The *Journal of* Immunology

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

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

---

References   This article **cites 28 articles**, 16 of which you can access for free at:
http://www.jimmunol.org/content/169/10/5796.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription   Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions   Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts   Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

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

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

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

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

fore, heightened tumor immunity in STAT6-deficient mice does not appear to be the result of the balance between CD4$^+$ Th1 and Th2 cells.

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

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

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

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

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

Materials and Methods

Mice, cells, and tumor inoculations

BALB/c, BALB/c STAT6$^{-/-}$ (henceforth called STAT6$^{-/-}$), and BALB/c CD1$^{-/-}$ (henceforth called CD1$^{-/-}$) mice were bred in the University of Maryland Biology Department animal facility from breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME), M. Grusby (Dana-Farber Cancer Institute, Boston, MA), and L. van Kaer (Washington University, St. Louis, MO), respectively. BALB/c IL-4Rα-deficient (henceforth called IL-4Rα$^{-/-}$) mice were purchased from The Jackson Laboratory. STAT6$^{-/-}$ and CD1$^{-/-}$ mice were originally made by targeted disruption of the STAT6 and CD1 genes, respectively, in 129 ES cells. Offspring were

backcrossed to BALB/c mice (10 backcross generations for STAT6$^{-/-}$; 9 backcross generations for CD1$^{-/-}$) (17–19). Female mice of 8–16 wk were used for all studies.

STAT6$^{-/-}$IFN-γ$^{-/-}$ mice were generated by crossing STAT6$^{-/-}$ mice with IFN-γ$^{-/-}$ BALB/c mice. Heterozygous offspring were intercrossed, and the F$_2$ were screened by PCR for homozygosity for STAT6$^{-/-}$ and IFN-γ$^{-/-}$. Ear punch tissue of individual mice was placed in a 0.5-ml microfuge tube, and 20 μl of 50 mM Tris-HCl (pH 8.0), 2 mM NaCl, 10 mM EDTA, 1% SDS, and 1 μl of 20 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) were added. Tubes were incubated at 55°C for 20 min, and after vigorous vortexing for 2 min, the mixture was incubated for an additional 20 min at 55°C. Sterile distilled water was then added to each tube to a final total volume of 200 μl, and the tubes were heated at 100°C for 5 min. The following primers were used: STAT6, 5′ primer, TGAGGTGGGGACCAGCCGG; STAT6, 3′ primer, GTGACCAGGACAC ACAGCGG; Neo-STAT6, primer, GCTACCCGTGATATTGCTGAAGAG; IFN-γ, 5′ primer, AGAAGTAAGTGGAAGGGCCCAGAAG; IFN-γ, 3′ primer, AGGGAAACTGGGAGAGGAGAAATAT; IFN-γ Neo, 5′ primer, TCAGCGCAGGGGCGCCCGGTTCTTT; IFN-γ Neo, 3′ primer, ATCGA CAAGACCGGCTTCCATCCGA. PCR conditions for both IFN-γ and STAT6 genes were: denature at 94°C for 5 min, denature at 94°C for 1 min, anneal at 59°C for 1 min, extend at 72°C for 1.3 min, repeat the last three steps 34 times, extend at 72º for 9 min. PCR products were electrophoresed on 2% agarose gels. The wild-type STAT6 and STAT6-deletion genes produce 100- and 250-kb bands, respectively (17). The wild-type IFN-γ and IFN-γ-deletion genes produce 220- and 375-bp bands, respectively (www.jax.org/resources/ documents/imr/protocols/Ifng_KO.html).

