TGF-β Contributes to the Shift Toward Th2-Type Responses Through Direct and IL-10-Mediated Pathways in Tumor-Bearing Mice

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In previous reports, we showed that tumor-derived TGF-β induced overproduction of IL-10, and these suppressive cytokines caused macrophage suppression in EL4-bearing mice. In this study, we have focused on the correlation between changes in Th1/Th2 balance and overproduction of these cytokines in tumor-bearing mice. Proliferation of T cells from EL4-bearing mice (EL4-T) was suppressed in parallel with the tumor progression and was dependent on IL-4, but not IL-2, whereas T cells from normal mice were responsive to IL-2. A balance between Th1- and Th2-type cytokine production in EL4-T in response to anti-CD3 Ab or phorbor myristate acetate plus A23187 shifted toward the Th2 dominant pattern. The prevention of TGF-β and IL-10 activities in vivo by administration of anti-IL-10 Ab (anti-IL-10) or anti-TGF-β Ab (anti-TGF-β) resulted in the reduction in EL4-T of both IL-4 dependent proliferation and Th2-dominant cytokine production induced by anti-CD3 stimulation. In addition, the anti-TGF-β treatment resulted in complete restoration in EL4-T of suppressed IL-2 responsiveness, IL-2R expression, and Th1-type cytokine production, whereas the anti-IL-10 treatment produced partial recovery. These results lead us to conclude that TGF-β drives the shift in the Th1/Th2 balance toward Th2 via IL-10-mediated development of the Th2 responses and via inhibition of the Th1-type responses directly in EL4-bearing mice. *The Journal of Immunology,* **1996, 156: 73–78.**

Differential cytokine production by two distinct types of Th cells plays an important role during an immune response. Th1 cells secreting IL-2 and IFN-γ induce cellular immune responses, whereas Th2 cells producing IL-4, IL-5, IL-6, and IL-10 promote humoral immune responses (1, 2). Recently, the correlation has been shown between the progression of some diseases and the balance between Th1 and Th2 responses (3–5). Studies in mice infected with *Leishmania major* confirm the importance of Th1/Th2 balance and protection against the parasite (6, 7). In contrast to pathogenic infections, several animal models of human inflammatory autoimmune diseases are demonstrated to show predominantly the Th1 response (8–10). However, to date, there are few reports identifying the balance between Th1 and Th2 responses in the anti-tumor immune system.

Immunosuppression in tumor-bearing hosts is one of the major obstacles in cancer therapy. To improve tumor-induced immunosuppression, the first approach is to identify the factor(s) mediating this suppression. Recent reports showed that two cytokines, TGF-β and IL-10, were involved in tumor-induced immunosuppression (11–14). Increased production of these cytokines by the tumor itself was demonstrated from clinical studies as well as animal models. Indeed, immunosuppressive factors in the culture supernatants of MOPC-315 and Meth-KDE were shown to be identical with TGF-β and IL-10 using neutralizing Abs to these cytokines in vitro (15–17).

We have identified TGF-β as an immunosuppressive factor in vivo using EL4 (mouse thymoma)-bearing mice. In our studies, treatment of tumor-bearing mice with neutralizing Ab to TGF-β resulted in the recovery of macrophage functions. This recovery was especially manifested in the manner of production of antitumor substances such as TNF-α and nitric oxide (18). In our model, EL4 produced TGF-β both in vitro and in vivo. In addition to TGF-β, we detected enhanced production of IL-10 by macrophages in tumor bearers. Furthermore, anti-TGF-β treatment resulted in reductions in both TGF-β and IL-10 levels, and the anti-IL-10 treatment reduced only IL-10 levels in the tumor-bearing mice, indicating that tumor-produced TGF-β causes both overproduction of IL-10 and progression of immunosuppressive states in the tumor-bearing mice (19).

Immunosuppression in tumor-bearing hosts occurs not only in macrophages but also in T cells. The enhanced production of IL-10 in EL4-bearing mice prompted us to investigate functions of T cells in EL4-bearing mice (EL4-T[2]), especially in terms of the cytokine production and proliferative responses. In the present study, we ascertain that profiles of cytokine production by EL4-T were shifted to the Th2 type in tumor-bearing mice in comparison with the T cells from normal mice (normal-T[2]). In addition, we examined the correlation between this shift and enhanced production of TGF-β and IL-10 in tumor-bearing mice using specific Abs. The results of these experiments indicated that TGF-β contributed to drive the shift in the Th1/Th2 responses toward Th2 by favoring the development of Th2-type cells via IL-10 overproduction and the inhibition of Th1-type responses by down-regulation of cytokine production and IL-2R expression in EL4-bearing mice.

