

Transforming Growth Factor- β Production and Myeloid Cells Are an Effector Mechanism through Which CD1d-restricted T Cells Block Cytotoxic T Lymphocyte-mediated Tumor Immunosurveillance: Abrogation Prevents Tumor Recurrence

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Abstract

Our previous work demonstrated that cytotoxic T lymphocyte (CTL)-mediated tumor immunosurveillance of the 15-12RM tumor could be suppressed by a CD1d-restricted lymphocyte, most likely a natural killer (NK) T cell, which produces interleukin (IL)-13. Here we present evidence for the effector elements in this suppressive pathway. T cell-reconstituted recombination activating gene (RAG)2 knockout (KO) and RAG2/IL-4 receptor α double KO mice showed that inhibition of immunosurveillance requires IL-13 responsiveness by a non-T non-B cell. Such nonlymphoid splenocytes from tumor-bearing mice produced more transforming growth factor (TGF)- β , a potent inhibitor of CTL, *ex vivo* than such cells from naive mice, and this TGF- β production was dependent on the presence *in vivo* of both IL-13 and CD1d-restricted T cells. *Ex vivo* TGF- β production was also abrogated by depleting either CD11b⁺ or Gr-1⁺ cells from the nonlymphoid cells of tumor-bearing mice. Further, blocking TGF- β or depleting Gr-1⁺ cells *in vivo* prevented the tumor recurrence, implying that TGF- β made by a CD11b⁺ Gr-1⁺ myeloid cell, in an IL-13 and CD1d-restricted T cell-dependent mechanism, is necessary for down-regulation of tumor immunosurveillance. Identification of this stepwise regulation of immunosurveillance, involving CD1-restricted T cells, IL-13, myeloid cells, and TGF- β , explains previous observations on myeloid suppressor cells or TGF- β and provides insights for targeted approaches for cancer immunotherapy, including synergistic blockade of TGF- β and IL-13.

Key words: TGF- β • NKT cells • immunologic surveillance • myeloid cells • IL-13

Introduction

Understanding and overcoming mechanisms of tumor escape from immunosurveillance have been a major focus of tumor immunology. During the last decade, multiple mechanisms of tumor escape from immunosurveillance have

been reported (1, 2). Immunosurveillance has been best documented in human tumors expressing foreign viral antigens, such as papillomavirus E6 and E7 in cervical cancers, HTLV-I antigens in T cell lymphoma/leukemia, or EBV antigens in B cell lymphoma/leukemia. Therefore, we have used a murine tumor expressing a viral protein as a

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Abbreviations used in this paper: D-NAME, N-nitro-D-arginine-methyl ester; L-NAME, N-nitro-L-arginine-methyl ester; NO, nitric oxide.

model for studying mechanisms of down-regulation of tumor immunosurveillance (3, 4). With this mouse tumor model, in which tumor shows a growth-regression-recurrence pattern, we demonstrated that regression was mediated by specific CD8⁺ CTLs (3). Tumor recurrence was the result of incomplete elimination of tumor cells by CTLs that were negatively regulated by IL-13 produced by CD4⁺ CD1d-restricted T cells, most likely NKT cells, through the IL-4R α -STAT6 signaling pathway, although this negative regulation was found to be independent of IL-4. CD1d-restricted T cells from tumor-bearing mice were shown to produce IL-13, and tumor recurrence was prevented by eliminating CD1d-restricted T cells or blocking IL-13 in vivo (4). This or a related pathway is likely to play a role in tumor immunosurveillance for other type of tumors, as STAT6 KO mice effectively reject a breast carcinoma and a mastocytoma in two different strains of mice (5, 6) and CD1 KO mice were resistant to a breast carcinoma (7). We have also observed similar inhibition of growth of a colon carcinoma and osteosarcoma in CD1 KO mice in preliminary studies. Thus, this pathway is possibly not limited to the gp160-expressing tumor under study.

However, it is known that T cells generally do not express IL-13 receptors (8). Therefore, we have hypothesized that intermediate cells may respond to IL-13 and suppress CTL-mediated tumor immunosurveillance, and that our earlier study had defined only the upstream half of the regulatory circuit. To test this hypothesis, we have now investigated the identity of this postulated intermediate cell and the mechanism by which it inhibits tumor immunosurveillance by CTL to define the effector elements in this suppressive pathway.

Previously, TGF- β has been suggested to play a role in tumor escape from immunosurveillance (9). Inhibition of TGF- β by antibody or soluble receptor inhibits tumor growth in vivo (9–13), but the mechanism has been unclear and not necessarily immunological. Recently Gorelik and Flavell (14) reported that mice with T cells that do not respond to TGF- β were resistant to implanted aggressive tumor cell lines B16 and EL4. This evidence suggests that direct action of TGF- β on T cells suppresses antitumor T cell activity and results in uncontrolled outgrowth of tumor cells. However, very little is known about what immune mechanisms induce TGF- β production in tumor-bearing animals or cancer patients, beyond production by the tumor cells themselves.

Here we have put these earlier observations in context by demonstrating that IL-13 and CD4⁺ CD1d-restricted T cells are required in vivo for TGF- β production by non-lymphoid cells of myeloid origin that express both CD11b and Gr-1. This intermediate cell and TGF- β were necessary for down-regulation of CD8⁺ CTL-mediated tumor immunosurveillance. Connecting myeloid cells and TGF- β to the CD1d-restricted T cell/IL-13 regulatory pathway puts previous separate observations on TGF- β or myeloid cells by themselves into the larger context of a regulatory

circuit, and provides an explanation for observations on their role in controlling tumor growth. Thus, we have now defined the downstream half of the immunoregulatory circuit and elucidated the effector mechanism of CD1d-restricted, T cell-mediated suppression of CTLs. Understanding the complete pathway suggests several potential therapeutic target sites at which intervention could increase antitumor immunity and inhibit tumor growth.

Materials and Methods

Mice. Female BALB/c mice were purchased from Charles River Laboratories. RAG2 KO (Taconic), RAG2/IL-4R α double KO, and CD1 KO mice (4) with BALB/c background were bred under pathogen-free conditions. Mice were maintained in a pathogen-free facility and used at 6–10 wk of age. Animal experiments were all approved by the National Cancer Institute (NCI) Animal Care and Use Committee.

Reagents. Purified rat anti-mouse CD4 mAb (GK1.5) was obtained from the Frederick Cancer Research and Development Center, NCI. A fusion protein of murine IL-13R α 2 and human IgG1 (sIL-13R α 2-Fc) was made by Genetics Institute (15). Monoclonal anti-TGF- β (1D11.16 specific for TGF- β 1, TGF- β 2, and TGF- β 3; reference 16) and isotype-matched control antibody (13C4; reference 17) were made by Genzyme. Anti-Ia, anti-CD11b, anti-CD11c, anti-B220, anti-DX5, anti-CD4, anti-CD8, and avidin-conjugated magnetic beads were purchased from Miltenyi Biotec, recombinant mouse IL-2 and IL-13 were purchased from R&D Systems, and recombinant human TGF- β 1 was purchased from PeproTech. PE-conjugated α -Galactosylceramide-loaded and empty mouse CD1d tetramer were provided by M. Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA; reference 18). PerCP-, PE-, and FITC-conjugated anti-CD11b (M1/70), FITC- and APC-conjugated CD11c (HL3), FITC and biotinylated anti-Gr-1 (RB6-8C5), APC-conjugated anti-CD31 (MEC 13.3), and PE-conjugated anti-IL-4R α (mIL-4R-M1) antibodies were purchased from BD Biosciences, and PE-conjugated anti-F4/80 antibody (A3-1) was purchased from Caltag Laboratories. *N*-nitro-L-arginine-methyl ester (L-NAME) and *N*-nitro-D-arginine-methyl ester (D-NAME) were purchased from Sigma-Aldrich.