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

Surgical removal of primary mammary tumors

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

Bone marrow chimeras

STAT6$^{-/-}$ mice containing BALB/c bone marrow (BALB/c→STAT6$^{-/-}$), BALB/c mice containing STAT6$^{-/-}$ bone marrow (STAT6$^{-/-}$→BALB/c), STAT6$^{-/-}$ mice containing CD1$^{-/-}$ bone marrow (CD1$^{-/-}$→STAT6$^{-/-}$), and CD1$^{-/-}$ mice containing STAT6$^{-/-}$ bone marrow (STAT6$^{-/-}$→ CD1$^{-/-}$) were constructed as follows using aseptic conditions. Donor mice were asphyxiated with CO$_2$ and immersed in 70% ethanol, and their hind legs were removed at the hip. Femurs were dissected away from the surrounding tissue, their ends were cut off, and the remaining bone was flushed three times with sterile PBS using a 30-ml syringe fitted with a 27-gauge needle. Bone marrow cells were collected in petri dishes and transferred to 15-ml conical tubes, and the aggregated material was allowed to gravity settle and was discarded. The remaining bone marrow cells were washed twice with PBS and resuspended in medium (RPMI, 1% penicillin, 1% streptomycin, 1% fungizone) at 200 μl per donor mouse. Recipient mice were taken off food the evening before bone marrow transfer. Between 0 and 2 h before bone marrow reconstitution, recipient mice were lethally irradiated (8.75 Gy, Cs-137 source, Gammator B; Kewaunee Scientific, Statesville, NC). Bone marrow was inoculated into recipient mice through the tail vein using a 1-ml syringe fitted with a 27-gauge needle. Each recipient received 100 μl of donor cells (bone marrow from one donor femur). Reconstituted mice received daily injections of gentamicin sulfate i.p. (100 μl of 5 mg/ml) for 7 days beginning 1 day before bone marrow reconstitution. Reconstituted mice were maintained on 2% tetracycline water starting 1–2 wk before bone marrow transfer and continuing for 6–8 wk after reconstitution. Eight to 12 wk after bone marrow reconstitution, chimeras were bled from the tail vein, and the blood was tested by PCR to ascertain hemopoietic genotype and reconstitution.

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

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

Treatment with soluble IL-13Rα2-Fc

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

CTL assays

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

Flow cytometry

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

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

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

Cytokine production

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

Statistical analyses

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

Results

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

To assess the potency of the STAT6 antitumor effect against established metastatic tumor, BALB/c and STAT6$^{-/-}$ mice were inoculated with 4T1 cells, and followed for development of metastasis and survival after surgical removal of primary tumor. Groups of BALB/c and STAT6$^{-/-}$ mice were inoculated in the abdominal mammary gland with 7000 4T1 tumor cells, and the tumors were allowed to grow progressively. Although primary tumors in STAT6$^{-/-}$ mice grow more slowly than primary tumors in

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

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

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

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

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

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

---

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

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

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

[a] BALB/c and STAT6$^{-/-}$ mice were inoculated with 7000 4T1 cells in the mammary gland. Two to 3 wk later, the primary tumors were surgically removed. On days 42–45 after initial 4T1 inoculation, mice were sacrificed, and the lungs, liver, and bone marrow were assayed by the clonogenic assay for the number of metastatic 4T1 cells. Average diameter of primary BALB/c vs STAT6$^{-/-}$ tumors at the time of surgery: 4.99 mm $\pm$ 1.42 vs 4.38 $\pm$ 1.31, respectively.
[b] Thirteen mice per group.
[c] Twenty mice per group.
[d] Number of metastatic cells determined by the clonogenic assay. Top number is the range of metastatic cells; number in parentheses is the average number of metastatic cells $\pm$ SD. Calculation includes only those mice with metastasis.
[e] A statistically significant difference between the STAT6$^{-/-}$ and BALB/c values ($p \leq 0.05$) for the number of metastatic cells per organ.

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

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

Enhanced tumor immunity requires both hemopoietic and nonhemopoietic components

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

3*A*, the chimeras and control BALB/c mice are dead by day 45 post-4T1 inoculation, while 46% of the STAT6$^{-/-}$ mice survive >350 days. STAT6$^{-/-}$ hemopoietic derived cells, therefore, are not sufficient for enhanced tumor immunity, suggesting that STAT6$^{-/-}$ nonhemopoietic derived cells or both hemopoietic and nonhemopoietic STAT6$^{-/-}$ cells are required.

