Suppression of Antitumor Immunity by IL-10 and TGF-β-Producing T Cells Infiltrating the Growing Tumor: Influence of Tumor Environment on the Induction of CD4$^+$ and CD8$^+$ Regulatory T Cells[1]

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We examined the hypothesis that a failure of the immune system to eradicate tumors is due to the immunosuppressive environment created by the growing tumor, which is influenced by the site of tumor growth. We demonstrated that T cell responses to a bystander Ag in mice were suppressed by a growing CT26 tumor. T cells purified from the growing tumor expressed mRNA for IL-10, TGF-β, and Foxp3. Intracellular cytokine staining revealed a high frequency of IL-10-secreting macrophages, dendritic cells, and CD4$^+$ and CD8$^+$ T cells infiltrating the tumor. In contrast, T cell IFN-γ production was weak and CD8$^+$ CTL responses were undetectable in mice with CT26 lung metastases and weak and transient following s.c. injection of CT26 cells, but were enhanced in the presence of anti-IL-10 and anti-TGF-β. Consistent with this, removal of CD8$^+$ T cells abrogated CTL responses and promoted progression of the s.c. tumor. However, in the lung model, depletion of CD8$^+$ T cells significantly reduced the tumor burden. Furthermore, depletion of CD4$^+$ or CD25$^+$ T cells in vivo reduced tumor burden in s.c. and lung models, and this was associated with significantly enhanced IFN-γ production by CD8$^+$ T cells. These findings suggest that tumor growth facilitates the induction or recruitment of CD4$^+$ regulatory T cells that secrete IL-10 and TGF-β and suppress effector CD8$^+$ T cell responses. However, CD8$^+$ T regulatory cells expressing IL-10 and TGF-β are also recruited or activated by the immunosuppressive environment of the lung, where they may suppress the induction of antitumor immunity. *The Journal of Immunology,* **2006, 177: 896–904.**

Tumor progression in normal immunocompetent individuals may reflect a failure of the immune system to recognize tumor Ags or may result from subversion of antitumor immune responses. Innate and adaptive immune response can be induced against tumors, and the protective effector cells include CD8$^+$ CTL, IFN-γ-producing CD4$^+$ and CD8$^+$ T cells, NK cells, and macrophages. Although T cells usually constitute a main immune cell population attracted to the tumor site, they are often ineffective at killing the tumor and recent evidence suggests that this may result from the function of regulatory T (Treg)[5] cells (1, 2).

Natural CD4$^+$CD25$^+$ Treg cells that express the transcriptional repressor Foxp3 and emerge as mature T cells from the thymus play a critical role in maintaining tolerance to self Ag and in preventing autoimmunity (3). Inducible Treg cells secreting IL-10 (termed type 1 Treg cells) or TGF-β (termed Th3 cells) are generated in the periphery in response to pathogen and self Ags (4). Natural and inducible Treg cells can be beneficial to the host during infection by preventing pathogen-induced immunopathology (5–7). However, the induction or activation of Treg cells by pathogens may be a strategy to subvert protective immunity, and depletion of CD4$^+$CD25$^+$ Treg cells enhances survival during certain infections (8). Depletion of CD4$^+$CD25$^+$ T cells can also enhance antitumor immunity (2, 9). However, the role of inducible Treg cells in antitumor immunity has received little attention.

Naive CD4$^+$ T cells can differentiate into effector Th1 or Th2 cells or Treg cells, and the selective induction of these distinct subtypes appears to be determined by a number of factors, including the maturation status of dendritic cells (DC) and regulatory cytokines secreted by cells of the innate immune system (4). The balance of effector vs Treg cells is also influenced by the tissue and environment where the immune response is generated. This may have a bearing on resolution or progression of tumor growth, especially for tumors in the respiratory or gastrointestinal mucosa, where the immune responses must be tightly regulated to maintain tolerance to inhaled and food Ags, respectively. The lung is an immunosuppressive environment, with high constitutive production of IL-10, which is necessary to protect the integrity of the delicate highly vascularized tissue, especially against excessive responses to allergens and pathology during infection (5, 10). DC in lungs secrete high levels of IL-10, and lymph nodes that drain the lung are less effective at activating naive T cells than DC from distal lymph nodes (10, 11). Therefore, the environment in the lung may not be conducive to the development of effective antitumor immune responses, and thereby facilitates tumor growth.

In this study, we have used the CT26 cell carcinoma, which can develop into a solid tumor when given s.c. or metastases in the

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[5] Abbreviations used in this paper: Treg, regulatory T; Cox-2, cyclooxygenase-2; DC, dendritic cell; IP-10, IFN-γ-inducible protein-10.

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lung when injected i.v., to examine the influence of the tumor environment on the relative development of effector and suppressor T cells during tumor growth. We found that CTL responses and IFN-γ-secreting T cells are suppressed during tumor growth, with a corresponding increase in IL-10-, TGF-β-, and Foxp3-expressing T cells. Depletion of $CD4^+$ or $CD4^+CD25^+$ T cells in vivo enhanced IFN-γ production by $CD8^+$ T cells and significantly reduced tumor growth and enhanced survival. In contrast, $CD8^+$ T cells had a protective role against the s.c. tumor, but surprisingly advanced the tumor growth in the lung. Our findings suggest the immunosuppressive environment of the lung is conducive to the development of $CD8^+$ as well as $CD4^+$ Treg cells, which suppress the development of effector T cell responses.

Materials and Methods

Mice

BALB/c mice were purchased from Harlan. DO.11.10 OVA TCR transgenic mice were bred in house. All mice were maintained under specific pathogen-free conditions and according to the regulations and guidelines of the Irish Department of Health.

Tumor models

The CT26 colon carcinoma-derived cell line was maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS. BALB/c mice were injected s.c. with 2×10^5 CT26 tumor cells, and tumor volume was monitored from day 7. Lung metastases were established by injecting BALB/c mice i.v. with 3×10^5 CT26 cells. To determine numbers of metastases, the lungs of sacrificed mice were stained by intertracheal administration of Indian ink, removed, added to Fekete's solution, and countered under a microscope.

Influence of tumor on proliferation and cytokine responses to an unrelated Ag

D0.11.10 mice were injected s.c. in the flank with 200 μg of OVA alone or with 2×10^5 CT26 cells. Mice were boosted 7 days later with 200 μg of OVA, and 14 days later lymph node cell suspensions were prepared and cultured at 2×10^6/ml in 96 microtiter round-bottom plates with OVA (50, 150, and 500 μg/ml) at 37°C in 5% CO_2. After 3 days, supernatants were removed and IFN-γ concentrations were determined by two-site ELISA. After 4 days of culture, 2 μCi of [^{3}H]thymidine (Amersham Biosciences) was added to each well. Plates were incubated for a further 6 h, after which cells were harvested (Tomtec Harvester 96 Mach III M) onto filtermats and cpm were determined using a beta counter (1450 Microbeta Trilux; Wallac).

T cell purification by magnetic cell sorting

Cell suspensions were prepared from lymph nodes, lungs, or surgically removed tumors. Tissue samples were washed with medium, finely chopped with a scalpel blade, and incubated with 0.1% solution of collagenase D (Sigma-Aldrich) in HBSS for 30 min at 37°C. Cell suspensions were then passed through a 70 μm cell strainer and RBC were lysed. Cells were washed, counted, and resuspended in 90 μl of MACS buffer with 10 μl of anti-CD4 or anti-CD8 magnetic beads (Miltenyi Biotec) and incubated at 4–8°C for 15 min. MACS buffer (2 ml) was added to each sample and then centrifuged at $300 \times g$ for 10 min. Each sample was then resuspended in 500 μl of MACS buffer and sorted using an AutoMacs (Miltenyi Biotec). The purities of $CD4^+$ and $CD8^+$ T cell fractions were 93–94% from lymph nodes and 82–85% from tumors.

