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Cutting Edge: STAT6-Deficient Mice Have Enhanced Tumor Immunity to Primary and Metastatic Mammary Carcinoma[1]

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STAT4 and STAT6 are essential for the development of $CD4^+$ Th1 and Th2 development, respectively. Tumor immunologists have hypothesized that Th1 cells are critical in tumor immunity because they facilitate differentiation of $CD8^+$ T cells, which are potent anti-tumor effectors. We have used $STAT4^{-/-}$ and $STAT6^{-/-}$ mice to test this hypothesis. BALB/c and knockout mice were challenged in the mammary gland with the highly malignant and spontaneously metastatic BALB/c-derived 4T1 mammary carcinoma. Primary tumor growth and metastatic disease are reduced in $STAT6^{-/-}$ mice relative to BALB/c and $STAT4^{-/-}$ mice. Ab depletions demonstrate that the effect is mediated by $CD8^+$ T cells, and immunized $STAT6^{-/-}$ mice have higher levels of 4T1-specific CTL than BALB/c or $STAT4^{-/-}$ mice. Surprisingly, Th1 or Th2 cells are not involved, because CD4 depletion does not diminish the anti-tumor effect. Therefore, deletion of the STAT6 gene facilitates development of potent anti-tumor immunity via a $CD4^+$-independent pathway. ***The Journal of Immunology,*** **2000, 165: 6015–6019.**

The STAT4 and STAT6 genes encode transcription factors that when phosphorylated by Janus kinases are activated and transported to the nucleus where they regulate cytokine-induced gene expression (1–3). Before mid-1999, production of IL-4, an essential cytokine for differentiation of Th2 cells, was thought to be completely dependent on signaling via STAT6, and early studies with STAT6 knockout ($STAT6^{-/-}$)[3] mice supported the hypothesis that $STAT6^{-/-}$ mice were completely deficient in Th2 production (4–7). However, more recent studies indicate there is an alternative pathway for differentiation of Th2 cells that is independent of STAT6 (8–11). Despite this alternative pathway, $CD4^+$ T cell differentiation in $STAT6^{-/-}$ mice defaults to a Th1-like response and a deficiency of functional Th2 cells (10, 12, 13). STAT4 was originally thought to be critical for development of Th1 cells (14) because it is a transcriptional regulatory molecule for IL-12, which is a potent differentiation agent for Th1 cells (15). However, recent studies demonstrate there are both STAT4-dependent and STAT4-independent pathways for the development of Th1 cells. The STAT4-dependent pathway involves induction of IL-12, while the STAT4-independent pathway does not involve IL-12 and is a default pathway that occurs in the absence of STAT6 signaling (16). Even though STAT4 knockout ($STAT4^{-/-}$) mice contain an alternative pathway for generating Th1 cells, the $CD4^+$ T cell response in these mice is strongly skewed toward a Th2 response, and $STAT4^{-/-}$ mice are deficient for Th1 cells.

Th1 cells are generally considered to provide "help" to $CD8^+$ T cells, whereas Th2 cells provide "help" to Ab-producing B cells (17). This paradigm has been extended to tumor immunity, and investigators have proposed that Th1 cells are the desired $CD4^+$ population because they facilitate differentiation of tumor-specific $CD8^+$ T cells (18, 19), which are potent anti-tumor effectors (20). Although the correlation between $STAT4^{-/-}$ and $STAT6^{-/-}$ mice and deficiencies in Th1 and Th2 cells, respectively, is not absolute, we speculated that these mice might provide useful information on the roles of Th1 and Th2 cells in anti-tumor immunity. We challenged BALB/c $STAT4^{-/-}$ and $STAT6^{-/-}$ mice in the mammary gland with the highly malignant, nonimmunogenic, and metastatic BALB/c-derived 4T1 mammary carcinoma (21–23). Our expectation was that $STAT6^{-/-}$ mice would have enhanced anti-tumor immunity because differentiation of $CD4^+$ T cells would be skewed toward a Th1 response and that $STAT4^{-/-}$ mice would have reduced anti-tumor immunity because they are deficient for Th1 cells. We observed that the 4T1 tumor grows and metastasizes less in $STAT6^{-/-}$ mice, suggesting that deletion of the STAT6 gene enhances anti-tumor immunity. Surprisingly, however, the

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[3] Abbreviations used in this paper: $STAT6^{-/-}$, BALB/c mouse knocked out for the STAT6 gene; $STAT4^{-/-}$, BALB/c mouse knocked out for the STAT4 gene; Tc1, $CD8^+$ T cytotoxic 1 cells; Tc2, $CD8^+$ T cytotoxic 2 cells; 4T1, BALB/c mouse mammary carcinoma.

