Distinct and Overlapping Roles of Interleukin-10 and CD25$^+$ Regulatory T Cells in the Inhibition of Antitumor CD8 T-Cell Responses

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Abstract

Lack of antitumor immunity is often related to impaired CD8 T-cell responses that could result from a poor priming capacity by tumor-infiltrating dendritic cells (TIDC) and/or further inhibition by regulatory T cells (T_{reg}). Interleukin-10 (IL-10) has been implicated in the inhibition of TIDC as well as in the generation and functions of T_{reg}. Here, we address some of the respective and possibly overlapping roles of IL-10 and CD25$^+$ T_{reg} in CD8 antitumor immunity. Whereas tumor antigen–specific CD8 T cells proliferated *in vivo* in the presence of IL-10 or T_{reg}, optimal effector functions were observed in mice lacking both IL-10 and T_{reg}. Indeed, tumors grown in normal but not in IL-10–deficient or CD25-depleted mice induced tumor antigen–specific CD8 suppressor T cells. Suppression involved transforming growth factor-β. Similarly, both IL-10 and T_{reg} were responsible for impaired CD8 T cell priming by TIDCs, but IL-12 production by TIDCs was prevented only by T_{reg}-independent IL-10. Subsequently, IL-10 defect and T_{reg} depletion were required to achieve optimal induction of CD8 T-cell effectors by TIDC following CpG activation. Our results point out major redundant and nonredundant roles for IL-10 and T_{reg} in the inhibition of TIDC-mediated generation of antitumor CD8 T-cell response. (Cancer Res 2005; 65(18): 8479-86)

Introduction

Tumors express defined antigens that can be recognized by cytotoxic CD8 T lymphocytes (1). However, this CD8 T-cell response is in general insufficient to lead to tumor eradication. Several mechanisms have been evoked to explain this deficiency, including (a) a defect in the priming phase of naive CD8 T cells; (b) a defect in the acquisition of effector functions such as IFN-γ production and cytotoxicity by tumor-associated antigen (TAA)–specific CD8 T cells; (c) the lack or loss of expression of relevant class I MHC tumor antigen peptides by tumor cells; and (d) the suppressive activity of cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) and, more recently, regulatory T cells (T_{reg}).

T_{reg} cells encompass several populations of regulatory T cells, defined by their distinct ontogeny, phenotype, or regulatory mechanism (2, 3). "Natural" T_{reg} refers to a population of CD4$^+$CD25$^+$ T cells selected in the thymus and involved in peripheral tolerance. Depletion of natural T_{reg} using CD25 antibody before tumor cell inoculation was able to enhance antitumor responses (4, 5), suggesting an antigen-independent inhibition mechanism. In contrast, other T_{reg} subsets such as Tr1 cells seem induced in an antigen-specific fashion to control ongoing immune responses (6, 7). T_{reg} can be associated with human tumors, although their precise origin in that case remains unclear (8, 9). Cytokines, and in particular IL-10 and TGF-β, play major roles in the generation and function of T_{reg} activity, although again their particular contributions to different T_{reg} subsets is still a matter of debate. An important recent finding was that either immature classic (10), or other less classic dendritic cell subsets referred as tolerogenic dendritic cells (11) could induce T_{reg}. In turn, CD25$^+$ T_{reg} cells can inhibit dendritic cell functions (12).

Dendritic cells might play a central role in the induction of tumor immunity, and especially in the initiation of CD8 T-cell responses, because they are able to cross-present MHC class I–restricted antigens captured from apoptotic bodies (13–15). Solid human tumors are frequently infiltrated by dendritic cells (16, 17) and a few animal studies have shown that tumor-infiltrating dendritic cells (TIDC) could *de novo* present TAA to naive CD8$^+$ T cells (18, 19). We and others have hypothesized that impaired TIDC functions could be responsible for the absence of an efficient antitumor response. In particular, the tumor milieu lacks strong dendritic cell–activating stimuli such as those delivered by microbes. Indeed, intratumoral delivery of Toll-like receptor (TLR) ligands can improve antitumor CD8 T-cell responses (20–22). We have described, however, that in several mouse models, TIDC could not produce IL-12 in response to the TLR-9 ligand CpG unless endogenous IL-10 was neutralized using IL-10R (19). Subsequently, anti-IL-10R antibody treatment *in vivo* allowed for a robust, CD8-dependent antitumor response following intratumoral CpG injection (19). We could not define, however, whether IL-10 was acting solely and directly at the level of TIDCs or through other regulatory mechanisms.

Given the roles of IL-10 in dendritic cells and T_{reg} biology, we wanted to further analyze the relative contribution of IL-10 and CD25$^+$ T_{reg} on tumor antigen–specific CD8 T-cell response. We show herein that IL-10 and T_{reg} act in concert, in particular at the level of TIDC, to impair antitumor CD8 T-cell effector differentiation and induce tumor antigen–specific suppressor CD8 T cells.

Materials and Methods

Mice. Female wild-type (WT) C57BL/6, C57BL/6 IL-10 gene-deficient (IL-10 KO; ref. 23), OT-I transgenic for αβ-TCR reactive with the H-2K^b-restricted 257-264 peptide of OVA (24), and C57BL/10 RAG KO mice were

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purchased from Charles River Laboratories (St. Germain sur l'Arbresle, France). All mice were housed in our pathogen-free facility and used between 5 to 10 weeks of age. The experiments were done in compliance with relevant European Economic Community laws and institutional guidelines.

Culture medium. Splenic CD11c$^+$ cells, TIDCs, and T cells were cultured in RPMI 1640 (Invitrogen, Cergy Pontoise, France) supplemented with 10% FCS (Invitrogen), 1 mmol/L HEPES (Invitrogen), 2 mmol/L L-glutamine (Invitrogen), Gentallin (Schering-Plough, Union, NJ), and 2×10^{-5} M β_2-mercaptoethanol (Sigma, St. Louis, MO). Tumor cells were cultured in DMEM (Invitrogen) with the same additives.

Tumor model and *in vivo* procedures. EL-4 H-2^b or EG-7 (10^5, EL-4 stably transfected to express ovalbumin) thymoma cells (American Type Culture Collection, ATCC, Rockville, MD) were s.c. injected in the right flank in 100 μL of RPMI 1640. Tumor growth was monitored by palpation and mice sacrificed when tumors reached 1 cm in diameter. For *in vivo* activation, mice were injected intratumorally with 10 μg of the TLR-9 ligand CpG 1668 (5′-TCCATGAGGTTCCTGATGCT-3′, MWG Biotech, Ebersberg, Germany) in 50 μL PBS (Invitrogen) and i.p. with 250 μg of endotoxin-free purified anti-IL-10R antibody (1B13A, DNAX Research Institute of Molecular and Cellular Biology Research Institute, Palo Alto, CA) in 200 μL PBS. When indicated, some groups of mice were i.p. injected with anti-CD25 supernatant (PC61-5-3, ATCC) at the day of tumor cell inoculation and then twice a week until adoptive transfer. The latter treatment allowed for the depletion of CD4$^+$CD25$^+$ splenocytes from 3% to <0.2% of CD4$^+$ splenocytes, as monitored by fluorescence-activated cell sorting (FACS) analysis using another anti-CD25 antibody (7D4).

