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Inhibition of Host Signal Transducer and Activator of Transcription Factor 6 Results in Cure With Cyclophosphamide and Interleukin 12 Immunotherapy

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Background: Interleukin (IL)-12 immunotherapy is highly effective against established immunogenic tumors. However, nonimmunogenic tumors fail to respond to IL-12 therapy. Analysis of tumor rejection of the immunogenic tumors shows that a preexisting antitumor immune response is required for an effective IL-12 response. It is not known whether this lack of a preexisting host antitumor immune response is a limiting factor for the lack of response to IL-12 therapy by nonimmunogenic tumors.

Methods: Experiments were done using the spontaneously arising nonimmunogenic metastatic murine breast 4T1 carcinoma in normal and STAT6 knockout BALB/c mice.

Results: 4T1 is nonimmunogenic in normal mice, and established subcutaneous tumors are resistant to immunotherapy with cyclophosphamide (Cy) plus IL-12. However, in STAT6 knockout mice, 4T1 becomes immunogenic, and established 4T1 tumors are eradicated by Cy plus IL-12. Adoptive transfer of spleen cells from normal mice into STAT6 knockout mice before tumor inoculation reduces both the immunogenicity and response to Cy plus IL-12 immunotherapy of 4T1 in the recipient mice.

Conclusions: Cy plus IL-12 immunotherapy can eradicate nonimmunogenic tumors as long as a preexisting immunity is established in the tumor-bearing host. Furthermore, the STAT6 pathway is likely involved in the suppression of the development of host antitumor immunity.

Key Words: Immunogenic—STAT6—Cyclophosphamide—Interleukin 12.

Recently we have demonstrated dramatic antitumor effects in murine immunogenic tumors with interleukin (IL)-12 immunotherapy. Well-established large subcutaneous and late-stage disseminated immunogenic tumors are completely eradicated by treatment with IL-12[1] or a single dose of cyclophosphamide (Cy) followed by IL-12.[2,3] In immunogenic tumor models, the presence of preexisting tumor-sensitized T cells is essential for a subsequent response to IL-12 therapy.[3] Sensitization of T cells by tumor occurs in immunogenic tumors, whereas it does not occur in hosts bearing nonimmunogenic tumors.[3,4] However, even if tumor-sensitized T cells can be raised through immunological manipulations in hosts bearing nonimmunogenic tumors, it is unclear whether this would lead to a better response to immunotherapy. To answer this question requires the establishment of a preexisting antitumor immunity in mice bearing nonimmunogenic tumors. Because this does not occur spontaneously, immunological manipulations are required.

Traditionally, the immunogenicity of a tumor is largely determined by the intrinsic characteristics of the tumor. However, recent studies suggest that certain host factors may also have an important role in determining tumor immunogenicity. For example,

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the immunogenicity of a poorly immunogenic tumor was shown to increase in mice lacking mature B cells.[5] Similarly, increased host antitumor immune responses were seen in mice lacking signal transducer and activator of transcription factor 6 (STAT6).[6–8] STAT6 is involved in the signal transduction pathway of IL-4 receptor α,[9,10] a common subunit shared by both IL-4 and IL-13 receptors.[11] In mice that lack STAT6, the T helper 2 (Th2) humoral immune response is impaired, and the Th1 cellular immune response becomes predominant. To examine the relationship between the immunogenicity of a tumor and its response to IL-12 immunotherapy, we took advantage of the increased immunogenicity of nonimmunogenic tumors in STAT6 knockout mice. The nonimmunogenic tumor 4T1 is derived from a spontaneously arising breast carcinoma of BALB/c mice.[12] The tumor is highly lethal in normal mice, in which as few as 1×10^4 cells kill the host in a few weeks through metastases to the lung and liver.[13] This tumor is nonimmunogenic, in that immunization of naive mice with inactivated tumor cells does not protect against subsequent challenge with live tumor cells.[14] 4T1 is resistant to several previous immunotherapy approaches,[15–17] in that no immunotherapy has been shown to be able to eradicate established subcutaneous 4T1 tumors. In this study, we show that the increased tumor immunogenicity of 4T1 tumors in STAT6 knockout mice is associated with an increased response to Cy plus IL-12 immunotherapy.

