

## CD8<sup>+</sup> Foxp3<sup>+</sup> Regulatory T Cells Mediate Immunosuppression in Prostate Cancer

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**Abstract** **Purpose:** Although elevated proportions of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells have been shown in several types of cancers, very little is known about the existence and function of CD8<sup>+</sup> Treg cells in prostate cancer. In this study, we investigated prostate tumor–derived CD8<sup>+</sup> Treg cells and their function. **Experimental Design:** Tumor-infiltrating lymphocytes (TIL) from fresh tumor specimens of patients with prostate cancer were generated and subjected to phenotypic and suppressive function analyses. In particular, we investigated the role and function CD8<sup>+</sup> Treg cells in prostate cancer. **Results:** We show that high percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells are probably present in the majority (70%) of prostate TILs. Remarkably, both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subpopulations possessed potent suppressive activity. T-cell cloning and fluorescence-activated cell sorting analyses showed the presence of CD8<sup>+</sup>CD25<sup>+</sup> Treg cell clones that expressed FoxP3 and suppressed naïve T-cell proliferation, in addition to the previously known CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. These CD8<sup>+</sup> Treg cells suppressed naïve T-cell proliferation mainly through a cell contact–dependent mechanism. Importantly, the suppressive function of CD8<sup>+</sup> Treg cells could be reversed by human Toll-like receptor 8 (TLR8) signaling. **Conclusion:** Our study shows that like CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, CD8<sup>+</sup> Foxp3<sup>+</sup> Treg cells present in prostate tumor–derived TILs suppress immune responses and that their suppressive function can be regulated by TLR8 ligands, raising the possibility that the manipulation of Treg cell function by TLR8 ligands could improve the efficacy of immunotherapy for prostate cancer patients.

Prostate cancer is the most common cancer among men in the United States and the second most common malignant cause of male deaths in the United States. There is currently no effective therapy for late-stage disease, whereas surgery or radiation therapy is the only choice for early-stage disease. Immunotherapy affords a promising approach to the treatment of various types of cancer, including prostate cancer (1–5). Although peptide- or dendritic cell–based vaccines can induce antigen-specific immune responses, objective clinical responses remain

infrequent and transient (3, 6). A possible explanation is that tumor cells may create an immunosuppressive environment in cancer patients. Thus, a better understanding of the interaction between tumor-infiltrating immune cells and cancer cells is critical to efforts to devise strategies that would enhance the therapeutic efficacy of immunologic interventions.

Recent studies indicate that preexisting CD4<sup>+</sup> regulatory T (Treg) cells at tumor sites may pose major obstacles to effective cancer immunotherapy, as these cells have a potent ability to suppress host immune responses (7–9). Indeed, increased proportions of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in the total CD4<sup>+</sup> T-cell populations have been documented in patients with different types of cancers, including lung, breast, and ovarian tumors (10–12). Our recent findings further show the presence of antigen-specific CD4<sup>+</sup> Treg cells at tumor sites, where they induce antigen-specific and local immune tolerance (7, 8). The removal or elimination of Treg cell populations with anti-CD25 monoclonal antibody (mAb) treatment results in effective rejection of transplanted tumors in animal models (13), further suggesting a functional role for these Treg cells in tumor progression and immunosuppression.

Because Treg cell–mediated immunosuppression exists at tumor sites, a new strategy for depletion of Treg cells or reversal of the suppressive function of Treg cells will be important in efforts to induce antigen-specific effector T cells. Thus, we recently showed that Toll-like receptor 8 (TLR8) ligands can specifically reverse the suppressive function of both antigen-specific and

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naturally occurring Treg cells (14). Treatment of Treg cells with polyguanosine (poly-G) oligonucleotides enhanced anti-tumor immunity in an animal model, but whether TLR8 signaling pathway can also control the suppressive function of other Treg cells, such as CD8<sup>+</sup> Treg and  $\gamma\delta$ <sup>+</sup> Treg cells, remains unclear.

A major obstacle to characterize immune components involved in prostate cancer has been the difficulty of growing tumor-infiltrating lymphocytes (TIL) from fresh tumor samples and the inability to determine the identity and prevalence of Treg cell subsets in prostate cancer. We hypothesize that prostate tumor cells may recruit and facilitate the accumulation of Treg cells, which, in turn, suppress antitumor immunity and promote tumor growth. The presence of high percentages of Treg cells at tumor sites may also be partially responsible for the poor growth of T cells associated with prostate tumors. Although a recent study shows that a high percentage of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were present in prostate cancer (15), it is not clear whether other subsets of Treg cells, such as CD8<sup>+</sup>, NKT, or  $\gamma\delta$  Treg cells, also play a role in mediating immunosuppression. In this study, we show that the majority (70%) of prostate TILs (PTIL) analyzed contained elevated proportions of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the total T-cell population. Besides CD4<sup>+</sup> T cells, we identified CD8<sup>+</sup> T-cell subpopulation that also had potent suppressive activity. T-cell cloning analysis confirmed the presence of CD4<sup>+</sup>CD25<sup>+</sup> FoxP3<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup> FoxP3<sup>+</sup> Treg cell clones in bulk PTIL lines. These Treg cells suppress immune responses mainly through a cell contact-dependent mechanism, although some inhibited naïve T-cell proliferation via unknown soluble factors [other than interleukin (IL)-10 and transforming growth factor- $\beta$ ]. The suppressive function of CD8<sup>+</sup> Treg cells could be reversed by human TLR8 signaling, suggesting that the reversal of their suppressive function by TLR8 ligands may improve the efficacy of immunotherapy for prostate cancer.

## Materials and Methods

**Generation of tumor-infiltrating T cells and T-cell cloning.** Prostate cancer tissues or melanoma samples were minced into small pieces followed by digestion with triple-enzyme mixture containing collagenase type IV, hyaluronidase, and DNase for 2 h at room temperature. After digestion, the cells were washed twice in RPMI 1640 and cultured in RPMI 1640 containing 10% human serum supplemented with L-glutamine and 2-mercaptoethanol and 1,000 U/mL of IL-2 for the generation of T cells over 2 to 3 weeks. Experiments for human materials and tumor sample collection is conducted under the institutional review board protocol (H-9086) approved by Baylor College of Medicine Institutional Review Board committee. T-cell clones were generated from TILs by the limiting dilution cloning method, as previously reported (7). T-cell clones were transferred to fresh 96-well plates and used in a functional assay to determine their ability to inhibit naïve T-cell proliferation. Some T-cell clones with suppressive activity were selected for further analyses. 1359 1E3 T cells generated from melanoma TIL 1359 suppressed naïve T-cell proliferation and served as a control for CD4<sup>+</sup> Treg cells.

**Fluorescence-activated cell sorting analysis of CD25 and GITR.** CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations were purified with specific antibody-coated beads. The expression of CD25 and GITR on Treg cells was determined by fluorescence-activated cell sorting (FACS) analysis after staining with specific antibodies (purchased from R&D Systems and BD Biosciences), as previously described (7, 8).

**Proliferation assays and transwell experiments.** CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $2 \times 10^5$ ) purified from human peripheral blood mononuclear cells (PBMC) by antibody-coated beads (Miltenyi Biotec) were cultured for 60 h in U-bottomed 96-well plates containing  $5 \times 10^4$  CD3-depleted antigen-presenting cells, 0.1  $\mu$ g/mL anti-CD3 mAb, and different numbers of CD4<sup>+</sup> regulatory or effector T cells. The proliferation of responder T cells was determined by the incorporation of [<sup>3</sup>H]thymidine for the last 16 h of culture, as previously described (7, 8). Cells were harvested, and the radioactivity was counted in a scintillation counter. All experiments were done in triplicate. Transwell experiments were done in 24-well plates with a pore size of 0.4  $\mu$ m (Corning Costar).