Cytotoxicity assay

Spleens were removed from mice 7, 14, or 21 days posttumor challenge, and a single-cell suspension was prepared. Spleen cells (1.5×10^7 cells) were cultured with irradiated (100 Gy) CT26 tumor cells at a ratio of 50:1. After 5 days, effector cells were cultured with ^{51}Cr-labeled CT26 target cells (10^4/well) in triplicate in a 96-well round-bottom microtiter plate at E:T ratios of 100:1, 50:1, and 25:1 and incubated at 37°C. After 4 h, 50 μl of supernatant was removed and added to 150 μl of scintillation fluid; ^{51}Cr release was determined using a Microbeta counter and percentage of lysis was calculated as follows: (sample cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100.

Cell depletion in vivo

Mice were depleted of $CD4^+$ or $CD8^+$ T cells by i.p. injection of 1 mg of anti-CD4 (GK1.5) or anti-CD8 (YTS169), respectively, at days 0, 3, 7, and 12. $CD25^+$ T cells were depleted by i.p. injection of 1 mg of anti-CD25 Ab (PC61) on days 0, 3, 7, and 12.

RT-PCR

RNA was extracted from tumor cells or purified $CD4^+$ and $CD8^+$ T cells using TriReagent (Sigma Aldrich) and reverse transcribed using Superscript II RT (Invitrogen Life Technologies) and oligo($dT_{12–18}$) primers (Invitrogen Life Technologies). Primers specific for murine TGF-β (sense, AGACGGAATACAGGGCTTTCGATTCA; antisense, CTTGGGCTT GCGACCCACGTAGTA), IL-10 (sense, CTGGACAACATACTGCTA ACCGAC; antisense, TTCATTCATGGCCTTGTAGACACC), Foxp3 (sense, CAGCTGCCTACAGTGCCCCTA; antisense, CATTTGCCAG CAGTGGGTAG), cyclooxygenase-2 (Cox-2) (sense, GTATCAGAAC CGCATTGCCTCTGA; antisense, CGGCTTCCAGTATTGAGGA GAACAGAT), IFN-γ-inducible protein-10 (IP-10) (sense, CGCACCTCCACATAGCTTACAG; antisense, CCTATCCTGCCC ACGTGTTGAG), IL-23p19 (sense, TCTCGGAATCTCTGCATGC; antisense, CTGGAGGAGTTGGCTGAGTC), IL-15 (sense, CATATG GAATCCAACTGGATAGATGTAAGATA; antisense, CATATGCTC GAGGGACGTGTTGATGAACAT), and β-actin (sense, GGACTC CTATGTGGGTGACGAGG; antisense, TGCCAATAGTGAT GACTTGGC) were used with 2 μg of sample cDNA and amplified with *Taq* polymerase (Promega) using a Peltier Thermal Cycler.

Flow cytometric analysis and intracellular cytokine staining

Single-cell suspensions were prepared from lymph nodes, lungs, and tumors. Lungs and tumors were digested in HBSS with 0.1% collagenase D (Sigma-Aldrich). Cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 1 h, and then brefeldin A (10 μg/ml) was added for 4 h at 37°C. Cells were resuspended with Abs specific for CD4 (Caltag Laboratories), CD8 (BD Pharmingen), CD11c (BD Pharmigen), F4/80 (Caltag Laboratories), or isotype control Abs (BD Pharmingen). Cells were then fixed with 50 μl of fixation medium A (Fix & Perm cell permeabilization kit; Caltag Laboratories) and incubated with 50 μl of the permeabilization medium B (Fix & Perm cell permeabilization kit; Caltag Laboratories) and 5 μl of anti-IL-10 or anti-IFN-γ Abs (BD Pharmingen). Fc receptor binding of Abs was minimized by preincubation with rat anti-mouse CD16/CD32 (FcRγIII/II) mAb (Mouse BD FC Block from BD Pharmingen). Immunofluorescence analyzed was performed on a FACSCalibur (BD Biosciences), with CellQuest software.

Results

CT26 tumor growth inhibits T cell responses to unrelated Ags

To test the possibility that the failure to generate effective adaptive immune responses during tumor growth may result from an immunosuppressive environment created by the growing tumor, we examined the influence of a growing tumor on T cell responses to unrelated Ag. OVA-TCR transgenic mice were injected s.c. with OVA in the presence or absence of CT26 cells, and mice were boosted with OVA after 7 days and sacrificed 14 days later. Draining lymph nodes were removed, and OVA-specific T cell proliferation and IFN-γ production were tested. Mice challenged with CT26 cells and OVA had a significant reduction in OVA-specific T cell proliferation when compared with mice injected with OVA only (Fig. 1*A*). Coadministration of the CT26 cells also significantly reduced OVA-specific IFN-γ production in the lymph node (Fig. 1*B*), demonstrating that the growing CT26 tumor suppresses immune responses to other Ags particularly at the site of injection.

Immunosuppressive cytokines are differentially induced by CT26 tumor at different sites

To examine the possible mediators of immunosuppression during tumor growth, we examined the expression of cytokine mRNA in the cultured tumor cells and the growing tumor ex vivo. Cultured

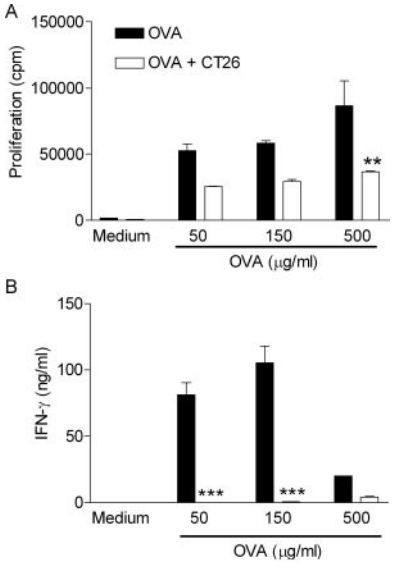

FIGURE 1. CT26 tumor cells inhibit T cell proliferation and IFN-γ production to a bystander Ag. DO.11.10 mice were injected s.c. with OVA (200 μg) alone or with 2×10^5 CT26 cells. Mice were boosted after 7 days with 200 μg of OVA, and 14 days later lymph node cells were restimulated with OVA, and proliferation (*A*) was examined after 4 days and IFN-γ (*B*) concentrations determined in supernatants removed after 3 days. Results are mean $\pm$ SD for five mice per group and assayed in triplicate. OVA vs OVA + CT26; **, $p < 0.01$; ***, $p < 0.001$ by ANOVA.