MATERIALS AND METHODS

Tumors and Mice

The murine 4T1 tumor is a cloned cell line derived from a spontaneously arising mammary carcinoma in the BALB/c strain of mice.[12] STAT6 knockout mice were also BALB/c (H-2d) mice. Tumor cells were maintained by cell culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM of glutamine, 100 μg/mL of streptomycin, 100 IU/mL of penicillin, and 5×10^{-5} M of
β-mercaptoethanol. Normal BALB/c mice were obtained from the Biological Testing Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health (Frederick, MD). All gene knockout mice were obtained from Jackson Laboratory (Bar Harbor, ME). Experiments were performed with 8- to 12-week-old female mice.

Prophylactic and Concomitant Immunity Tests

Concomitant immunity tests were performed as described previously.[4,18] Briefly, subcutaneous tumors were first established in naive mice on one flank with 5×10^5 tumor cells. At the indicated time points after the first tumor inoculation, a second inoculation of 5×10^5 tumor cells was given to naive or tumor-bearing mice on the opposite flank. The development of tumors from the second inoculation in both naive and tumor-bearing mice was assessed. Prophylactic immunity was tested by immunizing naive mice with 1×10^6 irradiated (50 gray) tumor cells subcutaneously once a week two times followed by challenge with 5×10^5 live tumor cells on the opposite flank 1 to 2 weeks after the second immunization. Naive mice were used as controls at the time of live tumor challenge.

Cy Plus IL-12 Immunotherapy

In subcutaneous tumor models, 5×10^5 tumor cells in .2 mL of saline were injected subcutaneously on the flank of naive mice. Tumor size was assessed with calipers. Cy plus IL-12 treatment was composed of a single intraperitoneal injection of 3 mg (120 mg/kg) of Cy (Sigma, St. Louis, MO) in .5 mL of saline followed 3 days later by recombinant murine IL-12 (Peprotech, Rocky Hill, NJ) administered intraperitoneally at a dose of 500 ng in .5 mL of 1% mouse serum in saline given once every other day for three doses. An additional single dose of IL-12 was given weekly after the initial IL-12 treatment during tumor regression.

Immunohistochemistry

Tumors harvested for immunohistochemistry were immediately frozen in OTC medium. Tumor sections at 6 μm were prepared and fixed in cold acetone as described previously.[2] The sections were blocked with 2% goat serum and 1% bovine serum albumin in phosphate-buffered saline and stained with antibodies specific to mouse CD4 (clone H129.19; Pharmingen, San Diego, CA), CD8 (clone 53-6.7; Pharmingen), Mac-1 (clone M1/70; Pharmingen), and inducible nitric oxide synthase (rabbit polyclonal; Calbiotech, Spring Valley, CA) as described previously.[2] Microscope images were captured with a digital camera (Spot II; Diagnostic Instruments, Sterling Heights, MI). Images were assembled and enhanced by using computer software (Adobe Photoshop version 5; Adobe, Mountain View, CA).