CFSE staining. Naive T cells or splenocytes (1×10^7) in 1 mL HBSS (Invitrogen) were labeled with CFSE (Molecular Probes, Eugene, OR) at 1 to 10 μmol/L according to different assays for 10 minutes at 37°C (25). Cells were washed twice in medium containing 10% FCS to block staining.

Flow cytometry analyses. Fc receptors were blocked using Fc-Block (BD Biosciences PharMingen, San Diego, CA). All antibodies used in this study were from BD Biosciences PharMingen: CD4 (L3T4), CD8β (Ly-3.2), CD11b (M1/70), CD11c (HL3), CD25 (7D4), CD62L (MEL-14), and CD28 (37.51). For intracellular detection of IL-12 p40/p70, cells incubated for 2 hours in Brefeldin A (Sigma) and stained with CD11c were processed with the Cytofix/Cytoperm Kit (BD Biosciences PharMingen) and stained with PE-labeled anti-IL-12 (C15.6). Variables were acquired on a FACSCalibur (BD Biosciences, San Jose, CA).

Naive T-cell and tumor-infiltrating dendritic cell isolation. Naive T cells were sorted from spleen cells on a FACSVantage based on the coexpression of CD8 and CD62L to >95% purity. In some experiments, CD8$^+$ T cells were enriched by negative selection using a CD8$^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch, Gladbach, Germany). TIDCs, or splenic dendritic cells as control, were purified using CD11c$^+$ Microbeads (Miltenyi Biotec), at a purity >92% (19).

Tumor-infiltrating dendritic cell induction of naive T-cell proliferation *in vitro*. Naive OT-I T cells were labeled with 7.5 μmol/L CFSE and cultured in 96-well plates at 1×10^6 cells/mL with 2×10^5/mL irradiated TIDC or splenic CD11c$^+$ cells in culture medium, with or without OVA peptide (10 nmol/L SIINFEKL peptide, Neosystem, Strasbourg, France), and/or activation stimulus. Activation consisted of 5 μg/mL of CpG 1668 ± 10 μg/mL anti-IL-10R antibody. After 5 days of culture, cell division was analyzed based on CFSE labeling intensity using the Modfit software (BD Biosciences).

Adoptive transfer of naive T cells. CFSE-labeled OT-I naive T cells ($8\text{-}10 \times 10^6$) were transferred into tumor-bearing mice via i.v. injection. Cell division and surface phenotype was analyzed in tumor-draining and contralateral lymph nodes by flow cytometry at 3 and 7 days following transfer.

Tumor-infiltrating dendritic cell induction of naive T-cell functions *in vivo*. Naive C57BL6/ mice were adoptively reconstituted with 8 to 10×10^6 CFSE-labeled OT-I naive T cells. One day later, 5×10^5 TIDC recovered from resting animals or animals treated 2 hours before with CpG 1668 ± anti-IL-10R were injected in the right footpad. Popliteal draining lymph nodes were harvested 5 days following TIDC injection and IFN-γ-secreting CD8 T cells numbered by ELISPOT. Briefly, 96-well nitrocellulose-bottom plates (Millipore, Molsheim, France) coated with rat anti-IFN-γ (clone R4-6A2, BD Biosciences PharMingen) were seeded with 2×10^5 lymph node cells and incubated with or without 10 nmol/L OVA class I peptide for 48 hours. Cytokine-producing cells were revealed by sequential incubations with anti-IFN-γ biotinylated antibody (clone XMG1.2, BD Biosciences PharMingen), peroxidase-streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL) and AEC substrate (Vector Laboratories, Burlingame, CA). Spots were quantified using an ELISPOT reader (Microvision Instrument, Evry, France).

***In vivo* CTL assay.** Spleen cells from C57BL/6 mice were pulsed with 10 nmol/L OVA class I peptide and labeled with CFSE at a final concentration of 1 μmol/L; unpulsed spleen cells were labeled at a final concentration of 10 μmol/L. The two cell populations were mixed at 1:1 ratio and 2×10^7 total cells were injected i.v. into tumor-bearing or naive mice. The percentage of antigen-specific lysis was determined 18 hours following target cell injection by analyzing the relative proportion of unpulsed versus pulsed targets based on CFSE labeling intensity in tumor-draining lymph node (TDLN) cells. FACS acquisition was done over 1 minute and the percentage of specific lysis calculated as follows: $[(M_2 - M_1) / M_2] \times R \times 100$, where M_2 = total unpulsed target events, M_1 = total pulsed target events, and R = the mean ratio of unpulsed/pulsed targets from three naive recipients.

Sorted CFSE$^+$ T-cell induction of naive T-cell proliferation *in vitro*. Three days following adoptive transfer, CFSE$^+$ cells were sorted from TDLNs. Proliferation assays were done by culturing 5×10^4 CD8$^+$CD62L$^+$-sorted naive T cells (OT-I mice) per well for 72 hours with various concentrations of CFSE$^+$-sorted T cells in flat-bottomed 96-well plates with irradiated APC (T cell–depleted splenocytes) and 10 nmol/L OVA class I peptide. In some experiments, cells were stimulated with 5 μg/mL plate-bound anti-CD3 (145-2C11, BD Biosciences). In some experiments, 30 μg/mL anti-TGF-β (1-D11, R&D Systems, Minneapolis, MN), or isotype control antibodies were added to the culture. Cells were pulsed with 1 μCi per well [^{3}H]thymidine (Amersham, Les Ulis, France) for the last 8 hours of culture, and proliferation was measured in a TopCount apparatus (Perkin-Elmer, Courtaboeuf, France).

Statistical analysis. Statistical analysis was done with Student's t test.

Results

CpG intratumor treatment cured established tumors in the absence of interleukin-10 or after depletion of CD25$^+$ T$_{reg}$ cells. As we previously reported for C26 colon carcinoma tumors (19), we show here that treatment with CpG 1668 + anti-IL-10R combination cured C57BL/6 mice bearing established s.c. tumors originated from the cell line EG-7, a variant of the EL-4 thymoma that stably expresses ovalbumin (OVA; ref. 26), whereas CpG alone had no effect on survival in this model (Fig. 1*A*). Unlike the temporary neutralization of IL-10 using anti-IL-10R that had no effect on tumor growth (Fig. 1*A*), in mice genetically deficient in IL-10 EG7 tumor growth was delayed and survival was increased by 2 weeks compared with WT C57BL/6 mice (Fig. 1*B*). Remarkably, treatment with CpG alone, which was ineffective in WT mice, induced 75% tumor rejection in EG-7 tumor-bearing IL-10 KO mice (Fig. 1*B*). *In vivo* injection of CD25 antibody, used to deplete the T$_{reg}$ subset (27, 28), also delayed tumor growth in C57BL/6 mice (Fig. 1*C*) and intratumor CpG treatment achieved a high rate of tumor rejection in CD25 antibody-treated mice (Fig. 1*C*). Interestingly, combination of T$_{reg}$ depletion and IL-10 blockade without CpG treatment induced tumor rejection in only a minor proportion of animals (Fig. 1*D*). Taken together, these results suggest that both IL-10 and T$_{reg}$ prevent the antitumor response induced by CpG.