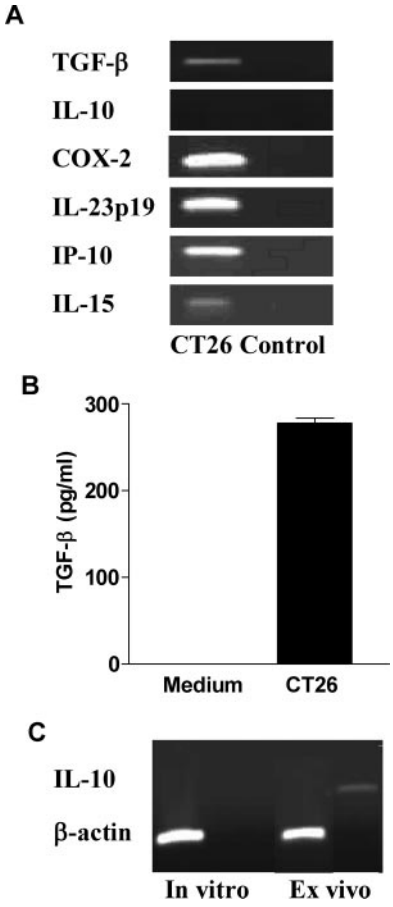

FIGURE 2. CT26 tumor cells secrete TGF-β in vitro. *A*, RNA was extracted from cultured CT26 cells and RT-PCR was performed using primers specific for IL-10, TGF-β, IL-23p19, IL-15, and IP-10. *B*, TGF-β protein in supernatants of cultured CT26 cells was quantified by ELISA. *C*, RNA was extracted from in vitro cultured CT26 cells or homogenized solid CT26 tumors excised from mice bearing s.c. CT26 tumors (RNA pooled from five mice), and RT-PCR was performed using primers specific for IL-10 and β-actin. Results are representative of three experiments.

CT26 tumor cells expressed mRNA for the anti-inflammatory cytokine, TGF-β (Fig. 2*A*). Furthermore, TGF-β protein was detected in supernatants of the growing tumor (Fig. 2*B*). Cox-2, IL-23p19, IL-15, and IP-10 mRNA were also expressed by CT26 cells (Fig. 2*A*), but we failed to detect expression of mRNA for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, IL-13, IL-18, IL-27p28, IL-27EB13, TNF-α, and IFN-γ (data not shown). Although IL-10 mRNA could not be detected in the in vitro cultivated tumor (Fig. 2*A*) even after stimulation of the cells with PMA (data not shown), it was detected in the tumor mass ex vivo (Fig. 2*C*), suggesting that cells infiltrating the tumor in vivo secreted IL-10. Therefore, we examined IL-10 and TGF-β mRNA expression by RT-PCR in $CD4^+$ and $CD8^+$ T cells purified from the lungs and cervical lymph nodes of mice with CT26 lung metastases and from solid tumor and inguinal lymph nodes of mice injected s.c. with CT26 cells.

In the lung model, IL-10 expression was considerably enhanced in both $CD4^+$ and $CD8^+$ T cells from the lungs of tumor-bearing mice compared with that found in T cells from the lungs of naive mice; however, constitutive expression of IL-10 mRNA was high in $CD4^+$ T cells from lungs compared with lymph nodes of naive mice (Fig. 3*A*). There was little change in IL-10 mRNA expression in the cervical lymph nodes of mice with lung metastases. In addition, TGF-β mRNA expression was dramatically higher in both $CD4^+$ and $CD8^+$ T cells purified from the lungs of mice with CT26 lung metastases when compared with naive control mice, with little change in the lymph nodes (Fig. 3*A*). These findings suggest that growth of CT26 tumor enhances the already immunosuppressive environment in the lung and promotes the infiltration of IL-10- and TGF-β-expressing $CD4^+$ and $CD8^+$ T cells to the tumor site.

In the s.c. model, IL-10 mRNA expression was weak or undetectable in $CD4^+$ and $CD8^+$ T cells purified from inguinal lymph nodes of normal and tumor-bearing mice, but was detectable at low levels in both $CD4^+$ and $CD8^+$ T cells purified from solid tumors from mice injected s.c. with CT26 (Fig. 3*B*). Although there was high constitutive expression of TGF-β mRNA in $CD4^+$ T cells and to a lesser extent $CD8^+$ T cells from the inguinal lymph nodes of naive mice, there was a small increase in TGF-β mRNA in $CD8^+$ T cells from the inguinal lymph nodes of mice s.c. tumors.

Foxp3 expression is enhanced in $CD4^+$ T cells within the CT26 tumor mass

Having demonstrated that T cells expressing IL-10 and TGF-β accumulate in the tumor during growth, we examined the possibility that natural Treg cells were recruited to the site of the tumor. The inguinal lymph nodes and solid tumors in the s.c. model and superficial cervical lymph nodes and lungs in the lung model were removed from BALB/c mice 3 and 14 days post-CT26 challenge, and cells were determined for expression of CD4 and CD25 using specific Abs and FACS analysis. Six to 8% of $CD4^+$ T cells from the inguinal and superficial cervical lymph nodes expressed CD25, and there was no significant change 3 or 14 days after tumor challenge (data not shown). In contrast, the frequency of $CD4^+CD25^+$ infiltrating the tumor increased dramatically during tumor growth (s.c. tumor, day 3, 1.4%, and day 14, 10.3%; lung tumor, day 3, 1.4%, and day 14, 2.9%).

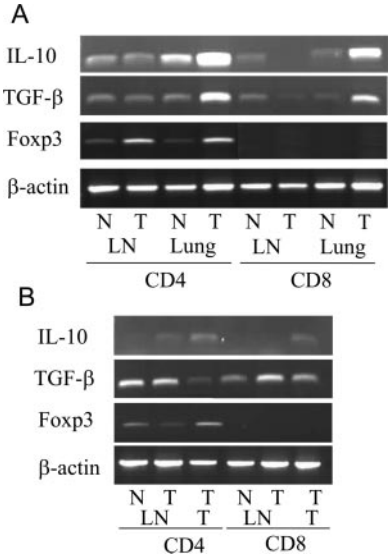

FIGURE 3. Enhanced IL-10, TGF-β, and Foxp3 mRNA expression in tumor-infiltrating T cells. BALB/c mice (five per group) were injected with CT26 cells either i.v. (*A*) or s.c. (*B*). Lymph nodes (LN) and s.c. tumor masses (T) or lungs were taken from naive (N) and tumor-bearing mice (T) 14 days after tumor challenge. $CD4^+$ and $CD8^+$ T cells were isolated using magnetic cell sorting, and RNA was isolated and RT-PCR was performed using primers specific for IL-10, TGF-β, and Foxp3.

Because CD25 is expressed on activated T cells as well as Treg cells, we used an additional marker for Treg cells based on expression of Foxp3. $CD4^+$ and $CD8^+$ T cells were purified from inguinal lymph nodes and solid tumors in the s.c. model and superficial lymph nodes and lungs in the lung model, and RT-PCR was performed using primers specific for Foxp3. In the lung model, Foxp3 expression was dramatically enhanced in $CD4^+$ T cell purified from lungs and the draining lymph nodes of tumor-bearing mice compared with the same tissues from naive mice (Fig. 3*A*). Foxp3 expression was also detected in $CD4^+$ T cells infiltrating the s.c. tumor mass (Fig. 3*B*). Foxp3 expression in tumor-infiltrating $CD4^+$ T cells was confirmed at the protein level by intracellular staining with an anti-Foxp3 Ab and FACS analysis (data not shown). Unlike the lung model, Foxp3 expression was not enhanced in the draining lymph node of mice with s.c. tumors. Foxp3 expression was not detected in $CD8^+$ T cells from any of the tissues from naive or tumor-bearing mice.

