The Journal of Immunology

RESEARCH ARTICLE | JANUARY 15 2006

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J Immunol (2006) 176 (2): 984–990.
https://doi.org/10.4049/jimmunol.176.2.984

Effect of $CD4^+CD25^+$ and $CD4^+CD25^-$ T Regulatory Cells on the Generation of Cytolytic T Cell Response to a Self but Human Tumor-Associated Epitope In Vitro[1]

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$CD4^+$ T cells naturally expressing CD25 molecules (natural T regulatory cells (Tregs)) have a role in maintaining self tolerance and in regulating responses to infectious agents, transplantation Ags, and tumor Ags. $CD4^+$ Tregs induced from $CD4^+CD25^-$ precursors (induced Tregs) also regulate immune responses in the periphery. However, which of these Tregs is a major impediment in generating antitumor CTL responses is not clear. We show that although the $CD4^+CD25^+$ subsets isolated from peripheral blood-derived lymphocytes do suppress the proliferation of $CD4^+CD25^-$ effector T cells, they do not suppress the activation and expansion of the self but melanoma-associated, melanoma Ag-reactive T cell 1 (MART-1)$_{27–35}$-specific $CD8^+$ T cells stimulated by the respective peptide-loaded matured dendritic cells in vitro. The $CD4^+CD25^-$ counterparts, in contrast, lead to the generation of $CD25^+$ glucocorticoid-inducible $TNFR^+$-Forkhead/winged helix transcription factor$^+$ populations and efficiently suppress the activation and expansion of the MART-1$_{27–35}$ epitope-specific CTLs. Our data suggest that when CTL precursors are optimally stimulated, natural Tregs are not a formidable constraint toward generating a robust antitumor CTL response, but induced Tregs could be. ***The Journal of Immunology,*** **2006, 176: 984–990.**

A role for T suppressor cells (1) in tolerance induction as well as suppression of immune responses in the periphery, once highly controversial (2), is now widely accepted (3–5), primarily due to a set of seminal observations on the role for a class of $CD4^+CD25^+$ T cells in preventing autoimmune pathologies after neonatal thymectomy in mice by Sakaguchi et al. (see Ref. 3 for a review). These cells are now called T regulatory (Treg)[3] cells, and because they naturally express CD25, they have also been described as natural Treg (nTreg) cells. In addition to maintaining self tolerance in the periphery, their role has been extended to the control of immune responses against infectious agents, transplantation Ags, and tumor-associated Ags (6). North et al. (7) were the first to show that T cells bearing the helper phenotype (Lyt $1^+2^-3^-$) can function as suppressor T cells in a mouse tumor model. A role for $CD4^+$ T cells in the regulation of immune responses in vitro was extended in the human tumor model thereafter (8–11). Recent studies, however, have led to a new paradigm that although $CD4^+CD25^-$ T cells can be induced to become suppressor or regulatory cells (i.e., induced Tregs (iTregs)), nTregs are the central players (5) in tolerance induction in the periphery.

The literature on Tregs is extensive (see Refs. 3–6 for a review). The literature on Tregs in tumor immunity is also quite substantial (see Refs. 12–28 for a review). It is now widely held that $CD4^+CD25^+$ nTregs are the major regulatory cells in antitumor immunity. However, given that IL-10-producing, Tr1-type $CD4^+$ T cells have been clearly implicated in the control of inflammatory bowel disease (29, 30) as well as in antitumor immunity (8–11), the time has come for a critical comparison of the suppressive properties of nTregs ($CD4^+CD25^+$ T cells) and iTregs ($CD4^+$ Tregs generated from $CD4^+CD25^-$ precursors) on the activation of relevant tumor epitope-specific CTL in a human model. In this study we show that although the fresh $CD4^+CD25^+$ subsets purified from peripheral blood-derived lymphocytes do suppress the proliferation of $CD4^+CD25^-$ effector T cells in a soluble anti-CD3 Ab-driven proliferation assay (31), they are ineffective in suppressing the activation and expansion of the melanoma Ag-reactive T cell-1 (MART-1)$_{27–35}$ epitope-specific $CD8^+$ T cells. The $CD4^+CD25^-$ subsets, in contrast, give rise to $CD4^+CD25^+$/GITR$^+$/Forkhead/winged helix transcription factor (Fox P3)$^+$ populations and suppress the activation and expansion of MART-1$_{27–35}$-epitope specific $CD8^+$ T cells quite efficiently. Our data suggest that when CTL precursors are optimally stimulated, nTregs are not a formidable constraint toward generating a robust antitumor CTL response, but iTregs could be.