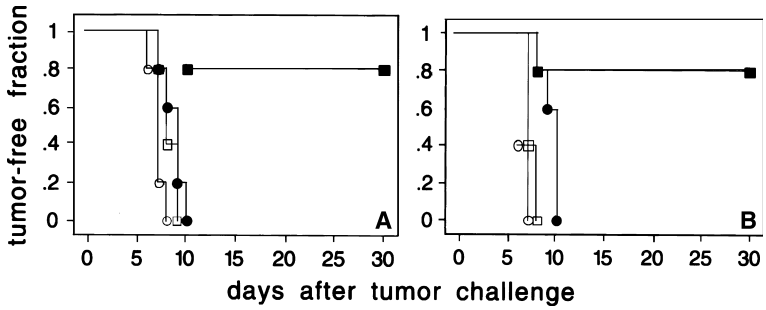

FIG. 1. The immunogenicity of 4T1 tumor by prophylactic (**A**) and concomitant (**B**) immunity tests in normal (open symbols) and signal transducer and activator of transcription factor 6 knockout (closed symbols) mice. For the prophylactic immunity test, mice were immunized with irradiated 4T1 cells in the flank subcutaneously. Naive control (circles) and immunized (squares) mice were then challenged with 5×10^5 live 4T1 cells subcutaneously on the opposite flank. Tumor development after challenge was monitored and expressed in the graph as the fraction of mice that lost their tumor-free status. For the concomitant immunity test, mice were challenged with 5×10^5 live 4T1 cells on one flank. Ten days later, naive control (circles) and tumor-bearing (squares) mice were challenged again with 5×10^5 4T1 cells on the opposite flank. Tumor development on the second challenge was observed and presented in the figure.

Adoptive Spleen Cell Transfer

Spleens from normal or STAT6-deficient mice were harvested and made into a single-cell suspension by mechanical separation. The red blood cells were lysed with osmotic shock, and the remaining lymphocytes were washed two times in saline. Cells were suspended in saline at one spleen per milliliter, and a half equivalent of spleen cells ($35–40 \times 10^6$) was injected intravenously into recipient mice via the tail vein.

RESULTS

4T1 Is an Immunogenic Tumor in STAT6-Deficient Mice

To compare the immunogenicity of 4T1 tumors in normal and STAT6 knockout mice, we examined two conditions during which protection against 4T1 tumor cell challenge was measured. In the prophylactic immunity test, naive normal and STAT6-deficient mice were immunized with radiation-inactivated 4T1 tumor cells. The immunized and naive control mice were subsequently challenged with a high dose (5×10^5) of live 4T1 tumor cells. In the concomitant immunity test, naive normal and STAT6-deficient mice were inoculated with 4T1 tumor cells to establish a primary subcutaneous tumor. Seven to ten days after tumor inoculation, when tumors were palpable, a second inoculation of 4T1 tumor cells (5×10^5) was given to tumor-bearing and naive control mice on the opposite flank. By these tests, 4T1 is nonimmunogenic in normal mice, in which immunization with irradiated 4T1 tumor cells failed to generate protection against subsequent challenge with live 4T1 tumor cells (Fig. 1A). Similarly, wild-type mice bearing primary 4T1 tumors failed to reject a second 4T1 challenge given 7 days later on the opposite flank (Fig. 1B). In contrast, 80% of immunized and 80% of tumor-bearing STAT6 knockout mice were protected against the same dose of tumor challenge under both prophylactic and concomitant immunity tests (Fig. 1); this indicates that 4T1 is immunogenic in STAT6 knockout mice and nonimmunogenic in wild-type mice. The increased immunogenicity of 4T1 tumors in STAT6 knockout mice was associated with an increased number of T cells infiltrating into the tumor. Whereas only a few sporadic $CD4^+$ T cells and almost no $CD8^+$ T cells were seen in 4T1 tumors from wild-type mice, significant numbers of $CD8^+$ T cells were found infiltrating into tumors from STAT6 knockout mice (Fig. 2).

