abdominal mammary gland, primary tumor grows progressively and spontaneously metastasizes to the lungs, liver, blood, lymph nodes, brain, and bone marrow (11, 12, 14, 15). Analogous to human mammary carcinoma, metastatic cells proliferate at distant sites while the primary tumor is in place, and continue to proliferate when the primary tumor is surgically removed (13). The 4T1 system allows us to focus on the development of metastatic disease after the surgical removal of primary tumor. This scenario has not been previously explored in animal models, and may be very relevant for human cancers such as breast cancer, in which metastatic

---

Received 2/19/02; accepted 5/24/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by grants from the United States Army Research and Development Command (DAMD17-94-J-4323 and DAMD-17-01-1-0312) and the NIH (RO1 CA52527 and R01CA84232). B. A. P. was supported by a postdoctoral fellowship from the United States Army Research and Development Command, DAMD17-97-1-7152.

[2] To whom requests for reprints should be addressed, at Department of Biological Sciences, University of Maryland, 1000 Hilltop Circle, Baltimore, MD 21250.

[3] The abbreviations used are: NK, natural killer; Mig, monokine induced by IFN-γ; IP-10, IFN-γ-inducible protein; TD, tumor diameter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent; IL, interleukin; mAb, monoclonal antibody; LPS, lipopolysaccharide; DC, dendritic cell; iNOS, inducible nitric oxide synthetase.

disease after the excision of primary tumor is the principal cause of death.

Our studies indicate that although several tumor- and host-derived IFN-γ-dependent factors regulate the growth of distant metastasis, host phagocytic cells play a major role. Furthermore, IFN-γ acts via host cells and/or factors and does not regulate metastatic growth by directly acting on tumor cells.

MATERIALS AND METHODS

Animals, Cell Lines, cDNA Expression Vectors, and Transfectants. Female BALB/c, BALB/c.IFNγ$^{-/-}$, and BALB/c.IL4$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). BALB/c.Pfp$^{-/-}$ mice were generated as described previously (16). All of the mice were maintained and bred in the University of Maryland-Baltimore County (UMBC) Biology Department animal facility and were used at 8–16 weeks of age. All of the animal procedures were reviewed and approved by the UMBC Institutional Animal Care and Use Committee and are in compliance with the NIH guidelines for the humane treatment of laboratory animals. 4T1, a 6-thioguanine-resistant, BALB/c-derived spontaneous mammary carcinoma (11), was cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine product (Hyclone, Logan, UT) and 1× Antibiotic-Antimycotic (Life Technologies, Inc.; Ref. 12). Wild-type tumor cells were transfected with the expression vector pEF2.mugR (5), containing the cDNA for a truncated IFN-γR$_{\alpha}$, by using Pfx-5 lipid (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were selected with 400 μg/ml G-418 (Life Technologies, Inc.), stained for surface antigen expression, and analyzed by flow cytometry (12). mAbs 34-5-8 (H-2D^d) and MKD6 (I-A^d) were affinity purified and used as previously described (12). mAb CD119 (IFN-γRα) was purchased from BD BioScience (San Diego, CA).

***In Vitro* Proliferation Assay.** 4T1 and 4T1/IRt transfectants at 1 × 10^4/ml were cultured with recombinant IFN-γ (Pierce Endogen, Rockford, IL) for 3 days, and proliferation was measured using MTT (13). The percentage of growth inhibition = $[(A_{0U/ml} - A_{nU/ml})/(A_{0U/ml})] \times 100\%$. 4T1/IRt transfectants that expressed low levels of CD119 remained responsive to IFN-γ.

***In Vitro* Induction of Mig.** 4T1 and 4T1/IRt cells were induced *in vitro* with 100 units/ml IFN-γ for 2 h. RNA was isolated, and Mig was detected using RT PCR (17).

Tumor Inoculation, Clonogenic Metastasis Assay, *in Vivo* Depletion, and Surgery. Mice were challenged s.c. in the abdominal mammary gland with 7 × 10^3 4T1 or transfected 4T1 tumor cells. Primary tumor growth and spontaneous metastasis in the lungs and liver using the clonogenic assay were measured as described previously (12). Mice were depleted of CD4$^+$ T, CD8$^+$ T, and NK cells and were monitored to ascertain depletion as described previously (17). Phagocytic cells were depleted by injecting mice i.p. with 2 mg carrageenan iota type (Ref. 18; Sigma, St. Louis, MO) on days −3 and −1 before tumor inoculation, followed by 1 injection every 14 days throughout the experiment. Depletion of macrophages by carrageenan treatment was evaluated by measuring reduced susceptibility to LPS-induced toxic shock syndrome. Primary tumors were surgically removed on days 21–24 after 4T1 inoculation as described (13).

Statistical Analyses. Data were analyzed using Student's *t* test for unequal variances (Microsoft Excel v5.0).

RESULTS

IFN-γ-deficient Mice Have a Significantly Reduced Survival Time after Inoculation of 4T1 Mammary Carcinoma Cells, Relative to IFN-γ-Competent Mice. We previously established that syngeneic BALB/c mice inoculated with 7 × 10^3 4T1 mammary carcinoma cells in the abdominal mammary gland develop disseminated metastasis within 10–21 days of 4T1 inoculation and will die from metastatic cancer even if the primary tumor is surgically removed (13). If IFN-γ reduces metastatic tumor growth, then IFN-γ-deficient (BALB/c.IFNγ$^{-/-}$) mice might have a decreased survival time relative to wild-type BALB/c mice. To test this hypothesis, BALB/c and BALB/c.IFNγ$^{-/-}$ mice were inoculated with 4T1 cells, had primary tumors surgically removed on days 21–24, and were followed for survival. The average survival time for BALB/c and BALB/c.IFNγ$^{-/-}$ mice was 44 (±6) and 36 (±2) days, respectively ($P < 0.001$). To determine whether the shortened survival of the BALB/c.IFNγ$^{-/-}$ mice was caused by more metastases arising from larger primary tumors, survival time was plotted as a function of TD at the time of surgery. The average sizes of the primary tumors in BALB/c and BALB/c.IFNγ$^{-/-}$ mice were similar at their time of removal (Fig. 1*A*). Therefore, BALB/c.IFNγ$^{-/-}$ mice are more susceptible to the 4T1 tumor, which suggests that metastatic disease progresses more rapidly in IFN-γ-deficient hosts.

BALB/c.IFNγ$^{-/-}$ Mice Develop Spontaneous Lung and Liver Metastases Earlier Than Wild-Type BALB/c Mice. To determine whether the deletion of IFN-γ results in earlier deaths because of increased metastatic disease, the number of metastatic tumor cells in 4T1-inoculated BALB/c and BALB/c.IFNγ$^{-/-}$ mice was measured. Mice were inoculated s.c. in the abdominal mammary gland with 7 × 10^3 4T1 tumor cells and killed on day 35, after which their lungs and livers were removed. The number of metastatic 4T1 tumor cells in these organs was quantified using the clonogenic assay. Primary tumors were not surgically removed for this experiment because

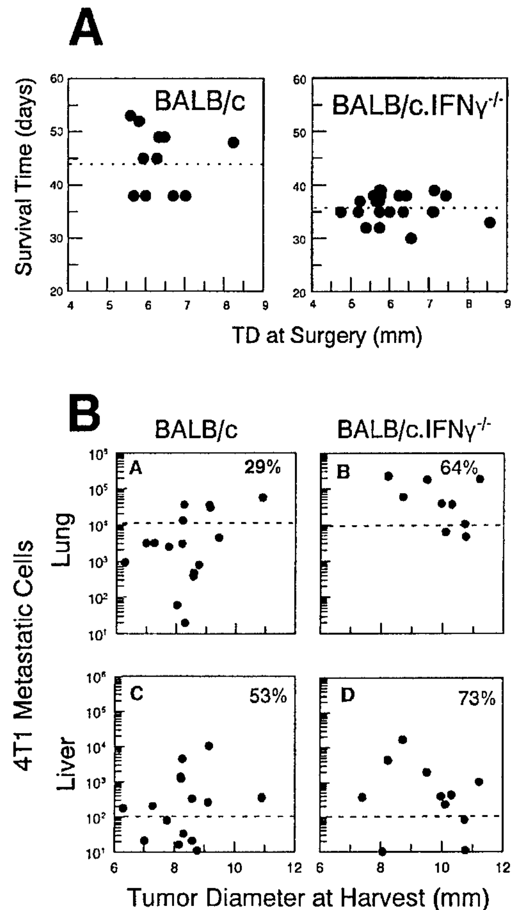


Fig. 1. BALB/c.IFNγ$^{-/-}$ mice have a shortened survival time and increased numbers of metastatic cells after removal of primary tumor, relative to wild-type BALB/c mice. BALB/c and BALB/c.IFNγ$^{-/-}$ mice were inoculated s.c. in the abdominal mammary gland with 7 × 10^3 4T1 cells on day 1. *A*, primary tumors were measured and surgically removed on days 21–24, and mice were followed for survival. *Data points*, the survival time of an individual mouse. · · · ·, the mean survival times. *B*, 35 days after 4T1 inoculation, mice were sacrificed, and the number of metastatic 4T1 cells in the lungs and liver quantified using the clonogenic assay. *Data points*, the number of 4T1 metastatic cells in an individual mouse. · · · ·, the lethal levels of metastatic disease in these organs.

surgical removal of primary tumor does not alter the survival time or the number of disseminated metastases (13).

To determine whether primary tumor size impacts the level of metastatic disease, the data were plotted as the number of clonogenic metastatic cells *versus* TD at the time of harvest (day 35). Because 10,000 and 100 tumor cells in the lungs and liver, respectively, indicated lethal levels of metastatic disease (12), a line indicating these values was included in the plots. As shown in Fig. 1*B* and Table 1, 64% of BALB/c.IFN$\gamma^{-/-}$ *versus* 29% of BALB/c mice contained >10,000 clonogenic lung metastases and 73% of BALB/c.IFN$\gamma^{-/-}$ *versus* 53% of BALB/c mice contained >100 clonogenic liver metastases. To determine whether increased metastasis is specific to the BALB/c.IFN$\gamma^{-/-}$ mice, BALB/c.IL-4$^{-/-}$ mice were also tested. BALB/c.IL-4$^{-/-}$ mice did not develop lung and liver metastases faster than BALB/c mice (data not shown). Thus, deletion of IFN-γ increases the number of spontaneous metastatic tumor cells, and this increase is most likely responsible for the heightened lethality of 4T1 in BALB/c.IFN$\gamma^{-/-}$.

Primary Tumor Growth Is Marginally Increased in BALB/c.IFN$\gamma^{-/-}$ Mice as Compared with Normal BALB/c Mice. Because previous immunotherapy studies with 4T1 showed disparate effects on primary and metastatic tumor cells (17), we also analyzed the effects of IFN-γ on primary tumor development in the mice used for Fig. 1. Primary tumors were palpable within 6–13 days of inoculation and although the TDs between BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice were significantly different at day 35 (P = 0.045), the overall kinetics of primary tumor growth did not differ (Table 2 and data not shown). Therefore, IFN-γ is a critical mediator of metastatic tumor progression and has only marginal effects on primary tumor growth.

Generation of IFN-γ-nonresponsive 4T1 Tumor Cells. Because IFN-γ has multiple immune and nonimmune targets *in vivo*, we tested for whether IFN-γ was inhibiting metastasis progression by acting directly on the tumor cells. Several studies have shown that IFN-γ can alter tumor cells directly *in vitro* with correlative reduction in primary tumorigenicity *in vivo* (19, 20). To examine this possibility, we generated 4T1 transfectants that were insensitive to IFN-γ (4T1/IRt transfectants). If IFN-γ affects metastatic tumor growth by directly

Table 1 *4T1 mammary tumor cells metastasize more rapidly to the lungs and liver of BALB/c.IFN$\gamma^{-/-}$ mice than of wild-type BALB/c mice*

BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice were inoculated s.c. in the abdominal mammary gland with 7 × 10^3 4T1 cells and were killed on day 35. The number of lung and liver metastatic cells was determined using the clonogenic assay.

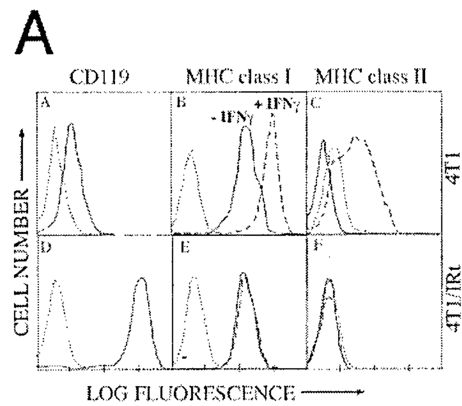
	BALB/c	BALB/c.IFN$\gamma^{-/-}$
Mice with >10,000 lung metastases	29% (5/17)	64% (7/11)
Mice with >100 liver metastases	53% (9/17)	73% (8/11)

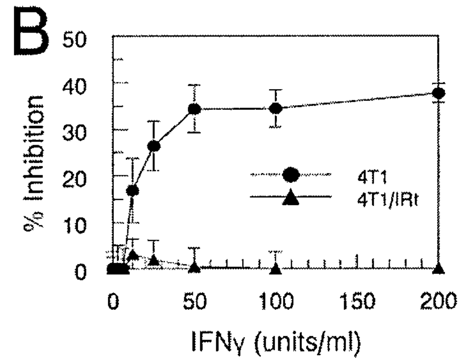
Table 2 *Primary 4T1 mammary tumors grow slightly faster in BALB/c.IFN$\gamma^{-/-}$ mice as compared with wild-type BALB/c mice; however, growth is not affected by depletion of CD4, CD8, NK, or phagocytic cells*

Mice were inoculated s.c. in the abdominal mammary gland with 7 × 10^3 4T1 cells. Primary tumors were measured every 3–4 days. Numbers in parentheses indicate the number of mice per group. Two separate experiments are shown.

Strain	Tumor onset (days)	Tumor diameter at day 35 (mm)
BALB/c (25)	6–13	8.5 ± 1.4
BALB/c.IFN$\gamma^{-/-}$ (11)	7–10	9.5 ± 1.3[a]
BALB/c (27)	7–12	9.3 ± 1.5
BALB/c.Pfp$^{-/-}$ (8)	10–12	8.5 ± 0.4[b]
anti-asialo GM1 (8)	7–9	9.5 ± 1.1
CD4-depleted (9)	7–10	9.2 ± 0.9
CD8-depleted (10)	10–14	8.5 ± 1.3
Carrageenan (9)	7–11	9.0 ± 0.7

[a] Statistically significantly different from that of control BALB/c mice (P < 0.05).
[b] Statistically significantly different from that of control BALB/c mice (P < 0.025).





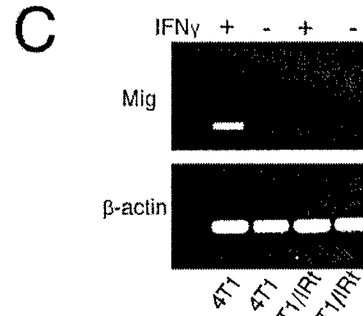


Fig. 2. 4T1 tumor cells expressing an IFN-γ receptor with a truncated α chain (4T1/IRt cells) are not responsive to IFN-γ. *A*, 4T1 and 4T1/IRt transfectants were stained by indirect immunofluorescence for IFN-γ receptor (CD119 mAb), MHC class I (34-5-8 mAb), or MHC class II (MKD6 mAb). · · · ·, staining by fluorescent conjugate alone; — — —, staining after a 3-day *in vitro* treatment with 100 units/ml IFN-γ; —, staining in the absence of IFN-γ treatment. The *X* axis shows four log cycles of fluorescence intensity. *B*, 4T1 and 4T1/IRt cells (1–10 × 10^3/well) were cultured *in vitro* in the presence of various levels of IFN-γ for 3 days, and the % inhibition determined using an MTT assay. *Error bars*, SDs of the mean. *C*, RNA from 4T1 and 4T1/IRt tumor cells cultured in the presence or absence of 100 units/ml IFN-γ for 2 h was reverse-transcribed and amplified using Mig-specific or β-actin-specific primers.

acting on the tumor cells, then the growth kinetics of 4T1/IRt in BALB/c mice will be similar to the growth kinetics of 4T1 in BALB/c.IFN$\gamma^{-/-}$ mice.

To generate an IFN-γ-insensitive cell line, 4T1 was transfected with the pEF2.mugR plasmid, which contains a cDNA for a truncated IFN-γR_α chain. High levels of truncated IFN-γR_α chain compete with endogenous full-length IFN-γR_α and yield cells that are nonresponsive to IFN-γ (5). Transfectants were selected in G418 and analyzed for overexpression of IFN-γR (CD119) using flow cytometry. As shown in Fig. 2*A*, the transfectants (4T1/IRt line) express approximately two logs more CD119 than the parental 4T1 cells. The sensitivity of 4T1/IRt cells to IFN-γ was first examined by measuring the

induction of MHC class I and class II molecules. Parental 4T1 cells were induced to express MHC class II and increased levels of MHC class I molecules after incubation with 100 units/ml IFN-γ for 3 days *in vitro*. In contrast, IFN-γ did not induce the 4T1/IRt line to express MHC class II or increase MHC class I levels.

To further evaluate the responsiveness of 4T1 and the insensitivity of 4T1/IRt to IFN-γ, proliferation and chemokine expression in the presence and absence of IFN-γ were determined. As shown in Fig. 2B, 100 units/ml IFN-γ inhibited the proliferation of wild-type 4T1 *in vitro* by 34.5% and has no effect on 4T1/IRt. Because chemokine expression by tumor cells can be induced by IFN-γ and has been suggested to regulate tumor growth *in vivo* through antiangiogenic mechanisms, induction of Mig in 4T1- and 4T1/IRt-IFN-γ-treated cells was also measured. IP-10 induction was not measured because 4T1 was previously shown not to express IP-10 in response to IFN-γ *in vitro* (17). 4T1 was induced by IFN-γ to express Mig, whereas Mig was not induced in IFN-γ-treated 4T1/IRt cells (Fig. 2C). Thus, 4T1/IRt cells are not responsive to IFN-γ and could be used to distinguish whether IFN-γ acts directly on the tumor cells or via the host's system *in vivo*.

IFN-γ Does Not Act Directly on 4T1 Tumor Cells. To determine whether IFN-γ directly acts on 4T1 tumor cells, 4T1 and 4T1/IRt cells were injected at 7×10^3 into BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice. As shown in Fig. 3*A*, there are no significant differences in the growth kinetics of primary tumors in any of the tumor-cell/mouse-strain combinations tested. Therefore, primary solid tumor growth is not affected by IFN-γ directly acting on 4T1 tumor cells.

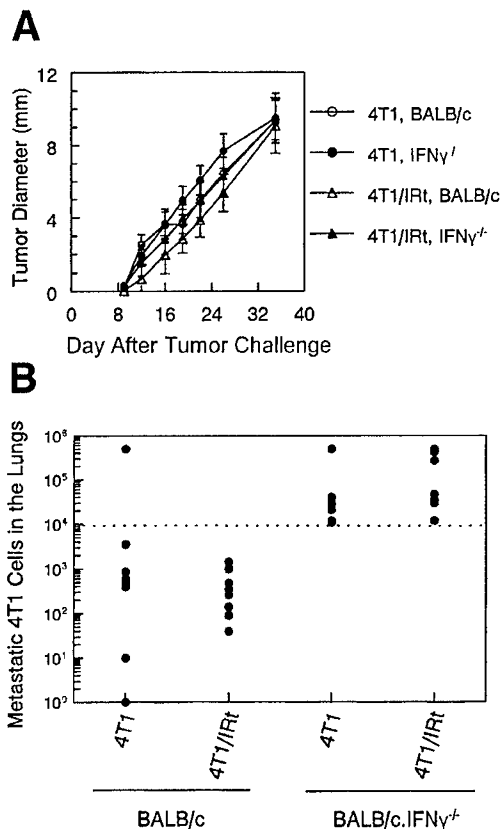
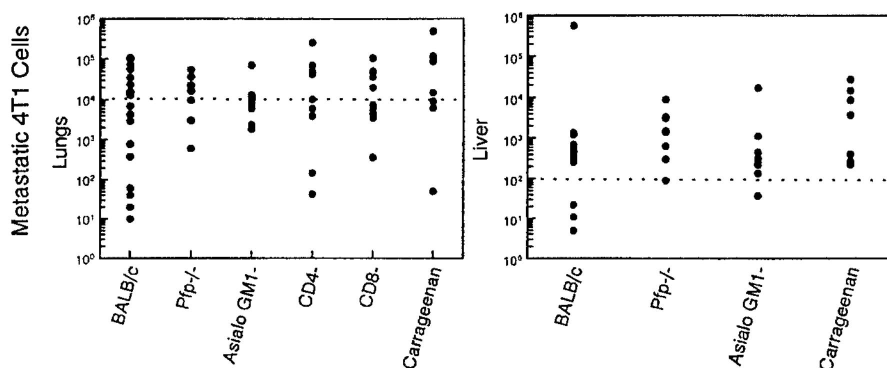


Fig. 3. 4T1 and 4T1/IRt cells have similar primary and metastatic growth kinetics. *A*, BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice were inoculated s.c. in the abdominal mammary gland with 7×10^3 4T1 or 4T1/IRt cells on day 1. Primary tumors were measured every 3–4 days thereafter. *Data points*, the average TD of 5–7 mice. *Error bars*, the SD of the mean. *B*, the mice used in *A* were sacrificed on day 35 after tumor cell inoculation, and the number of 4T1 or 4T1/IRt metastatic cells in their lungs was determined using the clonogenic assay. *Data points*, the number of metastatic 4T1 cells in the lungs of an individual mouse. · · · ·, the lethal level of metastatic cells in the lungs.

To determine whether IFN-γ acts directly on metastasizing 4T1 tumor cells to reduce metastatic disease, the BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice of Fig. 3A were sacrificed on day 35 after 4T1 inoculation, and the number of metastatic cells in their lungs determined using the clonogenic assay. 4T1 and 4T1/IRt tumor cells produced comparable numbers of metastatic cells in the lungs of BALB/c mice and much higher levels of metastatic cells in the lungs of BALB/c.IFN$\gamma^{-/-}$ mice (Fig. 3B). Hence, the antitumor effects of IFN-γ are most likely mediated by host-derived factors, rather than by IFN-γ directly altering tumor cell proliferation.

Perforin-mediated Effector Mechanisms Minimally Contribute to the IFN-γ-dependent Antitumor Immune Response. Several studies have shown that IFN-γ activates T and NK cell responses *in vitro* and *in vivo* (2). T and NK cells mediate their effects through perforin-mediated lysis (21). To determine whether T and/or NK cells play a role in IFN-γ-mediated responses to 4T1, BALB/c.Pfp$^{-/-}$ mice and BALB/c mice depleted for NK cells, CD4$^+$ or CD8$^+$ T cells were injected s.c. in the abdominal mammary gland with 7×10^3 parental 4T1 cells, and primary tumor growth monitored. Although a small decrease in tumor size was seen in the BALB/c.Pfp$^{-/-}$ *versus* wild-type BALB/c mice at day 35 ($P = 0.025$), the growth kinetics of 4T1 primary tumors in wild-type BALB/c, BALB/c.Pfp$^{-/-}$, and depleted BALB/c mice were very similar (Table 2 and data not shown). To ascertain whether T and/or NK cells are involved in the IFN-γ-mediated antimetastatic response, the numbers of clonogenic metastatic cells in the lungs and/or livers of these animals were analyzed (Fig. 4). Statistically significant differences were not seen in the anti-asialo-GM1-treated, CD4-depleted, and CD8-depleted BALB/c mice. Spontaneous lung and liver metastases in BALB/c.Pfp$^{-/-}$ mice were slightly increased relative to metastases in BALB/c mice (Table 3, $P = 0.049$ and $P = 0.007$, respectively). Survival time of BALB/c.Pfp$^{-/-}$ mice, however, does not differ from that of BALB/c mice (data not shown), suggesting that the minimal difference in lung and liver metastases is not physiologically important. Therefore, perforin-dependent mechanisms may modestly reduce the numbers of metastatic cells; however, the increased survival of BALB/c *versus* BALB/c.IFN$\gamma^{-/-}$ mice cannot be attributed to perforin-dependent effector activities. Likewise, because depletion of CD4$^+$, CD8$^+$, or NK cells did not affect tumor growth or survival time, these cell populations cannot account for the increased survival time of wild-type mice *versus* IFN-γ-deficient mice. Therefore, it is likely that non-perforin-dependent mechanisms and cells other than CD4$^+$, CD8$^+$, and NK cells are responsible for increased survival mediated by IFN-γ.

IFN-γ-dependent Phagocytic Cells Are Primarily Responsible for Controlling Spontaneous Metastatic Tumor Growth and Survival. IFN-γ also activates phagocytic cells, such as macrophages and dendritic cells (2). Macrophages possess potent tumoricidal activity (18), and dendritic cells are critical cells for antigen presentation (22). To determine whether these cells are responsible for increased spontaneous metastatic disease in BALB/c.IFN$\gamma^{-/-}$ mice, BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice were treated with carrageenan, a drug that depletes phagocytic cells (23). Mice were sacrificed at day 35, and the number of metastatic tumor cells in the lungs and liver was quantified using the clonogenic assay. As shown in Fig. 4*A* and Table 3, the number of metastatic cells in the lungs is significantly increased in carrageenan-treated BALB/c mice as compared with untreated BALB/c mice ($P = 0.017$). Although the number of metastatic cells in the livers of carrageenan-treated mice seems to be increased relative to BALB/c mice, the trend was not statistically significant ($P = 0.156$). Fig. 4*A* also shows that the levels of metastatic cells in



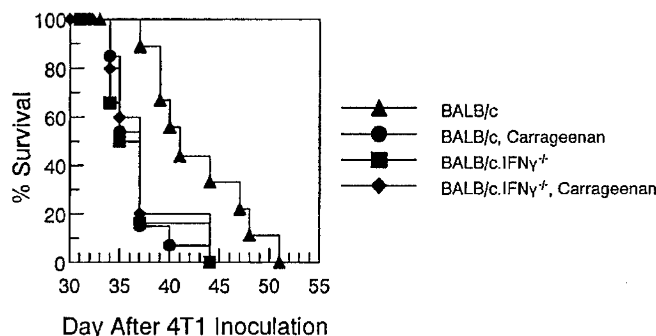


Fig. 4. Carrageenan-treated BALB/c mice have increased metastatic disease and decreased survival time. *A*, BALB/c, BALB/c.Pfp$^{-/-}$, anti-asialo-GM1-treated, CD4-depleted, CD8-depleted, and carrageenan-treated BALB/c mice were inoculated s.c. in the abdominal mammary gland with 7×10^3 4T1 cells on day 1. Mice were sacrificed on day 35, and the number of metastatic 4T1 tumor cells in the lungs and liver were quantified using the clonogenic assay. Data points, the number of metastatic 4T1 cells in an individual mouse. · · · ·, the lethal levels of metastatic cells in these organs. *B*, BALB/c (▲), BALB/c.IFNγ$^{-/-}$ (■), carrageenan-treated BALB/c (●), and carrageenan-treated BALB/c.IFNγ$^{-/-}$ (◆) mice were inoculated s.c. in the abdominal mammary gland with 7×10^3 4T1 tumor cells on day 1. Primary tumors were surgically removed on days 21–24, and the mice were followed for survival time.

carrageenan-treated BALB/c mice is similar to that found in BALB/c.IFN$^{-/-}$ mice (see Fig. 1*B*).

To determine whether the increase in metastatic cells in carrageenan-treated mice results in decreased survival, BALB/c and carrageenan-treated BALB/c mice were injected with 7×10^3 4T1 cells in the abdominal mammary gland, their primary tumors removed on days 21–24, and the mice followed for survival. A group of carrageenan-treated and a group of non-carrageenan-treated BALB/c.IFNγ$^{-/-}$ mice were also included to compare the effects of carrageenan treatment *versus* IFN-γ-deficiency. As shown in Fig. 4*B*, carrageenan-treated BALB/c mice died within 37 (±3) days, which is identical to the survival time of 4T1-challenged BALB/c.IFNγ$^{-/-}$ mice and significantly shorter than the survival time of wild-type BALB/c mice ($P = 0.005$). In contrast, the survival time of carrageenan-treated BALB/c.IFNγ$^{-/-}$ mice was the same as the survival time of carrageenan-treated BALB/c and BALB/c.IFNγ$^{-/-}$ mice. Therefore, the depletion of host phagocytic cells by carrageenan treatment resulted in accelerated tumor growth and decreased survival, which mimics the tumor-growth kinetics seen in BALB/c.IFN$^{-/-}$γ mice. Because the carrageenan-treatment and the IFN-γ-depletion effect were not additive, it is likely that these agents mediate their effects via a common mechanism.

Table 3 *Depletion of phagocytic cells by carrageenan treatment increases the number of metastatic cells in the lungs*

BALB/c, BALB/c.Pfp$^{-/-}$, anti-asialo-GM1-treated, CD4-depleted, CD8-depleted, or carrageenan-treated BALB/c mice were inoculated s.c. in the abdominal mammary gland with 7×10^3 4T1 cells. Mice were killed on day 35, and the numbers of metastatic cells in the lungs and liver were determined using the clonogenic assay.

Mice	Mice with >10,000 metastatic cells in the lungs	Mice with >100 metastatic cells in the liver
BALB/c	33% (9/27)	59% (16/27)
BALB/c.Pfp$^{-/-}$	63% (5/8)	88% (7/8)[a]
anti-asialo-GM1	63% (5/8)	88% (7/8)
CD4-depleted	56% (5/9)	ND[b]
CD8-depleted	40% (4/10)	ND
Carrageenan	80% (8/10)[a]	88% (7/8)

[a] Statistically significantly different from those of wild-type, untreated BALB/c mice ($P < 0.018$).
[b] ND, not determined.

DISCUSSION

Multiple strategies have been used to evaluate the role of IFN-γ in tumor immunity, *e.g.*, (*a*) tumor cells have been cultured with IFN-γ *in vitro* (3, 5, 7, 17, 23); (*b*) IFN-γ has been administered systemically to mice and patients (24–26); (*c*) tumor cell lines have been transfected with the genes encoding IFN-γ or IFN-γR (5, 7, 19, 20, 23); (*d*) antibodies to IFN-γ have been administered systemically to mice (23); and (*e*) knockout mice deficient for IFN-γ, IFN-γR, and IFN-γ-dependent transcription factors (STAT-1) have been challenged with transplantable syngeneic tumors (6–8, 27). Most of these studies have been conducted using primary solid-tumor models, have only examined a few aspects of immunity (*e.g.*, T cells or NK cells or angiogenesis), and/or have not discriminated as to whether IFN-γ is acting on host cells or directly on tumor cells. As a result, it is difficult to compare results from the various studies and to identify the relevant effector mechanisms that mediate the IFN-γ effect. The shortage of experimental studies in metastatic tumor models means that even less is understood about the role of IFN-γ in metastatic disease. In the present studies, we have used a realistic metastatic mammary carcinoma model in which the primary tumor is surgically removed to model the human disease situation, and have comprehensively examined multiple avenues of IFN-γ activity.

The significant reduction in the survival time and the increase in number of metastatic tumor cells in BALB/c.IFN$\gamma^{-/-}$ mice relative to BALB/c mice demonstrate that IFN-γ plays an important innate role in regulating mammary carcinoma metastasis. It is not surprising that primary 4T1 tumor growth does not differ between BALB/c.IFN$\gamma^{-/-}$ and BALB/c mice, because earlier studies with the 4T1 tumor in an immunotherapy setting demonstrated that primary and metastatic tumor cells frequently respond differently to immune effectors (13, 17).

