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Regulatory T Cells Prevent CD8 T Cell Maturation by Inhibiting CD4 Th Cells at Tumor Sites¹

Nathalie Chaput,^{2*} Guillaume Darrasse-Jèze,^{2*} Anne-Sophie Bergot,^{*} Corinne Cordier,[†] Stacie Ngo-Abdalla,^{*} David Klatzmann,^{*} and Orly Azogui^{3*}

Natural regulatory T cells (Tregs) are present in high frequencies among tumor-infiltrating lymphocytes and in draining lymph nodes, supposedly facilitating tumor development. To investigate their role in controlling local immune responses, we analyzed intratumoral T cell accumulation and function in the presence or absence of Tregs. Tumors that grew in normal BALB/c mice injected with the 4T1 tumor cell line were highly infiltrated by Tregs, CD4 and CD8 cells, all having unique characteristics. Most infiltrating Tregs expressed low levels of CD25Rs and Foxp3. They did not proliferate even in the presence of IL-2 but maintained a strong suppressor activity. CD4 T cells were profoundly anergic and CD8 T cell proliferation and cytotoxicity were severely impaired. Depletion of Tregs modified the characteristics of tumor infiltrates. Tumors were initially invaded by activated CD4⁺CD25⁻ T cells, which produced IL-2 and IFN- γ . This was followed by the recruitment of highly cytotoxic CD8⁺ T cells at tumor sites leading to tumor rejection. The beneficial effect of Treg depletion in tumor regression was abrogated when CD4 helper cells were also depleted. These findings indicate that the massive infiltration of tumors by Tregs prevents the development of a successful helper response. The Tregs in our model prevent Th cell activation and subsequent development of efficient CD8 T cell activity required for the control of tumor growth. *The Journal of Immunology*, 2007, 179: 4969–4978.

Most tumors are not efficiently rejected despite their recognition by CD4 and CD8 T cells (1, 2). The mechanisms that inhibit tumor rejection include those mediated by the tumor itself, such as production of tumor-derived TGF- β or soluble class I-related MHC molecules (3, 4). The tumor stroma may also prevent efficient T cell priming and expansion in both draining lymph nodes (DLNs)⁴ and at the tumor site (5). The absence of immune-mediated tumor rejection also includes inadequate host T cell responses that may be due to T cell anergy, T cell deletion (6, 7), and inhibition of T cell responses by suppressive cytokines such as IL-10. More recent studies revealed a potential major role of natural CD4⁺CD25⁺ regulatory T cells (Tregs) in controlling the immune responses to tumors.

Tregs, which express high levels of CD25Rs, have been shown to be a powerful inhibitor of T cell responses in several pathologies

such as organ-specific autoimmunity or chronic infection, and in tolerance to transplantation (8–11). Beside CD25 expression, Tregs constitutively express at high levels CTLA-4 and glucocorticoid-induced TNFRs (GITRs) (12), certain TLRs (13), and the $\alpha_E\beta_7$ integrin CD103 (14). They specifically express the transcription factor FoxP3 which is a master control gene for their development and suppressive function (15). In addition to naturally occurring Tregs, IL-10-induced T regulatory 1 (Tr1)-type cells have also been implicated in suppressor functions (16).

Tregs were reported to massively infiltrate tumors, suggesting that they could have some role in the control of local tumor immune responses (17, 18). This notion could explain anti-CD25 treatment inducing tumor rejection in animal models (19). It is, however, unclear where Tregs induce tolerance to tumor cell growth. In mice transferred with transgenic T cells specific for an Ag expressed by the tumor, it was suggested that Tregs act in the tumor DLNs by reducing the frequency of specific responding CD8 T cells (20). However, injections of anti-CD4 or -GITR Abs directly in tumor masses led to tumor regression, suggesting that Tregs may have a major local role, exerting their suppressive function at tumor sites (21, 22). The mechanisms by which regulatory cells mediate their suppressive function remain unclear. It has recently been reported that Treg cells have a direct effect on CD8 T cell function by suppressing the release of cytotoxic granules (23). However, Tregs have been shown to display a strong suppressive effect on CD4 responses suggesting that they could act at multiple levels depending on differences in experimental models.

To get further insight into the mechanisms involved in the control of antitumor immunity in the tumor site, we analyzed the effect of Tregs on the infiltration and activation of CD4 and CD8 T cells in a nontransgenic mouse model of cancer. We show that Tregs decrease antitumor CD8 cytotoxic response by limiting the early infiltration of CD4⁺ Th cells and thus prevent the generation of efficient cytotoxic CD8 T cells.

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⁴ Abbreviations used in this paper: DLN, draining lymph node; Treg, regulatory T cell; GTR, glucocorticoid-induced TNFR; TIL, tumor-infiltrating lymphocyte; NLN, normal lymph node; Tr1, T regulatory 1.

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Materials and Methods

Mice and 4T1 tumor samples

Female BALB/c mice were purchased from Charles River Laboratories and were between 6 and 8 wk of age. All mice were manipulated according to European Union guidelines for experimentation. The 4T1 tumor cell line derived from a BALB/c spontaneous mammary carcinoma was supplied by Dr. S. Ostrand-Rosenberg (Department of Biological Science, University of Baltimore, Baltimore, MD). 4T1 cells were cultured in IMDM (Invitrogen Life Technologies) supplemented with 10% FCS and 1% gentamicin. A total of 10^4 – 10^5 4T1 cells were injected s.c. in 100 μ l of PBS. After 6, 11, 17, and 28 days, mice were sacrificed and tumors were resected. To isolate lymphocytes from tumor tissues, comparable amounts of tumors were collected, cut into pieces, and digested in RPMI 1640 containing 600 μ g/ml liberase and 200 μ g/ml DNase for 45 min at 37°C until all the tumor tissue had resolved into a cell suspension.

In vivo depletion of T cells

Treg depletion was performed by a single injection of 90 or 200 μ g of purified anti-CD25 mAb (PC61) (24). Treatment led to 76% depletion of CD25⁺ cells as detected by flow cytometry by using the 7D4 CD25-specific mAb which recognizes a separate CD25 epitope from that recognized by PC61 (data not shown). CD4 and CD8 depletion was performed by one injection of 500 μ g of anti-CD4 (GK1.5) or anti-CD8 (53.6.7) mAbs 4 days after tumor cell inoculation. Depletions lasted at least 2 wk.

Flow cytometry analysis

FACS analysis were performed using FITC-, PE-, allophycocyanin-, PerCP, or biotin-conjugated Abs to mouse CD4 (RM4-5), CD8 (53-6.7), CD25 (7D4), CD69 (H1.2F3), CD62L (Mel-14; all BD Biosciences). For staining, cells were incubated with appropriate Abs in a standard procedure and analyzed by a FACScalibur cytometer. FACS data were analyzed with CellQuest (BD Biosciences) or FlowJo software (Tree Star).

Intracellular staining for cytokines and Foxp3 expression

To determine the percentages of IFN- γ - and IL-2-expressing cells, tumor-infiltrating lymphocytes (TIL) from anti-CD25-treated or untreated mice were stimulated for 4 h in cultures containing 1 μ g/ml PMA, 2 μ g/ml ionomycin (Sigma-Aldrich), and Golgi plug (BD Pharmingen). Then cells were washed, permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), and stained for intracellular proteins according to the manufacturer's protocol. Intracellular staining for Foxp3 proteins was performed by using fixation and permeabilization buffers provided by the Foxp3 kit (eBioscience). We verified that enzymatic treatment of TIL did not modify CD25 and Foxp3 expression.

