Cutting Edge: Differentiation of Antitumor CTL In Vivo Requires Host Expression of Stat1

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Several lines of evidence suggest that an IFN-γ-producing, Th1/Tc1 phenotype may be optimal for tumor rejection. Recent work has indicated that IFN signaling on tumor cells is important for protection against carcinogenesis. However, the potential involvement of IFN signaling among host immune cells has not been carefully examined. To this end, Stat1-deficient mice were employed as tumor recipients. In contrast to wild-type mice, Stat1−/− mice failed to reject immunogenic tumors and did not support regression of poorly immunogenic tumors when treated with an IL-12-based vaccine. T cells from immunized Stat1−/− mice produced 50% of the levels of IFN-γ and lacked cytolytic activity compared with wild-type mice, and NK lytic activity also was not observed. Lack of cytolytic function correlated with a failure to up-regulate serine esterase activity. Thus, IFN-mediated signaling on host cells is required for the development of antitumor lytic effector cells. The Journal of Immunology, 1999, 163: 4109–4113.

Although transfection of a multitude of cytokines into tumor cells has been shown to promote tumor regression in vivo, the host factors actually required for spontaneous rejection of immunogenic tumors are less well defined. Using neutralizing Abs, soluble ligand fusion proteins, or knockout mice generated by gene targeting, an important role for CD8+ T lymphocytes (1, 2), B7/CD28 interactions (2), IL-12 (3), lymphocyte function-associated Ag-1 (LFA-1) (4), perforin (5), and IFN signaling (6) in controlling tumor growth has been established. Collectively, these results suggest that induction of a lytic Th1/Tc1-like phenotype may be optimal for promoting tumor rejection in vivo. Consistent with this model, treatment of mice with vaccines incorporating IL-12 (7, 8), or in some cases with IL-12 alone (9), has eliminated established tumors in a variety of experimental systems. Given these preclinical observations, vaccines that incorporate rate IL-12 have entered clinical application with encouraging preliminary results (10).

The role of IFN-γ itself in antitumor immunity is not entirely clear. Mice deficient in IFN-γ signaling show increased susceptibility to tumor induction by chemical carcinogens (6), and tumors arising in those mice regain immunogenicity when IFN signaling is reconstituted. Thus, one role played by IFN-γ is at the level of the tumor cell, presumably by up-regulating the expression of MHC molecules, Ag processing machinery, and perhaps tumor Ags as well. However, a potential role for IFN signaling between host immune cells in promoting antitumor T cell responses has not been carefully examined.

Stat1-deficient mice have been generated by homologous recombination and show a specific deficiency in all measured effects of signaling by IFN-α/β and IFN-γ, with other cytokine-mediated responses remaining intact (11, 12). An advantage of using Stat1-deficient mice rather than IFN-γR-deficient mice is the elimination of a potential overlapping activity of IFN-α/β (13), some functions of which might compensate for the absence of IFN-γ responsiveness. In the current study, Stat1-deficient mice were examined for the ability to reject immunogenic P815 variants and to control the growth of poorly immunogenic P815 cells in response to IL-12-based vaccination. Defective tumor rejection was observed, which correlated with a failure to develop cytolytic T cells containing serine esterase activity. Thus, in addition to a potential role at the effector phase of an antitumor immune response, Stat1-dependent signaling can contribute to CTL generation.

Materials and Methods

Mice

Stat1-deficient mice were generated as described previously (12) and generously provided by Dr. David Levy (New York University). These were backcrossed for five generations with DBA/2 mice (The Jackson Laboratory, Bar Harbor, ME). Heterozygous mice were intercrossed to obtain Stat1 wild-type (+/+), heterozygous (+/−), and homozygous knockout (−/−) mice. All mice were maintained under specific pathogen-free conditions in a barrier facility at the University of Chicago. Mice between 6 and 10 wk of age were used for experiments. PCR analysis of tail DNA was used to identify mice carrying the targeted Stat1 gene. Tail DNA was prepared using standard methods. A set of three primers was used for PCR analysis: P1 (5’-GAGATAATTCACAATAGACAGAGAG-3’), P2 (5’-CTGATCCAGGGCAGGCTT-3’), and P3 (5’-TAAAGCTTCTAGGGATATCAGT-3’). Thirty-five cycles were performed, using an annealing temperature of 50°C. The PCR product was resolved using 1.5–2% agarose gels and visualized by ethidium bromide staining. Bands of distinct sizes corresponded to the wild-type and targeted alleles.