It is not known whether IFN-γ regulates tumor growth by acting directly on tumor cells or indirectly by modulating host cells and/or factors. Several mechanisms have been proposed by which IFN-γ directly reduces tumor cell growth: (*a*) tumor cells treated *in vitro* with IFN-γ have increased tryptophan metabolism, which leads to tumor cell starvation (3); (*b*) tumor cells treated with IFN-γ are induced to express chemokines that inhibit angiogenesis, thereby depriving the growing tumor of the requisite vasculature (5, 7); (*c*) IFN-γ-treated tumor cells have up-regulated levels of MHC class I and/or class II molecules, making them better targets for CD4$^+$ and CD8$^+$ T cells (23). These proposed mechanisms are not strongly supported by studies in the literature. Although demonstrated *in vitro*, tryptophan starvation has not been found *in vivo*. The results of several studies are consistent with the hypothesis that the production of antiangiogenic factors by tumor cells limits tumor growth (6, 7). However, none of these studies show decreased tumor rejection by blocking antiangiogenesis *in vivo*. Although increased MHC class I and II expression should lead to increased sensitivity to T cells (23), it is unlikely that this mechanism is responsible for the IFN-γ effect, because primary tumor rejection of many tumors is mediated by nonspecific effectors and not by T cells (19, 20). Although these studies suggest that tumor cells are not the immediate target for IFN-γ, they are indirect, and more definitive experiments are necessary. Our findings that 4T1/IRt cells have the same *in vivo* growth kinetics as wild-type 4T1 cells demonstrate that direct interactions of IFN-γ with tumor cells are not responsible for decreased metastatic disease. Therefore, direct action of IFN-γ on tumor cells is probably not involved in the IFN-γ effect on the 4T1 mammary carcinoma.

It is more likely that IFN-γ mediates its tumor effect by acting on host cells that secondarily produce factors that diminish tumor growth. CD119 is expressed on many cells (28), and IFN-γ is known to up-regulate the transcription of hundreds of genes (1), thereby producing multiple host-derived effector cells. Different studies support a role for IFN-γ-activated NK cells (8), CD4$^+$ and/or CD8$^+$ T cells (6, 7), B cells (9), macrophages (26), and/or non-hematopoietic-derived cells (6, 7). With the exception of IFN-γ-activated NK cells (8), a role for more than one cell type was implicated in each of these studies.

In contrast, the observation reported here that the deletion of phagocytic cells gives a tumor phenotype completely overlapping with IFN-γ-deficient mice strongly suggests that phagocytic cells are a central cell population for IFN-γ-mediated innate immunity. Because mice doubly depleted for phagocytic cells and IFN-γ (carrageenan-treated BALB/c.IFN$\gamma^{-/-}$ mice) have the same tumor phenotype as singly depleted mice, IFN-γ and phagocytic cells do not appear to act additively, which suggests that they control tumor growth via the same pathway. Therefore, phagocytic cells may be the critical cell population through which IFN-γ mediates its effects.

Previous studies have identified NK and/or NKT cells (8, 29) and perforin-mediated cytotoxicity (16) as critical components of innate immunity against metastatic tumor. In contrast, the present study did not find any effect of NK cells, and perforin-mediated mechanisms were only marginally involved. Differences in the present studies and previous studies may be the result of several factors: (*a*) previous studies used i.v.-induced experimental metastases (8). The present study uses spontaneous metastases. There may be significant physiological differences between metastases that are established as the result of spontaneous disease *versus* experimental metastases, and these differences may dictate the type of effector mechanisms to which the metastatic cells are susceptible; (*b*) in the present report, primary tumor was surgically removed, and spontaneous metastatic disease was subsequently assessed. In contrast, earlier studies were performed with primary tumor *in situ*, although one study used a postsurgery model followed by i.v.-induced metastases (8). Surgical removal of primary tumor may create a novel *in vivo* environment that is not present when the primary tumor remains in place. For example, large 4T1 primary tumor burdens are systemically immunosuppressive.[4] This immunosuppression may interfere with or block some effector mechanisms, but not others; (*c*) the 4T1 line used in the present report expresses high levels of MHC class I molecules (see Fig. 2*A*). This high level expression may render 4T1 resistant to NK cell lysis, and, hence, the deletion of NK cells does not greatly impact 4T1 growth. Indeed, 4T1 is not killed by NK cells in *in vitro* NK cell assays (14); and (*d*) earlier reports did not assess the potential involvement of macrophages, and additional experiments with these other tumor systems are needed to clarify the role of host phagocytic cells.

Carrageenan deletes/inactivates phagocytic cells such as macrophages, immature DCs, and neutrophils. A major role of DCs is to phagocytose antigen for presentation to T cells. The finding that T cells are largely unimportant in the IFN-γ effect implies that DCs are also not involved. Additional experiments assessing antigen presentation activity of DCs derived from BALB/c.IFN$\gamma^{-/-}$ *versus* BALB/c mice showed no diminished activity,[5] further indicating that DCs are not a key player.

Neutrophils and macrophages are also active phagocytic cells. Although neutrophils are key cells for Fas/FasL-mediated tumor rejection (30, 31), they principally ingest bacteria. Activated macrophages directly kill tumor cells by releasing incompletely reduced oxygen intermediates, such as hydrogen peroxide and nitric oxide, which are directly toxic to target cells (1). At least two observations support the hypothesis that IFN-γ mediates its antitumor effects via phagocytic cells releasing hydrogen peroxide and nitric oxide: (*a*) mice with iNOS-targeted mutations show reduced inflammatory responses to carrageenan and are resistant to LPS-induced mortality (1). Similarly, carrageenan-treated BALB/c mice have reduced LPS-induced mortality.[6] Therefore, carrageenan treatment and iNOS deficiency both result in macrophage dysfunction, which supports the idea that iNOS production and phagocytic cells are linked; (*b*) IL-13 suppresses innate immunity (32) and antagonizes IFN-γ-mediated induction of iNOS (1). These observations tie together IFN-γ, IL-13, iNOS production, and carrageenan-treatment, and suggest the following model for the role of IFN-γ in innate immunity to metastatic tumor. In the presence of IFN-γ, macrophages are activated to make iNOS and H_2O_2 that directly kill tumor cells. In carrageenan-treated mice, macrophages are eliminated therefore, iNOS is not produced, and metastatic tumor cells proliferate. In addition to activating macrophages, IFN-γ also activates NKT cells. Either activated NKT cells activate antigen-presenting cell to produce IL-12, which feeds back to induce more IFN-γ, or they produce IL-13, which blocks iNOS production. Therefore, up-regulation of IL-13 and/or treatment with carrageenan prevents iNOS production and limits macrophage-induced control of metastatic disease.

---

[4] E. Danna, M. Gilbert, B. Pulaski, and S. Ostrand-Rosenberg, unpublished observations.

[5] B. Pulaski and S. Ostrand-Rosenberg, unpublished results.

[6] B. Pulaski, E. Danna, and S. Ostrand-Rosenberg, unpublished results.

Although innate immunity triggered by IFN-γ succeeds in limiting tumor growth, it is not sufficient to mediate complete tumor destruction. Hence, immunocompetent mice display delayed metastatic disease and longer survival times relative to IFN-γ-deficient mice, but they still die from metastases. As with most immune responses, the optimal situation would be the development of an adaptive immune response against tumor that would take over when the innate response was no longer capable of curtailing tumor progression.

The immunosurveillance hypothesis states that the immune system destroys or inactivates newly transformed cells, thereby preventing the outgrowth of malignant tumors. This concept has been controversial; however, it has recently regained support through experiments with T-cell- deficient and knockout mice (27, 33). If immunosurveillance occurs, components of the innate immune response are likely to be involved. Although IFN-γ may not be exclusively responsible for tumor immunosurveillance, it is a strong candidate as a component of innate immunity that contributes to protection against the proliferation of transformed cells.

ACKNOWLEDGMENTS

We thank Tim Ford and Fran Baldwin for their graphic arts expertise and Sandy Mason for the excellent care given to our animals. The BALB/c.Pfp$^{-/-}$ mice were generously supplied by Dr. R. H. Wiltrout, NCI Frederick Cancer Research and Development Center (Frederick, MD) and the pEF2.mugR plasmid by Dr. Y. Patterson, University of Pennsylvania (Philadelphia, PA).

REFERENCES

1. Shtrichman, R., and Samuel, C. E. The role of γ interferon in antimicrobial immunity. Curr. Opin. Microbiol., *4:* 251–259, 2001.
2. Boehm, U., Klamp, T., Groot, M., and Howard, J. C. Cellular responses to interferon-γ. Ann. Rev. Immunol., *15:* 749–795, 1997.
3. Taylor, M. W., and Feng, G. S. Relationship between interferon-γ, indoleamine 2, 3-dioxygenase, and tryptophan catabolism. FASEB J., *5:* 2516–2522, 1991.
4. Gollob, J. A., Mier, J. W., Veenstra, K., McDermott, D. F., Clancy, D., Clancy, M., and Atkins, M. B. Phase I trial of twice-weekly intravenous interleukin 12 in patients with metastatic renal cell cancer or malignant melanoma: ability to maintain IFN-γ induction is associated with clinical response. Clin. Cancer Res., *6:* 1678–1692, 2000.
5. Coughlin, C., Salhany, K., Gee, M., LaTemple, C., Kotenko, S., Ma, X., Gri, G., Wysocka, M., Kim, J., Liu, L., Liao, F., Farber, J., Pestka, S., Trinchieri, G., and Lee, W. Tumor cell responses to IFN-γ affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. Immunity, *9:* 25–34, 1998.
6. Wigginton, J. M., Gruys, E., Geiselhart, L., Subleski, J., Komschlies, K. L., Park, J-W., Wiltrout, T., Nagashima, K., Back, T. C., and Wiltrout, R. H. IFNγ and Fas/FasL are required for the antitumor and antiangiogenic effects of IL-12/pulse IL-2 therapy. J. Clin. Investig., *108:* 51–62, 2001.
7. Qin, Z., and Blankenstein, T. CD4+ T-cell mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFNγ receptor expression by nonhematopoietic cells. Immunity, *12:* 677–686, 2000.
8. Smyth, M. J., Cretney, E., Takeda, K., Wiltrout, R. H., Sedger, L. M., Kayagaki, N., Yagita, H., and Okumura, K. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) contributes to interferon γ-dependent natural killer cell protection from tumor metastasis. J. Exp. Med., *193:* 661–670, 2001.
9. Nanni, P., Nicoletti, G., DeGiovanni, C., Landuzzi, L., DiCarlo, E., Cavallo, F., Pupa, S. M., Rossi, I., Colombo, M. P., Ricci, C., Astolfi, A., Musiani, P., Forni, G., and Lollini, P-L. Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER-2-neu transgenic mice. J. Exp. Med., *194:* 1–12, 2001.
10. Gingrich, J. R., Barrios, R. J., Morton, R. A., Boyce, B. F., DeMayo, F. J., Finegold, M. J., Angelopoulou, R., Rosen, J. M., and Greenberg, N. M. Metastatic prostate cancer in transgenic mouse. Cancer Res., *56:* 4096–4102, 1996.
11. Aslakson, C., and Miller, F. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res., *52:* 1399–1405, 1992.
12. Pulaski, B., and Ostrand-Rosenberg, S. MHC class II and B7.1 immunotherapeutic cell-based vaccine reduces spontaneous mammary carcinoma metastases without affecting primary tumor growth. Cancer Res., *58:* 1486–1493, 1998.
13. Pulaski, B., Terman, D., Khan, S., Muller, E., and Ostrand-Rosenberg, S. Cooperativity of *Staphylococcal aureus* enterotoxin B superantigen, major histocompatibility complex class II, and CD80 for immunotherapy of advanced spontaneous metastases in a clinically relevant postoperative breast cancer model. Cancer Res., *60:* 2710–2715, 2000.
14. Ostrand-Rosenberg, S., Grusby, M., and Clements, V. Cutting Edge: Stat6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. J. Immunol., *165:* 6015–6019, 2000.
15. Lelekakis, M., Moseley, J., Martin, T., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F., Slavin, J., and Anderson, R. A novel orthotopic model of breast cancer metastasis to bone. Clin. Exp. Metastasis, *17:* 163–170, 1999.
16. Smyth, M., Thia, K., Cretney, E., Kelly, J., Snook, M., Forbes, C., and Scalzo, A. Perforin is a major contributor to NK cell control of tumor metastasis. J. Immunol., *162:* 6658–6662, 1999.
17. Pulaski, B., Clements, V., Pipeling, M., and Ostrand-Rosenberg, S. Immunotherapy with vaccines combining MHC class II/CD80+ tumor cells with IL-12 reduces established metastatic disease and stimulates immune effectors and monokine-induced by interferon-γ. Cancer Immunol. Immunother., *49:* 34–45, 2000.
18. Debrick, J. E., Campbell, P. A., and Staerz, U. D. Macrophages as accessory cells for class I MHC-restricted immune responses. J. Immunol., *147:* 2846–2851, 1991.
19. Esumi, N., Hunt, B., Itaya, T., and Frost, P. Reduced tumorigenicity of murine tumor cells secreting γ-interferon is due to non-specific host responses and is unrelated to class I major histocompatibility complex expression. Cancer Res., *51:* 1185–1189, 1991.
20. McAdam, A. J., Pulaski, B. A., Storozynsky, E., Yeh, K. Y., Sickel, J. Z., Frelinger, J. G., and Lord, E. M. Analysis of the effects of cytokines (interleukins 2, 3, 4, and 6, granulocyte-monocyte colony-stimulating factor, and interferon-γ) on generation of primary cytotoxic T lymphocytes against a weekly immunogenic tumor. Cell Immunol., *165:* 183–192, 1995.
21. Jackson, P. G., and Evans, S. R. Intraperitoneal macrophages and tumor immunity: a review. J. Surg. Oncol. 146–154, 2000.
22. Mellman, I., and Steinman, R. M. Dendritic cells: specialized and regulated antigen processing machines. Cell, *106:* 259–262, 2001.
23. Dighe, A. S., Richards, E., Old, L. J., and Schreiber, R. D. Enhanced *in vivo* growth and resistance to rejection of tumor cells expressing dominant negative IFN γ receptors. Immunity, *1:* 447–456, 1994.
24. Haase, K. D., Lange, O. F., and Scheef, W. Interferon-γ treatment of metastasized malignant melanoma. Anticancer Res. 335–336, 1987.
25. D'Acquisto, R., Markman, M., Hakes, T., Rubin, S., Hoskins, W., and Lewis, J. L. A Phase I trial of intraperitoneal recombinant γ-interferon in advanced ovarian carcinoma. J. Clin. Oncol., *6:* 689–695, 1988.
26. Fidler, I. J., Fan, D., and Ichinose, Y. I. M. Potent *in situ* activation of murine lung macrophages and therapy of melanoma metastases by systemic administration of liposomes containing muramyltripeptide phosphatidylethanolamine and interferon γ. Invasion Metastasis, *9:* 75–88, 1989.
27. Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., and Schreiber, R. D. N. IFNγ and lymphocytes prevent primary tumour development and shape tumour immunity. Nature (Lond.), *410:* 1107–1111, 2001.
28. Farrar, M. A., and Schreiber, R. D. The molecular biology of interferon-γ and its receptor. Ann. Rev. Immunol., *11:* 571–611, 1993.
29. Smyth, M., Thia, Y., Street, S., Cretney, E., Trapani, J., Taniguchi, M., Kawano, T., Pelikan, S., Crowe, N., and Godfrey, D. Differential tumor surveillance by natural killer (NK) and NKT cells. J. Exp. Med., *191:* 661–668, 2000.
30. Arai, H., Gordon, D., Nabel, E. G., and Nabel, G. J. Gene transfer of Fas ligand induces tumor regression *in vivo*. Proc. Natl. Acad. Sci. USA, *94:* 13862–7, 1997.
31. Seino, K., Kayagaki, N., Okumura, K., and Yagita, H. Antitumor effect of locally produced CD95 ligand. Nat. Med., *3:* 165–170, 1997.
32. Terabe, M., Matsui, S., Noben-Trauth, N., Chen, H., Watson, C., Donaldson, D., Carbone, D., Paul, W., and Berzofsky, J. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat. Immunol., *1:* 515–520, 2000.
33. Smyth, M., Thia, K., Street, S., MacGregor, D., Godfrey, D., and Trapani, J. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J. Exp. Med., *192:* 755–760, 2000.

Resistance to Metastatic Disease in STAT6-Deficient Mice Requires Hemopoietic and Nonhemopoietic Cells and Is IFN-γ Dependent[1]

Suzanne Ostrand-Rosenberg,[2]* Virginia K. Clements,* Masaki Terabe,† Jong Myun Park,† Jay A. Berzofsky,† and Samudra K. Dissanayake*

Mice deficient for the STAT6 gene (STAT6$^{-/-}$ mice) have enhanced immunosurveillance against primary and metastatic tumors. Because STAT6 is a downstream effector of the IL-4R, and IL-13 binds to the type 2 IL-4R, IL-13 has been proposed as an inhibitor that blocks differentiation of tumor-specific CD8$^+$ T cells. Immunity in STAT6$^{-/-}$ mice is unusually effective in that 45–80% of STAT6$^{-/-}$ mice with established, spontaneous metastatic 4T1 mammary carcinoma, whose primary tumors are surgically excised, survive indefinitely, as compared with <10% of STAT$^{+/+}$ (BALB/c) mice. Surprisingly, STAT6$^{-/-}$ and BALB/c reciprocal bone marrow chimeras do not have increased immunosurveillance, demonstrating that immunity requires STAT6$^{-/-}$ hemopoietic and nonhemopoietic components. Likewise, CD1$^{-/-}$ mice that are NKT deficient and therefore IL-13 deficient also have heightened tumor immunity. However, STAT6$^{-/-}$ and CD1$^{-/-}$ reciprocal bone marrow chimeras do not have increased survival, suggesting that immunity in STAT6$^{-/-}$ and CD1$^{-/-}$ mice is via noncomplementing mechanisms. Metastatic disease is not reduced in BALB/c mice treated with an IL-13 inhibitor, indicating that IL-13 alone is insufficient for negative regulation of 4T1 immunity. Likewise, in vivo depletion of CD4$^+$CD25$^+$ T cells in BALB/c mice does not increase survival, demonstrating that CD4$^+$CD25$^+$ cells do not regulate immunity. Cytokine production and tumor challenges into STAT6$^{-/-}$ IFN-$\gamma^{-/-}$ mice indicate that IFN-γ is essential for immunity. Therefore, immunosurveillance in STAT6$^{-/-}$ mice facilitates survival against metastatic cancer via an IFN-γ-dependent mechanism involving hemopoietic and nonhemopoietic derived cells, and is not exclusively dependent on counteracting IL-13 or CD4$^+$CD25$^+$ T cells. *The Journal of Immunology,* **2002, 169: 5796–5804.**

Mice with a deleted STAT6 gene (STAT6$^{-/-}$ mice) have heightened immunity to syngeneic tumors. They are resistant to recurrence of a primary fibrosarcoma (1), reject a transplanted mastocytoma (2), and have significantly reduced metastatic disease following challenge with a spontaneously metastatic mammary carcinoma (3). STAT6$^{-/-}$ mice are deficient in responsiveness to IL-4 and IL-13, two cytokines essential for development of CD4$^+$ Th2 cells, and hence preferentially produce CD4$^+$ Th1 cells (4–6). It has been hypothesized that the development of Th1 cells optimizes CD8-mediated tumor immunity (7), and hence STAT6$^{-/-}$ mice were thought to be more tumor resistant because they predominantly make Th1 cells. In vivo depletion studies in the mammary carcinoma system, however, contradicted this hypothesis and demonstrated that although CD8$^+$ T cells are essential for tumor rejection by STAT6$^{-/-}$ mice, CD4$^+$ T cells are not involved (3). In contrast, depletion of CD4$^+$ cells increases immunity in the fibrosarcoma system; however, additional studies suggest that the enhancement is due to depletion of regulatory CD4$^+$ NKT cells, rather than CD4$^+$ Th cells (1). Therefore, heightened tumor immunity in STAT6-deficient mice does not appear to be the result of the balance between CD4$^+$ Th1 and Th2 cells.

As an alternative explanation to Th1 vs Th2 CD4$^+$ helper cell ratio, it has been proposed that STAT6-deficient mice have enhanced immunity because they lack an inhibitor that blocks the development of tumor-reactive CD8$^+$ T cells (1, 3). In the primary fibrosarcoma system, IL-13 produced by CD4$^+$ NKT cells has been hypothesized as the inhibitor (1). Three lines of evidence support a role for IL-13: 1) IL-4R$\alpha^{-/-}$ and STAT6$^{-/-}$, but not IL-4$^{-/-}$ mice show the enhanced immunity, and IL-13 is the only cytokine other than IL-4 known to use the IL-4Rα-chain and STAT6. 2) IL-13 is produced by NKT cells, and NKT cell-deficient CD1 knockout mice have enhanced immunity to primary, fibrosarcoma tumors. 3) Blockading of IL-13 with an IL-13 inhibitor results in heightened immunity to primary fibrosarcoma tumor. Because STAT6 is a downstream effector of the IL-4R and IL-13 binds to the type 2 IL-4R, deletion of STAT6 may be functionally equivalent to deletion of IL-13 and NKT cells, and therefore enhanced immunity to primary tumor in both CD1$^{-/-}$ and STAT6$^{-/-}$ mice may be the result of elimination of IL-13 (1). The cellular target for IL-13 is unclear. Although CD8$^+$ T cells are the antitumor effectors, they do not have receptors for IL-13. Therefore, IL-13 may act on an intermediate cell through a downstream STAT6-dependent pathway, and subsequently modulate CD8$^+$ T cell activation (1).

Another potential inhibitor of tumor immunity is the CD4$^+$CD25$^+$ T cell. The presence of these cells inhibits the differentiation of cytotoxic CD8$^+$ T cells against tumors (8), and their absence facilitates the development of autoimmunity (9–11).

Although STAT6-deficient mice have enhanced resistance to three independent mouse tumors, it is not clear whether the same

*Department of Biological Sciences, University of Maryland, Baltimore, MD 21250; and †Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Received for publication November 12, 2001. Accepted for publication September 11, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These studies were supported by grants from the U.S. Army Breast Cancer Research Program (DAMD17-01-1-0312) and National Institutes of Health (R01CA52527 and R01CA84232).

[2] Address correspondence and reprint requests to Dr. Suzanne Ostrand-Rosenberg, Department of Biological Sciences, University of Maryland, 1000 Hilltop Circle, Baltimore, MD 21250. E-mail address: srosenbe@umbc.edu

Copyright © 2002 by The American Association of Immunologists, Inc.

0022-1767/02/$02.00

mechanisms mediate heightened immunity to the fibrosarcoma, mastocytoma, and mammary carcinoma, or whether enhanced immunity to metastatic tumor occurs via the same mechanism as enhanced immunity to primary tumor. To address these questions, we have used the BALB/c-derived 4T1 mouse mammary carcinoma and assessed both primary tumor growth and metastastic disease in STAT6$^{-/-}$- and NKT-deficient CD1$^{-/-}$ mice. The 4T1 mouse tumor closely parallels human breast cancer in its growth kinetics, pathology, invasiveness, poorly immunogenic phenotype, and pattern of spontaneous metastasis to multiple distant organs (12, 13). Inoculation of small numbers of 4T1 tumor cells into the mammary gland of syngeneic BALB/c mice causes lethality due to lung, liver, bone marrow, and/or brain metastasis within 6–8 wk of inoculation. The 4T1 tumor also closely parallels human breast cancer in that progression of metastatic disease is not affected by surgical removal of primary tumor, so that mice whose primary tumors are removed after metastatic disease is established also die within 6–8 wk of initial tumor inoculation (13). Because 4T1 tumor cells are resistant to 6-thioguanine, the number of metastatic cells in distant organs can be quantified using a clonogenic assay (12, 14, 15). Metastatic disease following surgical removal of primary tumor is the principal cause of death in patients with solid tumors (16). Therefore, the 4T1 tumor also allows us to assess tumor immunity in an animal model that closely parallels human cancer, by assessing survival of mice whose primary tumors are surgically removed.

Our studies show that tumor immunity in STAT6$^{-/-}$ mice is unusually effective in that 45–80% of STAT6$^{-/-}$ mice with established, spontaneous metastatic disease, whose primary tumors have been surgically excised, survive indefinitely, as compared with <10% of wild-type BALB/c mice. Surprisingly, experiments with STAT6$^{-/-}$ and wild-type BALB/c reciprocal bone marrow chimeric mice and autologous bone marrow chimeras indicate that enhanced immunity requires STAT6$^{-/-}$ hemopoietic and nonhemopoietic derived components. Similar experiments with CD1$^{-/-}$ mice confirm earlier results that CD1$^{-/-}$ mice have heightened tumor immunity to primary tumors and demonstrate that they also are highly resistant to metastatic disease. However, STAT6$^{-/-}$ and CD1$^{-/-}$ reciprocal bone marrow chimeras do not have increased tumor immunity, suggesting that heightened immunity in STAT6$^{-/-}$ and CD1$^{-/-}$ mice is achieved via different mechanisms or different steps in a regulatory pathway. Studies aimed at clarifying the role of IL-13 in tumor immunity demonstrate that neither 4T1 primary tumor growth nor metastatic disease is reduced in mice treated with an IL-13 inhibitor, in contrast to the results with the fibrosarcoma primary tumor. Therefore, heightened tumor immunity in STAT6-deficient and CD1-deficient mice confers a distinct survival advantage on mice with established metastatic mammary cancer; however, the underlying mechanism of enhanced immunity differs from the mechanism responsible for increased immunity to primary fibrosarcoma tumor and is not solely dependent on elimination of IL-13 or CD4^{+}CD25^{+} T regulatory cells.

Materials and Methods

Mice, cells, and tumor inoculations

BALB/c, BALB/c STAT6$^{-/-}$ (henceforth called STAT6$^{-/-}$), and BALB/c CD1$^{-/-}$ (henceforth called CD1$^{-/-}$) mice were bred in the University of Maryland Biology Department animal facility from breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME), M. Grusby (Dana-Farber Cancer Institute, Boston, MA), and L. van Kaer (Washington University, St. Louis, MO), respectively. BALB/c IL-4Rα-deficient (henceforth called IL-4Rα$^{-/-}$) mice were purchased from The Jackson Laboratory. STAT6$^{-/-}$ and CD1$^{-/-}$ mice were originally made by targeted disruption of the STAT6 and CD1 genes, respectively, in 129 ES cells. Offspring were backcrossed to BALB/c mice (10 backcross generations for STAT6$^{-/-}$; 9 backcross generations for CD1$^{-/-}$) (17–19). Female mice of 8–16 wk were used for all studies.

STAT6$^{-/-}$IFN-γ$^{-/-}$ mice were generated by crossing STAT6$^{-/-}$ mice with IFN-γ$^{-/-}$ BALB/c mice. Heterozygous offspring were intercrossed, and the F_2 were screened by PCR for homozygosity for STAT6$^{-/-}$ and IFN-γ$^{-/-}$. Ear punch tissue of individual mice was placed in a 0.5-ml microfuge tube, and 20 μl of 50 mM Tris-HCl (pH 8.0), 2 mM NaCl, 10 mM EDTA, 1% SDS, and 1 μl of 20 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) were added. Tubes were incubated at 55°C for 20 min, and after vigorous vortexing for 2 min, the mixture was incubated for an additional 20 min at 55°C. Sterile distilled water was then added to each tube to a final total volume of 200 μl, and the tubes were heated at 100°C for 5 min. The following primers were used: STAT6, 5′ primer, TGAGGTGGGGACCAGCCGG; STAT6, 3′ primer, GTGACCAGGACAC ACAGCGG; Neo-STAT6, primer, GCTACCCGTGATATTGCTGAAGAG; IFN-γ, 5′ primer, AGAAGTAAGTGGAAGGGCCCAGAAG; IFN-γ, 3′ primer, AGGGAAACTGGGAGAGGAGAAATAT; IFN-γ Neo, 5′ primer, TCAGCGCAGGGGCGCCCGGTTCTTT; IFN-γ Neo, 3′ primer, ATCGA CAAGACCGGCTTCCATCCGA. PCR conditions for both IFN-γ and STAT6 genes were: denature at 94°C for 5 min, denature at 94°C for 1 min, anneal at 59°C for 1 min, extend at 72°C for 1.3 min, repeat the last three steps 34 times, extend at 72° for 9 min. PCR products were electrophoresed on 2% agarose gels. The wild-type STAT6 and STAT6-deletion genes produce 100- and 250-kb bands, respectively (17). The wild-type IFN-γ and IFN-γ-deletion genes produce 220- and 375-bp bands, respectively (www.jax.org/resources/documents/imr/protocols/Ifng_KO.html).

The 4T1 mammary carcinoma cells were maintained in culture and inoculated into the abdominal mammary gland, and mice were followed for survival, as described (12, 13). Primary tumors were measured using an electronic calipers. Reported measurements are the square root of the product of two perpendicular diameters. Numbers of metastatic cells in lung, liver, bone marrow, and brain were determined using the clonogenic metastasis assay in which dissociated organ cells were cultured in medium supplemented with 6-thioguanine (12–14).

Surgical removal of primary mammary tumors

BALB/c, STAT6$^{-/-}$, CD1$^{-/-}$, and chimeric mice were inoculated in the abdominal gland with 7000 4T1 cells, and primary, solid tumors were surgically removed, as described (13, 20), with the following modifications: primary tumors were removed 16–21 days after 4T1 inoculation when they were between 2 and 9 mm in diameter and well vascularized. More than 95% of mice survived surgery. Postsurgery mice were followed for metastasis development and/or survival. Mice in which primary tumors recurred at the site of the original tumor inoculation were omitted from the study. These mice were less than 5% of operated mice.

Bone marrow chimeras

STAT6$^{-/-}$ mice containing BALB/c bone marrow (BALB/c→STAT6$^{-/-}$), BALB/c mice containing STAT6$^{-/-}$ bone marrow (STAT6$^{-/-}$→BALB/c), STAT6$^{-/-}$ mice containing CD1$^{-/-}$ bone marrow (CD1$^{-/-}$→STAT6$^{-/-}$), and CD1$^{-/-}$ mice containing STAT6$^{-/-}$ bone marrow (STAT6$^{-/-}$→CD1$^{-/-}$) were constructed as follows using aseptic conditions. Donor mice were asphyxiated with CO_2 and immersed in 70% ethanol, and their hind legs were removed at the hip. Femurs were dissected away from the surrounding tissue, their ends were cut off, and the remaining bone was flushed three times with sterile PBS using a 30-ml syringe fitted with a 27-gauge needle. Bone marrow cells were collected in petri dishes and transferred to 15-ml conical tubes, and the aggregated material was allowed to gravity settle and was discarded. The remaining bone marrow cells were washed twice with PBS and resuspended in medium (RPMI, 1% penicillin, 1% streptomycin, 1% fungizone) at 200 μl per donor mouse. Recipient mice were taken off food the evening before bone marrow transfer. Between 0 and 2 h before bone marrow reconstitution, recipient mice were lethally irradiated (8.75 Gy, Cs-137 source, Gammator B; Kewaunee Scientific, Statesville, NC). Bone marrow was inoculated into recipient mice through the tail vein using a 1-ml syringe fitted with a 27-gauge needle. Each recipient received 100 μl of donor cells (bone marrow from one donor femur). Reconstituted mice received daily injections of gentamicin sulfate i.p. (100 μl of 5 mg/ml) for 7 days beginning 1 day before bone marrow reconstitution. Reconstituted mice were maintained on 2% tetracycline water starting 1–2 wk before bone marrow transfer and continuing for 6–8 wk after reconstitution. Eight to 12 wk after bone marrow reconstitution, chimeras were bled from the tail vein, and the blood was tested by PCR to ascertain hemopoietic genotype and reconstitution.