A high frequency of IL-10-secreting CD4 and CD8 T cells and low frequency of IFN-γ-secreting T cells infiltrate the growing tumor, especially in the lungs

Having demonstrated the presence of IL-10-secreting and $Foxp3^+$ T cells in the growing tumor, we used intracellular cytokine staining to examine the relative frequency of effector vs Treg cells recruited into the tumor mass and draining lymph nodes during tumor growth. Mice were challenged s.c. or i.v. with CT26 cells and tumors and lymph nodes were removed after 3 or 14 days; intracellular cytokine staining was performed for IL-10 and IFN-γ on T cells labeled for surface CD4 or CD8. A very high frequency (24–36%) of IL-10-secreting $CD4^+$ and $CD8^+$ T cells was detected in lungs of mice bearing CT26 metastases and in the tumor mass of mice injected s.c. with CT26 cells (Fig. 4*A*). In contrast, 6–27% of $CD4^+$ and $CD8^+$ T cells secreted IFN-γ (Fig. 4*A*). The frequency of IFN-γ-producing $CD8^+$ T cells in the inguinal lymph nodes increased 3 days after s.c. tumor challenge, but declined again after 14 days (Fig. 4*B*). Furthermore, the percentage of IFN-

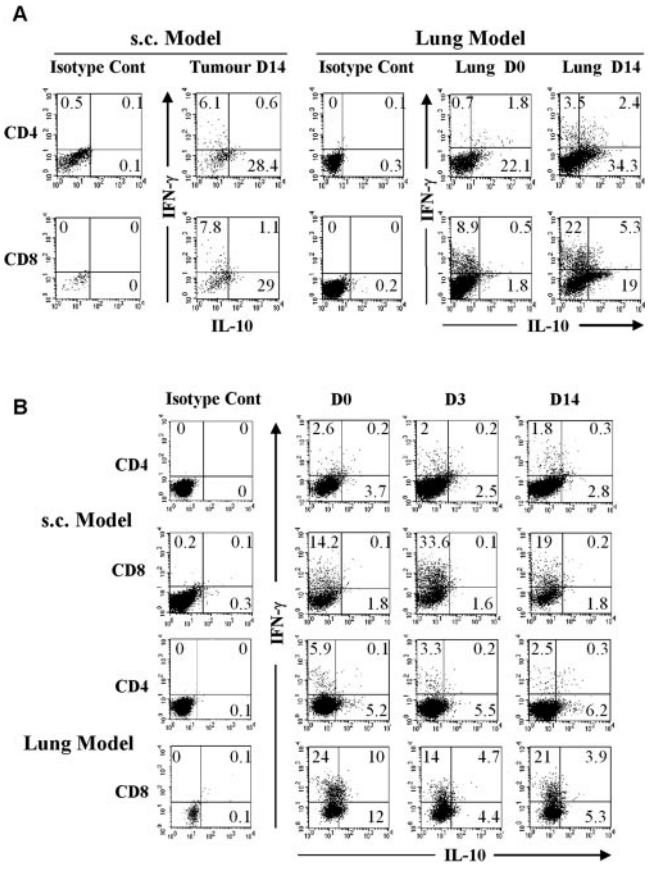

FIGURE 4. IFN-γ and IL-10 production by $CD4^+$ and $CD8^+$ T cells at the tumor site and lymph nodes following tumor challenge. BALB/c mice were injected either s.c. or i.v. with 2×10^5 or 3×10^5 CT26 colon carcinoma cells, respectively. Solid s.c. tumor or lungs (*A*) and inguinal and cervical lymph nodes (*B*) were excised at the days indicated. Cells were stimulated with PMA and ionomycin and labeled with Abs specific for surface CD4, CD8, and intracellular IL-10 and IFN-γ, and immunofluorescence analysis was performed. Staining with isotype control Abs was performed for each experimental group, and is only shown for cells from the naive control mice, but was similar for all groups. All quadrants are gated on isotype controls. Results are representative of four experiments.

γ-secreting $CD4^+$ and $CD8^+$ T cells in cervical lymph nodes declined as lung metastases developed, while IL-10 $CD4^+$-producing T cells increased (Fig. 4*B*). These findings are consistent with enhanced IL-10 and TGF-β mRNA expression in T cells infiltrating the tumor and suggest that IL-10- and TGF-β-producing Treg cells are recruited or activated in the growing tumor, and this may suppress the development of IFN-γ-secreting effector T cells. The findings also demonstrate a greater defect in IFN-γ-secreting T cells in lymph node draining the lung compared with s.c tumor.

IL-10-secreting macrophages and DC infiltrate the growing tumor

It has been reported that innate IL-10, secreted by macrophages and DC, plays a critical role in the differentiation of inducible Treg cells from naive T cells in the periphery (4). Having demonstrated recruitment of IL-10-secreting T cells to the lungs during development of CT26 metastases, we therefore examined the possibility that this may involve recruitment and activation of IL-10-producing macrophages or DC in the lungs. Mice were challenged i.v. with CT26 cells and lungs and cervical lymph nodes were removed after 3 or 14 days; intracellular cytokine staining was performed

for cells labeled for surface CD11c or F4/80. The development of CT26 tumors in the lungs was associated with significant recruitment of macrophages to the lungs; this was especially evident 14 days after tumor challenge (Fig. 5). Although our staining method, using anti-CD11c FITC, did not allow us to discriminate between myeloid and plasmacytoid DC or NK cells expressing CD11c, we did find evidence of infiltrating $CD11c^+$ cells in the lungs 14 days after tumor challenge. Furthermore, >40% of infiltrating macrophages and ~75% of infiltrating $CD11c^+$ cells secreted IL-10 on day 14 after tumor challenge. In contrast, the percentage of $CD11c^+$ and $F4/80^+$ cells was lower in the lymph node 3 and 14 days after tumor challenge when compared with naive control mice. Furthermore, a very low frequency of these cells secreted IL-10, and this did not change significantly after tumor challenge.

Defective CTL responses are restored by anti-IL-10 and TGF-β Abs

CTL play a key role in the antitumor immune response against the majority of tumors by directly killing tumor cells. As CT26 cells express MHC class I, rendering them susceptible to killing by CTL, the induction of functional CTL against CT26 was assessed during the course of tumor growth. Spleens were removed on days 7, 14, and 21 following tumor s.c. or i.v. challenge. CT26-specific CTL responses could not be detected in mice bearing CT26 lung metastases at any of the time points examined (data not shown). Following s.c. tumor challenge, CT26-specific CTL responses were undetectable 7 days post-s.c. tumor challenge, and although modest responses (22% lysis) were detected on day 14, this response was again undetectable on day 21 (Fig. 6*A*).

We have shown that the growing tumors can suppress proliferative responses and IFN-γ to bystander Ags, and that infiltrating T cells express IL-10 and TGF-β. Therefore, we examined the possibility that these anti-inflammatory cytokines may suppress CTL responses during tumor progression. Spleens from mice injected s.c. with CT26 were removed on day 14 and spleen cells were cultured with medium alone, irradiated CT26 cells (100 Gy) alone, or with anti-IL-10 and/or anti-TGF-β Abs. Neutralization of IL-10 in the cultures resulted in a 7% increase in CT26 lysis, whereas neutralization of TGF-β resulted in a 13% increase in CT26-specific killing (Fig. 6*B*). The combination

