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Human Prostate Tumor Antigen–Specific CD8$^+$ Regulatory T Cells Are Inhibited by CTLA-4 or IL-35 Blockade

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Regulatory T cells play important roles in cancer development and progression by limiting the generation of innate and adaptive anti-tumor immunity. We hypothesized that in addition to natural CD4$^+$CD25$^+$ regulatory T cells (Tregs) and myeloid-derived suppressor cells, tumor Ag–specific Tregs interfere with the detection of anti-tumor immunity after immunotherapy. Using samples from prostate cancer patients immunized with a DNA vaccine encoding prostatic acid phosphatase (PAP) and a *trans*-vivo delayed-type hypersensitivity (tvDTH) assay, we found that the detection of PAP-specific effector responses after immunization was prevented by the activity of PAP-specific regulatory cells. These regulatory cells were CD8$^+$CTLA-4$^+$, and their suppression was relieved by blockade of CTLA-4, but not IL-10 or TGF-β. Moreover, Ag-specific CD8$^+$ Tregs were detected prior to immunization in the absence of PAP-specific effector responses. These PAP-specific CD8$^+$CTLA-4$^+$ suppressor T cells expressed IL-35, which was decreased after blockade of CTLA-4, and inhibition of either CTLA-4 or IL-35 reversed PAP-specific suppression of tvDTH response. PAP-specific CD8$^+$CTLA-4$^+$ T cells also suppressed T cell proliferation in an IL-35–dependent, contact-independent fashion. Taken together, these findings suggest a novel population of CD8$^+$CTLA-4$^+$ IL-35–secreting tumor Ag–specific Tregs arise spontaneously in some prostate cancer patients, persist during immunization, and can prevent the detection of Ag-specific effector responses by an IL-35–dependent mechanism. ***The Journal of Immunology*****, 2012, 189: 5590–5601.**

The ability of the immune system to respond to infectious pathogens, noninherited Ags, and malignant tumor Ags is counterbalanced by an equally important system of regulatory immune responses that seek to limit the self-reactive potential of these effector responses. These responses can be mediated by a variety of cell types (including the recently identified myeloid-derived suppressor cells), but traditionally have been thought of as Ag-nonspecific CD4$^+$ T cells. CD4$^+$ regulatory T cells (Tregs) are broadly defined as either "natural" (CD4$^+$CD25$^+$) (1) or "induced" Tregs. Induced Tregs are generated as uncommitted CD4$^+$ T cells, which gain distinct suppressive functions based on particular antigenic stimulation and are characterized by their secretion of various cytokines: IL-10–secreting type 1 T-regulatory cells (2), TGF-β–secreting Th3 cells (3), and the recently identified IL-35–secreting iTr35 population (4). These iTr35 cells are particularly important to Treg function, as IL-35 expression (composed of a heterodimer of the IL-12p35 and Ebi3 subunits) has been shown to be required for the maximal regulatory function of murine and human Tregs and can propagate infectious tolerance in part by converting conventional CD4$^+$ T cells into iTr35 regulatory cells (4–7).

Although most reports of Treg populations have largely focused on their Ag-nonspecific function, the immune system has also been shown to have the ability to mobilize Ag-specific Treg responses, notably in several models of autoimmunity and transplantation (8–16). These Ag-specific populations require their cognate Ag to become activated, but once active can suppress effector responses in an Ag-nonspecific fashion (14–16). Emerging evidence has also shown a role for Ag-specific regulation in cancer, with the identification of CD4$^+$ T cells specific for various tumor Ags that have suppressive function (17–24). Much of this research has been conducted in patients with melanoma, with the identification of CD4$^+$ Tregs that recognize Ags such as LAGE1 (17), ARTC1 (18), or other tumor Ags (19). As in other models of Ag-specific Tregs, these responses required ligand-specific activation, but once activated could suppress proliferation in a nonspecific fashion. In two seminal reports by Wang and colleagues (17, 18), the blockade of either IL-10 or TGF-β did not abrogate the suppressive activity of Ag-specific Tregs, suggesting other cytokines or cell contact–dependent mechanisms are responsible for mediating suppression.

The association between regulatory immune responses and the development and progression of cancer has been well established in a number of malignancies, including prostate cancer (25–27). Numerous reports in both rodent models as well as patients have shown that prostate tumor–bearing individuals have increased frequencies of CD4$^+$ Tregs compared with healthy individuals,

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Abbreviations used in this article: DTH, delayed-type hypersensitivity; FDA, Food and Drug Administration; PAP, prostatic acid phosphatase; PSA, prostate-specific Ag; Treg, regulatory T cell; TT/D, tetanus/diphtheria toxoid; tvDTH, *trans*-vivo delayed-type hypersensitivity.

both in the periphery (3, 27–30) and infiltrating the tumor (27, 30–33). Tregs have also been shown to be associated with prostate cancer disease progression, as patients with advanced disease were found to have higher percentages of peripheral Tregs than individuals with early-stage disease (3, 29, 30). Immunosuppressive factors produced by Tregs, such as IL-10 and TGF-β, have been shown to contribute to prostate cancer development and progression (34, 35). Additionally, many current prostate cancer therapies have been shown to enhance Treg frequency, including radiation therapy (36), androgen deprivation (37, 38), and chemotherapy (39).

The use of anti-tumor immunotherapies for prostate cancer has seen several advances in recent years, with the Food and Drug Administration (FDA) approval of sipuleucel-T for advanced prostate cancer (40) and another randomized clinical trial evaluating a viral-based vaccine (PROSTVAC) showing a significant survival benefit (41). However, as in other established cancer therapies, regulatory immune responses have also been shown to have a profound detrimental effect on the immune and clinical success of these immunotherapies. For example, baseline effector T cell responses to a variety of prostate tumor–associated Ags have been shown to be suppressed by concurrent Treg responses, including effector responses to prostatic acid phosphatase (PAP) and prostate-specific Ag (PSA), the Ags targeted by sipuleucel-T and PROSTVAC, respectively (42). Furthermore, although immunotherapies aim to augment anti-tumor effector responses, several reports have found that they can also enhance regulatory immune responses, which can suppress concurrent effector responses (38, 43–47), including a report of a DNA vaccine in patients with either non–small cell lung, esophageal, or prostate cancer in which regulatory responses were found to suppress effector responses generated after immunization (48).

We recently reported the results of a phase I clinical trial evaluating a DNA vaccine encoding PAP in which patients with early recurrent prostate cancer received six biweekly immunizations (49). In this trial, 8 of 22 individuals developed PAP-specific effector IFN-γ–secreting T cells that persisted for several months after immunization, which correlated with a favorable change in PSA doubling time (a decrease in the rate of PSA rise), a possible prognostic marker in patients with early recurrent prostate cancer (50–54). However, three of these eight "responding" patients did not have an immune response 2 wk after the final immunization, and several other individuals were not found to have a detectable effector immune response up to a year after immunization. We hypothesized that the presence of PAP-specific regulatory cells might have prevented the detection of PAP-specific effector responses postimmunization. Consequently, in this report we used samples from this trial to investigate whether Ag-specific Treg responses existed and, if so, to characterize further the phenotype and function of this population.

Materials and Methods

Patient populations

Patient PBMCs used for the studies were obtained from individuals previously treated on a clinical trial at the University of Wisconsin Carbone Cancer Center (49). This included 21 subjects with PSA-recurrent prostate cancer, without radiographic evidence of metastases, and not receiving concurrent androgen deprivation. All subjects gave written, institutional review board–approved consent for the use of residual blood products to be used for immunological research. PBMCs had been collected by leukapheresis prior to, and 2 wk after, vaccination with six biweekly intradermal injections of a DNA vaccine encoding PAP, and by blood draw at 3-m intervals for 1 y after immunization. All subjects also received an i.m. tetanus booster immunization prior to receiving the DNA vaccinations, which was used as a recall Ag. PBMCs were cryopreserved in aliquots in liquid nitrogen until use.

Mice

CB-17 SCID mice were bred at the University of Wisconsin Gnotobiotic Laboratory facility. All animals were housed and treated in accordance with guidelines outlined by the University of Wisconsin and the National Institutes of Health under an institutional animal care and use committee–approved protocol.