Materials and Methods

Study population

HLA-A2-positive melanoma patients or healthy donors were included in this study after obtaining their informed consents.

Culture medium and reagents

The MART-1$_{27–35}$ peptide (AAGIGILTV) was purchased from Multiple Peptide Systems; β_2-microglobulin was purchased from Sigma-Aldrich.

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Received for publication August 18, 2005. Accepted for publication November 1, 2005.

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[1] This work was supported by U.S. Public Health Service Grants CA83130 and CA88059.

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[3] Abbreviations used in this paper used: Treg, T regulatory cell; DC, dendritic cell; FoxP3, Forkhead/winged helix transcription factor; iTreg, inducible Treg; IVC, in vitro coculture; mDC, myeloid mature DC; nTreg, natural Treg; MART, melanoma Ag-reactive T cell; GITR, glucocorticoid-inducible TNFR.

0022-1767/06/$02.00

Culture medium consisted of IMDM (Invitrogen Life Technologies) supplemented with 10% FBS (Gemini Bioproducts), 0.55 mM L-arginine, 0.24 mM L-asparagine (both from Invitrogen Life Technologies), 1.5 mM L-glutamine (Sigma-Aldrich), 50 U/ml penicillin, and 50 μg/ml streptomycin (both from Abbott Laboratories). This will be referred to as complete medium. The TAP-deficient line, T2, was a gift from Dr. P. Cresswell (Yale University, New Haven, CT). Recombinant human GM-CSF was purchased from Immunex. Recombinant human IL-4, IL-2, IFN-γ, and IL-15 were purchased from R&D Systems. LPS from *Escherichia coli* 055:B5 was purchased from Sigma-Aldrich. MART-$1_{27–35}$ (EAGIGILTV) tetramer labeled with allophycocyanin was purchased from Beckman Coulter. Purified anti-human IL-10R; anti-human HLA-DR, -DP, and -DQ; fluorochrome-labeled anti-human mAbs to CD4, CD8, and CD25; glucocorticoid-inducible TNFR (GITR); goat anti-mouse IgG; and Cytofix/Cytoperm reagent were purchased from BD Biosciences. Anti-human TGF-β mAb was obtained from R&D Systems. Rabbit anti-goat FITC was purchased from Santa Cruz Biotechnology. Anti-FoxP3 polyclonal Ab was purchased from Abcam. Ficoll-Hypaque and [^{3}H]TdR were purchased from Amersham Biosciences.

Generation of dendritic cells (DC) and isolation of T cell subsets

The procedures for generating myeloid mature DC (mDC) and isolation of T cell subsets by positive selection by Dynal beads (Dynal Biotech) have been described previously (32, 33). For the isolation of T cell subsets, $CD8^+$ T cells were first purified by positive selection. From the $CD8^-$ fractions, $CD4^+$ T cells were then isolated by positive selection. Thereafter, the $CD4^+CD25^+$ were isolated by positive selection, and the $CD4^+CD25^-$ subsets were obtained by negative selection by Dynal beads. Purity was determined by flow cytometry and routinely exceeded 92%.