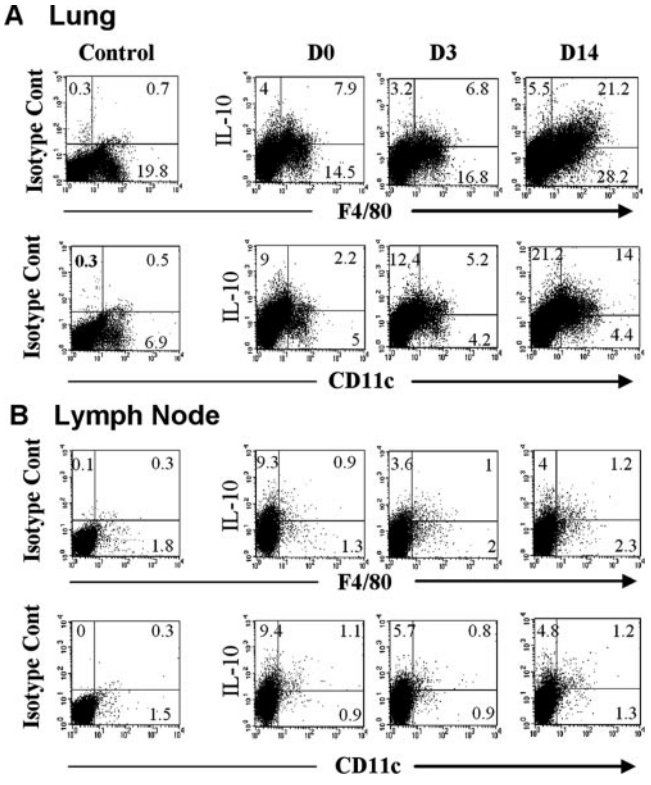

FIGURE 5. IL-10-secreting macrophages and DC infiltrate the growing tumor. BALB/c mice were injected i.v. with 3×10^5 CT26 colon carcinoma cells, and superficial cervical lymph nodes and lungs were removed from naive mice (day 0) or from mice 3 and 14 days posttumor challenge. Lung (*A*) or lymph node (*B*) cells were labeled with Abs specific for surface CD11c and F4/80 and for intracellular IL-10 or with isotype control Abs, and immunofluorescence analysis was performed. Staining with isotype control Abs is only shown for day 0 cells, but was similar at other time points.

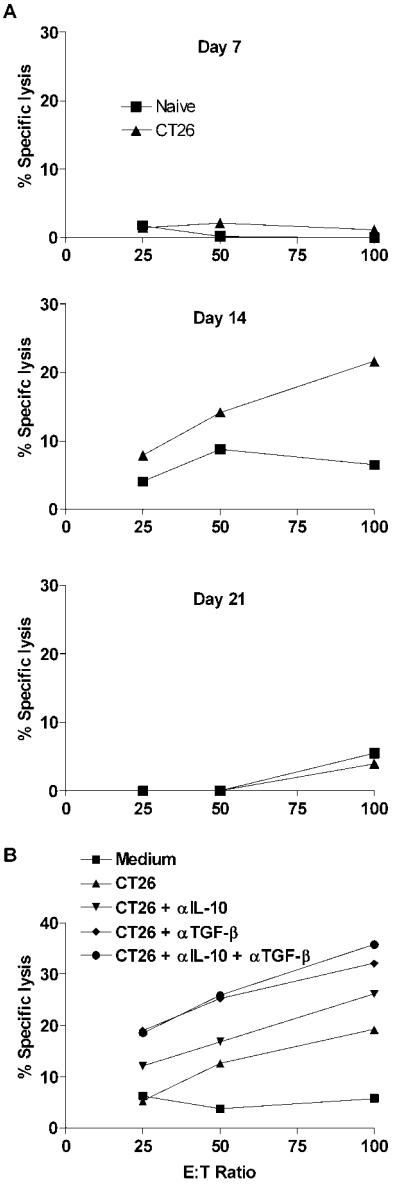

FIGURE 6. Tumor-specific $CD8^+$ CTL responses are inhibited by IL-10 and TGF-β. Mice were injected s.c. with 2×10^5 CT26 cells. *A*, Spleen cells, prepared 7, 14, and 21 days following tumor challenge or from naive control mice, were incubated with irradiated CT26 cells for 5 days, before testing for CTL activity against CT26 tumor targets using a ^{51}Cr release assay. *B*, Spleen cells removed from mice 14 days after s.c. tumor challenge were cultured with medium, irradiated CT26 cells (100 Gy) alone, or anti-IL-10 (6 μg/ml) or anti-TGF-β (1.6 μg/ml) alone or combined and tested for CTL activity. Results are mean values ± SEM for five mice at each time point and are representative of three experiments.

of anti-IL-10 and anti-TGF-β marginally improved CT26 killing to 36%, or 18% greater than that observed in the absence of added Abs, indicating that these anti-inflammatory cytokines produced by T cells in the growing CT26 tumor cells or present in the tumor environment can contribute to the inhibition of CTL activity.

Depletion of CD4$^+$ and CD8$^+$ T cells has differential effects on tumor growth and survival in s.c. and lung models

Our data suggest that a high frequency of IL-10-secreting CD4$^+$ and CD8$^+$ T cells is recruited into the growing tumor and that a lower frequency of infiltrating T cells secretes IFN-γ; we examined the role of CD4$^+$ and CD8$^+$ T cells in protection vs suppression of antitumor immunity by in vivo depletion with Ab before tumor challenge. In the s.c. model, depletion of CD4$^+$ T cells slowed CT26 tumor growth, although not significantly (Fig. 7*A*). The survival of CD4$^+$ T cell-depleted mice was also enhanced compared with that of nondepleted tumor-bearing control mice, with 50% remaining tumor free, whereas all nondepleted mice died as a result of tumor growth (Fig. 7*B*). In the lung model, removal of CD4$^+$ T cells resulted in a highly significant ($p < 0.001$) reduction in the number of CT26 lung metastases compared with nondepleted mice (Fig. 7*C*).

In contrast to the regulatory role of CD4$^+$, removal of CD8$^+$ T cells had distinct effects on growth of CT26 in the lungs and at an s.c. site. In the s.c. model, depletion of CD8$^+$ T cells resulted in a pronounced increase in tumor volume compared with nondepleted control mice (Fig. 7*D*). CD8$^+$ T cell-depleted mice all died within 21 days of tumor challenge, whereas nondepleted control mice died within 35 days (Fig. 7*E*). In contrast, in the lung model, depletion of CD8$^+$ T cells resulted in a highly significant reduction in the number of CT26 lung metastases compared with nondepleted mice (Fig. 7*F*). This was a consistent finding in three independent experiments involving a total of 40 mice, with mean tumor counts of 51.2 $\pm$ 8.8 vs 10.95 $\pm$ 9.6 for control and CD8 T cell-depleted mice, respectively ($p < 0.001$). These findings suggest that, unlike the s.c. model, both CD4$^+$ and CD8$^+$ T cells in the lungs can suppress protective immune responses against CT26 tumor.

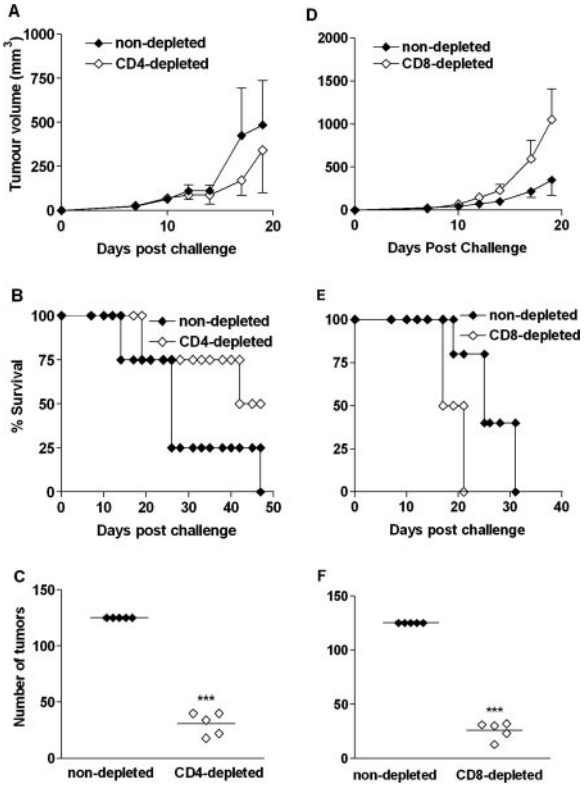

FIGURE 7. Depletion of CD4$^+$ or CD8$^+$ T cells has differential effects on development of s.c. and lung tumors. BALB/c mice were depleted of CD4$^+$ (*A–C*) or CD8$^+$ (*D–F*) T cells by injecting anti-CD4 Ab GK1.5 or anti-CD8 Ab YTS169 i.p. on days 0, 3, 7, and 12, and were challenged either s.c. or i.v. with 2×10^5 or 3×10^5 CT26, respectively. Tumor volume (*A* and *D*) and survival rates (*B* and *E*) were monitored following tumor challenge in the s.c. model. In mice challenged i.v. (*C* and *F*), tumor nodules on the large lung lobe were counted on day 14. All mice in the nondepleted group had greater than 125 tumor nodules on the large lung lobe. Results are representative of three experiments. ***, $p < 0.001$ by ANOVA compared with nondepleted mice.