Trans-*vivo delayed-type hypersensitivity*

PBMCs (7.5×10^6 to 10×10^6) obtained from patients prior to and after immunization were coinjected into the footpads of 6- to 8-wk-old SCID mice with 1 μg of recombinant human PAP protein (Fitzgerald Industries, Acton, MA) or recombinant human PSA (Fitzgerald Industries) as a nonspecific Ag control. The response to tetanus/diphtheria toxoid (TT/D; Aventis Pasteur, Bridgewater, NJ) recall Ag alone plus PBMCs was used as a positive control, and PBMCs plus PBS was used as a negative control. Ag-driven swelling was determined as previously described (55). Delayed-type hypersensitivity (DTH) reactivity after 24 h was measured as the change in footpad thickness in multiples of 10^{-4} inches, measured using a dial thickness gauge (Mitutoyo, Kawasaki, Japan), and net swelling is the Ag-specific swelling subtracted for the contribution obtained with PBMCs plus PBS. To determine the effect of neutralizing Abs, PBMCs were mixed with PAP or PSA Ag and injected into the footpads of SCID mice with 25 μg of either control IgG or rabbit anti-human TGF-β (R&D Systems, Minneapolis, MN), goat anti-human IL-10 (R&D Systems), or 1 μg of mouse anti-human CTLA-4 mAb (clone AS32; Ab Solutions, Mountain View, CA). The extent of bystander suppression was measured as inhibition of recall Ag response in the presence of PAP or PSA Ags and calculated as previously described (56). To reverse bystander suppression of DTH responses, PBMCs were first mixed with PAP protein and 25 μg of TT/D, and then combined with either control IgG, anti-human CTLA-4 mAb, MHC class I (clone W6/32; BioLegend, San Diego, CA), MHC class II Abs (clone L243; BioLegend), mouse anti-human IL-12p35 (clone 27537; R&D Systems), mouse anti-human IL-12p40 (clone 24901; R&D Systems), goat anti-human IL-23p19 (AF1716; R&D Systems), goat anti-mouse IL-27p28 (cross-reactive with human, AF1834; R&D Systems), or mouse anti-human Ebi3 [clone V1.4F5.25 (6)], and the mixture was then injected into the footpads of SCID mice. DTH reactivity was again measured after 24 h as described earlier. Results are expressed as the change over the swelling induced by injection of PBMCs plus PBS alone. Absolute net *trans*-vivo delayed-type hypersensitivity (tvDTH) response of $\geq 15 \times 10^{-4}$ inches, and changes in net tvDTH $>10 \times 10^{-4}$ inches to a particular Ag, were considered as consistent with the presence or gain of a DTH immune response (16, 55, 57). Given the nature of the testing, data shown are typically from single measurements, but with experiments repeated two to three times. We have previously demonstrated the reproducibility of this tvDTH assay (58). In separate experiments, PBMCs were depleted of $CD8^+$ T cells, or enriched/selected for $CD8^+$ T cells, by magnetic bead selection (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer's instructions. In other studies, $CD8^+$ enriched/selected T cells were further depleted of subpopulations using PE-labeled Abs specific for CTLA-4 (clone 14D3; eBioscience, San Diego, CA) or control Ag followed by magnetic bead selection (StemCell Technologies). For these T cell subset studies, the purity of $CD3^+CD8^+$ cells and effective depletion of $CD8^+$ T cells from PBMCs was determined by flow cytometry and was routinely found to be >94%. As a result of this separation, contaminating $CD4^+CTLA\text{-}4^+$ T cells among the added back $CD8^+$ T cells would be expected to be at most 0.06% of the total injected population.

IFN-γ ELISPOT assay

ELISPOT assay was performed as previously described (53). In brief, wells of nitrocellulose 96-well microtiter (ELISPOT) plates were coated with an anti–IFN-γ capture mAb (Endogen, Rockford, IL). Cryopreserved PBMCs, obtained at various times prior to or after vaccination, were then thawed and cultured for 48 h in the presence of media only (no Ag), 2 μg/ml PAP protein (Research Diagnostics, Flanders, NJ), 250 ng/ml tetanus toxoid (Calbiochem, San Diego, CA), or 5 μg/ml PHA (positive mitogenic control; Fisher, Pittsburgh, PA). ELISPOT plates were then washed and probed for 1.5 h with a biotinylated anti–IFN-γ Ab (Endogen), streptavidin-labeled alkaline phosphatase for 1 h, and then developed with BCIP/NBT colorimetric substrate (BioRad, Hercules, CA) for 15–30 min. The number of spots per well was determined with an automated ELISPOT reader (Autoimmun Diagnostika GmbH, Strassberg, Germany) and normalized to 10^6 PBMCs. The mean number of spots detected under media-only conditions at each time point was subtracted from the Ag-specific conditions to enumerate Ag-specific IFN-γ spot-forming units ± SD.

Comparison of experimental wells with control, no-Ag, wells was performed using a two-tailed *t* test, with $p \leq 0.05$ used to define a significant T cell response.

Protein stimulations, cell sorting, RNA, cDNA, and quantitative real-time PCR

PBMC samples were stimulated for 72 h with media alone (RPMI 1640 media supplemented with L-glutamine, penicillin, streptomycin, and 10% human AB serum) or either recombinant PSA (2 μg/ml; Chemicon) or recombinant human PAP (2 μg/ml; Chemicon). Furthermore, PSA- or PAP-stimulated cultures were also treated with either a blocking Ab specific for CTLA-4 (clone AS32) or a murine IgG control. Cultures were collected, stained with Abs specific for CD3, CD8, and CTLA-4, and sorted by flow cytometry (FACSAria; BD Biosciences), collecting the following populations: CD3$^+$CD8$^+$CTLA-4$^+$, CD3$^+$CD8$^+$CTLA-4$^-$, and CD3$^+$CD8$^-$ (CD3, clone OKT3; CD8, clone SK1; CTLA-4, clone 14-D3; all eBioscience). RNA was then collected from sorted cells using the Qiagen mRNA kit, and cDNA was reverse-transcribed with the iScript cDNA Synthesis kit (Bio-Rad). The cDNA samples were then subjected to 40 cycles of amplification with primers specific for *IL-12A, IL-12B, IL-23A, IL-27, Ebi3, IL-10* [as previously described (6)] or the ribosomal protein P0 as a control gene [as previously described (59)] in a MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad) and were quantified by the comparative cycling threshold method. Fold induction results were analyzed by the $2^{-\Delta\Delta Ct}$ method (60) relative to P0 expression and the media-only treatment group, and statistical differences between CTLA-4 and IgG-treated samples were determined using a two-tailed *t* test, with $p \leq 0.05$ used to define a significant T cell response.

In vitro PKH26 suppression assays

In vitro suppression assays were performed with modifications to a previously established protocol (4, 6). PBMC samples from patients with PAP-specific, CTLA-4 and IL-35–regulated CD8$^+$ suppressive T cells were stimulated for 72 h in the presence of PAP, PSA, or media alone. After stimulation, cultures were sorted for CD8$^+$CTLA-4$^+$ and CD8$^+$CTLA-4$^-$ T cells by flow cytometry (as earlier), and the sorted cells were labeled with CFSE (Sigma-Aldrich). For direct suppression assays, sorted cells were cocultured in 96-well round-bottom plates with unstimulated, autologous PBMCs labeled with PKH26 (Sigma-Aldrich). Sorted cells were added back at titrated amounts: either their natural frequency as determined by flow cytometry or 10-fold lower or higher. To set PKH26 gates as well as to calculate the percent suppression, a "No Add Back" control was included using PKH26-labeled unstimulated PBMCs alone. Cocultures were stimulated with media alone or a mixture of anti-CD3/anti-CD28–coated beads (clones OKT3 and CD28.2, respectively; BioLegend) and 20 U/ml recombinant human IL-2 (Fitzgerald Industries). Additionally, stimulated cultures were also treated with IL-35 blocking Abs [a combination of Abs directed against the IL-12p35 subunit (clone 27537) and Ebi3 (clone V1.4F5.25)] or a murine IgG control. After a 7-d incubation, cells were harvested and labeled with fluorescent Abs directed against CD3 (clone OKT3), CD4 (clone RPA-T4; BD Pharmingen), and CD8 (clone SK1). Lymphocytes were gated based on size, CFSE staining (to exclude sorted cells added back to the experiment), either CD4$^+$ or CD8$^+$ expression, and PKH26 staining. Percent suppression was calculated using the following formula: $100 - 100 \times [(Exp_{\alpha CD3/28} - Exp_{Media\ Alone}) / (No\ Add\ Back_{\alpha CD3/28} - No\ Add\ Back_{Media\ Alone})]$. For Transwell suppression assays, sorted cells were added to the top chamber of 96-well Transwell plates (96-well cell culture 0.4-μm insert plate; Millipore), and PKH26-labeled unstimulated, autologous PBMCs were added to the bottom chamber (96-well Feeder/Transport Tray; Millipore). Either media alone or anti-CD3/anti-CD28–coated latex beads along with 20 U/ml IL-2 were added to the bottom chamber, and cultures were incubated 7 d. After this incubation, insert plates were removed, and cells in the bottom chamber were collected and analyzed for proliferation as earlier.