All animal procedures have been reviewed and approved by the University of Maryland or National Cancer Institute Institutional Animal Care

and Use Committee, and comply with National Institutes of Health guidelines for the humane treatment of laboratory animals.

Treatment with soluble IL-13Rα2-Fc

BALB/c mice were inoculated with 7000 4T1 cells in the abdominal mammary gland on day 0 and given soluble IL-13Rα2-Fc (sIL-13Rα2-Fc)[3] (0.2 mg/200 μl/dose) every other day from day 0 to 14. Control mice were treated with human IgG having the same Fc as the Fc of the IL-13 inhibitor.

CTL assays

BALB/c, STAT6$^{-/-}$, and bone marrow chimeric mice were immunized with 50 Gy-irradiated 4T1 cells (1×10^6 cells i.p.) once every ~14 days for three to five immunizations. Splenocytes of immunized mice were harvested 5 days after the last immunization and used as effector cells in overnight (~16-h) assays. CTL assays were performed as described (3). Percent specific activity is the percentage of cytotoxicity against 4T1 targets minus percentage of cytotoxicity against B16 targets.

Flow cytometry

Mouse splenocytes were characterized by flow cytometry using the following mAbs: CD3 FITC, CD4 PE, B220 PE, Mac-1 FITC (Caltag, Burlingame, CA), as described (3).

CD4$^+$CD25$^+$ T cell depletions

The hybridoma PC61 secreting anti-CD25 mAb (IL-2Rα-specific, rat IgG1) (21) was obtained from American Type Culture Collection (Manassas, VA) and was purified by protein G affinity column from culture supernatants, as previously described (12). For in vivo depletions, mice were given 800 μg in 100 μl PBS i.p., as described (8), on day −4 and were inoculated on day 0 with 7000 4T1 cells in the abdominal mammary gland. For experiments monitoring survival after surgery, mice were given 800 μg PC6-5.3 in 100 μl PBS i.p. on day −4 after inoculation of 7000 4T1 cells; primary tumor was surgically removed on day 20; and mice were followed for survival. Efficiency of mAb depletion of CD4$^+$CD25$^+$ T cells was ascertained by double-staining splenocytes of treated mice with directly coupled CD25-specific mAb (CD25 FITC; BD PharMingen, San Diego, CA) and CD4-specific mAb (GK1.5 PE; BD PharMingen) 3 days and 1 wk after inoculation of mAb PC6-5.3. Undepleted mice showed ~10% of the splenocytes as CD4$^+$CD25$^+$; depleted mice had 1–2% of their cells as CD4$^+$CD25$^+$.

Cytokine production

Naive or surgery-survivor mice were inoculated in an abdominal mammary gland and/or i.p. with 10^5 or 10^6 live or irradiated 4T1 cells. Draining lymph nodes or spleens were removed 5 days later, and 1×10^6 lymphocytes were cocultured with 3×10^5 80 Gy-irradiated 4T1/B7.1 or B16 melF10 stimulator cells in a total volume of 1 ml in 24-well plates (RPMI, 10% FCS, 5×10^{-5} M 2-ME, 1% penicillin, 1% streptomycin). Supernatants were harvested 48 h later and tested in triplicate by ELISA for IL-2, IL-4, and IFN-γ, according to the manufacturer's directions (Pierce/Endogen, Rockford, IL). Specific cytokine release was determined by subtracting nonspecific release (B16 melF10 stimulators) from 4T1-stimulated release.

Statistical analyses

Data were analyzed using unpaired Student's *t* tests (Microsoft Excell, Redmond, WA).

Results

Following surgical removal of primary tumor, STAT6$^{-/-}$ mice reject lung, liver, and bone marrow metastasis and survive

To assess the potency of the STAT6 antitumor effect against established metastatic tumor, BALB/c and STAT6$^{-/-}$ mice were inoculated with 4T1 cells, and followed for development of metastasis and survival after surgical removal of primary tumor. Groups of BALB/c and STAT6$^{-/-}$ mice were inoculated in the abdominal mammary gland with 7000 4T1 tumor cells, and the tumors were allowed to grow progressively. Although primary tumors in STAT6$^{-/-}$ mice grow more slowly than primary tumors in BALB/c mice (3), most tumors were 2–9 mm in diameter within 2–3 wk of 4T1 inoculation. Previous studies demonstrated that mice with primary tumors >2 mm in diameter have established metastatic disease (13). Surgical removal of primary tumors was completed over a 7-day period so that sizes of primary tumors between the BALB/c and STAT6$^{-/-}$ groups could be matched. Because metastasis is not reliably established when primary tumors are smaller than 2 mm in diameter (13), mice with tumors <2 mm in diameter were omitted from the experiment. Three to four weeks after surgery (42–45 days post-4T1 inoculation), mice were sacrificed, and the lungs, liver, and bone marrow were isolated. The number of metastatic cells in each organ was determined using the clonogenic assay (12).

As shown in Table I, ≥60% of postsurgery STAT6$^{-/-}$ mice (12 of 20) do not have detectable metastasis in the lungs, liver, or bone marrow, while only 7.5% of intact BALB/c mice (1 of 13) are free of metastasis. Those STAT6$^{-/-}$ mice that have organ metastases have fewer metastatic cells per organ than BALB/c mice (a maximum of 3383 metastatic cells/organ in STAT6$^{-/-}$ mice vs 2.8×10^5 in BALB/c mice). Therefore, the majority of STAT6$^{-/-}$ mice are resistant to the outgrowth and development of 4T1 metastasis if the primary mammary tumor is surgically removed.

To determine whether the reduced number of metastatic cells in STAT6$^{-/-}$ mice translates into increased survival time, additional groups of BALB/c and STAT6$^{-/-}$ mice were inoculated with 4T1 cells in the mammary gland, and primary tumors were surgically removed as per the experiment of Table I. The resulting mice were observed for survival. As shown in Fig. 1*A*, 67% of STAT6$^{-/-}$ mice (12 of 18) survived >175 days, while only 8.3% of BALB/c mice (1 of 12) survived. Many postsurgery STAT6$^{-/-}$ mice, therefore, appear to have completely rejected or inhibited the growth of their metastatic tumors.

In earlier studies, it was noted that mice with larger primary tumors at the time of surgery tended to have more metastatic cells in their lungs, livers, and brains. To test whether the resistance of STAT6$^{-/-}$ mice is limited by the size of the primary tumor, we examined survival time in days vs tumor diameter (TD) at the time of surgery. The data shown in Fig. 1*B* are the pooled results of three independent experiments in which BALB/c and STAT6$^{-/-}$ mice, respectively, were inoculated in the mammary gland with 7000 4T1 cells, and primary tumors were surgically removed at 2.5–3 wk. In this aggregate group, 63% (24 of 38) of STAT6$^{-/-}$ mice survived indefinitely (>150 days), vs only 6% (2 of 36) of BALB/c mice. Survival of STAT6$^{-/-}$ mice did not correlate with size of primary tumor at the time of surgery, indicating that resistance in STAT6$^{-/-}$ mice is independent of primary tumor size. A large percentage of STAT6$^{-/-}$ mice, therefore, is resistant to spontaneous metastatic mammary carcinoma if the primary tumor is removed, regardless of the size of the primary tumor, while most wild-type BALB/c mice are highly susceptible.

STAT6$^{-/-}$ mice that have survived an initial 4T1 challenge are immune to subsequent spontaneous metastatic disease

In earlier studies, CD8$^+$ T cells were shown to be required for limiting tumor growth in STAT6$^{-/-}$ mice, indicating that tumor resistance is immune mediated (3). In these earlier experiments, primary tumors remained in place, and all mice eventually died from tumor, even though metastatic disease developed more slowly in STAT6$^{-/-}$ mice than in BALB/c mice. However, many STAT6$^{-/-}$ mice with established metastasis survive indefinitely if their primary tumor is surgically removed (Fig. 1*A*). To determine whether these survivors have enhanced immunity and long-term memory, they were rechallenged with 4T1 tumor. STAT6$^{-/-}$ mice whose primary tumors were removed and who survived ≥185

[3] Abbreviations used in this paper: sIL-13Rα2-Fc, soluble IL-13Rα2-Fc; TD, tumor diameter.

Table I. *The majority of STAT6$^{-/-}$ mice are metastasis free after surgical removal of the primary mammary tumor*[a]

Organ	BALB/c[b]		STAT6$^{-/-}$[c]	
	Mice with metastasis (%)[d]	Metastatic cells/organ[d]	Mice with metastasis (%)[d]	Metastatic cells/organ[d]
Lung	92.5	$1228 - 2.8 \times 10^5$ ($6 \times 10^4 \pm 9.2 \times 10^4$)	40	5–3383[e] (1158 ± 1315)
Bone marrow	54	1–612 (94 ± 176)	0	0
Liver	92	$5 - 1.9 \times 10^4$ (3431 ± 6164)	15	50–530 (214 ± 186)

[a] BALB/c and STAT6$^{-/-}$ mice were inoculated with 7000 4T1 cells in the mammary gland. Two to 3 wk later, the primary tumors were surgically removed. On days 42–45 after initial 4T1 inoculation, mice were sacrificed, and the lungs, liver, and bone marrow were assayed by the clonogenic assay for the number of metastatic 4T1 cells. Average diameter of primary BALB/c vs STAT6$^{-/-}$ tumors at the time of surgery: 4.99 mm ± 1.42 vs 4.38 ± 1.31, respectively.

[b] Thirteen mice per group.

[c] Twenty mice per group.

[d] Number of metastatic cells determined by the clonogenic assay. Top number is the range of metastatic cells; number in parentheses is the average number of metastatic cells ± SD. Calculation includes only those mice with metastasis.

[e] A statistically significant difference between the STAT6$^{-/-}$ and BALB/c values ($p \leq 0.05$) for the number of metastatic cells per organ.

days (STAT6$^{-/-}$ surgery survivors from the experiment shown in Fig. 1*A*) were rechallenged in the mammary gland with 7000 4T1 cells and followed for primary tumor development and survival. The single BALB/c surgery survivor from Fig. 1*A* was also rechallenged, as were naive BALB/c and STAT6$^{-/-}$ mice. A control group of STAT6$^{-/-}$ mice whose primary tumor was recently removed, but which had not as yet gone through long-term survival and was not reinoculated with 4T1 (STAT6$^{-/-}$ surgery group), was also included.

As shown in Fig. 2, the BALB/c surgery survivor and naive BALB/c mice are dead by day 50, while naive STAT6$^{-/-}$ mice die more slowly, but 100% are dead by day 88. In contrast, 75% of the STAT6$^{-/-}$ long-term surgery survivors survive >350 days after their second 4T1 challenge, whereas only 45% of STAT6$^{-/-}$ surgery mice survived long term. Surgical removal of primary tumor, therefore, results in induction of long-term antitumor immunity in up to 75% of STAT6$^{-/-}$ mice, or selects for animals that are more inherently resistant.

Enhanced tumor immunity requires both hemopoietic and nonhemopoietic components

We originally hypothesized that deletion of the STAT6 gene resulted in skewing of the CD4$^+$ T cell population toward a Th1 phenotype, thereby enhancing CD8-mediated tumor immunity. However, Ab depletion experiments demonstrated that although CD8$^+$ T cells are involved, CD4$^+$ T cells are not required (3). Deletion of the STAT6 gene, therefore, results in enhanced tumor immunity via a mechanism independent of Th1 CD4$^+$ T cells. To understand the mechanism of enhanced immunity in STAT6$^{-/-}$ mice, we need to identify the cells that must be STAT6 deficient. Because CD8$^+$ T cells are the effector cells against 4T1 tumor (3), we asked whether it was sufficient that CD8$^+$ T cells be knocked out for the STAT6 gene. To test this hypothesis, BALB/c mice with STAT6$^{-/-}$ bone marrow were prepared. Experimental chimeras (STAT6$^{-/-}$ bone marrow into lethally irradiated BALB/c mice; STAT6$^{-/-}$→BALB/c), control chimeras (BALB/c bone marrow into lethally irradiated STAT6$^{-/-}$ mice; BALB/c→STAT6$^{-/-}$), and control naive STAT6$^{-/-}$ and BALB/c mice were inoculated in the abdominal mammary gland with 7000 4T1 cells. Onset and progression of primary tumors did not significantly differ between the groups. Primary tumors were surgically removed 15–21 days later when they measured between 2.8 and 7 mm in diameter and when metastatic disease was firmly established. The mice were then followed for survival. As shown in Fig. 3*A*, the chimeras and control BALB/c mice are dead by day 45 post-4T1 inoculation, while 46% of the STAT6$^{-/-}$ mice survive >350 days. STAT6$^{-/-}$ hemopoietic derived cells, therefore, are not sufficient for enhanced tumor immunity, suggesting that STAT6$^{-/-}$ nonhemopoietic derived cells or both hemopoietic and nonhemopoietic STAT6$^{-/-}$ cells are required.

To test this hypothesis and to assure that lethal irradiation did not destroy an essential component for antitumor immunity, autologous bone marrow chimeras were prepared. BALB/c and STAT6$^{-/-}$ mice were lethally irradiated and reconstituted with syngeneic bone marrow (BALB/c bone marrow into BALB/c mice, BALB/c→BALB/c; and STAT6$^{-/-}$ bone marrow into STAT6$^{-/-}$ mice, STAT6$^{-/-}$→STAT6$^{-/-}$). These chimeras along with control naive BALB/c and STAT6$^{-/-}$ mice were inoculated with 7000 4T1 cells in the abdominal mammary gland. Primary mammary tumors were surgically removed at 2–3 wk when tumors were 3–6 mm in diameter, and the mice were followed for survival. As shown in Fig. 3*B*, 100% of the control naive BALB/c and 89.9% of the BALB/c→BALB/c chimeras died by day 47 post-4T1 inoculation. In contrast, 57.1% of the STAT6$^{-/-}$ mice and 75% of the STAT6$^{-/-}$→STAT6$^{-/-}$ chimeras survive ≥100 days. Enhanced immunity, therefore, requires cells and/or components derived from both hemopoietic and nonhemopoietic compartments.

Earlier in vivo depletion studies demonstrated that CD8$^+$ T cells are required for enhanced immunity to 4T1 mammary carcinoma in STAT6$^{-/-}$ mice. In vitro assays using splenocytes from BALB/c and STAT6$^{-/-}$ mice immunized with 4T1 showed a strong correlation between tumor rejection and the development of tumor-specific CD8$^+$ CTL (3). To further test whether CTL activity reflects antitumor activity, bone marrow chimeric mice were immunized with irradiated 4T1 tumor cells, and splenocytes were tested for CTL activity against 4T1 and irrelevant B16 melanoma target cells. As shown in Fig. 3*C*, 4T1-immunized STAT6$^{-/-}$mice have specific CTL activity, while STAT6$^{-/-}$→BALB/c, BALB/c→STAT6$^{-/-}$, and BALB/c mice do not. Splenocyte in vitro cytotoxic activity to tumor, therefore, correlates with in vivo tumor rejection, and STAT6$^{-/-}$ bone marrow reconstitution alone is not sufficient to generate tumor-specific cytotoxic activity.

CD1-deficient mice have enhanced immunity to metastatic mammary carcinoma, but not to primary mammary carcinoma

Earlier studies identified NKT cells and IL-13 as potential inhibitors of tumor immunity to the HIV gp160-transfected 15-12RM

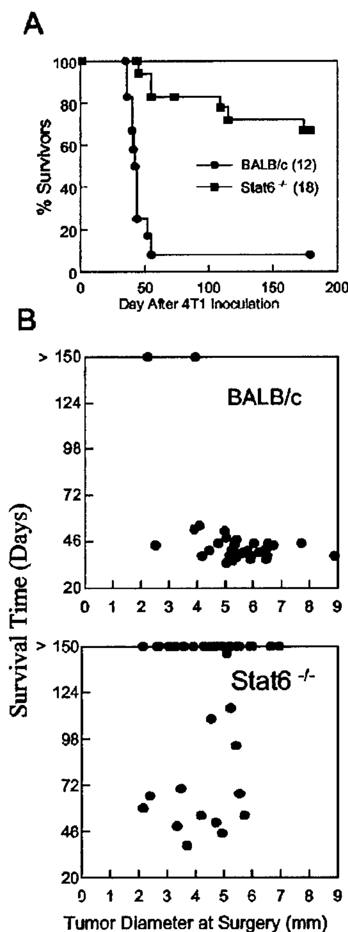


FIGURE 1. A majority of STAT6$^{-/-}$ mice survive metastatic mammary carcinoma following surgical removal of primary tumor. STAT6$^{-/-}$ and BALB/c mice were inoculated in the abdominal mammary gland with 7000 4T1 cells. Later (2–2.5 wk), primary tumors were surgically removed and mice were followed for survival. *A*, Average primary TD ± SD (mm) at the time of surgery were STAT6$^{-/-}$, 4.51 ± 1.27; BALB/c, 4.87 ± 1.62. Values in parentheses are the number of mice per group. *B*, Survival time plotted as a function of TD at the time of surgery. Data are pooled from three independent experiments. Each BALB/c or STAT6$^{-/-}$ group in each individual experiment contained 7–18 mice, for a total of 36 BALB/c and 38 STAT6$^{-/-}$ mice.

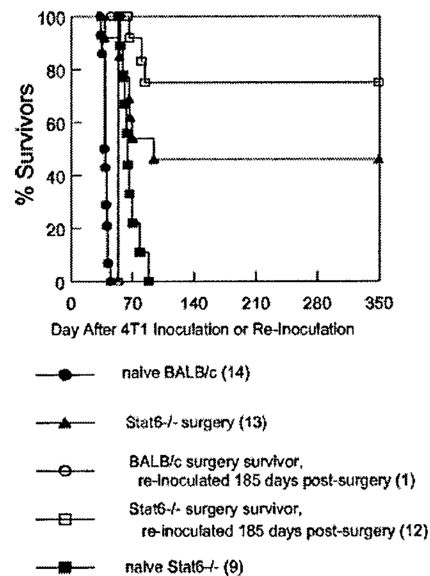


FIGURE 2. STAT6$^{-/-}$ survivor mice are immune to subsequent inoculations of 4T1 tumor. STAT6$^{-/-}$ and BALB/c survivor mice from Fig. 1*A* were reinoculated in the abdominal mammary gland with 7000 4T1 cells and followed for survival. Control and comparison groups included BALB/c and STAT6$^{-/-}$ mice whose primary tumors were not surgically removed (naive BALB/c and naive STAT6$^{-/-}$), and STAT6$^{-/-}$ mice whose primary tumors were removed, but not reinoculated with 4T1 (STAT6$^{-/-}$ surgery). Values in parentheses are the number of mice per group.

11 fibrosarcoma (1). To determine whether NKT cells and IL-13 also inhibit immunity to metastatic 4T1 tumor, NKT cell-deficient and IL-13-deficient CD1$^{-/-}$ mice were tested. CD1$^{-/-}$ mice were inoculated in the abdominal mammary gland with 7000 4T1 cells. Primary tumors were removed from the CD1$^{-/-}$ and control BALB/c and STAT6$^{-/-}$ mice at 2–3 wk, and the mice were followed for survival. As shown in Fig. 4*A*, 100% of control BALB/c mice were dead by day 52, while 80 and 60% of CD1$^{-/-}$ and STAT6$^{-/-}$ mice, respectively, survived >100 days. Elimination of NKT cells and accompanying reduction in IL-13, therefore, produce resistance to 4T1 metastasis.

To ascertain whether primary tumor growth is affected by NKT and IL-13 deficiency, the 4T1 solid tumors of the mice in Fig. 4 were measured at the time of surgery. As shown in Table II, 100% of CD1$^{-/-}$ and BALB/c mice develop primary tumors at the inoculation site (abdominal mammary gland) within 2 wk of inoculation of 7000 4T1 cells. The primary tumors in the CD1$^{-/-}$ mice are slightly larger than tumors in the BALB/c group ($p < 0.05$). In contrast, only 50% of STAT6$^{-/-}$ mice develop tumors. Therefore, primary 4T1 tumor growth in NKT-deficient mice is not reduced relative to primary tumor growth in BALB/c mice, indicating that deletion of NKT cells does not enhance immunity to primary mammary carcinoma.

Chimeric mice of STAT6$^{-/-}$ bone marrow in CD1$^{-/-}$ recipients, and vice versa, do not have enhanced tumor immunity to mammary carcinoma metastasis

As seen in the experiments of Fig. 3, enhanced immunity in STAT6$^{-/-}$ mice requires hemopoietic and nonhemopoietic derived cells. If STAT6$^{-/-}$ and CD1$^{-/-}$ mice share a common mechanism underlying their enhanced immunity, then chimeras of STAT6$^{-/-}$ or CD1$^{-/-}$ bone marrow and recipients may have enhanced immunity. To test this hypothesis, STAT6$^{-/-}$ recipients were reconstituted with CD1$^{-/-}$ bone marrow (CD1$^{-/-}$→STAT6$^{-/-}$ chimeras), and CD1$^{-/-}$ recipients were reconstituted with STAT6$^{-/-}$ bone marrow (STAT6$^{-/-}$→CD1$^{-/-}$). The chimeras, along with control STAT6$^{-/-}$, CD1$^{-/-}$, and wild-type BALB/c mice, were challenged with 7000 4T1 cells in the abdominal mammary gland, their primary tumors were removed 2–3 wk later, and the mice were followed for survival time. As shown in Fig. 4*B*, 70% of STAT6$^{-/-}$ and 100% of CD1$^{-/-}$ mice survived >150 days, while 100% of both chimeras died within 53 days. Therefore, STAT6$^{-/-}$ and CD1$^{-/-}$ hemopoietic derived cells are not equivalent in terms of tumor immunity, and it is likely that enhanced immunity in STAT6$^{-/-}$ and CD1$^{-/-}$ mice is mediated by different mechanisms, or that they have defects in distinct steps of the relevant regulatory pathway.

Inhibition of IL-13 in BALB/c mice does not facilitate tumor immunity to primary or metastatic 4T1 mammary carcinoma

As demonstrated by earlier studies, mice treated with an inhibitor for IL-13 (sIL-13Rα2-Fc) are resistant to recurrence of the 15-12RM gp160-transfected fibrosarcoma (1). This result, coupled with the observation that NKT-deficient CD1$^{-/-}$ mice, as well as

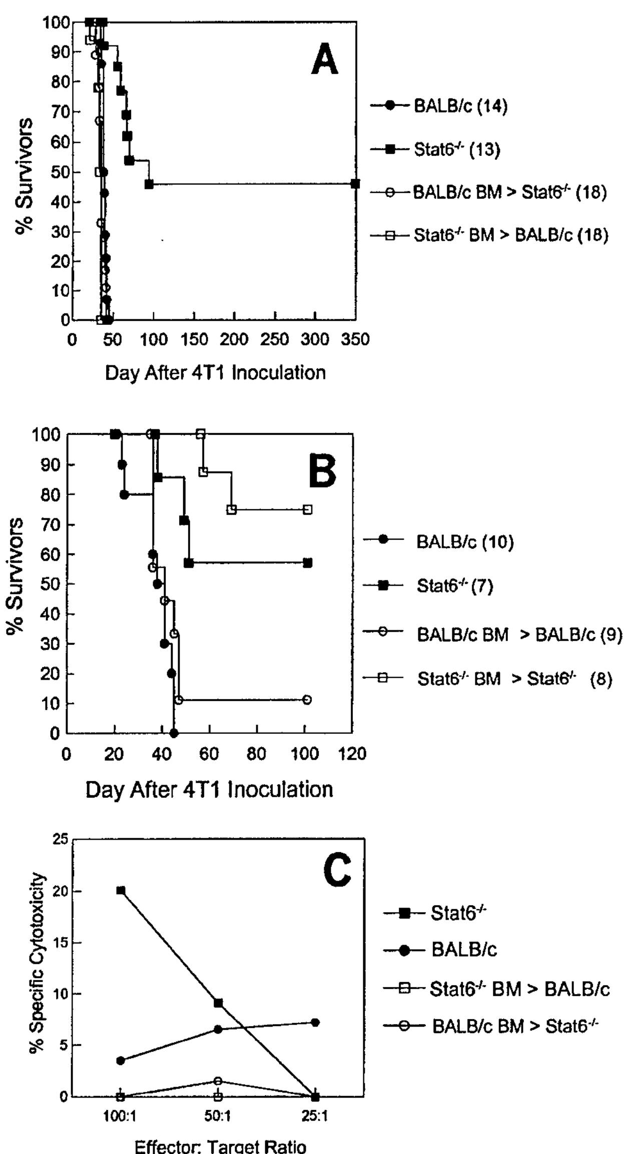


FIGURE 3. Enhanced immunity requires hemopoietic and nonhemopoietic components. Chimeric mice and untreated control BALB/c and STAT6$^{-/-}$ mice were inoculated in the abdominal mammary gland with 7000 4T1 tumor cells, and primary tumors were surgically removed 15–21 days later. Values in parentheses are the number of mice per group. *A*, Allogeneic chimeras. Average primary TD ± SD (mm) at the time of surgery were BALB/c, 5.41 ± 0.76; STAT6$^{-/-}$, 4.61 ± 1.51; BALB/c→STAT6$^{-/-}$, 4.68 ± 0.79; STAT6$^{-/-}$→BALB/c, 5.3 ± 0.9. *B*, Autologous chimeras. Average primary TD ± SD (mm) at the time of surgery were BALB/c, 5.13 ± 0.7; STAT6$^{-/-}$, 4.12 ± 0.52; BALB/c→BALB/c, 4.59 ± 0.74; STAT6$^{-/-}$→STAT6$^{-/-}$, 4.93 ± 0.47. *C*, STAT6$^{-/-}$, but not BALB/c, STAT6$^{-/-}$→BALB/c, or BALB/c→STAT6$^{-/-}$ chimeric mice contain CTL to 4T1 tumor cells. Chimeras, BALB/c, and STAT6$^{-/-}$ mice were multiply immunized i.p. with 10^6 irradiated 4T1 cells. Five days after the last immunization, splenocytes were removed and tested for cytotoxic activity against Cr-51-labeled 4T1 and B16 melanoma targets.

IL-13-nonresponsive STAT6$^{-/-}$ mice, and IL-4R$\alpha^{-/-}$ mice are also resistant to 15-12RM (1), supports the hypothesis that IL-13 produced by NKT cells blocks activation of CD8$^+$ T cells. Inhibition of IL-13 by sIL-13Rα2 therefore blocks IL-13 activity, and allows tumor-specific CD8$^+$ T cells to differentiate (1). To determine whether enhanced immunity to 4T1 in STAT6$^{-/-}$ mice is

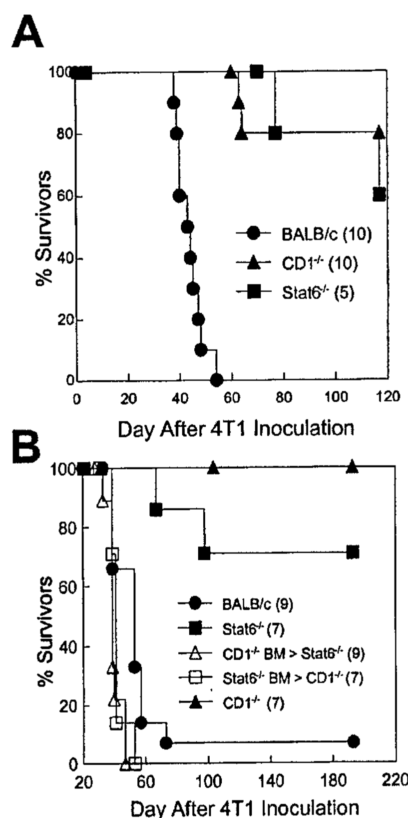


FIGURE 4. CD1$^{-/-}$ mice have enhanced immunity to 4T1 metastatic disease; however, CD1$^{-/-}$ and STAT6$^{-/-}$ bone marrow chimeras do not. *A*, CD1$^{-/-}$, STAT6$^{-/-}$, and BALB/c mice were inoculated in the abdominal mammary gland with 7000 4T1 cells. Two to three weeks later, primary tumors were surgically removed and mice were followed for survival. Average primary TD ± SD (mm) at the time of surgery were BALB/c, 4.26 ± 0.86; STAT6$^{-/-}$, 4.43 ± 1.13; CD1$^{-/-}$, 4.95 ± 0.64. *B*, Eight weeks after bone marrow reconstitution, mice were inoculated in the abdominal mammary gland with 7000 4T1 tumor cells. Primary tumors were surgically removed on days 18–26. Average primary TD ± SD (mm) at the time of surgery were BALB/c, 4.93 ± 0.98; STAT6$^{-/-}$, 4.47 ± 1.17; CD1$^{-/-}$→STAT6$^{-/-}$, 4.75 ± 0.65; STAT6$^{-/-}$→CD1$^{-/-}$, 4.52 ± 0.8; CD1$^{-/-}$, 4.9 ± 1.2. Values in parentheses are the number of mice per group.

due to nonresponsiveness to IL-13, BALB/c mice were treated with the IL-13 inhibitor, sIL-13Rα2-Fc, and inoculated with 4T1. Groups of BALB/c mice were either treated with sIL-13Rα2-Fc or a control human IgG starting on day 0, and inoculated in the abdominal mammary gland with 7000 4T1 cells. Inhibitor or control IgG treatment was continued for the first 2 wk of tumor growth. On day 26, primary tumors were surgically removed, and the mice were followed for survival. As shown in Fig. 5, neither

Table II. *CD1$^{-/-}$ mice have a high incidence of primary mammary carcinoma*[a]

Strain	Tumor Incidence	Diameter of Primary Tumor ± SD (mm)
BALB/c	10/10	4.26 ± 0.96
STAT6$^{-/-}$	5/10	4.42 ± 1.13
CD1$^{-/-}$	10/10	4.94 ± 0.67

[a] Mice were inoculated in the abdominal mammary gland with 7000 4T1 mammary carcinoma cells and followed for development of primary tumor at the site of injection. Tumor incidence is number of mice that developed solid tumor/total mice inoculated. Tumor-free mice did not develop tumors within a 70-day observation period. TD were measured at 2–3 wk after 4T1 inoculation at the time of surgery.

primary tumor progression (Fig. 5*A*) nor survival following surgical removal of primary tumor (Fig. 5*B*) is altered by sIL-13Rα2-Fc treatment, suggesting that inhibition of IL-13 does not yield enhanced tumor immunity to the 4T1 mammary carcinoma.

Depletion of $CD4^+CD25^+$ T cells in BALB/c mice does not enhance immunity to primary or metastatic 4T1 mammary carcinoma

$CD4^+CD25^+$ T cells have also been shown to inhibit the activation of $CD8^+$ T cytotoxic cells (8, 9, 11). To determine whether $CD4^+CD25^+$ T regulatory cells inhibit activation of 4T1-specific $CD8^+$ T cells, BALB/c mice were depleted for $CD4^+CD25^+$ T cells before inoculation with 4T1 mammary carcinoma. BALB/c mice were either untreated or given CD25 mAb starting on day −4, and inoculated with 7000 4T1 cells in the abdominal mammary gland on day 0. In one group of mice, progression of primary tumors was followed. In a second group of mice, primary tumors were surgically excised on day 21, and the mice were followed for survival. Depletion of $CD4^+CD25^+$ T cells does not alter growth of primary 4T1 (Fig. 6*A*) nor survival (Fig. 6*B*). Therefore, inactivation of $CD4^+CD25^+$ T regulatory cells is not responsible for enhanced immunity to 4T1 primary tumor or metastatic disease in $STAT6^{-/-}$ mice.

