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

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

E-Mail: srosenbe@umbc.edu

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13. ABSTRACT (Maximum 200 Words)

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.

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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

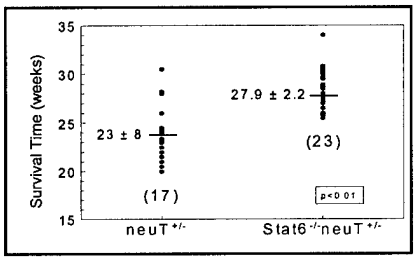

Figure 1: Deletion of the Stat6 gene extends survival of mice that spontaneously develop multifocal, metastatic mammary carcinoma.

During the 3rd year of this grant we have made the following progress:

Objective #1: ***Determine if survival of mice with spontaneous, metastatic mammary carcinoma is extended in Stat6-deficient mice that are at high risk for spontaneously developing mammary tumors.*** $NeuT^{+/-}$ mice spontaneously develop multifocal, metastatic mammary carcinoma (1). Last year we reported that tumor incidence and onset of tumor were reduced in $Stat6^{-/-}neuT^{+/-}$ mice vs. $neuT^{+/-}$ mice. As shown in **figure 1**, $Stat6^{-/-}neuT^{+/-}$ mice also have a significantly extended survival time relative to $neuT^{+/-}$ mice, demonstrating that deletion of the Stat6 gene also confers resistance to spontaneous metastatic mammary cancer.

Objective #3. Determine if the $Stat6^{-/-}$ effect is the result of a type 1 vs. Type 2 response? (In the original proposal these experiments were restricted to Type 1 and 2 $CD4^+$ and $CD8^+$ T cells. We have extended these studies to include type 1 and type 2 macrophages.)

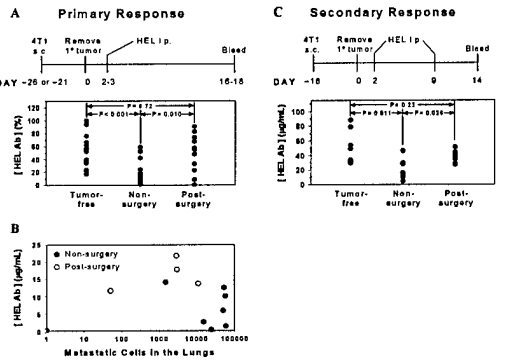

Figure 2: Surgical removal of bulky, primary mammary tumors reverses tumor-induced immune suppression of antibody responses to lysozyme.

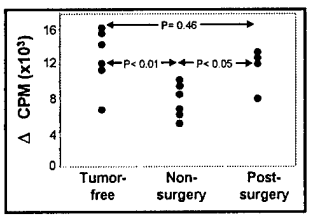

Figure 3: Surgical removal of primary tumor reverses tumor-induced immune suppression of T cell responses to lysozyme.

Mice with large, bulky primary mammary tumor are immune suppressed for B and T cell responses, but dendritic cell function is not affected. Immune responsiveness recovers after surgical removal of primary tumor. For over 20 years immunologists have noted that tumor-bearing individuals are often immunosuppressed and unable to respond to their tumors (2). Anecdotal results have suggested that the suppression may be reversible if the primary tumor is removed. To test this hypothesis BALB/c mice were inoculated with 4T1 tumor in the mammary gland and tumors were surgically

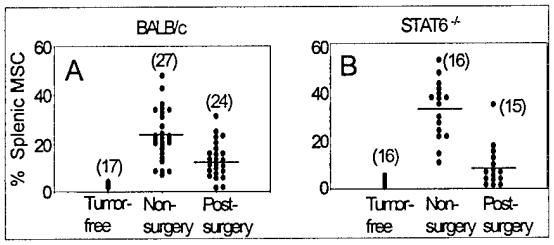

Figure 4. Myeloid suppressor cell (MSC) levels are elevated in mice with primary tumor, but regress to background levels in Stat6$^{-/-}$ mice after surgery, but remain elevated in BALB/c mice.

resected from one group of mice. Different groups were then immunized with hen eggwhite lysozyme (HEL) and antibody and T cell responses to HEL were assessed. Both antibody (**figure 2**) and T cell (**figure 3**) responses were significantly suppressed in tumor-bearing mice, but returned to normal levels after removal of primary tumor. In contrast, dendritic cell function is not impaired in mice with primary mammary tumors (data not shown).