Cells

Three variants of P815 were used in this study: P1.HTR, a highly transfectable variant of P815 (14); P198, an immunogenic tum− clone of P815 (15); and P511, a subclone of P815 known to express the tumor Ag P1A.
HTR.B7-1 and HTR.IL-12 were obtained by transfection of P1.HTR cells as described previously (16). The irrelevant syngeneic tumor L1210 and the NK-sensitive target YAC-1 were used as control targets.

**In vivo tumor experiments**

Cultured tumor cells were washed three times with DPBS, and 10^6 living cells were injected in 100 μl DPBS via a 27-gauge needle on the left flank. Tumor size was assessed twice per week using calipers, the longest and shortest diameters were measured, and a mean was calculated. Data from groups of three to five mice were analyzed at each time point, and a mean and SE were determined. Measurements were continued for 3–4 wk.

**Immunization with irradiated P1.HTR transfectants**

Cultured cells were washed three times in DPBS, adjusted to 10^6 cells/ml, and irradiated (10,000 cGy). Mice received 50 μl per hind footpad (5 × 10^6 cells) via a 27-gauge needle each week for 3 consecutive wk. In some experiments, naive mice were immunized, and, 2 wk after the last injection, splenocytes were stimulated in a mixed lymphocyte-tumor culture (MLTC) before assessing cytolytic activity. In other experiments, P1.HTR parental tumor cells were implanted first in the left flank of groups of mice, and, 1 wk later, immunization with irradiated P1.HTR transfectants was initiated with therapeutic intent.

**Lymph node stimulation and lymphokine assays**

Mice were injected into each hind footpad with 10^6 living HTR.IL-12 cells in 50 μl of DPBS; control mice received DPBS. After 5 days, the draining popliteal lymph nodes were harvested, and single cell suspensions were prepared. Cells (10^6) were incubated in the presence or absence of 2.5 × 10^5 irradiated (10,000 cGy) HTR.B7-1 cells, supernatants were harvested after 48 h, and residual cells were removed by centrifugation. IFN-γ and IL-4 concentrations were determined using an ELISA with Ab pairs obtained from PharMingen (San Diego, CA). Concentrations were expressed in U/ml or pg/ml as determined by the respective recombinant cytokines as standards.

**MLTC and cytolytic assay**

Splenocytes (5 × 10^6) from immunized or control mice were stimulated with irradiated (10,000 cGy) HTR.B7-1 cells (2.5 × 10^5) in a volume of 2 ml, and 5–6 days later effector activities were analyzed. For cytolytic assays, unfractionated cells from the MLTC were washed, adjusted to 2 × 10^6/ml, and titrated in duplicate in V-bottom microtiter plates along with 2000 ^3Cr-labeled target cells. Supernatants were collected after 4 h and transferred to 96-well luma plates (Packard, Meriden, CT). After overnight incubation to allow drying, radioactivity was measured using a microplate scintillation counter (Packard). Percentage specific lysis was calculated using standard methods.

**Serine esterase assay**

Determination of total serine esterase activity was done by measuring cellular content of N-a-benzoylxyloxycarbonyl-t-lysine-thiobenzyl (BLT) esterase as previously described (17). Briefly, T cells were purified by negative selection using a magnetic separation system (StemCell Technologies, Vancouver, Canada) from MLTC cultures generated as above. The purity of the eluted fraction, determined by flow cytometry using anti-Thy-1 antibodies, was greater than 95%. Cells were centrifuged, washed three times in DMEM, and lysed in 200 μl of DPBS containing 1% Triton X-100; 100 μl of this lysate was added in duplicate to 96-well plates. Assay solution (100 μl) consisting of Tris buffer (pH 7.2) with 0.44 mM 5.5-dithio-bis(2 nitro)-benzoic acid and 0.40 mM BLT (Calbiochem, La Jolla, CA) was then added. Plates were incubated (15–30 min) at 37°C, and absorbance was measured at 405 nm using an ELISA plate reader.

**Results**

Stat1-deficient mice fail to reject the immunogenic P815 tumor variant, P198

We previously reported that the spontaneous rejection of P198 cells by syngeneic DBA/2 mice was prevented by neutralization of endogenous IL-12 and correlated with generation of T cells producing high amounts of IFN-γ (3). To explore the role of signaling by IFN among host cells, Stat1^+/+, Stat1^+/−, and Stat1^-/- mice were challenged with immunogenic P198 cells, and tumor size was measured over time. As shown in Fig. 1, P198 was successfully rejected by Stat1^+/+ and Stat1^+/− mice following an initial growth phase. However, Stat1^-/- mice failed to reject, with all mice developing rapidly growing tumors and widespread metastases, leading to death. These results indicate that IFN signaling on host cells is necessary for the spontaneous rejection of this immunogenic tumor.