Influence of CD4$^+$ or CD8$^+$ T cell depletion on cytokine production and CTL responses

To determine the functional effect of CD4$^+$ and CD8$^+$ T cell depletions on responses of the reciprocal T cell population, and how this may relate to enhancement of protection or exacerbation of antitumor immunity, the frequency of CD4$^+$- or CD8$^+$-secreting IL-10 and IFN-γ was examined by intracellular cytokine staining.

In the s.c. model, depletion of CD4$^+$ T cells resulted in a modest enhancement of the frequency of IFN-γ-secreting CD8$^+$ T cells infiltrating the inguinal lymph node during tumor growth (representative experiment shown in Fig. 8*A*; summary of three experiments: 5.1 $\pm$ 0.1 and 8.5 $\pm$ 0.2% IFN-γ-secreting CD8$^+$ T cells in undepleted vs CD4-depleted mice, respectively). In the lung model, depletion of CD4$^+$ T cells also enhanced the frequency of IFN-γ-producing CD8$^+$ T cells in the cervical lymph nodes of mice with lung metastases (Fig. 8*A*; summary data: 1.7 $\pm$ 0.1 and 4.4 $\pm$ 0.2% IFN-γ-secreting CD8$^+$ T cells in undepleted vs CD4-depleted mice, respectively). We found an even more dramatic effect in the lung tissue, where depletion of CD4$^+$ T cells substantially increased the percentage of IFN-γ-secreting CD8$^+$ T cells (Fig. 8*A*; summary data: 13 $\pm$ 2 and 38 $\pm$ 8% IFN-γ-secreting CD8$^+$ T cells in undepleted vs CD4-depleted mice, respectively). Furthermore, the increase in frequency of IL-10-secreting CD8$^+$ T cells in the lung during the development of CT26 lung metastases was completely reversed by depletion of CD4$^+$ T cells (Fig. 8*A*; summary data: naive, 18 $\pm$ 3%; undepleted, 26 $\pm$ 5%; CD4 depleted, 14 $\pm$ 2%). Therefore, removal of CD4$^+$ T cells has a positive impact on effector cytokine production during CT26 tumor growth in the lung or at an s.c. site, which reflected the reduced tumor growth and enhanced survival following both routes of tumor challenge, which is consistent with a regulatory role for CD4$^+$ T cells in tumor immunity.

We also examined immune responses during tumor progression in mice depleted of CD8$^+$ T cells. In the s.c. model, depletion of CD8$^+$ T cells abrogated CTL responses in tumor-bearing mice (data not shown). Furthermore, depletion of CD8$^+$ T cells resulted in a reduced frequency of IFN-γ-secreting CD4$^+$ T cells in inguinal lymph nodes of mice with s.c. tumors (Fig. 8*B*; summary data: 6 $\pm$ 1 and 2.5 $\pm$ 0.5% IFN-γ-secreting CD4$^+$ T cells in undepleted vs CD8-depleted mice, respectively). In contrast, in the lung model, depletion of CD8$^+$ T cells did not affect the numbers of IFN-γ-secreting CD4$^+$ T cells (Fig. 8*B*), but significantly enhanced the percentage of non-T cells secreting IFN-γ (data not shown). Furthermore, an examination of T cells from the lungs revealed that depletion of CD8$^+$ T cells enhanced IFN-γ-secreting CD4$^+$ T cells (Fig. 8*B*; summary data: 2.5 $\pm$ 0.5 and 6.5 $\pm$ 1% IFN-γ-secreting CD4$^+$ T cells in undepleted vs CD8-depleted mice, respectively) and reduced the frequency of IL-10-secreting CD4$^+$ T cells by almost 50% (Fig. 8*B*; summary data: 25 $\pm$ 4 and 13 $\pm$ 3% IL-10-secreting CD4$^+$ T cells in undepleted vs CD8-depleted mice,

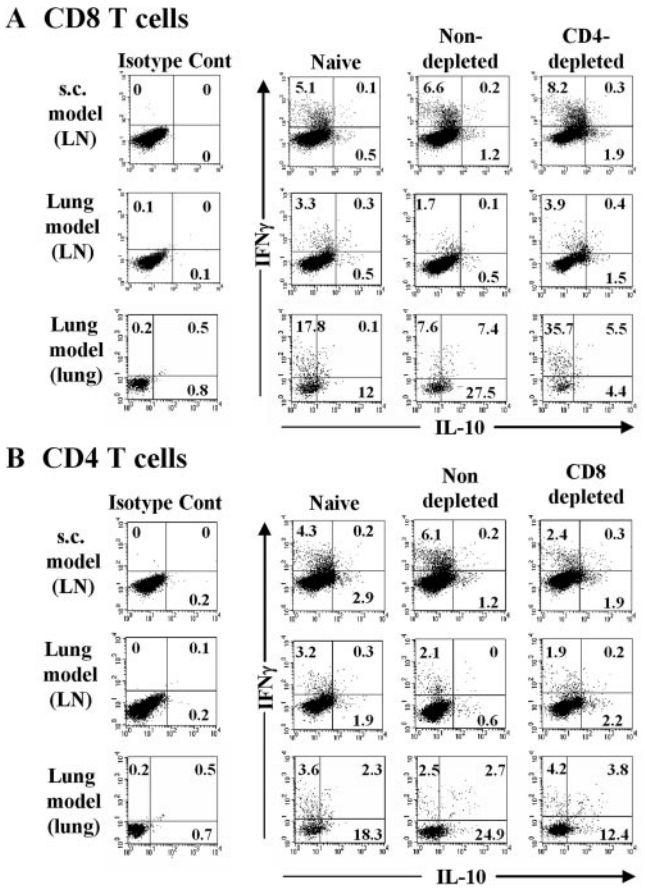

FIGURE 8. Depletion of $CD4^+$ or $CD8^+$ T cells enhances IFN-γ by the reciprocal subtype. BALB/c mice were depleted of $CD4^+$ (*A*) and $CD8^+$ (*B*) cells before s.c. or i.v. challenge with CT26 cells, as described in Fig. 7. Inguinal lymph nodes, cervical lymph nodes, or lungs were removed on day 14. Cells were labeled with Abs specific for surface CD4, CD8, and intracellular IL-10 and IFN-γ, and immunofluorescence analysis was performed. Staining with isotype control Abs was performed for each experimental group, and is only shown for cells from the naive control mice, but was similar for all groups. All quadrants are gated using isotype controls. Results are representative of three experiments.

respectively). Furthermore, $CD8^+$ T cells purified from the lungs of tumor-bearing mice exerted suppressor activity when cocultured with naive T cells in vitro (proliferative response of anti-CD3- and anti-CD28-stimulated naive T cells alone, 140,770 ± 1,867 vs 72,144 ± 6,795 for naive T cells alone or in presence of $CD8^+$ T cells; $p < 0.001$). These findings suggest that $CD4^+$ T cells can exert regulatory control on $CD8^+$ T cells during tumor growth, but also suggested that in addition to a protective role of effector $CD8^+$ T cells, evident in the s.c. model, $CD8^+$ Treg cells that secrete IL-10 are recruited or activated by immunosuppressive environment in the lung, where they can have suppressed antitumor immunity.