Myeloid suppressor cell levels are elevated when primary tumor is present, but return to baseline in Stat6-deficient mice following surgical removal of primary tumor. To determine if deletion of the Stat6 gene affects myeloid suppressor cell (MSC) levels in 4T1 mammary 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, and stained and analyzed by flow cytometry for Gr1^{+}CD11b^{+} MSC ("non-surgery" group). Because immune suppression has been shown to decrease if primary tumor is removed, we also assessed MSC levels in mice whose primary tumors were surgically resected (post-surgery group). For the post-surgery group, primary tumors were resected on day 21-28 after initial tumor inoculation, and splenic MSC levels were assessed 9-11 days later. As shown **in figure 4**, tumor-free BALB/c and Stat6$^{-/-}$ mice have <8% splenic MSC, while BALB/c and Stat6$^{-/-}$ mice with primary tumors in place ("non-surgery" groups) have similar high levels of splenic MSC. Although both post-surgery groups show decreases in splenic MSC, 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 less than 8% splenic MSC 11 days after surgery. Therefore, although BALB/c and Stat6-deficient mice have comparable high levels of splenic MSC while primary tumor is present, after surgery Stat6$^{-/-}$ mice have fewer MSC. (note: last year we reported preliminary results for this finding)

Reduced MSC levels in post-surgery Stat6-deficient mice correlate with reduced metastatic disease. Tumor cells are known to secrete cytokines that stimulate the accumulation of MSC. Therefore, the different levels of MSC in post-surgery BALB/c vs. Stat6-deficient mice could be due to differences in cytokine production by metastatic 4T1 tumor cells. To test this hypothesis, supernatants from 4T1 cells were tested for cytokines production. Although supernatants from ex vivo cultured primary and metastatic 4T1 cells contained variable levels of IL-6, GM-CSF, VEGF, and activated TGFβ, there 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 MSC; however, the difference in MSC levels in post-surgery BALB/c and Stat6$^{-/-}$ mice is not due to differential secretion of these cytokines. Since tumor load may affect MSC levels, we have quantified lung metastatic disease in the non-surgery and post-surgery BALB/c and Stat6-deficient mice. Post-surgery Stat6$^{-/-}$ mice have significantly fewer metastatic tumor cells than post-surgery BALB/c mice (data not shown). Therefore, the decrease in MSC levels in Stat6$^{-/-}$ mice correlates with reduced metastatic disease and may be one of the factors contributing to reduced metastatic disease in Stat6-deficient animals.

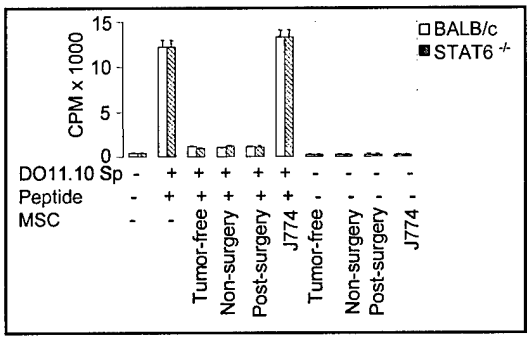

Figure 5: MSC from tumor-free, non-surgery, and post-surgery BALB/c and Stat6 $^{-/-}$ mice are equally immunosuppressive.

MSC from Stat6$^{-/-}$ and BALB/c tumor-bearing mice are phenotypically and functionally equivalent and their suppressive activity is due to arginase production. Qualitative differences between MSC of BALB/c and Stat6$^{-/-}$ mice may also contribute to the increased anti-tumor immunity of Stat6-deficient animals. To test this hypothesis, purified MSC from

the two mouse strains were phenotyped by antibody staining. Although the MSC from both strains are phenotypically similar, MSC from Stat6-deficient mice express more CD16/CD32 and CD80, while MSC from BALB/c mice express more CD11c, DEC205, and CD8 (data not shown). Therefore, there are subtle differences between the MSC of tumor-bearing BALB/c and Stat6-deficient mice. To determine if the MSC of BALB/c and Stat6-deficient mice have similar immunosuppressive activity, equal numbers of MACS-sorted $Gr1^+CD11b^+$ cells from tumor-free, non-surgery, or post-surgery mice were co-cultured with ovalbumin-peptide-pulsed splenocytes from DO11.10 transgenic mice and T cell proliferation was measured by ^{3}H-thymidine incorporation. As shown in **figure 5**, $Gr1^+CD11b^+$ cells from all three groups and from both strains are highly suppressive, while control J774 cells do not inhibit T cell activation. Therefore, the suppressive capacity of $Gr1^+CD11b^+$ cells on a per cell basis is similar in BALB/c and Stat6-deficient mice and is independent of whether tumor is present.