Materials and Methods

Mice

Inbred 6- to 10-wk-old female C57BL/6 mice were purchased from Charles River Japan (Tokyo, Japan) and were maintained under specific pathogen-free conditions.

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[2] Abbreviations used in this paper: EL4-T, purified T cells from EL4-bearing mice; normal-T, purified T cells from normal mice; PMA, phorbor myristate acetate.

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Tumor cells and preparation of tumor-bearing mice

EL4 tumor cells, the mouse thymoma cell line, were maintained by i.p. passage of 1×10^7 cells every 7 days in 8- to 10-wk old C57BL/6 mice. For preparation of EL4-bearing mice, EL4 cells were collected from the peritoneal cavity of i.p. inoculated mice and were s.c. inoculated (1×10^4) in C57BL/6 mice.

Antibodies, cytokines, and cytokine ELISA

Neutralizing anti-human TGF-β1 Ab (anti-TGF-β) and control chicken IgY were purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant murine IL-2 and IL-4, monoclonal rat anti-mouse IL-10 Ab (anti-IL-10), control rat IgG, and mAbs to mouse IL-2 (anti-IL-2) and IL-4 (anti-IL-4) were purchased from Genzyme (Cambridge, MA). Monoclonal anti-mouse CD3 Ab (anti-CD3) and FITC-conjugated Abs to mouse Thy1.2 and IL-2 receptor (anti-IL-2R-FITC) were purchased from PharMingen (San Diego, CA).

IL-2, IFN-γ, IL-4, IL-6, and IL-10 concentrations in samples were determined by specific ELISA kits according to the manufacturer's guidelines. All kits were purchased from Endogen (Boston, MA).

T cell purification from whole spleen cells

Before analysis of T cells from EL4-bearing mice, we separated T cells from whole spleen cells, avoiding contamination of EL4 cells that revealed high metastatic potential in vivo. Purified T cell populations were obtained by a Mouse T Cell Enrichment Column (R&D Systems). Briefly, total spleen cells suspended in PBS containing 5% fetal bovine serum (HyClone Laboratories, Logan, UT) were loaded onto a T Cell Enrichment Column to remove B cells via F(ab)-surface Ig interactions and to remove monocytes via Fc interactions. Enriched T cell populations were contained in the column elute (>85% Thy1.2$^+$). The eluted T cells were washed twice with serum-free RPMI 1640 medium, resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and used for proliferation and cytokine production assays and flow cytometry.

T cell proliferation assay

Purified normal-T and EL4-T (2×10^5/well) were plated onto 96-well flat-bottom plates and cultured with the indicated concentrations of anti-CD3 in the presence or the absence of rIL-2 (200 U/ml), rIL-4 (0.4, 2, or 10 ng/ml), or 10 μg/ml of anti-IL-2 or anti-IL-4 for 72 h at 37°C. Cells were then pulsed with 0.5 μCi [^{3}H]thymidine (Amersham Japan, Tokyo, Japan) during the final 4 h of culture, and the incorporation was measured by Beta Plate (Pharmacia, Hamburg, Germany).

Flow cytometry

Flow cytometric analysis was performed by Cyto ACE-150 (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Purified normal-T and EL4-T were cultured with anti-CD3 (10 μg/ml) for 24 h, and were stained with FITC-conjugated rat anti-mouse CD25 (IL-2R), fixed with 10% formaldehyde solution, and analyzed. Data from 10,000 events were collected.

Cytokine production assay

T cells (2×10^6/well) were plated onto 24-well flat-bottom plates and cultured in the presence or the absence of anti-CD3 (10 μg/ml) or phorbor myristate acetate (PMA[2] 10 nM; Sigma Chemical Co., St. Louis, MO) plus A23187 (1 μM; Sigma Chemical Co.) for 24 h at 37°C. Cellfree culture supernatants were collected, filtered, and measured for cytokine contents by specific ELISA kits.

Antibody administration experiments

EL4-bearing mice were injected i.p. with anti-IL-10 (250 μg/mouse·day) and anti-TGF-β (500 μg/mouse·day) for 4 consecutive days from day 4. No direct mitogenic or cytotoxic effects on normal macrophages of these Abs used in this study were observed in vitro (data not shown). Administration of the control Abs (rat IgG and chicken IgY) did not affect the T cell functions tested in this study (data not shown). One day after the final injection (on day 8), spleen T cells were purified on the columns described above and used for proliferation, cytokine production assays, and flow cytometry analysis. Serum samples were also obtained from each group. As control, a serum sample from normal mice was used. Serum samples were measured for their IL-4 contents by specific ELISA.