Depletion of $CD25^+$ cells reduced IL-10 and enhances IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the lungs and draining lymph nodes

Having demonstrated that depletion of $CD4^+$ T cells enhanced antitumor immunity and that Foxp3 expression was enhanced in T cells within the tumor, we examined the possibility that depletion of $CD4^+CD25^+$ T cells may have a more dramatic effect than depleting the whole $CD4^+$ T cell population. In the s.c. model, depletion of $CD25^+$ T cells resulted in a significant decrease in tumor volume (Fig. 9*A*) and a significant increase in survival of mice, with 75% remaining tumor free (Fig. 9*B*). In the lung model, depletion of $CD25^+$ T cells resulted in a significant reduction in the number of metastases compared with nondepeleted control mice (Fig. 7*C*). These findings suggest that removal of Treg cells allows the generation of effective antitumor immunity.

We examined the effect of $CD25^+$ T cell depletion on cytokine production by $CD4^+$ and $CD8^+$ T cells. In the lung model, IL-10 production from both $CD4^+$ and $CD8^+$ T cells in the lungs and cervical lymph nodes was significantly increased 14 days post-i.v. tumor challenge, but this was substantially reduced with the depletion of $CD25^+$ T cells (Fig. 10*A*). This suggests that $CD4^+$ $CD25^+$ T cells are the source of IL-10 or that they induce other T cells, such as $CD8^+$ T cells to produce IL-10 during tumor growth. Conversely, the frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the superficial cervical lymph nodes and in the lungs was dramatically increased following depletion of $CD4^+CD25^+$ T cells (Fig. 10*A*). In the s.c. model, depletion of $CD25^+$ T cells resulted in a decrease in the frequency of IL-10-secreting $CD4^+$ T cells and a modest increase in the percentage of $CD8^+$ T cells secreting IFN-γ (Fig. 10*B*). These findings demonstrate the potent effects of relatively small numbers of $CD4^+CD25^+$ cells in establishing the conditions conducive to tumor growth, especially in the immunosuppressive environment of the lung.

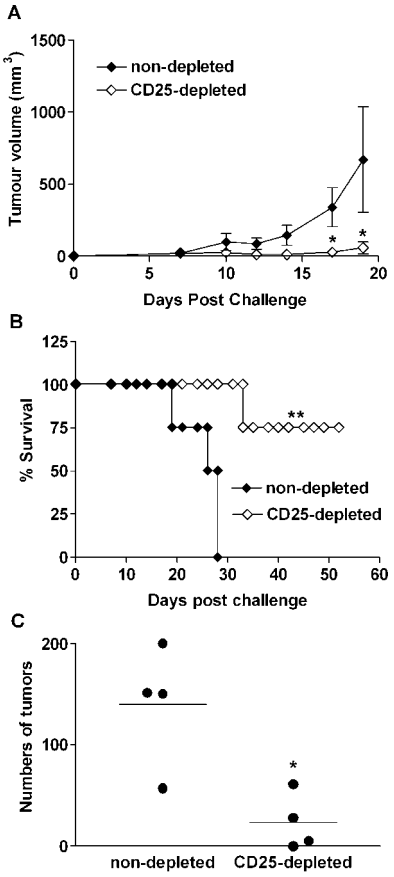

FIGURE 9. Depletion of $CD25^+$ T cells confers protection against s.c. and i.v. CT26 tumor challenge. BALB/c mice were depleted of $CD25^+$ T cells by injecting anti-CD25 Ab PC61 i.p. on days 0, 3, 7, and 12. Mice were injected either s.c. or i.v. with 2×10^5 or 3×10^5 CT26, respectively, on day 2. Tumor volume (*A*) and survival rates (*B*) were monitored following s.c. tumor challenge, and tumor nodules on the large lung lobe were counted on day 14 post-i.v. challenge (*C*). *, $p < 0.05$; **, $p < 0.01$ by ANOVA compared with nondepleted mice. Results are representative of three experiments.

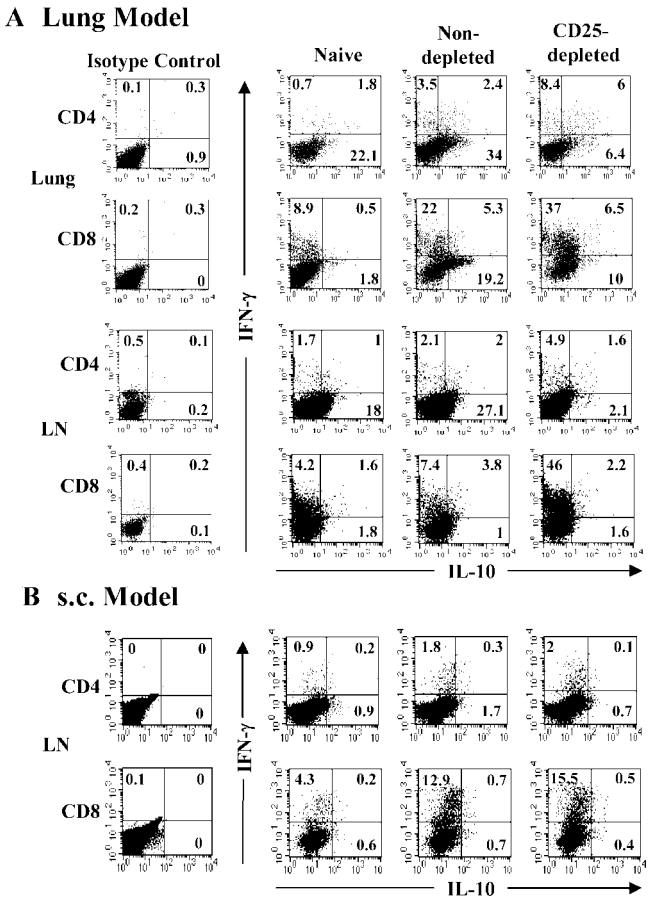

FIGURE 10. Enhanced IFN-γ and reduced IL-10 production by $CD4^+$ and $CD8^+$ T cells in mice depleted of $CD25^+$ T cells. BALB/c mice were depleted of $CD25^+$ T cells and injected i.v. (*A*) or s.c. (*B*) with CT26, as described in Fig. 9. Lungs and cervical lymph nodes (*A*) or inguinal lymph nodes (*B*) were removed from naive or nondepleted or CD25-depleted 14 days after CT26 challenge; cell suspension was labeled with Abs specific for surface CD4 and CD8 and intracellular IL-10 and IFN-γ; and immunofluorescence analysis was performed. Staining with isotype control Abs was performed for each experimental group, and is only shown for cells from the naive control mice, but was similar for all groups. All quadrants are gated using isotype controls. Results are representative of three experiments.

Discussion

The significant new findings of this study are that $CD4^+$ and $CD8^+$ T cells producing IL-10 and TGF-β can contribute to suppression of antitumor immunity and that this may be influenced by the site of the tumor growth. We observed enhanced expression of Foxp3, TGF-β, and IL-10 in $CD4^+$ T cells in growing CT26 tumors in mice, as well as a high frequency of infiltrating $CD8^+$ T cells that secreted IL-10 and a relatively low frequency of $CD4^+$ and $CD8^+$ T cells secreting IFN-γ. Depletion of $CD4^+$ or $CD25^+$ T cells enhanced antitumor immune responses and prolonged survival, whereas depletion of $CD8^+$ T cells enhanced growth of the s.c. tumor, but reduced the number of tumors in the lungs. This is the first description of a regulatory role for $CD8^+$ T cells in antitumor immune responses in the lungs.