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STAT6$^{-/-}$ effect is independent of Th1 or Th2 cells, suggesting that the STAT6 gene negatively regulates tumor growth via a CD4$^+$ T cell-independent mechanism.

Materials and Methods

Mice and tumor challenges

BALB/c STAT6$^{-/-}$ (5) and STAT4$^{-/-}$ (14) mice were bred in the University of Maryland Baltimore County (UMBC) Biology Department animal facility. These mice were generated by targeted disruption of the STAT6 and STAT4 genes, respectively, in 129 strain mice. Offspring were backcrossed for 10 generations to BALB/c mice. STAT6$^{-/-}$ and STAT4$^{-/-}$ mice have no detectable STAT6 or STAT4 protein, respectively. BALB/c mice were either purchased from The Jackson Laboratory (Bar Harbor, ME), and/or bred at UMBC and maintained in accordance with National Institutes of Health guidelines for the humane treatment of laboratory animals. Mice were challenged in the abdominal mammary gland with 7×10^3 wild-type 4T1 tumor cells. Primary tumors at the site of injection were measured using an electronic calipers and tumor diameters calculated as the square root of the length $\times$ width of the tumor as previously described (24). Number of clonogenic lung metastases was determined by plating dissociated lung cells in medium supplemented with 6-TG, as previously described (24). Data presented are representative of three independent primary tumor challenge experiments and two independent metastasis experiments.

Immunofluorescence

Immunofluorescence staining was performed as previously described (24). To determine percent CD4$^+$ and CD8$^+$ T cells in naive and immunized STAT4$^{-/-}$, STAT6$^{-/-}$, and BALB/c mice, splenocytes were double labeled for CD3 plus CD4 or CD3 plus CD8. Mice were immunized using the identical schedule and number of immunizations as the donors for effectors in the CTL assays; however, the cells were not from the same animals used in the CTL assays. Reported values for percent CD4$^+$ and CD8$^+$ T cells are averages from at least five animals per group.

CD4$^+$ and CD8$^+$ T cell depletions

Mice were in vivo depleted for CD4$^+$ or CD8$^+$ T cells using mAbs to CD4 (GK1.5; Ref. 25) or CD8 (2.43; Ref. 26) on days -6, -3, and -1, and one to two times a week thereafter, where day 0 is the day of tumor challenge, as previously described (22). All mice were checked by indirect immunofluorescence at the end of the experiment (day 42) for completeness of depletions. In the experiments shown in Figs. 2 and 3, CD4 and CD8 levels in depleted mice were $<1\%$, except for one of the CD4-depleted mice which had 8% CD4$^+$ T cells. For CTL assays, immunized donors were depleted for CD4$^+$ or CD8$^+$ T cells using mAbs to CD4 (GK1.5) or CD8 (2.43) before the last immunization using the same schedule as above, where day 0 is the day of the final immunization and spleens were removed on day 5. Data presented are representative of two independent experiments.

CTL assays

CTL assays were performed as previously described (27) with the following modifications. Target cells (4T1 and melF10) were labeled at up to 10^7 cells/500 μl FCS with 100 μC ^{51}Cr (NEN, Boston, MA) for 1.5 h at 37°C. Labeled cells were washed with excess PBS, incubated an additional 30 min at 37°C, and used at 5×10^4 cells per well. For the CTL experiment of Fig. 4, splenic effector cells were obtained from mice that were immunized six times with 5000 rad irradiated 5×10^5 4T1/A^d/CD80 (24) plus 5×10^5 4T1/SEB (23) cells. The first immunization was performed on day 1, and subsequent immunizations on days 16, 28, 35, 49, and 62 (approximately every 2 wk). Spleens were removed 5 days after the last immunization and used directly in the assays. Labeled targets were incubated with the indicated number of effector cells in 96-well round-bottom plates at 37°C for ~18 h. Percentage cytotoxicity = [(cpm experimental $-$ cpm spontaneous)/(cpm total $-$ cpm spontaneous)] $\times$ 100%. Net cytotoxicity is the percent cytotoxicity of the specific targets (4T1) minus the percent cytotoxicity of the irrelevant targets (B16 melF10). Data presented are representative of two independent experiments. (For the second experiment, mice were immunized five, instead of six, times.) An additional CTL experiment was performed using splenocytes from BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice immunized five times with wild-type 4T1 cells (same schedule and number of immunizing cells as above.) Each CTL experiment was conducted with splenic effectors from BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice that were immunized concurrently using the identical immunization schedule and number of immunizing cells. The effectors were assayed concurrently on the same batch of target cells prepared for each experiment.