**PCR analysis of FoxP3 and TLR8.** Total RNA was extracted from  $1 \times 10^7$  T cells using TRIzol reagent (Invitrogen, Inc.). A SuperScript II reverse transcription kit (Invitrogen) was used to perform reverse transcription, in which 20  $\mu$ L of the reverse transcription mixture, containing 2  $\mu$ g of total RNA, were incubated at 42°C for 1 h. FoxP3 mRNA levels were quantified by real-time PCR using ABI/PRISM7000 sequence detection system (PE Applied Biosystems, Inc.). The PCR reaction was done with primers and an internal fluorescent TaqMan probe specific for FoxP3 or HPRT, all purchased from PE Applied Biosystems. FoxP3 mRNA levels in each sample were normalized with the relative quantity of HPRT. All samples were run in triplicate. For analysis of TLR8, we did PCR analysis with the TLR8 forward primer, 5'-TTTCCCACC-TACCCTCTGGCTT-3', and the reverse primer, 5'-TGCTCTGCATGAGGTTGTCGGATGA-3'. The primers for reverse transcription-PCR of Foxp3 were Foxp3-5P, 5'-GCCCTTGGACAAGACCCGATG-3' and Foxp3-3P, 5'-CATTGCCCAGCAGTGGGTAGGAG-3'. PCR amplification for glyceraldehyde 3-phosphate dehydrogenase served as a PCR control (forward primer, 5'-CGAGATCCCTCCAAAATCAA-3'; reverse primer, 5'-TGTGGT-CATGAGTCCTTCCA-3').

**Immunohistochemical staining of tumor tissues.** Prostate cancer tissues and normal prostate tissues were stained with mouse anti-human CD8 (1:10 dilution, BD PharMingen) and goat anti-human Foxp3 IgG (1:100 dilution, Novus Biologicals), followed by Alexa Fluor 633-conjugated goat anti-mouse IgG and Alexa Fluor 488 donkey anti-goat IgG (2  $\mu$ g/mL; Molecular Probes). Immune staining-positive cells were detected with a fluorescence confocal microscopy.

**Toll-like receptor ligands and proliferation assays.** Naïve CD4<sup>+</sup> T cells were purified from PBMCs by use of microbeads (Miltenyi Biotec). Naïve CD4<sup>+</sup> T cells ( $10^5$ /well) were cultured with Treg cells at a ratio of 10:1 in OKT3 (2  $\mu$ g/mL)-coated, U-bottomed 96-well plates containing the following ligands. Lipopolysaccharide (100 ng/mL), imiquimod (10  $\mu$ g/mL), loxoribine (500  $\mu$ mol/L), copolymer of polyinosinic and polycytidylic acids (25  $\mu$ g/mL), ssRNA40/LyoVec (3  $\mu$ g/mL), ssRNA33/LyoVec (3  $\mu$ g/mL), pam3CSK4 (200 ng/mL), and flagellin (10  $\mu$ g/mL) were all purchased from Invivogen. CpG-A (3  $\mu$ g/mL), CpG-B (3  $\mu$ g/mL), and poly-G oligonucleotides (3  $\mu$ g/mL) were synthesized by Integrated DNA Technologies.

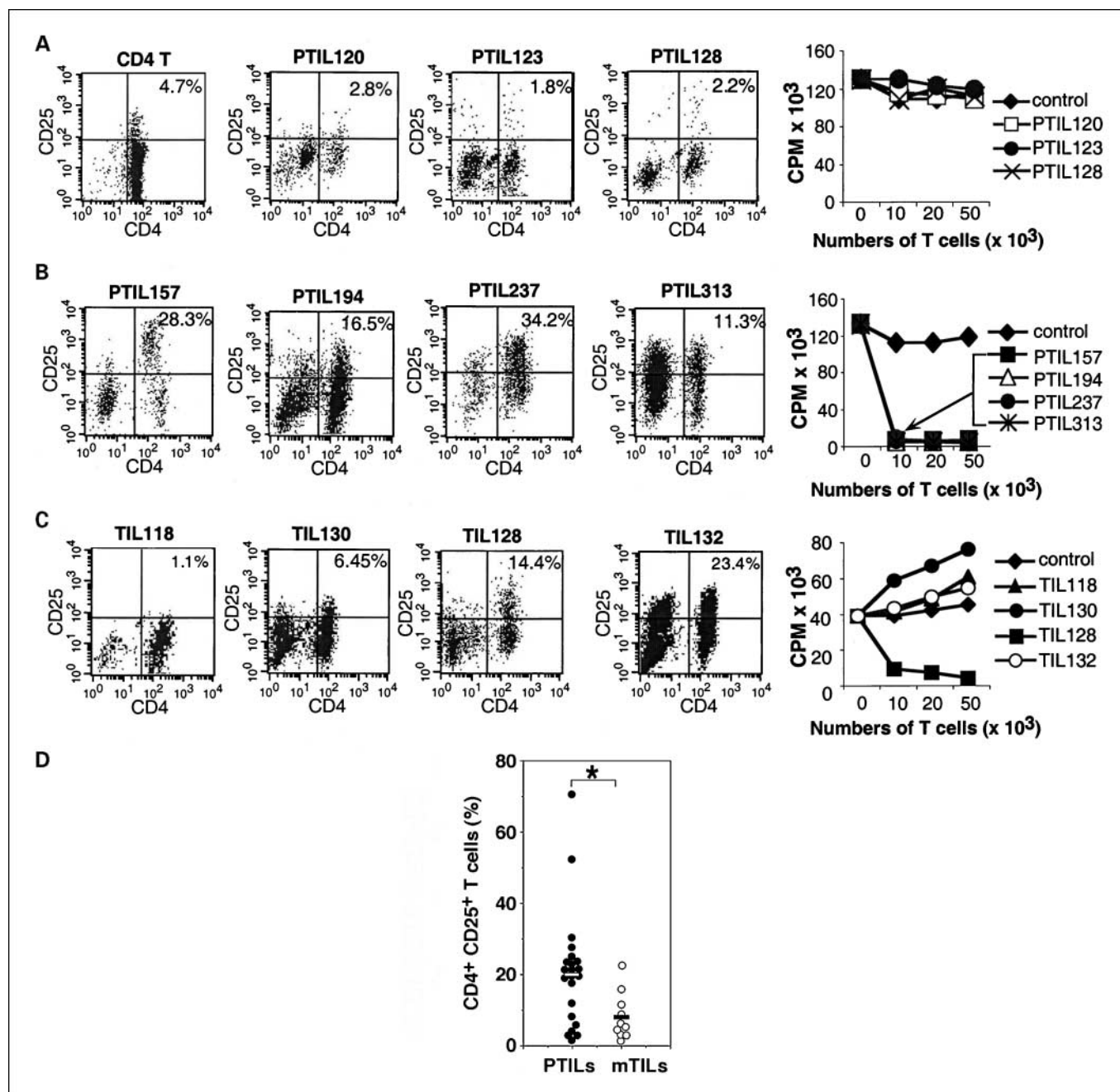
## Results

**Treg cells in prostate cancer.** Increased percentages of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells have been found in several types of cancers; however, very little is known about Treg cells in human prostate cancer. We therefore established 52 TIL cell lines from 200 prostate tumor samples and maintained them in culture for 3 to 4 weeks to obtain enough number of cells for further analysis. FACS analysis of 22 TIL lines identified two discrete subsets based on the expression of CD4 and CD25 molecules. Six (28%) of 22 TILs contained <5% CD4<sup>+</sup>CD25<sup>+</sup> T cells in the total T-cell population, and did not produce a suppressive effect on naïve T-cell proliferation, while the remaining 16 TILs (72%), including PTIL157, PTIL194, PTIL237, and PTIL313, contained elevated percentages (11-77%) of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the total T-cell population, and showed a potent

ability to suppress naïve T-cell proliferation. Representative data are shown in Fig. 1A and B.