IFN-γ has a protective role in antitumor immunity, exerting antiproliferative, antiangiogenic, and proapoptotic effects on a wide variety of tumor cells (12, 13). In this study, a low frequency of IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells infiltrated the tumor mass or was recruited to the draining lymph nodes. In the s.c. model, IFN-γ-secreting $CD8^+$ were detected in the tumor-draining inguinal lymph nodes after 3 days, but their frequency declined after 7–14 days. CTL activity was transiently detected in mice with s.c. tumor, but was undetectable in mice with lung metastases. The failure to generate type 1 responses to the tumor was also reflected in suppression of immune responses to an unrelated Ag. Injection of tumor cells into OVA TCR transgenic mice inhibited T cell proliferation and IFN-γ production by draining inguinal lymph node cells in response to immunization with OVA. This suggests that the growing CT26 tumor created an immunosuppressive environment that suppresses T cell responses to both tumor and bystander Ags.

In an attempt to explain the immunosuppressive effects of the tumor, we found that CT26 cells expressed Cox-2 and TGF-β mRNA and also secreted TGF-β protein in vitro. Tumor-derived Cox-2 induces expression of Foxp3 and enhances the activity of $CD4^+CD25^+$ Treg cells (14) while TGF-β has potent anti-inflammatory properties and has been implicated in the induction and function of natural and inducible Treg cells (15, 16). An examination of T cells purified from the tumor mass revealed high expression of TGF-β and IL-10 mRNA, and intracellular cytokine staining revealed a very high frequency of IL-10-producing T cells, but also macrophages and DC. Innate IL-10 and TGF-β production, together with a semimature DC phenotype, are associated with the induction of IL-10-producing Treg cells in response to infection (17, 18) and to tumors (19). Therefore, TGF-β and other factors secreted by the growing tumor activate DC to secrete IL-10 and undergo partial maturation, resulting in selective induction of Treg cells. The direct immunosuppressive effects of anti-inflammatory cytokines secreted by macrophages and DC or their ability to drive the induction of IL-10 and TGF-β Treg cells may explain the defective IFN-γ-secreting $CD4^+$ or $CD8^+$ T cells and CTL responses and suppression of T cell responses to unrelated Ags. This is consistent with the demonstration that in vitro neutralization of anti-TGF-β and IL-10 enhanced CT26-specific CTL responses. The suppressive function of $CD4^+$ T cells was also demonstrated in vivo, as $CD4^+$ T cell depletion augmented IFN-γ production by $CD8^+$ T cells and slowed the rate of tumor progression in the lungs and at an s.c. site.

There is growing evidence that natural $CD4^+CD25^+$ T cells may play a critical role in progression of a number of cancers (2, 9, 20). There is an increase in the number of $CD4^+CD25^+$ Treg cells in the peripheral blood of cancer patients, and this is believed to play a major part in the failure of many immunotherapies against tumors (21). In patients with non-small cell lung cancer, natural Treg cells from the tumors have been shown to inhibit proliferation of autologous, but not allogeneic T cells (1). In the present, we found a small increase in the number of $CD4^+CD25^+$ T cells, but a substantial increase in Foxp3 expression in $CD4^+$ T cells at the tumor site in s.c. and lung tumor models. Foxp3 mRNA expression was also enhanced in $CD4^+$ T cells from the draining lymph nodes of mice with CT26 lung metastases. In human ovarian cancer, $CD4^+CD25^+Foxp3^+$ natural Treg cells are attracted by the chemokine CCL22, produced by tumor cells and macrophages (22), suggesting that Treg cells may be attracted by the local tumor microenvironment and once there, exert immunosuppressive effects on immune effector cells. We found that depletion of $CD25^+$ T cells in the s.c. and the lung metastasis models resulted in significant decreases in tumor growth and a significant increase in survival and a dramatic enhancement of IFN-γ production from both $CD4^+$ and $CD8^+$ T cells. In contrast, depletion of $CD25^+$ T cells almost completely eliminated IL-10 production by T cells in the lung and draining lymph nodes, suggesting that $CD25^+$ T cells may be the predominant source of $CD4^+$ T cell-derived IL-10 in tumor-bearing mice. However, we also observed

an enhanced frequency of IL-10-producing $CD8^+$ T cells in mice with CT26 tumors, which was significantly reduced following depletion of $CD4^+CD25^+$ T cells. Because removal of $CD25^+$ T cells unmasks effective antitumor immune responses, these findings suggest that recruitment of $CD4^+CD25^+Foxp3^+$ T cells to the site of the tumor may help to skew tumor-specific T cells responses from an effector to a regulatory subtype and thereby subvert protective antitumor immunity.

In addition to the role of $CD4^+$ Treg cells, we also found evidence for the induction and suppressive function of IL-10- and TGF-β-expressing $CD8^+$ Treg cells during development of tumors in the lung. The traditional view on the role of $CD8^+$ T cells in antitumor immunity has been one of protection, through CTL activity and secretion of IFN-γ. Indeed, in the s.c. tumor model, we found that removal of $CD8^+$ cells led to a substantial increase in CT26 tumor growth and a decrease in survival of the mice. Furthermore, CT26 tumor-specific $CD8^+$ CTL responses were transiently generated in nondepleted mice during tumor growth and enhanced by neutralizing IL-10 and TGF-β. However, in the lung model, $CD8^+$ CTL responses were undetectable, whereas there was a high frequency of IL-10-secreting $CD8^+$ T cell in the lungs. Depletion of $CD8^+$ T cells significantly reduced the number of lung metastases. Furthermore, $CD8^+$ T cells purified from the lungs of mice with lung metastases suppressed proliferation of naive T cells in vitro. Collectively, our findings suggest that IL-10-secreting $CD8^+$ Treg cells are induced during tumor growth and that these cells subvert antitumor immunity in the lung.

Our demonstration of a regulatory role for $CD8^+$ T cells in the lung is also consistent with studies on non-small cell lung cancer, which demonstrated low concentrations of IFN-γ and elevated IL-10 and TGF-β at the tumor site (23) and high numbers of tumor-infiltrating $CD8^+$ T cells, which did not correlate to patient survival (24). $CD8^+$ Treg cells have also been reported in the lungs in an airways hyperreactivity model (25), and during mycoplasma infection (26). Furthermore, suppressive IL-10-producing $CD8^+$ T cells have been found in the tumor environment of patients with ovarian cancer; their induction was independent of $CD4^+CD25^+$ T cell, but dependent on plasmacytoid DC (27). Our data support the suggestion that Treg cells in the lung may be induced under the influence of IL-10-secreting DC and macrophages, found at high numbers in the lungs during tumor growth. This is also consistent with reports that lung DC in mice exposed to respiratory Ags transiently secrete IL-10 and promote the induction of IL-10-secreting Treg cells (10). In contrast to the lung, peripheral sites are more responsive to inflammatory insults and can therefore allow optimal priming and activation of cells of the innate and adaptive immune systems. Indeed, one study reported that 10- to 100-fold more tumor-specific T cells are required to protect against an i.v. tumor challenge, compared with an equivalent s.c. tumor challenge (11). Taken together with the findings of our study, this suggests that the environment in the lung is not conducive to effective antitumor immune responses, and this may be explained in part by selective induction of $CD4^+$ and $CD8^+$ Treg cells during tumor development.

Disclosures

K. H. G. Mills is a cofounder, director and shareholder in Opsona Therapeutics Limited, a university campus company involved in the development of anti-inflammatory therapeutics.

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