Tumor Cell Lines and Tumor Inoculation. Fibrosarcoma 15-12RM and 18Neo (3) were maintained as previously described (3). 10⁶ 15-12RM cells were injected s.c. on the right flank. To deplete CD4⁺ cells in vivo, mice were inoculated i.p. with 0.5 mg anti-CD4 mAb or control rat IgG (Sigma-Aldrich) 3 d in a row from day 0 and twice a week thereafter. In some experiments, mice were treated i.p. on days 0, 1, and 3 with 0.2 mg sIL-13R α 2-Fc or every other day for 10 d with 0.1 mg of either anti-TGF- β mAb or control mAb. For Gr-1⁺ cell depletion, mice were injected i.p. with 200, 100, 10, or 1 μ g anti-Gr-1 antibody (Cedarlane Laboratories Ltd.) on days 5, 6, 10, 15, and 20 after tumor injection. Where indicated, mice received 0.2 mg L-NAME or D-NAME every day for 2 wk after tumor injection.

Mouse colon carcinoma line CT26 was maintained in complete T cell medium (3). 20,000 CT26 cells were injected via the tail vein into BALB/c mice. The mice were inoculated i.p. with 0.1 mg of either anti-TGF- β mAb or control mAb every other day for 2 wk. At the time when control mice had developed a sufficient number of tumor metastases (~3 wk after tumor inoculation), all the mice were killed and evaluated for pulmonary me-

tastases by counting nodules in the lungs perfused with a 15% solution of India ink and fixed with Feke's solution.

T Cell Purification and In Vivo Transfer. T cells were negatively isolated from the spleen cells of wild-type or IL-4R α KO BALB/c mice. Single cell suspensions of spleen cells were depleted for Ia $^+$, CD11b $^+$, CD11c $^+$, and DX5 $^+$ cells by using magnetic beads (Miltenyi Biotec). 50 million purified T cells were inoculated into RAG2 KO or RAG2IL-4R α double KO mice i.v. 1 wk before 15-12RM injection.

CTL Generation and CTL Assay. Spleen cells from 10 7 PFU vPE16-injected BALB/c mice were stimulated with 1 μ M P18-IIIIB and used for CTL assay as previously described (4). In some experiments, non-T non-NK cells were prepared from spleen cells from both naive and 15-12RM-injected BALB/c mice by depleting CD4 $^+$, CD8 $^+$, and DX5 $^+$ cells by magnetic beads conjugated with anti-CD4, anti-CD8, and anti-DX5 antibodies (Miltenyi Biotec). After being pulsed with 1 μ M P18-IIIIB peptide, the cells were admixed with T cells from vPE16-injected mouse spleen cells purified with magnetic beads (Miltenyi Biotec) in complete T cell medium (3) with 20 U/ml IL-2 in the presence or absence of 50 μ g/ml anti-TGF- β antibody (1D11) as indicated. After 1 wk of culture, viable cells were harvested and used as effector cells for the CTL assay as previously described (4).

Isolating Nonlymphoid Cells and Measurement of Ex Vivo TGF- β 1 Production. Non-T non-B non-NK cells, referred to as non-lymphoid cells, were purified from spleen cells of BALB/c mice by depletion of T, B, and NK cells using magnetic beads coated with anti-CD4, anti-CD8, anti-B220, and anti-DX5 (Miltenyi Biotec). In some experiments, CD11b $^+$, CD11c $^+$, or Gr-1 $^+$ cells were depleted using magnetic beads coated with anti-CD11b, anti-CD11c, or streptavidin (Miltenyi Biotec) along with biotinylated anti-Gr-1 antibody (BD Biosciences). 2 \times 10 5 cells were cultured in vitro without stimulation in a 96-well plate in 200 μ l X Vivo 20 medium (BioWhittaker, Inc.). At the indicated times, 100 μ l culture medium was harvested from each well and stored at -80°C until TGF- β 1 measurement. After acidification, to convert all TGF- β 1 to the active form, the concentration of total TGF- β 1 in the culture supernatant was determined by ELISA kit (R&D Systems). All samples were assayed in triplicate. Without acidification, no TGF- β 1 was detected, indicating that the TGF- β was all in the latent form. The amount of TGF- β 1 production ex vivo by 10 6 cells was calculated as follows: concentration of culture supernatant (pg/ml) \times 0.2 (ml)/(2 \times 10 5 /10 6) = pg produced per million cells.

Flow Cytometric Analysis. Spleen cells were obtained from naive and 15-12RM-injected mice. The cells were stained with antibodies for 30 min after blocking CD16/CD32 (2.4G2; BD Biosciences) for 15 min. After washing, the cells were analyzed by FACSscan™ or FACSCaliber™ by using CELLQuest™ software (BD Biosciences).

Morphological Study of Gr-1 $^+$ CD11b $^+$ Cells. Nonlymphoid cells prepared from BALB/c spleen cells were stained with FITC-conjugated anti-Gr-1 and APC-conjugated anti-CD11b antibodies for 30 min after blocking CD16/CD32 (BD Biosciences) for 15 min. After washing, the cells were gated on Gr-1 $^{\text{hi}}$ CD11b $^+$ and Gr-1 $^{\text{int}}$ CD11b $^+$ cell populations and sorted by FACS Vantage™ (BD Biosciences). After washing, sorted cells were collected by cytopspin onto glass slides and dried completely. The cells were stained with Wright-Giemsa stain using a Diff-Quik stain set (Dade Behring Inc.).

Statistical Analysis. Statistical analysis was performed by a log-rank test, one-way analysis of variance, or Student's *t* test. Data were considered significant at *P* < 0.05.

Results

Non-T Non-B Cells Are the Cells Responding to IL-13 That Down-regulate Tumor Immunosurveillance. In BALB/c mice injected s.c. with the fibrosarcoma 15-12RM, tumors show a growth regression recurrence pattern. The spontaneous regression was mediated by CD8 $^+$ CTLs (3). As we showed previously, both IL-13 and CD4 $^+$ CD1d-restricted T cells, most likely NKT cells, the only known CD1d-restricted T cells in the mouse (19), were necessary to down-regulate this CTL-mediated tumor immunosurveillance (4). To understand the mechanism of down-regulation of tumor immunosurveillance induced by IL-13, first we examined the direct effect of IL-13 on CTLs in vitro. Spleen cells from mice immunized with recombinant vaccinia vPE16, which expresses HIV gp160, were stimulated with P18 peptide, the immunodominant epitope peptide of the V3 loop of HIV gp160 responsible for much of the CTL activity against the 15-12RM tumor (3). The cells were maintained for 1 wk with various doses of IL-13 in addition to IL-2, and used as effector cells in a CTL assay (Fig. 1 A). IL-13 during stimulation had no effect on CTL activity (Fig. 1 A), nor did adding IL-13 during the CTL assay (not depicted). These results are consistent with published reports that T cells lack IL-13 receptors (8) and suggest that although IL-13 is necessary for down-regulation of CTL tumor immunosurveillance, it could not directly affect CTL induction or