Because melanoma is a relatively immunogenic human cancer and the associated TILs are relatively easy to grow in culture, we decided to compare the percentages of Treg cells from prostate tumors with those from melanoma. Of 10 melanoma-derived TILs, three showed an increased propor-

tion of CD4<sup>+</sup>CD25<sup>+</sup> T cells; the remaining melanoma-derived TILs contained low or normal percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the total T-cell population. In contrast to the suppressive activity of bulk prostate TILs with a high percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells, most melanoma TILs, regardless of percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells, did not have a suppressive effect on naïve T-cell proliferation (Fig. 1C). The mean



**Fig. 1.** FACS and functional analysis of bulk TILs derived from prostate cancers. *A*, low percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells in PTIL120, PTIL123, and PTIL128. Prostate tumor-derived TILs were stained with FITC-conjugated anti-CD4 mAb and phycoerythrin-conjugated anti-CD25 mAb; healthy donor-derived T cells served as a control. PTIL120, PTIL123, and PTIL128 contained low percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells and did not suppress the proliferative response of naïve CD4<sup>+</sup> T cells. *B*, high percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells in PTIL157, PTIL194, PTIL237, and PTIL313. These PTILs suppressed the proliferative response of naïve CD4<sup>+</sup> T cells, whereas control CD4<sup>+</sup> T cells did not. *C*, analysis of CD4<sup>+</sup>CD25<sup>+</sup> T cells in melanoma-derived TILs. Most melanoma-derived TILs contained low percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells and did not suppress the proliferative response of naïve CD4<sup>+</sup> T cells. *D*, percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the total T-cell population of prostate tumor samples ( $n = 22$ ) was significantly higher than that of melanoma ( $n = 10$ ; \*,  $P < 0.01$ ).

frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells in melanoma-derived TILs was significantly lower than that in prostate tumor-derived TILs (Fig. 1D). These results suggest that the majority of prostate cancer-derived TILs, but only a small percentage of melanoma-derived TILs, contained elevated proportion of CD4<sup>+</sup>CD25<sup>+</sup> T cells and exhibited suppressive activity, which may explain why melanoma-derived T cells are relatively easy to grow and expand *in vitro*.

**Suppression of naïve T-cell proliferation by CD4<sup>+</sup> and CD8<sup>+</sup> subsets of Treg cells.** To determine the subsets of Treg cells responsible for the observed suppression of naïve T-cell proliferation, we selected four bulk TIL cell lines (PTIL157, PTIL194, PTIL237, and PTIL313) for further analysis. CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subpopulations were purified from bulk T-cell lines with anti-CD4 or anti-CD8 antibody-coated magnetic beads and tested for their ability to inhibit the proliferation of naïve CD4<sup>+</sup> T cells. As expected, CD4<sup>+</sup> T-cell population showed a marked suppressive effect; however, we also found that CD8<sup>+</sup> T populations isolated from four TILs were suppressive, suggesting that the purified CD8<sup>+</sup> T-cell population contained CD8<sup>+</sup> Treg cells (Fig. 2A).

To determine whether CD4<sup>+</sup> and CD8<sup>+</sup> subsets of Treg cells express Foxp3, we did real-time PCR to evaluate the mRNA expression level of *FoxP3* in CD4<sup>+</sup> and CD8<sup>+</sup> populations. Both CD4<sup>+</sup> and CD8<sup>+</sup> subsets of PTIL194 and PTIL237 cells expressed *FoxP3* much higher than the control effector T cells (Fig. 2B). 1359 1E3 clone served as a positive control for *FoxP3* expression in Treg cells. Bulk TIL lines and Treg cell clones secreted a large amount of IFN- $\gamma$ , but little or no IL-2, IL-4, or IL-10 after stimulation with anti-CD3 antibody (data not shown). To exclude the possibility that Foxp3 expression in PTIL CD4<sup>+</sup> and CD8<sup>+</sup> subsets is due to *in vitro* culture with IL-2, we isolated RNA from primary CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets from tumor samples before T-cell culture. Reverse transcription-PCR experiments showed that both primary CD4<sup>+</sup> and CD8<sup>+</sup> T cells also expressed Foxp3 (Fig. 2C). However, the expression level of Foxp3 in CD4<sup>+</sup> and CD8<sup>+</sup> subsets was lower than that in CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. This difference may be due to the fact that CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations contained Treg cells as well as effector T cells.

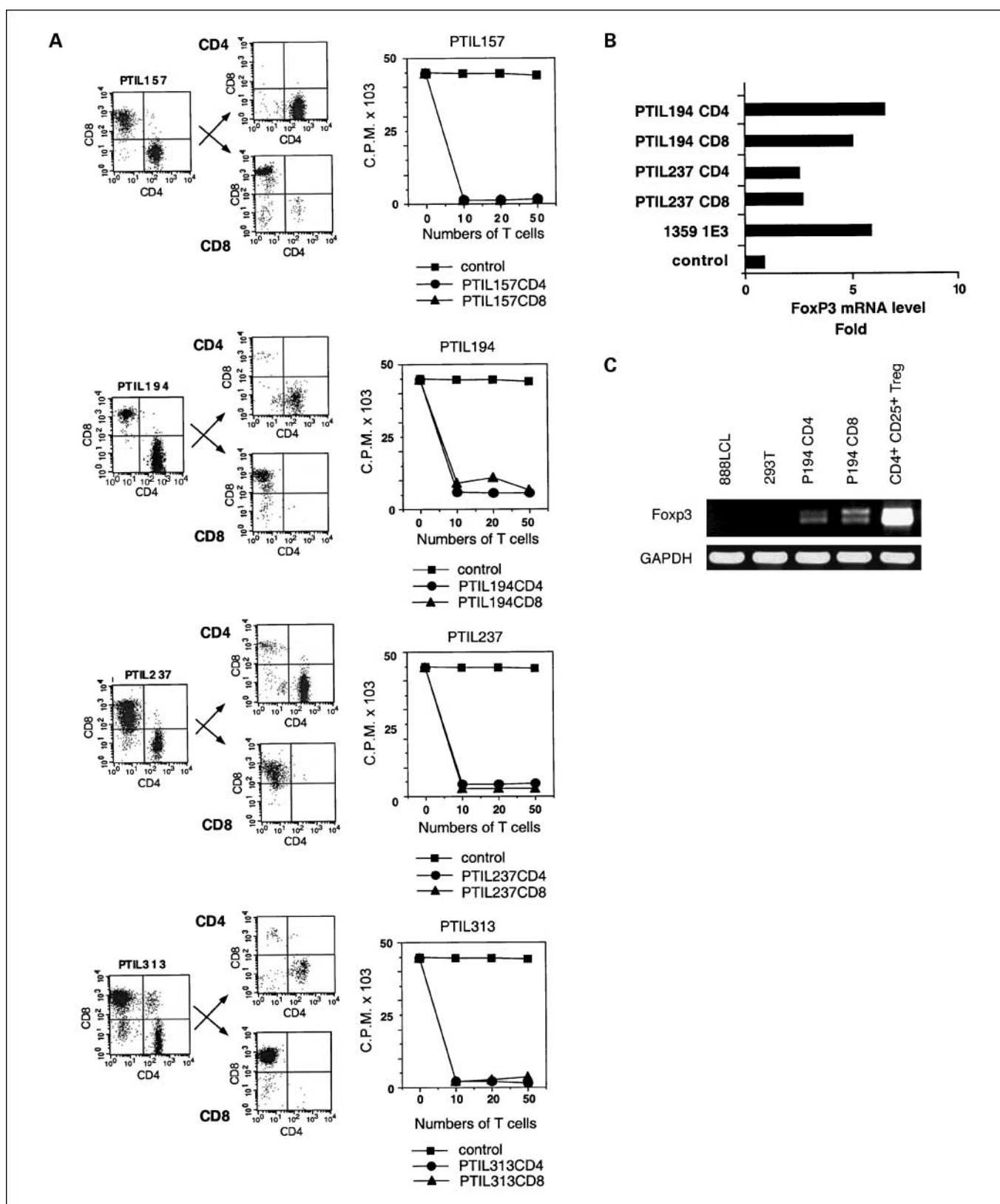
Although CD4<sup>+</sup> Treg cells have been shown to express Foxp3 at the RNA and protein levels, Foxp3 expression by CD8<sup>+</sup> T cells remains to be shown. To further confirm that Foxp3 protein was expressed in these prostate tumor-derived CD8<sup>+</sup> Treg cells, we did a FACS analysis of prostate tumor-derived T-cell lines PTIL194 and PTIL237 as well as PBMCs obtained from healthy donors and prostate cancer patients. Representative data in Fig. 3A show that PTIL194 and PTIL237 bulk lines contained 12.9% and 18% Foxp3<sup>+</sup> CD8<sup>+</sup> T cells, respectively. By contrast, the PBMCs of healthy donors and prostate cancer patients contained <0.1% Foxp3<sup>+</sup> CD8<sup>+</sup> T cells (Fig. 3B). As expected, Foxp3<sup>+</sup> CD4<sup>+</sup> T cells were readily detected in the PBMCs of healthy donors and prostate cancer patients but no differences were found in the percentages of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells among PBMCs, whether from healthy donors or prostate cancer patients (Fig. 3B). Similar results were obtained with additional PBMCs from five healthy donors and five prostate cancer patients (data not shown). In addition, the expression level of Foxp3 in CD8<sup>+</sup> Treg cells is comparable with that in CD4<sup>+</sup> Treg cells based on the fluorescence intensity of Foxp3 staining in PTIL194 and PTIL237 bulk lines (Fig. 3A),