Cell surface staining and single-cell sorting

Cells for FACS sorting were prepared from tumors and lymph nodes. The used mAbs were obtained from BD Pharmingen. T cells were sorted as CD4⁺CD25⁺ cells using allophycocyanin-conjugated (anti-CD4) and PE-conjugated Abs (anti-CD25). Cells were sorted using allophycocyanin-conjugated anti-CD4, PE-conjugated anti-CD25, and FITC-conjugated anti-CD8 Abs. Single cells were directly sorted into PCR tubes containing 5 μ l of PBS and immediately stored at –80°C until use in PCR.

Single-cell RT-PCRs

Single cells were lysed before the reverse transcription of RNA by heating tubes for 2 min at 70°C and then cooling at 4°C. RNA of each cell was reverse transcribed using a standard protocol of specific reverse transcription (Invitrogen Life Technologies) in a final volume of 15 μ l containing 3 μ M of antisense primers R1 designed as shown in Table I. The reverse transcriptase was inactivated by heating for 5 min at 70°C. PCR amplifications were conducted in two rounds: for the first round, the reaction contained all 5' primers in combination with the same 3' primers used for specific reverse transcription. Amplification was performed in a final volume of 80 μ l by touch-down PCR over five cycles starting with an annealing temperature at 68°C followed by 25 cycles consisting of 30 s at 94°C, 20 s at 58°C, and 1 min at 72°C. For the second round of PCR, 2- μ l aliquots of the first amplification products were transferred into six separate reactions, each containing a single 5' primer used in the first PCR in combination with a nested 3' primer. All PCR contained dNTPs at 200 μ M each, 1 U of TaqDNA polymerase, and PCR buffer (1.5 mM MgCl₂) in a final volume of 20 μ l. Five minutes of extension at 72°C terminated each PCR and final amplified products were then analyzed on 1% agarose gels stained with ethidium bromide. The specificity of each PCR product was verified by DNA sequence analysis.

Table I. PCR primer sequences used for multiplex RT-PCR

Sequences	
5'	
CD3 ϵ	GCCTCAGAAGCATGATAAG
FoxP3	CTAGTCATGGTGGCACCCTC
CTLA-4	AACTGCAGCTGCCTTCTAGGAC
GITR	CATGCTGTATGGAGTCTCGATGC
IL-10	AAGGGTTACTTGGGTTGCCAAGC
IFN- γ	ACACTGCATCTTGGCTTTGCAGC
Granzyme B	GATGAAGATCCTCTCTGCTACTGC
Perforin	CAGCTGAGAAGACCTATCAGGAC
3' primers	
CD3 ϵ	CCTTGGCCTTCCTATTCTT
FoxP3	GATGATCTGCTTGGCAGTGCTTGA
CTLA-4	TTCCACCTTGCAGAGGTACAGT
GITR	CTTGTTCCAGGGAACATGGTGA
IL-10	TTAGAGAGCTCTGTCTAGGTCCT
IFN- γ	CGACTCCTTTTCCGCTTCCT
Granzyme B	CCAACGACCCACATAGCACACAT
Perforin	TACTTCGACGTGACGCTCACGGTA
3' nested primers	
CD3 ϵ	TGACCATCAGCAAGCCAG
FoxP3	GAAACTCTTCTGGCTCCTCGAAG
CTLA-4	AACAGCTCTCAGTCTTGGATG
GITR	AGTTGTCCAAAGTCTGCAGTGAC
IL-10	GTCCAGCAGACTCAATACACACT
IFN- γ	CACATCGGATGAGCTCATTGAATGC
Granzyme B	CACATCTCCTGGCTTCACATTGA
Perforin	GTATTCACTGGAGACGCTGGCTTG

In vitro T cell suppression assay

Different T cell populations were sorted with a mean purity of 98–99% using a FACS Vantage (BD Biosciences). Dilutions of CD4⁺CD25^{low} and CD4⁺CD25^{high} T cells that were sorted from normal lymph nodes (NLN) of BALB/c mice, tumor DLNs, and TILs were cocultured in 96-well plates with sorted CD4⁺CD25⁺ cells from naive mice (2.5×10^4 /well) plus 0.5 μ g/ml anti-CD3 ϵ Abs and 4×10^3 MACS-sorted (Miltenyi Biotec) CD11c⁺ dendritic cells. After 72 h of culture at 37°C, cells were pulsed with 1.5 μ Ci/well [³H]thymidine for 12–16 h and [³H]thymidine incorporation was measured by a scintillation counter (Beckman).

Cytotoxicity assay

TIL and DLN were harvested at day 17 or 28 of tumor growth; lymph nodes from normal mice were also harvested and used as negative controls. CD8⁺ T cells were magnetically selected using anti-CD8-Biot from BD Biosciences and biotin beads (Miltenyi Biotec) according to the manufacturer's instructions. Viable trypan blue-excluded CD8 T cells were counted and used as effector cells. Cytotoxicity of CD8 T cells was measured in a standard 4-h ⁵¹Cr-release assay at 37°C using Na₂⁵¹CrO₄-labeled 4T1 (2.10^3 cells/well), MC26, and B16F10 targets. Experiments were conducted in duplicate at various E:T ratios. Spontaneous release was assessed from wells that contained labeled target cells alone, and maximum ⁵¹Cr release was assessed by addition of 2% Triton X-100 (Sigma-Aldrich). Specific cytotoxicity was calculated as percentage of ⁵¹Cr release = (cpm experimental – cpm spontaneous) \times 100 (cpm maximum – cpm spontaneous).

Statistical analysis

Results are expressed as means \pm SEM. Groups were compared by using ANOVA followed by multiple comparisons of means with Fisher's least significance procedure. We evaluated statistical significance with Prism software (GraphPad Software). Values of $p < 0.05$ were considered significant.

Results

Phenotype and suppressive activity of tumor-infiltrating CD4 T cells

To study the immune response in tumor-bearing mice, we used the 4T1 mouse mammary carcinoma, a BALB/c-derived transplantable tumor. This tumor is reported to induce tumor growth when