Statistical analysis

Statistical significance between any two groups was analyzed by Student's *t*-test.

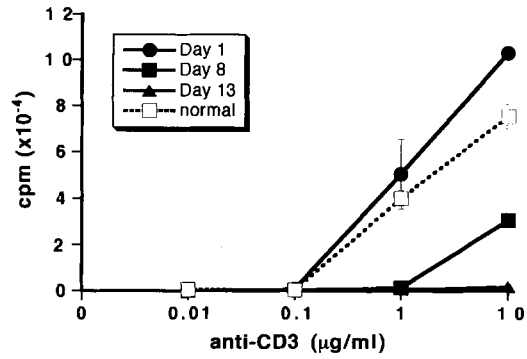

FIGURE 1. Changes in proliferative responses of EL4-T to anti-CD3 Ab stimulation. Purified normal-T or EL4-T (2×10^5) obtained from the tumor-bearing mice on the indicated days were cultured with various concentrations of anti-CD3 Ab for 72 h in vitro. [^{3}H]TdR was added during the final 4 h of culture. Results are shown as the mean and SD of triplicate cultures.

Results

Proliferation of EL4-T in response to anti-CD3 Ab stimulation in vitro

In the first experiments, normal-T and EL4-T obtained on days 1, 8, and 13 after tumor inoculation were stimulated with anti-CD3 to estimate their proliferative responses in vitro. As shown in Figure 1, proliferative responses of EL4-T to anti-CD3 stimulation were reduced below normal levels on day 8 and further reduced on day 13. These results indicate that EL4-T are suppressed in their proliferative activity in parallel with the tumor progression.

Th2 type cytokine production profiles in EL4-T

We also examined profiles of Th1- and Th2-type cytokine production by EL4-T obtained on days 1 and 8. EL4-T were stimulated with anti-CD3 for 24 h, and cytokine contents in the supernatant were measured and assessed as a percentage of normal-T cytokine production. As shown in Figure 2, cytokine production by EL4-T on day 1 was observed to undergo only a slight change; however, marked changes in cytokine production were observed on day 8. Both IFN-γ and IL-2 (Th1-type cytokines) productions by EL4-T were reduced to 60% of those by normal-T; on the other hand, IL-4 and IL-6 (Th2-type cytokines) productions were much enhanced to >200% of those by normal-T. IL-10 production was also enhanced, but not so dramatically as the enhancement of production of IL-4 and IL-6. To further confirm the above findings, EL4-T obtained on day 8 was also stimulated with PMA and A23187, a bypass of TCR-mediated signaling, for 24 h in vitro, and cytokine production profiles were analyzed. The stimulation with PMA plus A23187 also induced Th2-dominant cytokine production in EL4-T. Actual cytokine production values for the normal control are as follows: IFN-γ, 499.5 ± 22.1 pg/ml; IL-2, 530.1 ± 90.3 pg/ml; IL-4, 30.8 ± 5.2 pg/ml; IL-6, 192.9 ± 30.9 pg/ml; and IL-10, 1.9 ± 0.23 U/ml.

Effects of administration of anti-TGF-β or anti-IL-10 Abs on proliferative responses in EL4-T

To examine the growth factor dependencies of normal-T and EL4-T obtained on day 8, blocking mAbs specific for IL-2 or IL-4, or recombinant murine IL-2 and/or IL-4 were added to the culture during the proliferation assays. Figure 3*A* demonstrates that while anti-CD3-induced proliferative responses of normal-T were enhanced by the addition of rIL-2 (200 U/ml), they were little affected by the addition of rIL-4 (0.4, 2, or 10 ng/ml). Proliferation

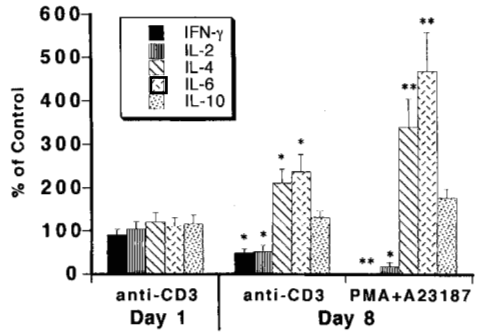

FIGURE 2. Changes in cytokine production by EL4-T cells obtained on days 1 and 8. EL4-T (2×10^6) obtained on days 1 and 8 after the tumor inoculation were stimulated with anti-CD3 (10 μg/ml) or PMA (10 nM) plus A23187 (1 μM) for 24 h at 37°C, and cytokine contents in the culture supernatant were measured by specific ELISA kits. Results are shown as a percentage of cytokine production by normal-T as controls (actual values for cytokine production for the normal control are as follows: IFN-γ, 499.5 ± 22.1 pg/ml; IL-2, 530.1 ± 90.3 pg/ml; IL-4, 30.8 ± 5.2 pg/ml; IL-6, 192. 9 ± 30.9 pg/ml; IL-10, 1.9 ± 0.23 U/ml). *, $p < 0.01$; **, $p < 0.001$ (compared with EL4-T obtained on day 1).