Endogenous interleukin-10 or CD25$^+$ T$_{reg}$ cells limit the proliferation of tumor-specific CD8 T cells *in vivo*. We have shown that CD8 T cells were involved in the rejection of tumors

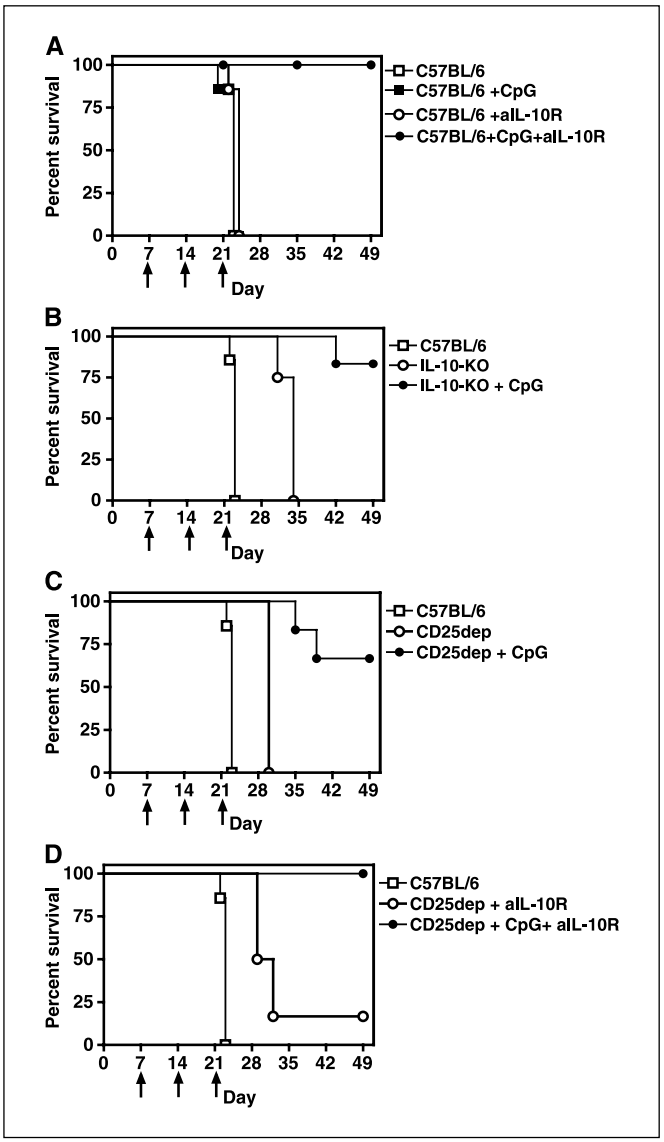

Figure 1. Lack of IL-10 or CD25 depletion licenses CpG to induce tumor rejection. Groups of six mice were injected s.c. at day 0 with 1×10^5 EG-7 tumor cells. *A,* EG-7 tumor-bearing C57BL/6 mice were treated at days 7, 14, and 21 (*arrows*) with nothing (□), 10 μg CpG 1668 injected intratumorally (■), 250 μg anti-IL-10R antibody injected i.p. (○), or combination of both (●). *B,* EG-7 tumor-bearing IL-10 KO mice were treated with nothing (○) or CpG (10 μg intratumorally, ●) at days 7, 14, and 21 (*arrows*). *C-D,* tumor-bearing C57BL/6 mice were injected with anti-CD25 at day 0 then twice a week until day 21 (○ or ●) and treated with nothing (*C*, ○) or combinations of CpG and anti-IL-10R antibody as in (*A*) (*C*, ●: CpG; *D*, ○: anti-IL-10R, ●: CpG plus anti-IL-10R). Mice were monitored thrice a week and sacrificed according to institutional guidelines, and survival time defined by the day of sacrifice.

mediated by CpG plus anti-IL-10R treatment (19). To better define the respective roles of IL-10 and T_{reg} cells in the inhibition of tumor antigen–specific CD8 T-cell response, we adoptively reconstituted EL-4 or OVA-expressing EG7 tumor-bearing mice with CD8 T cells from OT-I mice that express a TCR specific for the SIINFEKL peptide of OVA (24, 26), using CFSE labeling to monitor the transferred cells.

Three days following adoptive transfer in EL-4 tumor-bearing mice, we detected approximately the same percentage of $CFSE^+$ $CD8^+$ cells in TDLNs and in the contralateral lymph nodes (CLN; Fig. 2*B*). In contrast, antigen-driven local accumulation of CD8 T cells was observed in EG-7 tumor-bearing mice in which 2.5 times more $CFSE^+$ $CD8^+$ T cells on average were retrieved at day 3 from TDLN than in CLN. At this time, only a very low percentage of cells showed decreased CFSE labeling, indicative of cell division, in C57BL/6 mice (Fig. 2*A*). At day 7 after transfer, the analysis of CFSE intensity indicated a high level of cell division and an even greater accumulation of $CFSE^+CD8^+$ cells in the TDLN of EG-7 tumor-bearing animals than EL-4 tumor-bearing animals. These results strongly suggested that cell division was at least partly responsible for the accumulation (Fig. 2*A*).

Cell division was accompanied by the loss of expression of CD62L (Fig. 2*A*) and, in a proportion of $CFSE^+CD8$ T cells, expression of CD25 (data not shown). Unlike in WT C57BL/6 mice, OT-I CD8 T cells transferred in IL-10 KO mice divided as early as day 3 (Fig. 2*A*), resulting in a significantly higher (P = 0.002) proportion of $CFSE^+$ cells among $CD8^+$ cells in TDLN (Fig. 2*B*). CD8 T-cell proliferation was antigen dependent because it was not observed in EL-4 tumor-bearing mice (Fig. 2*B*).

CD25 antibody treatment also resulted in a greater antigen-specific CD8 T-cell accumulation in the TDLN at day 3 (P = 0.03) in C57BL/6 mice (Fig. 2*B*). Treatment of IL-10 KO mice with CD25 antibody did not show an additive effect (Fig. 2*B*). These data suggest that both IL-10 and T_{reg} cells prevent the optimal proliferation of tumor antigen–specific CD8 T cells *in vivo* in a redundant fashion.