which is consistent with reverse transcription-PCR data in Fig. 2C. These results suggest that despite the scant fraction of Foxp3<sup>+</sup> CD8<sup>+</sup> T cells in the PBMCs of healthy donors and prostate cancer patients, high percentages of Foxp3 and CD8<sup>+</sup> T cells are present in the prostate tumor-derived T-cell population.

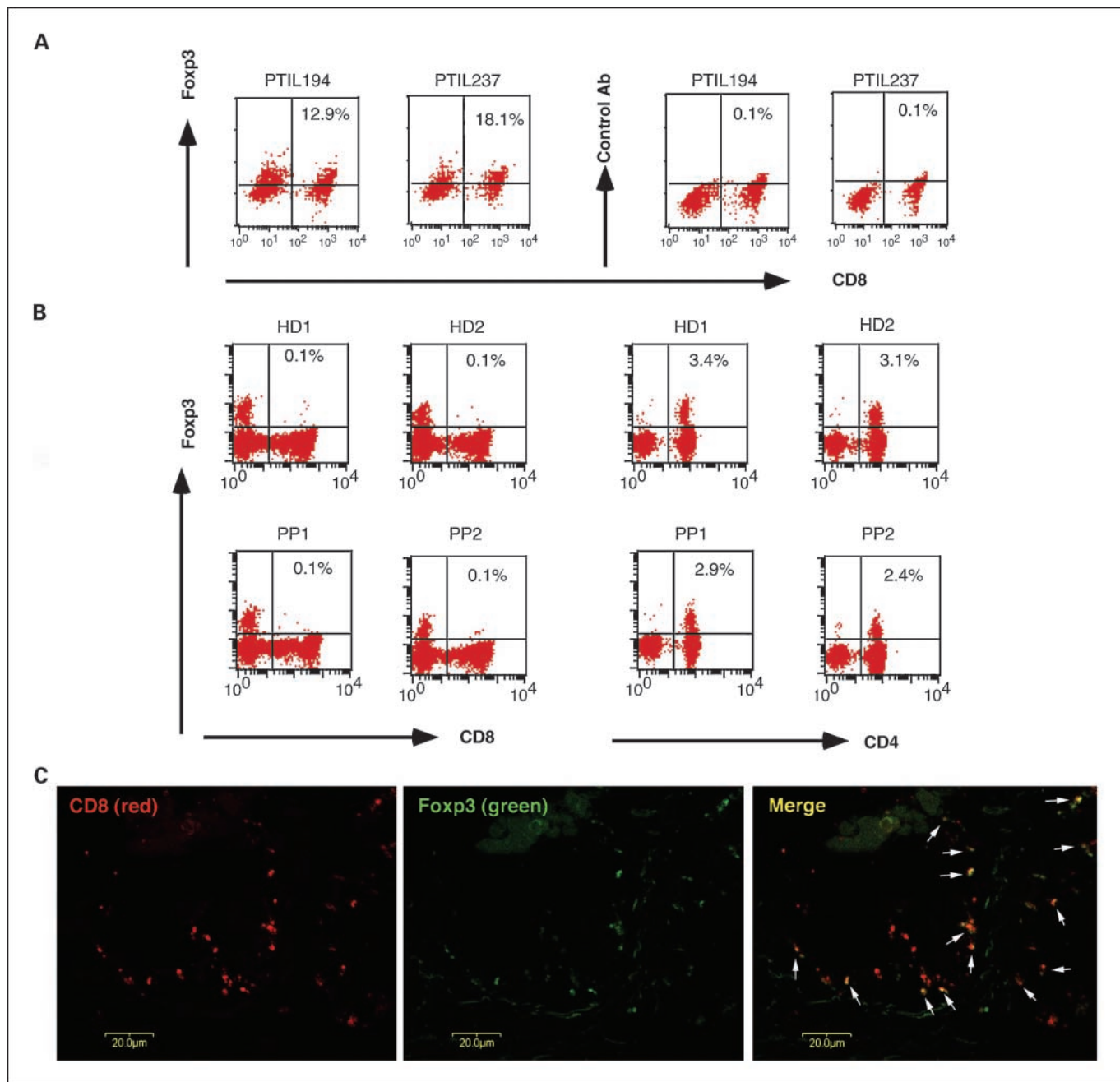
To provide direct evidence that tumor-derived CD8<sup>+</sup> T cells can express Foxp3 *in vivo*, we stained prostate tumor tissues with anti-CD8 and anti-Foxp3 antibodies and detected immunofluorescence with a confocal microscope. Representative tissues staining with specific antibodies are shown in Fig. 3C. CD8<sup>+</sup> T cells were readily detected in tumor tissues. Importantly, some of the CD8<sup>+</sup> T cells were also positive for Foxp3, whereas others were negative for Foxp3, suggesting that Foxp3<sup>+</sup> CD8<sup>+</sup> Treg cells and Foxp3<sup>-</sup> CD8<sup>+</sup> effector T cells coexist in tumor tissues. Similar results were obtained with tumor tissues obtained from several different prostate cancer patients, but not with normal prostate tissues (data not shown). Together, these results clearly indicate that prostate tumor-derived CD8<sup>+</sup> Treg cells express Foxp3 protein *in vitro* and *in vivo* and suppress the proliferation and function of effector T cells.

**Generation of CD8<sup>+</sup> Treg cell clones with suppressive function.** To show the presence of CD8<sup>+</sup> Treg cells in prostate cancer-derived TILs, we established T-cell clones from PTIL194 by limiting dilution cloning. More than 100 T-cell clones were obtained and analyzed for their ability to inhibit naïve T-cell proliferation in a functional assay. A representative set of data is shown in Fig. 4A. Among 94 T-cell clones, 51 had strong suppressive activity, whereas 43 had little or no suppressive activity. These results are consistent with data in Fig. 3C showing both Foxp3<sup>+</sup> and Foxp3<sup>-</sup> T cells present in prostate tissues. To determine the cell phenotype of clones with suppressive activity, we did FACS analysis. As expected, some of T-cell clones with a suppressive function were CD4<sup>+</sup> T cells that expressed CD25, GITR, CD122 (IL-2 receptor  $\beta$  chain), and Foxp3 molecules, whereas the remaining clones were the suppressive CD8<sup>+</sup> T cells that expressed CD25, CD122, and Foxp3, but were negative for GITR. Representative data for both CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells (two for each cell type) are shown in Fig. 4B. Nonsuppressive CD4<sup>+</sup> or CD8<sup>+</sup> T cells were negative for CD25 and Foxp3 molecules (data not shown). It has been reported that CCR4 is expressed in Treg cells (11). Interestingly, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells express CCR4 molecules, whereas CD8<sup>+</sup>CD25<sup>+</sup> Treg cells did not (Fig. 4B). It should be noted that the expression pattern of all CD4<sup>+</sup> Treg cells were the same, whereas all suppressive CD8<sup>+</sup> Treg cell clones were identical to those CD8<sup>+</sup> T-cell clones shown in Fig. 4B. FACS as well as real-time PCR analyses showed that both CD4<sup>+</sup> and CD8<sup>+</sup> Treg cell clones expressed Foxp3 at RNA and protein level (Fig. 4B and C). The expression level of Foxp3 in CD8<sup>+</sup>CD25<sup>+</sup> Treg cell clones was comparable with that in CD4<sup>+</sup>CD25<sup>+</sup> Treg cell clones. Similar results were obtained with Treg cell clones established from PTIL237 (data not shown). Taken together, we conclude that besides CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> T-cell clones, CD8<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> T-cell clones are present in PTILs and function as Treg cells for immunosuppression.