of normal-T was reduced to approximately 40% of that of control by the addition of 10 μg/ml of anti-IL-2. In contrast, Figure 3*B* indicates that EL4-T showed suppressed proliferative responses even in the presence of 10 μg/ml of anti-CD3, and the suppression was not restored by the addition of rIL-2 or anti-IL-2. However, the addition of rIL-4 enhanced the proliferation of EL4-T in response to anti-CD3 in a dose-dependent manner, and anti-IL-4 completely blocked the proliferative response.

To clarify the correlation between enhanced production of TGF-β and IL-10 and a dominant Th2-type response, the proliferative responses of EL4-T from mice treated with anti-TGF-β or anti-IL-10 were also investigated. EL4-bearing mice were administered with anti-TGF-β or anti-IL-10 i.p., and subsequently, EL4-T were obtained from each group on day 8, followed by analysis of anti-CD3-induced proliferation. Anti-IL-10 treatment revealed dramatic effects on the IL-4-dependent proliferation of EL4-T (Fig. 3*C*). IL-4 dependence of EL4-T proliferation completely disappeared; the addition of exogenous rIL-4 or anti-IL-4 had no effect on their proliferation. The inability to proliferate in response to rIL-2 of EL4-T was partially recovered. Furthermore, EL4-T from mice treated with anti-TGF-β showed proliferative responses similar to those from normal mice (Fig. 3*D*). The suppressed IL-2 responsiveness in EL4-T was restored to normal levels by the administration with anti-TGF-β. These results suggest that both TGF-β and IL-10 contribute to Th2-type proliferative responses of EL4-T in tumor-bearing mice.

Effects on cell surface IL-2R expression in EL4-T of administration of anti-TGF-β or anti-IL-10 Abs

To further analyze the unresponsiveness of EL4-T to IL-2, we investigated IL-2R expression in normal-T and EL4-T by flow cytometry. Normal-T and EL4-T obtained on day 8 were cultured in the presence or the absence of anti-CD3 for 24 h in vitro, followed by staining with anti-IL-2R-FITC. As shown in Figure 4, IL-2R expression was strongly induced by anti-CD3 treatment in normal-T (normal/untreated, 75.6% positive). However, significantly reduced expression of IL-2R was detected in EL4-T from the tumor-bearing controls even after anti-CD3 stimulation (EL4/untreated, 31.2% positive). In contrast to EL4-T from the tumor-bearing controls, IL-2R expression was induced by anti-