Results

PAP-specific T effector immune responses after immunization are suppressed by PAP-specific Tregs in a CTLA-4–dependent fashion

Patients with early recurrent prostate cancer were vaccinated six times biweekly with a DNA vaccine encoding PAP (49, 53). Eight of 22 patients experienced at least a 2-fold increase in the PSA doubling time, and this was significantly associated with the development of long-term PAP-specific IFN-γ–secreting immune responses (53). One of these patients (ID007) is highlighted in Fig. 1A, showing the development of a durable PAP-specific IFN-γ–secreting immune response. Although this patient eventually developed a PAP-specific immune response many months after

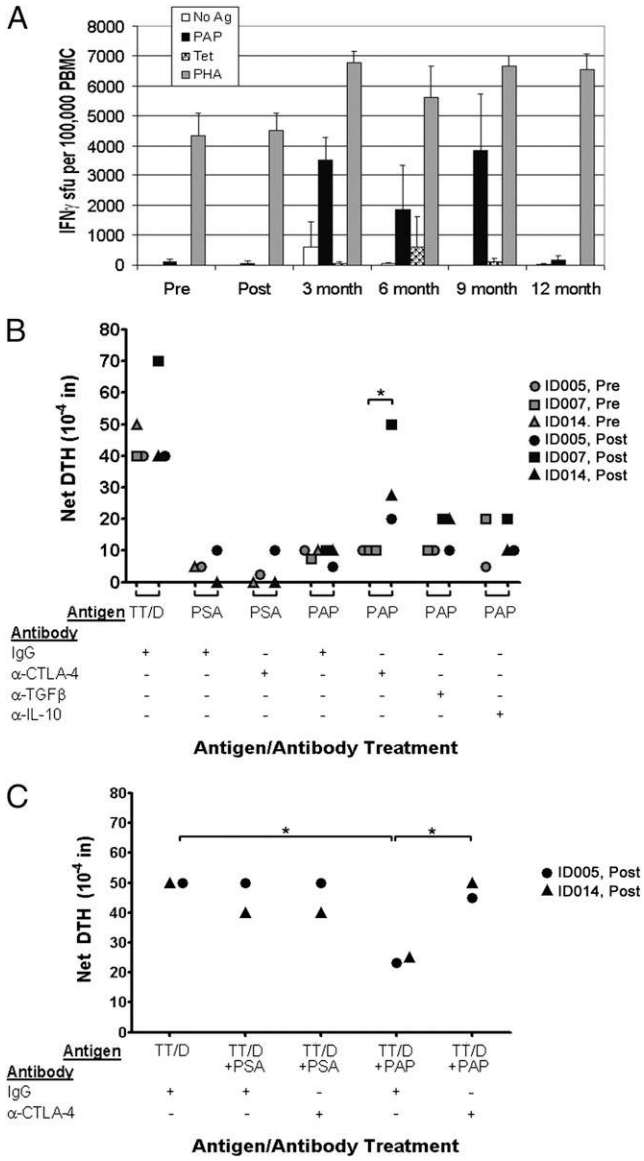

FIGURE 1. PAP Ag–specific T effector immune responses after immunization are suppressed by PAP-specific Tregs in a CTLA-4–dependent fashion. (**A**) IFN-γ ELISPOT responses of patient ID007 prior to (Pre) or 2 wk after (Post) six biweekly immunizations, and 3-mo intervals thereafter. PBMCs were evaluated for IFN-γ responses after stimulation with media alone (No Ag, unfilled), PAP (black), tetanus/diphtheria toxoid (Tet, hatched), or a PHA-positive control (gray). Shown are the mean and SD of quadruplicate samples in spot-forming units (sfu). (**B**) Preimmunization (gray) or postimmunization (black) PBMCs from three patients (ID005, ID007, and ID014) were injected into the footpads of SCID mice with the indicated Ags (TT/D, PAP, or PSA) and blocking Abs (IgG control, anti–CTLA-4, anti–TGF-β, or anti–IL-10). DTH swelling responses (10^{-4} in.) were measured after 24 h. Data shown are representative of at least two independent experiments. Differences in mean tvDTH between groups were analyzed by Student *t* test. *$p < 0.05$. (**C**) Postimmunization samples from patient ID005 and ID014 were used in tvDTH assays measuring responses to TT/D alone or in combination with PAP or PSA and in the presence or absence of a CTLA-4 blocking Ab. Data shown are representative of at least two independent experiments. Differences between mean DTH were compared using a Student *t* test. *$p < 0.05$.

immunization, he had no detectable IFN-γ–secreting immune response 2 wk after the final immunization. A similar finding of delayed T effector responses (but no response 2 wk after the final immunization) was also seen in two other patients who experienced a greater than 2-fold increase in PSA doubling time (ID005 and ID014). Although conceivable that an effector immune response might have taken months to develop, we reasoned that an alternative explanation might be that PAP-specific effector T cell responses were augmented during vaccination, but that the detection of these effector responses was prevented by a concurrent regulatory immune response after immunization.

To distinguish between these two possibilities, we used the tvDTH assay, a method used extensively to identify Ag-specific Treg responses in human organ transplant recipients (55). This technique involves injecting patient peripheral blood samples into the footpads of SCID mice along with the Ag of interest and measuring footpad swelling 24 h later as an indicator of an inflammatory immune response. As shown in Fig. 1B, although these three patients had baseline recall responses to tetanus toxoid that remained after a booster immunization (given prior to the priming PAP DNA immunization), PAP-specific tvDTH immune responses were not detected either preimmunization or 2 wk after the final immunization. These findings agree with previously published results using standard T cell proliferation and IFN-γ ELISPOT assays (49, 53). However, when a blocking Ab specific for CTLA-4 was coadministered, PAP-specific immune responses were uncovered in all three patients 2 wk after the final immunization (Fig. 1B). Coadministration of blocking Abs to the immunosuppressive cytokines IL-10 and TGF-β did not uncover a PAP-specific effector response (Fig. 1B).

We reasoned that the uncovering of effector responses upon treatment with a CTLA-4 blocking Ab could be a result of targeting CTLA-4 expressed on effector cells after activation or alternatively could be a result of blocking CTLA-4 expressed by regulatory cells (61). To distinguish between these possibilities, we evaluated the same patients for bystander suppression of a recall tetanus response, a technique we have routinely used to identify Ag-specific Tregs in models of transplant tolerance (55). This method involves coinjecting patient peripheral blood samples with a recall Ag (either TT/D or inactivated EBV) and an experimental Ag being evaluated for regulatory immune responses. As with other Ag-specific regulatory immune responses, the experimental Ag–specific regulatory cells require their cognate Ag for activation, but once activated can suppress in a nonspecific fashion (thus suppressing the TT/D bystander immune response). Although samples were unavailable to conduct this analysis with patient ID007, postimmunization samples from patients ID005 and ID014 were found to generate a strong immune response to TT/D. This response was unaffected by coadministration of a control protein (PSA; Fig. 1C). However, when PAP was injected along with TT/D, the bystander immune response to TT/D was significantly diminished. Furthermore, this PAP-specific bystander suppression could be alleviated with coadministration of a blocking Ab directed against CTLA-4. Blocking CTLA-4 did not have an effect on DTH in animals treated with TT/D and the control protein PSA, indicating that this blocking Ab was not targeting the intrinsic effector activity of TT/D-specific effector immune responses. These results suggest that PAP-specific regulatory cells were present along with PAP-specific effector immune responses after immunization and that these regulatory cells mediate suppression via CTLA-4.