**Suppressive mechanisms of prostate tumor-derived CD8<sup>+</sup> Treg cells.** We next sought to determine the suppressive mechanisms of prostate tumor-derived CD8<sup>+</sup> Treg cells. Although both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations from PTIL194 and PTIL237 inhibited naïve CD4 T-cell proliferation in the



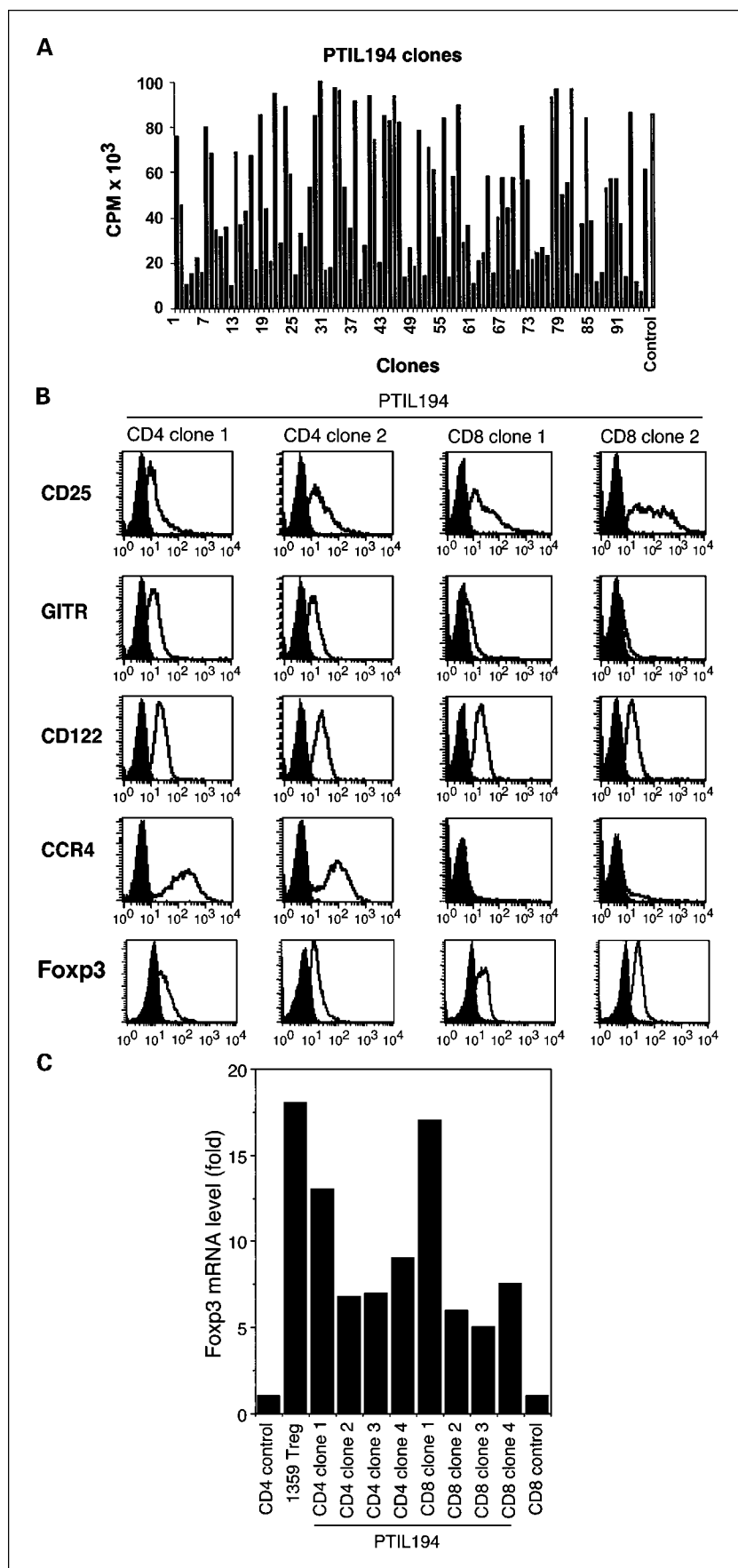
**Fig. 2.** Functional analysis and Foxp3 expression of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations. *A*, CD4<sup>+</sup> or CD8<sup>+</sup> T cells were purified from bulk PTIL lines with a bead-coated anti-CD4 or anti-CD8 antibody (*left panels*). The purity of CD4<sup>+</sup> or CD8<sup>+</sup> T cells was >95%. The suppressive function of each CD4<sup>+</sup> or CD8<sup>+</sup> T-cell population was tested for their ability to suppressive naïve T-cell proliferation (*right panels*). *B*, determination of Foxp3 mRNA in PTIL194 and PTIL237 by real-time PCR. HPRT served as an internal control. *C*, evaluation of Foxp3 expression in primary CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Reverse transcription-PCR was done using RNAs isolated from primary CD4<sup>+</sup> (P194 CD4) and CD8<sup>+</sup> T cells (P194 CD8) before T-cell expansion. Two isoforms of Foxp3 mRNA can be detected with the primers that are located in exons 1 and 3 of Foxp3 mRNA. 888LCL and 293T served as negative controls, whereas CD4<sup>+</sup>CD25<sup>+</sup> Treg cells served as a positive control.