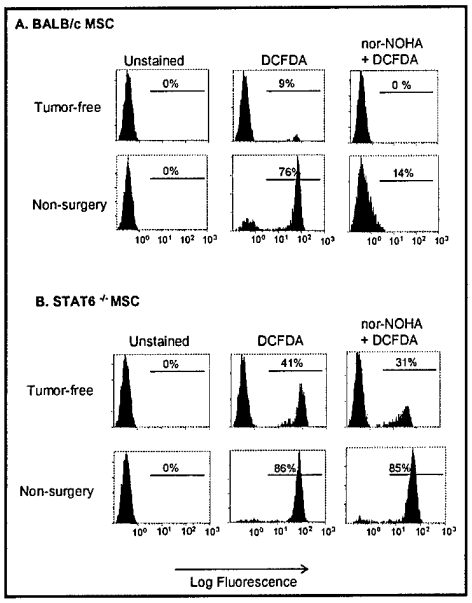

Figure 6: MSC from tumor-bearing BALB/c and Stat6$^{-/-}$ mice produce ROS; however, the ROS of BALB/c MSC is arginase-dependent.

MSC suppressive activity is mediated by the production of reactive oxygen species (ROS) (3). To determine if differences in anti-tumor immunity between BALB/c and Stat6$^{-/-}$ mice could be due to differential expression of ROS, purified $Gr1^+CD11b^+$ MSC from tumor-free and non-surgery BALB/c and Stat6-deficient mice were treated with DCFDA or DHE and 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. Likewise, DHE is oxidized by superoxide to a fluorescent species. As shown in **figure 6**, MSC from tumor-bearing non-surgery BALB/c and Stat6-deficient mice contain more ROS than MSC from tumor-free mice. Neither MSC population stains with DHE (data not shown), indicating that the MSC do not make superoxide. To determine if arginase is required for ROS production in the 4T1-induced MSC, BALB/c and Stat6$^{-/-}$ MSC were treated with the arginase inhibitor N-hydroxy-L-arginine (Nor-NOHA) prior to staining with DCFDA. Interestingly, Nor-NOHA blocks the production of ROS from MSC of BALB/c non-surgery mice, but has no effect on ROS expression by MSC from non-surgery STAT6$^{-/-}$ mice. Therefore, MSC from tumor-bearing BALB/c and Stat6-deficient mice make similar ROS; however, the ROS produced by BALB/c mice are arginase-dependent, while the ROS produced by Stat6-deficient MSC are independent of arginase.

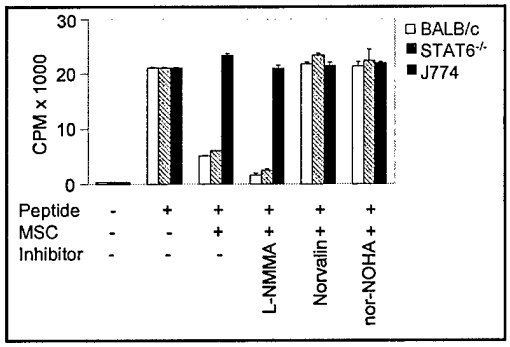

Figure 7: BALB/c and Stat6$^{-/-}$ MSC suppress via the production of arginase.

To determine if MSC from tumor-bearing BALB/c and Stat6-deficient mice differ because of selective expression of arginase and/or iNOS, as reported (4, 5), ovalbumin-peptide-pulsed splenocytes from DO11.10 transgenic mice were co-cultured with MSC from BALB/c or Stat6-deficient mice in the presence or absence of inhibitors of arginase or iNOS. As shown in **figure 7**, BALB/c and Stat6$^{-/-}$ MSC inhibit DO11.10 proliferation, and this inhibition is reversed by the arginase inhibitors norvalin and Nor-NOHA, but not by the iNOS inhibitor, N^G-monomethy-L-argine (L-NMMA). Therefore, MSC from both BALB/c and Stat6-deficient mice mediate their suppression through arginase, and iNOS is not involved.