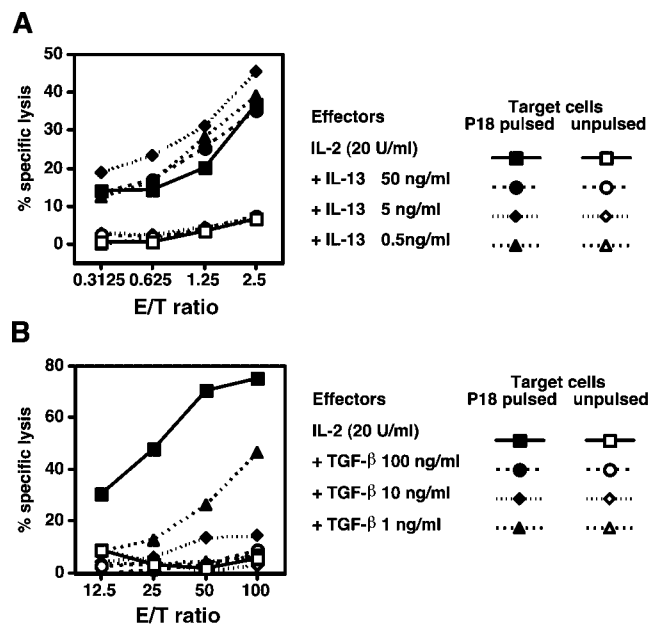


Figure 1. TGF- β 1 but not IL-13 can directly suppress CTL induction in vitro. 4 \times 10 6 spleen cells from mice immunized with vPE16 were stimulated in vitro with 10 6 P18-pulsed spleen cells for 6 d. During in vitro stimulation, in addition to IL-2 (■ and □), in A the cells were maintained with 50 ng/ml (● and ○), 5 ng/ml (◇ and ◆), or 0.5 ng/ml (▲ and △) of IL-13 or in B, 100 ng/ml (● and ○), 10 ng/ml (◇ and ◆), or 1 ng/ml (▲ and △) of TGF- β was added. The cultured cells were used for CTL assay at the E/T ratios shown. Targets were 18Neo fibroblasts either without peptide (open symbols) or pulsed with 1 μ M P18-IIIIB peptide (solid symbols). The data shown are representative of three independent experiments.

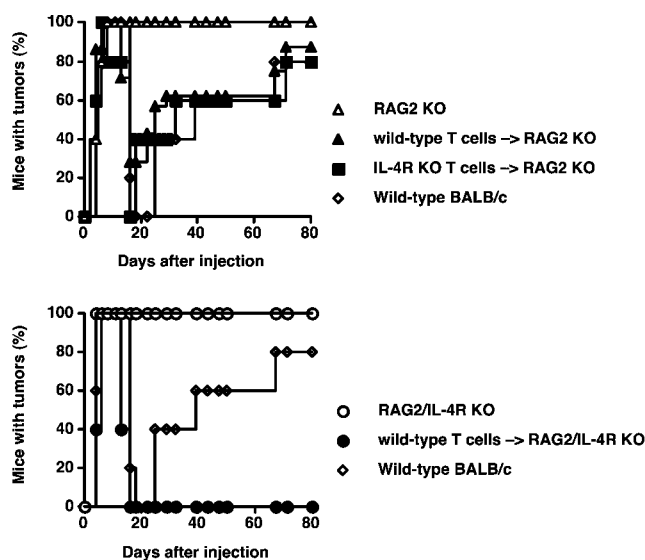


Figure 2. Non-T non-B cells are the cells responding to IL-13 that down-regulate tumor immunosurveillance. RAG2 KO and RAG2/IL-4R α KO mice were injected i.v. with 5×10^7 purified T cells from the spleens of wild-type or IL-4R α KO mice. These T cells were purified by depleting cells expressing MHC class II, CD11b, CD11c, and DX5 cells from spleen cells by using magnetic beads conjugated with antibodies against each molecule. 1 wk later, the mice were injected s.c. with 10^6 15-12RM tumor cells. RAG2 KO or RAG2/IL-4R α KO mice lack T and B cells. T cells from IL-4R α KO mice and nonlymphoid host cells from RAG2/IL-4R α double KO mice cannot respond to IL-4 or IL-13. Five mice were used for each group. The result shown is representative of three experiments.

activity and that there must be other downstream steps that directly down-regulate CTL activity.

To identify an intermediate cell that responds to IL-13 and then acts on CTLs to down-regulate immunosurveillance, we performed a T cell transfer experiment using RAG2 KO and RAG2/IL-4R α double KO recipient mice (Fig. 2). IL-4R α is required for response to IL-13. Splenic T cells were purified by negative selection for cells expressing MHC class II, CD11b, CD11c, and DX5. The latter is expressed on NK but not NKT cells (19). The purified T cells contained 2–3% CD1d tetramer⁺ cells (NKT cells), compared with 1–1.5% in spleen cells. 50 million T cells purified from wild-type or IL-4R α KO mice were injected i.v. into RAG2 KO and RAG2/IL-4R α double KO mice 1 wk before 15-12RM injection. RAG2 KO or RAG2/IL-4R α double KO mice did not reject tumors at all because they lack T and B cells. However, RAG2 KO mice receiving transferred T cells, regardless of the expression of IL-4R α by the T cells, behaved like wild-type BALB/c mice, implying that both CD8⁺ effector cells and CD1d-restricted T regulatory cells were successfully reconstituted. Further, the fact that T cells from IL-4R α KO mice reconstitute as well as wild-type T cells implies that neither the effector nor the regulatory T cells themselves need to be able to respond to IL-13. In contrast, in RAG2/IL-4R α double KO mice that received the same wild-type T cells, the tumor did not recur after initial growth and regression, even though the transferred T cells expressed IL-4R α .

Thus, it is the recipient RAG2 KO cells that must express IL-4R α to repress immunosurveillance. We have shown previously that IL-4 was neither necessary nor sufficient for down-regulation of tumor immunosurveillance in this system, and that the cytokine responsible for signaling through IL-4R α to regulate tumor immunosurveillance was IL-13 (4). Therefore, the cells that respond to IL-13 to mediate the down-regulation of tumor immunosurveillance must derive from the RAG2 KO host, which lacks T and B cells. We conclude that the intermediate cells responding to IL-13 and down-regulating CTL activity are non-T non-B cells.

TGF- β 1 Is Necessary for the Down-regulation of Tumor Immunosurveillance. To understand how non-T non-B cells respond to IL-13 and suppress CTL induction in tumor-bearing mice, we considered that TGF- β 1 is known for its ability to suppress lytic activity of CD8⁺ T cell function (20). To examine the ability of TGF- β 1 to suppress CTL function, we stimulated spleen cells of vPE16-immunized mice in vitro for 1 wk with P18 peptide in the presence of various doses of TGF- β and then tested these in a CTL assay (Fig. 1 B). When vPE16-immunized spleen cells were stimulated in vitro in the presence of TGF- β 1, cytolytic activity was suppressed. TGF- β had no effect in the lytic assay itself (not depicted). These results showed that in contrast to IL-13, TGF- β 1 directly down-regulates CTL induction.

If TGF- β 1 is the cytokine induced by IL-13 in tumor-bearing mice to down-regulate tumor immunosurveillance, TGF- β 1 production should be increased in non-T non-B cells of tumor-bearing mice. To test whether TGF- β 1 production is up-regulated in tumor-bearing mice in vivo, we purified splenic nonlymphoid cells (Thy1.2⁻ B220⁻ DX5⁻ cells) from both naive and 15-12RM-injected mice at day 3 after tumor injection, and examined TGF- β 1 production ex vivo without in vitro stimulation (Fig. 3 A). Latent TGF- β 1, although not detected at 3 h of culture (not depicted), was already detected in the culture supernatants of the nonlymphoid cells from 15-12RM-injected mice after only 6 h of culture but was undetected in cells from naive mice at that time point ($P < 0.005$ by Student's *t* test). This earliest time point probably reflects the in vivo activity of these cells at the time they were removed from the mouse, without any in vitro stimulation. By 12 h in tissue culture plastic, even the cells from nontumor-bearing mice were making some TGF- β , suggesting a nonspecific stimulation by the tissue culture plates or medium. Nevertheless, even at this later time point, the cells from 15-12RM-injected mice still produced more TGF- β 1 than those from naive mice. We also examined the kinetics of TGF- β 1 production by nonlymphoid cells (Fig. 3 B). The TGF- β 1 production by nonlymphoid cells was increased until at least day 10 after tumor injection. Therefore, to test the hypothesis that TGF- β 1 was necessary for tumor recurrence in vivo, we injected i.p. either a neutralizing anti-TGF- β or control mAb (Fig. 3 C). Because we have previously shown that the first 10 d after 15-12RM injection were very critical for negative regulation of tumor immunosur-