Immunization increases the frequency of CTLA-4–regulated Ag-specific effector responses

To determine if the development of PAP-specific effector responses regulated by CTLA-4 is a common phenomenon among patients immunized with a PAP DNA vaccine, we studied remaining preimmunization and postimmunization samples from 21 patients

Table I. PAP-specific effector responses regulated by CTLA-4

	PAP					
	Preimmunization (Net DTH, 10^{-4} in.)			Postimmunization (Net DTH, 10^{-4} in.)		
Subject ID	Plus IgG[a]	Plus Anti–CTLA-4[a]	CTLA-4–Regulated PAP Response	Plus IgG[a]	Plus Anti–CTLA-4[a]	CTLA-4–Regulated PAP Response
02	10 ± 0.0	15 ± 5.8	5	10	35	25[b]
03	30	20	−10	30	26.7 ± 5.8	−3.3
04	12.5 ± 5	25 ± 10	**12.5**[b]	15 ± 5.8	13.3 ± 2.9	−1.7
05	11.25 ± 2.5	12.5 ± 5	1.25	8.3 ± 5.8	16.7 ± 5.8	**8.4**[b]
06	20 ± 0	22.5 ± 5	2.5	18.3 ± 2.9	45 ± 8.7	**36.7**[b]
07	5	10	5	10	50	**40**[b]
08	25	25	0	25	20	−5
09	30	40	10	35	35	0
10	26.7 ± 5.8	27.5	0.8	20	15	−5
11	10	10	0	0	5	5
12	10	20	10	5	0	−5
13	30	30	0	30	20	−10
14	10 ± 0	16.7 ± 5.8	6.7	10 ± 0	26.7 ± 2.9	**16.7**[b]
15	20 ± 0	17.5	−2.5	27.5	10	−17.5
16	10 ± 0	10 ± 0	0	5 ± 5	30 ± 0	**25**[b]
17	10	10	0	5	20	**15**[b]
18	20	20 ± 0	0	5	15	10
19	20	20	0	20	30	10
20	30	40	10	5	15	10
21	20	20	0	10	0	−10
22	8 ± 4.5	10 ± 5	2	8.75 ± 2.5	26.7 ± 7.6	**17.95**[b]

Bold indicates the individuals who had significant regulated responses.

[a]Shown are the mean ± SD values calculated from triplicate assays. When sample availability precluded triplicate experimental assays, shown are the average experimental values.

[b]CTLA-4–regulated PAP response >15 in.$^{-4}$ in at least one assay.

who received six biweekly immunizations with a DNA vaccine targeting PAP. As shown in Table I, PAP-specific effector responses regulated by CTLA-4 were uncommon prior to immunization, with only one patient (ID004) showing a CTLA-4–regulated PAP-specific effector response. However, after immunization, eight individuals (ID002, ID005, ID006, ID007, ID014, ID016, ID017, and ID022) were found to have PAP-specific effector responses that were regulated by CTLA-4. The frequency of patients with these CTLA-4–regulated effector responses was significantly higher after immunization than pretreatment ($p = 0.021$, Fisher's exact test). Similar to the patients evaluated in Fig. 1, PAP-specific effector responses were restored by blocking CTLA-4, but were unaffected by the coadministration of Abs specific for IL-10 or TGF-β (Supplemental Fig. 1).

PAP-specific regulatory responses are present prior to immunization and are CD8$^+$CTLA-4$^+$ T cell dependent

To determine if PAP-specific regulatory cells were present in prostate cancer patients prior to immunization, samples from patients obtained prior to immunization were evaluated for PAP-specific bystander suppression of TT/D immune responses. As shown in Fig. 2A, preimmunization samples from patients identified as having CTLA-4–regulated PAP-specific effector responses (see Table I) lacked effector responses to PAP or PSA. However, although these patients had strong immune responses to TT/D, cotreatment with PAP resulted in a significant suppression of the TT/D immune response in 6 of 6 individuals. This suppression was alleviated by the coadministration of a blocking Ab specific for CTLA-4 (Fig. 2A) and was Ag-specific, as coadministration of PSA did not result in suppression of the TT/D immune response. Moreover, the suppression of responses to TT/D was found not to be due to the PAP protein itself, as samples from other patients without CTLA-4–regulated responses did not demonstrate this bystander suppression (data not shown).

After identifying a PAP-specific regulatory cell population, we sought to identify the cell population mediating this suppression. Surprisingly, when CD8$^+$ T cells were depleted from PBMC samples of patients with regulatory responses, we found that PAP-specific bystander suppression was lost and a TT/D-specific response could be detected (Fig. 2B). Furthermore, when purified CD8$^+$ T cells were added back to CD8-depleted PBMCs, this PAP-specific bystander regulation was reestablished. To evaluate whether these CD8$^+$ T cells were being directly activated as a result of encountering PAP-derived epitopes bound to MHC class I, we conducted bystander regulation assays in the presence of blocking Abs targeting MHC class I or II. As the TT/D recall response is mediated primarily by CD4$^+$ T cells, blocking MHC class II (but not MHC class I) abrogated a TT/D effector response (Fig. 2C). However, whereas cotreatment of TT/D and PAP resulted in the suppression of the TT/D immune response, blocking MHC class I relieved this suppression. When this CD8$^+$ T cell was further interrogated based on surface expression of CTLA-4, the addition of CD8$^+$CTLA-4$^+$ cells to CD8-depleted PBMCs restored the suppression of TT/D immune responses (Fig. 2D), whereas the addition of CD8$^+$CTLA-4$^-$ T cells did not, suggesting the presence of a PAP-specific population of CD8$^+$CTLA-4$^+$ suppressive T cells.

CTLA-4 blockade decreases the expression of IL-35 by Ag-specific CD8$^+$CTLA-4$^+$ T cells

After finding that IL-10 and TGF-β did not play a role in mediating PAP-specific regulatory cell function (Fig. 1C), we explored the potential contribution of IL-35. Preimmunization PBMC samples from a patient with a CTLA-4–regulated immune response (ID002) were stimulated for 72 h with PAP in the presence of either CTLA-4 blocking Ab or an IgG control. CD3$^+$CD8$^+$CTLA-4$^+$ cells were then isolated by cell sorting (flow gating schema shown in Supplemental Fig. 2A) and assessed for cytokine expression by quantitative real-time PCR. As shown in Fig. 3A, stimulation with PAP resulted in a 20- to 30-fold induction in expression of *IL-12A* and *Ebi3* subunits of IL-35 in CD8$^+$CTLA-4$^+$ T cells compared with PSA-treated cells. When regulatory responses were inhibited using an anti–CTLA-4 Ab, the expression of the IL-35 subunits was significantly decreased in a PAP-specific fashion. An Ag-specific, CTLA-4–regulated increase in IL-35 expression was not observed in other T cell populations (Fig. 3B, 3C). Additionally, we did not detect any CTLA-4–regulated expression of other components of the IL-12 family (*IL-12B, IL-23A*, and *IL-27*; Fig. 3). Consistent with previous results, we did not detect significant expression, nor a CTLA-4–regulated decrease in expression, of IL-10 by quantitative real-time PCR (Fig. 3A), ELISA, or multiplex cytokine analysis (data not shown). Similar results were also observed in each of the other two patients that had been found to have PAP-specific CD8$^+$ suppressive immune responses (Supplemental Fig. 3).

CD8$^+$CTLA-4$^+$ T cells suppress T cell proliferation in an IL-35–dependent, contact-independent fashion

To assess directly the suppressive function of PAP-specific CD8$^+$ CTLA-4$^+$ T cells, PBMCs were stimulated with PAP or the control Ag PSA and sorted for CD8$^+$CTLA-4$^+$ and CD8$^+$CTLA-4$^-$ T cells (shown in Supplemental Fig. 3B). These sorted cells were then assessed for their ability to suppress the proliferation of autologous T cells in the presence or absence of Abs blocking IL-35. As shown in Fig. 4A, PAP-stimulated CD8$^+$CTLA-4$^+$ T cells had a marked, titratable suppressive effect on the proliferation of autologous CD4$^+$ and CD8$^+$ T cells, an effect that was abrogated by the addition of Abs blocking both subunits of IL-35. This suppression was not seen when CD8$^+$CTLA-4$^-$ T cells were added, nor cells sorted from cultures stimulated with either PSA or media alone (graphically represented in Fig. 4B). Similar results were obtained from other patients with PAP-specific CD8$^+$ suppressive immune responses (Supplemental Fig. 4A).