4T1 Responds to Cy Plus IL-12 in STAT6 Knockout, but Not Wild-Type, Mice

Because we have previously demonstrated that established immunogenic tumors completely respond to Cy plus IL-12 therapy[3] and because the above-described experiments suggested that 4T1 is immunogenic in STAT6 knockout mice, we evaluated the response of 4T1 to Cy plus IL-12 therapy in both normal and STAT6 knockout mice. Treatment of day 12 established (3- to 7-mm) 4T1 tumors in wild-type BALB/c mice with Cy plus IL-12 decreased tumor growth without inducing tumor regression (Fig. 3A). In contrast, Cy plus IL-12 therapy in STAT6 knockout mice caused complete regression of day 12 established 4T1 tumors (Fig. 3A). This complete

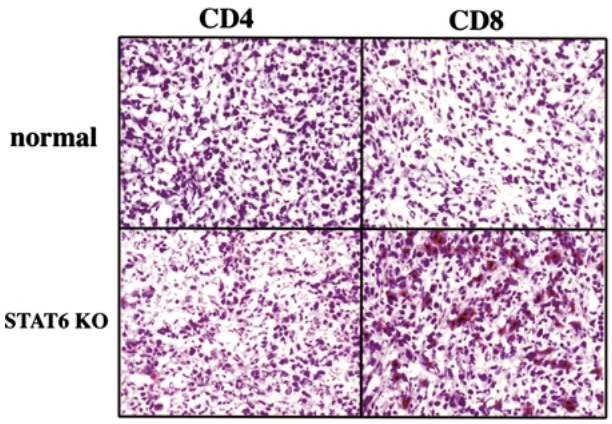

FIG. 2. Photomicrographs of subcutaneous 4T1 tumors collected from normal and signal transducer and activator of transcription factor 6 (STAT6) knockout (KO) mice 10 days after challenge with 5×10^5 tumor cells. Frozen sections of tumors were analyzed with antibodies to CD4 and CD8 markers by immunohistochemistry. The positive cells are red (original magnification, ×200).

eradication of primary tumors resulted in 100% long-term survival as compared with 4T1-bearing wild-type mice that died of disseminated disease between 5 and 7 weeks (Fig. 3B). Treating tumor-bearing STAT6-knockout mice with Cy alone did not result in tumor regression. Treating 4T1 tumors in normal wild-type mice with Cy plus IL-12 therapy as early as day 8 after tumor establishment when tumor sizes were <2 mm also failed to yield any tumor regression. Conversely, tumor regression and the 40% rate of cure were still observed when Cy plus IL-12 therapy was initiated 16 days after tumor establishment in STAT6 knockout mice. The regression of 4T1 tumors in STAT6 knockout, but not wild-type, mice was associated with an increased immune cell infiltration at the site of tumors (Fig. 4). $CD8^+$ T cells and macrophages were especially prominent in the regressing tumors. As observed in regressing tumors treated with IL-12 and Cy plus IL-12 in previous studies,[2,19] tumor-infiltrating macrophages that were located next to the T cells expressed high levels of inducible nitric oxide synthase, thus indicating a highly active state. The STAT6 knockout mice that were cured of 4T1 tumors were subsequently rechallenged with 5×10^5 4T1 tumor cells, and each completely rejected tumor rechallenge.

Inhibition of Antitumor Immunity and Response to Cy Plus IL-12 Therapy in STAT6 Knockout Mice by Spleen Cell Transfer From Wild-Type Mice

The difference in tumor immunogenicity and response to Cy plus IL-12 immunotherapy between