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

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

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

Earlier studies identified NKT cells and IL-13 as potential inhibitors of tumor immunity to the HIV gp160-transfected 15-12RM
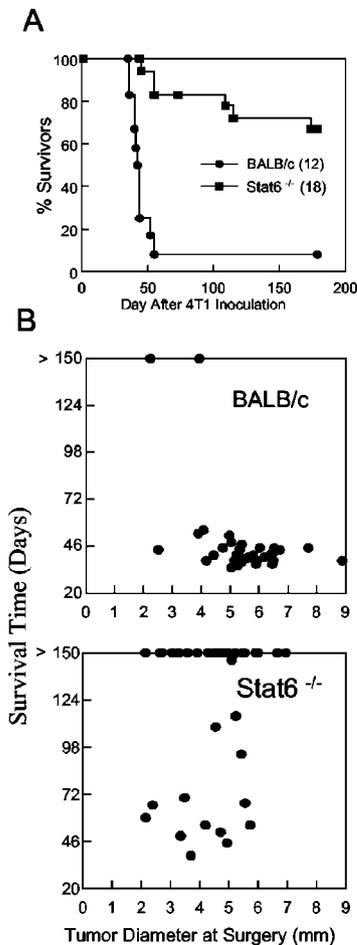
FIGURE 2. STAT6$^{-/-}$ survivor mice are immune to subsequent inoculations of 4T1 tumor. STAT6$^{-/-}$ and BALB/c survivor mice from Fig. 1A were reinoculated in the abdominal mammary gland with 7000 4T1 cells and followed for survival. Control and comparison groups included BALB/c and STAT6$^{-/-}$ mice whose primary tumors were not surgically removed (naive BALB/c and naive STAT6$^{-/-}$), and STAT6$^{-/-}$ mice whose primary tumors were removed, but not reinoculated with 4T1 (STAT6$^{-/-}$ surgery). Values in parentheses are the number of mice per group.

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

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

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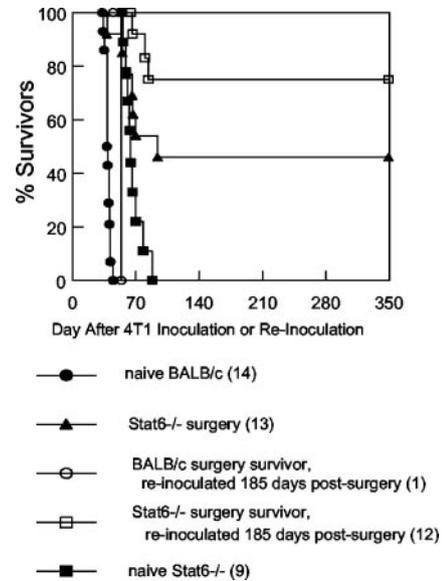
relative to primary tumor growth in BALB/c mice, indicating that deletion of NKT cells does not enhance immunity to primary mammary carcinoma.

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

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

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

As demonstrated by earlier studies, mice treated with an inhibitor for IL-13 (sIL-13Rα2-Fc) are resistant to recurrence of the 15-12RM gp160-transfected fibrosarcoma (1). This result, coupled with the observation that NKT-deficient CD1$^{-/-}$ mice, as well as
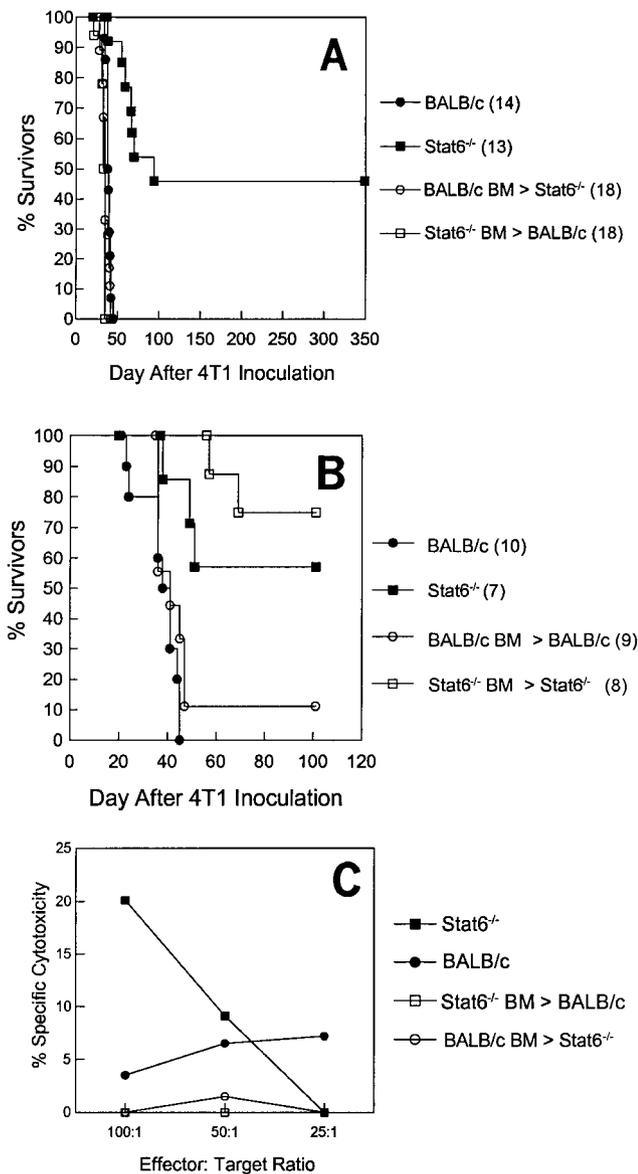
FIGURE 3. Enhanced immunity requires hemopoietic and nonhemopoietic components. Chimeric mice and untreated control BALB/c and STAT6$^{-/-}$ mice were inoculated in the abdominal mammary gland with 7000 4T1 tumor cells, and primary tumors were surgically removed 15–21 days later. Values in parentheses are the number of mice per group. *A*, Allogeneic chimeras. Average primary TD ± SD (mm) at the time of surgery were BALB/c, 5.41 ± 0.76; STAT6$^{-/-}$, 4.61 ± 1.51; BALB/c→STAT6$^{-/-}$, 4.68 ± 0.79; STAT6$^{-/-}$→BALB/c, 5.3 ± 0.9. *B*, Autologous chimeras. Average primary TD ± SD (mm) at the time of surgery were BALB/c, 5.13 ± 0.7; STAT6$^{-/-}$, 4.12 ± 0.52; BALB/c→BALB/c, 4.59 ± 0.74; STAT6$^{-/-}$→STAT6$^{-/-}$, 4.93 ± 0.47. *C*, STAT6$^{-/-}$, but not BALB/c, STAT6$^{-/-}$→BALB/c, or BALB/c→STAT6$^{-/-}$ chimeric mice contain CTL to 4T1 tumor cells. Chimeras, BALB/c, and STAT6$^{-/-}$ mice were multiply immunized i.p. with 10^6 irradiated 4T1 cells. Five days after the last immunization, splenocytes were removed and tested for cytotoxic activity against Cr-51-labeled 4T1 and B16 melanoma targets.