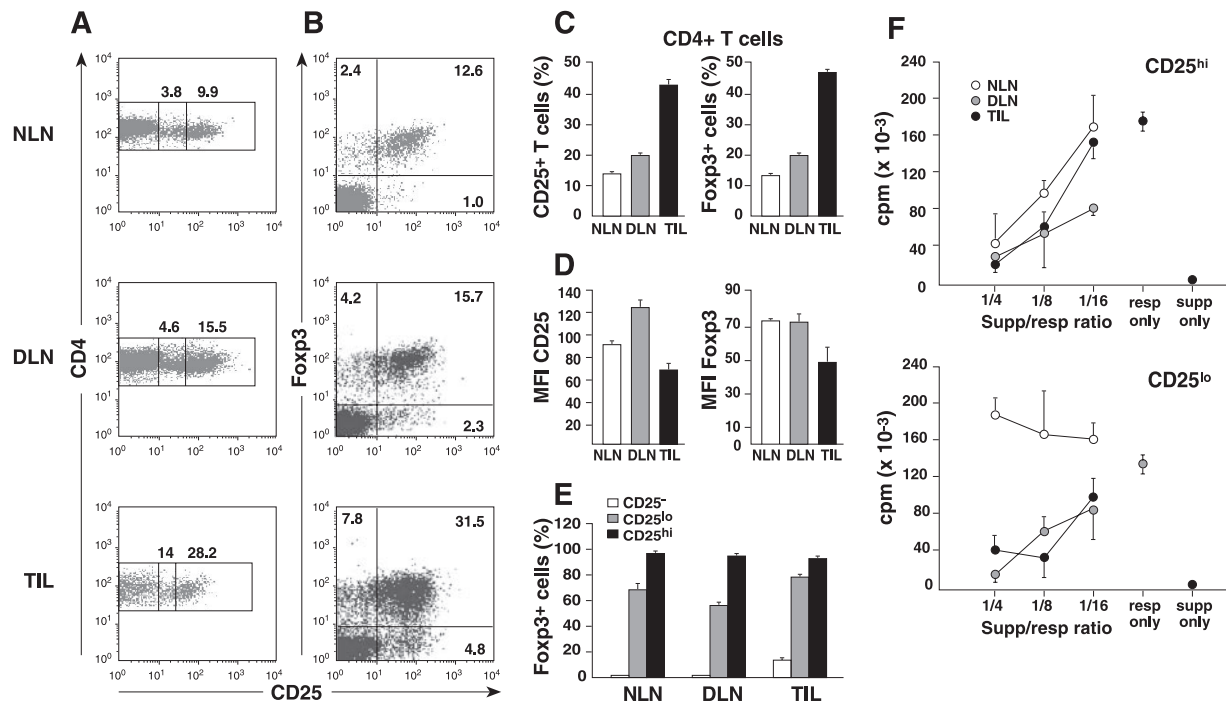


FIGURE 1. Increased suppressive activity of tumor-infiltrating CD4⁺CD25^{low} T cells at a late stage of tumor progression. *A* and *B*, Flow cytometry of CD4⁺ T cells from NLN, DLN, and TIL. Cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 Abs. Numbers in outlined areas or quadrants indicate the percentage of cells in the indicated gates. *C*, Average percentage of CD25⁺ and Foxp3⁺ cells among CD4⁺ T cells. *D*, Mean fluorescence intensity for CD25 expression on CD4⁺ T cells (*left*) and Foxp3 expression on CD4⁺CD25⁺ T cells (*right*). *E*, Average percentage of Foxp3⁺ cells among CD4⁺CD25⁻, CD4⁺CD25^{low}, and CD4⁺CD25^{high} T cells. Error bars indicate SD of the mean. Data are representative of two experiments with at least three mice. *F*, Suppressive activity of purified CD4⁺CD25^{high} and CD25^{low} T cells was tested at the indicated ratio to suppress proliferation of 2.5×10^4 purified CD4⁺CD25⁻ T cells. Data are presented as mean [³H]thymidine incorporation in triplicate cultures. Error bars indicate SD of the mean. Data are representative of two separate experiments.

low numbers of cells are injected and to induce metastasis even after the injection of such low cell numbers (25). Indeed, on s.c. inoculation of BALB/c mice with 4T1 cells, reliable establishment of tumors required injection of only 10^4 to 10^5 4T1 cells per mouse.

Using this model, we first compared the percentages of regulatory cells in the tumors (TIL) and their DLN with those of corresponding lymph nodes obtained from normal BALB/c mice (NLN). To distinguish between Treg and other CD4⁺ T cells, we evaluated CD25 cell surface levels as well as the expression of Foxp3 within these cell types (Fig. 1, *A* and *B*). The percentages of CD4⁺ expressing high or low levels of CD25 (CD25^{high} and CD25^{low}) as well as Foxp3 were increased in DLN when compared with NLN, but were even higher in TIL (Fig. 1*C*). However, as indicated by the diminished mean fluorescence intensity of CD25⁺ and Foxp3⁺ TIL (Fig. 1*D*), CD25 and Foxp3 expression levels were lower than that found in DLN, suggesting the existence of a CD4⁺CD25^{low}Foxp3^{low} subpopulation specific to TIL. It must be noted that these low expressions were not a consequence of the enzymatic treatment. Indeed, we compared CD25 and Foxp3 expression in spleen cells isolated mechanically or by enzymatic treatment. We found the same expression levels in both circumstances, indicating that those markers were not affected by our technical isolation (data not shown). Despite the low Foxp3 expression in TIL, the percentages of Foxp3⁺ cells in both CD25^{high} and CD25^{low} subpopulations were approximately equivalent to that of LN (Fig. 1*E*). Taken together, these findings show that Tregs selectively accumulate inside the tumor and constitute nearly half of CD4⁺ TIL. Similar data of CD25⁺ T cell accumulation at the tumor site were also observed in another model of B16

melanoma in B6 mice (data not shown) and in other previous reports (26, 27).

A recent study has shown that high expression of Foxp3 is required for the Treg-suppressive function (28). It was therefore possible that the lower Foxp3 and CD25 expression rendered CD4⁺CD25⁺ TIL unable to function as suppressor cells. Thus, according to the gates shown in Fig. 1*A*, we directly compared the ability of purified CD4⁺CD25^{high} and CD4⁺CD25^{low} cells recovered from different tissues to suppress nonregulatory CD4⁺CD25⁻ T cell proliferation. The suppressive activity of CD25^{high} TIL was equivalent to that of CD25^{high} NLN but was lower to that of CD25^{high} DLN (Fig. 1*F*). In contrast, TIL and DLN CD25^{low} T cells showed equivalent suppressive activity. Moreover, these CD25^{low} cells that were particularly abundant in TIL, showed a much-increased suppressor activity when compared with CD25^{low} cells recovered from NLN. Thus, total CD25⁺ TIL were fully able to suppress T cell proliferation independent of their level of CD25 expression. In this particular situation of tumor immunity, the reduced Foxp3 expression levels are not associated with reduced suppressive function.

Tregs are activated at tumor sites

The suppressive function of CD25⁺ regulatory cells depends on the expression of Foxp3 and other associated molecules as CTLA-4 and GITR. Moreover, T cells may also mediate suppressor functions by the secretion of IL-10. The study of the coexpression of these molecules in CD4 T cells by flow cytometry is complex. On one hand, the T cell activation required to detect IL-10 reduces the expression of CD3, CD4, and Foxp3. On the other