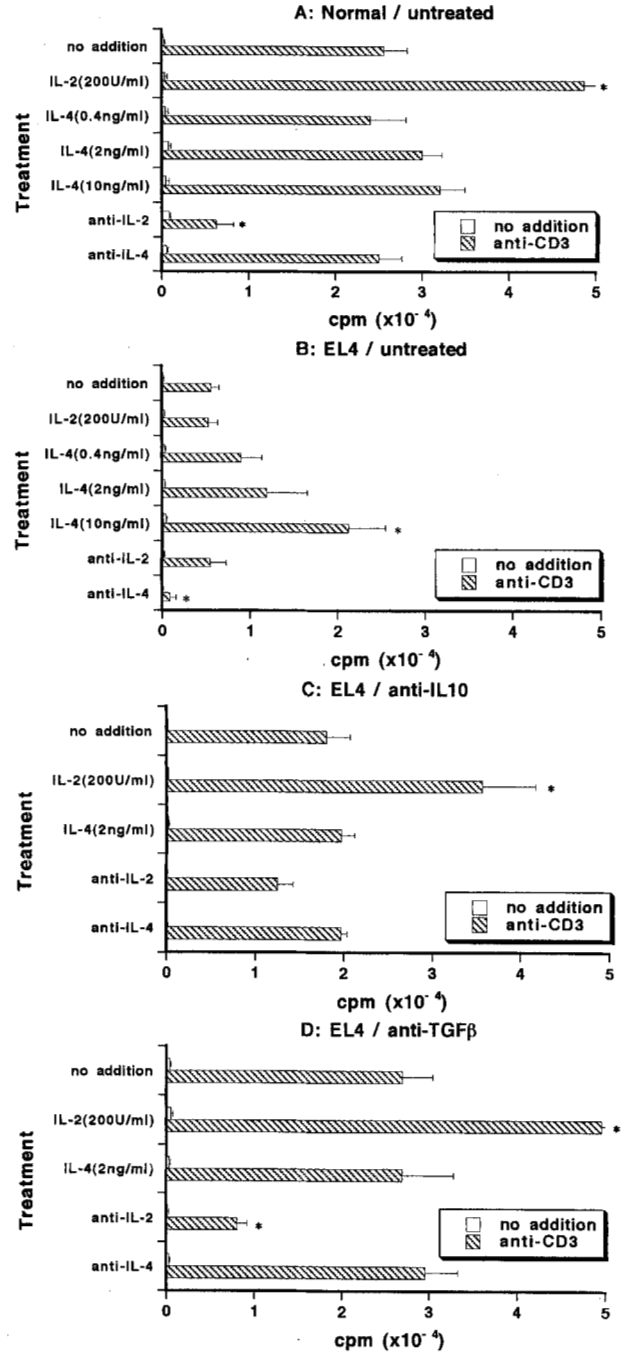

FIGURE 3. Proliferative responses of T cells from normal and EL4-bearing mice with or without treatment of anti-TGF-β or anti-IL-10 Abs. Anti-CD3-induced proliferation assay of normal-T (*A*) or EL4-T obtained from untreated (*B*), anti-IL-10-treated (*C*), or anti-TGF-β-treated (*D*) EL4-bearing mice were performed in the presence or the absence of rIL-2 (200 U/ml), rIL-4 (0.4, 2, or 10 ng/ml), or 10 μg/ml of anti-IL-2 or anti-IL-4 for 72 h in vitro. [^{3}H]TdR was added during the final 4 h of culture. Results are shown as the mean and SD of triplicate cultures. *, $p < 0.01$ compared with [^{3}H]TdR incorporation by the anti-CD3-stimulated control.

CD3 stimulation in EL4-T from anti-TGF-β-treated mice (EL4/anti-TGF-β, 70.0% positive), as observed in normal-T, whereas anti-IL-10 treatment had little effect on the restoration of IL-2R expression (EL4/anti-IL10, 37.7% positive). These results demonstrate that EL4-T fail to express IL-2R in response to anti-CD3 stimulation, mainly due to TGF-β.

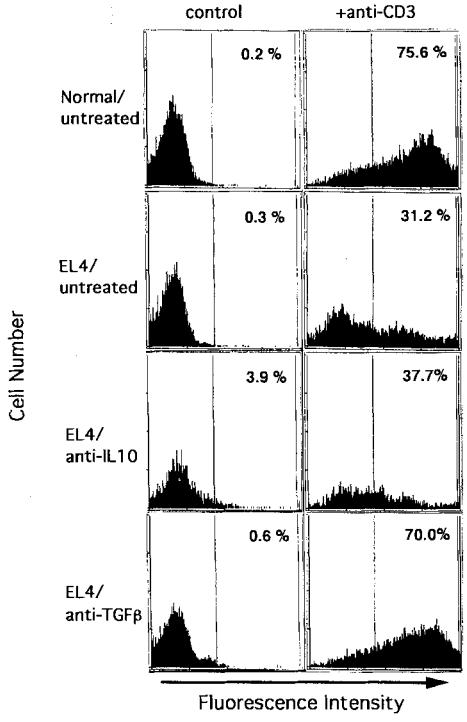

FIGURE 4. Effects of treatment with anti-TGF-β or anti-IL-10 Abs on anti-CD3-induced IL-2R expression by EL4-T. Normal-T (normal) and EL4-T obtained from EL4-bearing controls (untreated), EL4-bearing mice treated with anti-IL-10 (anti-IL-10), and the mice treated with anti-TGF-β (anti-TGF-β) were cultured in the absence (control) or the presence of 10 μg/ml of anti-CD3 (+anti-CD3) for 24 h, followed by immunostaining with FITC-conjugated rat anti-mouse CD25 (IL-2R) Ab. Analysis was based upon 10,000 cells collected.

