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SYMPOSIUM IN WRITING

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

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

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

Stat6-deficient mice preferentially make T_H1 responses

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Stat6-deficient mice are resistant to spontaneously arising mammary tumors

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

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

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

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

Resistance requires IFN-γ

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

IL-13 as an inhibitor of type 1 tumor immunity

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

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

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

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

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

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

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

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

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

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

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

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

Other mechanisms

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

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

Conclusions

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

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