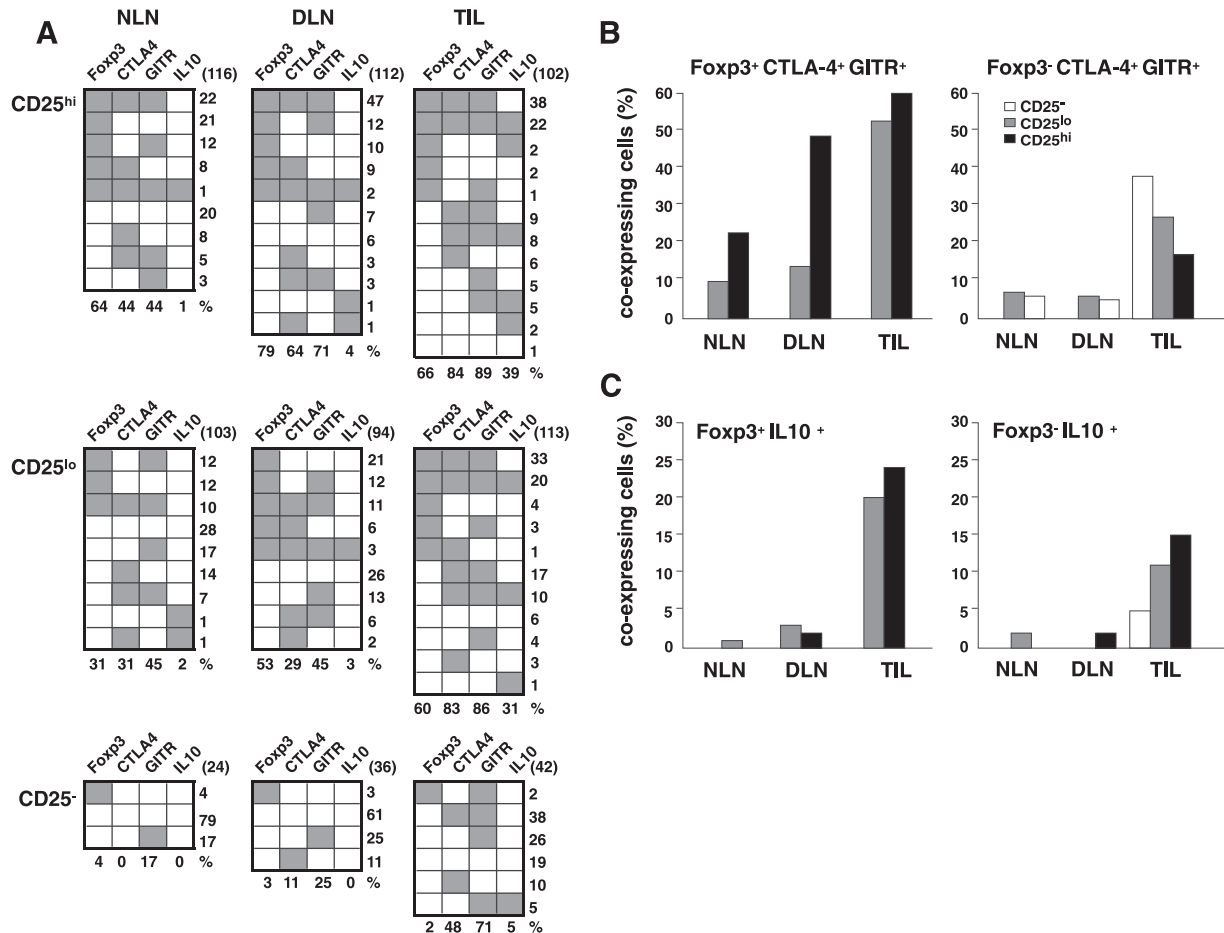


FIGURE 2. Increased number of activated Treg cells at the tumor site. Coexpression of Foxp3, CTLA-4, GITR, and IL-10 mRNA were analyzed in “individual cells.” A, cDNAs from single CD4⁺CD25^{high}, CD4⁺CD25^{low}, and CD4⁺CD25⁻ T cell isolated from TIL, DLN, and NLN were amplified by two rounds of nested PCR. Detection of PCR products is indicated by shading the boxes. The percentage of cells that express more than one gene is shown at the right; the frequency of cells expressing each product is shown along the bottom. The total number of cells studied is indicated in the right corner. B, Percentage of cells that coexpress CTLA-4 and GITR in FoxP3⁺ cells (right) and FoxP3⁻ cells (left). C, Percentage of cells that coexpress IL-10 in FoxP3⁺ and FoxP3⁻ cells. The data shown in A were used to determine these percentages.

hand, the low CTLA4 expression levels prevent clear discrimination in between low expressers and negative cells. To further discriminate the possible heterogeneity of Tregs isolated from different tissues, we studied individual cells and in “each cell,” we determined the coexpression of *Foxp3*, *CTLA-4*, *GITR*, and *IL-10* genes.

For this purpose, single CD4⁺CD25^{high}, CD4⁺CD25^{low}, and CD4⁺CD25⁻ T cells were sorted from TIL, DLN, and NLN. Single cells were lysed and the mRNA was reverse-transcribed and amplified by two rounds of PCR using CD3ε-, FoxP3-, CTLA-4-, GITR-, and IL-10-specific primers (Table I). The mRNA CD3ε constitutively expressed in T cells was used as an internal control and results are presented only for mRNA CD3ε⁺ samples (Fig. 2A). Consistent with previous data (29), Foxp3⁺ cells could be detected in small numbers of CD25⁻ T cells (2–4%). The percentage of FoxP3⁺ cells among CD4⁺CD25^{high} T cells was significantly higher in DLN than in TIL and NLN (79 vs 66% and 64%; $p < 0.02$). Strikingly, FoxP3⁺ cells were found in CD25^{low} TIL, with proportions close to those observed in CD25^{high} T cells from TIL and DLN, but higher than those found in NLN (60 and 53% vs 32%; $p < 0.001$).

Differences in coexpression of *FoxP3* genes in combination with CTLA-4 and GITR or IL-10 may have distinct effects on the suppressive activity mediated by Treg cells, which in turn

could explain the increased suppressive activity that is seen in TIL and DLN (Fig. 1F). Most of Foxp3⁺CD25^{high} Tregs isolated from TIL and DLN coexpressed both CTLA-4 and GITR, as did most of the Foxp3⁺CD25^{low} cells from TIL, but not those from DLN (Fig. 2B). In contrast, the majority of Foxp3⁺CD25^{low} cells from NLN did not coexpress these molecules. It should be noted that among the TIL that did not express FoxP3, a significant proportion of CD4⁺CD25⁻ cells expressed CTLA-4 and GITR (38%), suggesting that these cells were activated effectors. Finally, a significant number of IL-10-expressing cells could be detected only in TIL, both in FoxP3⁺- and FoxP3⁻-activated T cells (Fig. 2C).

Collectively, these results showed that Tregs present in DLN and TIL were activated when compared with NLN. Moreover, these data showed that Treg cells present at the tumor site had different molecular characteristics than DLN because a substantial fraction of Foxp3⁺ and Foxp3⁻ TIL expressed *IL-10* genes.