Changes in cytokine production profiles of EL4-T by administration of anti-TGF-β or anti-IL-10 Abs

We next investigated the effects of anti-IL-10 and anti-TGF-β treatment on cytokine production profiles of EL4-T. As shown in Figure 5*A*, reduced production of Th1-type cytokines (IFN-γ, not detected; IL-2, 38.0 ± 10.0%) by EL4-T was significantly restored by treatment with anti-TGF-β (IFN-γ, 191.0 ± 21.9%; IL-2, 81.1 ± 17.5%), while anti-IL-10 treatment had little effect (IFN-γ, 32.2 ± 11.0%; IL-2, 52.4 ± 14.7%).

In the case of Th2-type cytokines, enhanced production of IL-4 and IL-6 was reduced by treatment with anti-TGF-β or anti-IL-10 (Fig. 5*B*). Enhanced production of IL-4 and IL-6 was detected in EL4-T (IL-4, 199.0 ± 21.4; IL-6, 178.0 ± 21.3). Anti-TGF-β treatment reduced Th2-type cytokine production significantly (IL-4, 62.7 ± 14.0%; IL-6, 50.1 ± 8.5%), except for IL-10. Anti-IL-10 treatment also had a reducing effect on Th2-type cytokine production of EL4-T (IL-4, 110.0 ± 28.6%; IL-6, 84.5 ± 9.8%), but was less effective than the anti-TGF-β treatment. EL4-T produced IL-10 at a slightly higher level (128.0 ± 22.6%) than normal-T (Fig. 5*B*), and treatment with anti-IL-10 reduced IL-10 production by EL4-T to below the normal level (62.6 ± 14.2%), while anti-TGF-β treatment had only a slight effect (97.4 ± 15.8%). These results suggest that both TGF-β and IL-10 favor Th2 development, and TGF-β mainly contributes to the prevention of Th1-type cytokine production in EL4-bearing mice.

Effects on in vivo IL-4 production of the administration of anti-TGF-β or anti-IL-10 Abs

Finally, to determine whether the production of Th2-type cytokine was enhanced in vivo, we also measured IL-4 levels in sera from

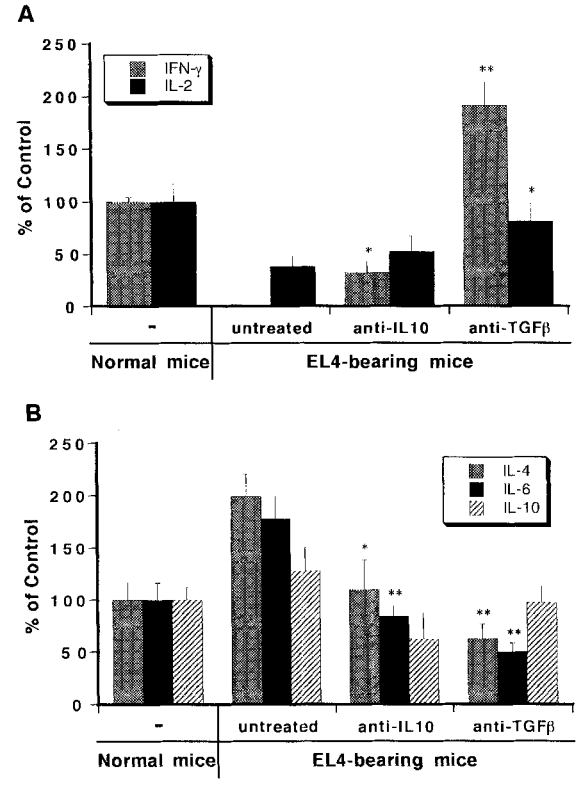

FIGURE 5. Effects of anti-IL-10 or anti-TGF-β treatment on cytokine production by EL4-T. Normal-T or EL4-T obtained from untreated, anti-IL-10-treated, or anti-TGF-β-treated EL4-bearing mice were cultured with anti-CD3 (10 μg/ml) for 24 h. The levels of Th1-type cytokines (*A*; IFN-γ and IL-2) and Th2-type cytokines (*B*; IL-4, -6, and -10) in the culture supernatants were measured by specific ELISA kits. Results are shown as a percentage of cytokine production by normal-T as controls. *, $p < 0.005$; **, $p < 0.001$ (compared with untreated EL4-bearing mice).

EL4-bearing mice on day 8. As shown in Figure 6, the IL-4 level increased approximately fourfold in the serum of EL4-bearing mice compared with that in normal mice (EL4-bearing, 954.0 ± 110.5 pg/ml; normal, 218.0 ± 29.5 pg/ml). However, the increased IL-4 levels in serum of EL4-bearing mice were significantly reduced to normal levels by treatment with either anti-IL-10 (278.5 ± 76.5 pg/ml) or anti-TGF-β (199.0 ± 46.0 pg/ml) Abs.