To evaluate the contact dependence of this regulation, similar in vitro suppression assays were performed using a Transwell system (6, 7). In this system, PAP-stimulated CD8$^+$CTLA-4$^+$ T cells physically separated from the responding autologous T cell population were found to mediate similar levels of suppression compared with direct suppression assays, and this suppression could be alleviated upon cotreatment with Abs blocking both subunits of IL-35 (Fig. 5). T cells lacking CTLA-4$^+$ expression lacked this suppressive activity, as did cells isolated from unstimulated PBMCs or PSA-stimulated cultures. Similar results were obtained from another patient with PAP-specific CD8$^+$CTLA-4$^+$ suppressive immune responses (Supplemental Fig. 4B), showing that PAP-specific CD8$^+$CTLA-4$^+$ suppressive immune responses can mediate suppression in an IL-35–dependent, contact-independent fashion.

PAP-specific regulatory responses mediate tvDTH suppression via IL-35

After identifying PAP-specific IL-35 expression in CD8$^+$CTLA-4$^+$ T cells and finding that this expression is decreased by inhibiting CTLA-4–mediated regulatory responses, we studied the role of IL-35 in mediating PAP-specific suppressive responses in the tvDTH in vivo setting. Samples from three patients with preexisting PAP-specific CD8$^+$CTLA-4$^+$ regulatory responses were evaluated for bystander suppression in the presence of various

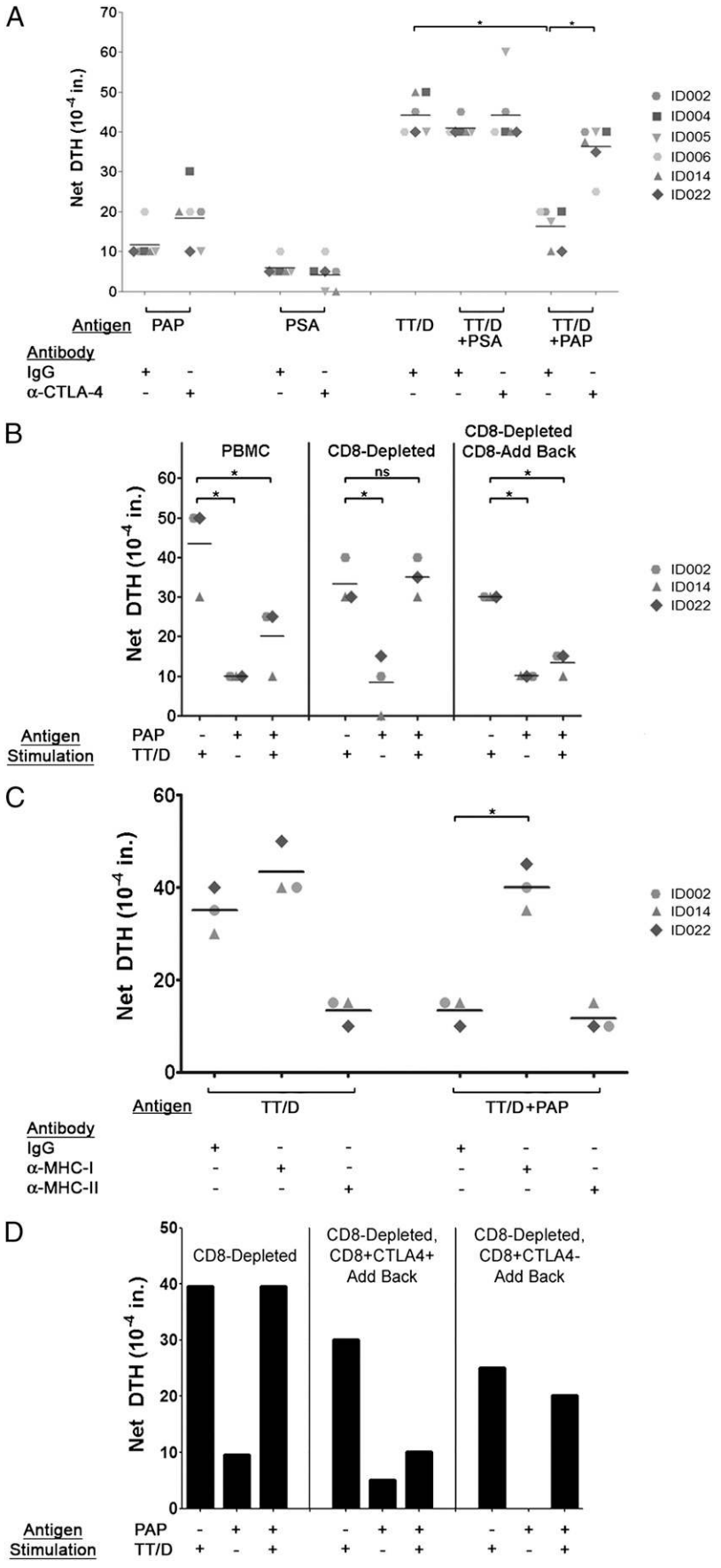

FIGURE 2. PAP-specific regulatory cells exist prior to immunization and are $CD8^+CTLA\text{-}4^+$. (**A**) PBMC samples from multiple prostate cancer patients were tested for preexisting PAP-specific regulatory responses by tvDTH. PBMCs were stimulated with the described Ags and Ab treatments, and tvDTH reactivity was measured. Data shown are representative of at least two independent experiments. Differences in mean tvDTH among different Ag/Ab treatment groups (indicated by solid lines) were compared using a Student *t* test. $*p \leq 0.05$. (**B**) PAP-specific regulatory responses were evaluated in tvDTH studies using samples from patients identified in (A). These studies were conducted using whole PBMCs (*left*), PBMC samples that had been depleted of $CD8^+$ T cells (*center*), or PBMC samples that had been depleted of $CD8^+$ T cells and were subsequently supplemented with autologous $CD8^+$ T cells (*right*). Data shown are representative of at least two independent experiments. Statistical differences between group mean DTH values (indicated by solid lines) were analyzed using a Student *t* test. $*p \leq 0.05$. (**C**) PBMCs from patients with CD8-dependent regulatory responses identified in (B) were evaluated for MHC dependency in tvDTH bystander suppression assays using Abs blocking MHC class I or MHC class II. Statistical differences between group mean DTH values (indicated by solid lines) were analyzed using a Student *t* test. $*p \leq 0.05$. (**D**) PBMC samples from patient ID022 were evaluated for tetanus bystander suppression by tvDTH. PBMC samples were depleted of $CD8^+$ T cells and tested alone (*left*) or supplemented with magnetic bead–sorted $CD8^+CTLA\text{-}4^+$ (*center*) or $CD8^+CTLA\text{-}4^-$ T cells (*right*) along with Ags described.