**Immunization with irradiated IL-12 transfectants induces rejection of established tumors in wild-type but not Stat1^-/-DBA/2 mice**

We previously reported that P1.HTR cells transfected to express IL-12 (HTR.IL-12) were rejected in vivo, and that immunization with irradiated HTR.IL-12 cells induced regression of preestablished P1.HTR tumors (16). To investigate whether this rejection depended on IFN signaling, P1.HTR tumors were implanted subcutaneously in Stat1^+/+ or Stat1^-/- DBA/2 mice. One week after tumor implantation, mice were treated weekly with either irradiated HTR.IL-12 or with PBS. As shown in Fig. 2, immunization with irradiated HTR.IL-12 cells controlled tumor growth in Stat1^+/+ but not in Stat1^-/- mice. Similarly, living HTR.IL-12 cells were rapidly rejected by wild-type mice but grew progressively in Stat1^-/- mice (data not shown). Thus, efficacy of IL-12-facilitated antitumor immunity requires IFN signaling on host cells.

**Defective T cell differentiation in Stat1^-/- mice**

The lack of tumor rejection in Stat1^-/- mice could have been secondary to deficient T cell differentiation, or due to a defect at the effector phase of the immune response via IFN signaling on other cells. To examine whether a primed T cell phenotype was generated in the absence of Stat1, HTR.IL-12 tumor cells were injected in the hind footpads of Stat1^+/+ or Stat1^-/- DBA/2 mice, and the draining popliteal lymph node cells were harvested and restimulated to measure secreted cytokines. As shown in Fig. 3, although T cells from both wild-type and Stat1-deficient mice were primed to produce IFN-γ, Stat1^-/- T cells produced approximately 50% of the level of IFN-γ seen with wild-type T cells. Of note, there was not a corresponding increase in IL-4 production, which was secreted only minimally by T cells from either group of mice (Fig. 3B). In parallel experiments, a similar pattern of IFN-γ
production was observed following stimulation with anti-CD3 mAb, consistent with T cells being the source of the IFN-γ produced (data not shown).

It was conceivable that acquisition of cytolytic activity was more severely impaired than was acquisition of IFN-γ production. Indeed, in the above studies of T cells directly out of tumor-draining lymph nodes, approximately 15% specific lysis was observed in wild-type mice, but no specific lysis was detected from Stat1−/− mice (data not shown). To elicit higher levels of lytic activity for comparison, Stat1+/+ or Stat1−/− DBA/2 mice were immunized with living HTR.IL-12 cells by three weekly injections of irradiated HTR.IL-12 cells in the hind foot pads. This regimen had been observed to induce cytolytic activity previously (16). Splenocytes from immunized or control mice were restimulated in vitro, and cytolytic activity was measured against P815 and the NK target YAC-1. As shown in Fig. 4A, immunization with HTR.IL-12 generated P815-specific CTL activity, as well as NK-mediated cytotoxicity, in Stat1+/+ but not in Stat1−/− mice. Lysis against L1210 control cells was less than 10% in all cases, demonstrating specificity (data not shown).

The failure to observe cytolytic activity in Stat1−/− mice was likely a result of poor differentiation into a lytic phenotype. To examine this possibility more directly, serine esterase activity was analyzed in purified T cells from immunized and control mice. This enzymatic activity correlates with the presence of cytotoxic granules (18, 19). As can be seen in Fig. 4B, T cells from immunized Stat1+/+ showed increased serine esterase activity compared with control T cells. In contrast, up-regulation of serine esterase activity was not induced in Stat1-deficient T cells. Collectively, these results indicate that IFN signaling through Stat1 is required for the acquisition of a cytolytic phenotype by antitumor T cells and NK cells in vivo.

**Discussion**

Much evidence suggests that an IFN-γ-producing, Th1/Tc1-type T cell response may be important for optimal rejection of established tumors. In vivo, neutralization of B7/CD28 interactions or of IL-12 prevents CTL generation and blocks spontaneous tumor rejection (2, 3). Conversely, coadministration of IL-12 augments the efficacy of a variety of tumor Ag vaccines (7, 8, 20, 21), and provision of IL-12 by other modalities also promotes antitumor immunity (9, 16, 22). The fact that some biologic effects of IL-12 can be blocked upon neutralization of IFN-γ (23), and that tumor rejection has been observed to be deficient in perforin knockout mice (5), have suggested that the effector functions of a differentiated Tc1 phenotype are important for tumor elimination. In fact, Tc1 cells have been shown to be quantitatively superior to Tc2 cells as an adoptive immunotherapy for established tumors (24). Our present results suggest that IFN signaling among host cells is in fact necessary for the development of antitumor cytolytic activity, revealing a surprisingly critical role for IFN in peripheral T cell differentiation. A recent report indicated that IL-4-deficient mice exhibited augmented CTL activity (25), consistent with our present results.