Statistical analyses

A Student's *t* test for unequal variances was performed using Microsoft Excel (Redmond, WA) to determine the statistical significance of indicated data.

Results

Primary tumor growth is reduced and delayed in STAT6$^{-/-}$ mice

The BALB/c-derived 4T1 mouse mammary carcinoma closely models human breast cancer in that it grows progressively in the anatomically correct site (mammary gland) and spontaneously metastasizes to a variety of target organs (21, 24, 28, 29). 4T1 is highly malignant in that as few as 7×10^3 cells implanted into the mammary gland lead to progressively growing primary and metastatic tumor in $>95\%$ of inoculated mice. 4T1 is also nonimmunogenic in that immunization with irradiated 4T1 cells provides no protection against wild-type tumor (23, 24). To assess the effects of the STAT4 and STAT6 genes on tumor growth, female BALB/c, STAT6$^{-/-}$, and STAT4$^{-/-}$ mice were inoculated in the mammary gland with 4T1 tumor cells. As shown in Fig. 1, following inoculation of 7×10^3 4T1 cells, onset of primary tumor growth and growth rate are slowed in STAT6$^{-/-}$ mice relative to STAT4$^{-/-}$ or wild-type BALB/c mice. Therefore, deletion of the STAT6 gene reduces primary mammary tumor growth, suggesting that the presence of a functional STAT6 gene facilitates tumor cell proliferation.

Metastatic disease is reduced in STAT6$^{-/-}$ mice

Following inoculation into the mammary gland, 4T1 tumor cells spontaneously metastasize to the lungs, liver, brain, lymph nodes, blood, and bone marrow (21, 24, 29). The kinetics of metastasis varies with the target organs; however, the earliest metastases are found in the lymph nodes draining the site of primary tumor growth and the lungs. Within 2–3 wk of inoculation into the mammary gland, ~95% of mice have metastatic cells in their lungs (23, 24). Because 4T1 cells are 6-thioguanine resistant, metastatic tumor burden in target organs can be accurately quantified (24). To determine whether metastatic spread is affected by the STAT4 and/or STAT6 genes, BALB/c, STAT6$^{-/-}$, and STAT4$^{-/-}$ mice were inoculated in the mammary gland with 7×10^3 4T1 cells, mice were sacrificed 42 days later, and the number of 4T1 cells in their lungs were measured. As shown in Fig. 2, metastatic cells in the lungs of BALB/c mice range between 0 and 4×10^6 per mouse, while four of five STAT4$^{-/-}$ mice have $>2 \times 10^4$ cells

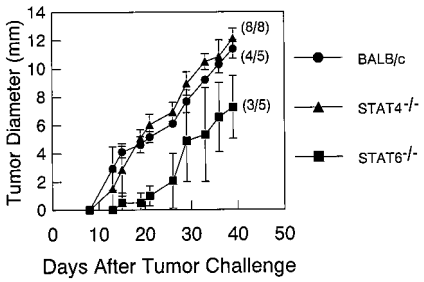

FIGURE 1. STAT6$^{-/-}$ mice have delayed and reduced primary mammary carcinoma growth relative to wild-type BALB/c or STAT4$^{-/-}$ mice. Mice were challenged in the mammary gland with 7×10^3 4T1 mammary carcinoma cells and followed for tumor growth. Numbers to the right of each line indicate the number of mice with tumor per total number of mice challenged.