Reduction of MSC levels and decrease in metastatic disease in Stat6$^{-/-}$ mice is IFNγ-dependent. Previous studies established that resistance to the 4T1 tumor in Stat6-deficient mice requires IFNγ since Stat6$^{-/-}$IFNγ$^{-/-}$ mice were just as susceptible to metastatic disease as BALB/c mice (6). If tumor resistance in post-surgery

Stat6$^{-/-}$ mice is dependent on the rapid decrease in MSC and if IFNγ is involved in that decrease, then post-surgery Stat6$^{-/-}$ IFNγ$^{-/-}$ mice should have relatively high levels of MSC. To test this hypothesis, Stat6$^{-/-}$IFNγ$^{-/-}$ mice were inoculated with 4T1 tumor on day 1,and tumors were either left in place non-surgery group) or they were removed 21-28 days later (post-surgery group). Nine to eleven days after the surgery date, all micewere sacrificed and splenocytes stained with the Gr1 and CD11b antibodies, and the lungs of the non-surgery and post-surgery groups were assayed by the clonogenic assay for metastatic 4T1 tumor cells. As shown in **figure 8A**, there is a modest decrease in MSC in post-surgery Stat6$^{-/-}$IFNγ$^{-/-}$ mice; however, the decrease is comparable to that seen in tumor-susceptible BALB/c mice (14% and 21% have <8% MSC, respectively), and does not approach the larger decrease seen in Stat6$^{-/-}$ mice in which 67% of mice have <8% MSC. In agreement with the high MSC count, the number of metastatic cells in the Stat6$^{-/-}$IFNγ$^{-/-}$ mice is also elevated (**figure 8B**). Therefore, resistance to metastatic disease in Stat6$^{-/-}$ mice requires IFNγ and IFNγ is essential for the rapid decrease in MSC in post-surgery Stat6$^{-/-}$ mice.

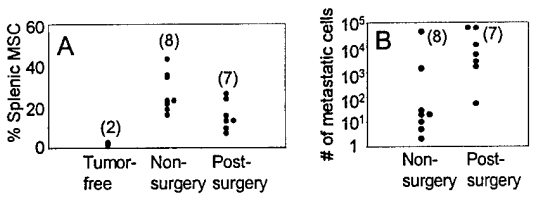

Figure 8: A, MSC levels do not return to baseline (A) and metastatic disease remains high (B) in Stat6$^{-/-}$IFNγ$^{-/-}$ mice after removal of primary tumor from.

Stat6$^{-/-}$ macrophages produce high levels of iNOS and ROS, are cytotoxic for 4T1 tumor cells, and do not produce arginase. In addition to cytotoxic CD8$^+$ T cells, macrophages have been shown to have significant tumoricidal activity. Type 1 macrophages (M1 macrophages), which make iNOS and do not make arginase, are particularly tumoricidal, while M2 macrophages, which make arginase and do not make iNOS are not tumoricidal (7-9). Previous studies indicated that phagocytic cells are an important component of immune surveillance against the 4T1 tumor (10), suggesting that macrophages may be involved in the enhanced immunity of Stat6-deficient mice. To determine if qualitatively distinct macrophage populations could be responsible for the heightened immunity in Stat6-deficient mice, bone marrow-derived macrophages (BMDM) were prepared from tumor-free, non-surgery, and post-surgery BALB/c and Stat6$^{-/-}$ mice, and tested in vitro for arginase, iNOS, and ROS production, and for cytotoxicity against 4T1 tumor cells. As shown in **figure 9A**, both activated and non-activated BALB/c macrophages from non-surgery and post-surgery mice synthesize arginase, as measured by urea production, whereas Stat6$^{-/-}$ macrophages, regardless of activation state, do not produce arginase. iNOS production, as measured by NO, also differs between BALB/c and Stat6$^{-/-}$ macrophages (**figure 9B**), although non-activated macrophages from either strain do not produce iNOS. Activated macrophages from tumor-free BALB/c mice produce high levels of iNOS, whereas macrophages from non-surgery and post-surgery BALB/c mice produce significantly less iNOS. In contrast, activated macrophages from Stat6$^{-/-}$ mice produce uniformaly high levels of iNOS regardless of the presence or absence of 4T1 tumor. Tumoricidal activity of activated macrophages, as measured by cytotoxicity against in vitro cultured 4T1 cells, is significantly higher in Stat6$^{-/-}$ mice than in BALB/c mice (**figure 9C**). Collectively, these data indicate that BALB/c mice make an M2 macrophage response that is ineffective in destroying 4T1 tumor, while Stat6-deficient mice make an M1 macrophage response that results in macrophage-mediated destruction of 4T1 tumor cells.