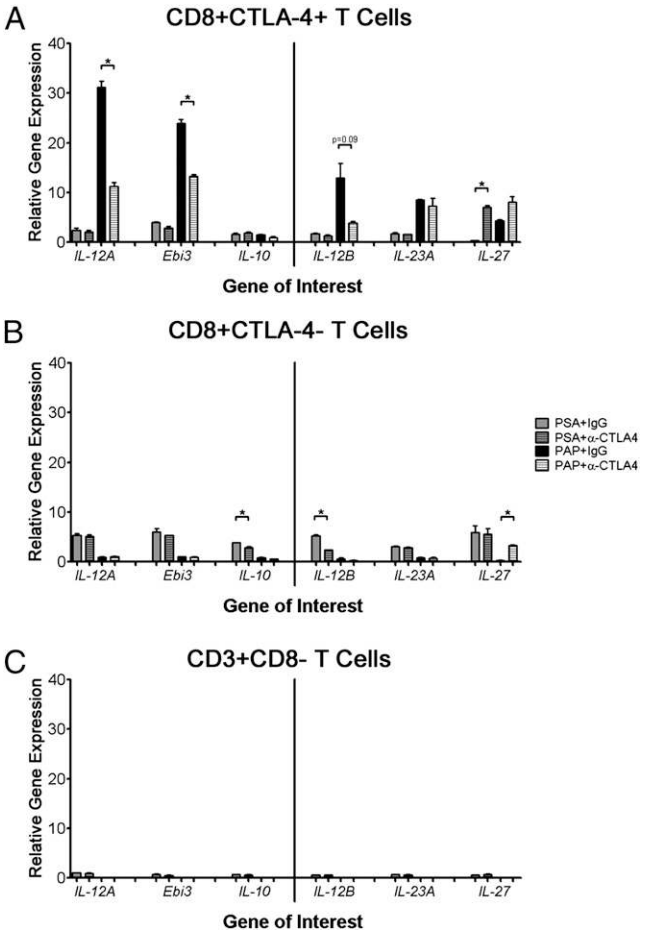

FIGURE 3. CTLA-4 blockade decreases expression of IL-35 by Ag-specific CD8$^+$CTLA-4$^+$ T cells. Samples from a patient with CTLA-4–regulated PAP-specific immune responses (ID002) were stimulated for 72 h with PAP, PSA, or media alone in the presence of an anti–CTLA-4 Ab or an IgG control (PSA plus IgG, gray; PSA plus anti–CTLA-4, hatched gray; PAP plus IgG, black; PAP plus anti–CTLA-4, hatched white). Cells were then sorted via flow cytometry for the following populations: CD3$^+$CD8$^+$ CTLA-4$^+$ (**A**), CD3$^+$CD8$^+$CTLA-4$^-$ (**B**), or CD3$^+$CD8$^-$ (**C**). Relative mRNA expression of *IL-12A, Ebi3, IL-10, IL-12B, IL-23A,* and *IL-27* was determined by quantitative RT-PCR and was normalized against the ribosomal protein P0 as an internal control and the media-alone cell-sorted sample. Data shown are representative of three independent experiments. Differences between control IgG and anti–CTLA-4 treatment group means were calculated using a Student *t* test. $*p \leq 0.05$.

blocking Abs. Specific neutralization of IL-35 (by blocking IL-12p35 or Ebi3, alone or in combination) significantly relieved the PAP-specific bystander suppression of tetanus immune response in 3 of 3 patients tested (Fig. 6). Treatment with blocking Abs targeting the other members of the IL-12 family (IL-12p40, IL-23p19, or IL-27p28), as well as blocking the immunosuppressive cytokines IL-10 and TGF-β, did not relieve this PAP-specific bystander suppression, further illustrating that PAP-specific regulatory cells mediate suppression via CTLA-4 and IL-35.

Discussion

Whereas cancer vaccines have shown promise in eliciting anti-tumor immune responses, albeit with modest clinical benefits, the effector responses these vaccines aim to augment are often countered by concurrent regulatory responses. Although reports evaluating the effects of regulatory responses on vaccine efficacy have focused on CD4$^+$CD25$^+$ Treg responses and myeloid-derived suppressor cells, in this study we show that CD8$^+$ Ag-specific suppressive responses also exist in individuals with prostate cancer, and these regulatory responses can have a profound effect in limiting the detection of Ag-specific effector T cell responses. Using samples from a recently completed clinical trial evaluating a DNA vaccine targeting PAP, we identified individuals who had Ag-specific effector immune responses after immunization that were suppressed by concurrent regulatory responses. CTLA-4–regulated PAP-specific effector responses were found to be common after immunization, but such effectors were rare prior to immunization. However, PAP-specific Tregs, revealed by bystander suppression of a recall Ag response, were identified prior to immunization in nearly 30% (6 of 21) of prostate cancer patients. PAP-specific Tregs were found to mediate suppression via IL-35 in a contact-independent fashion and were not found to rely on more extensively characterized suppressive cytokines such as IL-10 or TGF-β (62). The data indicate that tumor Ag–specific immunosuppressive responses mediated by CD8$^+$CTLA-4$^+$ IL-35–expressing T cells arise spontaneously in some patients and that these responses affect the detection (and possibly the function) of effector immune responses resulting from immunization.

Our results suggest that Ag-specific CD8$^+$ cells might be a significant obstacle in efforts to augment effective anti-tumor immune responses to prostate tumor–associated Ags. This is of particular importance with regard to PAP, as it is the target of the FDA-approved sipuleucel-T immunotherapy for patients with advanced prostate cancer (40). Tumor Ag–specific CD8$^+$ T cell responses to tumor-associated Ags were reported by Andersen and colleagues (63), who identified peripheral CD8$^+$ Tregs in patients with melanoma or renal or breast cancer that were specific for heme oxygenase-1, a protein involved in the termination of inflammatory responses. They found that heme oxygenase-1–specific CD8$^+$ Tregs had enhanced suppressive function compared with CD4$^+$ Tregs and that this suppression was contact-independent. However, they did not detect secretion of either IL-10 or TGF-β by these CD8$^+$ regulatory cells, leaving open a role for other immunosuppressive cytokines such as IL-35.

Although we show that CD8$^+$CTLA-4$^+$ T cells specific for PAP have suppressive activity in vivo and in vitro, the specific target and mechanism of action of this suppression remains unclear. Our ability to isolate these CD8$^+$CTLA-4$^+$ Tregs 2 wk after the final immunization suggests that CTLA-4 expression may be sustained in this population (as opposed to the transient expression of CTLA-4 by effector T cells). As such, the expression of CTLA-4$^+$ itself could potentially have a suppressive effect by preventing effector T cells from having access to B7 ligands on APCs. However, IL-35 appears to be the central mediator of contact-independent tolerance in this model, which could occur via a number of mechanisms. It could be that IL-35 produced by this regulatory population is acting directly on effector T cells, including the PAP-specific effector cells identified after immunization and thus preventing their detection after immunization. IL-35 could also be acting to propagate these regulatory responses through the generation of additional induced Tregs, as has previously been identified (4, 5). Alternatively, these CD8$^+$CTLA-4$^+$ Tregs could be acting on a population of APCs, dampening their ability to activate effector responses. Identifying the precise target of suppression of this regulatory population will lead to a better understanding of how these regulatory cells may effect the generation of effector responses after immunization.

Although we focus on the characterization of PAP-specific regulatory CD8$^+$ T cells, our results certainly do not exclude the possibility that PAP-specific CD4$^+$ regulatory cells exist. A recent report has found that PAP-specific immune responses in prostate cancer patients are enhanced by depletion of CD4$^+$CD25$^+$ Tregs