Inasmuch as Stat1−/− mice are deficient in signaling by IFN-αβ as well as IFN-γ, either or both of these cytokines might be important for CTL development. Both IFN-α and IFN-γ may contribute to T cell skewing toward a Th1-like phenotype (13, 26–28). Moreover, IFN-α is a potent inducer of NK function and has a clear role in promoting anti-viral CD8+ T cell responses (13, 29). On the other hand, IFN-α treatment has been reported to enhance IL-10 production by monocytes (30) and to inhibit IL-12 production by dendritic cells (31), both of which might be expected to decrease Th1-like differentiation. Formal dissection of whether signaling by IFN-αβ or IFN-γ is required for CTL generation will require additional experiments utilizing mice deficient in IFN-γR, IFN-αβR, or both.

The mechanism by which IFN signaling contributes to CTL maturation is not entirely clear. It seems likely that Stat1−/− T cells have an intrinsic defect in acquiring cytolytic activity. In support of this notion, preliminary experiments have indicated that

![FIGURE 2. Failure to control P1.HTR tumor growth in Stat1−/− mice by immunization with HTR.IL-12 cells. Stat1+/+ (circles) or Stat1−/− (triangles) DBA/2 mice (five mice per group) were inoculated s.c. with living P1.HTR cells. They then were immunized weekly in the hind footpad with irradiated HTR.IL-12 cells (filled symbols) or with PBS (open symbols). Tumor sizes were measured on the indicated days. Similar results were observed in two independent experiments.](image)

![FIGURE 3. T cells from Stat1−/− mice immunized with HTR.IL-12 cells produce reduced levels of IFN-γ, but do not produce IL-4. Three individual Stat1+/+ and Stat1−/− mice were inoculated with 10⁶ living HTR.IL-12 cells (gray bars) or with PBS (black bars) in the hind footpad. The draining popliteal lymph node cells were then removed and restimulated for assessment of IFN-γ (A) and IL-4 (B) content by ELISA. Similar results were obtained in three experiments.](image)
immunization with tumor cells expressing B7-1, IL-12, and ICAM-1, which should replace the essential signals normally provided by professional APCs, still failed to induce specific CTL (data not shown). Stat1-dependent transcription may be vital for the expression of one of several genes encoding cytotoxic granule proteins. The observation that NK lytic activity also was defective supports the hypothesis of a generalized inability to develop cytolytic effector machinery in the absence of Stat1. However, an indirect role for Stat1 signaling via non-T cells in mediating this effect has not been excluded.

Previous work has indicated that IFN signaling at the level of the tumor cell contributes to antitumor immunity (6). Human lung cancer cells and melanoma cells deficient in IFN-γR components or Stat1 have been observed with high frequency (32). However, our present data indicate an additional role for IFN signaling on host cells for the development of effective antitumor immunity. A recent report correlated the immunosuppressive effects of the chemotherapy drug fludarabine with down-regulation of Stat1 signaling in mononuclear cells (33). Collectively, these observations suggest that defects in Stat1 activation by IFN treatment of host lymphocytes should be explored as a potential mechanism of immunosuppression in cancer-bearing patients.

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References


FIGURE 4. Deficient CTL maturation in Stat1−/− mice. A, Stat1+/+ (light gray bars) or Stat1−/− (medium gray bars) DBA/2 mice (five per group) were immunized weekly with irradiated HTR-IL-12 cells in the hind footpads for 3 wk. PBS-injected Stat1−/− mice (black bars) are shown as a control. Two weeks after the last injection, splenocytes were restimulated in MLTC, and cytolytic activity was assessed from individual mice against the positive target P511, the NK-sensitive target YAC-1, and the negative control syngeneic target L1210. Each bar represents the mean lytic activity obtained from five individual mice at an E:T ratio of 100:1. Lysis against L1210 was less than 10% (data not shown). B, In the same experiment after MLTC, T cells were purified by negative selection and assayed for the presence of BLT esterase activity. Similar results were obtained in two independent experiments. Parallel experiments with naive Stat1−/− T cells revealed low BLT esterase activity comparable to that seen in naive wild-type T cells (data not shown).