IFN-γ is essential for tumor resistance in $STAT6^{-/-}$ mice

To determine whether tumor resistance correlated with differential cytokine production, draining lymph node cells of $STAT6^{-/-}$ and BALB/c mice were assayed. Mice were inoculated in an abdominal mammary gland with 4T1 cells, and 5 days later draining inguinal lymph nodes were removed and cocultured with irradiated 4T1/B7.1 or irrelevant stimulators. A total of 21 $STAT6^{-/-}$ and 17 BALB/c mice were tested in seven separate experiments. Fig. 7*A* shows the pooled results of these experiments. $STAT6^{-/-}$, but not BALB/c mice produce high levels of IFN-γ, while BALB/c mice produce more IL-4 than $STAT6^{-/-}$ mice. Both strains produce low levels of IL-2. If 100 pg/ml of IFN-γ is used as a cutoff for responders, then 62% of the $STAT6^{-/-}$ mice produce IFN-γ, which is approximately equal to the percentage of $STAT6^{-/-}$ mice that survive in a typical surgery experiment.

To ascertain whether IFN-γ production is essential for enhanced tumor resistance in $STAT6^{-/-}$ mice, double knockout $STAT6^{-/-}$ $IFN\text{-}\gamma^{-/-}$ mice were inoculated in the abdominal mammary gland with 4T1 cells, and followed for survival after surgical removal of primary tumor. As shown in Fig. 7*B*, 100% of $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ and $IFN\text{-}\gamma^{-/-}$ mice die by day 62, while 87% of the $STAT6^{-/-}$ mice survive. Therefore, IFN-γ is essential for enhanced tumor resistance in $STAT6^{-/-}$ mice.

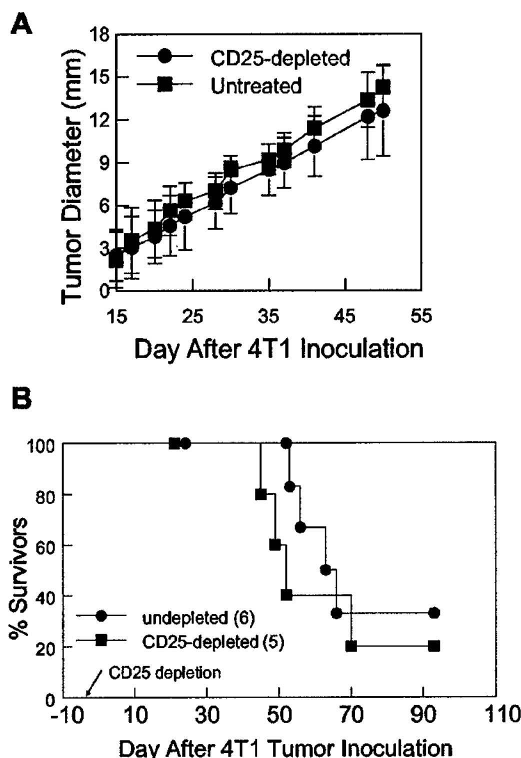


FIGURE 6. Depletion of $CD25^+$ cells does not alter primary tumor growth or metastatic disease. BALB/c mice were treated or not treated on day −4 with mAb PC61 to CD25 and inoculated on day 0 with 7000 4T1 cells. *A*, Primary tumor growth. *B*, Survival following surgical removal of primary tumor on day 21. Average primary TD ± SD (mm) at the time of surgery were CD25 depleted, 6.42 ± 1.33; not depleted, 5.21 ± 1.39. The numbers in parentheses are the number of mice per group.

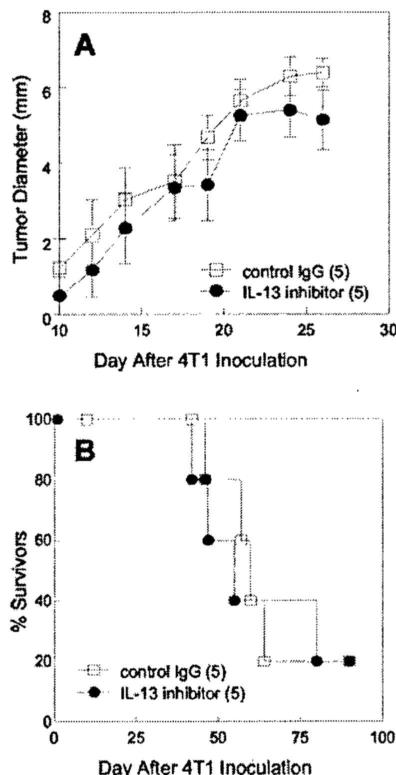


FIGURE 5. Inhibition of IL-13 with the sIL-13Rα2-Fc does not alter primary tumor growth or metastatic disease. BALB/c mice were inoculated on day 0 with 7000 4T1 cells and treated with sIL-13Rα2-Fc or a control Ig every other day from day 0 to 14. *A*, Primary tumor growth. *B*, Survival following surgical removal of primary tumor on day 26. Average primary TD ± SD (mm) at the time of surgery were: IL-13-inhibitor treated, 5.14 ± 0.79; control IgG treated, 6.38 ± 0.37. The numbers in parentheses are the number of mice per group.

Discussion

To evaluate the antitumor effect of STAT6 deficiency on metastatic disease, we have used an animal system that closely models advanced, human metastatic disease. These experiments demonstrate that if primary tumor is surgically removed, then 45–80% of $STAT6^{-/-}$ mice survive indefinitely and develop a potent immunity to tumor. These observations are notable for several reasons. 1) Metastatic disease in distant organs in this animal model is

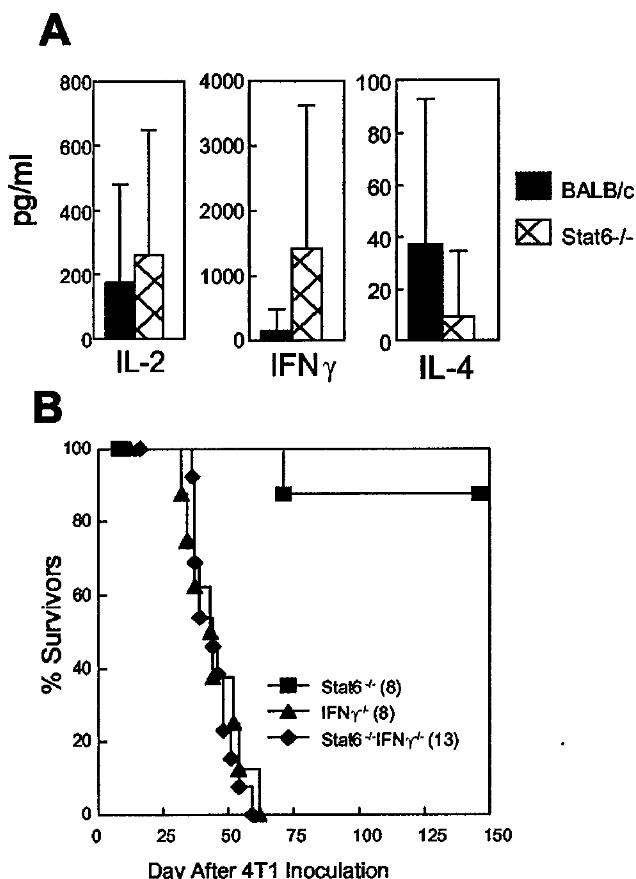


FIGURE 7. Tumor resistance in STAT6$^{-/-}$ mice is dependent on IFN-γ. *A*, STAT6$^{-/-}$ (cross-hatched bars) and BALB/c (filled bars) mice were inoculated with 4T1 cells. Draining lymph nodes were removed 5 days later and cocultured with irradiated 4T1/B7.1 or irrelevant B16 melF10 cells. Supernatants were harvested 2 days later and tested by ELISA for IL-2, IL-4, and IFN-γ. These data are the pooled results of 21 STAT6$^{-/-}$ and 17 BALB/c mice. *B*, STAT6$^{-/-}$IFN-γ$^{-/-}$ mice were inoculated in the abdominal mammary gland with 7000 4T1 cells. Primary tumors were removed on day 21, and mice were followed for survival. Average primary TD ± SD (mm) at the time of surgery were STAT6$^{-/-}$, 5.59 ± 0.7; IFN-γ$^{-/-}$, 5.93 ± 0.81; STAT6$^{-/-}$IFN-γ$^{-/-}$, 6.25 ± 1.62. The numbers in parentheses are the number of mice per group.

firmly established as early as 2 wk post-4T1 inoculation and/or when primary tumors are >2 mm in diameter (13). Therefore, at the time of surgery (2.5–3 wk after 4T1 inoculation), mice have extensive, established metastatic disease. 2) Tumor immunity following surgery is very effective whether the primary tumor is relatively small (2–4 mm in diameter), or large (4–7 mm in diameter). Earlier studies established that the extent of metastatic disease is approximately proportional to the size of primary tumor (13). Therefore, immunity in STAT6$^{-/-}$ mice is effective against a large number of metastatic cells. 3) Mice that survive inoculation of 4T1 must eliminate tumor cells in multiple sites because the 4T1 tumor metastasizes to the lungs, liver, bone marrow, brain, lymph nodes, and blood (12, 14, 22). Therefore, tumor immunity in STAT6$^{-/-}$ mice is systemic, and is effective against metastatic cells regardless of their location. 4) 4T1 is a poorly immunogenic tumor that spontaneously arose in BALB/cfC3H mice that were carrying an exogenous mouse mammary tumor virus (23). Its tumor Ags are likely to be self molecules to which BALB/c and STAT6$^{-/-}$ mice are tolerant. Therefore, tolerance in STAT6$^{-/-}$ mice does not preclude the development of immunity to 4T1. Functional elimination of the STAT6 gene, therefore, allows the development of a CD8$^+$ T cell-mediated immunity that protects mice against continued development of dispersed, metastatic disease.

Because any immune system cells that might be involved are bone marrow derived, we expected that reconstitution of BALB/c mice with STAT6$^{-/-}$ bone marrow would generate mice that were as tumor resistant as STAT6$^{-/-}$ mice. Surprisingly, STAT6$^{-/-}$→BALB/c chimeras were just as susceptible as BALB/c mice. Because the STAT6 gene is deleted in all cells of STAT6$^{-/-}$ mice (17), the bone marrow chimera data are consistent with the hypothesis that both hemopoietic and nonhemopoietic cells contribute to the antitumor phenotype. STAT6$^{-/-}$ bone marrow may not give a tumor immune phenotype in BALB/c recipients because STAT6$^{-/-}$ stem cells may require a STAT6$^{-/-}$ thymus for appropriate development. STAT6$^{-/-}$ thymic epithelium may provide different signals during positive selection that result in positive selection of a different T cell repertoire than that generated in wild-type STAT6$^{+/+}$ mice. Alternatively, negative selection may be impacted by the STAT6 deletion and result in a T cell repertoire that includes CD8$^+$ T cells that would normally be deleted during negative selection in STAT6$^{+/+}$ mice. In either case, the novel T cell repertoire could contain CD8$^+$ T cells that when activated are more effective against metastatic tumor. A third alternative is that the regulatory pathway requires a nonhemopoietic cell, or a cell that survives the radiation treatment used to prepare the chimeras.

The mechanism underlying tumor resistance to 4T1 in STAT6$^{-/-}$ mice remains unclear. It was originally hypothesized that enhanced immunity in STAT6$^{-/-}$ mice is due to preferential production of CD4$^+$ Th1 cells. However, in vivo depletion of CD4$^+$ T cells does not reduce tumor resistance, indicating that CD4$^+$ T cells are not required for enhanced immunity (3). However, IFN-γ, a cytokine that is pivotal for Th1 cell differentiation, is produced early after 4T1 inoculation and is essential for enhanced immunity because IFN-γ-deficient STAT6$^{-/-}$ mice are as susceptible to 4T1 as are wild-type BALB/c mice. IFN-γ is a highly pleiotropic cytokine that has many functions in addition to its role in Th1 differentiation (24, 25), and any of these additional activities could facilitate tumor rejection in STAT6$^{-/-}$ mice.

CD4$^+$CD25$^+$ T cells have also been proposed as inhibitors of tumor immunity. Inhibitory CD4$^+$ T cells were first described by North et al. (26) over 17 years ago. More recently, immunosuppressive T cells have been phenotyped, when studies in autoimmune systems led to the identification of CD4$^+$CD25$^+$ T cells that regulate/suppress autoreactive CD8$^+$ T effector cells (9–11). The inhibitory effects of CD25$^+$CD4$^+$ T cells on tumor immunity have also been demonstrated in several tumor systems (8, 27). Although it is likely that STAT6$^{-/-}$ mice have enhanced immunity because of deletion of an inhibitor, the CD25-depletion studies performed in this work demonstrate that CD4$^+$CD25$^+$ T cells are not the relevant inhibitor in STAT6$^{-/-}$ mice.

Earlier studies using the 15-12RM fibrosarcoma and CD1$^{-/-}$ and STAT6$^{-/-}$ mice led to the hypothesis that IL-13, secreted by NKT cells, inhibits the differentiation of tumor-specific CD8$^+$ T cells by acting on an intermediate cell through a STAT6-dependent pathway (1). Although our studies confirm that NKT-deficient CD1$^{-/-}$ mice also have enhanced immunity to 4T1 tumor, inhibition of IL-13 alone is not sufficient because treatment of BALB/c mice with the IL-13 inhibitor, sIL-13Rα-Fc, does not produce 4T1-resistant mice. Because STAT6$^{-/-}$ mice are also deficient for response to IL-4 activity, IL-4 is another candidate inhibitor. However, previous studies using BALB/c IL-4$^{-/-}$ mice demonstrated that these mice also do not have enhanced immunity to 4T1 primary tumor or metastatic disease (28) or to 15-12RM (1). Therefore, neither loss of response to IL-13 nor loss of response to IL-4

alone is sufficient for the resistance of $STAT6^{-/-}$ mice to 4T1 metastatic disease. Furthermore, neither $STAT6^{-/-} \rightarrow CD1^{-/-}$ nor $CD1^{-/-} \rightarrow STAT6^{-/-}$ bone marrow chimeras have enhanced immunity to 4T1 metastatic disease, so it is likely that resistance in $STAT6^{-/-}$ and $CD1^{-/-}$ mice occurs via noncomplementing steps in the same regulatory pathway or via different mechanisms.

STAT6 transduces the signal from both IL-4 and IL-13, and IL-13 and IL-4 bind to the same receptor (type II IL-4R consisting of IL-4Rα and IL-13Rα1 chains) (29). Therefore, elimination of the activity of both cytokines simultaneously may be necessary for enhanced immunity because IL-4 may compensate for the absence of IL-13 and vice versa (30). In preliminary experiments, we have tested this hypothesis in BALB/c mice depleted of IL-4 by in vivo treatment with an anti-IL-4 mAb (11B11) and simultaneously treated with the IL-13 inhibitor. These mice showed no enhanced immunity to 4T1 in a postsurgery setting (M. Terabe, J. M. Park, and J. A. Berzofsky, unpublished results). Similarly, IL-4R$\alpha^{-/-}$ mice, which are incapable of transmitting either IL-13 or IL-4 signals, showed no enhanced immunity to 4T1 (Clements and Ostrand-Rosenberg, unpublished results). Simultaneous elimination of IL-4 plus IL-13, therefore, does not result in immunity to primary and/or metastatic 4T1 tumor.

As a transcriptional regulatory factor, STAT6 is well positioned to modulate expression of numerous critical inhibitory molecules. The role of STAT6 protein in IL-13 and IL-4 activity is well known; however, STAT6 may also play a role in the expression or activity of as yet uncharacterized cells and/or cytokines and/or other molecules that inhibit tumor immunity. For example, STAT6 may activate a novel factor that stimulates $CD25^{-}$ regulatory T cells (suppressor cells), which in turn inhibit differentiation of tumor-specific $CD8^{+}$ T lymphocytes. $STAT6^{-/-}$ mice, therefore, would not contain the inhibitory T cells, and tumor-specific $CD8^{+}$ T cells would be produced and mediate tumor regression. Alternatively, NKT cells may secrete a novel molecule (in addition to IL-4 or IL-13) that acts via the STAT6 pathway to block tumor-specific $CD8^{+}$ T cell differentiation. If this novel molecule uses a receptor other than IL-4Rα, then $CD1^{-/-}$ and $STAT6^{-/-}$ mice would show enhanced immunity because one strain would not produce and the other strain would not respond to the inhibitory molecule. Although we cannot at present distinguish between these hypothetical mechanisms, it is intriguing to speculate that a novel molecule/cell/cytokine produced by or in response to NKT cells and operating via a STAT6 pathway negatively regulates tumor immunity. Such a factor could be responsible for the absence of effective tumor immunity in tumor-bearing or tumor-immunized individuals, and could be a target for future immunotherapies.

Acknowledgments

We appreciate the excellent care of our animals by Sandy Mason. We thank Dr. Beth Pulaski for breeding the $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice, Drs. Michael Grusby and Luc van Kaer for generously supplying multiple mating pairs of $STAT6^{-/-}$ and $CD1^{-/-}$ mice, respectively, and Dr. Debra Donaldson (Wyeth Laboratories, Boston, MA) for providing the IL-13 inhibitor.

References

1. Terabe, M., S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D. Donaldson, D. Carbone, W. Paul, and J. Berzofsky. 2000. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat. Immun. 1:515.*
2. Kacha, A., F. Fallarino, M. Markiewicz, and T. Gajewski. 2000. Spontaneous rejeciton of poorly immunogenic P1.HTR tumors by Stat6-deficient mice. *J. Immunol. 165:6024.*
3. Ostrand-Rosenberg, S., M. Grusby, and V. Clements. 2000. Cutting edge: Stat6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. *J. Immunol. 165:6015.*
4. Jankovic, D., M. Kullberg, N. Noben-Trauth, P. Caspar, W. Paul, and A. Sher. 2000. Single cell analysis reveals that IL-4 receptor/STAT6 signaling is not required for the in vivo or in vitro development of $CD4^{+}$ lymphocytes with a Th2 cytokine profile. *J. Immunol. 164:3047.*
5. Ohmori, Y., and T. Hamilton. 1998. STAT6 is required for the anti-inflammatory activity of IL-4 in mouse peritoneal macrophages. *J. Biol. Chem. 273:29202.*
6. Stamm, L., A. Raisanen-Sokolowski, M. Okano, M. Russell, J. David, and A. Satoskar. 1998. Mice with STAT6-targeted gene disruption develop a Th1 response and control cutaneous leishmaniasis. *J. Immunol. 161:6180.*
7. Shurin, M., L. Lu, P. Kalinski, A. Stewart-Akers, and M. Lotze. 1999. Th1/Th2 balance in cancer, transplantation and pregnancy. *Springer Semin. Immunopathol. 21:339.*
8. Sutmuller, R., L. van Duivenvoorde, A. van Elsas, T. Schumacher, M. Wildenberg, J. Allison, R. Toes, R. Offringa, and C. Melief. 2001. Synergism of CTLA-4 blockade and depletion of $CD25^{+}$ regulatory T cells in anti-tumor therapy reveals alternative pathways for suppression of auto-reactive CTL responses. *J. Exp. Med. 194:823.*
9. Sakaguchi, S., M. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol. 155:1151.*
10. Salomon, B., D. Lenschow, L. Rhee, N. Ashourian, B. Slingh, A. Sharpe, and J. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^{+}CD25^{+}$ immunoregulatory T cells that control autoimmune diabetes. *Immunity 12:431.*
11. Piccirillo, C., and E. Shevach. 2001. Cutting edge: control of $CD8^{+}$ T cell activation by $CD4^{+}CD25^{+}$ immunoregulatory cells. *J. Immunol. 167:1137.*
12. Pulaski, B., and S. Ostrand-Rosenberg. 1998. MHC class II and B7.1 immunotherapeutic cell-based vaccine reduces spontaneous mammary carcinoma metastases without affecting primary tumor growth. *Cancer Res. 58:1486.*
13. Pulaski, B., D. Terman, S. Khan, E. Muller, and S. Ostrand-Rosenberg. 2000. Cooperativity of SEB superantigen, MHC class II, and CD80 in immunotherapy of advanced metastases in a clinically relevant post-operative breast cancer model. *Cancer Res. 60:2710.*
14. Aslakson, C., and F. Miller. 1992. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res. 52:1399.*
15. Pulaski, B., V. Clements, M. Pipeling, and S. Ostrand-Rosenberg. 2000. Immunotherapy with vaccines combining MHC class II/$CD80^{+}$ tumor cells with IL-12 reduces established metastatic disease and stimulates immune effectors and monokine induced by interferon-γ. *Cancer Immunol. Immunother. 49:34.*
16. DeVita, V., S. Hellman, and S. Rosenberg. 2001. *Cancer: Principles & Practice of Oncology*, 6th Ed. Lippincott-Raven, New York.
17. Kaplan, M., U. Schindler, S. Smiley, and M. Grusby. 1996. STAT6 is required for mediating responses to IL-4 and for the development of Th2 cells. *Immunity 4:313.*
18. Seino, K., K. Fukao, K. Muramoto, K. Yanagisawa, Y. Takada, S. Kakuta, Y. Iwakura, L. Van Kaer, K. Takeda, T. Nakayama, et al. 2001. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc. Natl. Acad. Sci. USA 98:2577.*
19. Mendiratta, S., W. Martin, A. Boesteanu, S. Joyce, and L. Van Kaer. 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity 6:469.*
20. Pulaski, B., and S. Ostrand-Rosenberg. 2000. Mouse 4T1 breast tumor model. In *Current Protocols in Immunology*. J. Coligan, D. Margulies, E. Shevach, W. Strober, and A. Kruisbeek, eds. Wiley, Hoboken, NJ, p. 20.2.1.
21. Lowenthal, J., P. Corthesy, C. Tougne, R. Lees, and H. MacDonald. 1985. High and low affinity IL-2 receptors: analysis by IL-2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61. *J. Immunol. 135:3988.*
22. Lelekakis, M., J. Moseley, T. Martin, D. Hards, E. Williams, P. Ho, D. Lowen, J. Javni, F. Miller, J. Slavin, and R. Anderson. 1999. A novel orthotopic model of breast cancer metastasis to bone. *Clin. Exp. Metastasis 17:163.*
23. Miller, F., B. Miller, and G. Heppner. 1983. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. *Invasion Metastasis 3:22.*
24. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon-γ. *Annu. Rev. Immunol. 15:749.*
25. Shtrichman, R., and C. E. Samuel. 2001. The role of γ interferon in antimicrobial immunity. *Curr. Opin. Microbiol. 4:251.*
26. North, R., and I. Bursuker. 1984. Generation and decay of the immune response to a progressive fibrosarcoma. I. $Ly\text{-}1^{+}2^{-}$ suppressor T cells down-regulate the generation of $Ly\text{-}1^{-}2^{+}$ effector cells. *J. Exp. Med. 159:1295.*
27. Shimizu, J., S. Yamazaki, and S. Sakaguchi. 1999. Induction of tumor immunity by removing $CD25^{+}CD4^{+}$ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol. 163:5211.*
28. Pulaski, B. A., M. J. Smyth, and S. Ostrand-Rosenberg. 2002. IFNγ-dependent phagocytic cells are a critical component of innate immunity to metastatic mammary carcinoma. *Cancer Res. 62:4406.*
29. Zurawski, S., F. Vega, B. Huyghe, and G. Zurawski. 1993. Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. *EMBO J. 12:2663.*
30. Zurawski, S., and J. de Vries. 1994. Interleukin-13, an interleukin-4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol. Today 15:19.*

APPENDIX 7

Cancer Immunol Immunother (2004) 53: 86–91
DOI 10.1007/s00262-003-0446-z

SYMPOSIUM IN WRITING

Suzanne Ostrand-Rosenberg · Pratima Sinha
Virginia Clements · Samudra I. Dissanayake
Seth Miller · Cordula Davis · Erika Danna

Signal transducer and activator of transcription 6 (Stat6) and CD1: inhibitors of immunosurveillance against primary tumors and metastatic disease

Received: 5 June 2003 / Accepted: 7 August 2003 / Published online: 30 October 2003
© Springer-Verlag 2003

Abstract Many tumor immunologists favor the hypothesis that optimal anti-tumor activity is mediated by type 1 $CD4^+$ and $CD8^+$ T cells, and that the production of type 2 $CD4^+$ T cells may be counterproductive for effective anti-tumor immunity. Since Stat6-deficient or "knockout" mice lack the signal transducer and activator of transcription-6 protein and are unable to transmit signals initiated by the type 2 cytokines, IL-4 and IL-13, they have been studied to confirm the T_H1 vs T_H2 paradigm. Using transplantable tumor cells that cause primary solid tumors and metastatic disease, as well as a spontaneous transgenic tumor model, multiple studies have demonstrated that $Stat6^{-/-}$ mice are able to reject or delay primary tumor growth, prevent recurrence of primary tumors, and/or reject established, spontaneous metastatic disease. Deletion of the Stat6 gene, therefore, provides significantly enhanced immunosurveillance. Comparable experiments with CD1-deficient mice, which lack NKT cells and hence are deficient for IL-13, give similar results and suggest that removal of NKT cells also enhances immunosurveillance. Because immunity is enhanced in the absence of Stat6 or CD1, it has been hypothesized that these deletions result in the removal of an inhibitor that blocks constitutive immunosurveillance. Several mechanisms have been tested as potential inhibitors, including $CD4^+CD25^+$ T regulatory cells, IL-13, a T_H2 shift, and myeloid suppressor cells. Although the first three mechanisms do not appear to be relevant, regression of myeloid suppressor cells in Stat6-deficient and CD1-deficient mice may be responsible for enhanced immunosurveillance. Although additional studies are clearly needed to clarify the mechanism(s) underlying improved anti-tumor immunity in $Stat6^{-/-}$ and $CD1^{-/-}$ mice, deletion of these genes results in a potent anti-tumor immunity and may be a basis for an immunotherapy strategy.

Abbreviations *Stat6* signal transducer and activator of transcription 6 · *MSC* myeloid suppressor cell · *BALB/c NeuT* transgenic mice that spontaneously develop mammary carcinoma · *$Stat6^{-/-}NeuT^{+/-}$* Stat6-deficient, BALB/c NeuT mice · *$Stat6^{-/-}IFN\gamma^{-/-}$* Stat6-deficient, interferon-γ-deficient BALB/c mice

This article forms part of the Symposium in Writing "Inhibitors of immunosurveillance and anti-tumor immunity," published in Vol. 53. These studies were supported by NIH R01CA52527 and R01CA84232, and by DOD US Army Research and Materiel Command DAMD17-01-1-0312. Reported animal studies adhered to NIH guidelines for the humane treatment of laboratory animals, and have been approved by the UMBC IACUC.

S. Ostrand-Rosenberg (✉) · P. Sinha · V. Clements
S. I. Dissanayake · S. Miller · C. Davis · E. Danna
Department of Biological Sciences, University of Maryland, Baltimore County, Baltimore, MD 21250 USA
E-mail: srosenbe@umbc.edu
Tel.: +1-410-4552237
Fax: +1-410-4553875

Stat6-deficient mice preferentially make T_H1 responses

Many tumor immunologists believe that optimal anti-tumor immunity is mediated by type 1 $CD8^+$ T lymphocytes [6, 7], and is dependent on "help" from type 1 $CD4^+$ T cells (T_H1) [10, 34]. In contrast, type 2 $CD4^+$ T cells are thought to preferentially provide "help" to B cells for antibody production [5]. Investigators have speculated that activation of type 2 $CD4^+$ T cells may even be detrimental in tumor immunity, because polarization of the response towards a type 2 phenotype may limit the opportunities for generating a type 1 response [2, 16], although this assumption is controversial [20].

Signal transducer and activator of transcription 6 (stat6) is a cytosolic protein that when phosphorylated by Janus kinases 1 and 2 is activated and migrates to the nucleus where it binds to DNA and regulates cytokine production (reviewed in [9, 11, 17]). This signaling pathway is activated when the cytokines IL-4 and/or IL-13 bind to their common type II IL-4R receptor, which consists of IL-4Rα plus IL-13Rα1 or IL-13Rα2

chains [22]. Activation of this pathway maintains production of IL-4 and/or IL-13, and in turn polarizes immunity towards a type 2 response.

Because Stat6 protein is essential for responsiveness to IL-4 and IL-13, Stat6-deficient mice do not make significant amounts of type 2 $CD4^+$ T cells, and their $CD4^+$ T cells are polarized towards type 1 responses [15, 33]. This observation led to the suggestion that Stat6-deficient mice might have heightened immunosurveillance against tumors because their default type 1 response might provide more efficacious tumor immunity. Several studies have examined this assumption. Although there is uniform consensus that Stat6-deficient mice have dramatically enhanced anti-tumor immunity, there is no agreement on the mechanism(s) underlying the improved immunity, or that type 1 vs type 2 responses are responsible for the effect.

In this article we will summarize the data showing that Stat6-deficient mice have heightened immunosurveillance against transplanted primary tumors, spontaneous primary tumors, and metastatic disease. We will then discuss the mechanisms to which this enhanced immunity has been attributed.

Stat6-deficient mice are resistant to transplanted primary, solid tumors

Three independent BALB/c-derived tumors have been studied in Stat6-deficient BALB/c mice. These include the 15-12RM BALB/c fibrosarcoma [36], the P815 mastocytoma [14], and the 4T1 mammary carcinoma [12, 24, 25]. Studies with all three tumors noted reduction in primary tumor growth in Stat6-deficient vs wild-type BALB/c mice, although the magnitude of the response differed.

In the 15-12RM tumor system, tumor cells were transfected with HIV gp160 as a model antigen. Following s.c. inoculation into wild-type BALB/c mice, this transfected tumor initially grows, then regresses, and then recurs and grows progressively. Depletion of $CD4^+$ T cells protected BALB/c mice from recurrence of the tumor, suggesting that $CD4^+$ T cells were inhibiting the activity of $CD8^+$ effectors [19]. These investigators suspected that T_H cell subpopulations and/or their cytokines might be involved, so they inoculated Stat6-deficient mice with the 15-12RM tumor. As expected, in Stat6-deficient mice, the 15-12RM tumor initially grew and then permanently regressed, indicating that deletion of the Stat6 gene removed an inhibitor of immunosurveillence [36].

Stat6-deficient mice are also resistant to a mammary carcinoma, as originally reported by Ostrand-Rosenberg et al. [24]. Tumor 4T1 is a transplantable mammary carcinoma derived from BALB/c mice [1, 21]. It is very poorly immunogenic and spontaneously metastasizes following inoculation in the mammary gland [27, 28]. When a small number of 4T1 cells are inoculated in the abdominal mammary gland of Stat6-deficient mice, primary tumors grow, but growth is significantly retarded relative to growth in Stat6-competent, BALB/c mice. Antibody depletion experiments demonstrated that reduced growth requires $CD8^+$ T cells. Depletion studies also demonstrated that tumor resistance in Stat6-deficient mice did not involve $CD4^+$ T lymphocytes [24]. However, unlike the 15-12RM system, depletion of $CD4^+$ T cells in BALB/c mice did not result in improved anti-tumor immunity (Clements and Ostrand-Rosenberg, unpublished).

Despite the delayed growth of primary tumors in Stat6-deficient mice, as long as the primary tumor is left undisturbed, Stat6-deficient mice eventually die of metastatic disease, as do BALB/c mice [25]. As described below, if the primary tumor is surgically removed, then a high percentage of Stat6-deficient mice survive indefinitely, whereas $>90\%$ of Stat6-competent BALB/c mice die.

Jensen et al. [12] have recently confirmed the observations of Ostrand-Rosenberg and colleagues. However, they inoculated mice s.c. in the flank instead of in the abdominal mammary gland, and found complete rejection of 4T1 tumors by most Stat6-deficient mice. The difference in tumor growth between the two studies may be due to the difference in inoculation site. Perhaps a mammary tumor is less immunogenic in situ than when present ectopically. Regardless of this discrepancy, both studies demonstrate that Stat6-deficient mice have enhanced immunity to this mammary carcinoma.