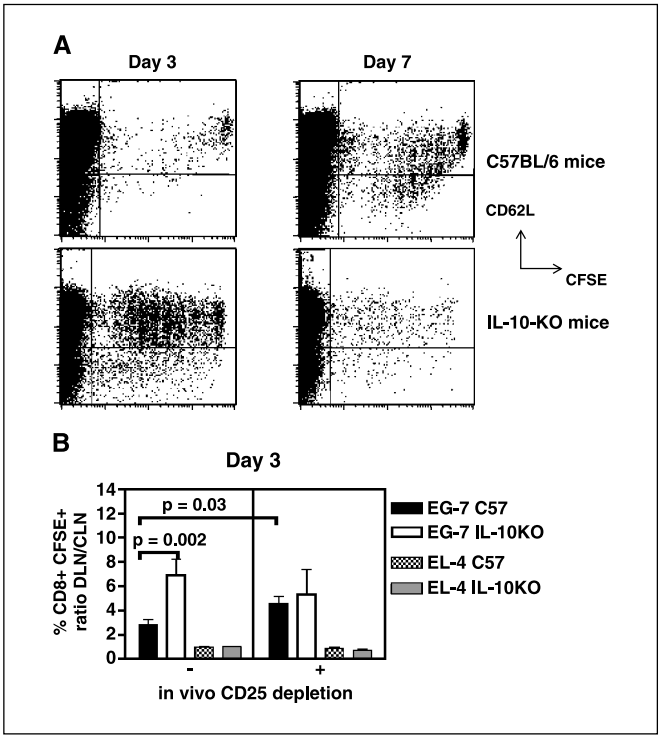

Figure 2. Adoptively transferred CD8 T cells specifically accumulate and proliferate in EG-7 TDLNs. Mice were treated with anti-CD25 at the day of tumor inoculation and twice a week until adoptive transfer. *A,* CFSE and CD62L expression was analyzed 3 and 7 days following adoptive T-cell transfer in EG-7 tumor-bearing C57BL/6 or IL-10 KO mice as indicated. Flow cytometry dot plots show the expression of CFSE and CD62L within gated CD8 T cells (log scale). Representative of four similar experiments. *B,* CD8 T-cell accumulation was analyzed at day 3 by measuring the ratio % of CFSE $CD8^+$ T cells in TDLN: % $CFSE^+CD8^+$ T cells in the CLN in EG-7 or EL-4 tumor-bearing C57BL/6 (*filled* and *checkered columns*) or IL-10 KO (*open* and *gray columns*) mice with (*right*) or without (*left*) *in vivo* CD25 depletion. *Columns,* mean ratios of 8 to 10 individual mice, from four independent experiments; *bars,* ±SE.

Interleukin-10 and CD25$^+$ T_{reg} cells decrease *in vivo* tumor-specific CD8 T-cell functions. We observed a modest but significant ($P < 0.01$) increased frequency of OVA antigen-specific IFN-γ-producing cells in the TDLN of EG-7 tumor-bearing IL-10 KO mice compared with C57BL/6 mice (Fig. 3*B, right*). Much more evident results were observed after adoptive transfer of OT-I CD8 T cells in EG-7 tumor-bearing mice. Three days following transfer, the frequency of OVA-specific IFN-γ-secreting cells in TDLN was significantly higher in IL-10 KO mice and in mice treated with CD25 antibody than in untreated WT C57BL/6 mice (Fig. 3*A, left*). The absence of IFN-γ-producing cells in TDLN from EL-4 tumor-bearing mice or when OVA peptide was omitted during *in vitro* restimulation confirmed the antigen specificity of the response (Fig. 3*B*). Treatment of IL-10 KO mice with CD25 antibody did not significantly increase the frequency of IFN-γ-producing cells (Fig. 3*A, left*).

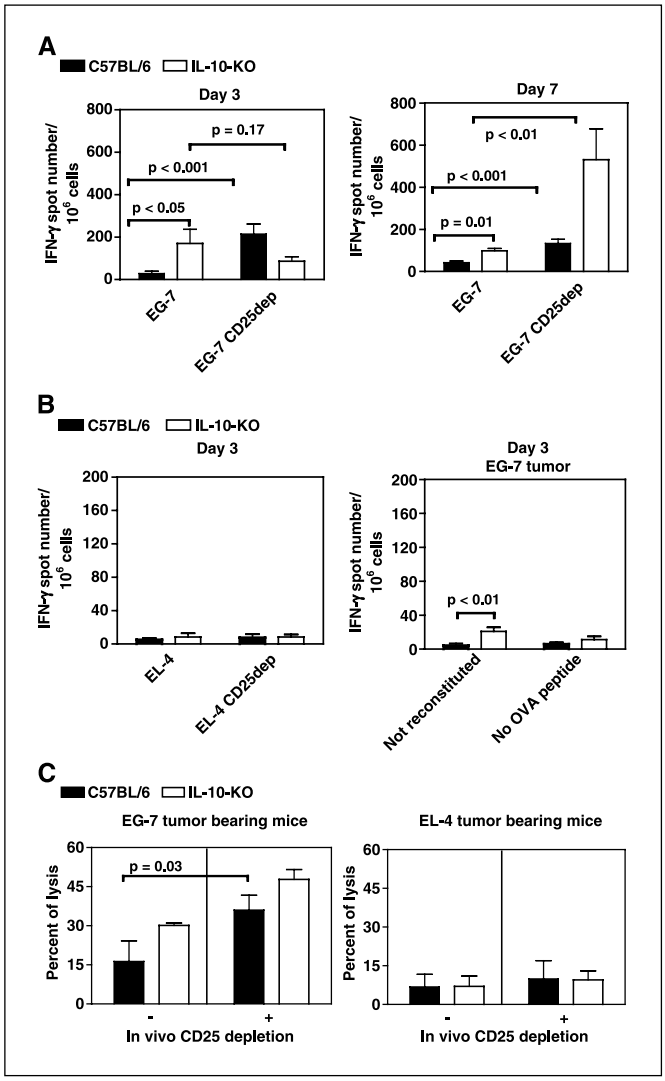

Figure 3. CD25 depletion and/or lack of IL-10 improve CD8 T-cell capacity to secrete IFN-γ and increase tumor antigen–specific CTL. Tumor antigen–specific IFN-γ-secreting cells were analyzed in TDLN by ELISPOT according to Materials and Methods. *A,* TDLN cells from EG-7 tumor-bearing C57BL/6 (*black columns*) or IL-10 KO (*open columns*) mice, with (*right*) or without (*left*) *in vivo* CD25 depletion, at days 3 (*left*) and 7 (*right*) following adoptive transfer. *B,left,* TDLN cells from EL-4 tumor-bearing C57BL/6 (*black columns*) or IL-10 KO (*open columns*) mice, with (*right*) or without (*left*) *in vivo* CD25 depletion 3 days following adoptive transfer. *Right,* TDLN cells from unreconstituted (*left*) EG-7 tumor-bearing C57BL/6 (*black columns*) or IL-10 KO (*open columns*) mice or without *in vitro* OVA peptide stimulation (*right*). *Columns,* mean spot numbers from nine to 12 individual mice from three to four independent experiments; *bars,* ±SE. *C,* a 1:1 mixture of spleen cells from C57BL/6 mice pulsed with 10 nmol/L OVA class I peptide and unpulsed spleen cells were labeled with CSFE at two different intensities then injected i.v. into EG-7 or EL-4 tumor-bearing C57BL/6 (*black columns*) or IL-10 KO (*open columns*) mice, with (*right*) or without (*left*) *in vivo* CD25 depletion according to Materials and Methods and in the absence of adoptive transfer. The cytotoxic activity was measured 24 hours after injection in TDLN. *Columns,* mean % antigen-specific lysis calculated as described in Materials and Methods in three individual mice; *bars,* ±SE. Two separate experiments were done with similar results.