Intratumor Tregs and CD4 helper cells are unresponsive to IL-2

It has been previously shown that in both CD4 and CD8 T cells, the induction of T cell tolerance (also called T cell anergy) was associated with progressive defects in the response of T cells. First, T cells were not able to produce IL-2, but could still respond to TCR stimulation when exogenous IL-2 was provided (30). We

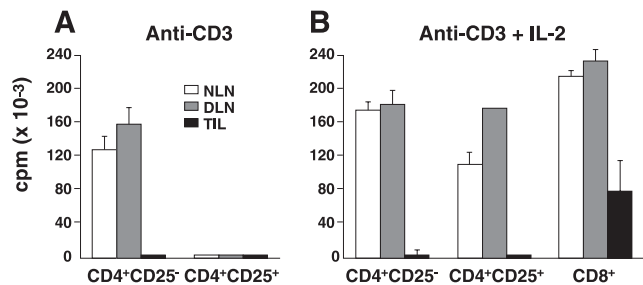


FIGURE 3. T cells from 4T1 tumors do not proliferate and do not respond to exogenous IL-2. A total of 2.5×10^4 CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells from TIL, DLN, and NLN were purified 28 days after tumor challenge and cultured with anti-CD3 Abs and CD11c⁺ DCs (A) or in the presence of IL-2 (B) for 3 days. Data are presented as mean [³H]thymidine incorporation in duplicate cultures. Error bars indicate SD of the mean. Data are representative of two separate experiments.

studied the proliferative responses of TIL and LN T cells after polyclonal stimulation with anti-CD3ε Abs, with or without the addition of exogenous IL-2. CD4⁺CD25⁻ T cells from NLN and DLN proliferated in the absence of IL-2 (Fig. 3A) while both CD4⁺CD25⁺ T cells and CD8⁺ T cells recovered from these tissues required exogenous IL-2 to divide (Fig. 3B). In contrast, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from TIL did not respond to stimulation even in the presence of high doses of IL-2 while TIL CD8⁺ T cells moderately proliferated only in the presence of IL-2. In agreement with data already reported for T cells (31), these data show that all T cell types isolated from TILs are less responsive to IL-2 than T cells in DLN and are profoundly anergic. Moreover, the nonresponse of Tregs in the presence of IL-2 could mean that these cells cannot be expanded at tumor sites at the late stage of tumor growth.

Low cytolytic activity of CD8 T cells at the tumor site

Previous studies suggest that Treg cells suppress IFN-γ secretion (32) and cytolytic function of CD8 T cells in tumor DLNs (33). To address this question in TILs, we evaluated the lytic activity of “ex vivo” tumor-infiltrating CD8 T cells in a chromium-release assay. Freshly isolated CD8 T cells from growing tumors at day 28 and their corresponding DLN were tested against the specific 4T1 tumor cell line used as target cells (Fig. 4A) and against the nonspecific B16F10 and MC26 tumor cell lines used as target cell controls. Whereas enriched CD8 T cells obtained from DLN showed weak cytotoxic activity against 4T1 targets (12% at 70:1 E:T ratio), CD8 T cells enriched from TIL were found to be cytotoxic for 4T1 targets. Even though low, this cytotoxic activity was found to be specific for 4T1 targets as illustrated by the fact that at lower ratios the two nonspecific targets were not killed.

Activation of CD8 T cells leads to IFN-γ gene expression, and the cytotoxic activity needs coexpression of granzyme B and perforin, but Abs detecting native perforin in mice are not available. To determine whether the low cytolytic activity was due to low numbers of CTLs in TIL, coexpression of IFN-γ, granzyme B, and perforin genes was studied in series of “individual cells” (Fig. 4B). We adapted PCR-based techniques, previously developed for single-cell analysis of Treg cells to detect perforin, granzyme B, and IFN-γ transcripts in CD8⁺ T cells freshly isolated from TIL and DLN. We show that the three genes were coexpressed by most CD8 T cells from TIL (55%) whereas only a minority of this cell subset derived from DLN expressed perforin mRNAs (21%) without coexpression of IFN-γ and granzyme B. Collectively, these data show that ac-

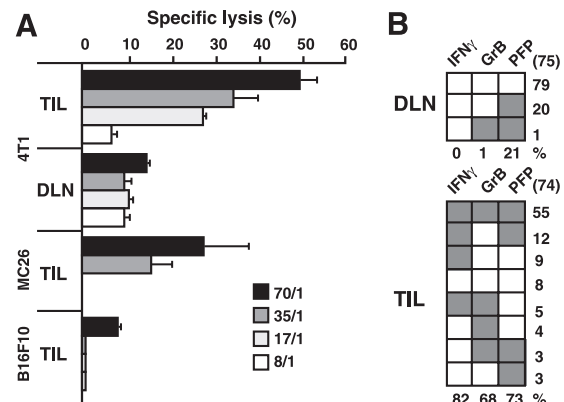


FIGURE 4. Low cytolytic activity of TIL is not linked to low numbers of CTLs at the late stage of tumor development. A, The efficacy of ex vivo cytolytic activity of TIL and DLN was measured 28 days after tumor challenge in a ⁵¹Cr-release assay using specific 4T1 targets cells and the two nonspecific melanoma B16F10 and colon carcinoma MC26 tumor cell lines. B, At the same time point, the frequency of individual cells expressing IFN-γ, granzyme B (GrB), and perforin (PFP) was studied as described in *Materials and Methods*. The percentage of cells coexpressing more than one gene is shown on the right and the percentage of cells expressing each PCR product is shown below the panels. The total number of cells studied is indicated in the right corner.

tivated CD8 T cells were not detectable in DLN, whereas tumor sites harbor activated CD8 T cells with low levels of specific cytotoxic activity. Therefore, at the late stage of tumor progression (day 28), the presence of Treg at the tumor site does not fully abrogate CD8 effector functions.

Treg depletion leads to tumor infiltration of activated CD4⁺CD25⁻ T cells and increased numbers of CD8 T cells

The influence of Tregs on tumor infiltration and activation of CD4 and CD8 T cells has not been clearly studied. To address this point, the initial experiments focused on the effect of CD25 depletion on tumor infiltration by CD4 and CD8 T cells and their activation in treated and untreated mice at various days after s.c. inoculation of 4T1 cells. One injection of anti-CD25 mAb (PC61 clone) before tumor challenge induced a transient depletion of CD25⁺ T cells because their percentages dropped from 12 to 2.2% in DLN 6 days after injection and increased slowly to 6% at day 17 (data not shown). This injection was sufficient to induce tumor regression in 63% (19 of 30) and 80% (24 of 30) of the mice studied in two independent experiments. In these mice, tumors grew, although slower, and regressed within 20–25 days (see also below in Fig. 7).

In PC61-treated mice challenged with 4T1 tumor cells and analyzed on days +6, +11, and +17, we observed that the percentages of CD4⁺CD25⁺ cells in TIL were substantially reduced and that this reduction lasted at least until day 17. Although to a lesser degree, the frequency of Foxp3⁺ cells was reduced at least 2-fold in tumors at all three time points (Fig. 5, A and B) and most Foxp3⁺ cells were CD25⁻ or CD25^{low}.

The reduction of Tregs induced by anti-CD25 treatment did not induce a parallel reduction of total CD4 T cells recovered from tumor tissues. The percentage of total CD4 T cells was similar while CD8 T cell prevalence significantly increased with time to day 17 (Fig. 5C). Moreover, as related to tumor mass, the total number of both CD4 and CD8 T cells was significantly higher in anti-CD25-treated mice (Fig. 5D). These results suggested that in tumor sites, Tregs were substituted by another T cell type, likely activated T cells that are known to have the capacity to migrate to