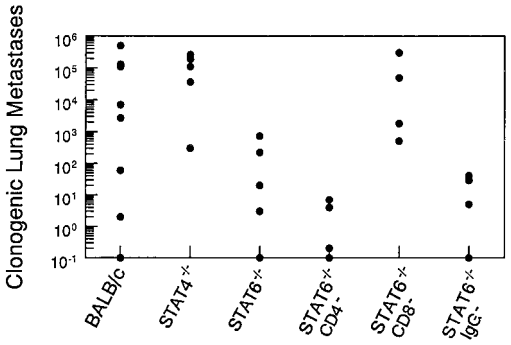

FIGURE 2. STAT6$^{-/-}$ mice challenged with 4T1 mammary tumor cells have reduced metastatic disease relative to wild-type BALB/c or STAT4$^{-/-}$ mice. Reduced disease is due to CD8$^+$ T cells. Mice were challenged with 4T1 tumor as in Fig. 1. Four weeks after tumor challenge, mice were sacrificed, lungs were removed, and the number of lung metastases was quantified. In some experiments, STAT6$^{-/-}$ mice were in vivo depleted with Abs to CD4$^+$ or CD8$^+$, or irrelevant Abs (IgG$^-$ group), before 4T1 challenge, and the lungs were assayed on day 42. The number of clonogenic cells in the lungs was quantified using the 6-thioguanine resistance assay (24). Each point represents the number of clonogenic metastatic cells from an individual animal. The CD8-depleted STAT6$^{-/-}$ group, but not the CD4-depleted STAT6$^{-/-}$ group, is significantly different ($p < 0.01$) from the undepleted STAT6$^{-/-}$ and irrelevant control IgG-depleted STAT6$^{-/-}$ groups. These data are pooled from two experiments.

each, and none of the STAT6$^{-/-}$ mice have $>10^3$ metastatic cells. Therefore, STAT6$^{-/-}$ mice have fewer metastatic cells in their lungs than either STAT4$^{-/-}$ or wild-type BALB/c mice, suggesting that expression of a functional STAT6 gene favors metastatic tumor growth.

Reduced primary and metastatic tumor growth is mediated by CD8$^+$ T cells and CD4$^+$ T cells are not involved

To identify the effector cells responsible for the reduced tumor burdens of STAT6$^{-/-}$ mice, mice were in vivo depleted for CD4$^+$ or CD8$^+$ T cells before tumor inoculation into the mammary gland. Depletions were initiated on day −6 before tumor challenge and continued through day 42 of tumor growth. Primary tumor growth was measured throughout the 42-day period. As shown in Fig. 3, control untreated BALB/c and CD8-depleted STAT6$^{-/-}$ mice have rapidly and progressively growing primary mammary tumors, while CD4-depleted STAT6$^{-/-}$ and control IgG-depleted STAT6$^{-/-}$ mice have more slowly growing primary tumors. CD4- and CD8-depleted STAT6$^{-/-}$ mice were also followed for metastatic disease. Depletions were started on day −6, mice were inoculated with 4T1 tumor in the mammary gland on day 0, and depletions were continued until day 42 when the mice were sacrificed and the number of clonogenic metastatic cells in the lungs were quantified by the 6-TG assay. As shown in Fig. 2, STAT6$^{-/-}$ mice depleted of CD8$^+$ T cells have higher levels of metastatic tumor cells than nondepleted STAT6$^{-/-}$ mice. In contrast, CD4-depleted and control IgG-depleted STAT6$^{-/-}$ mice have very low levels of metastatic cells in the lungs, comparable to nondepleted STAT6$^{-/-}$ mice. Therefore, depletion of CD8$^+$ T cells, but not CD4$^+$ T cells in STAT6$^{-/-}$ mice, negates the anti-tumor effect, indicating that CD8$^+$ T cells are critical for tumor rejection and that CD4$^+$ T cells are not involved.

Tumor-specific CD8$^+$ CTL develop in STAT6$^{-/-}$ mice, but not in wild-type BALB/c mice

The results of Figs. 2 and 3 demonstrate that CD8$^+$ T cells are responsible for the reduced primary tumor growth and metastases in STAT6$^{-/-}$ mice. To determine whether the difference in tumor susceptibility between STAT6$^{-/-}$, STAT4$^{-/-}$, and BALB/c mice is due to differential activation of CD8$^+$ T cells, CTL assays were performed using splenocytes from immunized mice and ^{51}Cr-labeled 4T1 cells as specific targets and B16 melF10 melanoma cells as irrelevant targets. In initial experiments, BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice were immunized with irradiated wild-type 4T1 cells. Only STAT6$^{-/-}$ effectors had significant cytotoxic activity (net cytotoxicity of 0, 12, and 56% for BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ effectors, respectively, at a ratio of 120:1).