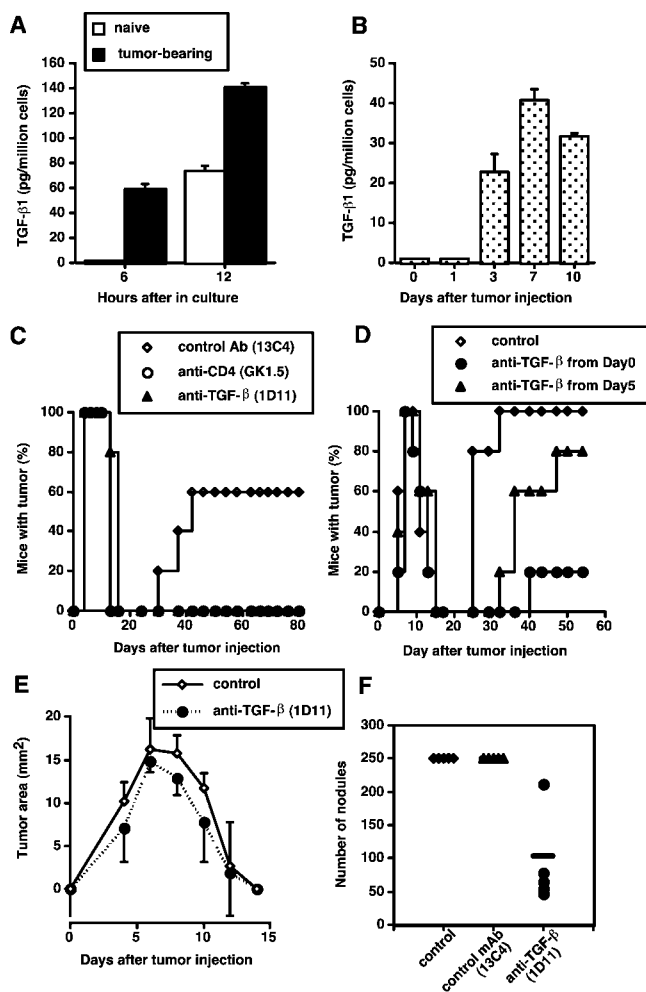


Figure 3. TGF- β 1 production was increased in nonlymphoid spleen cells (non-T non-B non-NK cells) from 15-12RM-injected BALB/c mice and anti-TGF- β antibody prevents tumor recurrence. (A) On day 3 after 15-12RM injection, freshly isolated nonlymphoid cells from naive BALB/c (open bar) and 15-12RM tumor-injected BALB/c mice (solid bar) were examined for TGF- β 1 production ex vivo. Nonlymphoid cells were purified from spleen cells of BALB/c mice by depletion of T cells, B cells, and NK cells. 2×10^5 cells were cultured in vitro without stimulation in a 96-well plate in 200 μ l X Vivo 20 medium. The concentration of TGF- β 1 was determined by ELISA. Each value shows the average \pm SD of triplicate assay. $P < 0.005$ at 6 h and $P < 0.01$ at 12 h with Student's t test between naive and tumor-bearing groups. Error bars not shown are too small to be seen on this scale. This experiment is representative of 10 experiments with similar results. (B) Freshly isolated nonlymphoid cells were prepared from 15-12RM-injected mice at different time points after tumor injection, and were examined for TGF- β 1 production ex vivo as described in A. (C) Anti-CD4-treated mice (\circ), anti-TGF- β -treated mice (\blacktriangle), or control isotype-matched antibody-treated (\diamond) BALB/c mice (five per group) were injected with 10^6 15-12RM cells s.c. 0.5 mg of the anti-CD4 mAb was inoculated on days 0, 1, 2, 6, and 10 after tumor injection. 100 μ g anti-TGF- β mAb or isotype-matched control mAb were injected every other day from days 0 to 10. $P < 0.05$ with log-rank test between the control group and the anti-TGF- β group. This experiment is representative of three experiments with similar results. (D) After s.c. inoculation of 15-12RM cells, the mice (five per group) were treated with 100 μ g anti-TGF- β antibody from day 0 (\bullet) or day 5 (\blacktriangle), or control isotype-matched antibody (\diamond) every other day for 10 d. (E) Size of primary tumors in 15-12RM-injected BALB/c mice treated with 100 μ g anti-TGF- β mAb (\bullet) i.p. every other day for 10 d or without any antibody treatment (\diamond). The mice were inoculated s.c. with 10^6 15-12RM cells.

veillance (4), we treated mice with mAb for the first 10 d after tumor injection. The mice treated with anti-TGF- β were protected from recurrence of tumor similarly to anti-CD4-treated mice. The effect of anti-TGF- β was seen only in the recurrent phase when the difference in tumor size was enormous (>400 mm 2 in the controls vs. none in the anti-TGF- β -treated mice). In contrast, there was little difference in the primary tumors. Although the sizes of the primary tumors during the initial growth period in anti-TGF- β -treated mice were slightly smaller than those of tumors in control mice in all the experiments, the difference was not significant (Fig. 3 E). These results demonstrate that TGF- β 1 is necessary for tumor recurrence. Even though increased TGF- β production was observed through day 10 (Fig. 3 B), starting the anti-TGF- β treatment at 5 d was too late to make an impact on late tumor recurrence (Fig. 3 D). These results suggest that TGF- β 1 produced early after tumor injection plays a critical role in down-regulation of antitumor immunosurveillance. Therefore, we decided to measure ex vivo TGF- β production by the nonlymphoid cells on day 3 after tumor injection. Furthermore, the fact that blockade of TGF- β for the first 10 d is sufficient suggests that it is critical only during the priming of CTLs, consistent with our observation that TGF- β inhibits the induction of CTLs (Fig. 2) but not their effector function (not depicted).

For further confirmation of the role of TGF- β made by the nonlymphoid cells from 15-12RM-injected mice in down-regulation of CTLs, we examined the effect of splenic non-T non-NK cells from 15-12RM-injected mice on CTL induction in vitro. Purified splenic T cells from mice immunized with recombinant HIV gp160-expressing vaccinia vPE16 were stimulated with P18 peptide-pulsed non-T non-NK spleen cells (depleted of CD4 $^+$, CD8 $^+$, and DX5 $^+$ cells by magnetic beads) from naive or 15-12RM-injected mice at day 3 after injection. The cells were maintained with IL-2 and with or without 50 μ g/ml anti-TGF- β antibody (1D11) for 1 wk, and used as effector cells in a CTL assay (Fig. 4). The CTL cultured with non-T non-NK cells from 15-12RM-injected mice showed lower cytolytic activity than those cultured with the cells from naive mice. However, this suppression was fully overcome by adding anti-TGF- β antibody during the culture. This result indicates that TGF- β made ex vivo by nonlymphoid cells (Fig. 3, A and B) has the potential to directly suppress CTL induction.

Tumor size was measured in two perpendicular dimensions with calipers every other day. The vertical axis shows tumor area measured as the product of these two dimensions. This experiment is representative of four independent experiments. (F) BALB/c mice (five per group) injected with 2×10^5 CT26 were treated with 0.1 mg anti-TGF- β mAb or isotype-matched control mAb every other day for 2 wk. On day 21, the mice were killed and the lungs were perfused with a 15% solution of India ink. After fixation by Fekete's solution, the number of nodules was macroscopically counted. The maximum number of the nodules counted per lung was 250. The mean number of nodules is indicated as horizontal bars. $P < 0.0001$ with one way analysis of variance test for the anti-TGF- β group compared with control and control mAb groups. Similar results were obtained in another experiment.