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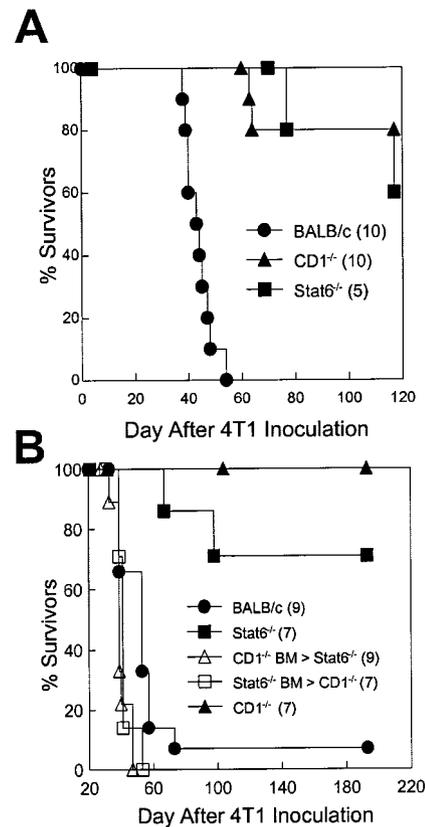

FIGURE 4. CD1$^{-/-}$ mice have enhanced immunity to 4T1 metastatic disease; however, CD1$^{-/-}$ and STAT6$^{-/-}$ bone marrow chimeras do not. *A*, CD1$^{-/-}$, STAT6$^{-/-}$, and BALB/c mice were inoculated in the abdominal mammary gland with 7000 4T1 cells. Two to three weeks later, primary tumors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

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

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

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

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

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

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

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

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

To determine whether tumor resistance correlated with differential cytokine production, draining lymph node cells of STAT6$^{-/-}$ and

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

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

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

Discussion

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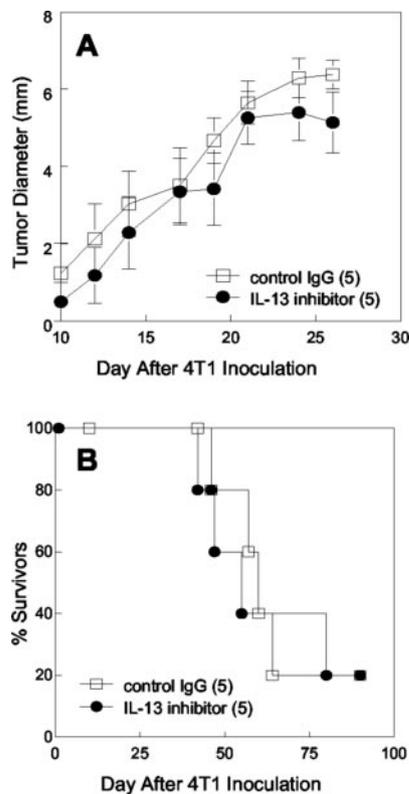