Seven days following adoptive transfer, the frequency of tumor antigen–specific IFN-γ-producing cells was still significantly ($P = 0.01$) increased in IL-10 KO mice and CD25 antibody–treated mice, compared with untreated C57BL/6 recipients (Fig. 3*A, right*). Intriguingly, the frequency of tumor antigen–specific IFN-γ-producing cells at day 7 was lower than that observed at day 3 in IL-10 KO mice or CD25 antibody–treated WT C57B/6 mice but strikingly higher than at day 3 in IL-10 KO mice with CD25 antibody (Fig. 3*A, right*).

We also investigated the generation of tumor-specific CTL activity as measured *in vivo* against OVA class I peptide-pulsed fluorescently labeled spleen cells. Unlike IFN-γ secretion, *in vivo* CTL activity was measured in tumor-bearing mice in the absence of OT-I cell–adoptive transfer. Tumor antigen–specific lysis was increased only modestly and not in a statistically significant way in IL-10 KO compared with C57BL/6 recipient mice and significantly ($P = 0.03$) following CD25 antibody treatment of C57Bl/6 mice (Fig. 3*C*). Furthermore, treatment of IL-10 KO mice with CD25 antibody yielded the highest lysis rate (Fig. 3*C*). The presence of antigen-expressing tumor was required because only background lytic activity was observed in EL-4 tumor-bearing mice (Fig. 3*C*). Collectively, these data strongly suggest that both IL-10 and T_{reg} prevent the optimal early development of tumor antigen–specific CD8 T-cell functions, in a nonredundant fashion.

Interleukin-10 and CD25$^+$ T_{reg} cells are required for *in vivo* generation of tumor-induced antigen-specific regulatory CD8 T cells. Although we could not detect significant CD8 effector functions at the steady state, EG-7 but not EL-4 tumors were able to trigger modest OT-I CD8 T-cell proliferation. This suggested that antigen-specific priming had occurred. We further investigated whether these primed CD8 T cells would have acquired regulatory properties *in vivo*, characterized by an anergic state and the capacity to inhibit naive T-cell proliferation. To do so, we retrieved, 3 days following adoptive transfer, OT-I cells from TDLN using cell sorting based on CFSE labeling.

We first tested the capacity of the sorted cells to proliferate *in vitro* in response to anti-CD3 stimulation (Fig. 4*A*). We observed that OT-I cells parked in EL-4 tumor-bearing mice proliferated in response to anti-CD3 even to a greater extent than freshly isolated naive OT-I CD8 T cells or total C57BL/6 CD8 T cells. In marked contrast, OT-I cells parked in EG-7 tumor-bearing mice poorly proliferated in response to anti-CD3. Although we could not completely rule out that the inability of proliferate was due to clonal exhaustion, this result as well as the poor proliferation of OT-I cells *in vivo* (Fig. 2) argues in favor of a tumor-induced, antigen-specific anergy in these CD8 T cells.

We then investigated whether the parked cells had acquired regulatory properties, using cocultures with naive OT-I cells in the presence of irradiated APC and OVA class I peptide. The addition of OT-I cells parked in EL-4 tumor-bearing mice to naive OT-I CD8 T cells resulted in a proliferative response higher than that observed with naive OT-I cells alone, likely due to the proliferation