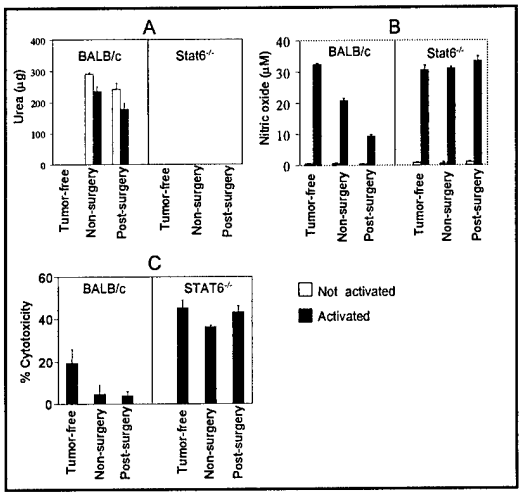

Figure 9: A, BALB/c, but not Stat6$^{-/-}$ macrophages produce arginase. B, Stat6$^{-/-}$ macrophages consistently produce more iNOS than BALB/c macrophages, regardless of the presence of primary tumor or metastais. C, Stat6$^{-/-}$, but not BALB/c, macrophages are tumoridical for 4T1 tumor cells.

Proposed model for enhanced tumor-specific immunity in Stat6$^{-/-}$ mice. **Figure 10** shows a model of how

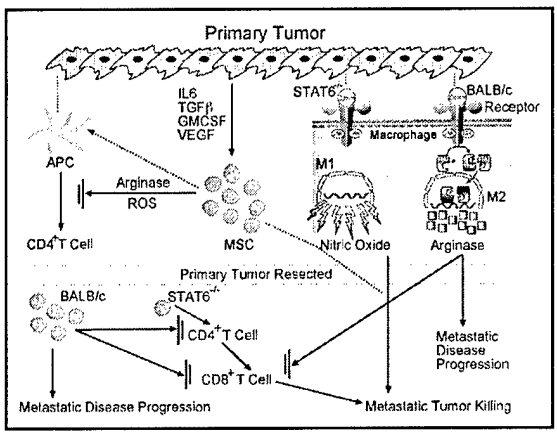

Figure 10: Proposed model for Stat6$^{-/-}$ resistance to metastatic mammary carcinoma. See text for explanation of model.

these effectors and inhibitors may interact to mediate mammary 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 MSC. MSC are thought to inhibit T cell function by secreting arginase which depletes L-arginine causing a loss of CD3ζ chain expression (11, 12). Therefore, the high levels of arginase-producing MSC block activation of tumor-reactive CD4$^+$ T cells which would normally be activated to tumor antigens by cross-presentation by professional antigen presenting cells (APC). In the absence of functional CD4$^+$ T cells, potent tumor-specific CD8$^+$ T cells are not generated and tumor growth progresses. However, following surgical removal of 4T1 primary tumor from Stat6-deficient mice, MSC levels decrease to baseline. The post-surgery decrease in MSC is likely due to two factors: (i) a decrease in tumor-produced cytokines that induce MSC; and (ii) macrophage production of nitric oxide that stimulates differentiation of the MSC to normal myeloid-derived cells (13, 14). In the absence of elevated MSC, CD4$^+$ T cells are no longer inhibited, and cytotoxic tumor-specific CD8$^+$ T cells are activated. In contrast, MSC levels in post-surgery Stat6-competent BALB/c mice do not decrease to baseline because BALB/c macrophages produce high levels of arginase and much less NO, so CD4$^+$ T cells remain suppressed and effector CD8$^+$ T cells are not generated. In addition to their ability to stimulate MSC differentiation, macrophages of Stat6-deficient mice are also tumoricidal because they make NO, whereas BALB/c macrophages are not cytotoxic and make arginase which supports tumor growth (30). 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. This model is consistent with the results presented in this report, and also explains our earlier studies in which phagocytic cells, presumably macrophages, were shown to be important for effective immunosurveillance against the 4T1 tumor (10).