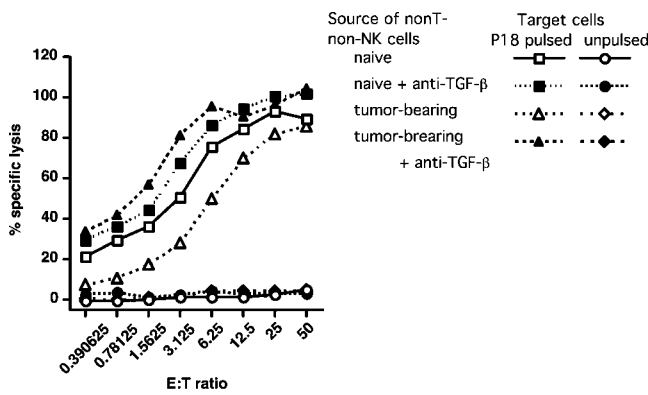


Figure 4. CTL induction was down-regulated by non-T non-NK cells from 15-12RM-injected mice through TGF- β . On day 3 after 15-12RM injection, non-T non-NK cells were prepared from spleen cells of tumor-bearing (\blacktriangle and \triangle , and \blacklozenge and \lozenge) and naive BALB/c mice (\blacksquare and \square , and \bullet and \circ) by negatively depleting CD4⁺, CD8⁺, and DX5⁺ cells, and pulsed with 1 μ M P18 peptide. Splenic T cells from vPE16-immunized mice were stimulated in vitro in the presence of these non-T non-NK cells for 1 wk. In addition to IL-2 added to all cultures, 50 μ g/ml anti-TGF- β was added (solid symbols) or omitted (open symbols) during in vitro stimulation. The cultured cells were harvested and used for CTL assay. Targets were 18Neo fibroblasts pulsed either with P18 peptide (\blacksquare and \square , and \blacktriangle and \triangle) or without peptide (\bullet and \circ , and \blacklozenge and \lozenge). A similar result was obtained in another independent experiment.

Recently we found that the negative immunoregulatory pathway involving CD1d-restricted T cells and IL-13, which we originally studied using the 15-12RM mouse tumor, is also active in the mouse lung metastasis model of syngeneic colon carcinoma CT26, to be reported elsewhere (unpublished data). Therefore, we also tested the effect of anti-TGF- β on tumor growth in vivo by using this tumor system (Fig. 3 F). Anti-TGF- β treatment significantly reduced the number of lung metastases. This result indicates that the role of TGF- β in down-regulation of CTL-mediated tumor immunosurveillance is not unique to the 15-12RM fibrosarcoma.

TGF- β 1 Production Is Induced by IL-13 or CD1-restricted T Cells in Tumor-bearing Mice. Although the results suggested that TGF- β 1 might be induced by IL-13 and act downstream to down-regulate CTL-mediated tumor immunosurveillance, they did not rule out the possibility that both IL-13 and TGF- β 1 were necessary and acting in parallel to inhibit tumor immunosurveillance, albeit on different cell populations. To distinguish between these possibilities, we tested the effect of in vivo IL-13 inhibitor treatment, which protects mice from tumor recurrence, on ex vivo TGF- β 1 production of splenic nonlymphoid cells from tumor-bearing mice 3 d after tumor inoculation (Fig. 5 A). The IL-13 inhibitor treatment reduced the TGF- β 1 level almost to that seen in naive mice. As we have previously shown that CD1d-restricted T cells were responsible for IL-13 production induced in tumor-bearing mice and CD1 KO mice that lack these cells resisted tumor recurrence (4), we also examined the TGF- β 1 production in the splenic nonlymphoid cells from CD1 KO mice 3 d after tumor injection. The nonlymphoid cells from CD1 KO

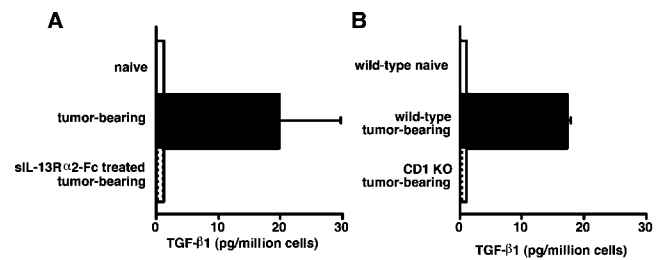


Figure 5. In vivo TGF- β 1 production was down-regulated in IL-13 inhibitor treated wild-type mice or CD1 KO mice injected with 15-12RM tumor cells. (A) On day 3 after 15-12RM tumor injection, freshly isolated nonlymphoid cells from naive (open bar), 15-12RM tumor-injected mice (solid bar), and 15-12RM tumor-injected mice treated with IL-13 inhibitor (sIL-13R α 2-Fc) were examined for TGF- β 1 production ex vivo. 0.2 mg IL-13 inhibitor (sIL-13R α 2-Fc) was administered i.p. on days 0, 1, and 2 after tumor injection. (B) Naive wild-type BALB/c (open bar), 15-12RM tumor-injected wild-type BALB/c (solid bar), and tumor-injected CD1 KO BALB/c mice (gray bar) were used as a source of nonlymphoid cells on day 3 of tumor injection. As in Fig. 3 A, in both A and B, 2×10^5 cells purified were cultured in vitro without stimulation. The culture supernatant was collected at 6 h and the concentration of TGF- β 1 was determined by ELISA. Each value shows the average \pm SD of triplicate assay. Error bars not shown are too small to be seen on this scale. Similar results were obtained in three independent experiments.

mice failed to produce TGF- β 1 immediately ex vivo, in contrast to the cells from wild-type BALB/c mice (Fig. 5 B). Thus, TGF- β 1 expression by nonlymphoid cells of tumor-bearing animals is dependent in vivo on IL-13 and CD1d-restricted T cells.

CD11b⁺ Gr-1⁺ Cells Were the Major Source of TGF- β 1 in Tumor-bearing Mice and Necessary for Tumor Recurrence. The splenic nonlymphoid cells include CD11b⁺ ($45.33 \pm 10.23\%$ in naive mice, $33.72 \pm 8.61\%$ in tumor-bearing mice), CD11c⁺ ($3.78 \pm 1.21\%$ in naive mice, $3.47 \pm 1.76\%$ in tumor-bearing mice), F4/80⁺ ($23.75 \pm 9.80\%$ in naive mice, $17.83 \pm 7.21\%$ in tumor-bearing mice), and Gr-1⁺ cells ($45.09 \pm 7.59\%$ in naive mice, $35.92 \pm 11.88\%$ in tumor-bearing mice). Among the CD11b⁺ cells, almost 80% ($37.52 \pm 7.72\%$ and $28.16 \pm 10.68\%$ of nonlymphoid spleen cells in naive and tumor-bearing mice) were positive for Gr-1 and $>50\%$ ($23.75 \pm 9.8\%$ and $17.83 \pm 7.21\%$) were positive for F4/80, but $<10\%$ were CD11c⁺. Most of the CD11c⁺ cells were myeloid dendritic cells that also expressed CD11b (Fig. 6).