CTL generation assay in in vitro coculture (IVC)

The in vitro CTL generation assay with purified $CD8^+$ T cells stimulated by peptide-loaded mDCs and flow cytometric analyses of epitope-specific T cells have been previously described (32, 33). Briefly, $CD8^+$ T cells from Ficoll-Hypaque gradient separated blood mononuclear cells by Dynal magnetic beads. The purity of $CD8^+$ T cells was routinely in excess of 92%. In vitro activation/expansion of MART-1-specific CTL precursors was conducted by coculturing purified $CD8^+$ T cells with mDC loaded with the relevant peptide (100 μg/ml) and 5 μg/ml β_2-microglobulin at a $CD8^+$ T cell to DC ratio of 100. IL-2 (50 U/ml) and IL-15 (1000 U/ml) were added on day 3, medium and cytokines were replaced every third day, and expansion/activation were read out in tetramer-based flow cytometric and functional assays on days 7–10.

Treg assays

Treg function was assessed in the standard proliferation assay using $CD4^+CD25^-$ effector cells as described by Thornton and Shevach (31) with minor modification (we used autologous DC as accessory cells). In addition, to determine the effects of $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets on MART-$1_{27–35}$ epitope-specific CTL generation, in vitro CTL generation assays were set up in the presence or the absence of the respective $CD4^+$ subsets at multiple CD8:CD4 ratios, and the outcome was assessed in tetramer assays, IFN-γ response assays, and CTL assays.

CTL and IFN-γ response assays

The chromium release microcytotoxicity assay and the IFN-γ response assay have been described previously (32).

Phenotypic analysis and Western blotting

The method for phenotypic analyses in flow cytometry and the method for quantitative analysis of epitope-specific T cells by tetramer staining have been described previously (32, 33). Intracellular staining for FoxP3 was performed using Cytofix/Cytoperm reagent (BD Biosciences) according to the manufacturer's protocol. For Western blotting, samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and then incubated with primary Abs. The membranes were washed, reincubated with alkaline phosphatase-conjugated secondary Abs, then developed by alkaline phosphatase color developing reagent according to the manufacturer's instructions (Promega).

Statistical analysis

Statistical analyses of data were performed using Student's *t* test.

Results

To examine the regulatory properties of the two Treg subsets on the generation of antitumor CTL responses, we performed all experiments in an autologous setting using the MART-$1_{27–35}$ peptide as a model for a human tumor-associated epitope and with cellular reagents from donors harboring easily detectable levels of MART-$1_{27–35}$ peptide-specific CTL precursors. Fig. 1 shows the phenotypic and functional characterization of the two $CD4^+$ subsets used