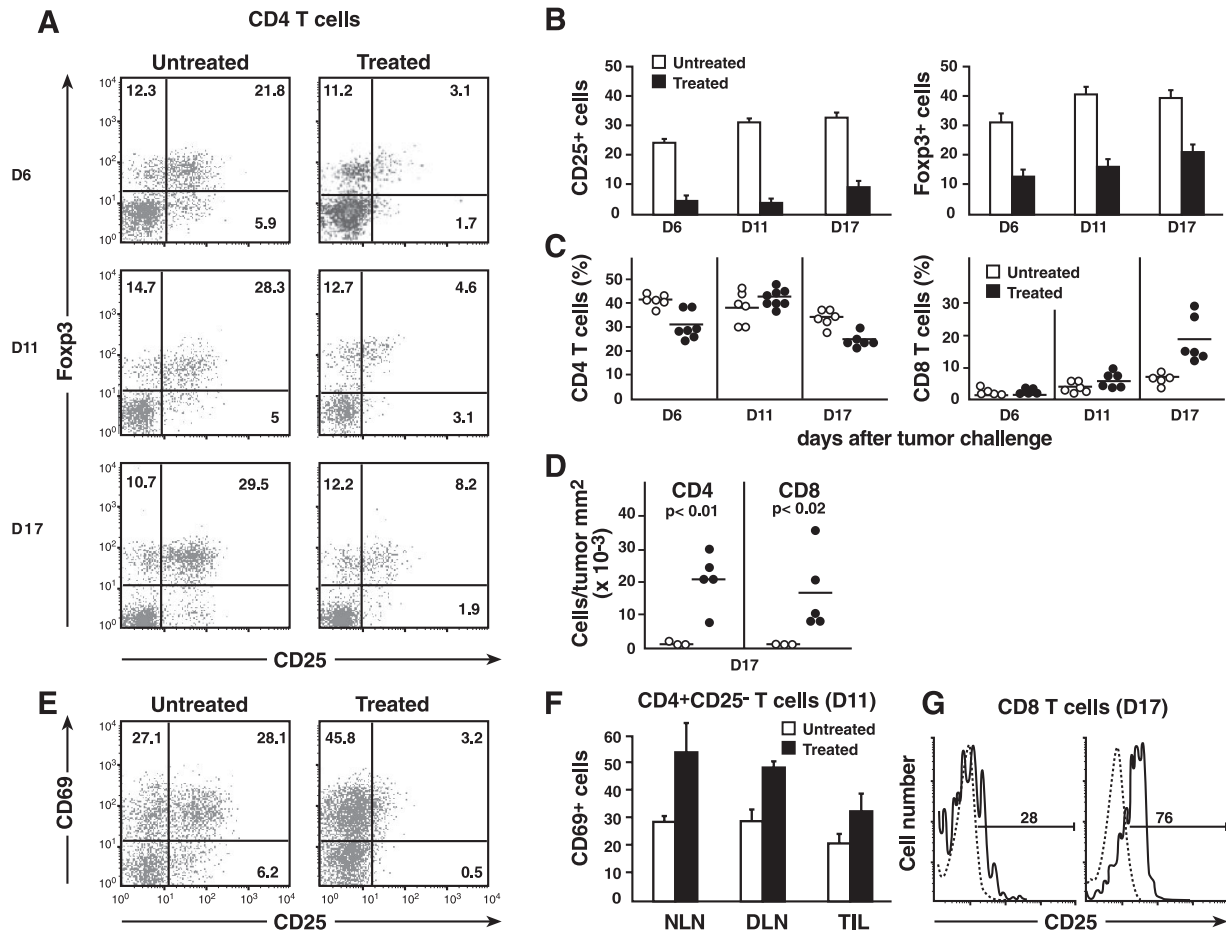


FIGURE 5. Early activation of CD4⁺CD25⁻ T cells and increased number of CD8 T cells in tumors of Treg-depleted mice. **A**, TILs from untreated or anti-CD25-treated mice were extracted from their tumor tissues after 6, 11, and 17 days and stained with anti-CD4, -CD25, and -Foxp3. **B**, Average percentages of CD25⁺ and Foxp3⁺ T cells among TIL CD4⁺ T cells. **C**, Percentages of CD4 and CD8 T cells within regressive vs progressive tumors. **D**, Percentages and calculated numbers of CD4 and CD8 T cells among total tumor-infiltrating mononuclear cells. **E**, Data represent expression of CD69. Numbers in quadrants indicate the percentage of cells gated on total CD4⁺ TIL. **F**, Average percentages of CD4⁺CD69⁺ T cells gated on CD4⁺ TIL. **G**, Percentage of CD25⁺ cells observed in treated and untreated mice. Histograms are gated on CD8⁺ T cells extracted from tumors at day 17. Data are representative of two separate experiments.

tumor sites. To evaluate the percentage of activated T cells, we have analyzed the expression of the early activation marker CD69 in intratumor CD4⁺ cells from treated mice and untreated mice (Fig. 5, *E* and *F*). At day 11 after tumor inoculation, the proportion of CD4⁺CD25⁻ TILs expressing CD69 was significantly increased in treated mice. We also observed that CD25 expression in CD8 T cells was increased in later phase (day 17) of tumor regression (Fig. 5*G*).

TIL functional capacity is increased after Treg depletion

The depletion of Tregs also modified the functional properties of T cells infiltrating the tumor sites. At day 11 after tumor challenge, the percentages of CD4 T cells able to secrete IFN- γ and IL-2 significantly increased (Fig. 6*A*). In addition, a detailed study of CD8 function in treated and untreated mice showed an earlier development of specific cytotoxic CD8⁺T cells in treated mice (Fig. 6*B*). Thus, TIL isolated from treated mice had increased specific lytic activity especially at lower E:T ratios, i.e., 10:1 and 2:1.

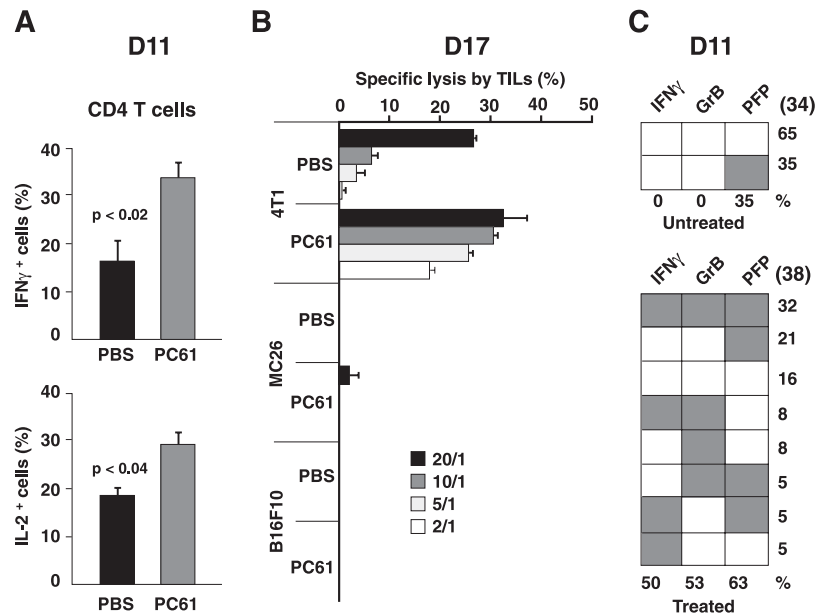
This increased killer capacity is corroborated by the coexpression of genes associated with cytotoxic function. In treated mice, CD8⁺CD25⁺ T cells coexpressed IFN- γ , granzyme B, and perforin genes suggesting a cytotoxic potential for these T cells even at an early time point (day 11) (Fig. 6*C*). In contrast, in untreated

mice, CD8 T cells did not coexpress these molecules indicating they were poor killers at the same time point.