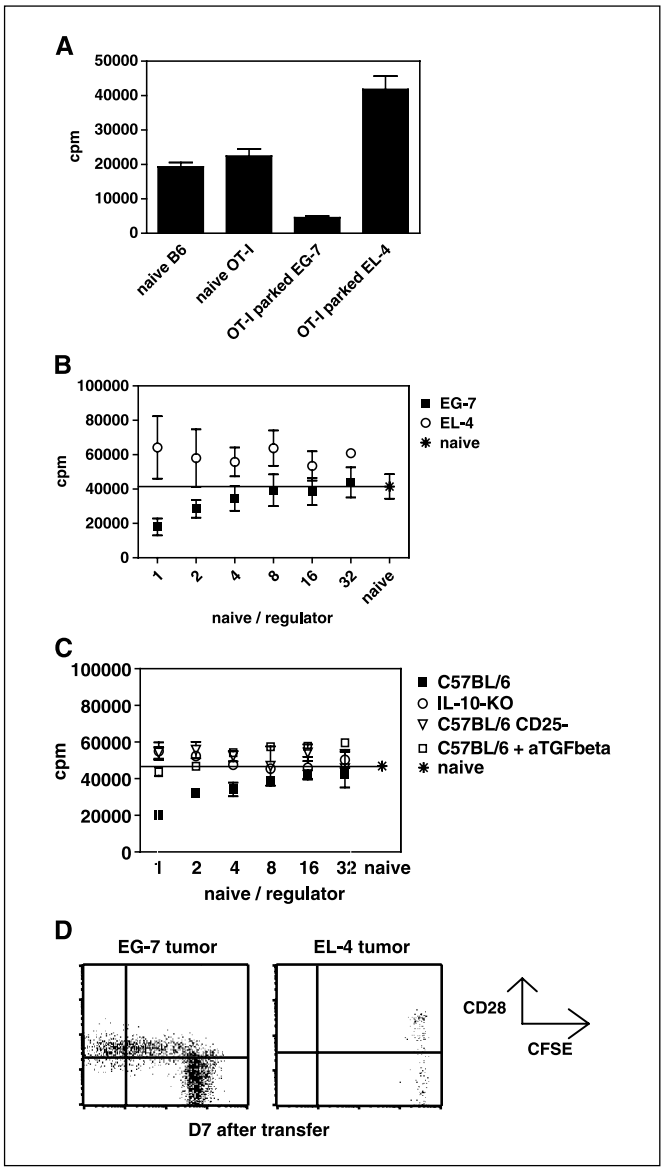

Figure 4. Antigen-specific induction by EG-7 tumors of suppressive CD8 T cells. *A,* naive wild type, OT-I spleen cells or CFSE$^+$ cells sorted from TDLN 3 days following adoptive transfer of CFSE-labeled naive OT-I cells were cultured in the presence of plate-bound anti-CD3 antibody for 72 hours. *Columns,* mean [^{3}H]thymidine incorporation during the last of 8 hours of culture for triplicates; *bars,* ±SE. *B-C,* sorted CFSE$^+$ cells from TDLN (*regulator*) were cultured in the presence of irradiated APC, OVA class I peptide, and naive OT-I CD8 T cells (*naive*) at the indicated ratios for 72 hours. *, naive cells alone. *Points,* mean [^{3}H]thymidine incorporation during the last of 8 hours of culture for triplicates; *bars,* ±SE. *B,* proliferation from cocultures of sorted CFSE$^+$ cells from EG-7 (■) or EL-4 (○) tumor-bearing C57BL/6 mice. *C,* proliferation from cocultures of sorted CFSE$^+$ cells from EG-7 tumor-bearing untreated C57BL/6 with (□) or without (■) anti-TGF-β, IL-10 KO mice (○) or C57BL/6 mice treated with CD25 antibody at the day of tumor inoculation and then twice a week (▽). Two to three experiments were done with similar results. *D,* CD28 versus CFSE expression from TDLN 7 days following adaptive transfer of CFSE-labeled OT-I cells. *Left,* sorted CFSE$^+$ cells from EG-7 TDLN; *right,* gated CFSE$^+$ cells from EL-4 TDLN.

of both populations. In marked contrast, the addition of OT-I cells parked in EG-7 tumor-bearing mice suppressed the proliferation of naive OT-I cells in a dose-dependent fashion (Fig. 4*B*). This indicates that tumors induced regulatory CD8 T cells in a tumor antigen–specific fashion. Such suppressive activity was not observed with OT-I cells parked in IL-10 KO or CD25-depleted mice (Fig. 4*C*). Although we cannot completely rule out that some level of suppressive activity was masked by effector T cells present in those samples, these results suggest that both IL-10 and T_{reg} favor the development of tumor antigen–specific regulatory CD8 T cells. Furthermore, neutralizing anti-TGF-β antibody (Fig. 4*C*) but not anti-CTLA4 or anti-IL-10R (data not shown) added during culture abrogated the suppressive activity. Interestingly, whereas the vast majority of OT-I cells parked in EL-4 tumor-bearing mice as well as dividing OT-I cells parked in EG-7 tumor-bearing mice expressed CD28, most OT-I cells parked in EG-7 tumor-bearing mice that did not undergo division were CD28 negative (Fig. 4*D*), a feature of some populations of regulatory CD8 T cells (29).

Endogenous interleukin-10 suppresses the ability of tumor-infiltrating dendritic cell to produce interleukin-12 while CD25$^+$ T_{reg} cells do not. We addressed the functional status of TIDC. We had previously shown that dendritic cells infiltrating mouse tumors were impaired in their capacity to produce IL-12 in response to CpG (19). This was reversed by the addition of anti-IL-10R antibody during stimulation. Here, we observed a similar inability to produce IL-12 in TIDC from EG-7 tumor-bearing WT C57BL/6 mice (Fig. 5). However, TIDC from EG-7 tumors implanted in IL-10 KO mice displayed robust IL-12 production in response to CpG alone (Fig. 5). The vast majority of TIDC were $CD11c_{low}CD11b^+$ and this dendritic cell subset was responsible for IL-12 production in response to CpG, as previously described (19). We also examined whether the impairment of TIDC to produce IL-12 in C57BL/6 mice could be mediated by T_{reg} cells. We observed that TIDC from RAG-KO mice, deficient for all T cells including T_{reg}, required anti-IL-10R treatment to achieve optimal CpG-induced IL-12 secretion (Fig. 5). Thus, the ability of the tumor environment to inhibit TIDC IL-12 production requires endogenous IL-10 but it is not mediated by T_{reg}.

Endogenous interleukin-10 and T_{reg} cells inhibit tumor-infiltrating dendritic cell–mediated tumor-antigen specific CD8 T-cell responses. We first verified that in our model, TIDC could prime tumor-specific CD8 T cells. We showed, *in vitro*, that TIDC isolated from EG-7 but not from EL-4 tumors were efficient in inducing the proliferation of OVA-specific naive OT-I CD8 T cells (Fig. 6*A*). The presentation of OVA class I peptide was due to TIDC and not to contaminating EG-7 cells, because adding increasing numbers of EG-7 cells to EL-4 TIDC did not induce CD8 T-cell proliferation (Fig. 6*B*). These results indicate that TIDC were capable of presenting to naive CD8 T cells tumor antigens acquired *in vivo* from tumor cells. In addition, *in vitro* activation of TIDC with CpG in the presence of anti-IL-10R antibody further increased the proliferation of OT-I cells (Fig. 6*A*).

Furthermore, EG-7 TIDC isolated from IL-10 KO mice or mice treated with CD25 antibody induced more efficiently the proliferation of naive OT-I cells than those isolated from WT C57B/6 mice (Fig. 6*C, black columns*). In each condition, *in vivo* activation of TIDC by CpG treatment 2 hours before isolation improved their priming capacity *in vitro* (Fig. 6*C, white columns*).

Following injection in naive mice reconstituted with OT-I cells, only TIDC isolated from WT C57B/6 mice treated *in vivo* with CpG 2 hours before isolation induced IFN-γ production. The ability to induce IFN-γ production was enhanced in IL-10 KO mice or CD25 antibody–treated WT mice and was highest with TIDC isolated from CD25 antibody–treated IL-10 KO mice (Fig. 6*D*).

Thus, both endogenous IL-10 and T_{reg} cells impaired the ability of TIDC to induce antigen-specific CD8 T-cell responses. Whereas IL-10 and T_{reg} cells were redundant in suppressing the ability of