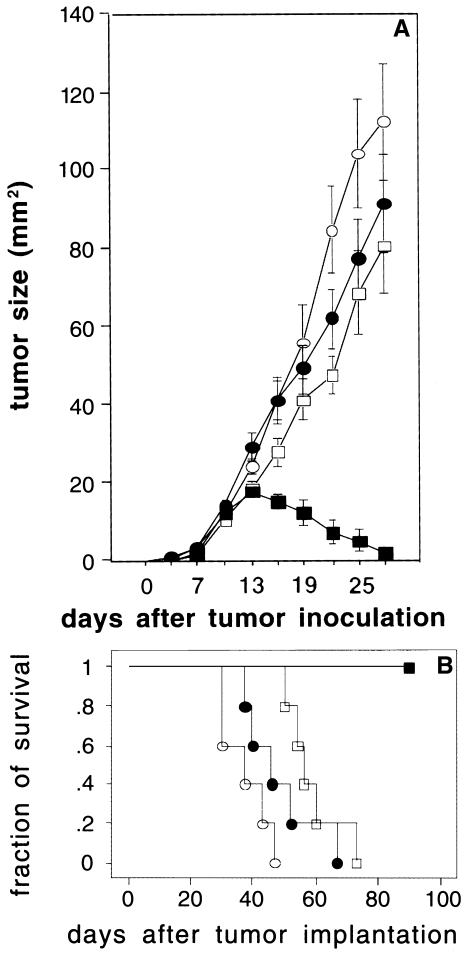

FIG. 3. Response to cyclophosphamide (Cy) plus interleukin (IL)-12 therapy by palpable 4T1 tumors in normal and signal transducer and activator of transcription factor 6 (STAT6)-deficient mice. Normal (open symbols) and STAT6 knockout (closed symbols) mice received 5×10^5 4T1 cells subcutaneously. Twelve days after tumor inoculation, when tumors were 3 to 5 mm in diameter, treatment with saline (circles) or Cy plus IL-12 (squares) was initiated. Tumor sizes (length × width) were measured and are presented (**A**). The survival times of various treatment groups were plotted, and the Kaplan-Meier curves are presented (**B**).

wild-type and STAT6 knockout mice suggests that 4T1 tumor cells are better recognized by host T cells in the absence of the STAT6 pathway. To further delineate the mechanism by which the lack of STAT6 affects tumor immunogenicity, we tested the development of concomitant immunity and response to immunotherapy of 4T1 tumors in STAT6 knockout mice that had received intravenous spleen cells from normal wild-type BALB/c mice 1 day before tumor inoculation. The results show that infusion of spleen cells from wild-type mice into STAT6 knockout mice completely abolished the concomitant immunity induced by primary 4T1 tu-

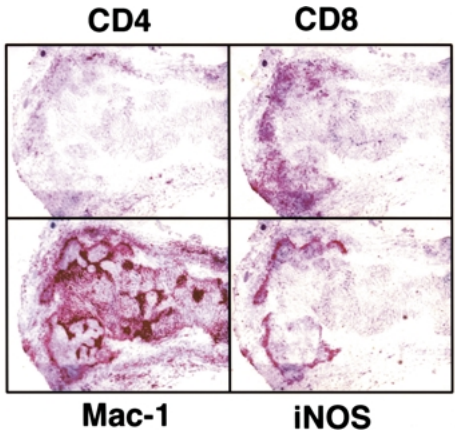

FIG. 4. Photomicrograph of immune cell infiltration of regressing 4T1 tumors after cyclophosphamide (Cy) plus interleukin (IL)-12 treatment. Signal transducer and activator of transcription factor 6 knockout mice bearing 12-day-established palpable 4T1 subcutaneous tumors were treated with Cy plus IL-12. Two days after the last IL-12 injection, regressing tumors were collected, and frozen tumor sections were analyzed by immunohistochemistry with antibodies to specific markers as indicated. Positive cells are red (original magnification, ×40). iNOS, inducible nitric oxide synthase.