For other studies, we have developed 4T1 cell-based vaccines as therapy agents for the treatment of 4T1 tumors in wild-type BALB/c mice. The vaccines consist of wild-type 4T1 cells transfected with syngeneic MHC class II and CD80 genes (4T1/A^d/CD80) (24) or *Streptococcus aureus* enterotoxin B gene (4T1/SEB) (23). To determine whether STAT6$^{-/-}$ mice respond selectively to the vaccines, BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice were immunized with 4T1/A^d/CD80 plus 4T1/SEB cells, and CTL assays were performed. As shown in Fig. 4, STAT6$^{-/-}$ mice have strong 4T1-specific CTL activity, while effectors from STAT4$^{-/-}$ mice have modest activity and BALB/c mice have minimal activity. Depletion of CD8$^+$, but not CD4$^+$, T cells from

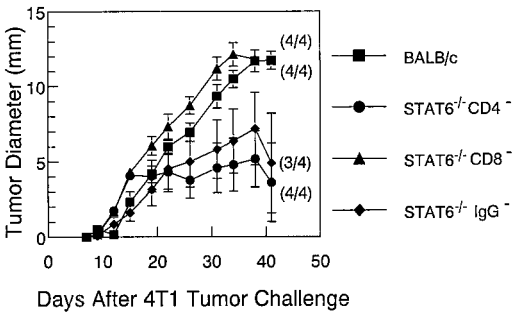

FIGURE 3. CD8$^+$ T cells mediate reduced primary tumor growth in STAT6$^{-/-}$ mice. Mice were challenged with 4T1 tumor, and primary tumor growth was monitored as in Fig. 1. Some groups of mice were in vivo depleted with Abs to CD4$^+$ or CD8$^+$ T cells, or irrelevant Abs (IgG$^-$ group), before 4T1 challenge.

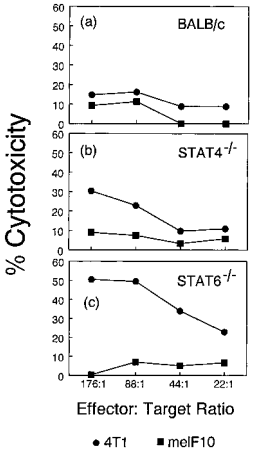

FIGURE 4. STAT6$^{-/-}$ mice have higher levels of tumor-specific CTL following immunization with 4T1 tumor cells than BALB/c or STAT4$^{-/-}$ mice. BALB/c (*a*), STAT4$^{-/-}$ (*b*), or STAT6$^{-/-}$ (*c*) mice were immunized with irradiated 4T1 tumor cells, and splenocytes were tested as effector cells against ^{51}Cr-labeled 4T1 or irrelevant melF10 targets.

immunized STAT6$^{-/-}$ donors before harvesting splenocytes for CTL assays, eliminates 4T1-specific cytotoxicity, demonstrating that the CTL effect is CD8$^+$ T cell-mediated (data not shown).

The increased CTL activity in 4T1-immunized STAT6$^{-/-}$ mice may be due to differences in quantities of CD4$^+$ and CD8$^+$ T cells between BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice. To test this possibility, splenocytes from naive and immunized BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice were stained for CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells and analyzed by flow cytometry. Percentages of CD4$^+$ and CD8$^+$ T cells in naive BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice do not differ between the strains (CD4$^+$ T cells in naive BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice: 24.5, 29.3, and 26.9%, respectively; CD8$^+$ T cells: 9.4, 9.3, and 7.8%, respectively). Likewise, the percentages of CD4$^+$ and CD8$^+$ T cells in immunized BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice do not differ from those in naive mice (CD4$^+$ T cells in immunized BALB/c, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice: 25.0, 28.2, and 25.8%, respectively; CD8$^+$ T cells: 8.1, 8.9, and 9.4%, respectively). Therefore, the knockout mice do not have significantly more CD8$^+$ T cells, and the increased tumor-specific CTL activity of STAT6$^{-/-}$ mice cannot be explained because of higher quantities of CD8$^+$ cytotoxic cells.

Therefore, significant tumor-specific CD8$^+$ CTL activity develops in STAT6$^{-/-}$ mice, but does not develop in BALB/c or STAT4$^{-/-}$ mice, even when the genetically modified tumor vaccines are the immunizing agent.