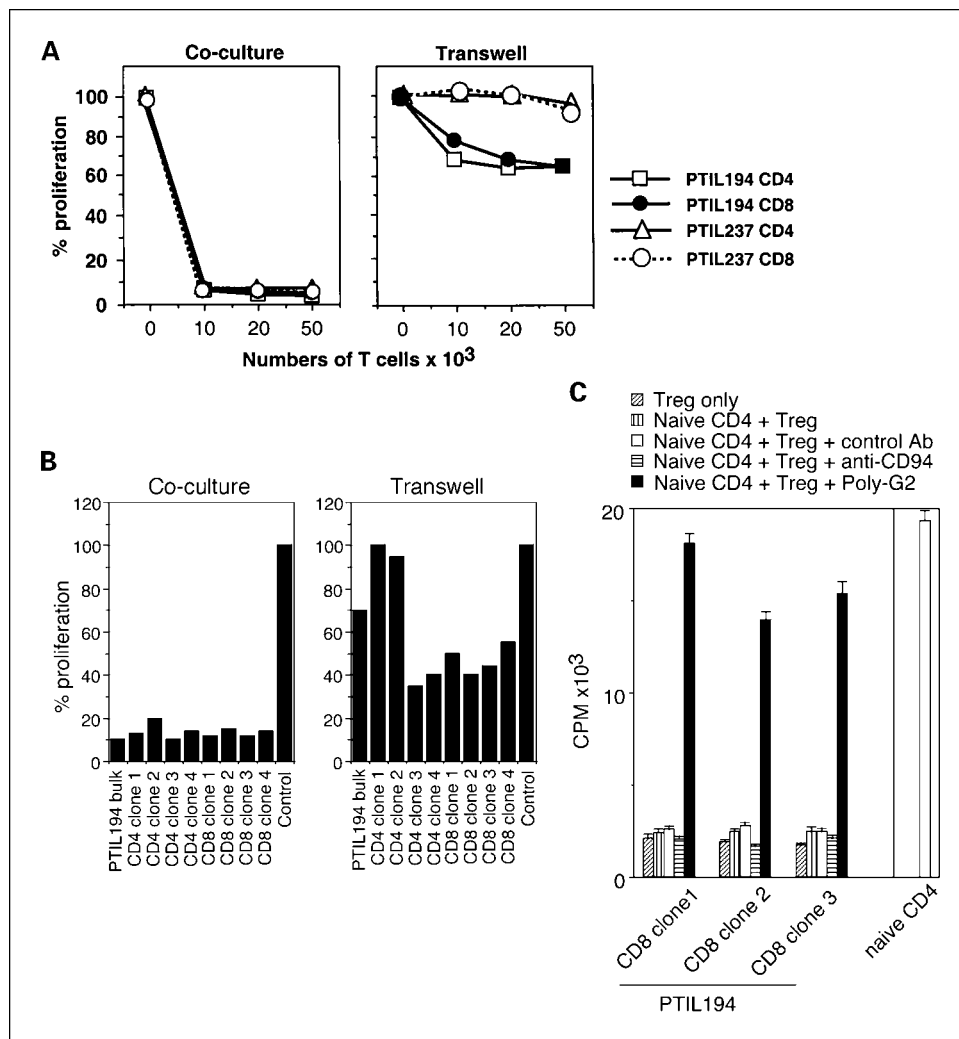
**Fig. 3.** Determination of Fopx3 protein expression in CD8<sup>+</sup> T cells by FACS and immunohistochemistry analyses. **A**, FACS analysis of PTIL194 and PTIL237 with anti-Fopx3 and anti-CD8 antibodies. Prostate tumor – derived PTIL194 and PTIL237 cells were stained with anti-CD8 mAb and anti-Fopx3 mAb. An isotype-matched control antibody served as a control for staining and FACS gating. **B**, frequency of Fopx3<sup>+</sup> CD8<sup>+</sup> T cells and Fopx3<sup>+</sup> CD4<sup>+</sup> T cells in the PBMCs of healthy donors (*HD1* and *HD2*) and prostate cancer patients (*PP1* and *PP2*). PBMCs were obtained from healthy donors and prostate cancer patients and stained with anti-Fopx3, anti-CD8, and anti-CD4 antibodies. Similar results were obtained with additional PBMCs (five healthy donors and five prostate cancer patients). **C**, immunohistochemical staining for CD8 and Fopx3 expression in prostate tumor tissues. Frozen prostate tumor tissues were stained with anti-CD8 (red) and anti-Fopx3 (green) and detected with a confocal microscope. Fluorescence images were merged. Arrows, cells express both CD8 (red) and Fopx3 (green) molecules to generate yellow fluorescence. Similar results were obtained with two repeated experiments and three different prostate tumor tissues.

coculture assay condition, T cells from PTIL237 could not suppress naïve CD4 T-cell proliferation in a transwell system (Fig. 5A), suggesting that cell-to-cell contact is required for immunosuppression by PTIL237. Similarly, we found that like CD4<sup>+</sup> Treg cells, PTIL237-derived CD8<sup>+</sup> Treg cell clones suppressed naïve T-cell proliferation in a cell contact-dependent manner (data not shown). However, T cells from

PTIL194 showed partial inhibition of naïve T cells in a transwell system (Fig. 5A), indicating that some Treg cells in PTIL194 inhibit naïve T-cell proliferation through a cell contact-dependent mechanism, whereas others suppress immune responses via soluble factors (IL-10 and/or transforming growth factor- $\beta$ ). Indeed, we found that some Treg cell clones from PTIL194 could not inhibit naïve CD4 T-cell proliferation in a



**Fig. 4.** Identification and characterization of Treg cell clones derived from PTIL194. *A*, generation of Treg cell clones with suppressive activity. T-cell clones were screened for their suppressive activity in a proliferation assay. Fifty-one clones showed strong suppressive activity, whereas 43 clones did not or weakly suppressed the proliferation of naive CD4<sup>+</sup> T cells. *B*, phenotypic analysis of T-cell clones by FACS. CD4<sup>+</sup> T-cell clones and CD8<sup>+</sup> T-cell clones with suppressive activity were analyzed for expression of CD25, GITR, CD122, CCR4, and Foxp3 molecules. Foxp3 was detected by intracellular antibody staining. Similar expression patterns were observed for all CD4<sup>+</sup> Treg cell clones, whereas all CD8<sup>+</sup> Treg cells shared the same expression patterns. Effector T cells of CD4<sup>+</sup> or CD8<sup>+</sup> T cells were negative for CD25 and Foxp3 molecules (not shown). Representative data for two T clones are shown. *C*, determination of Foxp3 expression levels in CD4<sup>+</sup> and CD8<sup>+</sup> Treg cell clones by real-time PCR. *HPRT* served as an internal control.



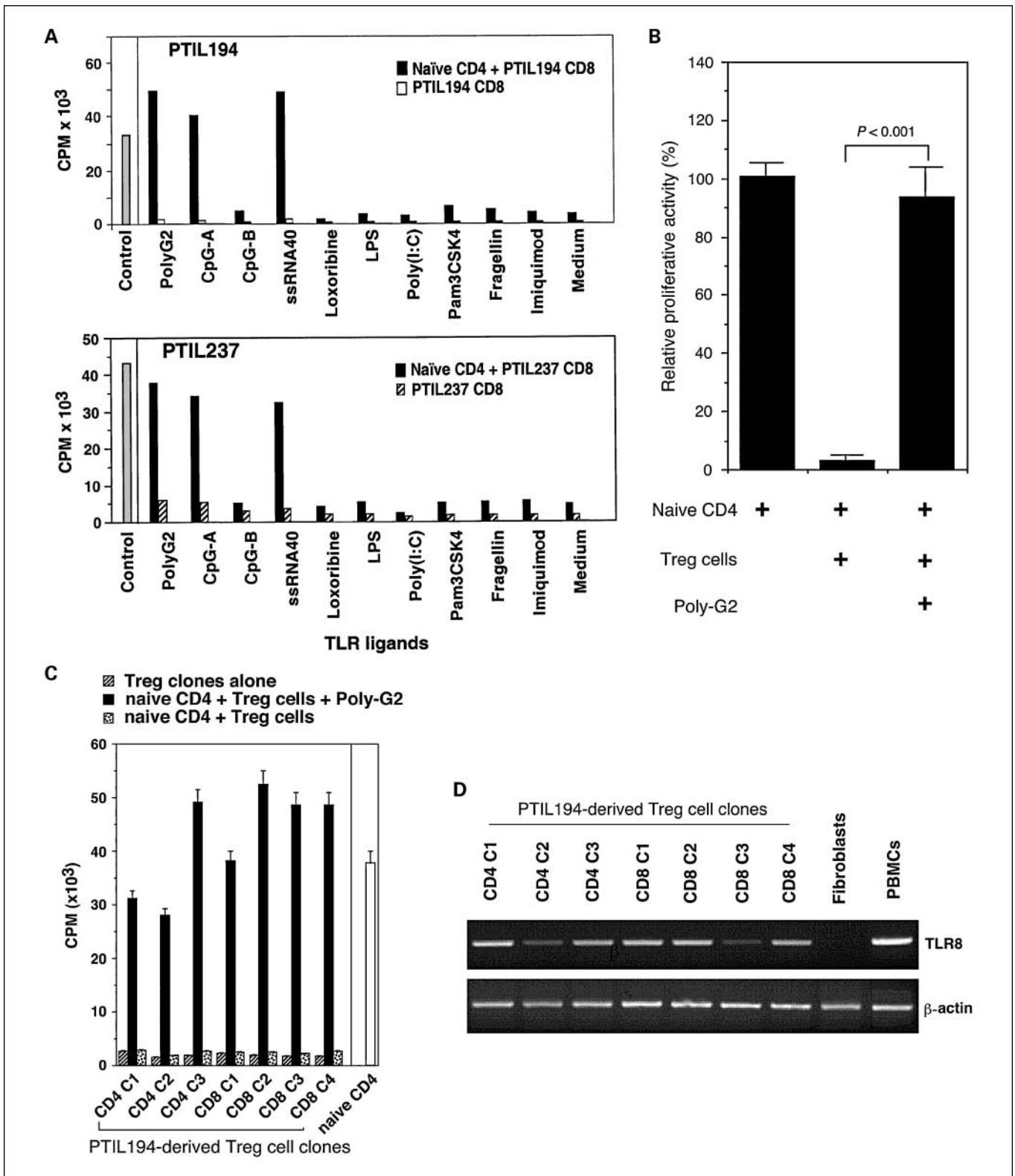
**Fig. 5.** Cell contact – dependent or soluble factor – dependent inhibition by CD8<sup>+</sup> Treg cells. **A**, cell-to-cell contact is required for Treg suppression. Equal numbers of CD4<sup>+</sup> responding T cells were cultured in the outer wells, whereas PTIL194 or PTIL237 were cultured in the inner wells of a transwell plate. Cocultured cells served as the positive control for PTIL194 and PTIL237. **B**, evaluation of suppressive activity of the responding CD4<sup>+</sup> T cells by CD4<sup>+</sup> or CD8<sup>+</sup> Treg cell clones generated from PTIL194 in coculture and transwell assays. **C**, interaction of HLA-E and CD94 molecules is not involved in CD8<sup>+</sup> Treg cell – mediated suppression. Functional suppression assays were done in the presence of anti-CD94 antibody. Poly-G2 oligonucleotide treatment served as a control.