Infiltration of activated CD4⁺CD25⁻ T cells promotes CD8 function during tumor regression

Our results indicate that the Treg depletion modified both the CD4 and CD8 T cells present in the tumor sites, increasing their number and functional capacity. These effects could be due to a direct suppression of CD8 and CD4 T cell functions. Alternatively, the effect of Treg depletion on CD8 T cells could be indirect, by removing the helper activity required for optimal CD8 function. To investigate this alternative as well as the role of CD4 and CD8 T cells in the control of tumor growth, we studied T cell function and tumor regression in mice where different T cell populations were removed by the injection of depleting Abs. In agreement with previous studies (19), depletion of Tregs induced tumor regression (Fig. 7*A*). In these experiments, the mice were sacrificed at day 17 and others allowed to survive showed a complete tumor regression at day 25. This regression was fully abrogated when CD8 T cells were also removed. These results demonstrate the fundamental role of CD8 T cells in the control of tumor growth in this model, as it was described in other models (21, 34).

FIGURE 6. Treg depletion leads to increased percentages of IFN- γ -expressing cells and increased anti-tumor cytotoxic function. *A*, Results show intracellular staining for IFN- γ and IL-2 after in vitro stimulation with PMA and ionomycin of TIL isolated by day 11 after tumor inoculation. Represented histograms were gated on CD4 $^{+}$ T cells. Error bars indicate SEM. *B*, Increased efficacy of ex vivo cytolytic activity by CD8 $^{+}$ TIL in anti-CD25-treated mice. Lytic activity of CD8 $^{+}$ TILs was determined by evaluation of mean-specific lysis \pm SE in a ^{51}Cr -release assay using 4T1-specific targets cells and MC26 and B16F10 used as nonspecific targets. *C*, Single CD8 $^{+}$ T cells enriched from indicated tumor samples were analyzed for expression of IFN- γ , granzyme B (*GrB*), and perforin (*PFP*) genes at day 11 after tumor inoculation. Data are representative of at least two separate experiments.



However, CD4 helper cells appeared fundamental for this CD8 function. Depletion of CD4 cells also prevented tumor regression (Fig. 7*A*, right). This effect occurred when CD4 T cells were depleted before (not shown) or after tumor challenge. This was not due to a failure in recruitment of CD8 cells into the tumor sites. Indeed, the number of CD8 cells in TILs increased after Treg depletion, but the further removal of helper CD4 cells

did not significantly modify numbers of infiltrated CD8 T cells (Fig. 7*B*). In contrast, the presence of CD4 cells fully modified CD8 function, because the capacity of CD8 T cells to secrete IFN- γ was significantly increased when CD4 helper cells were present in the tumor sites (Fig. 7*C*). This experiment ascribes a primarily role of the CD4 Th cell in the process of the tumor regression.

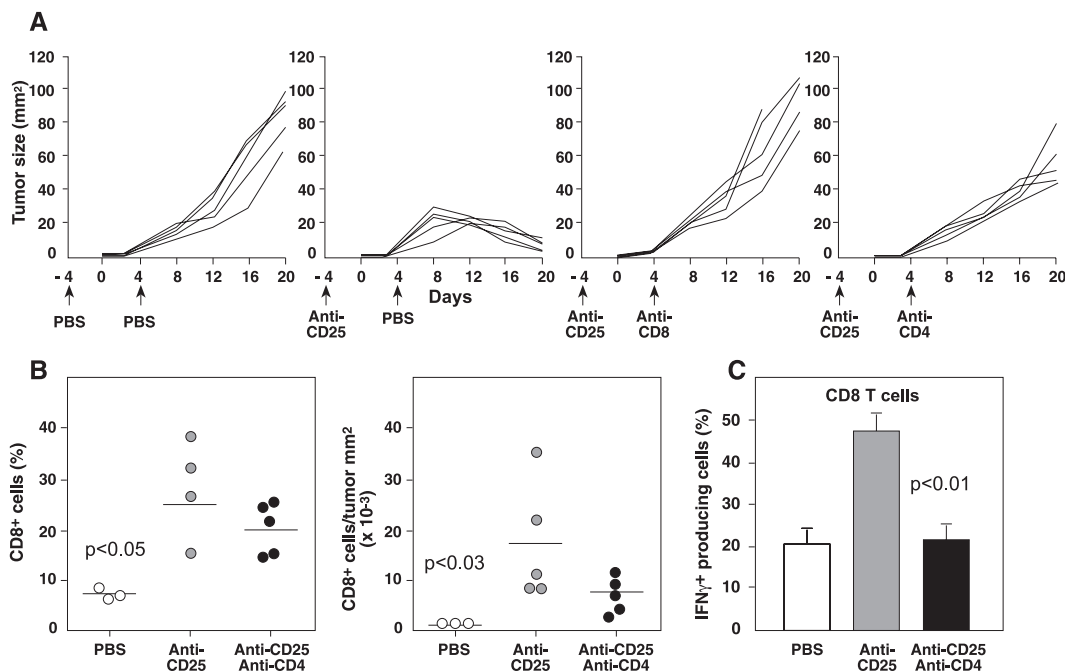


FIGURE 7. CD4 T cell depletion prevents tumor regression. Mice were inoculated with 4T1 cells. *A*, The left graph represents growth of tumors from untreated mice, the second graph shows tumor sizes from mice treated with anti-CD25 abs (PC61), the two last graphs show growth of tumor mice treated first with anti-CD25 at day 4 before tumor inoculation followed by selective elimination of either CD4 (GK1.5) or CD8 (53.6.7) Abs 4 days after tumor challenge. The difference between tumor sizes in mice receiving PBS vs anti-CD25 treatment was statistically significant at day 16 ($p = 0.02$). *B*, On day 17, mice were sacrificed and the percentages of CD4 and CD8 T cells and absolute numbers related to tumor size were calculated in different groups of untreated or treated mice. The percentage and numbers of CD4 and CD8 T cells were determined among total tumor-infiltrating mononuclear cells at indicated time points after tumor inoculation in anti-CD25-treated or untreated mice. *C*, TIL were incubated with PMA and ionomycin and stained for IFN- γ and surface markers as described in *Materials and Methods*. Histograms represent the percentage of IFN- γ -expressing cells among CD8 $^{+}$ T cells isolated from different groups of mice. Data are representative of at least two separate experiments with at least four mice.

Discussion

The failure of the immune response against tumors has been studied in transgenic models and has been attributed to active suppression mediated by CD4⁺CD25⁺ Tregs in the lymph node draining tumor sites (20). However, several studies have shown that CD4⁺CD25⁺ Treg cells also invaded tumor masses in animal models of cancer (19, 35) and in cancer patients (17, 18, 36), but the link between these infiltrating Tregs and the local effector immune response is poorly understood. In our study, we used a mouse model of a highly progressive and invasive 4T1 tumor. A closer examination of TILs in this model indicated that the failure of the immune response was related to the accumulation of activated Tregs that might suppress CD4⁺CD25[−] infiltrating T cells at tumor sites and finally constitute a barrier, which prevented the infiltration and differentiation of efficient CD8 T cells.