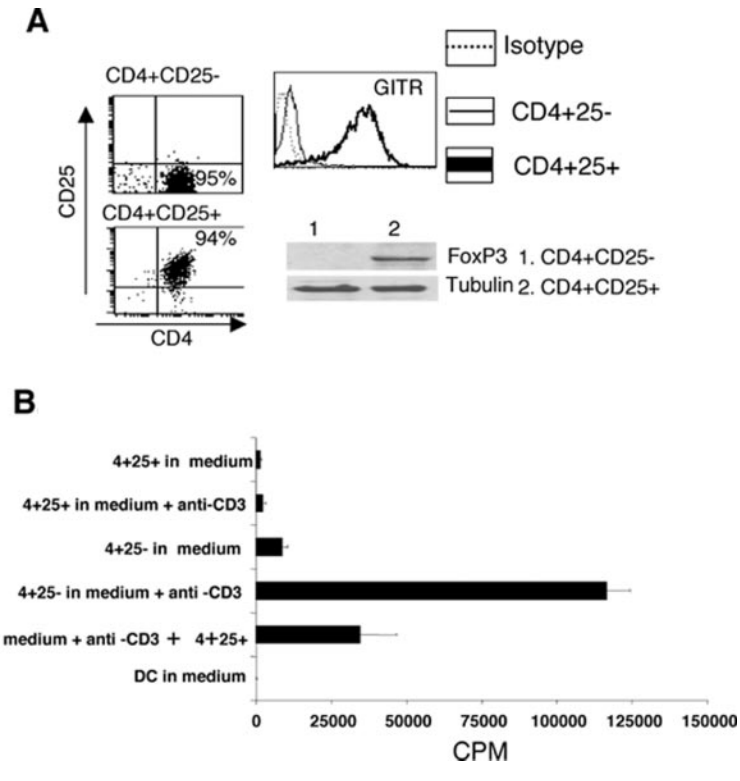

FIGURE 1. Phenotype and function of the two $CD4^+$ subsets. *A*, Phenotypes of the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells used in this study. GITR expression was analyzed by flow cytometry, and FoxP3 expression was assessed by Western blot analysis. *B*, In vitro Treg assay. Data are expressed as the mean ± SD of three replicate samples. Both $CD4^+$ T cell subsets were stimulated by 5 μg/ml soluble anti-CD3 mAb in the presence of irradiated autologous DCs (T cell:DC ratio, 100). The $CD4^+CD25^-$ T cells were also similarly stimulated in the presence of $CD4^+CD25^+$ T cells at a $CD25^-$: $CD25^+$ cell ratio of 2.

in these studies. The freshly isolated $CD4^+CD25^+$ cells used as nTregs were of considerable purity. They expressed GITR and FoxP3 (Fig. 1*A*). Fig. 1*B* shows that these $CD4^+CD25^+$ cells were anergic to stimulation by soluble anti-CD3 mAb, but they efficiently suppressed the proliferation of $CD4^+CD25^-$ T cells in the standard soluble anti-CD3 Ab-driven proliferation assay that has now been widely used as a nTreg assay (31).

We then examined the relative regulatory effects of nTregs and iTregs on CTL activation/expansion using an in vitro CTL generation protocol against the MART-$1_{27–35}$ epitope-loaded, autologous, fully matured DCs (32, 33). The phenotype of the mDC in LPS and IFN-γ was similar to that we have previously shown (32, 33). The mDC markedly up-regulated CD83, MHC class II molecules, CD80, and CD86 (data not shown). Fig. 2*A* shows a representative experiment demonstrating the effects of the $CD4^+CD25^+$ and the $CD4^+CD25^-$ subsets on the activation and expansion of MART-$1_{27–35}$ peptide-specific CTLs. The epitope-specific CTLs expanded in coculture with the peptide-loaded mDC (Fig. 2*A*) and were functionally activated (synthesized IFN-γ when restimulated; Fig. 2*B*). The $CD4^+CD25^+$ T cells did not significantly affect their expansion or their functional activation (Fig. 2, *A–C*). Both expansion and functional activation were, however, markedly suppressed by the $CD4^+CD25^-$ subset (Fig. 2, *A–C*). Individual experiments were repeated two or three times with similar results. The inability of $CD4^+CD25^+$ T cells to suppress the expansion of epitope-specific CTLs was also observed with higher ratios of $CD4^+CD25^+$:$CD8^+$ T cells (Fig. 2*D*). Of interest, $CD4^+CD25^-$ T cells did not affect the number of epitope-specific CTLs that were preactivated and did not suppress their IFN-γ-producing ability after overnight coculture (data not shown).

The reduced expansion of epitope-specific CTLs in the presence of $CD4^+CD25^-$ T cells could have resulted from preferential proliferation of other cells. As shown in Fig. 2*A*, the number of CD8-positive and MART-1 tetramer-negative subpopulations (*right*