Kacha et al. [14] have also found that growth of a primary tumor is diminished in Stat6-deficient mice. They used the P1.HTR tumor which is a P1A-expressing variant of the P815 mastocytoma that grows progressively in syngeneic DBA/2 mice [8]. Although P1.HTR tumors initially grow in Stat6-deficient mice, they rapidly regress while comparable tumors in wild-type DBA/2 mice grow progressively. Additional experiments using P1A-immunized mice and Stat1-deficient mice suggest that tumor regression is mediated by $CD8^+$ T cells and is IFN-γ-dependent. A possible complicating factor in interpreting these experiments is the potential genetic complexity of the Stat6-deficient mice used in the studies. Because P1.HTR is a DBA/2-derived tumor, the authors backcrossed BALB/c Stat6-deficient mice to DBA/2 mice for six generations, and then intercrossed the sixth generation to obtain "DBA/2 Stat6-deficient mice." In reality, these "DBA/2 Stat6-deficient mice" retain considerable BALB/c genetic material so they are not completely syngeneic with respect to the P1.HTR tumor. Indeed, minor histocompatibility differences between the Stat6-deficient mice and the P1.HTR may facilitate tumor rejection independent of the Stat6 effect.

Stat6-deficient mice reject spontaneous metastatic tumor cells and survive indefinitely

Immunity to disseminated metastatic cancer cells would be highly desirable since metastatic disease is often resistant to conventional therapies. To determine if the

Stat6 gene influences immunity to metastatic cancer, the 4T1 mammary carcinoma has been studied. Stat6-deficient and Stat6-competent BALB/c mice were inoculated with 4T1 in the mammary gland, and spontaneous metastases to the lungs, liver, brain, bone marrow, blood, and lymph nodes were allowed to develop. Mice were then sacrificed and the number of tumor cells in the lungs determined using a quantitative assay based on 4T1 resistance to 6-thioguanine [27]. Stat6-deficient mice had two–three logs fewer metastatic cells in their lungs compared with BALB/c mice. In vivo antibody deletion experiments showed that the reduction required $CD8^+$ T cells and was independent of $CD4^+$ T cells [24]. Similar studies using experimental metastases (i.v. inoculation of 4T1) also showed a reduction in lung metastases in Stat6-deficient mice [12].

Studies have also been done to determine if Stat6 deficiency increases survival time of mice with metastatic 4T1. Because mice with 4T1 primary tumors are globally immunosuppressed (Danna, Gilbert, Pulaski, and Ostrand-Rosenberg, submitted), 4T1 primary tumors were surgically removed after spontaneous metastatic disease was established, and mice were followed for survival. Sixty to ninety percent of Stat6-deficient mice survived >185 days under these conditions and >60% of mice had no detectable tumor cells in their lungs, liver, or bone marrow. In contrast, less than 10% of BALB/c mice survived [25] and 50–90% had metastatic cells in these organs. Therefore, deletion of the Stat6 gene provides potent protection against spontaneous metastatic disease and allows for long-term survival.

Stat6-deficient mice are resistant to spontaneously arising mammary tumors

Although enhanced resistance to transplanted solid tumors is strong evidence that Stat6 deficiency is protective, it does not necessarily follow that Stat6 deficiency allows for improved immunity to spontaneously arising tumors, and subsequent increased survival time. To test this hypothesis, Ostrand-Rosenberg and colleagues have studied the effects of Stat6 deficiency on mice that spontaneously develop mammary carcinoma.

There are several transgenic mouse models in which animals spontaneously develop mammary carcinoma. The inbred strain, BALB-NeuT, are transgenic mice that are heterozygous for the activated HER-2/*neu* oncogene under control of the mouse mammary tumor virus LTR. Female BALB/c NeuT mice spontaneously develop atypical mammary hyperplasia by approximately 10 weeks of age, carcinoma in situ by approximately 15 weeks of age, and palpable mammary carcinoma nodules by approximately 20 weeks of age [3, 18]. To determine if Stat6 deficiency provides enhanced immunity to spontaneous mammary carcinoma, Stat6 knockout ($Stat6^{-/-}$) mice were bred to BALB/c NeuT mice. Since the BALB-NeuT mice are $Stat6^{+/+}$ and $NeuT^{+/-}$, the F1s were screened for $NeuT^+$ and backcrossed to $Stat6^{-/-}$ to obtain $Stat6^{-/-}NeuT^{+/-}$ mice. The resulting $Stat6^{-/-}NeuT^{+/-}$ mice were then observed for tumor development and followed for survival time. In agreement with the studies with transplantable tumors, $Stat6^{-/-}NeuT^{+/-}$ mice have increased resistance to spontaneous disease. Relative to BALB/c NeuT mice, $Stat6^{-/-}NeuT^{+/-}$ mice live longer, develop mammary tumors later, and have fewer tumors (Ostrand-Rosenberg, Dissanayake, Miller, and Davis, unpublished results).

Possible mechanisms of resistance in $Stat6^{-/-}$ mice

Although there is strong experimental consensus that Stat6 deficiency allows for the development of potent anti-tumor immunity, there is little consensus on the mechanism(s) by which this immunity is enhanced. Most investigators believe that the Stat6 gene produces a factor that inhibits the development of anti-tumor immunity, so that when the Stat6 gene is deleted, successful immunosurveillance occurs. The following sections describe the mechanisms that have been proposed, and the data supporting and contradicting their involvement in tumor immunity.

Resistance requires IFN-γ

IFN-γ is a pleiotropic cytokine that regulates hundreds of genes, including many genes that regulate immunity. Several studies have shown that IFN-γ is involved in heightened immunity in Stat6-deficient mice. For example, tumor-primed draining lymph node cells of Stat6-deficient or CD1-deficient mice secrete higher levels of IFN-γ than lymph node cells from Stat6-competent mice [12, 14, 25, 36]. In addition, double deficient $Stat6^{-/-}IFN\gamma^{-/-}$ mice do not have heightened immunity to primary tumor, and die from metastatic disease with the same kinetics as Stat6-competent mice [25]. Therefore, IFN-γ is essential for enhanced immunity to primary, solid tumors, and for resistance to metastatic disease in Stat6-deficient mice.

IL-13 as an inhibitor of type 1 tumor immunity

As described above, Stat6 protein is essential for signal transduction through the IL-4R, and hence, Stat6-deficient individuals are not responsive to IL-4 and/or IL-13. This observation has led Terabe et al. [36] to hypothesize that IL-13 is an inhibitor that blocks the development of anti-tumor immunity, and that Stat6-deficient mice have enhanced tumor immunity because they are not responsive to IL-13.

The role of IL-13 as an inhibitor is supported by several additional observations made by Terabe et al. They first demonstrated that deletion of IL-4 alone is not sufficient for enhanced immunity because the

15-12RM tumor recurred in IL-4-deficient mice. In contrast, the tumor did not recur in IL-4R mice, suggesting that a cytokine other than IL-4, but acting through the IL-4R, inhibited anti-tumor immunity. The logical candidate was IL-13, since it also binds to the IL-4R. To determine if IL-13 is an inhibitor, Terabe et al. treated wild-type and IL-4-deficient BALB/c mice with a soluble competitor for IL-13 (sIL-13Rα2-Fc), before and after inoculation with 15-12RM tumor cells. Tumor recurrence did not occur in mice treated with the IL-13 inhibitor, indicating that IL-13 is a potent blocker of immunity to solid, subcutaneous tumor [36]. These investigators also found that the 15-12RM tumor does not recur in CD1-deficient BALB/c mice, indicating that CD1-deficient mice also have enhanced anti-tumor immunity. CD1 is a nonclassical MHC class I molecule that binds and presents glycolipids to NKT cells, which are a rich source of IL-13 [13]. Based on these results, Terabe et al. proposed that CD1 mice are resistant to tumor growth because they lack NKT cells and hence do not make IL-13. Taken together, these data indicate that IL-13 produced by $CD4^+$ NKT cells inhibits immunosurveillance and that Stat6-deficient mice have enhanced immunity because they cannot signal through the Stat6 pathway, and hence do not respond to IL-13 [36].

To determine if IL-13 acts as an inhibitor in the 4T1 tumor system, 4T1 growth was studied in $CD1^{-/-}$ mice. Although a very high percentage of CD1-deficient mice survived 4T1 challenge after surgical removal of primary tumor, neither primary tumor growth nor metastatic disease was inhibited by treatment with the IL-13 inhibitor. Additional experiments in IL-4-deficient mice [25] and in mice nonresponsive or deficient to both IL-4 and IL-13 (Clements and Ostrand-Rosenberg, unpublished) demonstrated that simultaneous elimination of both IL-4 and IL-13 responsiveness also did not yield tumor-resistant animals.

Therefore, although IL-13 appears to play a critical negative regulatory role in immunity to the 15-12RM fibrosarcoma, IL-13 alone is not responsible for inhibiting immunity to the 4T1 mammary carcinoma.

Reversal of myeloid suppressor cell levels in Stat6-deficient and CD1-deficient mice

Tumor-mediated immune suppression is common in individuals with malignancies [23], and surgical removal of the tumor frequently reverses the suppression [30]. Indeed, the 4T1 mammary carcinoma induces a strong global immunosuppression of both B- and T-cell responses within 3 weeks of inoculation (Danna, Gilbert, Ostrand-Rosenberg, manuscript in preparation). Because Stat6-deficient mice whose primary tumors have been surgically removed have a very high survival rate, Sinha and colleagues have suggested that Stat6-deficiency may favor a very rapid recovery from immune suppression. They have specifically focused on suppression by myeloid suppressor cells (MSCs) because MSC blockade of anti-tumor immunity is widespread in tumor bearers [4, 31].

In tumor-free mice less than 8% of splenocytes are MSCs, as measured by flow cytometry using Gr-1 and CD11b antibodies. In Stat6-competent, Stat6-deficient, or CD1-deficient mice with established 4T1 primary tumors, MSC levels are similar, and can be up to 50% of splenocytes. However, following surgical removal of primary 4T1 tumors, MSC levels in most Stat6-deficient and CD1-deficient mice regress rapidly, while MSC levels in Stat6-competent mice remain elevated. The percentage of postsurgery Stat6-deficient and CD-1-deficient mice with low levels of MSCs agrees well with the number of these mice that survive indefinitely after primary tumor is resected. The reduction in MSC is IFN-γ-dependent, since MSC levels do not revert to normal in $Stat6^{-/-}IFN\gamma^{-/-}$ mice (Sinha, Danna, Clements, and Ostrand-Rosenberg, unpublished). Therefore, a rapid regression of MSCs after surgery in Stat6-deficient and CD1-deficient mice correlates with survival, suggesting that Stat6 deficiency or CD1 deficiency can overcome immune suppression provided the bulky primary tumor is removed.

Alternatively, rather than causing enhanced immunity, the reduced number of MSCs in Stat6-deficient and CD1-deficient mice may be the result of decreased tumor burden. Interestingly, following surgical removal of primary tumor, Stat6-deficient mice have relatively low levels of metastatic cells, while CD1-deficient mice have very high levels of metastatic cells in the lungs (Sinha, Danna, Clements, and Ostrand-Rosenberg, unpublished). Since both strains have very low levels of MSCs and survive, a reduction in MSCs alone is not sufficient for reducing metastatic disease.

Additional experiments are needed to clarify the role of MSCs in survival and reduction of metastatic disease. For example, to determine if MSC levels are the cause or effect of increased survival, it will be necessary to adoptively transfer MSCs from BALB/c mice into Stat6-deficient mice that have low levels of endogenous MSCs, and follow these individuals for tumor progression.

$CD4^+CD25^+$ T regulatory cells are not responsible for enhanced immunity

$CD4^+CD25^+$ T regulatory cells suppress the activation of $CD8^+$ T cells by blocking the production of IL-2 [32]. These cells are critical for preventing autoimmunity [26, 29] and for inhibiting anti-tumor immunity [35]. In several tumor systems, enhanced anti-tumor immunity and subsequent tumor regression have been attributed to removal of $CD4^+CD25^+$ T regulatory cells (Wei et al., this volume). However, in vivo antibody depletion of $CD4^+CD25^+$ T cells from Stat6-competent BALB/c mice had no effect on 4T1 primary tumor growth or progression of metastatic disease [25]. Therefore, Stat6-deficient mice do not have heightened tumor immunity because they are deficient for $CD4^+CD25^+$ T cells.

Other mechanisms

Jenson and colleagues [12] have suggested that Stat6-deficient mice have heightened immunity because they lack Stat6 protein and hence respond to Stat6 protein of tumors as a "foreign antigen." They make a similar argument for CD1-deficient mice and CD1 protein (B. Fox, personal communication). All of the transplanted tumors studied in Stat6-deficient mice (4T1, 15-12RM, P815) express Stat6 protein ([12]; Clements and Ostrand-Rosenberg, unpublished); however, the 4T1 and 15-12RM tumors do not express CD1 protein (Terabe and Berzofsky, unpublished). Likewise, the spontaneous tumors of $Stat6^{-/-}NeuT^{+/-}$ mice do not contain Stat6 protein. In addition, CTLs from 4T1-immunized Stat6-deficient mice are not cytotoxic for other $H\text{-}2^{d}$ tumors that express Stat6 protein (e.g., P815 tumor) (Clements and Ostrand-Rosenberg, unpublished). If the effective immunity in Stat6-deficient mice were specific for Stat6 protein, then one would expect to find significant cross-reactivity to other MHC-matched, Stat6-expressing cells.

Jensen et al. find complete rejection of 4T1 primary tumors at doses for which Ostrand-Rosenberg and colleagues predominantly find only reduced growth rates [24, 25]. The apparent increased immunogenicity of the 4T1 tumor in the experiments of Jensen et al. could be due to divergence in the 4T1 tumors between the two labs. If the Jensen et al. variant contains more Stat6 protein, this might explain their findings of heightened Stat6-peptide reactivity in immunized mice. Therefore, although Jensen et al. find strong Stat6-peptide-specific reactivity in 4T1-immunized mice, it is unlikely that reactivity to the deleted protein is responsible for the increased immunosurveillance seen in Stat6-deficient or CD1-deficient mice.

Conclusions

The enhanced immunosurveillance of Stat6-deficient and CD1-deficient mice is effective in reducing primary tumor growth, in preventing recurrence of primary tumor, and in mediating rejection of established, metastatic disease. Indeed, the indefinite survival of mice with established, disseminated metastatic disease, and the lack of recurrence of primary tumors demonstrate that Stat6-deficiency may be a potent strategy for immunotherapy. Whether this immunity is the result of polarization towards a type 1 response remains unclear. Given the differences between the various tumor systems studied, it appears that the Stat6 protein may affect tumor immunity via multiple, divergent mechanisms. A better understanding of the mechanism(s) responsible for the dramatic reductions in tumor growth should be a high priority, since this knowledge could lead to effective, novel immunotherapies.

Acknowledgements The authors thank Dr Guido Forni for generously providing breeding pairs of the BALB/c NeuT mice used in these studies. We also thank Dr Beth Pulaski for setting up the BALB/c NeuT $\times$ $Stat6^{-/-}$ matings, and Ms Sandy Mason for her capable care of the mice.

References

1. Aslakson C, Miller F (1992) Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 52:1399
2. Bellone G, Turletti A, Artusio E, Mareschi K, Carbone A, Tibaudi D, Robecchi A, Emanuelli G, Rodeck U (1999) Tumor-associated transforming growth factor-beta and interleukin-10 contribute to a systemic Th2 immune phenotype in pancreatic carcinoma patients. Am J Pathol 155:537
3. Boggio K, Nicoletti G, Di Carlo E, Cavallo F, Landuzzi L, Melani C, Giovarelli M, Rossi I, Nanni P, De Giovanni C, Bouchard P, Wolf S, Modesti A, Musiani P, Lollini PL, Colombo MP, Forni G (1998) Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. J Exp Med 188:589
4. Bronte V, Serafini P, Apolloni E, Zanovello P (2001) Tumor-induced immune dysfunctions caused by myeloid suppressor cells. J Immunother 24:431
5. Constant S, Bottomly K (1997) Induction of the TH1 and TH2 $CD4^{+}$ T cell responses: alternative approaches. Annu Rev Immunol 15:297
6. Dobrzanski MJ, Reome JB, Dutton RW (1999) Therapeutic effects of tumor-reactive type 1 and type 2 CD8+ T cell subpopulations in established pulmonary metastases. J Immunol 162:6671
7. Dobrzanski MJ, Reome JB, Dutton RW (2001) Role of effector cell-derived IL-4, IL-5, and perforin in early and late stages of type 2 CD8 effector cell-mediated tumor rejection. J Immunol 167:424
8. Gajewski TF, Fallarino F, Uyttenhove C, Boon T (1996) Tumor rejection requires a CTLA4 ligand provided by the host or expressed on the tumor: superiority of B7–1 over B7–2 for active tumor immunization. J Immunol 156:2909
9. Heim M (1999) The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. J Recept Signal Transduct Res 19:75
10. Hu HM, Urba WJ, Fox BA (1998) Gene-modified tumor vaccine with therapeutic potential shifts tumor-specific T cell response from a type 2 to a type 1 cytokine profile. J Immunol 161:3033
11. Ihle J, Nosaka T, Thierfelder W, Quelle F, Shimoda K (1997) Jaks and Stats in cytokine signaling. Stem Cells [Supp 1]:105
12. Jensen SM, Meijer SL, Kurt RA, Urba WJ, Hu HM, Fox BA (2003) Regression of a mammary adenocarcinoma in STAT6-/- mice is dependent on the presence of STAT6-reactive T cells. J Immunol 170:2014
13. Joyce S, Van Kaer L (2003) CD1-restricted antigen presentation: an oily matter. Curr Opin Immunol 15:95
14. Kacha AK, Fallarino F, Markiewicz MA, Gajewski TF (2000) Cutting edge: spontaneous rejection of poorly immunogenic P1.HTR tumors by Stat6-deficient mice. J Immunol 165:6024
15. Kaplan M, Grusby M (1998) Regulation of T helper cell differentiation by STAT molecules. J Leukoc Biol 64:2
16. Kobayashi M, Kobayashi H, Pollard RB, Suzuki F (1998) A pathogenic role of Th2 cells and their cytokine products on the pulmonary metastasis of murine B16 melanoma. J Immunol 160:5869
17. Liu K, Gaffen S, Goldsmith M (1998) JAK/STAT signaling by cytokine receptors. Curr Opin Immunol 10:271
18. Lucchini F, Sacco MG, Hu N, Villa A, Brown J, Cesano L, Mangiarini L, Rindi G, Kindl S, Sessa F et al (1992) Early and multifocal tumors in breast, salivary, harderian and epididymal

tissues developed in MMTY-Neu transgenic mice. Cancer Lett 64:203
19. Matsui S, Ahlers JD, Vortmeyer AO, Terabe M, Tsukui T, Carbone DP, Liotta LA, Berzofsky JA (1999) A model for CD8+ CTL tumor immunosurveillance and regulation of tumor escape by CD4 T cells through an effect on quality of CTL. J Immunol 163:184
20. Mattes J, Hulett M, Xie W, Hogan S, Rothenberg ME, Foster P, Parish C (2003) Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. J Exp Med 197:387
21. Miller F, Miller B, Heppner G (1983) Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. Invasion Metastasis 3:22
22. Murata T, Obiri NI, Puri RK (1998) Structure of and signal transduction through interleukin-4 and interleukin-13 receptors (review). Int J Mol Med 1:551
23. North RJ, Digiacom A, Dye E (1987) Suppression of antitumor immunity. In: Den Otter W, Ruitenberg EJ (eds) Tumor immunology: mechanisms, diagnosis, therapy. Elsevier, Amsterdam, p 125
24. Ostrand-Rosenberg S, Grusby MJ, Clements VK (2000) Cutting edge: STAT6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. J Immunol 165:6015
25. Ostrand-Rosenberg S, Clements VK, Terabe M, Park JM, Berzofsky JA, Dissanayake SK (2002) Resistance to metastatic disease in STAT6-deficient mice requires hemopoietic and nonhemopoietic cells and is IFN-gamma dependent. J Immunol 169:5796
26. Piccirillo CA, Shevach EM (2001) Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. J Immunol 167:1137
27. Pulaski B, Ostrand-Rosenberg S (1998) MHC class II and B7.1 immunotherapeutic cell-based vaccine reduces spontaneous mammary carcinoma metastases without affecting primary tumor growth. Cancer Res 58:1486
28. Pulaski B, Ostrand-Rosenberg S (2000) Mouse 4T1 breast tumor model. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (eds) Current protocols in immunology. Wiley, New York, p 20.2.1
29. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155:1151
30. Salvadori S, Martinelli G, Zier K (2000) Resection of solid tumors reverses T cell defects and restores protective immunity. J Immunol 164:2214
31. Serafini P, De Santo C, Marigo I, Cingarlini S, Dolcetti L, Gallina G, Zanovello P, Bronte V (2003) Derangement of immune responses by myeloid suppressor cells. Cancer Immunol Immunother (in press)
32. Shevach EM (2002) CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389
33. Shimoda K, van Deursen J, Sangster M, Sarawar S, Carson R, Tripp R, Chu C, Quelle F, Nosaka T, Vagnali D, Doherty P, Grosveld G, Paul W, Ihle J (1996) Lack of IL-4-induced Th2 response and IgE class switching in mice with distrupted STAT6 gene. Nature 380:630
34. Shurin M, Lu L, Kalinski P, Stewart-Akers A, Lotze M (1999) Th1/Th2 balance in cancer, transplantation and pregnancy. Springer Semin Immunopathol 21:339
35. Sutmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP, Toes RE, Offringa R, Melief CJ (2001) Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 194:823
36. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, Carbone DP, Paul WE, Berzofsky JA (2000) NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol 1:515

APPENDIX 8

Tumor Immunity: A Balancing Act Between T Cell Activation, Macrophage Activation and Tumor-induced Immune Suppression

by

Pratima Sinha, Virginia K. Clements, Seth Miller, and Suzanne Ostrand-Rosenberg

Department of Biological Sciences

University of Maryland Baltimore County

Baltimore, MD 21250

Corresponding author: S. Ostrand-Rosenberg, Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250; <srosenbe@umbc.edu>, 410 455-2237 (voice); 410 455-3875 (FAX)

Key Words: tumor-induced immune suppression, immune surveillance, M1 macrophages, metastatic breast cancer, cell-mediated tumor immunity

SUMMARY

The mouse 4T1 mammary carcinoma is a BALB/c-derived tumor that spontaneously metastasizes and induces immune suppression. Although >95% of wild type BALB/c mice die from metastatic 4T1 tumor even if the primary mammary tumor is surgically removed, >65% of BALB/c mice with a deleted Signal Transducer Activator of Transcription 6 (STAT6) gene survive post-surgery. STAT6-deficiency also confers enhanced immunity against spontaneously developing breast cancer since NeuT$^{+/-}$ mice that are STAT6-deficient develop mammary tumors later and survive longer than NeuT$^{+/-}$ mice that are STAT6-competent. Rejection of metastastic disease and survival of STAT6-deficient mice after removal of primary tumor involve three mechanisms: i) The generation of M1 type macrophages that produce nitric oxide and are tumoricidal; ii) A decrease to normal in the elevated levels of myeloid suppressor cells that accumulate during primary tumor growth; and iii) CD8^{+} tumor-specific T lymphocytes. STAT6-deficient, but not wild type BALB/c, mice generate nitric oxide producing macrophages because they lack the STAT6 transcription factor which is necessary for signaling through the type 2 IL-4Rα complex, and which induces the production of arginase instead of nitric oxide.

Signal Transducer Activator of Transcription 6 deficient (STAT6$^{-/-}$) mice have enhanced immunity to transplanted tumors.

The STAT6 gene transmits IL-4 and IL-13 signals via the IL-4R$\alpha^{-/-}$ and is required for the generation of CD4^{+} Th2 lymphocytes. As a result, STAT6$^{-/-}$ mice have their CD4^{+} T cells polarized towards a Type 1 phenotype [11]. We [21] and others [10, 30] have hypothesized that STAT$^{-/-}$ mice might have enhanced immunity because they preferentially generate CD4^{+} Th1 cells

that faciliate $CD8^+$-mediated tumor rejection. Studies conducted in multiple laboratories using three different transplanted tumors (mammary carcinoma, fibrosarcoma, and mastocytoma) demonstrated that $STAT6^{-/-}$ mice have heightened tumor immunity [9, 10, 21, 30]. Our studies used the BALB/c-derived mouse 4T1 mammary carcinoma [18]. This tumor closely models human breast cancer in its growth in the mammary gland, its pattern of disease progression, and it's ability to metastasize to a variety of target organs (brain, bone marrow, liver, lungs, blood, lymph nodes) while the primary tumor is present, as well as after the primary tumor is surgically removed [23, 24]. Tumor resistance of $STAT6^{-/-}$ mice was particularly effective after primary mammary tumors were excised, with >65% of $STAT6^{-/-}$ mice surviving indefinitely, while >95% of wild type BALB/c mice died from metastatic disease [22].

$STAT6^{-/-}$ mice have enhanced resistance to spontaneously arising mammary carcinoma.

$NeuT^{+/-}$ mice are transgenic for the transforming rat her2/neu gene and spontaneously develop multifocal and metastatic mammary carcinoma starting at approximately week 6-8 of age [2]. To determine if deletion of the STAT6 gene also protects against spontaneous cancer, $neuT^{+/-}$ males were crossed to $STAT6^{-/-}$ females and the female F1's PCR screened and selected for neuT expression ($neuT^{+/-}$). These heterozygotes ($STAT6^{+/-}neuT^{+/-}$) were then backcrossed to $STAT6^{-/-}$ females, and the offspring PCR screened for neuT expression and homozygous deletion of STAT6 ($STAT6^{-/-}neuT^{+/-}$ mice). Female $STAT6^{-/-}neuT^{+}$ mice were then observed weekly for a minimum of six months for mammary tumor development and survival. As seen in **Figure 1A,** mammary tumor onset, diameter (TD) of individual tumors, and total tumor mass is delayed in $STAT6^{-/-}neuT^{+/-}$ mice vs. STAT6-competent $neuT^{+/-}$ mice. Similarly, the survival time of $STAT6^{-}$

$^{/-}$neuT$^{+/-}$ mice is statistically longer than that of neuT$^{+/-}$ mice by approximately one month (**Figure 1B**). Therefore, deletion of the STAT6 gene facilitates rejection of metastatic disease, and also promotes survival of mice with spontaneous mammary carcinoma.

Myeloid-derived suppressor cells inhibit T cell activation and immunity in mice with large, primary mammary tumors.

Myeloid-derived cells that suppress the immune system have been identified in many patients and experimental animals with tumors [1, 4, 8, 12, 14, 27]. These so-called myeloid suppressor cells (MSC) are immature myeloid cells that suppress the activation of CD4$^+$ and CD8$^+$ T lymphocytes and thereby inhibit immune surveillance [3, 8, 15, 17]. The accumulation of MSC in the spleen and blood of tumor-bearing individuals is associated with increased tumor burden. Since removal of primary 4T1 tumor partially restores immunocompetence [5], we have hypothesized that immunity in STAT6$^{-/-}$ mice with primary tumor is inhibited by the presence of MSC. To test this hypothesis, BALB/c and STAT6$^{-/-}$ mice were inoculated with 4T1 tumor in the mammary gland and their splenocytes tested by flow cytometry for the presence of Gr1$^+$CD11b$^+$ MSC. In some groups, the primary tumor was surgically removed according to the schedule shown in **Figure 2A,** and 10-12 days after surgery spleens were removed and tested for MSC. Mice that were never exposed to tumor have less than 8% Gr1$^+$CD11b$^+$ cells in their spleens. In contrast, BALB/c and STAT6$^{-/-}$ mice with primary 4T1 mammary carcinomas have 30-60% Gr1$^+$CD11b$^+$ splenocytes. Although these levels decline after surgery, 80% of BALB/c mice retain elevated levels of MSC, while only 33% of STAT6$^{-/-}$ mice have above normal levels of Gr1$^+$CD11b$^+$ splenocytes (**figure 2B**). Therefore, the retention of high levels of MSC after

surgery is associated with shortened survival, while a decrease to baseline levels of MSC is associated with resistance to metastatic disease

Although a reduction in MSC after removal of primary tumor is associated with resistance to metastatic disease, it alone is not sufficient for resistance since BALB/c mice treated with all trans retinoic acid [13] have greatly reduced levels of MSC, but still die from metastatic 4T1 [29]. This finding has led us to examine other effector mechanisms that might be responsible for resistance of $STAT6^{-/-}$ mice.

$CD8^+$ T cells are required for immunity to metastatic disease in post-surgery $STAT6^{-/-}$ mice.

In earlier studies we noted that $STAT6^{-/-}$ mice have a modest immune response against primary tumor, and in vivo antibody depletion experiments demonstrated that this immunity was mediated by $CD8^+$ T cells, and that $CD4^+$ T cells were not involved [21]. The lack of involvement of $CD4^+$ T cells was surprising and demonstrated that our original hypothesis that heightened immunity was due to polarization towards a type 1 $CD4^+$ T cell response was incorrect. In addition, depletion of $CD4^+CD25^+$ T regulatory cells had no impact on 4T1 tumor growth in BALB/c mice, demonstrating that regulatory T cells were also not involved [22].

Since immunity after removal of primary tumor is much more effective than immunity in mice with primary tumor in place, we have also monitored T cell activity in post-surgery $STAT6^{-/-}$ mice that are resistant to 4T1 metastatic disease. $STAT6^{-/-}$ and control BALB/c mice were inoculated in the mammary gland with 4T1 cells according to the schedule shown in **Figure 2A**, and concomitantly in vivo depleted for $CD4^+$ or $CD8^+$ T cells using antibodies to CD4 and CD8 as previously described [21]. All BALB/c mice died by day 47, regardless of antibody treatment,

and all CD8-depleted STAT6$^{-/-}$ mice died by day 66. In contrast, all of the CD4-depleted STAT6$^{-/-}$ mice survived (**figure 2C**, left-hand panel). Therefore, CD8^{+}, but not CD4^{+}, T cells are essential for immunity to metastatic disease in STAT6$^{-/-}$ mice.

Cytotoxic nitric oxide producing M1 macrophages are required for immunity to metastatic disease in post-surgery STAT6$^{-/-}$ mice.

Macrophages can also be key players in tumor immunity. Macrophages polarized towards an M1 phenotype produce nitric oxide (NO) and are cytotoxic for tumor cells, whereas M2 macrophages produce arginase which facilitates tumor growth and progression [16, 19, 20]. Since earlier studies demonstrated that macrophages are involved in immune surveillance against the 4T1 tumor [25], we have examined the role of macrophages in STAT6$^{-/-}$ mice. To determine if macrophages are required for resistance to metastatic 4T1 tumor, STAT6$^{-/-}$ mice were inoculated with 4T1 cells and primary tumors removed and mice followed for survival according to the schedule shown in **figure 2A**. One group of mice was also treated with carrageenan, which depletes for phagocytic cells such as macrophages [29]. Macrophage/phagocytic cell depletion was monitored by measuring reduced susceptibility to lipopolysaccharide-induced toxic shock [25]. Seventy-five percent of the non-carrageenan treated STAT6$^{-/-}$ mice survived; whereas only 45% of the carrageenan-treated mice survived (**figure 2C**, right-hand panel). Mice in the carrageenan-treated group also developed more rapidly growing tumors than the mice in the non-carrageenan-treated group. Therefore, macrophages appear to be required for resistance to metastatic disease in STAT6$^{-/-}$ mice.