KEY ACCOMPLISHMENTS

- Demonstrated that the presence of primary 4T1 mammary carcinoma induces extensive immune suppression of antibody production and T cell responses, but not dendritic cell function.
- Demonstrated that surgical removal of primary tumor reverses T and B cell immune suppression even if metastatic survival time of individuals at high risk of developing mammary carcinoma is reduced by deletion of the Stat6 gene.
- Demonstrated that myeloid suppressor cell levels return to background after surgical removal of primary tumor from Stat6$^{-/-}$ mice, despite the presence of metastatic disease.
- Demonstrated that MSC of both tumor-susceptible BALB/c and tumor-resistant Stat6$^{-/-}$ mice mediate suppession via the production of arginase.
- Demonstrated that macrophages of Stat6$^{-/-}$, but not BALB/c, mice produce iNOS and are cytotoxic for mammary carcinoma cells.
- Demonstrated that IFNγ is essential for reduced myeloid suppressor cell levels in post-surgery Stat6$^{-/-}$ mice.

REPORTABLE OUTCOMES

- Review article entitled *Signal transducer and activator of transcription 6 (Stat6) and CD1: inhibitors of immunosurveillance against primary tumors and metastatic disease* was published in *Cancer Immunology and Immunotherapy 53:86-91, 2004* (paper is included in Appendix materials)

- Peer-reviewed article entitled *Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease* was published in *Cancer Research 64:2205-2211, 2004* (paper is included in the Appendix materials)

- Review article entitled *Animal models of tumor immunity, immunotherapy and cancer vaccines* was published in *Current Opinion in Immunology 16:143-150, 2004* (paper is included in Appendix materials)

- Poster presentation of the reported results was presented at the *95th annual American Association of Cancer Research conference*, Orlando, Fl, March 2004.

- Poster presentation of the reported results was presented at the American Association of Immunology Experimental Biology 2004 conference in Washington, DC, April 2004

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. Progress has been made in both of these areas. The studies demonstrating that mice at high risk of developing *spontaneous* breast cancer ($neuT^{+/-}$ mice) are protected by deletion of the Stat6 gene strongly supports previous findings with a transplantable mammary carcinoma, and demonstrates the potency of the Stat6 effect. The studies demonstrating that myeloid suppressor cell levels in $Stat6^{-/-}$ mice, but not in BALB/c mice, decrease after surgery, and that $Stat6^{-/-}$, but not BALB/c, macrophages produce iNOS and do not produce arginase, strongly suggest that tumoricidal macrophages may be a key element in tumor resistance in $Stat6^{-/-}$ mice. These results are significant because they demonstrate that effective immunity against metastatic mammary cancer is the result of two phenomena: 1) The reduction of tumor-induced immune suppression; and 2) the development of iNOS producing macrophages. These findings have direct implications for the development of effective therapies against metastatic disease.

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APPENDICES

The following published papers are attached:

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**.

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**.

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

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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

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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

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

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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

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.

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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.

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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.

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

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:

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

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:

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

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:

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

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.

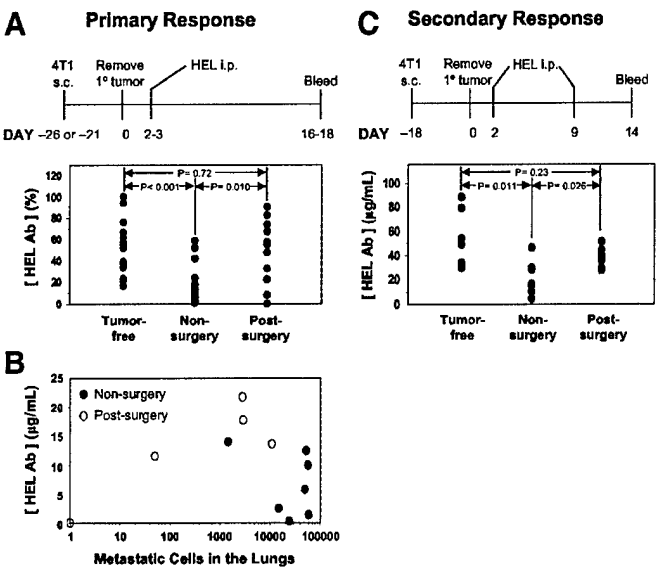

Fig. 1. Antibody production is reduced in 4T1 tumor-bearing mice but recovers following primary tumor removal despite the presence of metastatic disease. BALB/c mice without tumors ("tumor-free"), with a primary tumor in place ("nonsurgery"), or with their primary tumors surgically removed ("postsurgery") 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.