Discussion

Recently, a correlation between tumor-induced immunosuppression and the increased production of suppressor cytokines by tumor cells was reported by Gorelik et al. using MOPC-315 tumor (16). They demonstrated that MOPC-315 produced IL-10 and TGF-β in vitro and caused the suppression of CTL functions. Administration of the anti-cancer drug, melphalan, to MOPC-315-bearing mice reduced the production of both TGF-β and IL-10, and restored the suppressed CTL function by enhancing IFN-γ production. These results suggest that TGF-β and IL-10 evoked a Th2 pattern in tumor-bearing hosts in vivo, but clear evidence, especially in vivo, was not obtained.

Previously, we also found that immunosuppression in EL4-bearing mice was due to tumor-produced TGF-β (18), which induced enhanced IL-10 production in macrophages (19). In the present study, we indicate that Th2-type responses occurred predominantly in EL4-bearing mice from the following observations. 1) Production by EL4-T of Th2-type cytokines (IL-4 and IL-6) was enhanced, while that of Th1 type cytokines (IFN-γ and IL-2) was

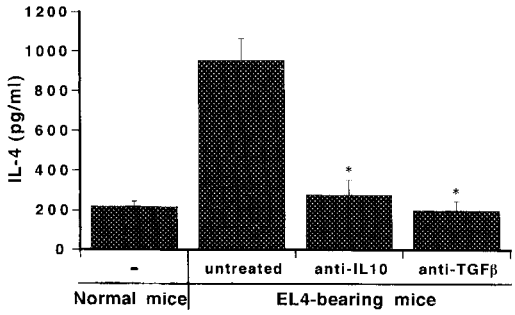

FIGURE 6. IL-4 levels in serum from EL4-bearing mice treated with Abs to IL-10 or TGF-β. IL-4 levels in serum of normal mice and the untreated, anti-IL-10-treated, or anti-TGF-β-treated EL4-bearing mice were measured by IL-4 specific ELISA kits. Results are shown as the mean and SD of four or five individuals from each group. *, $p < 0.005$ compared with untreated EL4-bearing mice.

suppressed in EL4-bearing mice. 2) Anti-CD3-induced proliferation of EL4-T was dependent on IL-4 rather than IL-2. 3) Serum IL-4 levels in vivo increased in EL4-bearing mice.

Mizoguchi et al. reported that T cells from tumor-bearing mice contained low amounts of CD3γ and completely lacked the ζ-chain (20). Therefore, to investigate whether the Th2-dominant cytokine production by EL4-T in response to anti-CD3 was also due to abnormalities in TCR complex expression, we analyzed cytokine production profiles in EL4-T stimulated by PMA plus A23187, a bypass of TCR complex-mediated signaling. Similar to the effects of anti-CD3, PMA plus A23187 stimulation induced EL4-T to produce a Th2-dominant cytokine, suggesting the Th2-dominant response in EL4-T may be not related to TCR structural abnormalities. Therefore, the intricate nature of the Th1/Th2 balance in EL4-bearing mice has provided new understanding of the potential roles of IL-10 and TGF-β as Th1/Th2 regulators.

To identify a role of IL-10 in the Th2-dominant responses in EL4-bearing mice, we administered anti-IL-10 to EL4-bearing mice. We have already proven that anti-IL-10 treatment caused the reduction of IL-10 to undetectable levels in vivo (19). In the anti-CD3-induced proliferation assay, EL4-T obtained from untreated mice showed suppressed proliferative responses compared with normal-T (Fig. 1). EL4-T showed IL-4-dependent proliferative responses (Fig. 3*B*). However, anti-IL-10 treatment restored IL-2-dependent proliferation and completely abolished IL-4-dependent proliferation of EL4-T (Fig. 3*C*). These results demonstrate that IL-10 affects the generation of Th2-type cells that proliferate in response to IL-4 in tumor-bearing mice.

Anti-IL-10 treatment also resulted in changes in the balance of Th1 and Th2 cytokine production profiles of EL4-T in response to anti-CD3. Th2-type cytokine production, especially that of IL-4 and IL-6, by EL4-T was significantly reduced, and IL-10 production was also lowered below that by normal-T by anti-IL-10 administration to EL4-bearing mice (Fig. 5*B*). However, marked changes in Th1-type cytokine production were not observed (Fig. 5*A*). These results of proliferation and cytokine production assays indicate that IL-10 favors dominant Th2-type responses by regulation of Th2-type cell generation rather than by prevention of Th1-type responses in EL4-bearing mice. This hypothesis was supported by the fact that no changes were observed in the reduced IL-2R expression by EL4-T after anti-IL-10 treatment (Fig. 4).