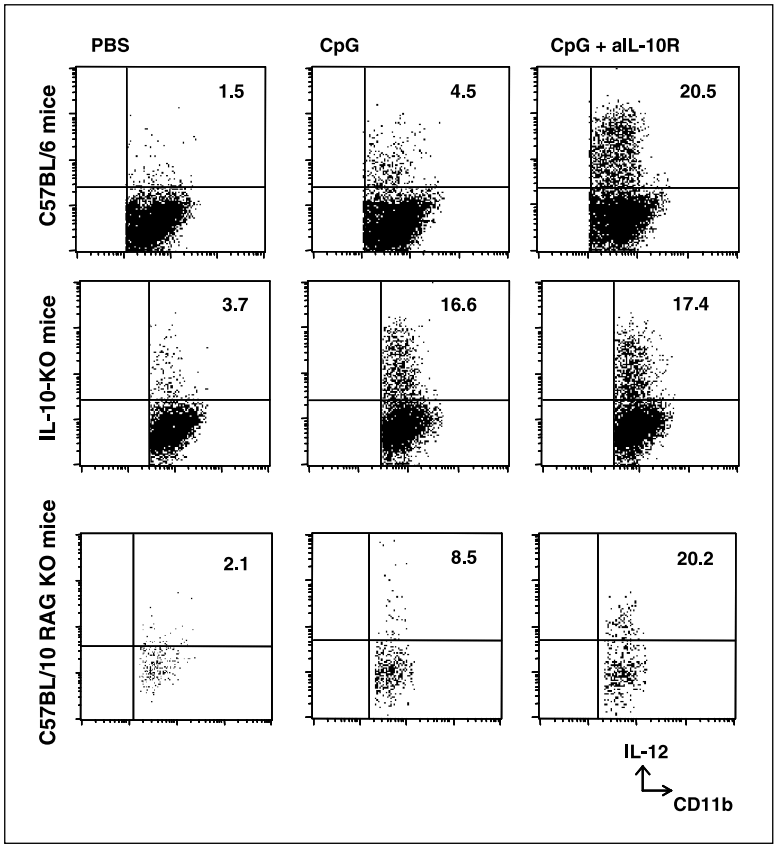

Figure 5. Lack or neutralization of IL-10 enhances TIDC IL-12 secretion. TIDC enriched from C57BL/6, IL-10 KO, or C57BL/10 RAG KO EG-7 tumor-bearing mice were cultured 24 hours with medium, 10 μg/mL CpG 1668, or CpG plus 10 μg/mL anti-IL-10R antibody. The ability of TIDC to produce IL-12 was analyzed by intracellular staining for IL-12 p40/p70 together with surface CD11b staining (log scale). Percentages of $CD11b^+$ cells expressing intracellular IL-12 (*top right quadrant*). Representative of two similar experiments.

TIDC to induce CD8 T-cell proliferation, they seemed to act synergistically in suppressing the ability of activated TIDC to induce IFN-γ production *in vivo*. Although the level of IL-12 production is likely to be one of the factors determining the ability of TIDC to induce IFN-γ production, the mechanism(s) through which TIDC induced a better proliferative effect remain(s) to be determined because the expression of costimulatory molecules such as CD40, CD80, and CD86 by TIDC was not significantly modified (data not shown).

Discussion

Many factors are responsible for the ability of tumors to escape the immune response. We showed here that both IL-10 and T_{reg} cells play major roles in antitumor CD8 T-cell immune suppression, in particular by acting in concert to inhibit TIDC functions.

In the absence of IL-10, tumor-specific CD8 T-cell proliferation and function as well as the ability of TIDC to prime naive CD8 T cells were dramatically enhanced. Indeed, better proliferation or function was observed even in the absence of activation. This was ascribed to the artificially high frequency of TAA-specific CD8 T cells in adoptively transferred animals. Our observations are in line with the broad immunosuppressive functions of IL-10 (reviewed in ref. 30). However, the remarkable restoration of TIDC and antitumor CD8 T-cell functions in IL-10 KO mice suggests that IL-10 has a much more central and unique role than previously hypothesized. The reversion of the suppression in IL-10 genetically deficient mice bearing IL-10-sufficient tumors suggests that endogenous and not tumor-derived IL-10 is responsible for immunosuppression, at least in our model.

Depletion of T_{reg} cells had beneficial effects similar to IL-10 deficiency or neutralization when treating tumor-bearing mice with CpG. Because IL-10 was shown to allow the development and support the functions of the Tr1 subset of T_{reg} cells (reviewed in ref. 7), it was tempting to hypothesize that IL-10 was playing a major role in T_{reg} immune suppression in our model. However, our data showed that the immunosuppressive functions of T_{reg} and IL-10 were not superimposable. This was best exemplified by the fact that endogenous IL-10 was able to inhibit TIDC IL-12 secretion in a T_{reg}-independent way, whereas both IL-10 and T_{reg} depletion increased the capacity of TIDC to prime CD8 effector T cells. Furthermore, results obtained in RAG-KO mice suggest that non-T cells such as tumor-associated macrophages or TIDC could be the main source of immunosuppressive IL-10 (reviewed in refs. 30, 31). Intriguingly, we observed lower numbers of IFN-γ secreting cells and, possibly, lower accumulation of transferred OT-I CD8 T cells at day 7 than at day 3 in IL-10 KO mice. Although this could be related to a migration of tumor antigen–specific cells outside the TDLN, we did not observe an increase of the transferred OT-I CD8 T cells within the tumor (data not shown). Another possible explanation is that IL-10, whereas detrimental to the initiation of the CD8 T-cell response, would help to maintain it at later times, as shown elsewhere (32).

Although not affecting TIDC capacity to produce IL-12, we showed that T_{reg} suppress the capacity of TIDC to induce effector CD8 T cells *in vitro* and *in vivo*. Indeed, human $CD25^+$ T_{reg} have been shown to decrease the capacity of dendritic cells to stimulate allogeneic naive T cells (12). The induction of the expression of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) via CTLA4/B7 interactions in dendritic cells has been proposed as

a mechanism to explain the propagation of T_{reg} activity, including in the tumor setting. Indeed, CTLA4 provided by Tr1 cells can up-regulate IDO expression in dendritic cells, whereas IDO-expressing dendritic cell–induced T_{reg} (33–35). We have observed by immunohistochemistry that TIDC express IDO, in WT as well as in IL-10 KO mice (data not shown), and we are currently investigating IDO expression in TIDC according to the different treatments.

Depletion of $CD25^+$ cells, including $CD4^+$ and possibly $CD8^+$ T_{reg}, increased both the *in vivo* proliferation of tumor antigen–specific CD8 T cells and their capacity to differentiate into effector cells producing IFN-γ and lysing tumor cells. Besides inhibiting TIDC functions, T_{reg} cells could directly act on both the proliferation and functional activation of CD8 T cells through mechanisms distinct from IL-10 such as TGF-β and CTLA4 (reviewed in refs. 2, 36), although it has been suggested that part of the tumor immunosuppressive effects of CTLA4 could be attributed to IL-10 (37).

Our results identified a novel mechanism of tumor escape: the *de novo* generation of tumor-induced regulatory CD8 T cells that are specific for an antigen expressed only in the tumor. The suppressive effect of these CD8 T cells was mediated by TGF-β but not by IL-10 or CTLA4. Both endogenous IL-10 and $CD25^+$ T_{reg} cells were involved in the differentiation of these CD8 regulatory cells. Although most studies on T_{reg} cells are restricted to CD4 T cells (reviewed in ref. 2), there are also several examples of regulatory CD8 T cells. (38). $CD8^+$ T_{reg} cells expressing the Foxp3 transcription factor thus resembling "natural" $CD4^+$ T_{reg} cells, were described in rats (39), but we did not observe Foxp3 expression in our suppressor CD8 T cells by PCR (data not shown). In humans, non-antigen-specific regulatory CD8 T cells can be generated from $CD28^-$ CD8 T cells in a process involving IL-10 (40). Interestingly, we have observed that the nonproliferating fraction of OT-I cells parked in EG-7 tumor-bearing mice were mostly $CD28^-$. Antigen-specific CD8 T_{reg} cells can also be induced, as it was shown following injection of antigen-loaded immature dendritic cells into human volunteers (41). Our tumor model is also reminiscent of chronic hepatitis C virus infection, in which virus-specific liver CD8 T cells can suppress T-cell responses via IL-10.