Discussion

Although 4T1 tumor growth and metastasis are rapid and progressive in syngeneic BALB/c mice, 4T1 growth in STAT6$^{-/-}$ BALB/c mice is significantly delayed and reduced. The delayed onset and reduced growth rate of primary tumor and large reduction in metastatic tumor burden are particularly noteworthy because 4T1 mammary carcinoma is highly malignant, spontaneously metastatic, and nonimmunogenic (24). Because depletion of CD8$^+$ T cells in STAT6$^{-/-}$ mice restores primary tumor growth and metastasis to levels of wild-type BALB/c mice, the anti-tumor effect in STAT6$^{-/-}$ mice is mediated by T lymphocytes. Surprisingly, however, depletion of CD4$^+$ T cells does not impact tumor growth in STAT6$^{-/-}$ mice, indicating that CD4$^+$ T cells are not involved in tumor reduction. Therefore, the STAT6 effect is not due to an increase in tumor-specific Th1 cells, as originally hypothesized. The lack of involvement of Th1 cells is also supported by the finding that STAT4$^{-/-}$ mice have the same growth rate of primary tumor and metastasis formation as wild-type BALB/c mice. If Th1 generation limited tumor growth, then mice deficient for Th1 cells would be expected to have more rapid tumor growth and metastasis formation than wild-type STAT4$^{+/+}$ mice.

If preferential development of tumor-specific Th1 cells is not responsible for enhanced tumor immunity in STAT6$^{-/-}$ mice, why does deletion of the STAT6 gene have such a profound effect on tumor growth? A trivial explanation is that the STAT6$^{-/-}$ mice are sufficiently genetically disparate from BALB/c mice that the 4T1 tumor is essentially an allograft and therefore immunogenic in STAT6$^{-/-}$ mice. This explanation is unlikely for at least two reasons. First, STAT6$^{-/-}$ mice have been backcrossed 10 generations to BALB/c mice, making them >99.99% BALB/c, and making it very unlikely that there are significant histocompatibility differences between 4T1 tumor cells and STAT6$^{-/-}$ mice. Second, because the STAT4$^{-/-}$ mice have been similarly backcrossed to BALB/c, if reduced tumor growth is due to genetic discrepancies, then STAT4$^{-/-}$ mice should also exhibit reduced tumor growth; however, their pattern of tumor progression is very similar to that of wild-type BALB/c mice.

Because CD4$^+$ T cells are not obviously involved, deletion of the STAT6 gene must enhance tumor immunity via a mechanism independent of CD4$^+$ T cells. There are several possible alternative mechanisms. First, STAT6$^{-/-}$ mice may preferentially produce Tc1 CD8$^+$ T cells that are more efficacious than Tc2 cells in reducing tumor cell growth. Second, the STAT6 gene may be involved in a signaling pathway that produces an inhibitor that blocks CD8$^+$ T cell-mediated anti-tumor immunity. Elimination of this inhibitor results in enhanced development of tumor-specific CD8$^+$ T effector cells. Third, CD8$^+$ T cells are one component of the enhanced anti-tumor effect, but other factors, such as anti-angiogenic factors, are also involved. Inactivation of the STAT6 gene favors the development of anti-angiogenic mechanisms that limit primary tumor and metastatic tumor growth. Therefore, STAT6$^{-/-}$ mice may show reduced tumor growth due to immunologic and nonimmunologic mechanisms.

In earlier studies, the 4T1 vaccines (4T1/A^d/CD80 plus 4T1/SEB) have shown significant therapeutic efficacy in wild-type BALB/c mice (23, 24), and in vivo CD8$^+$ T cell depletion experiments have shown that the effect is at least partially due to CD8$^+$ T cells. Therefore, it is surprising that the vaccines only stimulate a significant CD8$^+$ T cell response in STAT6$^{-/-}$ mice, and not in BALB/c mice.

Regardless of the mechanism by which STAT6$^{-/-}$ mice have enhanced immunity, the reduction in primary tumor growth and metastatic disease is large and is unusual in that knocking out a gene results in a gain-of-function phenotype. This observation raises the possibility that inactivation of the STAT6$^{-/-}$ gene might enhance the development of tumor-specific immunity and facilitate tumor rejection and/or limit malignant cell proliferation.

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