We also investigated whether TGF-β was relevant to driving the shift from Th1 to Th2 responses in EL4-bearing mice. In the proliferation assay, as shown in Figure 3*D*, anti-TGF-β treatment was effective at restoring the IL-2-dependent proliferation of EL4-T stimulated with anti-CD3. Th2-like, IL-4-dependent proliferative responses dramatically disappeared in EL4-T after anti-TGF-β treatment. Also, in the case of IL-2R expression, EL4-T failed to express IL-2R in response to anti-CD3 in vitro; however, EL4-T treated with anti-TGF-β expressed IL-2R at the same level as normal-T (Fig. 3). The inhibitory effects of TGF-β on IL-2R expression in vitro have been reported by several investigators (21, 22). We further confirmed these reports by the in vivo experiments (Fig. 6). Taken together, these results demonstrate that TGF-β mainly inhibits Th1-type proliferative responses, at least partly by down-regulation of IL-2R expression on T cells in EL4-bearing mice.

With regard to cytokine production profiles, TGF-β was a potent suppressive factor on Th1 development in EL4-bearing mice. In contrast to anti-IL-10, anti-TGF-β treatment was more effective in restoration of Th1-type cytokine production by EL4-T in response to anti-CD3 (Fig. 5*A*). Although EL4-T produced an undetectable level of IFN-γ in response to anti-CD3, EL4-T treated with anti-TGF-β produced twofold higher IFN-γ production than normal-T. The dominant Th2-type cytokine profiles of EL4-T were also reduced by anti-TGF-β treatment (Fig. 5*B*). These findings indicate that TGF-β can favor Th2-type responses as a consequence of a loss of Th1-like function of EL4-T in tumor-bearing mice.

From our findings reported in the present and previous studies, it is reasonable to deduce that TGF-β drives the shift from a Th1 to a Th2 response both directly and indirectly through an IL-10-mediated pathway. Anti-TGF-β treatment diminished the increase in IL-10 levels in ascites of EL4-bearing mice, but anti-IL-10 treatment did not affect TGF-β levels in the same model, indicating that tumor-produced TGF-β mediates overproduction of IL-10 in EL4-bearing mice (19). Moreover, as shown in the present study, anti-TGF-β treatment was more effective on the restoration of both proliferative responses and cytokine production profiles of EL4-T than anti-IL-10 treatment in the tumor-bearing mice. These findings suggest that the dominant Th2 responses may have occurred as a consequence of an incontinent increase in IL-10 induced by TGF-β. If TGF-β drives the shift toward Th2 solely via an IL-10-mediated pathway, anti-IL-10 treatment should result in the same effects on EL4-T functions as anti-TGF-β treatment. However, anti-IL-10 treatment was less effective in 1) recovery of proliferative responses, 2) restoration of IL-2R expression, and 3) favoring a dominant Th1 cytokine production in EL4-T (Figs. 3, 4, and 5*A*). These differences in efficacies between anti-TGF-β and anti-IL-10 treatment in vivo suggest that TGF-β drives the switch from Th1- to Th2-responses not only via an IL-10-dependent pathway(s) but also via a direct pathway(s) that is not dependent on IL-10 production. In the IL-10-independent pathway, TGF-β can inhibit Th1 functions directly in EL4-bearing mice. Therefore, anti-TGF-β treatment may be more effective in the restoration of Th1-type cytokine production by EL4-T (Fig. 5, *A* and *B*). Involvement of TGF-β in a shift from Th1 to Th2 was suggested in several studies (7, 23–27). Barral-Netto et al. demonstrated that *Leishmania*-infected mice showed predominantly Th2-type responses; mRNA expression of IL-4 was reduced and that of IFN-γ was enhanced by administration of anti-TGF-β Ab in the infected mice (7). Our results further confirm their conclusion in the tumor-bearing model and demonstrate the involvement of a new TGF-β-IL-10 pathway in the regulation of Th1/Th2 balance.

In conclusion, we speculate that in our model, regulation of the shift from Th1- to Th2-type responses occurred as follows. EL4 produces excessive amounts of TGF-β, which promotes the enhancement of IL-10 production from macrophages. TGF-β inhibits the development of Th1 responses by suppressing the production of IFN-γ from T cells, while TGF-β-induced IL-10 increases the

ratio of Th2 cells in parallel with tumor progression, which drives the shift from Th1- to Th2-type responses. Finally, the dominant Th2-type responses occur as a consequence of the progressive loss of Th1-type responses in EL4-bearing mice.

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