To determine which among the splenic nonlymphoid cells make TGF- β 1, we depleted CD11b⁺, CD11c⁺, or Gr-1⁺ cells from splenic nonlymphoid cells and measured TGF- β 1 levels in the supernatants of the ex vivo cultures (Fig. 7, A and B). Surprisingly, depletion of either CD11b⁺ or Gr-1⁺ cells almost completely abrogated TGF- β 1 production by nonlymphoid cells from tumor-bearing mice. However, removing CD11c⁺ cells did not have an effect on the TGF- β 1 level at 12 h, although it did at 6 h, whereas the effect of either CD11b or Gr-1 depletion persisted at 12 h, indicating that CD11c⁺ cells were less critical than CD11b⁺ Gr-1⁺ double positive cells for TGF- β production (Fig. 7 A). These results suggested that although CD11c⁺ cells may play some role, among the cells express-

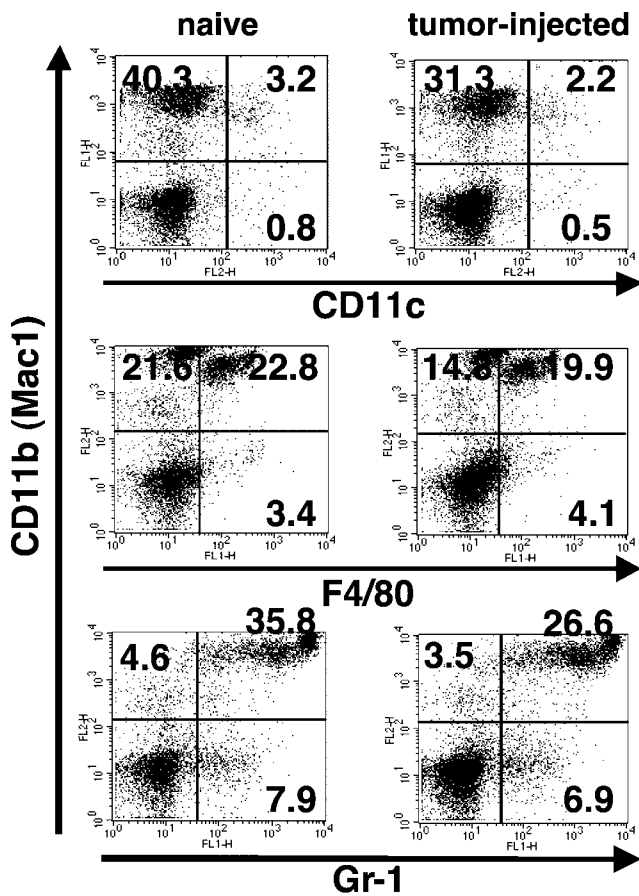


Figure 6. Flow cytometric staining of splenic nonlymphoid cells from naive and 15-12RM tumor-injected BALB/c mice. Nonlymphoid cells were prepared from spleen cells of naive and 15-12RM-injected mice (day 3) by depleting CD4⁺, CD8⁺, B220⁺, and DX5⁺ cells with magnetic beads. The cells were stained with PE- and FITC-conjugated anti-CD11b, PE-conjugated anti-CD11c, FITC-conjugated anti-F4/80, and FITC-conjugated anti-Gr-1, washed, and analyzed by flow cytometry. This experiment is representative of 10 experiments with similar results.

ing CD11b, CD11b⁺ Gr-1⁺ double positive cells were the major source of TGF- β 1 production by nonlymphoid cells from 15-12RM-injected mice. We detected these cells primarily in the spleen, not draining lymph nodes, where their numbers were inadequate for study.

Therefore, we examined the role of Gr-1⁺ cells in down-regulation of tumor immunosurveillance in vivo. Mice were treated with various doses (200, 100, 10, and 1 μ g) of anti-Gr-1. Among the doses, the group of mice treated with 1 μ g antibody from days 5–20 after tumor injection were protected from tumor recurrence (Fig. 7 C). (At high doses of anti-Gr-1 mAbs, tumor never even regressed, presumably due to immunodeficiency from depletion of antigen-presenting cells, so the recurrence could not be studied [not depicted]). Thus, Gr-1⁺ cells were not only necessary for ex vivo TGF- β 1 production, but also in fact necessary for down-regulation of immunosurveillance in vivo.

The results indicated that the intermediate cell in the downstream effector arm of the circuit is the Gr-1⁺ CD11b⁺ cell. To better characterize these cells, we exam-

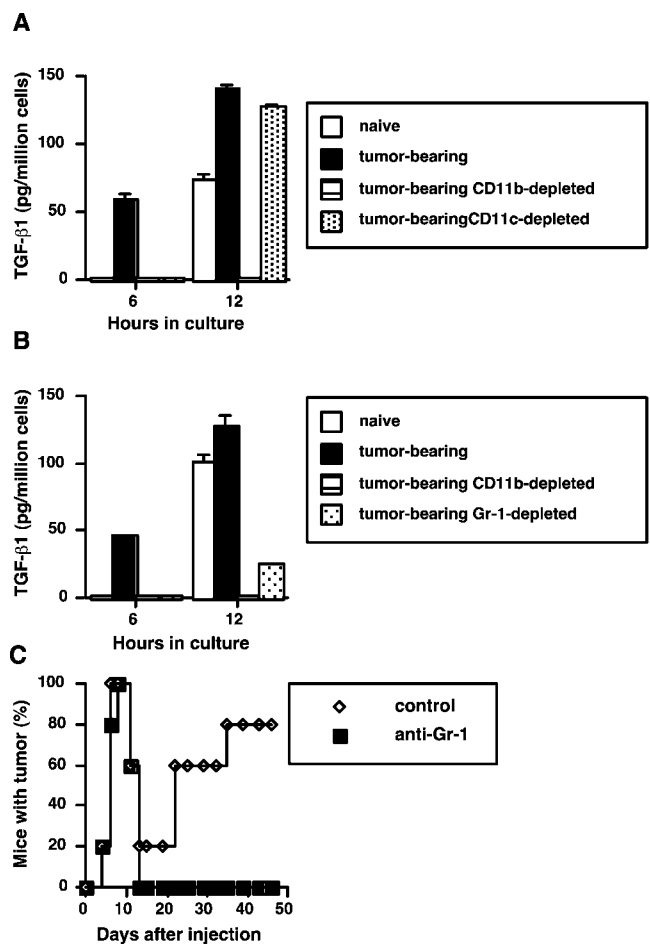


Figure 7. CD11b⁺ cells and Gr-1⁺ cells are the major source of TGF- β 1 and necessary for down-regulation of tumor immunosurveillance. (A and B) On day 3 after 15-12RM injection, nonlymphoid spleen cells were purified from naive (open bar) and tumor-injected BALB/c mice (solid bars) as in Fig. 3 A. CD11b⁺ (hatched bars) or CD11c⁺ (tightly dotted) cells were depleted with magnetic beads during purification of the cells from tumor-injected mice in A. CD11b⁺ (hatched bars) or Gr-1⁺ (dotted bars) cells were depleted during purification of the cells from tumor-injected mice, the latter using biotinylated antibody and avidin-coated magnetic beads in B. As in Fig. 3 A, in both A and B, 2×10^5 cells were cultured in vitro without stimulation. The concentration of TGF- β 1 in media was determined by ELISA. Each value shows the average \pm SD of triplicate assay. CD11b⁺- and Gr-1⁺-depleted cells produced significantly less TGF- β 1 ($P < 0.001$, respectively) than the untreated cells from tumor-bearing mice. Error bars not shown are too small to be seen on this scale. This experiment is representative of three experiments with similar results. (C) Anti-Gr-1-treated mice (■) or control BALB/c mice (◇; five per group) were injected with 10^6 15-12RM cells s.c. The anti-Gr-1 antibody (1 μ g) was inoculated on days 5, 6, 10, 15, and 20 after 15-12RM injection. $P < 0.05$ with log-rank test between the control group and the anti-Gr-1-treated group. This experiment is representative of three experiments with similar results.