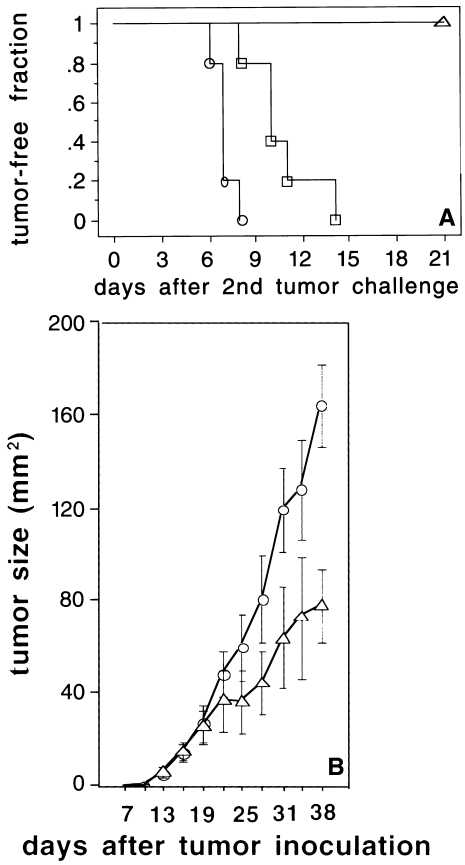

FIG. 5. Reduction of tumor immunogenicity and response to cyclophosphamide (Cy) plus interleukin (IL)-12 therapy by adoptive transfer of normal spleen cells into signal transducer and activator of transcription factor 6 (STAT6)-deficient mice. (**A**) Naive STAT6 knockout mice were given half spleen equivalents of cells from normal mice. One day after the adoptive cell transfer, naive (triangles) and recipient (squares) mice were inoculated with 5×10^5 4T1 cells on one flank. Ten days after the first tumor inoculation, naive (circles) and tumor-bearing (triangles and squares) mice were given a second tumor inoculation with 5×10^5 4T1 cells on the opposite flank. Tumor development of the second tumor inoculation is shown. (**B**) Subcutaneous 4T1 tumors were established in STAT6 knockout mice that received normal spleen cells. Twelve days after tumor establishment, treatment with saline (circles) or Cy plus IL-12 (triangles) was initiated. Tumor sizes (length × width) were measured and are shown.

mors (Fig. 5A). Tumors from STAT6 knockout mice who lost concomitant immunity as a result of wild-type spleen cell transfer showed a lack of T-cell infiltration similar to that in tumors established in normal mice (not shown). Correlating with this loss of concomitant immunity to tumor cell rechallenge, 4T1 tumors established in STAT6 knockout mice that received normal wild-type spleen cells did not respond to Cy plus IL-12 therapy (Fig. 5B). In contrast, transfer of spleen cells from naive STAT6 knockout mice into STAT6 knockout recipients resulted in delayed tumor development, fully preserved concomitant immunity, and a response to Cy plus IL-12 therapy (not shown).

DISCUSSION

The nonimmunogenic tumor 4T1 has been resistant to various forms of immunotherapy in previous studies. It is not known whether this resistance is the result of a weak immune response to the tumor or a resistance of the tumor to an immune destruction/effector mechanism. Findings demonstrated here suggest that the former explanation is correct. This study has shown that established palpable 4T1 tumors can be completely eradicated by a host antitumor immune response under certain conditions; thus, the 4T1 breast tumor does not seem to be intrinsically resistant to the immune effector mechanism. This study is also consistent with our previous hypothesis[3] that there is a direct link between tumor immunogenicity and response to IL-12–based immunotherapy. Thus 4T1 tumors established in wild-type mice are nonimmunogenic and are resistant to Cy plus IL-12 therapy, whereas the same tumor established in STAT6 knockout mice is immunogenic and responsive to immunotherapy. Furthermore, when the immunogenicity of 4T1 tumors in STAT6 knockout mice is lost as a result of the transfer of spleen cells

from wild-type mice, the response to Cy plus IL-12 is also abolished.

The immunogenicity of a tumor is shown by this study to be a major factor affecting the response to IL-12 immunotherapy. Because high immunogenicity represents strong host T-cell recognition of the tumor, a key strategy to improve the success of immunotherapy against nonimmunogenic tumors is to raise host recognition of the incipient tumor. However, immune recognition by itself may not be enough to achieve tumor eradication. One of the important implications from this study is that successful immunotherapy against nonimmunogenic tumors requires the combination of two manipulations: adequate host recognition of the incipient tumor and the amplification and nurturing of the initial immune recognition into a strong antitumor T-cell response. This may explain why previous efforts of vaccination against nonimmunogenic tumors have not been successful in eradicating established tumors. Immunization with various forms of tumor vaccines may result in an immune response that requires a second manipulation. This initial response alone is inadequate to cause the rejection of established tumors, because even immunogenic tumors progress in the presence of an antitumor immunity strong enough to reject a second tumor challenge.[3,4,20] The addition of a second immunological manipulation is necessary to amplify the immunity raised through the initial immunization into a strong T cell–mediated antitumor response. The use of Cy plus IL-12 in this study satisfies this second requirement, possibly by Cy-mediated removal of downregulatory cellular elements and IL-12–mediated amplification of a Th1 response. In immunogenic tumor models, Cy plus IL-12 seems to be the most effective, consistent immunological treatment identified thus far.[2,3,21]