Our results show major differences between the characteristics of T cells residing in the tumor and in DLNs. First, CD25 and Foxp3 expression levels in TIL were lower than in other Treg populations located either in DLN and NLN. In two different situations, it has been shown that Treg cells lose the expression of CD25 upon adoptive transfer into lymphopenic hosts (37, 38). Thus, the low level of CD25 expression on Treg cells that we observed could be explained by a lesser amount of IL-2 available within the tumors due to the anergic state we find in infiltrating CD4 helper cells which cannot produce IL-2. In mice expressing different Foxp3 levels, it was shown that a lower Foxp3 expression resulted in lower suppressive activity (28). However, the lower Foxp3 levels of Tregs located in the tumor site did not hinder their suppressive activity. On the contrary, these cells had an increased level of suppression; in particular, we could reveal the existence of a unique subpopulation of CD25^{low} cells, expressing low levels of Foxp3 and showing an important suppressive activity. Thus, in this tumor response, a reduction of Foxp3 expression does not lead to a reduced suppression. This unusual CD25^{low} phenotype of suppressor cells in TIL underlines the difficulty of characterizing Treg cells based on CD25 cell surface phenotype alone. Moreover, in a recent report, CD25[−] Treg cells have been shown to suppress non-Tregs at the same level as CD25⁺ Treg cells (39). Because TIL had an increased expression of CTLA-4 and GITR (reported to be associated with suppression), it is possible that the expression of these additional molecules compensates for the reduced Foxp3 levels.

The various characteristics of TIL regulatory cells may be the signature of their increased activation status. Indeed, CTLA-4 and GITR molecules are usually expressed by all activated T cell types. This CD25^{low} phenotype of TIL was also observed in a previous study of allograft rejection in which “in vivo” expanded Treg cells became CD25^{low} but sustained their suppressive activity (40). In an autoimmune model, it was reported that depletion of CD25⁺ cells resulted in an in vivo decrease in IL-10 production and autoimmunity and suggested that activated Tregs may secrete IL-10 (41). Our results show directly that in addition to CTLA-4 and GITR, TIL Foxp3⁺ Tregs co-express *IL-10* genes. It has been shown that activation by self-Ags can result in tolerance of both CD4 and CD8 T cells during tumor progression (42, 43). Thus, it is tempting to suggest that IL-10⁺ infiltrating Tregs were overstimulated by the persistence of tumor cell Ags. Therefore, our data are compatible with the notion that activated Treg cells are preferentially located at tumor sites, and that their IL-10 production may also contribute to their suppressive function.

It has recently been shown that IL-2 signaling is not required for the Treg-suppressive function but seemed to be involved in sur-

vival or expansion of these cells (38, 39). It is noteworthy that intratumor Treg cells not only retained the capacity to suppress, but have also lost the ability to proliferate in the presence of IL-2 in culture. This type of behavior, as well as the IL-10 expression, was previously described in peripheral T cells stimulated in vivo with massive doses of Ag in both TCR-transgenic models and normal mice submitted to massive virus loads and was named “peripheral high-dose anergy” (30). In addition, other T cell types localized within the tumor showed major defects in their capacity to proliferate. In cultures supplemented with IL-2, the CD8 response was reduced while CD4⁺CD25[−] did not proliferate at all. Thus, anergy was also observed in intratumor CD4⁺ cells which did not express *FoxP3* genes. These cells were also extensively activated as shown by their coexpression of *CTLA-4* and *GITR* genes and IL-10.

Depletion of Tregs was shown to induce tumor regression by increasing the percentage and number of CD8 T cells in tumor sites (26). To understand the suppressive function of Tregs one must observe both changes in CD4 and CD8 populations. In two different models of antitumor immunity, it was proposed that Tregs diminished CD8 activity either by consuming IL-2 (38) or by preventing cytotoxic granule release (23). In a model of autoimmunity, other authors propose that dendritic cells are central to Treg cell inhibition in vivo by attenuating priming of CD4 Th cells (44). Yet, another group has shown that close contact between Tregs and Th cells is required (45). It is probable that various combinations of several mechanisms are operating, depending on the environment and the type of immune responses. We found major defects of CD4 function suggesting that TIL regulatory cells could have other roles. To address this issue, we first determined the kinetics of tumor infiltration by CD4 and CD8 T cells and their activation state after in vivo depletion of CD25⁺ T cells by treatment of mice with PC61 Abs before tumor inoculation. These findings revealed several aspects that had not been reported previously. First, CD4 cells residing in tumor sites showed major changes. The percentage of CD4 cells remained the same, but they were predominantly CD4⁺CD25[−] T cells expressing high levels of CD69 and higher percentages of IL-2- and IFN- γ -producing cells than in untreated mice. These results suggested that Tregs and other anergic CD4 T cells were substituted by functional-reactive helper cells in the tumors of treated mice. The anti-CD25 treatment modified CD8 function and numbers. In early tumors, CD8 T cells remained low but showed a considerable functional improvement. They matured faster because the coexpression of perforin, granzyme B, and IFN- γ was already observed at day 11 after tumor implantation, while the expression of these molecules was very rare in untreated mice. Cytotoxic capacity of CD8 T cells was also much increased. This kinetics where the infiltration of CD4 helper cells precedes CD8 accumulation and the modifications of CD8 function suggest that CD8 needed CD4 help for their functional maturation. In such case, the effect of regulation could be indirect, by preventing the differentiation of CD4 cells into adequate helper cells.

Comparison of “ex vivo” CD8⁺ T cell cytotoxic function in the presence or absence of Treg cells suggested that when Treg cells are replaced by CD4⁺ Th cells including IFN- γ - and IL-2-producing cells at tumor sites, intratumor CD8⁺ T cells were much more efficient for eliminating tumor cells. This hypothesis was confirmed by additional data in which elimination of not only Treg cells but also CD4 Th cells after tumor challenge abrogated tumor regression. Together, the modifications of infiltration, activation, and lytic activities emphasized the major role of CD4 Th cells in efficient antitumor immunity and showed that at tumor sites, Treg

cells favor the tumor progression by suppressing CD4 Th cell effect. The effect of Tregs in directly controlling CD4 help and indirectly affecting CD8 differentiation does not preclude further direct roles of Tregs on other aspects of the CD8 response. Indeed, it was reported that Tregs suppressed CD8 cytotoxicity directly by preventing release of cytotoxic granules (23), but in this system, transgenic CD8 T cells were transferred to normal mice and thus endogenous CD4 help was present during CD8 activation and differentiation.

Our data could explain previous reports on the role of CD4 helper cells in promoting tumor rejection induced by vaccination (46). The mechanisms used by CD4 helper cells to promote CD8-improved immunity have yet to be determined but several nonexclusive alternatives can be envisaged. CD4 help may contribute directly to CD8 function by increasing CD8 T cell survival (47, 48) or promoting improved division and effector functions (7). CD4 help may also modify directly the trafficking and selection of CD8 T cells into tumor sites, allowing the accumulation of Ag-specific CD8 T cells expressing high-affinity TCRs. Indeed, antitumor therapy using naive CD8 T cells transfected with tumor-reactive TCRs showed that only high-avidity T cells were efficient to kill specific tumor targets (49).

Finally, our results emphasize the importance of the quality of the CD8 response, and the contribution of CD4 help to that response at the tumor site. It will, therefore, be important to characterize the subpopulation of CD4 Th cells that could rescue the differentiation of CD8 T cells at tumor sites which helps tumor immunity against persisting tumor Ags and subsequently breaks tolerance at the local level.

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Disclosures

The authors have no financial conflict of interest.

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