transwell assay, whereas others could partially inhibit naïve T-cell proliferation in a transwell system (Fig. 5B). However, the addition of anti-IL10, anti-transforming growth factor- $\beta$ , or both antibodies could not restore naïve T-cell proliferation (data not shown). It has been shown that murine CD8<sup>+</sup> suppressor T cells suppress immune response through interaction between Qa-1 and CD94 molecules (16). To exclude the possibility that human HLA-E or CD94 molecules are involved in CD8<sup>+</sup> Treg cell-mediated immunosuppression, we did a functional assay in the presence of anti-CD94 or control antibody and found that neither anti-CD94 nor control antibody affected the suppressive function of CD8<sup>+</sup> Treg cells (Fig. 5C). These results suggest that like CD4<sup>+</sup> Treg cells, CD8<sup>+</sup> Treg cells suppressed immune responses through both soluble factor-dependent and cell contact-dependent suppressive mechanisms, which are distinct from murine Qa-1-restricted CD8<sup>+</sup> suppressor cells.

**Reversal of CD8<sup>+</sup> Treg cell function by TLR8 ligands.** We recently showed that the suppressive function of CD4<sup>+</sup> Treg cells can be reversed by TLR8 ligands (14); however, it was not clear whether activation of TLR8 signaling can also reverse the suppressive function of CD8<sup>+</sup>CD25<sup>+</sup> Treg cells. To test this possibility, we purified CD8<sup>+</sup> Treg cells from PTIL194 and tested for their suppressive effect on naïve T-cell proliferation in

the presence and absence of TLR8 ligands (poly-G2 and ssRNA40) or ligands for other TLRs. We found that the suppressive activity of CD8<sup>+</sup> Treg cell population was abolished by TLR8 ligand poly-G2 and ssRNA40 but was not affected by ligands for other TLRs (Fig. 6A). Figure 6B is the summarized data obtained with CD8<sup>+</sup> Treg cells derived from eight prostate cancer patients, suggesting that TLR8 ligand poly-G2 can uniformly reverse the suppressive function of CD8<sup>+</sup> Treg cells, in addition to previously known CD4<sup>+</sup> Treg cells. To further test whether poly-G2 oligonucleotides could reverse the suppressive function of CD8<sup>+</sup> Treg cell clones, we did a functional assay and found that like CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, CD8<sup>+</sup>CD25<sup>+</sup> Treg cell clones responded to poly-G2 treatment. Treatment of poly-G2 oligonucleotides completely restored naïve T-cell proliferation (Fig. 6C), suggesting that TLR8 signaling controls the suppressive function of CD8<sup>+</sup> Treg cells. Consistent with this observation, we found that TLR8 expression could be detected in CD8<sup>+</sup> Treg cell clones, CD4<sup>+</sup> Treg cell clones, and PBMCs, whereas fibroblasts did not express TLR8 (Fig. 6D). Thus, besides their similarities in phenotypic and suppressive mechanisms between CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells, they also share a common pathway for their functional regulation through the TLR8 signaling. These results raise a possibility that manipulation of TLR8 signaling pathway can





**Fig. 6.** Reversal of the suppressive function of PTIL194 and PTIL237 by TLR ligands *A*, restoration of CD8<sup>+</sup> Treg cell – suppressed proliferation of naïve CD4<sup>+</sup> T cells by TLR8 ligands (poly-G2, CpG-A, and ssRNA40). Ligands for other TLRs showed no effect on suppression of naïve T-cell proliferation by CD8<sup>+</sup> Treg cells. *B*, restoration of naïve CD4<sup>+</sup> T-cell proliferation in the presence of CD8<sup>+</sup> Treg cells by poly-G2 oligonucleotide treatment. Columns, mean relative proliferative activity (%) for experiments with T-cell lines derived from eight different cancer patients; bars, SD. Proliferative activity of naïve T cells without Treg cells serves as a basis (100%) to calculate relative proliferation of naïve T cells plus Treg cells in the absence or presence of poly-G2. *P* value was determined by a one-tailed Student's *t* test. *C*, reversal of suppressive function of CD8<sup>+</sup> Treg cell clones by poly-G2. CD4<sup>+</sup> Treg cells served as controls for functional reversal by poly-G2. *D*, expression of *TLR8* mRNA in CD4<sup>+</sup> Treg and CD8<sup>+</sup> Treg cell clones generated from PTIL194 by reverse transcription-PCR. Fibroblast RNA was used as a negative control, whereas PBMC RNA served as a positive control for TLR8 expression.

reverse immunosuppression mediated by different subsets of Treg cells and improve therapeutic potential of cancer vaccines directed to cancer and/or infectious diseases.

## Discussion

Increasing evidence indicates that tumor-infiltrating immune cells play a major role in combating cancer and their activity correlates with disease prognosis and survival (17, 18). In most cases, however, tumor-specific T cells ultimately fail to control tumor growth. However, the clinical response rate could be improved to 50% if patients scheduled to receive adoptive T-cell therapy were first conditioned with cyclophosphamide for 2 days to remove whole-body lymphocytes (19). These results suggest that tumor-specific T cells may be suppressed by Treg cells in the tumor microenvironment, and their removal by cyclophosphamide may enhance antitumor immunity.

Although elevated proportions of Treg cells have been reported in patients with other cancers (10, 11), relatively less is known about Treg cells in prostate cancer. Analysis of the prevalence of different Treg cell subpopulations as well as their suppressive mechanisms in prostate cancer patients are critical to our understanding immunosuppression in prostate cancer and to devising new strategies for improving the efficacy of cancer vaccines. Several lines of evidence suggest that prostate-derived TILs are distinct from those derived from other types of cancers. First, we found that the majority of prostate tumor-derived TILs contained high percentages of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (Fig. 1). Further experiments show, however, that the frequency of CD4<sup>+</sup> Foxp3<sup>+</sup> and CD8<sup>+</sup> Foxp3<sup>+</sup> T cells in normal PBMCs is comparable with that in PBMCs of cancer patients (Fig. 3), suggesting that Treg cells are recruited to tumor sites. By contrast, <30% of TILs derived from melanoma had a high percentage of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Our results are not only consistent with a recent report showing that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells are enriched in prostate cancer (15), but also point out that prostate cancer-derived TILs contain more suppressive Treg cells than the counterparts of melanoma. Using a transgenic mouse model of prostate tumor, Tien et al. (20) found an increased frequency and number of CD4<sup>+</sup>CD25<sup>+</sup> T cells and an enhanced production of inhibitory cytokines during tumor progression. The second unique feature of our study is that prostate tumor-derived CD8<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells suppressed immune responses. Thus, prostate tumor environment seems to contain both CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells that can inhibit antitumor immunity, which may explain, at least in part, why prostate cancer is poorly immunogenic and why their associated T cells are generally difficult to grow *in vitro*. Third, our study clearly shows the presence of Treg cells in prostate cancer at both population as well as clonal levels. Finally, we show that the suppressive function of different subsets of Treg cells (CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup>) can be regulated through TLR8 ligands.