FIGURE 5. Inhibition of IL-13 with the sIL-13Rα2-Fc does not alter primary tumor growth or metastatic disease. BALB/c mice were inoculated on day 0 with 7000 4T1 cells and treated with sIL-13Rα2-Fc or a control Ig every other day from day 0 to 14. *A*, Primary tumor growth. *B*, Survival following surgical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

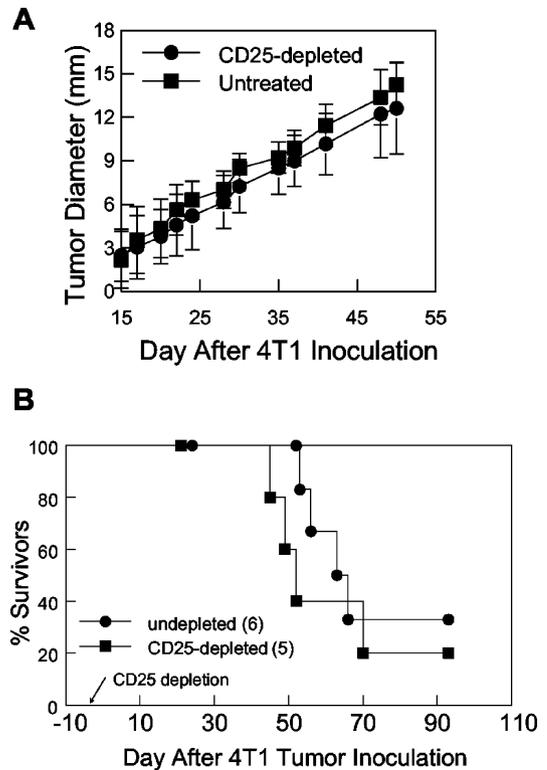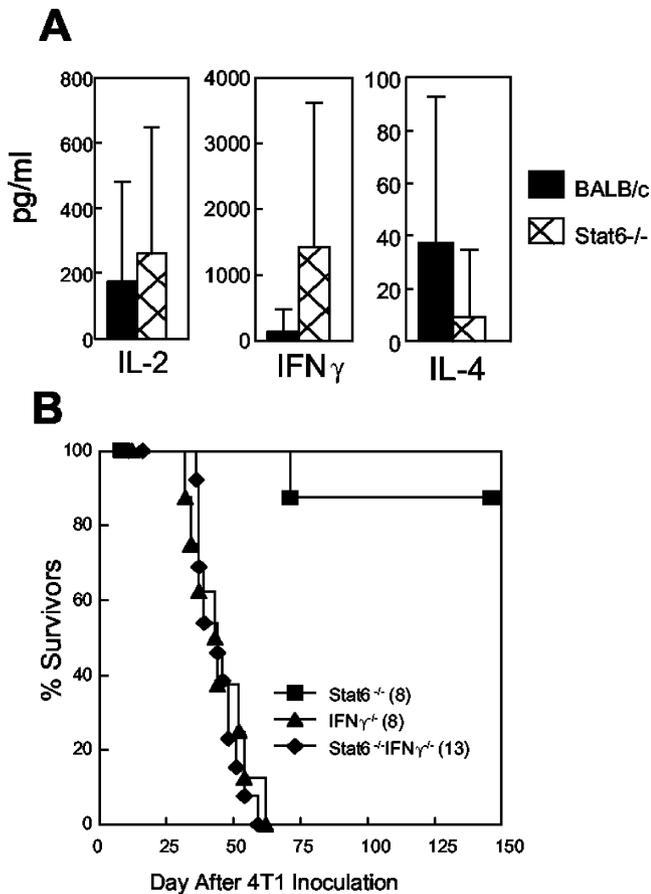## A

B

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

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

of the STAT6 gene, therefore, allows the development of a CD8$^+$ T cell-mediated immunity that protects mice against continued development of dispersed, metastatic disease.

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

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

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

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

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

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

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

Acknowledgments

References

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