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.

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

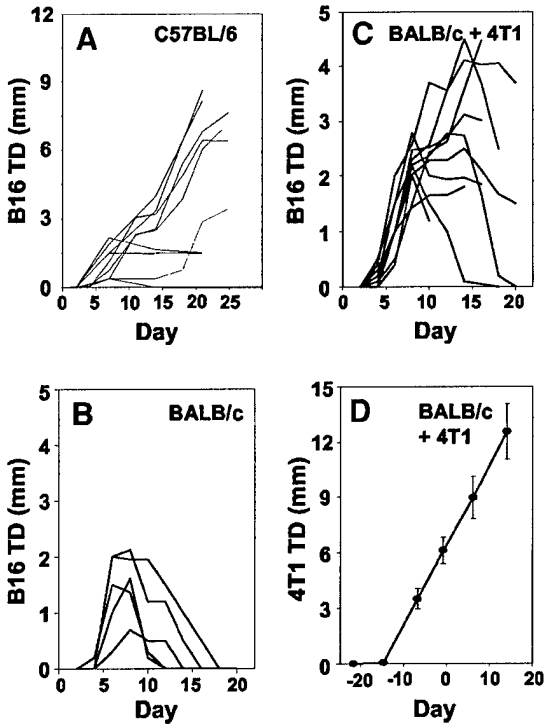

Fig. 2. 4T1 tumor-bearing mice do not efficiently reject allogeneic tumor. B16 tumor growth in C57BL/6 (*A*) or BALB/c (*B*) mice inoculated s.c. in the flank on day 0 with 1×10^6 B16 tumor cells (*H-2*^{b}). *C*, B16 tumor growth in BALB/c mice inoculated s.c. in the mammary gland on day −22 with 7×10^3 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

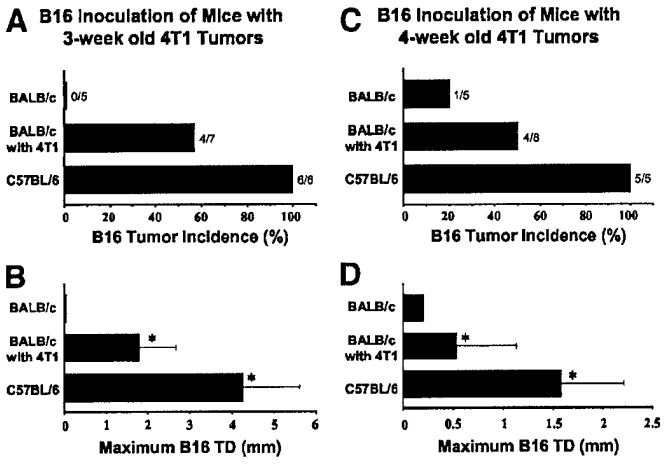

Fig. 3. Incidence and growth of allogeneic tumor are greater in 4T1 tumor-bearing mice than in tumor-free mice. BALB/c, C57BL/6, or 4T1 tumor-bearing nonsurgery BALB/c mice were inoculated s.c. with 5×10^5 B16 cells and followed for incidence of B16 tumor growth (*A* and *C*) and maximum B16 tumor diameter (*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 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.