In contrast to CD4<sup>+</sup> Treg cells, much less is known about CD8<sup>+</sup> Treg cells (21). CD8<sup>+</sup> Treg cells have been identified to mediate immunosuppression in an antigen-dependent manner (16, 21, 22). CD8<sup>+</sup> Treg cells suppress antigen-activated CD4<sup>+</sup> T cells in a TCR-specific manner restricted by the MHC class Ib molecule, Qa-1 (16, 23). However, recent studies showed that CD8<sup>+</sup>CD122<sup>+</sup> (IL-2/IL-15 receptor  $\beta$  chain) Treg cells can prevent the development of abnormally activated

T cell-mediated disease in CD122-deficient mice (24). CD8<sup>+</sup>CD25<sup>+</sup> Treg cells have recently been isolated from human PBMCs (25–27). Recently, it has been reported that ovarian tumor ascites plasmacytoid dendritic cells induced interleukin-10<sup>+</sup> CCR7<sup>+</sup> CD45RO<sup>+</sup> CD8<sup>+</sup> Treg cells (28). These CD8<sup>+</sup> Treg cells can suppress immune responses in an antigen-nonspecific manner. In this study, we show that prostate tumor-derived CD8<sup>+</sup> Treg cells expressed CD25 and Foxp3 molecules (Figs. 3 and 4), which are shared by CD4<sup>+</sup> Treg cells. Our study further showed that prostate tumor-derived CD8<sup>+</sup> Treg cells also expressed CD122 molecules (Fig. 3B). It seems that CD8<sup>+</sup> Treg cells can be induced by different cell types such as plasmacytoid dendritic cells, immature dendritic cells, or tumor cells (16, 21, 22, 28). Therefore, the phenotypic markers and suppressive mechanisms of CD8<sup>+</sup> Treg cells may be different depending on cytokines and tumor environments. Foxp3 has proved to be a relative specific marker for Treg cells and critical to the development of Treg cells (29, 30). It should be noted that Foxp3 expression in mouse is restricted to CD4<sup>+</sup> Treg cells but little or no expression in CD8<sup>+</sup> T cells and other cell population (31). In contrast, Foxp3 expression in human is not so restricted and has been detected in many subsets of T cells (32–34), although its expression level in Treg cells is higher than that in effector cells. Although it is well recognized that CD4<sup>+</sup> Treg cells suppress immune responses through cell contact-dependent mechanisms (35), prostate tumor-derived CD8<sup>+</sup> Treg cells inhibit naïve T-cell proliferation through a cell contact-dependent as well as soluble factor-dependent mechanisms, but do not require the involvement of Qa-1 and CD94 interaction, which is critical for murine CD8<sup>+</sup> suppressor T cells (16, 23). Hence, both human CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells share some phenotypic markers and suppressive mechanisms.

These findings raise an intriguing question: What is the mechanism that allows CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells to accumulate in the prostate tumor microenvironment? There may be sequential events occurred in the process. First, recent studies have linked chronic inflammation to cancer development and progression (36–38). Prostate cancer is concomitantly associated with prostatitis or inflammation that may create a specific cytokine environment favoring the expansion of Treg cells (39, 40). Suppressive cytokines such as IL-10 and transforming growth factor- $\beta$  and chemokines such as CCL22 secreted by tumor cells or tumor-infiltrating macrophages, myeloid suppressor cells, and dendritic cells not only recruit Treg cells to tumor sites but also favor the conversion of nonsuppressive T cells into Treg cells with suppressive function (11, 41). This notion is supported by our findings showing that prostate tumor-derived CD4<sup>+</sup>CD25<sup>+</sup> Treg cells express CCR4, a receptor for CCL22. However, CD8<sup>+</sup>CD25<sup>+</sup> Treg cells do not express CCR4 molecules. Second, it is likely that tumor cells may actively recruit, activate, and expand Treg cells by either directly or indirectly presenting antigenic peptides for their recognition. Indeed, our previous studies showed that tumor cells express tumor-specific antigens such as LAGE1 and ARTC1 and directly stimulate antigen-specific Treg cells (7, 8). Because some prostate tumor-derived TILs had tumor-specific recognition (data not shown), it is reasonable to believe that tumor antigens expressed by prostate tumor cells may play a critical role in the recruitment, activation, and maintenance of Treg cells at tumor sites. Thus, antigens expressed by tumor

cells, soluble factors, and cytokines/chemokines in tumor microenvironments may play a critical role in recruiting, expanding, and maintaining Treg cells at tumor sites (42).

Regardless of how Treg cells accumulate near the tumor sites of prostate, the depletion or removal of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells could be expected to improve antitumor immune responses. The fact that both suppressive and nonsuppressive CD8<sup>+</sup> T cells coexist at tumor sites based on T-cell cloning and immunohistochemical staining of tumor tissues suggests that immune responses to prostate cancer occur *in vivo*. Thus, it is possible to develop effective immunotherapy for prostate cancer if Treg cell numbers or their suppressive function can be controlled. Our study further extended analysis of subsets of Treg cells in prostate cancer to their functional regulation. Although the depletion of such Treg cells using anti-CD25 mAb in mice enhanced antitumor immunity *in vivo* (13, 20), testing of this concept in clinical trials using an IL-2/diphtheria toxin fusion protein (denileukin diftitox, or Ontak) indicated that the depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells by Ontak could enhance antitumor activity, although Ontak also depleted newly activated CD4<sup>+</sup>CD25<sup>+</sup> effector cells (43). However, another study by Attia et al. (44) showed that Ontak was ineffective in depleting CD4<sup>+</sup>CD25<sup>+</sup> T cells. Hence, even if this fusion protein is capable of depleting CD25<sup>+</sup> T cells, its use may limit to prevaccination treatment. We recently showed that TLR8 signaling could reverse the suppressive function of naturally occurring Treg as well as tumor-specific Treg cells (14). Here, we

show that TLR8 ligands not only reversed the suppressive function of CD4<sup>+</sup> Treg cells but also blocked CD8<sup>+</sup> Treg cell-suppressive function (Fig. 6), implying that CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells may share a common suppressive mechanism, which can be reversed by triggering TLR8 signaling pathway. Because TLR8 is not functional in mice (45), the regulation of murine Treg cells through TLR signaling may differ from the functional control of human Treg cells. Indeed, it was recently reported that TLR2 promoted the proliferation of both murine CD4<sup>+</sup> Treg and effector cells and transiently abrogated the suppressive function of murine CD4<sup>+</sup> Treg cells (46, 47). Regardless of these differences in the functional regulation of Treg cells between humans and mice, it may be possible to use TLR ligands to manipulate Treg cell-suppressive function as well as effector T cells, thus shifting the balance between Treg and effector cells. Because the suppressive function of both CD4<sup>+</sup> and CD8<sup>+</sup> subsets of Treg cells can be reversed by TLR8 ligands, it is likely that manipulating Treg cell function by TLR8 ligands may prove useful in improving the efficacy of cancer vaccines against prostate and other cancers.

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# Clinical Cancer Research

## CD8<sup>+</sup> Foxp3<sup>+</sup> Regulatory T Cells Mediate Immunosuppression in Prostate Cancer

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