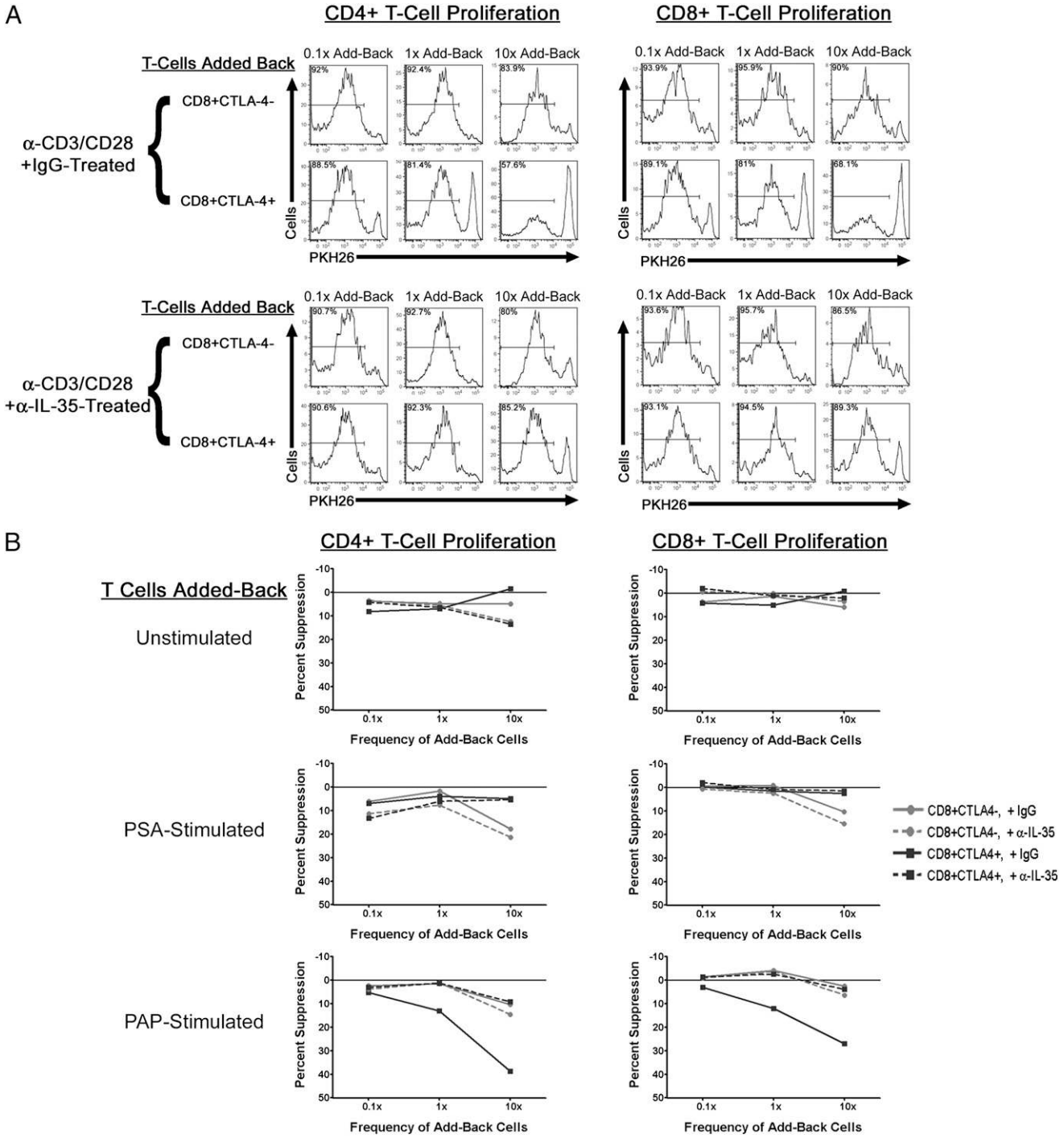

FIGURE 4. $CD8^+CTLA\text{-}4^+$ T cells suppress T cell proliferation in an IL-35–dependent fashion . PBMCs from a patient with CTLA-4– and IL-35–regulated PAP-specific bystander suppressive immune response (ID014) were stimulated for 3 d with PAP, PSA, or media alone. Cells were sorted by flow cytometry for $CD8^+CTLA\text{-}4^+$ or $CD8^+CTLA\text{-}4^-$ T cells, labeled with CFSE, and added back to autologous PKH26-labeled PBMCs at either their natural frequency or a fold higher or lower. Cocultures were stimulated for 7 d with either media alone, anti-CD3/anti-CD28–coated beads along with IgG, or anti-CD3/CD28–coated beads along with anti–IL-35 blocking Abs. After this incubation, the proliferation of $CFSE^-$ $CD4^+$ and $CD8^+$ T cells was measured by PKH26 dilution, and percent suppression was calculated compared with the proliferation of unstimulated PBMCs without any cells added back. (**A**) Proliferation of $CD4^+$ (*left*) and $CD8^+$ (*right*) to which were added increasing frequencies of PAP-stimulated $CD8^+CTLA\text{-}4^+$ or $CD8^+CTLA\text{-}4^-$ sorted T cells (indicated next to *y*-axis). Cocultures were also treated with either a control IgG (*top*) or IL-35 blocking Abs (*bottom*; indicated to *far left* of *x*-axis). (**B**) Graphical representation of suppression assays conducted using sorted $CD8^+CTLA\text{-}4^+$ (black) or $CD8^+CTLA\text{-}4^-$ (gray) T cells that were stimulated with either PAP (*bottom row*), PSA (*middle row*), or media alone (*top row*). Cocultures were treated with anti-CD3/CD28–coated beads along with IgG (solid lines) or IL-35 blocking Abs (dashed lines). After this incubation, the proliferation of $CFSE^-$ $CD4^+$ (*left*) and $CD8^+$ (*right*) T cells was measured by PKH26 dilution. Data shown are representative of at least two independent experiments from the same patient.

(42), and immunization of rodents with a vaccine targeting PAP was found to elicit IL-10–secreting immune responses (64). Indeed, preliminary data from our studies indicate that $CD4^+$ T cells may also contribute to PAP Ag–specific regulation, as one of the patients we identified as having PAP-specific $CD8^+$ regulatory responses also had $CD4^+CD25^{hi}$ T cells that could suppress T cell

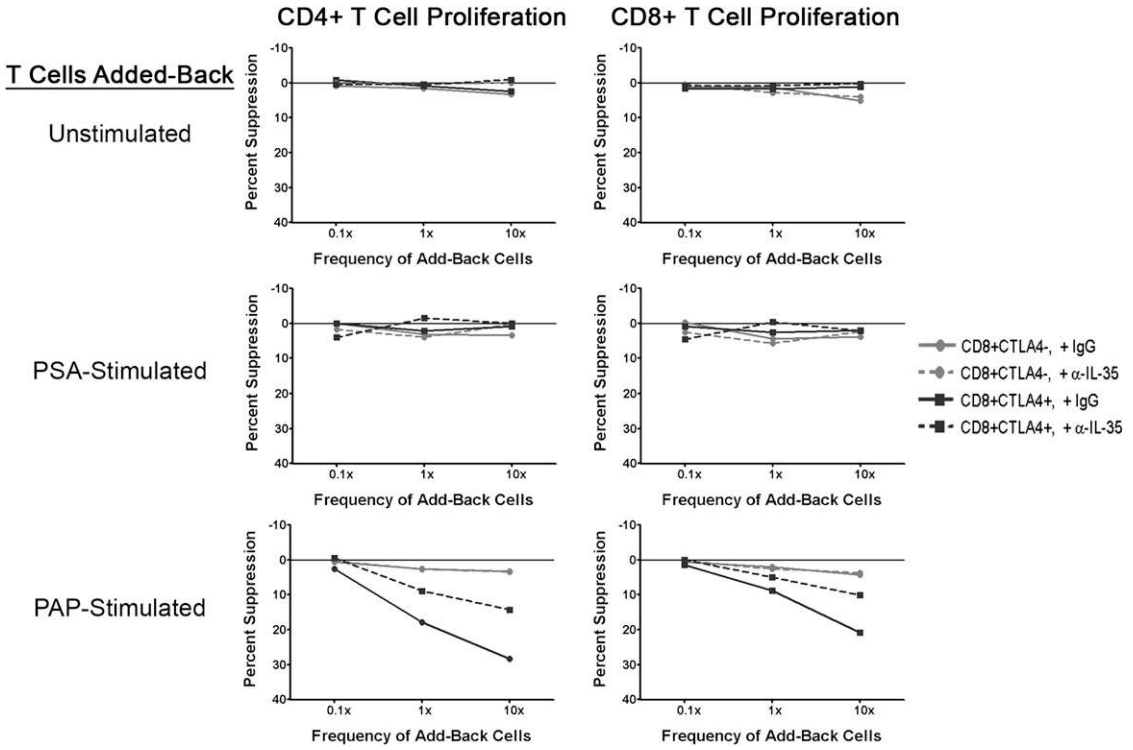

FIGURE 5. CD8$^+$CTLA-4$^+$ T cells suppress T cell proliferation in a contact-independent fashion. PBMCs from a patient with CTLA-4– and IL-35–regulated PAP-specific bystander suppressive immune response (ID002) were stimulated for 3 d with PAP (*bottom row*), PSA (*middle row*), or media alone (*top row*). Cells were sorted by flow cytometry for CD8$^+$CTLA-4$^+$ (black) or CD8$^+$CTLA-4$^-$ (gray) T cells and added back to the top chamber of a 96-well Transwell plate at either their natural frequency or a fold higher or lower (indicated along the *x*-axis). Autologous PKH26-labeled PBMCs were added to the bottom chamber of the Transwell plates and stimulated for 7 d with either media alone, anti-CD3/anti-CD28–coated beads along with IgG (solid lines), or anti-CD3/anti-CD28–coated beads along with anti–IL-35 blocking Abs (dashed lines). After this incubation, cells were collected from the bottom chamber and measured for the proliferation of naive CD4$^+$ (*left*) and CD8$^+$ (*right*) T cells by PKH26 dilution, and percent suppression was calculated compared with the proliferation of naive PBMCs without any cells added back. Data shown are representative of at least two independent experiments.

proliferation in vitro (B. Olson, D. McNeel, and W. Burlingham, unpublished observations). The impact of PAP Ag–specific regulatory immune responses (both CD8$^+$ and potentially CD4$^+$ T cells) on the generation of PAP effector responses will be evaluated in future studies.