ined the expression of CD11c, CD31, and IL-4R α on Gr-1⁺ CD11b⁺ cells (Fig. 8 A) and their morphology (Fig. 8 B). F4/80 was expressed on \sim 75% of the Gr-1⁺ CD11b⁺ cells from both naive and 15-12RM-injected mice. CD31 was expressed relatively higher on the cells from tumor-bearing mouse compared with the cells from naive mice (averages among several experiments were 24 and 19% in tumor-

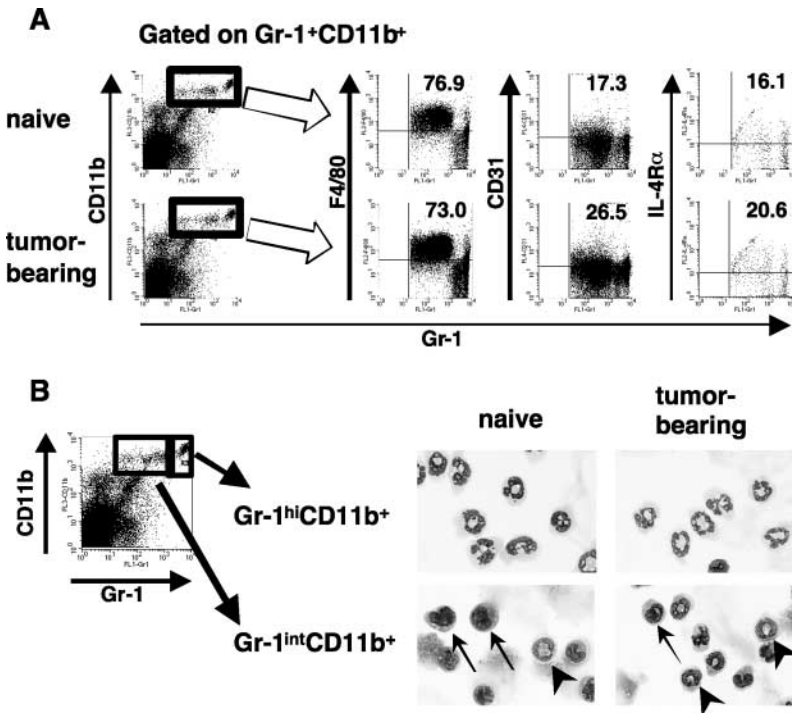


Figure 8. Characterization of Gr-1⁺ CD11b⁺ cells. On day 3 after 15-12RM injection, single cell suspensions of spleen cells from tumor-bearing and naive BALB/c mice were prepared. (A) The cells were stained with FITC-conjugated anti-Gr-1, Per-CP-conjugated anti-CD11b antibodies in combination with PE-conjugated anti-F4/80, APC-conjugated anti-CD31, or PE-conjugated anti-IL-4R α antibodies and analyzed by flow cytometry. During the analysis of the data acquired, we first gated on Gr-1⁺ CD11b⁺ cells and analyzed for the expression of other molecules. The numbers indicated are the percentage of the cells positive for each marker among the Gr-1⁺ CD11b⁺ cells. (B) Nonlymphoid cells were stained with FITC-conjugated anti-Gr-1, Per-CP-conjugated anti-CD11b antibodies. The cells were preparatively sorted by flow cytometry to select Gr-1^{hi} CD11b⁺ (top) and Gr-1^{int} CD11b⁺ populations (bottom). The sorted cells were collected by cytopspin on glass slides, dried overnight, and stained with Wright-Giemsa stain. Pictures were taken under a microscope at $\times 20$. Arrows indicate monocytes and arrowheads indicate immature granulocytes with band morphology.

bearing and naive mice, respectively), although the difference was not significant. IL-4R α , a component of the IL-13 receptor, was also expressed on the Gr-1⁺ CD11b⁺ cells almost at the same level on naive and tumor-bearing mice-derived cells (15–20%). Expression of IL-4R α on the Gr-1⁺ CD11b⁺ cells suggested that the Gr-1⁺ CD11b⁺ cells express IL-13 receptor. This result is consistent with the result in Fig. 5 A that in vivo blockade of IL-13 reduced ex vivo TGF- β production by these cells.

We also looked at the morphology of Gr-1⁺ CD11b⁺ cells after purifying the cells by preparative sorting and staining by the Wright-Giemsa method (Fig. 8 B). Because there were two distinct cell populations among the Gr-1⁺ CD11b⁺ cells based on the fluorescent intensity of Gr-1 staining, which we referred to as Gr-1^{hi} CD11b⁺ and Gr-1^{int} CD11b⁺, we sorted each cell population and examined its morphology. Gr-1^{hi} CD11b⁺ cells from both naive and 15-12RM-injected mice were primarily mature and some immature neutrophils, and no difference was observed between the cells from naive and 15-12RM-injected mice (Fig. 8 B, top). Gr-1^{int} CD11b⁺ cells (Fig. 8 B, bottom) represented primarily immature myeloid cells, so-called “bands” (Fig. 8 B, arrowheads), with some immature monocytes (Fig. 8 B, arrows). The population from the 15-12RM-injected mice contained relatively fewer monocytes than the cells from the naive mice.

Because some studies reported that Gr-1⁺ CD11b⁺ myeloid cells induced by tumors suppress T cell responses with nitric oxide (NO), we tested a possible role of NO in vivo by treating mice with L-NAME (21), which is reported to inhibit inducible NO synthase in vivo. The mice were treated with 0.2 mg of either L-NAME or D-NAME as a

control every day for 2 wk after tumor inoculation. L-NAME treatment did not alter tumor growth in vivo (Fig. 9). Therefore, in 15-12RM-injected mice, NO is not necessary for negative regulation of CD8⁺ CTLs.

Taken together, our results show that in tumor-bearing mice, IL-13 and CD1d-restricted T cells upstream are necessary for TGF- β 1 production by CD11b⁺ Gr-1⁺ cells downstream, which negatively regulates CTL-mediated immunosurveillance of tumors.

Discussion

We have previously shown that IL-13 made by CD4⁺ CD1d-restricted T cells, most likely NKT cells, is necessary

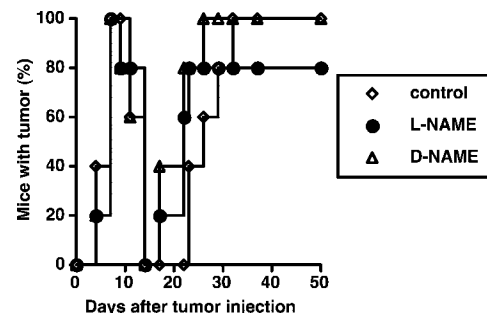


Figure 9. In vivo blockade of NO production did not alter tumor growth. BALB/c mice (five per group) were injected with 10^6 15-12RM cells s.c. Where indicated, mice were treated with 0.2 mg L-NAME (●) or D-NAME (Δ) every day for 2 wk. Control mice were inoculated with tumors without other treatment (◇). Similar results were obtained in an independent experiment.