Thus, our tumor therapy model points to the multiplicity of mechanisms through which immune tolerance to tumors may arise and propagate. The initiation of effector CD8 T cells by TIDC may be impeded by the presence of "natural" T_{reg} and/or the presence of IL-10 in the tumor milieu. Consequently, TIDC in the presence of IL-10 may induce regulatory CD8 T cells and possibly other T_{reg} such as Tr1 that will further inhibit the response, leading to infectious immune tolerance. Particular subsets of dendritic cells have been suggested to be able to induce Tr1 cells (42), but those cells were characterized by high CD45RB expression, unlike TIDC in our system (data not shown). We are currently investigating the possible differentiation of Tr1 cells using TIDC.

Toll-like receptor-mediated signals to dendritic cells, at least in part acting through IL-6 release, have been shown to be able to prevent the T suppressive activity of T_{reg} (43) and break T_{reg}-mediated CD8 tolerance *in vivo* (44). Moreover, CpG activation, as well as CD40L ligation, can release dendritic cells from the suppressive activity of T_{reg} to some extent (45). In the tumor setting, however, Toll-like receptor signals may not be sufficient to fully alleviate T_{reg} activity (31). Our results show that whereas CpG alone has little antitumor activity, it can induce tumor rejection when IL-10 or T_{reg} are blocked. However, even when IL-10 was blocked and T_{reg} deleted, treatment with CpG was still needed to achieve tumor rejection. We observed that CpG activation was required to detect some level of IFN-γ-secreting CD8 T cells following transfer of TIDC into WT mice, whereas these cells were readily detectable in the TDLN of IL-10 K0 or CD25-depleted EG-7

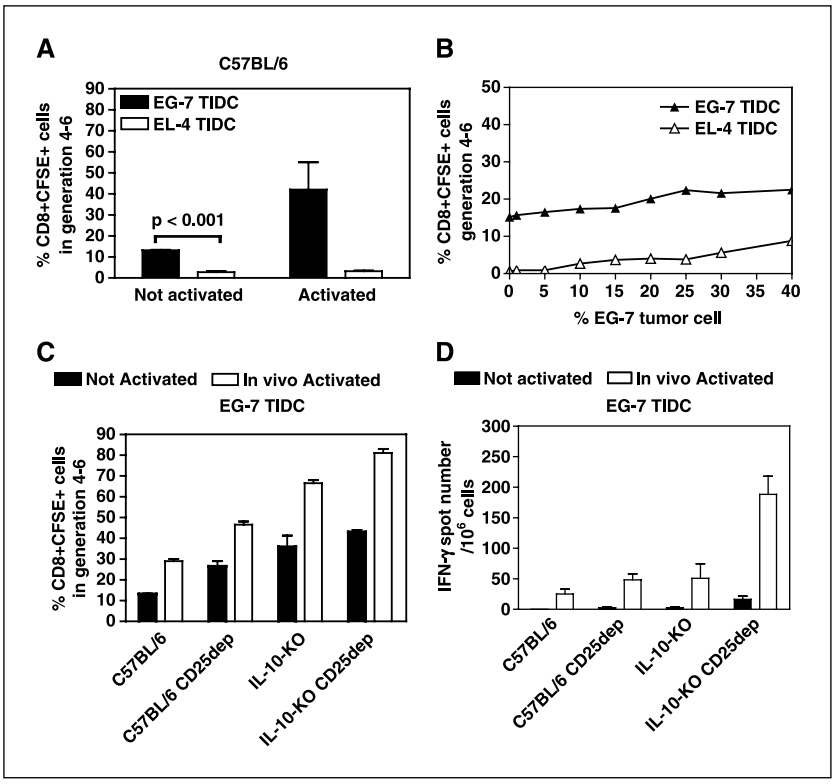

Figure 6. EG-7 TIDC present tumor-derived MHC class I peptide antigen but endogenous IL-10, $CD25^+T_{reg}$ and lack of activation inhibit TIDC-mediated tumor-antigen specific CD8 T-cell responses. *A,* irradiated EG-7 or EL-4 TIDC from C57BL/6 were cultured in the presence of CFSE-labeled naive OT-I T cells and proliferation assessed by the analysis of CFSE expression and determination of the percentage of cells in each generation as described in Materials and Methods. *A,* mean percentage of triplicate cultured cells in generations 4 to 6 after stimulation with EG-7 (*black columns*) or EL-4 (*open columns*) TIDC, without (*left*) or with *in vitro* activation with CpG and anti-IL-10R antibody; *bars,* ±SE. Representative of three separate experiments. *B,* percentage of $CFSE^+$ cells in generations 4 to 6 in the presence of a fixed number of EG-7 (▲) or EL-4 (△) TIDC and increasing proportions of irradiated EG-7 tumor cells. *C-D,* TIDC recovered from CpG-treated (*open columns*) or not (*black columns*) C57BL/6 or IL-10 KO EG-7 tumor-bearing mice, with or without *in vivo* CD25 depletion (as indicated), were analyzed for their capacity to prime antigen-specific T-cell responses. CpG treatment was done 2 hours before TIDC isolation. *C,* analysis of naive OT-I cell proliferation *in vitro* based on CFSE expression as described in Materials and Methods. *Columns,* mean percentage of $CFSE^+$ cells in generation 4 to 6 from three individual experiments; *bars,* ±SE. *D,* analysis by ELISPOT of the capacity of TIDC to induce OVA-specific IFN-γ-producing cells following injection in mice adoptively reconstituted 1 day before TIDC injection with naive OT-I cells. *Columns,* mean IFN-γ spot number in TIDC injection-draining lymph nodes as described in Materials and Methods from two separate experiments with three individual mice in each; *bars,* ±SE.

tumor-bearing mice. TIDC activated *in vivo* with CpG were, however, consistently more active than nonactivated TIDC in inducing IFN-γ-secreting CD8 T cells when adoptively transferred. This is in part explained by the fact that CpG activation is required to obtain high levels of IL-12 secretion by TIDC. In other tumor models we have shown that the antitumor effect of the CpG plus anti-IL-10R antibody combination could be ascribed to both CD8 T cells and natural killer cells and that mechanisms of innate resistance and adaptive immunity are sequentially playing an essential role in the tumor eradication obtained with this treatment (19, 46). Thus, we conclude that T_{reg} and anti-IL-10, through both distinct and overlapping mechanisms, prevent the development of an effective CD8 T-cell antitumor response in tumor-bearing mice, as well as the CpG-induced antitumor innate and adaptive immune responses. Such results have strong implications for the design and implementation of successful cancer immune therapies.

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