Since M1 macrophages are associated with tumor regression while M2 macrophages are

associated with tumor progression, we have analyzed the phenotype of macrophages from tumor-bearing and post-surgery BALB/c and STAT6$^{-/-}$ mice. Although non-activated bone marrow-derived macrophages (BMDM) from either strain had no NO or arginase activity, lipopolysaccharide and IFNγ-activated macrophages from STAT6$^{-/-}$ mice made high levels of NO, while activated macrophages from BALB/c mice produced arginase [29]. Therefore, STAT6$^{-/-}$ mice produce M1 macrophages which are essential for resistance to established metastatic disease, while BALB/c mice which are not resistant, produce M2 macrophages.

STAT6$^{-/-}$ mice generate M1 macrophages because they cannot transmit IL-13 signals which polarize macrophages towards an M2 phenotype.

The production of arginase, which is a characteristic of M2 macrophages, is induced by IL-4 and/or IL-13 when these cytokines bind to the IL-4Rα$^{-/-}$ and signal through the JAK3/STAT6 pathway [26, 31]. Since STAT6$^{-/-}$ mice are deficient for STAT6, this signaling pathway is inoperative in STAT6$^{-/-}$ mice. Hence, arginase production does not occur. In other studies we have observed that IL-4Rα$^{-/-}$ mice, which also cannot transmit IL-4 and/or IL-13 signals because they lack the requisite receptor, make M1 macrophages that produce NO (Sinha and Ostrand-Rosenberg, unpublished results). Interestingly, although IL-4Rα$^{-/-}$ mice make M1 macrophages, they are not resistant to metastatic 4T1 tumor because they retain high levels of MSC after removal of primary tumor (Sinha and Ostrand-Rosenberg, unpublished results). Therefore, STAT6$^{-/-}$ mice have M1 macrophages because they lack the signaling machinery to stimulate arginase production; however, the generation of M1 macrophages without concomitant reduction in MSC is not sufficient for resistance to metastatic disease.

IFNγ is essential for resistance and is required for the reduction in MSC and may be required for the activation of M1 macrophages.

IFNγ is a pleiotropic cytokine that affects a wide variety of genes and is instrumental in immune surveillance [6, 7, 28]. To determine if IFNγ is also required for resistance to metastatic disease in $STAT6^{-/-}$ mice we have crossed $STAT6^{-/-}$ mice with BALB/c $IFN\gamma^{-/-}$ mice and intercrossed the F1's to obtain double knockout $STAT6^{-/-}IFN\gamma^{-/-}$mice. The $STAT6^{-/-}IFN\gamma^{-/-}$mice were then inoculated with 4T1 in the mammary fat pad, primary tumors removed and mice followed for survival according to the schedule shown in **Figure 2A**. Not surprisingly, the $STAT6^{-/-}IFN\gamma^{-/-}$mice have the same survival times as wild type BALB/c mice, indicating that IFNγ is essential for $STAT6^{-/-}$ resistance to metastatic disease (**figure 2B**).

Experiments tracking MSC in $STAT6^{-/-}$ mice demonstrate that the decrease to normal levels after removal of primary tumor is dependent on IFNγ because MSC levels remain highly elevated in post-surgery $STAT6^{-/-}IFN\gamma^{-/-}$ mice [29]. In addition to its role in reducing MSC, IFNγ may also drive M1 macrophage production in $STAT6^{-/-}$ mice since it is required in vitro to activate macrophages from $STAT6^{-/-}$ mice [29]. Therefore, IFNγ appears to be a critical regulatory molecule in the induction of resistance to metastatic disease and it mediates it's effects by reducing MSC levels and activating M1 macrophages.

Concluding remarks.

Figure 3 shows a schematic model of how M1 macrophages, MSC levels, and activated $CD8^{+}$ T cells may interact to provide effective immune surveillance against metastatic disease. Under ideal conditions, tumor antigens of primary tumor cells would be processed and presented

by professional antigen presenting cells (APC) and activate tumor-specific $CD8^+$ T cells. However, many tumors, including the 4T1 mammary carcinoma, produce cytokines and/or growth factors that up-regulate $Gr1^+CD11b^+$ MSC in both BALB/c and $STAT6^{-/-}$ mice. The MSC produce arginase and reactive oxygen species (ROS) which then inhibit T cell activation; thereby blocking immune surveillance and favoring tumor progression. Concomitantly, in BALB/c mice IL-4 and IL-13 induce the production of M2 macrophages which also promote tumor progression. In contrast, $STAT6^{-/-}$ mice generate M1 macrophages because they lack the machinery to transmit IL-4 and/or IL-13 signals. Although the M1 macrophages are cytotoxic for tumor cells, they alone are insufficient for tumor rejection. When primary tumor is surgically removed, the quantities of tumor-produced cytokines and/or growth factors decrease and the levels of MSC decrease to baseline in $STAT6^{-/-}$ mice, permitting tumor-specific $CD8^+$ T cells to differentiate. However, the level of MSC does not decrease sufficiently in BALB/c mice after surgery so tumor-specific $CD8^+$ T cells do not develop. The combination of activated, tumor-specific $CD8^+$ T cells and M1 macrophages in $STAT6^{-/-}$ mice is then sufficient to mediate complete rejection of metastatic disease. Therefore, effective immune surveillance requires a decrease to baseline levels of MSC coupled with the activation of tumor-specific $CD8^+$ T cells and cytotoxic M1 macrophages.

ACKNOWLEDGMENTS

We thank Ms. Cordula Davis for help monitoring tumor progression in the $neuT^{+/-}$ and $STAT6^{-/-}neuT^{+/-}$ mice and Dr. Beth Pulaski for performing the carrageenan experiment. We appreciate the excellent care given to our mouse colony by Ms. Sandra Mason. These studies were supported by NIH grants R01 CA52527 and R01 CA84232, and by U.S. Army Breast Cancer Program Grant DAMD-17-01-1-0312.

REFERENCES

1. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP,Gabrilovich DI (2001) Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol 166:678.

2. Boggio K, Nicoletti G, Di Carlo E, Cavallo F, Landuzzi L, Melani C, Giovarelli M, Rossi I, Nanni P, De Giovanni C, Bouchard P, Wolf S, Modesti A, Musiani P, Lollini PL, Colombo MP,Forni G (1998) Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. J Exp Med 188:589.

3. Bronte V, Apolloni E, Cabrelle A, Ronca R, Serafini P, Zamboni P, Restifo NP,Zanovello P (2000) Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. Blood 96:3838.

4. Bronte V, Serafini P, Apolloni E,Zanovello P (2001) Tumor-induced immune dysfunctions caused by myeloid suppressor cells. J Immunother 24:431.

5. Danna EA, Sinha P, Gilbert M, Clements VK, Pulaski BA,Ostrand-Rosenberg S (2004) Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease. Cancer Res 64:2205.

6. Dunn GP, Bruce AT, Ikeda H, Old LJ,Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3:991.

7. Dunn GP, Old LJ,Schreiber RD (2004) The immunobiology of cancer immunosurveillance and immunoediting. Immunity 21:137.

8. Gabrilovich DI, Velders MP, Sotomayor EM,Kast WM (2001) Mechanism of immune

dysfunction in cancer mediated by immature Gr-1+ myeloid cells. J Immunol 166:5398.

9. Jensen SM, Meijer SL, Kurt RA, Urba WJ, Hu HM,Fox BA (2003) Regression of a mammary adenocarcinoma in STAT6-/- mice is dependent on the presence of STAT6-reactive T cells. J Immunol 170:2014.

10. Kacha AK, Fallarino F, Markiewicz MA,Gajewski TF (2000) Cutting edge: spontaneous rejection of poorly immunogenic P1.HTR tumors by Stat6-deficient mice. J Immunol 165:6024.

11. Kaplan MH, Schindler U, Smiley ST,Grusby MJ (1996) Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity 4:313.

12. Kusmartsev S,Gabrilovich DI (2002) Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother 51:293.

13. Kusmartsev S, Cheng F, Yu B, Nefedova Y, Sotomayor E, Lush R,Gabrilovich D (2003) All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. Cancer Res 63:4441.

14. Kusmartsev S,Gabrilovich DI (2003) Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. J Leukoc Biol 74:186.

15. Liu Y, Van Ginderachter JA, Brys L, De Baetselier P, Raes G,Geldhof AB (2003) Nitric oxide-independent CTL suppression during tumor progression: association with arginase-producing (M2) myeloid cells. J Immunol 170:5064.

16. Mantovani A, Sozzani S, Locati M, Allavena P,Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23:549.

17. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, Zanovello P,Segal DM (2002) Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol 168:689.

18. Miller FR, Miller BE,Heppner GH (1983) Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. Invasion Metastasis 3:22.

19. Mills CD, Kincaid K, Alt JM, Heilman MJ,Hill AM (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol 164:6166.

20. Mills CD (2001) Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. Crit Rev Immunol 21:399.

21. Ostrand-Rosenberg S, Grusby MJ,Clements VK (2000) Cutting edge: STAT6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. J Immunol 165:6015.

22. Ostrand-Rosenberg S, Clements VK, Terabe M, Park JM, Berzofsky JA,Dissanayake SK (2002) Resistance to metastatic disease in STAT6-deficient mice requires hemopoietic and nonhemopoietic cells and is IFN-gamma dependent. J Immunol 169:5796.

23. Pulaski BA, Clements VK, Pipeling MR,Ostrand-Rosenberg S (2000a) Immunotherapy with vaccines combining MHC class II/CD80+ tumor cells with interleukin-12 reduces established metastatic disease and stimulates immune effectors and monokine induced by interferon gamma. Cancer Immunol Immunother 49:34.

24. Pulaski BA, Terman DS, Khan S, Muller E,Ostrand-Rosenberg S (2000b) Cooperativity of Staphylococcal aureus enterotoxin B superantigen, major histocompatibility complex

class II, and CD80 for immunotherapy of advanced spontaneous metastases in a clinically relevant postoperative mouse breast cancer model. Cancer Res 60:2710.

25. Pulaski BA, Smyth MJ,Ostrand-Rosenberg S (2002) Interferon-gamma-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma. Cancer Res 62:4406.

26. Rutschman R, Lang R, Hesse M, Ihle JN, Wynn TA,Murray PJ (2001) Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. J Immunol 166:2173.

27. Serafini P, De Santo C, Marigo I, Cingarlini S, Dolcetti L, Gallina G, Zanovello P,Bronte V (2004) Derangement of immune responses by myeloid suppressor cells. Cancer Immunol Immunother 53:64.

28. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ,Schreiber RD (2001) IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 410:1107.

29. Sinha P, Clements VK,Ostrand-Rosenberg S (2005) Reduction of myeloid-derived suppressor cells and induction of m1 macrophages facilitate the rejection of established metastatic disease. J Immunol 174:636.

30. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, Carbone DP, Paul WE,Berzofsky JA (2000) NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol 1:515.

31. Wei LH, Jacobs AT, Morris SM, Jr.,Ignarro LJ (2000) IL-4 and IL-13 upregulate arginase I expression by cAMP and JAK/STAT6 pathways in vascular smooth muscle cells. Am J Physiol Cell Physiol 279:C248.

Figure Captions

Figure 1. Deletion of the STAT6 gene delays tumor progression and extends survival time of mice that spontaneously develop mammary carcinoma. $NeuT^{+/-}$ mice, which spontaneously develop multifocal breast cancer, were crossed and backcrossed to $STAT6^{-/-}$ mice to obtain $STAT6^{-/-}neuT^{+/-}$ mice. The $STAT6^{-/-}neuT^{+/-}$ and $neuT^{+/-}$ mice were observed weekly for **(A)** the number of primary mammary tumors per mouse, the mean tumor diameter (TD) of individual tumors, and the sum of the diameters of all tumors per mouse; and **(B)** survival time.

Figure 2. Resistance to metastatic mammary carcinoma requires M1 macrophages and $CD8^+$ T cells and is counter-acted by myeloid suppressor cells (MSC). **(A)** Mice are inoculated in the mammary gland on day 0 with 7000 4T1 mammary carcinoma cells; primary tumors are surgically removed on day 21-28; and mice are either followed for survival or sacrificed ten days after surgery and their spleens analyzed for MSC or their bone-marrow-derived macrophages assayed for arginase and iNOS activity. **(B)** BALB/c, $STAT6^{-/-}$, and $STAT6^{-/-}IFN\gamma^{-/-}$ mice were treated according to the schedule shown in part A, and their splenocytes were analyzed by flow cytometry for $Gr1^+CD11b^+$ MSC. Data are shown as percent of mice that have normal levels of MSC (<8% of splenocytes are $Gr1^+CD11b^+$). **(C)** $STAT6^{-/-}$ mice were treated according to the schedule in part A and concomitantly depleted for $CD4^+$ or $CD8^+$ T cells (left hand panel), or depleted for phagocytic cells/macrophages (right-hand panel).

Figure 3. Proposed pathways for immunological resistance to metastatic mammary carcinoma in post-surgery mice. Resistance requires three mechanisms: i) Reduction in tumor-induced

myeloid suppressor cells (MSC); ii) Activation of tumor-specific $CD8^+$ T lymphocytes; and iii) Activation of tumoricidal M1 macrophages. See text for detailed description.

Figure 1

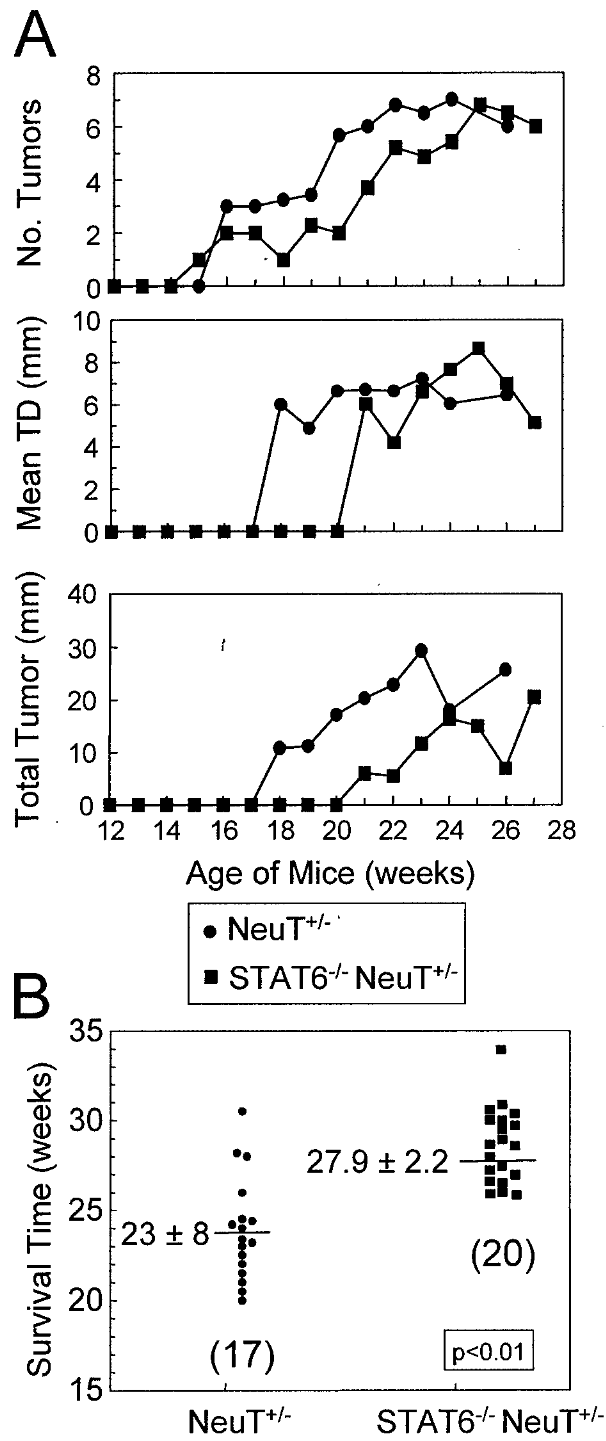
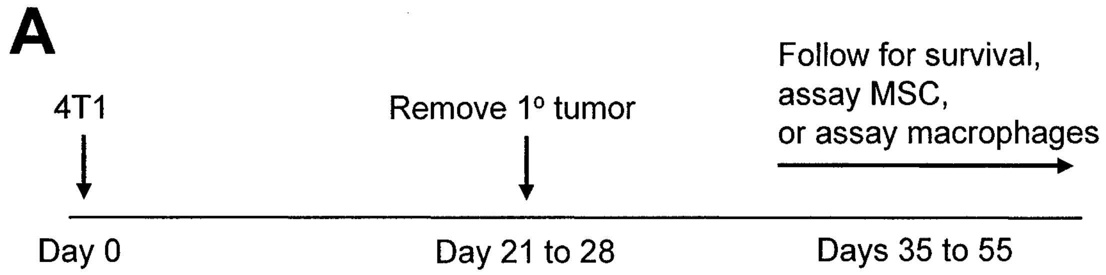
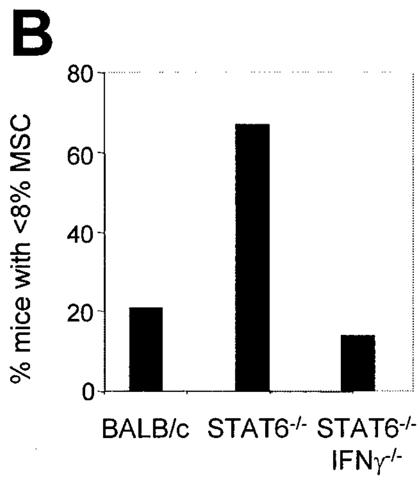


Figure 2





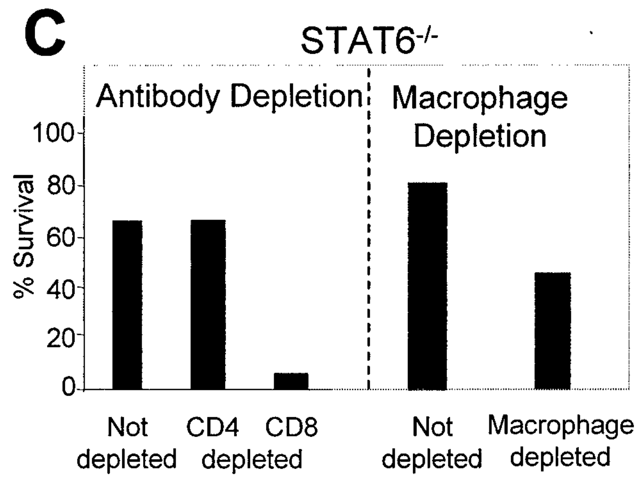
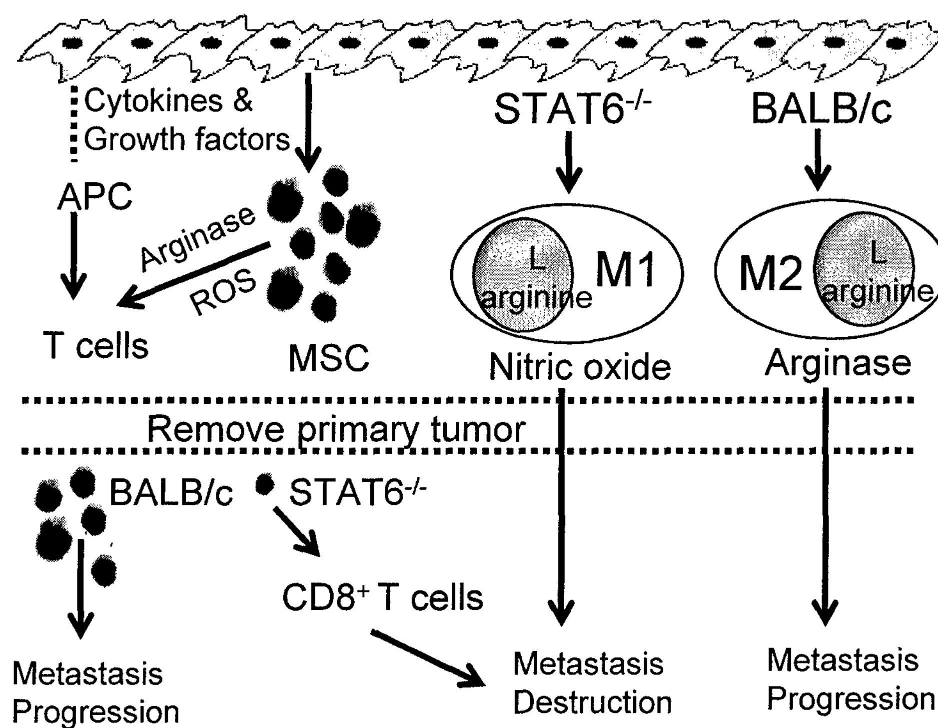


Figure 3



APPENDIX 9

IL13-regulated M2 Macrophages in Combination with Myeloid Suppressor Cells Block Immune Surveillance Against Metastasis

by

Pratima Sinha, Virginia K. Clements, and Suzanne Ostrand-Rosenberg

Department of Biological Sciences

University of Maryland Baltimore County

Baltimore, MD

Running Title: M1 macrophages and MSC in resistance to metastatic disease

Key Words: immune surveillance; tumor-induced immune suppression; activated macrophages; metastatic mammary carcinoma

Grant support: R01CA84232 and R01CA52527 from the NIH and DAMD 17-01-0312 from the United States Army Research and Development Command Breast Cancer Program (SOR).

Requests for reprints: Dr. S. Ostrand-Rosenberg, Department of Biological Sciences, U. of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250; Phone: 410 455-2237; FAX 410 455-3875; <srosenbe@umbc.edu>

Abbreviations used in this report:

DC,	dendritic cell(s)
DCFDA,	dichlorodihydrofluorescein diacetate
DHE,	dihydroethidium
DMEM,	Dulbecco's modified Eagle's medium
FBS,	fetal bovine serum
HA,	influenza virus hemagglutinin
HEL,	hen eggwhite lysozyme
IMDM,	Isacove's modified Dulbecco's medium
LPS,	lipopolysaccharide
MSC	myeloid suppressor cell(s)
NO,	nitric oxide
nor-NOHA,	N^{w}-hydroxyl-nor-L-arginine
OVA,	ovalbumin
ROS,	Reactive oxygen species

STAT6	signal transducer and activator of transcription 6
TCR,	T cell receptor
TD,	tumor diameter
TGFβ	transforming growth factor beta
6-TG,	6-thioguanine

ABSTRACT

CD1-deficient mice reject established, disseminated 4T1 metastatic mammary cancer and survive indefinitely if their primary mammary tumors are surgically removed. This highly effective immune surveillance is due to three interacting mechanisms: i) The generation of iNOS-producing M1 macrophages that are tumoricidal for 4T1 tumor cells; ii) A rapid decrease in myeloid-derived $Gr1^+CD11b^+$ suppressor cells that are elevated and down-regulate CD3ζ chain when primary tumor is present and that suppress T cells by producing arginase; and iii) Production of activated lymphocytes. Macrophages from wild type BALB/c mice are polarized by IL-13 towards a tumor-promoting M2 phenotype, thereby inhibiting the generation of tumoricical M1 macrophages. In contrast, $CD1^{-/-}$ mice, which are deficient for IL-13 because they lack IL-13-producting NKT cells, generate M1 macrophages that are cytotoxic for 4T1 via the production of nitric oxide. Although tumoricidal macrophages are a necessary component of immune surveillance in $CD1^{-/-}$ mice, they alone are not sufficient for tumor resistance since IL-$4R\alpha^{-/-}$ mice have M1 macrophages and retain high levels of myeloid suppressor cells after surgery, and are susceptible to 4T1 metastatic disease. These results demonstrate that effective immune surveillance against established metastatic disease is negatively regulated by IL-13 and requires the induction of tumoricidal M1 macrophages and lymphocytes combined with a reduction in tumor-induced myeloid suppressor cells.

INTRODUCTION

Recent studies have resurrected the hypothesis that immunosurveillance occurs in vivo and protects individuals against spontaneously arising malignant cells (1-4). Various effector mechanisms have been proposed as mediating immunosurveillance, including $CD4^+$ and $CD8^+$ T lymphocytes , NK cells, antibodies, and NKT cells (4-6). In addition to their role in protecting against tumor, NKT cells have also been implicated in facilitating tumor progression (7) by their production of the cytokine IL-13 (8, 9). Most of the data supporting an inhibitory role for NKT cells derive from experiments using CD1-deficient ($CD1^{-/-}$) mice. $CD1^{-/-}$ mice lack the non-classical MHC class I CD1d MHC molecule which is required for thymus selection of NKT cells (10). Hence, CD1-deficient mice lack NKT cells (11). Since NKT cells are a major producer of IL-13, $CD1^{-/-}$ mice are also IL-13-deficient (12).

We (13) and others (8, 9, 14) have reported that CD1-deficient ($CD1^{-/-}$) mice have enhanced immune surveillance against tumors, and have proposed that deletion of the CD1d gene removes an inhibitor that blocks anti-tumor immunity. In our studies we have used the spontaneously metastatic BALB/c-derived 4T1 mammary carcinoma (15-17). This tumor closely models human breast cancer in many of its characteristics including its pattern of metastatic spread (18). Also similar to many human cancers (19-23), 4T1 induces a profound immune suppression which can be partially reversed if the primary tumor is removed (24). Our finding that $CD1^{-/-}$ mice whose primary tumors are surgically removed survive indefinitely, despite the presence of metastatic disease, has led us to hypothesize that immune surveillance is blocked in wild type mice by two factors: i) an inhibitor that is regulated by the CD1d gene, and ii) immune suppression induced by primary tumor. Berzofsky and colleagues, using the 15-

12RM fibrosarcoma, have also concluded that wild type mice contain an inhibitor of immune surveillance, and have identified the inhibitor as the cytokine IL-13. They argue that IL-13 blocks immune surveillance by activating $Gr1^+CD11b^+$ myeloid-derived suppressor cells (MSC) that secrete the immunosuppressive cytokine TGFβ (8, 9). Although the immune suppression present in mice with 4T1 primary tumors is also mediated by $Gr1^+CD11b^+$ MSC, unlike the 15-12RM tumor system, 4T1-induced MSC are not induced by IL-13 (present report) and do not produce TGFβ (25), indicating that resistance to the 15-12RM and 4T1 tumors is mediated by different mechanisms.

Previous studies with the 4T1 tumor in $STAT6^{-/-}$ mice demonstrated that in addition to MSC, macrophages also regulate tumor growth (25). Macrophages polarized towards an M2 phenotype, produce arginase and support tumor growth. In contrast M1 macrophages, which produce iNOS, are tumoricidal and mediate tumor regression (26).

It is important to clarify the mechanisms that promote immune surveillance and facilitate tumor regression since a better understanding of these mechanisms may lead to strategies that enhance tumor-specific immunity. Therefore, we have studied the pathways leading to effective immune surveillance against the 4T1 mammary carcinoma in CD1-deficient mice whose primary tumors have been surgically removed, but who retain disseminated, metastatic disease. We find that effective immune surveillance requires a combination of three conditions: i) The generation of iNOS-producing tumoricidal M1 macrophages that are produced because $CD1^{-/-}$ mice are deficient for IL-13 which polarizes macrophages to an M2 phenotype; ii) A rapid decrease in the quantity of myeloid-derived $Gr1^+CD11b^+$ suppressor cells that are elevated when primary tumor is present and that suppress $CD4^+$ and $CD8^+$ T cell activation via the production of arginase and

reactive oxygen species; and iii) The activation of functional lymphocytes.

MATERIALS AND METHODS

Mice. CD1-deficient ($CD1^{-/-}$) (11), $3A9^{+/-}$ TCR-transgenic, (Vβ8.2 T cell receptor (TCR) specific for hen eggwhite lysozyme (HEL) restricted to $I\text{-}A^{k}$ (27), DO11.10 TCR-transgenic (Vβ8-TCR restricted to chicken ovalbumin (OVA) peptide 323-339 restricted by $I\text{-}A^{d}$ (28), Signal Transducer and Activator of Transcription 6-deficient ($STAT6^{-/-}$), and BALB/c mice were obtained as described (25). IL-4 receptor alpha-deficient ($IL\text{-}4R\alpha^{-/-}$) and RAG2-deficient ($RAG^{-/-}$) were from The Jackson Laboratory, Bar Harbor, ME and Taconic Farms (Germantown, MD), respectively. All strains are on a BALB/c background. Female mice of 8-16 wk were used for all studies. Mice were maintained and/or bred in the University of Maryland Baltimore County (UMBC) animal facility according to the NIH guidelines for the humane treatment of laboratory animals. All animal procedures are approved by the UMBC IACUC.

Reagents and Antibodies. Sodium thioglycolate and LPS were from Difco (Detroit, Michigan); recombinant mouse IFNγ from Pierce-Endogen (Rockford, IL); dichlorodihydrofluorescein diacetate (DCFDA) and dihydroethidium (DHE) from Molecular Probes (Eugene, OR). HA peptide 518-526 and OVA peptide 323-339 were synthesized and HPLC purified in the Biopolymer Core Facility at the University of Maryland, Baltimore.

Vβ8.1,2-PE, CD1d1.1-PE, Gr1-PE, rat IgG2a-PE isotype, and rat IgG2a-FITC isotype were from BD Pharmingen (San Jose, CA). CD3ζ-FITC was from Abcam Cambridge, MA.

CD11b-FITC and KJ1-26, an anti-clonotypic mAb that recognizes the DO11.10 TCR (29), were from Caltag (Burlingame, CA). MAb to arginase 1, and rat anti-mouse Gr-1 antibody for MACS sorting (clone RB6-8C5) were from BD Transduction Laboratories (San Jose, CA) and Pharmingen, respectively.

Cell Lines, Tumor Challenges, Surgery, and Metastasis Assay

The J774 macrophage cell line (American Type Culture Collectio, Manassas, VA) was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Biofluids, Rockville, MD) (25). Mice were inoculated in the abdominal mammary gland with 7000 4T1 cells, and primary tumor growth and lung metastases were measured as described (17) (18, 24). Tumor size was measured on the day of surgery and tumor diameter (TD) was calculated as the square root of length × width. Primary tumors were surgically removed as described (30). For experiments comparing non-surgery vs. post-surgery groups, mice were inoculated with 4T1 on day 0 and TDs were measured on the day of surgery. Mice were then divided into two groups so that the average TD for the groups were not significantly different. Primary tumors were removed from one group ("post-surgery") and left in place for the other group ("non-surgery").

T Cell and Macrophage Depletions. Mice were depleted for $CD4^+$ (mAb GK1.5) or $CD8^+$ (mAb 2.43) T cells or with irrelevant antibodies as described (31). Liposomes loaded with clondronate or control liposomes without clondronate were prepared and used to deplete macrophages as described (32). Briefly, mice were injected i.p. on days 1and 4 after surgery with 0.2 ml of clodronate or control PBS liposomes, and thereafter once a week with 0.1 ml of

clodronate or control PBS liposomes. Treatment continued until all of the experimental mice were moribund.

Flow Cytometry. Live cells were labeled for cell surface molecules by direct immunofluorescence(18). Samples were analyzed on an Epics XL flow cytometer (Beckman Coulter, Miami, FL) and analyzed using Expo32 ADC software (Beckman Coulter).

Myeloid Suppressor Cells (MSC) and Reactive Oxygen Species (ROS). Splenic MSC were positively purified by magnetic bead sorting using LS columns and rat anti-mouse Gr1 antibody with anti-rat IgG microbeads (25) (Miltenyi Biotec, Auburn, CA). Purifed MSC were assayed by flow cytometry and were > 90% $Gr1^{+}CD11b^{+}$. ROS production was measured by DCFDA and DHE as described (25).