Although ours is the first report to our knowledge to identify IL-35–secreting Ag-specific Tregs in patients with prostate cancer, CD8$^+$ Tregs have previously been identified in both murine models of prostate cancer and patients with prostate tumors. Kiniwa and colleagues (65) identified human prostate tumor–infiltrating CD8$^+$ T cells that could suppress the nonspecific activation of CD4$^+$ T cells. An intriguing finding from this study was that they report a contact-independent mechanism of suppression that was not mediated by either IL-10 or TGF-β (65). Additionally, another report from Shafer-Weaver and colleagues (66) identified murine prostate tumor–infiltrating CD8$^+$ T cells that were induced to become regulatory cells once at the site of tumor, although it remains unclear whether this conversion was mediated by tumor-derived factors or possibly through tumor-infiltrating regulatory cells. This study also found that CD8$^+$ Tregs mediate suppression in a contact-independent fashion, and whereas they identified a role for TGF-β in mediating this suppression, they found that blocking TGF-β did not completely alleviate contact-independent suppression (66). Given recent findings suggesting the crucial importance of IL-35 in propagating CD4$^+$ Treg–mediated suppression (4–6), our findings suggest that IL-35 may also play an important role in mediating suppression by CD8$^+$ Tregs.

Whereas CD8$^+$ suppressive T cells have been detected in multiple model systems, the origin of these CD8$^+$ suppressor cells remains unclear. Possible origins include i) a distinct thymic lineage like "natural" CD4$^+$CD25$^+$ Tregs, ii) an uncommitted population of naive CD8$^+$ T cells, or iii) conversion from traditional

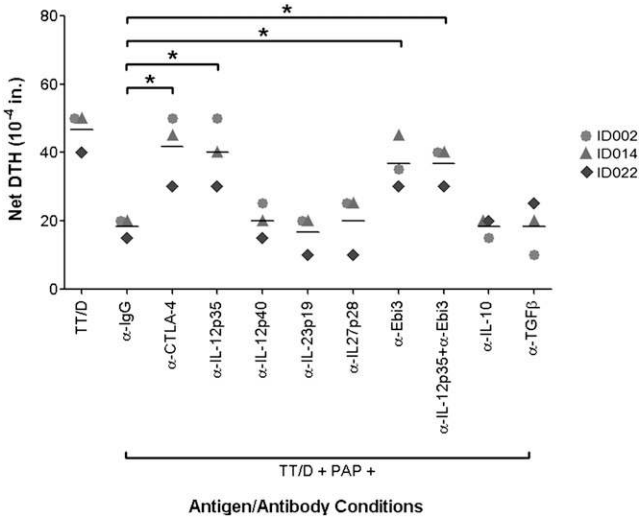

FIGURE 6. PAP-specific CD8$^+$ regulatory responses mediate tvDTH suppression via IL-35. PBMCs from patients with CTLA-4–regulated PAP-specific bystander suppressive immune responses were stimulated with TT/D alone or TT/D and PAP in the presence of either an IgG control or blocking Abs directed against CTLA-4, IL-12p35, IL-12p40, IL-23p19, IL-27p28, Ebi3, a combination of IL-12p35 and Ebi-3 (both IL-35 subunits), IL-10, or TGF-β. Data shown are representative of at least two independent experiments. Differences between treatment group mean DTH values (indicated by solid lines) were compared using a Student *t* test. $^*p < 0.05$.

effector CD8$^+$ T cells. We previously identified populations of CD8$^+$CTLA-4$^+$ suppressive T cells that mediate maternal/offspring tolerance to minor histocompatibility Ags, HA-1 and HY-1. In this model, a distinct population of tetramer^{dim} CD8$^+$CTLA-4$^+$ suppressive T cells was identified, whereas Ag-specific CD8$^+$ effector T cells were found to be tetramer^{bright} (16, 67, 68). This may suggest that distinct lineages of CD8$^+$ T cells arise whose functions are based on TCR avidity, which could be addressed in future studies using previously identified HLA-A2–restricted T cell epitopes specific for PAP (69). Although this model of distinct regulatory and effector Ag–specific T cell populations would account for the presence and function of Ag-specific regulatory cells, it does not account for the influence of the environment on T cell function (6, 34, 66, 70). Research into T cell plasticity has shown that T cells can change their functional phenotype depending on the conditions within the microenvironment, perhaps by regulatory CD4$^+$ and/or CD8$^+$ T cells or by the tumor itself, including a report of CD8$^+$ T cells being induced into a regulatory phenotype in a murine model of prostate cancer (66). Additionally, iTr35 cells have also been shown to be induced by the tumor microenvironment in murine models of both melanoma and colorectal adenocarcinoma (4). This concept of "local tolerance" (tolerance being induced within the microenvironment, rather than dichotomous central and peripheral tolerance) and a potential role for IL-35 in contributing to this induction of local tolerance warrants further investigation, as does the prevalence of CD8$^+$CTLA-4$^+$ IL-35–secreting Tregs within prostate tumors.

It remains unclear whether the presence of Ag-specific regulatory responses is beneficial or not in the context of immunization. A priori, the presence of Ag-specific regulation might predict for individuals unlikely to respond to an Ag-specific vaccine. However, we were able to detect this type of response in individuals who subsequently had evidence of PAP-specific IFN-γ–secreting T cells. Moreover, of the eight individuals in whom CTLA-4–regulated PAP responses were detected after immunization, five subsequently experienced at least a 200% increase in PSA doubling time (53). Consequently, it is conceivable that the presence of Ag-specific regulation belies the existence of prior effector or memory Ag–specific cells whose function becomes actively suppressed, suggesting that the presence of Ag-specific regulation might indicate individuals with existing effector cells that might be more readily augmented with immunization. As such, we believe that the detection of Ag-specific regulatory responses should be included in the baseline evaluation of patients receiving Ag-specific vaccines, and potentially at times thereafter. Furthermore, although we focused on PAP Ag–specific regulatory immune responses and did not detect regulatory responses for another prostate tumor Ag (PSA), we certainly do not exclude the possibility that Ag-specific regulatory immune responses exist for other prostate cancer tumor–associated Ags that are targets for immunotherapeutic vaccines. The association of these findings with immunological response, specifically whether the presence of Ag-specific CD8$^+$CTLA-4$^+$ IL-35–secreting regulatory cells affects the generation and kinetics of T cell effector responses, and more importantly clinical benefit, will be evaluated in sufficiently powered future studies. Such associations might ultimately be used to determine whether a particular individual is likely or not to respond to a particular Ag-specific vaccine. This is of particular relevance for the PAP tumor Ag, as this is the Ag target of the recently FDA-approved sipuleucel-T immunotherapy for patients with advanced prostate cancer (40).

Given the potentially detrimental impact regulatory responses can have on anti-tumor vaccines, there has been great interest in combining vaccination with strategies aimed at depleting regulatory responses in many different malignancies, including prostate cancer (46, 71–74). A recent report of a phase I trial by Fong and colleagues (74) in which prostate cancer patients received GM-CSF and/or a CTLA-4–blocking Ab found that a combination of both treatments acted synergistically to increase expansion of activated CD8$^+$ T cells. Several groups have reported the detection of Ag-specific T cells after CTLA-4 blockade, even years after Ag-specific vaccination, suggesting that Ag-specific T cell regulation might be a mechanism that could exist for long periods of time but be directly targeted (75). Furthermore, the central role of IL-35 in mediating suppression suggests that Abs directed against IL-35 may also have efficacy targeting the function of regulatory cells and may be a suitable target for future clinical evaluation. By identifying individuals with these preexisting regulatory responses, it may be possible to prioritize patients who may respond ideally to these combinatorial therapies.

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Disclosures

D.G.M., W.J.B., and D.A.A.V. have submitted patents that are pending and are entitled to a share in net income generated from licensing of these patent rights for commercial development. The other authors have no financial conflicts of interest.

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