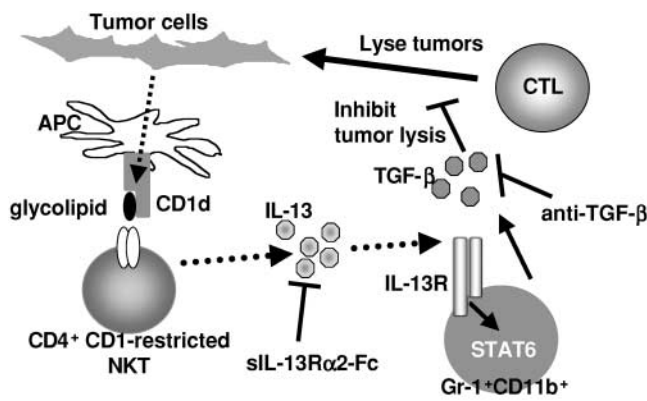


Figure 10. Proposed model of negative immunoregulatory circuit of CTL-mediated tumor immunosurveillance mediated by TGF- β , myeloid cells, IL-13, and CD1d-restricted T cells, probably NKT cells. Tumor antigen (glycolipid) presented by antigen-presenting cells via the CD1d molecule is recognized by and activates CD1d-restricted CD4⁺ NKT cells. The activated CD4⁺ NKT cell produces IL-13, which acts on Gr-1⁺ CD11b⁺ myeloid cells that express the IL-13 receptor. The Gr-1⁺ CD11b⁺ myeloid cell produces TGF- β to suppress CD8⁺ CTLs (CTL) that can kill tumor cells, thereby down-regulating tumor immunosurveillance. This pathway is able to be blocked by IL-13 inhibitor and anti-TGF- β antibody.

for down-regulation of CD8⁺ CTL-mediated tumor immunosurveillance through a IL-4R α -STAT6 signal pathway (4). We also recently reported that removing this negative regulatory pathway improves efficacy of antiviral vaccines (22). However, because IL-13 could not directly act on T cells, we attempted to find the missing link to define the downstream effector half of this novel immunoregulatory pathway. Here we have shown that TGF- β produced by CD11b⁺ Gr-1⁺ cells induced by IL-13 and CD1d-restricted T cells is necessary for down-regulation of CTL-mediated tumor immunosurveillance, completing this regulatory circuit (Fig. 10).

Blocking TGF- β by neutralizing antibody *in vivo* protected mice from tumor recurrence, but not from primary tumor growth. Because the primary tumor growth is necessary to elicit a CTL response, the absence of CTLs at early time points may explain the inability to suppress primary tumor growth.

Recent reports showed that blocking TGF- β by antibody or soluble receptor inhibited tumor growth, but the mechanism was unclear and not shown to be CTL mediated (9–13). In addition, the mechanisms of immunologic induction of TGF- β production and the source of this cytokine in tumor models have not been well studied, except for the fact that tumor cells are known to make this cytokine. This study defines the immunologic source and puts TGF- β in the context of a broader regulatory circuit. In our tumor model, although 15-12RM cells can make TGF- β (unpublished data), TGF- β produced by tumor cells cannot play a critical role in the negative regulation of tumor immunosurveillance because mice deficient for CD4⁺ T cells or CD1d-restricted T cells were resistant to tumor recurrence (3, 4), even though in both cases tumor

cells could still make TGF- β . Therefore, we hypothesized that a host cell induced by IL-13 might be responsible for relevant TGF- β production, as we have now confirmed. Analogously, in mice expressing the Tsk mutation (TSK/+ mice), which results in a scleroderma-like syndrome characterized by cutaneous hyperplasia, emphysema, cardiac hypertrophy, and autoantibodies specific for topoisomerase I, RNA polymerase I, and fibrillin-1, IL-4 acting through STAT6 induces TGF- β production by fibroblasts (23). It is likely that CTL priming occurs in the draining lymph nodes and/or spleen, where we have observed TGF- β production by myeloid cells, and that TGF- β production by the tumor itself is insufficient to affect this distant response.

CD11b⁺ Gr-1⁺ cells, which are myeloid cells or granulocytes (24), were the major source of TGF- β in tumor-bearing mice. Some CD11b⁺ Gr-1⁺ cells are also known as natural suppressor cells (25). Similar cells have been found under many different conditions: recombinant virus-immunized mice (26, 27), tumor-bearing mice (28–30), superantigen-induced T cell tolerance (31–33), chemotherapy with cyclophosphamide (34, 35), parasite infection (36–39), and vitamin A deficiency (40). Some myeloid cells have also been described to inhibit T cells in human cancer patients (41, 42). Consistent with our observation that some Gr-1⁺ CD11b⁺ cells express the IL-4R α chain, also necessary for signal transduction of IL-13, it has been reported that this cell is also induced *in vitro* by IL-4 (27). However, an immunologic mechanism by which myeloid cells are activated in tumor-bearing mice was unknown. Here we show the novel CD1d-restricted T cell/IL-13 regulatory circuit activates these cells to produce TGF- β . Although these myeloid cells have been described in other contexts above, and the relationship between IL-13 and TGF- β production has been seen in nontumor systems (see below), we believe that the tumor plays a role in initiating this pathway. We have previously shown that CD1d-restricted T cells from tumor-bearing mice express more IL-13 than CD1d-restricted T cells from control mice (4), and this study (Figs. 3 and 5) shows that TGF- β production by nonlymphoid cells is increased in tumor-bearing mice. Although the mechanism by which tumor growth induces these cytokines is unknown, we speculate that a tumor glycolipid is presented by CD1d on dendritic cells to activate CD1d-restricted T cells to make IL-13 to initiate the process. Indeed there have been several reports demonstrating that glycolipids from tumors were presented by CD1d molecules and able to stimulate NKT cells (43, 44). We believe the most likely interpretation of all the results together is that in tumor-inoculated mice, first, inoculated tumor cells induce IL-13 production by CD4⁺ CD1d-restricted T cells (4), and second, this IL-13 is necessary for CD11b⁺ Gr-1⁺ cells expressing IL-13 receptor to produce TGF- β that inhibits CTL induction. IL-13 may induce TGF- β production by myeloid cells but not increase their numbers, as suggested by the lack of increase in CD11b⁺ or Gr-1⁺ cells in the

spleens of tumor-bearing mice. It appears that IL-13 is necessary but not sufficient, as we have been unable to mimic the in vivo results by simply adding IL-13 to spleen cell cultures (unpublished data).

The mechanism of immunosuppression by CD11b⁺ Gr-1⁺ cells has been unclear, with different candidate molecules made by this cell population reported including TGF- β (27, 33), NO (29, 30, 34, 35, 38), prostaglandin (38), and peroxide (29, 41). Most of these mediators were studied in vitro. Thus, although the potential roles of the other immunosuppressive mediators are difficult to study in vivo, it is clear that in our tumor model, at least TGF- β is necessary but NO is not necessary for down-regulation of tumor immunosurveillance in vivo. Further, whereas TGF- β (14) and Gr-1⁺ myeloid cells (25) have each been found separately to play a role in inhibiting tumor immunosurveillance, as cited above, neither the immune source for the TGF- β nor the mechanism for activation of the myeloid suppressor cells was known. Here we show that Gr-1⁺ myeloid cells activated by IL-13 are the main immunologic source of differential TGF- β production in tumor-bearing and nontumor-bearing mice, but more importantly, that the two together represent the downstream effector half of a larger immunoregulatory pathway, putting each component in a larger context (Fig. 10).

Two findings in unrelated systems support our conclusions and indicate that this pathway is not confined to tumor immunity. In lung fibrosis (45), organ-specific overexpression of IL-13 transgene selectively up-regulated TGF- β production by macrophages that caused fibrosis in the lung. In helminth infection, treating mice with the IL-13 inhibitor blocked the development of hepatic and lung fibrosis (46) and there is some evidence suggesting TGF- β involvement (47). Thus, an IL-13-TGF- β axis may play a critical role not only in immune responses but also in other physiological processes.

In conclusion, we have now closed the loop in defining a novel immunoregulatory circuit, identifying the downstream effector mechanism in the proposed pathway (Fig. 10). Tumor growth induces CD1d-restricted CD4⁺ NKT cells to produce IL-13, which is necessary for CD11b⁺ Gr-1⁺ cells to produce TGF- β , and this TGF- β directly represses antitumor activity of CTLs. Neutralizing TGF- β or depleting immature myeloid cells may provide a unique opportunity for designing optimal immunotherapy for cancer. Alternatively, neutralizing both TGF- β and IL-13 may achieve synergy. Thus, identification here of this stepwise regulation of tumor immunosurveillance may provide insights for new approaches for cancer immunotherapy.

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