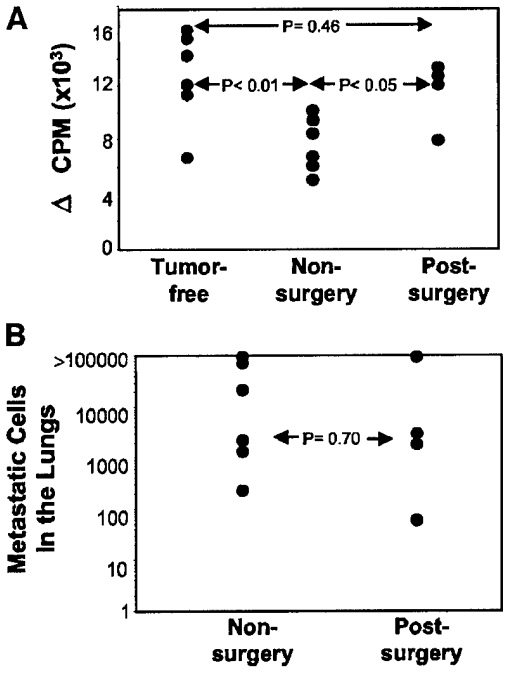

Fig. 4. Hen egg white lysozyme (HEL)-specific T-cell responses are reduced in 4T1 tumor-bearing mice but recover following primary tumor removal despite the presence of metastatic disease. *A*, tumor-free, nonsurgery, and postsurgery BALB/c mice were immunized with HEL. Nine days later, their spleens 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.

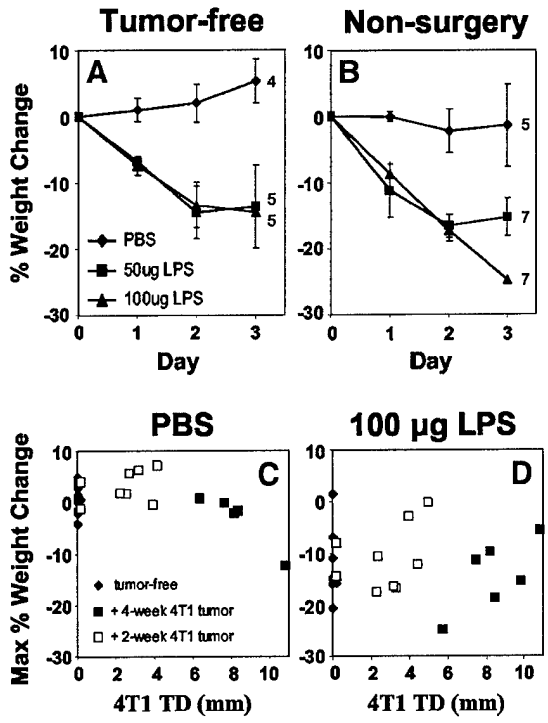

Fig. 5. 4T1 tumor burden does not affect macrophage activity. *A* and *B*, tumor-free BALB/c mice (*A*) and BALB/c mice inoculated s.c. with 4T1 on day −28 (nonsurgery; *B*) were inoculated with lipopolysaccharide (*LPS*) or with PBS on day 0. Weight loss was tracked daily or until mice died. At day 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.

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-

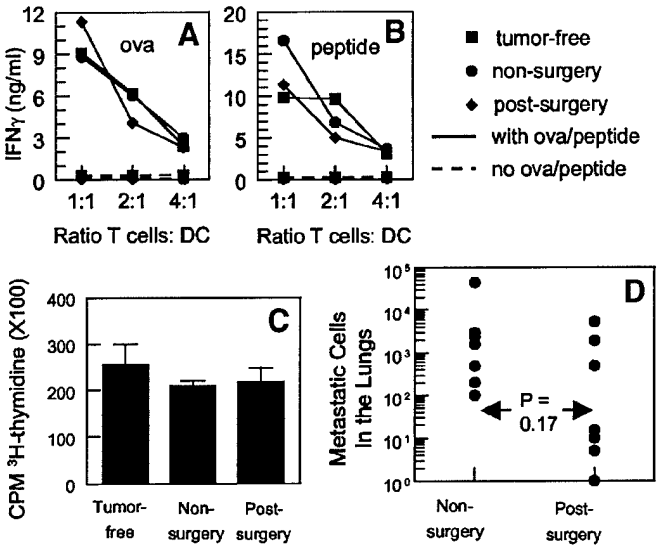

Fig. 6. Dendritic cell (*DC*) activity is not suppressed in tumor-bearing mice. Splenic DCs were purified from tumor-free, nonsurgery, and 10-day postsurgery BALB/c mice. DCs were pulsed with ovalbumin protein (*A*) or ovalbumin peptide 323–339 (*B*) and cocultured at varying ratios with I-A^d-restricted $ova_{323-339}$-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).

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ELSEVIER

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

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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.

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