Macrophage Isolation and Functional Assays. Peritoneal macrophages were generated by injecting 1 ml of sterile 3% Brewer thioglycolate medium (Difco) in distilled water i.p. Five days later, mice were euthenized by CO_2 asphyxiation, their abdomens wiped with 70% alcohol, and 10 ml of sterile PBS was injected into the peritoneal cavity and the resulting peritoneal fluid was withdrawn aseptically using a 10 ml syringe. Contaminating RBC were lysed with Gey's solution, and the peritoneal exudate cells were washed twice with DMEM with 10% FCS. Cells were plated at 1.5×10^{6}/ml in 0.5 ml DMEM containing 10% FCS in the wells of 24 well plates. Non-adherent cells were removed after a 3 hr incubation at 37°C in 5% CO_2. The resulting macrophages were activated by culturing with IFNγ and LPS at final concentrations of 2 and

100 ng/ml, respectively for 16 hrs. In some experiments macrophages were stimulated with IL-4 or IL-13 at a final concentration of 50 ng/ml for 16 hrs in DMEM containing 5% FBS prior to their activation with IFNγ and LPS.

Western Blots. Cultured macrophages were washed with excess PBS and resuspended in 200 μl of lysis buffer (one tablet of proteinase inhibitor mix (Roche), 2 mM PMSF, 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 5% Triton in 10 ml H_20). Lysates were microfuged (3000 g for 10 min at 4°C), the clarified supernatants electrophoresced in 12% SDS-PAGE gels, and the proteins blotted onto Hybond-PVDF membranes (Amersham, Piscataway, NJ), and immunoblotted with mAbs to arginase 1 (33). Proteins were detected using Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Nitric Oxide (NO) and CytotoxicityAssays. NO was measured using Griess (34) as described (25). Data are the mean ± SD of triplicate wells. Macrophage cytotoxicity was determined by the procedure of (35) as described (25). Values are the average of triplicates ± SD. Background values for media were subtracted from each point. Activated and non-activated macrophages without 4T1 were routinely run and gave no LDH release.

Percent specific lysis = 100% × [(A490 Experimental - A490 Spontaneous)/(A490 Maximum)]

T cell Proliferation Assay: T cell proliferation and transwell experiments were performed as described (25). All points were run in triplicates. Data are expressed as:

% suppression = 100% [1-(cpm of spleen+peptide+MSC/cpm of spleen+ peptide)].

CD3ζ Expression. Cells were mixed with peptide and with or without irradiated MSC (5000 Rads) in 24 well plates (5×10^5 T cells, 10^6 MSC, in 500 μl HL1 culture medium (Biowhittaker)/well). After 3 days of culture, cells were harvested, labeled for cell surface markers (KJ1-26-tricolor mAb for D011.10 with CD4-PE; or Vβ8-PE mAb for Clone 4 with CD8-tricolor (all at a 1:50 dilution), fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with a 1:20 dilution of CD3ζ-FITC mAb. Labeled cells were analyzed for expression of CD3ζ by gating on double positive ($CD4^+KJ1\text{-}26^+$ or $CD8^+V\beta8^+$) cells.

Statistical Analysis. Student's t test for unequal variance was performed using Microsoft Excell 2000.

RESULTS

CD1-deficient Mice Survive Indefinitely After Surgical Removal of Primary 4T1 Mammary Carcinoma. The 4T1 mammary carcinoma is a BALB/c-derived tumor that spontaneously metastasizes following inoculation into the mammary gland. Similar to human breast cancer, metastatic disease progresses while the primary tumor is present, as well as after the primary tumor is surgically removed. We have previously used this tumor system to study tumor immunity in a setting comparable to that of breast cancer patients whose primary tumors have been removed, but who have residual, disseminated metastatic disease (18, 30, 36). To confirm our earlier findings that CD1-deficient mice are resistant to 4T1 metastatic disease,

CD1-deficient and control syngeneic CD1-competent BALB/c mice were injected s.c. in their abdominal mammary gland with 7000 4T1 cells, primary tumors were either left in place (non-surgery group) or surgically removed 2-3 weeks later (post-surgery group), and mice were followed for survival (TD at the time of surgery, BALB/c: 4.93 ± 0.98; $CD1^{-/-}$: 4.9 ± 1.2 mm). As shown in **figure 1A**, 100% of post-surgery $CD1^{-/-}$ mice survived >180 days, whereas 89% of the BALB/c mice died with a mean survival time (MST) of 53.4 days. To determine if the differential in survival time between CD1-deficient and BALB/c mice was due to differences in metastatic disease burden, the lungs of non-surgery and post-surgery CD1-deficient and BALB/c mice were removed 30-39 days after 4T1 challenge (9-11 days after surgery for the surgery groups), and tested by the clonogenic assay for the number of metastatic tumor cells. Non-surgery and post-surgery CD1-deficient and BALB/c mice have very similar levels of metastatic cells in their lungs (**figure 1B**). Therefore, despite the presence of high levels of metastatic tumor, $CD1^{-/-}$ mice whose primary tumors are removed survive, while BALB/c mice die. To determine if $CD1^{-/-}$ mice survive because they eliminate metastatic cells, lung metastases were quantified by the clonogenic assay in long-term (4-10 month) $CD1^{-/-}$ survivors. These mice had no detectable 4T1 cells and splenic MSC levels were in the normal range (<8%), indicating that post-surgery $CD1^{-/-}$ mice are resistant because they reject 4T1 tumor cells (data not shown).

A trivial explanation for the resistance of $CD1^{-/-}$ mice to 4T1 tumor is that 4T1 tumor cells contain CD1 protein that functions as an alloantigen in the $CD1^{-/-}$ mice. To eliminate this possibility, 4T1 tumor cells were tested by flow cytometry for CD1 expression. As shown in **figure 1C**, 4T1 cells do not contain CD1. Therefore, rejection of 4T1 tumor and survival of $CD1^{-/-}$ mice is not due to an immune response against the knocked-out gene product.

Myeloid Suppressor Cell Levels Return to Normal in CD1-deficient Mice After Removal of Primary Tumor. Myeloid suppressor cells (MSC) accumulate in some tumor-bearing patients and animals and are potent inhibitors of cell-mediated, tumor-specific immunity (19-23). These cells are immature cells that are in the process of differentiating into mature granulocytes, dendritic cells (DC), or macrophages, and are identified by their expression of Gr1 and CD11b. In previous studies we have found that 4T1 tumor progression is associated with the accumulation of MSC in tumor-susceptible mice (25). To determine if the resistance of CD1-deficient mice is related to MSC activity, MSC levels were measured in tumor-bearing CD1-deficient and CD1-competent mice. $CD1^{-/-}$ and wild type BALB/c mice were inoculated with 4T1 tumor cells, and splenocytes were harvested 30-39 days later and analyzed for $CD11b^{+}$ $Gr1^{+}$ cells by flow cytometry (TD at the time of surgery, BALB/c: 6.05 ± 0.75 mm; $CD1^{-/-}$: 6.38 ± 0.8 mm). Tumor-free BALB/c and $CD1^{-/-}$ mice have <8% splenic MSC, while tumor-bearing (non-surgery) mice have elevated levels of MSC (**figure 2A**) (BALB/c: 23 ± 11 %; $CD1^{-/-}$: 26 ± 5 %). Therefore, non-surgery CD1-deficient and CD1-competent mice both have elevated levels of MSC relative to tumor-free mice.

To determine if surgical removal of primary tumor differentially affects MSC levels, BALB/c and CD1-deficient mice were inoculated with 4T1 tumor, primary tumors were removed 21-28 days later, and splenocytes were analyzed 9-11 days later (day 30-39 after initial tumor inoculation) (TD at surgery: BALB/c: 6.12 ± 0.81 mm; $CD1^{-/-}$: 5.99 ± 0.90 mm). After surgery, MSC levels in 90% of post-surgery $CD1^{-/-}$ mice are within the normal range (< 8%), while only 21% of post-surgery BALB/c mice have < 8% MSC (**figure 2A**). Therefore, while

MSC levels are high in the spleens of both BALB/c and $CD1^{-/-}$ mice when primary tumor is present, MSC levels drop to the normal level in most post-surgery $CD1^{-/-}$ mice.

The accumuation of MSC is most likely driven by factors secreted by tumor cells (37). Therefore, the dramatic decrease in post-surgery CD1-deficient mice may be because they have less metastatic disease than wild type BALB/c mice. To test this hypothesis, the numbers of metastatic cells in the lungs of the post-surgery mice of figure 1A were graphed with the percent of splenic MSC from the corresponding mice (**figure 2B**). Both wild type BALB/c and CD1-deficient mice have extensive metastatic disease, and there is no correlation between percentage of MSC and the number of metastatic cells. Therefore, the decrease in MSC in CD1-deficient mice after surgery is independent of metastatic tumor burden.

Although MSC levels are driven by tumor-secreted factors, lymphocytes may also play a role. To determine if the decrease in MSC is lymphocyte-dependent, splenic MSC levels were determined for BALB/c $RAG^{-/-}$ mice inoculated with 4T1 according to the schedule for BALB/c and $CD1^{-/-}$ mice in figure 2A (TD on the day of surgery, non-surgery: 5.07 ± 1.2 mm; post-surgery: 5.57 ± 0.95 mm). Likewise, TD of non-surgery $RAG^{-/-}$, BALB/c, and $CD1^{-/-}$ mice were similar at day 30-39 after tumor inoculation when splenic MSC levels were measured ($RAG^{-/-}$: 9.3 ± 1.5 mm; BALB/c: 9.5 ± 0.73; $CD1^{-/-}$: 10.24 ± 1.10). The baseline level of $Gr1^{+}CD11b^{+}$ splenocytes in tumor-free $RAG^{-/-}$ mice is <8%, while non-surgery $RAG^{-/-}$ mice have significantly ($p < 0.01$) more MSC than BALB/c or $CD1^{-/-}$ mice (**figure 2**) ($RAG^{-/-}$ MSC: 51.8% ± 6). After surgery, MSC in $RAG^{-/-}$ mice remain significantly higher than in BALB/c or $CD1^{-/-}$ mice ($p<0.01$).

To determine if $CD4^{+}$ and/or $CD8^{+}$ T cells are also involved in immune surveillance,

post-surgery $CD1^{-/-}$ mice were in vivo depleted for $CD4^+$ or $CD8^+$ T cells or treated with irrelevant antibodies. Both $CD4^+$ and $CD8^+$ T cells are required for tumor resistance since 100% of CD4-depleted (3/3) and 80% of CD8-depleted (4/5) post-surgery $CD1^{-/-}$ mice, but none of the irrelevant antibody treateded mice (3/3), die. Therefore, lymphoid cells are essential for tumor rejection and may act by reducing the amounts of MSC in post-surgery BALB/c mice.

MSC Inhibit T Cells by an Arginase-dependent Mechanism. $CD1^{-/-}$ mice may have greater tumor immunity because their MSC are less suppressive than MSC of BALB/c mice. To test this possibility, splenocytes from non-surgery BALB/c and $CD1^{-/-}$ mice were MACS purified for Gr1 (>91% and 93% $Gr1^+CD11b^+$ for BALB/c and $CD1^{-/-}$, respectively). The resulting MSC were then co-cultured with antigen-specific $CD4^+$ or $CD8^+$ syngeneic T cells or $CD4^+$ allogeneic T cells plus the appropriate peptide ($H\text{-}2^d$ D011.10 with OVA-peptide, $H\text{-}2^d$ clone 4 with HA-peptide, or $H\text{-}2^k$ 3A9 with HEL, respectively), and T cell activation measured by ^{3}H-thymidine uptake (**Figure 3A**). On a per cell basis, purified BALB/c and $CD1^{-/-}$ MSC were equally capable of suppressing syngeneic $CD4^+$ or $CD8^+$, or allogeneic $CD4^+$ T cells.

MSC are thought to mediate their effects via the production of arginase and/or inducible nitric oxide synthase (iNOS) (38). To ascertain if $CD1^{-/-}$ MSC mediate suppression by either of these molecules, DO11.10 transgenic T cells were co-cultured with $CD1^{-/-}$ MSC in the presence of OVA-peptide and the arginase inhibitor nor-NOHA, or the iNOS inhibitor L-NMMA, and T cell proliferation measured by ^{3}H-thymidine uptake. The arginase inhibitors, but not the iNOS inhibitor, reverses the suppression (**figure 3B**). Therefore, $CD1^{-/-}$ MSC inhibit T cell activation via arginase production.

To determine if suppression requires direct contact between the MSC and T cells, $CD1^{-/-}$ MSC were suspended in transwell chambers in wells containing OVA-peptide-pulsed DO11.10 T cells (**figure 3C**). Proliferation of DO11.10 cells was not inhibited when the MSC were separated from the T cells by a semi-permeable membrane. Therefore, suppression requires direct contact between the MSC and the affected T cells.

MSC Down-regulate T Cell Receptor-associated Zeta Chain in $CD4^+$, but not $CD8^+$ T Cells. Ochoa and colleagues have demonstrated that T cell dysfunction caused by macrophages or bacteria is associated with the down-regulation of the TCR-associated $CD3\zeta$ chain (39, 40). To determine if MSC induce suppression by this mechanism, OVA peptide-pulsed $CD4^+$ DO11.10 T cells were co-cultured with MSC from BALB/c or $CD1^{-/-}$ mice. Following three days of incubation, the cultures were harvested, and the cells triple labeled for $CD3\zeta$, CD4, and the D011.10 clonotype (KJ1-26). The cells were analyzed by flow cytometry by gating on the $DO11.10^+$ $CD4^+$ double positive population and assessing $CD3\zeta$ expression. Fifty-three percent of D011.10 transgenic T cells co-cultured with OVA peptide have elevated levels of $CD3\zeta$ chain (**figure 4**, top two panels). If BALB/c or $CD1^{-/-}$ MSC are added to the cultures, then only 17% and 15% of the T cells, respectively, have elevated $CD3\zeta$ expression. Therefore, BALB/c and $CD1^{-/-}$ MSC reduce $CD3\zeta$ chain expression which probably inhibits T cell activation by inhibiting signal transduction.

To determine if MSC also suppress the activation of $CD8^+$ T cells via the down-regulation of $CD3\zeta$, $CD8^+$ clone 4 T cells were cultured as per the $CD4^+$ DO11.10 T cells but with HA peptide. The resulting cells were gated on the $CD8^+$ $V\beta8^+$ double positive population

and analyzed for CD3ζ expression (**figure 4,** bottom two panels). Similar to the $CD4^+$ T cells, more than half of the activated $CD8^+$ T cells had elevated levels of CD3ζ. However, in contrast to the $CD4^+$ T cells, CD3ζ levels did not decrease following co-culture with either BALB/c or $CD1^{-/-}$ MSC. Therefore, BALB/c and $CD1^{-/-}$ MSC suppress $CD4^+$ T cells by down-regulating CD3ζ chain, but suppress $CD8^+$ T cells via a different mechanism.

BALB/c and $CD1^{-/-}$ MSC Produce Reactive Oxygen Species (ROS). Gabrilovich and colleagues have shown that production of ROS is a characteristic of MSC (41) and we have previously noted that ROS production characterizes different populations of MSC (25). To determine if ROS are differentially expressed in BALB/c vs. $CD1^{-/-}$ MSC, splenic MSC were MACS purified from tumor-free and non-surgery mice and analyzed by flow cytometry for ROS. Staining with DHE, which measures superoxide, was negative (data not shown). Staining with DCFDA, which measures hydrogen peroxide, hydroxyl radical, peroxynitrile, and superoxide, demonstrates that $Gr1^+CD11b^+$ splenic cells from non-surgery BALB/c (**figure 5A**) and $CD1^{-/-}$ (**figure 5B**) mice contain more ROS than MSC from the corresponding tumor-free mice. To assess if arginase is involved in ROS production, the arginase inhibitor nor-NOHA was added to the purified $Gr1^+CD11b^+$ cells prior to their staining with DCFDA. Although nor-NOHA has no effect on ROS expression in $CD1^{-/-}$ MSC, it inhibits ROS expression in BALB/c MSC. Therefore, MSC from both BALB/c and $CD1^{-/-}$ mice contain ROS; however, ROS expression in the $CD1^{-/-}$ MSC is arginase-independent, while in BALB/c MSC it is arginase-dependent.

$CD1^{-/-}$ Mice Have Tumoricidal M1 Macrophages. iNOS-producing M1 macrophages

are associated with heightened anti-tumor immunity and inhibition of tumor progression (26, 42, 43). IL-4 and IL-13 are known to polarize macrophages away from an M1 phenotype and towards an M2 phenotype (26, 43). Since $CD1^{-/-}$ mice lack NKT cells, which are a major source of IL-13 (11, 12, 44, 45), they may preferentially generate M1 macrophages, which may contribute to tumor resistance. To test this hypothesis, peritoneal macrophages from BALB/c and $CD1^{-/-}$ mice were activated in vitro with LPS and IFNγ and assayed for iNOS production. LPS and IFNγ activated macrophages from $STAT6^{-/-}$ and $IL\text{-}4R\alpha^{-/-}$ mice were used as controls. The IL-4Rα is a common chain that is shared between the receptors for IL-4 and IL-13 and hence is required for transmitting signals for both of these cytokines (46, 47). STAT6 is a transcription factor that transmits signals through the IL-4Rα (48-50). Therefore, $STAT6^{-/-}$ and $IL\text{-}4R\alpha^{-/-}$ macrophages should make iNOS regardless of the presence or absence of IL-4 and/or IL-13 (51). Macrophages from all four strains that are activated in vitro with LPS and IFNγ in the absence of IL-4 or IL-13, produce iNOS (**figure 6**). However, if the macrophages are treated with IL-4 or IL-13 prior to activation with LPS and IFNγ, then BALB/c and $CD1^{-/-}$ macrophages make much less iNOS, whereas iNOS production by $STAT6^{-/-}$ and $IL\text{-}4R\alpha^{-/-}$ is unaffected. Since BALB/c mice will produce IL-4 and/or IL-13 in vivo, their macrophages will not make significant levels of iNOS, and hence BALB/c mice will not have M1 macrophages. In contrast, $CD1^{-/-}$ mice will have iNOS-producing M1 macrophages in vivo because they have diminished levels of IL-4 and IL-13 since they lack NKT cells.

The production of arginase has been associated with M2 type macrophages which are thought to promote tumor progression (26, 42, 43). To determine if arginase production by macrophages is associated with tumor progression, BALB/c, $CD1^{-/-}$, and $IL\text{-}4R\alpha^{-/-}$ peritoneal

macrophages were isolated and tested for arginase by western blot (**figure 6B**). Macrophages were either not activated (lane 1), activated with LPS plus IFNγ (lane 2), pretreated with IL-4 before LPS and IFNγ activation (lane 3), pretreated with IL-13 before LPS and IFNγ activation (lane 4), unactivated and treated with IL-4 (lane 5), or not activated and treated with IL-13 (lane 6). BALB/c and $CD1^{-/-}$ macrophages, regardless of treatment, contain arginase, whereas IL-$4R\alpha^{-/-}$ macrophages contain very little, if any arginase.

Macrophage tumoricidal activity is attributed to iNOS production (26), so $CD1^{-/-}$ macrophages may be tumoricidal even though they also contain arginase. To test this hypothesis, BALB/c, $CD1^{-/-}$, and IL-$4R\alpha^{-/-}$ peritoneal macrophages were harvested, activated in vitro with LPS and IFNγ and tested for cytotoxic activity against 4T1 tumor cells. $CD1^{-/-}$ and positive control IL-$4R\alpha^{-/-}$ macrophages are significantly more cytotoxic than BALB/c macrophages (**figure 6C**) ($p<0.05$). The cytotoxicity is due to iNOS, since addition of the iNOS inhibitor, L-NMMA, eliminates the cytotoxic effect, whereas the inactive inhibitor D-NMMA has no effect. Therefore, although $CD1^{-/-}$ macrophages contain both iNOS and arginase, they have strong tumoricidal activity indicating they are polarized towards the M1 phenotype. To confirm the role of M1 macrophages in tumor resistance, macrophages were depleted from post-surgery $CD1^{-/-}$ mice by treatment with liposomes loaded with clodronate. Macrophage-depleted (3/3) mice were dead by 42 days after injection of primary tumor, whereas, mice treated with PBS loaded liposomes survived (MST > 83 days). Therefore, NO-producing M1 macrophages are essential for the survival of post-surgery $CD1^{-/-}$ mice.

IL-$4R\alpha^{-/-}$ Mice are Tumor Susceptible and Maintain Elevated Levels of MSC After

Surgery. If the presence of M1 macrophages is sufficient for tumor resistance, then IL-4R$\alpha^{-/-}$ mice, which have tumoricidal M1 macrophages, may survive after removal of primary tumor. To test this possibility, BALB/c and IL-4R$\alpha^{-/-}$ mice were inoculated with 4T1, primary tumors surgically removed 2-3 weeks later, and the mice followed for survival. IL-4R$\alpha^{-/-}$ mice are just as susceptible as BALB/c mice (5/6 IL-4R$\alpha^{-/-}$ vs. 7/8 BALB/c mice die), indicating that despite the presence of M1 macrophages, IL-4R$\alpha^{-/-}$ mice do not have heightened tumor immunity. Because tumor immunity in CD1$^{-/-}$ (see figure 2) and STAT6$^{-/-}$ (25) mice is associated with a rapid decrease to baseline in MSC, we assessed MSC levels in tumor-bearing non-surgery and post-surgery IL-4R$\alpha^{-/-}$ mice. (TD at the time of MSC assessment for non-surgery mice, BALB/c: 6.1 ± 1.7 mm; IL-4R$\alpha^{-/-}$: 7.1 ± 1.1 mm). (TD at the time of surgery for the post-surgery groups, BALB/c: 6.5 ± 1 mm; IL-4R$\alpha^{-/-}$: 7.5 ± 0.43 mm). Non-surgery IL-4R$\alpha^{-/-}$ mice have elevated levels of MSC (**figure 7A**), and MSC remain elevated after surgery similar to BALB/c ($p \geq 0.05$), with only 14% of IL-4R$\alpha^{-/-}$ mice having normal levels (<8% MSC). Likewise, post-surgery IL-4R$\alpha^{-/-}$ mice contain high levels of metastatic cells (**figure 7B**). Therefore, although IL-4R$\alpha^{-/-}$ mice generate tumoricidal M1 macrophages, they are not tumor resistant and they have elevated levels of MSC, even after removal of primary tumor.

DISCUSSION

Despite the presence of extensive metastatic mammary carcinoma, CD1-deficient mice survive indefinitely if 4T1 primary tumor is surgically removed. Resistance is associated with three phenomena: i) the production of iNOS-producing M1 macrophages; ii) a rapid decrease to

baseline in the levels of MSC; and iii) the presence of functional lymphocytes. Resistance to metastatic disease requires the coordinate interaction of the three conditions; neither mechanism alone is sufficient to mediate tumor rejection.

iNOS-producing M1 macrophages with tumoricidal activity have been described in numerous tumor systems (26, 42, 43). They are cytotoxic because iNOS converts arginine and oxygen to NO, which is toxic. The tumoricidal macrophages of $CD1^{-/-}$ mice are unusual in that they produce iNOS and arginase. Typically, M1 macrophages produce less arginase because it degrades arginine and therefore limits the amount of substrate available for conversion to NO (52, 53). Despite the co-expression of arginase by $CD1^{-/-}$ macrophages, these cells produce sufficient NO to mediate tumor cell destruction, indicating that tumoricidal M1 macrophages can coordinately express iNOS and arginase. El-Gayar et al (53) have demonstrated that IL-13 prevents iNOS production thereby polarizing macrophages towards a M2 phenotype. Since $CD1^{-/-}$ mice lack NKT cells, which are a major producer of IL-13, it is likely that $CD1^{-/-}$ mice have M1 macrophages because they are deficient for IL-13. This hypothesis is supported by two findings: i) Addition of IL-13 to cultures of $CD1^{-/-}$ macrophages polarizes them towards a M2 phenotype; and ii) Macrophages from IL-4R$\alpha^{-/-}$ mice, which lack the receptor for IL-13, are also M1 type iNOS-producers and tumoricidal. Although both IL-13 and IL-4 signal through the IL-4Rα and STAT6, it is unlikely that IL-4 is the relevant inhibitor of M1 macrophages generation in vivo since IL-4 is produced by activated Th2 cells in addition to NKT cells (54, 55), and $CD1^{-/-}$ mice are only deficient for NKT cells. Therefore, $CD1^{-/-}$ mice which are deficient for IL-13 (12), constitutively generate iNOS-producing M1 macrophages which are cytotoxic for 4T1 tumor, while wild type BALB/c macrophages are polarized towards a M2 phenotype under the induction

of IL-13.

Previous studies using macrophage-depleted mice demonstrated that macrophages are essential for immune surveillance against the 4T1 tumor (25, 56). Although M1 macrophages are necessary, their presence alone is not sufficient for tumor rejection. For example, IL-4R$\alpha^{-/-}$ mice, which have tumoricidal M1 macrophages, die from metastatic disease, and CD1-deficient mice are only resistant if their primary tumor is removed, even though tumoricidal M1 macrophages are present before surgery. In pre- and post-surgery IL-4R$\alpha^{-/-}$ mice and in pre-surgery CD1$^{-/-}$ mice, MSC levels are elevated, suggesting that M1 macrophages are ineffective in the presence of large quantities of MSC. In contrast, post-surgery CD1$^{-/-}$ mice with M1 macrophages have baseline levels of MSC and reject metastatic disease. Depletion of M1 macrophages from post-surgery CD1$^{-/-}$ mice makes them susceptible to tumor. Therefore, effective immune surveillance against metastatic tumor requires the production of M1 macrophages coupled with baseline levels of MSC, a condition that only exists in post-surgery CD1$^{-/-}$ mice.

Resistance to 4T1 metastatic disease in CD1$^{-/-}$ mice is reminiscent of resistance to 4T1 in STAT6$^{-/-}$ mice (13, 25, 31). In both strains, tumoricidal M1 macrophages are produced, MSC levels decrease drastically after surgery, and lymphocytes are required. It is likely that IL-13 plays the same role in both strains since IL-13 signaling through the IL4Rα is via the STAT6-JAK3 pathway (57). Although IL-13 plays an important role in blocking the production of M1 macrophages, it is not involved in accumulation of MSC or maintaining elevated MSC levels, since non-surgery CD1$^{-/-}$ or IL-4R$\alpha^{-/-}$ and post-surgery IL-4R$\alpha^{-/-}$ mice have high levels of MSC. Therefore, in addition to their effect on the IL-13/IL4Rα pathway, CD1- and STAT6-deficiencies also impact another pathway that regulates MSC cell retention.

MSC are present in many patients and experimental animals with cancer, and they are uniformly immunosuppressive (19-23). Although MSC from different individuals share the ability to suppress, they appear to be a heterogeneous population of cells which mediate suppression via a variety of mechanisms. Down-regulation of the CD3-associated ζ chain and the resulting dysfunction of T cells is a common phenomenon in many patients and experimental mice with cancer (reviewed by (58). Ochoa and colleagues have demonstrated that such a down-regulation is mediated by macrophages (39, 59). Our findings support this mechanism for the suppression of $CD4^+$ T cells by MSC. However, $CD8^+$ T cells are not down-regulated for CD3ζ chain in the experiments presented here, suggesting that there are additional mechanisms by which MSC inhibit T cell activation. Other studies also support the concept that MSC are a functionally heterogenous population of cells. For example, some MSC inhibit the activation of $CD4^+$ T cells and not $CD8^+$ T cells (60), while others inhibit $CD8^+$ T cells and have no effects on $CD4^+$ T cells (20, 22, 61), and others inhibit both $CD4^+$ and $CD8^+$ T cells (25) (the current report). The heterogeneity of MSC is further supported by the varied phenotypes that have been reported for these cells. Although many mouse MSC are characterized by their expression of the markers Gr1 and CD11b, other mouse MSC express CD31 and do not express Gr1 and/or CD11b (19). Some MSC express MHC class II, B220, F4/80, CD86, CD16/32, and DEC205 (38), while others express MHC class I and do not express MHC class II or costimulatory molecules (20), and others express MHC I and costimulatory molecules, but not MHC II (25). Differences also exist in ROS production between the different MSC populations studied. Kusmartsev et al. (41) have demonstrated that ROS production by MSC is arginase-dependent. ROS production by the BALB/c MSC described in this report are arginase-dependent, whereas ROS production by $CD1^{-/-}$

MSC are arginase independent. These phenotypic differences probably characterize sub-populations of MSC, and it is possible that the different sub-populations have different target cells (e.g. $CD4^+$ vs. $CD8^+$ T cells), thereby explaining the functional heterogeneity observed in the different tumor systems.

Others have also shown that $CD1^{-/-}$ mice have enhanced tumor immune surveillance (8, 9, 14, 62), supporting the concept that a deficiency in variant or invariant NKT cells facilitates tumor immunity. Preliminary data with $J\alpha18^{-/-}$ mice, which are deficient for invariant NKT cells, indicates that both variant and invariant NKT cells inhibit tumor immunity (Sinha and Ostrand-Rosenberg, unpublished results). Berzofsky and colleagues also observe that blocking of IL-13 or interfering with signal transduction through the IL4Rα is sufficient to cause rejection of the CT26 colon carcinoma or block recurrence of the 15-12RM fibrosarcoma. In the 15-12RM fibrosarcoma, they have shown that IL-13 inhibits tumor immunity by inducing MSC that produce high levels of the immunosuppressive cytokine TGFβ (9). Three findings demonstrate that this mechanism is not responsible for resistance to the 4T1 tumor: i) 4T1-induced MSC do not produce TGFβ (25); ii) IL-4R$\alpha^{-/-}$ mice, which are unable to respond to IL-13 because they lack the requisite receptor, are susceptible to 4T1 metastatic disease; and iii) wild type BALB/c mice treated with an inhibitor of IL-13 remain susceptible (13). Therefore, although we concur with Berzofsky and colleagues that IL-13 is a potent inhibitor of tumor immunity, we find that suppressing IL-13 is not sufficient for generating effective immune surveillance. We also do not concur with Berzofsky and colleagues that IL-13 inhibits immune surveillance by inducing MSC that produce high levels of TGFβ, because 4T1-derived MSC do not contain TGFβ (25). Our data indicate that IL-13 counteracts immune surveillance by polarizing macrophages away from a

tumoricidal M1 phenotype. Although these differences in interpretation of IL-13 function may be due to differences in the tumor systems used, they may also indicate that IL-13 is a pleiomorphic cytokine that blocks immune surveillance via multiple mechanisms.

These studies demonstrate that immune surveillance can eliminate metastatic disease in a post-surgery setting. Although effective immunity is a complex process that requires the activation of multiple effector cells (macrophages and lymphocytes) coupled with the down-regulation of suppressive/inhibitory cells (MSC), a better understanding of these mechanisms may reveal strategies for facilitating tumor immunity and extending survival.

ACKNOWLEDGMENTS

We thank Drs. L. van Kaer (Vanderbilt), Bill Wade (Dartmouth), and Ephraim Fuchs (Johns Hopkins) for providing breeding pairs of the $CD1^{-/-}$, 3A9, and clone 4 mice, respectively. We appreciate the excellent care given to our mouse colony by Ms. Sandra Mason.

REFERENCES

1. Smyth, M. J., Godfrey, D. I., and Trapani, J. A. A fresh look at tumor immunosurveillance and immunotherapy. Nat Immunol, *2:* 293-299, 2001.

2. Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., and Schreiber, R. D. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature, *410:* 1107-1111, 2001.

3. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., and Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol, *3:* 991-998, 2002.

4. Dunn, G. P., Old, L. J., and Schreiber, R. D. The immunobiology of cancer immunosurveillance and immunoediting. Immunity, *21:* 137-148, 2004.

5. Smyth, M. J., Thia, K. Y., Street, S. E., Cretney, E., Trapani, J. A., Taniguchi, M., Kawano, T., Pelikan, S. B., Crowe, N. Y., and Godfrey, D. I. Differential tumor surveillance by natural killer (NK) and NKT cells. J Exp Med, *191:* 661-668, 2000.