However, IL-12 therapy alone is ineffective against established nonimmunogenic tumors without the introduction of adequate preexisting immunity.[3] This may explain the outcome differences seen in the preclinical animal models and cancer patients, because most human cancers are likely to resemble nonimmunogenic tumors such as the 4T1 breast cancer studied here. It will be important to determine whether a combination approach of immunization with tumor vaccine and Cy plus IL-12 therapy will result in better responses in human tumors.

It is not clear why tumor immunogenicity is enhanced in STAT6-deficient mice. Although the role of STAT6 in development of a Th2 type of immune response has been well documented and Th2 inhibits Th1 development, there is no evidence to suggest that a Th1/Th2 relationship determines immunogenicity in the 4T1 tumor model. In addition, although STAT6 is the downstream signal transducer of the IL-4/IL-13 signal pathway,[10,22] the enhanced tumor immunogenicity and response to Cy plus IL-12 therapy does not seem to be due to the loss of this entire pathway. A recent study suggests that STAT6 expressed by tumor cells may become a foreign antigen in STAT6 knockout mice.[23] However, the presence of a new antigen alone may not explain the increase in immunogenicity of a nonimmunogenic tumor. Some previous studies that have attempted to introduce strong viral antigens into nonimmunogenic tumors have not been able to increase immunogenicity.[24,25] It seems that the presence of STAT6 may be responsible for suppression of the host immune response to incipient tumors. This view is supported by results from a previous study[6] and from the spleen cell transfer experiment from this study (Fig. 5). Various cell populations from wild-type spleen may exert a suppressive effect upon transfer into STAT6 knockout mice. Besides the role of B cells in suppressing Th1 antitumor immunity,[5] recently described $CD4^+CD25^+$ suppressor T cells may also be responsible for the downregulation of a Th1 response to incipient tumors.[26,27] Further experiments are needed to identify the specific cell population in the spleens of normal wild-type mice that are capable of exerting suppression of antitumor immunity when transferred into STAT6-deficient mice and the method through which these cells work. Regardless of the mechanism, the model described in this study helps to further explain why tumors are either immunogenic or nonimmunogenic.

This study has also demonstrated an entirely new approach of manipulating host factors (instead of tumor) for enhancing immune recognition by tumor-bearing mice and providing a better response to subsequent IL-12 immunotherapy. Despite the traditional view that the immunogenicity of a given tumor is determined largely by the intrinsic characteristics of that tumor, recent studies,[5–7] including this one, clearly show that certain host factors may play a critical role in determining the immunogenicity of a tumor. In fact, the effects of the manipulation of host factors on tumor immunity may be more pronounced than the manipulation of tumor cells. In the case of the 4T1 tumor model, various manipulations of the tumor cell in previous studies have failed to demonstrate the induction of adequate antitumor immunity.[15,17] Strategies to target individual tumor cells for manipulation of the immune response may be time and effort consuming, with inconsistency, whereas

manipulation of host factors that influence tumor immunogenicity may be more effective and consistent across multiple tumor models. This approach, if proven applicable to multiple tumor models, will likely improve cancer immunotherapy in patients when combined with other treatment modalities, such as Cy plus IL-12. These results suggest that manipulation of the STAT6 pathway may enhance immune recognition of incipient tumors.

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