6. Smyth, M. J., Crowe, N. Y., and Godfrey, D. I. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol, *13:* 459-463, 2001.

7. Moodycliffe, A. M., Nghiem, D., Clydesdale, G., and Ullrich, S. E. Immune suppression and skin cancer development: regulation by NKT cells. Nat Immunol, *1:* 521-525, 2000.

8. Terabe, M., Matsui, S., Noben-Trauth, N., Chen, H., Watson, C., Donaldson, D. D., Carbone, D. P., Paul, W. E., and Berzofsky, J. A. NKT cell-mediated repression of tumor

immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol, *1:* 515-520, 2000.

9. Terabe, M., Matsui, S., Park, J. M., Mamura, M., Noben-Trauth, N., Donaldson, D. D., Chen, W., Wahl, S. M., Ledbetter, S., Pratt, B., Letterio, J. J., Paul, W. E., and Berzofsky, J. A. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. J Exp Med, *198:* 1741-1752, 2003.

10. Kronenberg, M. and Gapin, L. The unconventional lifestyle of NKT cells. Nat Rev Immunol, *2:* 557-568, 2002.

11. Mendiratta, S. K., Martin, W. D., Hong, S., Boesteanu, A., Joyce, S., and Van Kaer, L. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. Immunity, *6:* 469-477, 1997.

12. Yoshimoto, T., Bendelac, A., Watson, C., Hu-Li, J., and Paul, W. E. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. Science, *270:* 1845-1847, 1995.

13. Ostrand-Rosenberg, S., Clements, V. K., Terabe, M., Park, J. M., Berzofsky, J. A., and Dissanayake, S. K. Resistance to metastatic disease in STAT6-deficient mice requires hemopoietic and nonhemopoietic cells and is IFN-gamma dependent. J Immunol, *169:* 5796-5804, 2002.

14. Terabe, M., Park, J. M., and Berzofsky, J. A. Role of IL-13 in regulation of anti-tumor immunity and tumor growth. Cancer Immunol Immunother, *53:* 79-85, 2004.

15. Dexter, D. L., Kowalski, H. M., Blazar, B. A., Fligiel, Z., Vogel, R., and Heppner, G. H. Heterogeneity of tumor cells from a single mouse mammary tumor. Cancer Res, *38:* 3174-3181, 1978.

16. Miller, F. R., Miller, B. E., and Heppner, G. H. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. Invasion Metastasis, *3:* 22-31, 1983.

17. Aslakson, C. J. and Miller, F. R. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res, *52:* 1399-1405, 1992.

18. Pulaski, B. A. and Ostrand-Rosenberg, S. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. Cancer Res, *58:* 1486-1493, 1998.

19. Serafini, P., De Santo, C., Marigo, I., Cingarlini, S., Dolcetti, L., Gallina, G., Zanovello, P., and Bronte, V. Derangement of immune responses by myeloid suppressor cells. Cancer Immunol Immunother, *53:* 64-72, 2004.

20. Gabrilovich, D. I., Velders, M. P., Sotomayor, E. M., and Kast, W. M. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. J Immunol, *166:* 5398-5406, 2001.

21. Bronte, V., Serafini, P., Apolloni, E., and Zanovello, P. Tumor-induced immune dysfunctions caused by myeloid suppressor cells. J Immunother, *24:* 431-446, 2001.

22. Bronte, V., Apolloni, E., Cabrelle, A., Ronca, R., Serafini, P., Zamboni, P., Restifo, N. P., and Zanovello, P. Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor

capable of activating or suppressing CD8(+) T cells. Blood, *96:* 3838-3846, 2000.

23. Almand, B., Clark, J. I., Nikitina, E., van Beynen, J., English, N. R., Knight, S. C., Carbone, D. P., and Gabrilovich, D. I. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol, *166:* 678-689, 2001.

24. Danna, E. A., Sinha, P., Gilbert, M., Clements, V. K., Pulaski, B. A., and Ostrand-Rosenberg, S. Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease. Cancer Res, *64:* 2205-2211, 2004.

25. Sinha, P., Clements, V. K., and Ostrand-Rosenberg, S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. J. Immunol., 2005.

26. Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol, *23:* 549-555, 2002.

27. Ho, W. Y., Cooke, M. P., Goodnow, C. C., and Davis, M. M. Resting and anergic B cells are defective in CD28-dependent costimulation of naive CD4+ T cells. J Exp Med, *179:* 1539-1549, 1994.

28. Murphy, K. M., Heimberger, A. B., and Loh, D. Y. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. Science, *250:* 1720-1723, 1990.

29. Haskins, K., Kubo, R., White, J., Pigeon, M., Kappler, J., and Marrack, P. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a

monoclonal antibody. J Exp Med, *157:* 1149-1169, 1983.

30. Pulaski, B. A., Terman, D. S., Khan, S., Muller, E., and Ostrand-Rosenberg, S. Cooperativity of Staphylococcal aureus enterotoxin B superantigen, major histocompatibility complex class II, and CD80 for immunotherapy of advanced spontaneous metastases in a clinically relevant postoperative mouse breast cancer model. Cancer Res, *60:* 2710-2715, 2000.

31. Ostrand-Rosenberg, S., Grusby, M. J., and Clements, V. K. Cutting edge: STAT6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. J Immunol, *165:* 6015-6019, 2000.

32. Van Rooijen, N. and Sanders, A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods, *174:* 83-93, 1994.

33. Qi, L. and Ostrand-Rosenberg, S. H2-O inhibits presentation of bacterial superantigens, but not endogenous self antigens. J Immunol, *167:* 1371-1378, 2001.

34. Green, S. J., Aniagolu, J., and Raney, J. J. Oxidative metabolism of murine macrophages. *In:* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (eds.), Current Protocols In Immunology, Vol. 3, pp. 14.15.11-14.15.11: Wiley, 2003.

35. Decker, T. and Lohmann-Matthes, M. L. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods, *115:* 61-69, 1988.

36. Pulaski, B. A., Clements, V. K., Pipeling, M. R., and Ostrand-Rosenberg, S. Immunotherapy with vaccines combining MHC class II/CD80+ tumor cells with

interleukin-12 reduces established metastatic disease and stimulates immune effectors and monokine induced by interferon gamma. Cancer Immunol Immunother, *49:* 34-45, 2000.

37. Kusmartsev, S. and Gabrilovich, D. I. Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother, *51:* 293-298, 2002.

38. Bronte, V., Serafini, P., De Santo, C., Marigo, I., Tosello, V., Mazzoni, A., Segal, D. M., Staib, C., Lowel, M., Sutter, G., Colombo, M. P., and Zanovello, P. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J Immunol, *170:* 270-278, 2003.

39. Rodriguez, P. C., Zea, A. H., DeSalvo, J., Culotta, K. S., Zabaleta, J., Quiceno, D. G., Ochoa, J. B., and Ochoa, A. C. L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. J Immunol, *171:* 1232-1239, 2003.

40. Zabaleta, J., McGee, D. J., Zea, A. H., Hernandez, C. P., Rodriguez, P. C., Sierra, R. A., Correa, P., and Ochoa, A. C. Helicobacter pylori arginase inhibits T cell proliferation and reduces the expression of the TCR zeta-chain (CD3zeta). J Immunol, *173:* 586-593, 2004.

41. Kusmartsev, S., Nefedova, Y., Yoder, D., and Gabrilovich, D. I. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J Immunol, *172:* 989-999, 2004.

42. Mills, C. D. Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. Crit Rev Immunol, *21:* 399-425, 2001.

43. Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., and Hill, A. M. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol, *164:* 6166-6173, 2000.

44. Smiley, S. T., Kaplan, M. H., and Grusby, M. J. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. Science, *275:* 977-979, 1997.

45. Chen, Y. H., Chiu, N. M., Mandal, M., Wang, N., and Wang, C. R. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. Immunity, *6:* 459-467, 1997.

46. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K., and Sugamura, K. Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. Science, *262:* 1874-1877, 1993.

47. Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E., and et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. Science, *262:* 1880-1883, 1993.

48. Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T., and Akira, S. Essential role of Stat6 in IL-4 signalling. Nature, *380:* 627-630, 1996.

49. Kaplan, M. H., Schindler, U., Smiley, S. T., and Grusby, M. J. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity, *4:* 313-319, 1996.

50. Shimoda, K., van Deursen, J., Sangster, M. Y., Sarawar, S. R., Carson, R. T., Tripp, R. A., Chu, C., Quelle, F. W., Nosaka, T., Vignali, D. A., Doherty, P. C., Grosveld, G., Paul, W. E., and Ihle, J. N. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature, *380:* 630-633, 1996.

51. Takeda, K., Kamanaka, M., Tanaka, T., Kishimoto, T., and Akira, S. Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. J Immunol, *157:* 3220-

3222, 1996.

52. Bronte, V., Serafini, P., Mazzoni, A., Segal, D. M., and Zanovello, P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol, *24:* 302-306, 2003.

53. El-Gayar, S., Thuring-Nahler, H., Pfeilschifter, J., Rollinghoff, M., and Bogdan, C. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. J Immunol, *171:* 4561-4568, 2003.

54. Paul, W. E. Interleukin-4: a prototypic immunoregulatory lymphokine. Blood, *77:* 1859-1870, 1991.

55. Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J., and Paul, W. E. The IL-4 receptor: signaling mechanisms and biologic functions. Annu Rev Immunol, *17:* 701-738, 1999.

56. Pulaski, B. A., Smyth, M. J., and Ostrand-Rosenberg, S. Interferon-gamma-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma. Cancer Res, *62:* 4406-4412, 2002.

57. Wei, L. H., Jacobs, A. T., Morris, S. M., Jr., and Ignarro, L. J. IL-4 and IL-13 upregulate arginase I expression by cAMP and JAK/STAT6 pathways in vascular smooth muscle cells. Am J Physiol Cell Physiol, *279:* C248-256, 2000.

58. Baniyash, M. TCR zeta-chain downregulation: curtailing an excessive inflammatory immune response. Nat Rev Immunol, *4:* 675-687, 2004.

59. Rodriguez, P. C., Quiceno, D. G., Zabaleta, J., Ortiz, B., Zea, A. H., Piazuelo, M. B., Delgado, A., Correa, P., Brayer, J., Sotomayor, E. M., Antonia, S., Ochoa, J. B., and Ochoa, A. C. Arginase I production in the tumor microenvironment by mature myeloid

cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res, *64:* 5839-5849, 2004.

60. Mazzoni, A., Bronte, V., Visintin, A., Spitzer, J. H., Apolloni, E., Serafini, P., Zanovello, P., and Segal, D. M. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol, *168:* 689-695, 2002.

61. Liu, Y., Van Ginderachter, J. A., Brys, L., De Baetselier, P., Raes, G., and Geldhof, A. B. Nitric oxide-independent CTL suppression during tumor progression: association with arginase-producing (M2) myeloid cells. J Immunol, *170:* 5064-5074, 2003.

62. Park, J. M., Terabe, M., van den Broeke, L. T., Donaldson, D. D., and Berzofsky, J. A. Unmasking immunosurveillance against a syngeneic colon cancer by elimination of CD4(+) NKT regulatory cells and IL-13. Int J Cancer, 2004.

FIGURE CAPTIONS

Figure 1. $CD1^{-/-}$ mice are resistant to disseminated metastatic disease. $CD1^{-/-}$ and BALB/c mice were inoculated in the mammary gland on day 0 with 7000 4T1 tumor cells, and primary tumors left in place (non-surgery group) or removed 2-3 weeks later (post-surgery group). **(A)** Post-surgery mice were followed for survival. Numbers indicate the number of mice surviving > 180 days /total mice. Data are from one of two independent experiments. **(B)** Lungs were harvested and the number of metastatic cells quantified by the clonogenic assay. Each symbol represents an individual mouse. Data are pooled from two independent experiments. **(C)** BALB/c or $CD1^{-/-}$ splenocytes, or 4T1 cells were stained with FITC-conjugated CD1d mAb (filled histograms) or isotype matched control mAb (open histograms) and analyzed by flow cytometry. Data are from one of two independent experiments.

Figure 2. Splenic MSC levels return to baseline after removal of primary tumor from $CD1^{-/-}$ mice. BALB/c or $CD1^{-/-}$ (A and B) or $RAG^{-/-}$ (C) mice were inoculated on day 0 with 4T1 tumor cells and primary tumors left in place (non-surgery groups) or removed on day ~28 (post-surgery groups). Tumor-free groups were not given 4T1. On day ~38 lungs and spleens were harvested, splenocytes were labeled with Gr1-PE and CD11b-FITC to determine percent MSC **(A and C)**, and the number of metastatic cells in the lungs quantified by the clonogenic assay **(B)**. Each symbol represents an individual mouse. Numbers in ( ) indicate the number of mice per group. Dotted lines indicate the maximum amount of MSC in tumor-free mice (<8%). Data are pooled from two independent experiments. * and ** indicates statistically significantly different values

($p<0.05$)

Figure 3. MSC inhibit T cell activation by an arginase-dependent mechanism. **(A)** Syngeneic DO11.10 or clone 4 or allogeneic 3A9 transgenic splenocytes were stimulated with $OVA_{323-339}$, $HA_{518-526}$, or HEL protein, respectively, in the presence of non-surgery BALB/c (open bars) or $CD1^{-/-}$ (filled bars) MSC. **(B)** The experiment of part A was repeated with or without the inhibitors for iNOS (L-NMMA) or arginase (nor-NOHA). MSC from 2-3 mice were pooled for each group. Control J774 cells gave no suppression (not shown). **(C)** DO11.10 T cells were stimulated with $OVA_{323-339}$ peptide and co-cultured in the same well with $CD1^{-/-}$ MSC, or the MSC were contained in a transwell chamber suspended in the well containing the T cells. Data are from one of two independent experiments.

Figure 4. MSC down-regulate CD3ζ chain in $CD4^+$, but not $CD8^+$, T cells. $CD4^+$ DO11.10 or $CD8^+$ clone 4 transgenic splenocytes were stimulated with $OVA_{323-339}$ or $HA_{518-526}$ peptide, respectively, in the presence of non-surgery BALB/c or $CD1^{-/-}$ MSC. Cultures were harvested and cells stained with CD3ζ-FITC, KJ1-26-tricolor, and CD4-PE ($CD4^+$ T cells) or CD3ζ-FITC, Vβ8-PE, and CD8-tricolor ($CD8^+$ T cells). CD3ζ expression was determined by gating on double positive cells ($CD4^+KJ1\text{-}26^+$ or $CD8^+V\beta8^+$, respectively). Dotted lines are unstimulated T cells (no peptide); solid lines are stimulated T cells (with peptide); filled histograms are stimulated T cells with MSC. Data are from one of two independent experiments.

Figure 5. ROS production by BALB/c, but not CD1$^{-/-}$, MSC is arginase-dependent. Purified BALB/c **(A)** and CD1$^{-/-}$ **(B)** MSC from tumor-free and non-surgery mice were unstained, or incubated with DCFDA in the presence or absence of the arginase inhibitor, nor-NOHA. Data are from one of two independent experiments.

Figure 6. CD1$^{-/-}$ mice have tumoricidal iNOS-producing M1 macrophages. **(A)** Peritoneal macrophages from BALB/c, CD1$^{-/-}$, IL-4R$\alpha^{-/-}$, or STAT6$^{-/-}$ mice were not activated, activated with LPS and IFNγ, and/or treated with IL-4 or IL-13 in the presence or absence of the iNOS inhibitor, L-NMMA or its inactive enantiomere, D-NMMA. NO was measured using the Griess reagent. **(B)** Peritoneal macrophages were prepared as in 6A, and cell lysates western blotted with mAb to arginase. Lane 1: not-activated; lane 2: activated with LPS and IFNγ; lane 3: pre-treated with IL-4 before activation with LPS and IFNγ; lane 4: pre-treated with IL-13 before activation with LPS and IFNγ; lane 5: pre-treated with IL-4, not activated; lane 6: pre-treated with IL-13, not activated. **(C)** LPS and IFNγ activated BALB/c, CD1$^{-/-}$, or IL-4R$\alpha^{-/-}$ peritoneal macrophages were co-cultured with 4T1 cells in the presence or absence of the iNOS inhibitor L-NMMA, and percent cytotoxicity measured by LDH release. Activated CD1$^{-/-}$, or IL-4R$\alpha^{-/-}$ macrophages are significantly more cytotoxic than BALB/c macrophages ($p < 0.05$).

Figure 7. IL-4R$\alpha^{-/-}$ mice are not resistant to 4T1 metastatic disease and retain elevated levels of MSC after surgery. IL-4R$\alpha^{-/-}$ and BALB/c mice were inoculated in the mammary gland on day 0 with 7000 4T1 tumor cells, and primary tumors left in place (non-surgery groups) or removed on day ~28 (post-surgery groups). Tumor-free mice were not given 4T1. **(A)** Lungs and spleens

were harvested on day ~38 and splenocytes were labeled with Gr1-PE and CD11b-FITC to determine percent MSC. **(B)** The number of metastatic cells in the lungs was quantified by the clonogenic assay. Data for (A) and (B) are pooled from two independent experiments and include 6 mice/group. Dotted lines indicate the maximum amount of MSC in tumor-free mice (<8%). Each symbol represents an individual mouse.

Figure 1

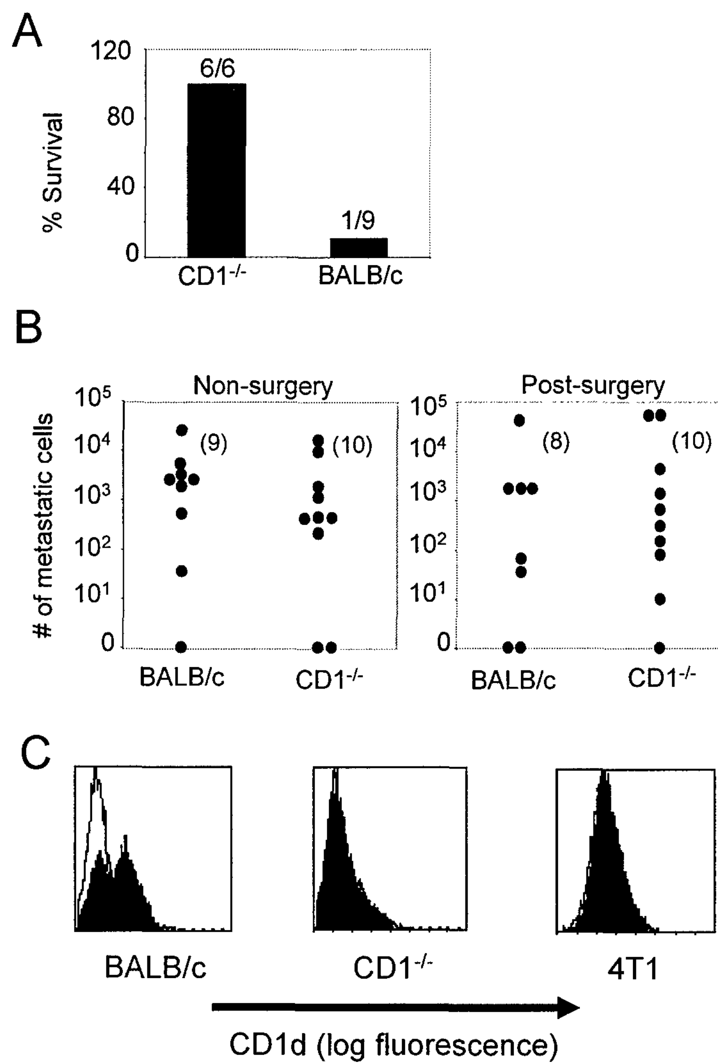


Figure 2

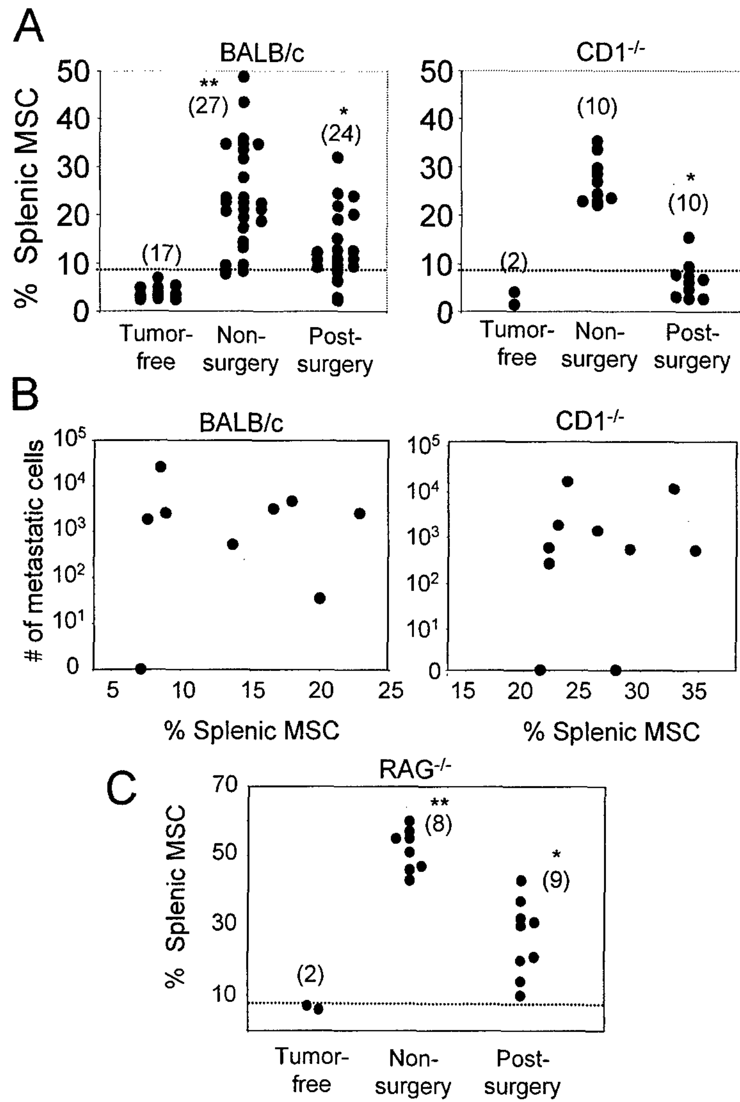


Figure 3

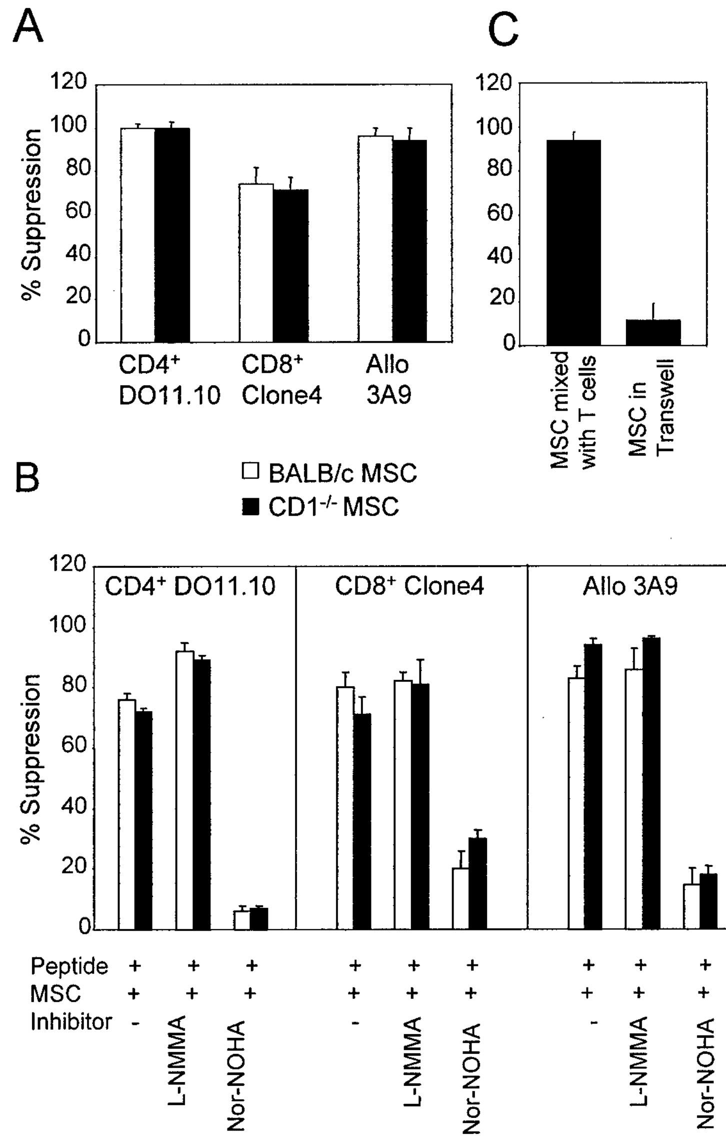


Figure 4

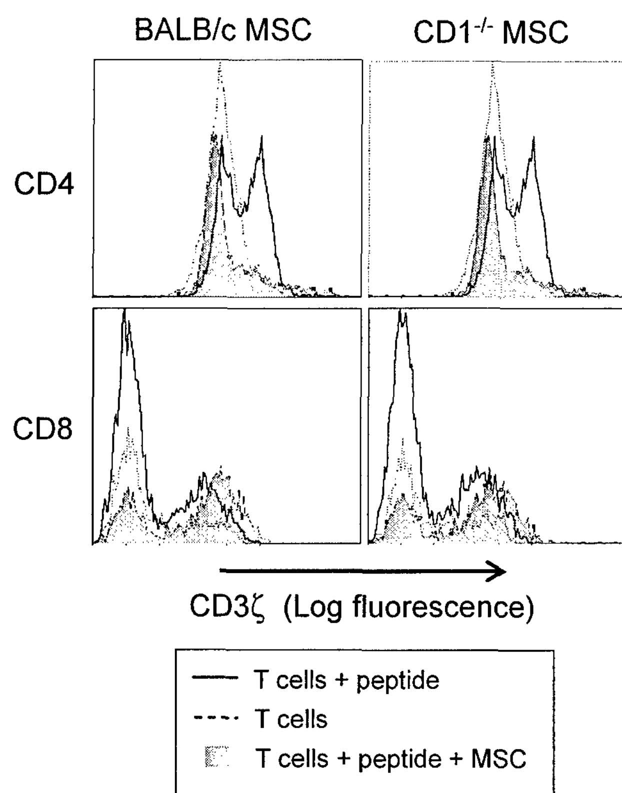


Figure 5

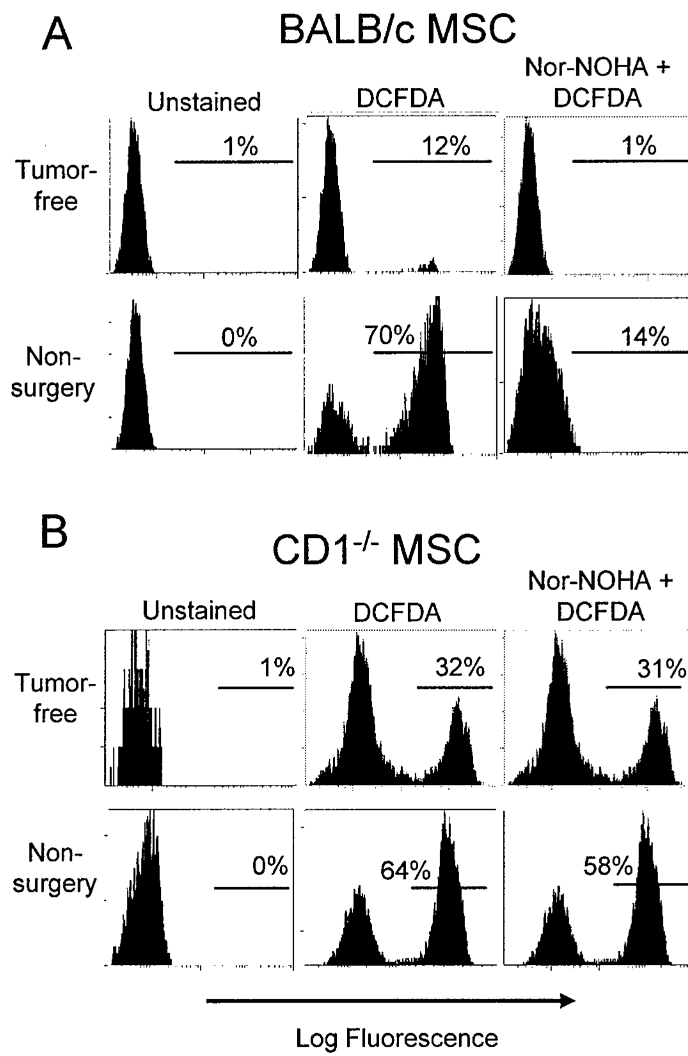
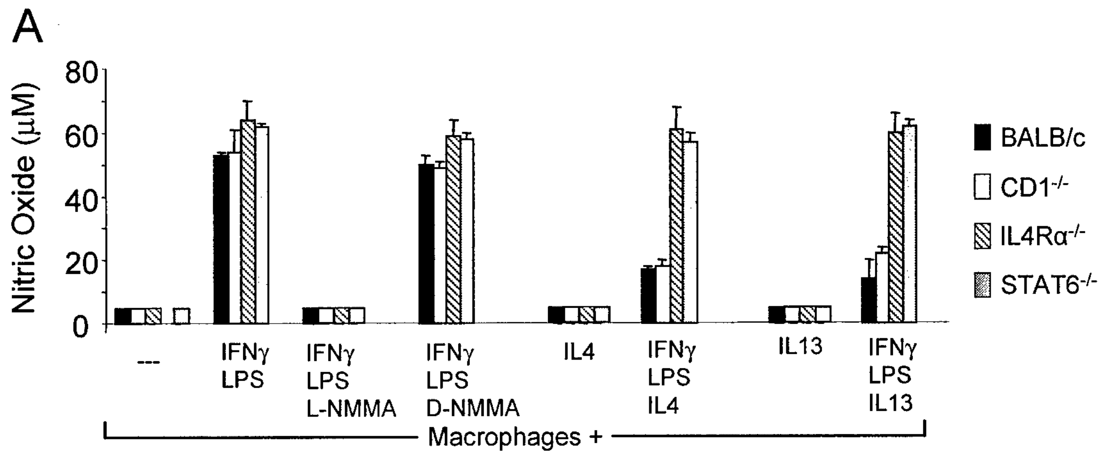
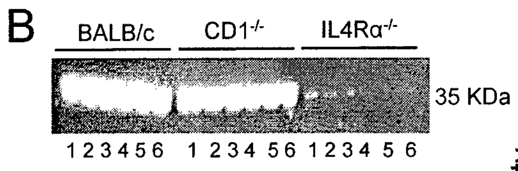


Figure 6





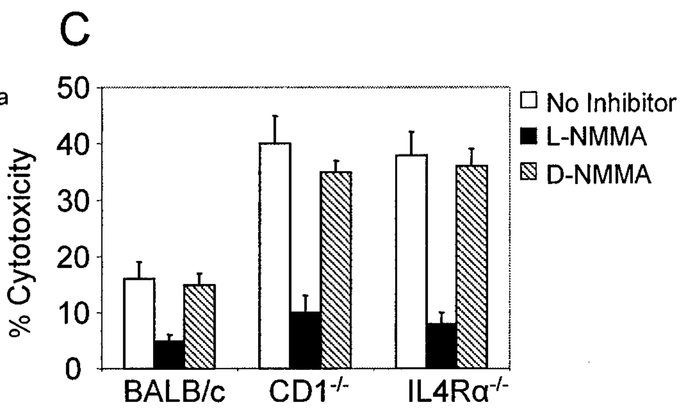


Figure 7