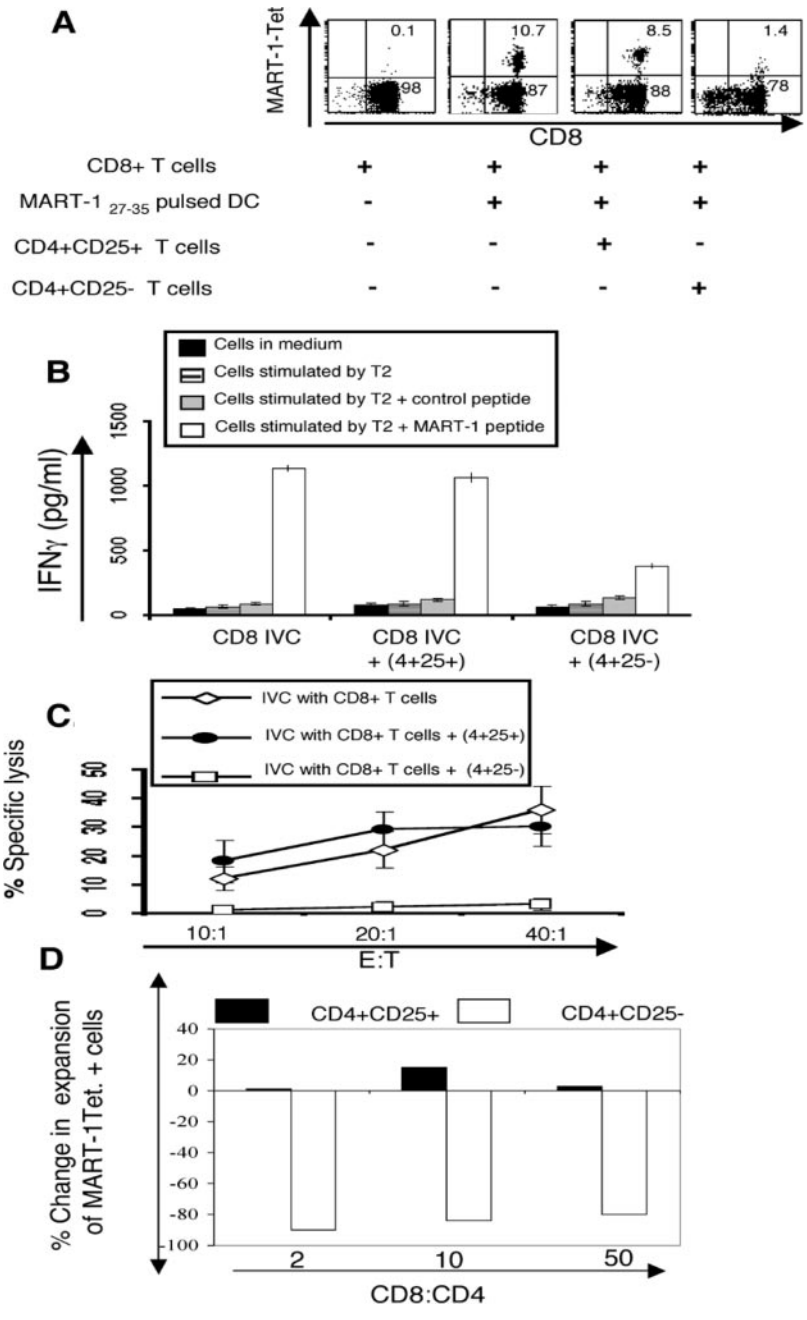

FIGURE 2. Effects of the two $CD4^+$ subsets on the activation and expansion of MART-$1_{27–35}$ epitope-specific CTL in vitro. *A*, MART-$1_{27–35}$ epitope-specific CTL expansion assay in the presence of the two $CD4^+$ T cell subsets (CD8:CD4 cell ratio, 50). *B*, Effects of the two $CD4^+$ T cell subsets on functional activation of the epitope-specific CTLs measured in an IFN-γ response assay. IFN-γ assays were conducted after the $CD8^+$ T cells were activated in the CTL generation protocol in the absence or in the presence of the two $CD4^+$ T cell subsets as described in *A*. Data are expressed as the mean $\pm$ SD of three replicate samples. *C*, Effects of the two $CD4^+$ T cell subsets on the cytolytic function of the epitope-specific CTL generated in the absence or the presence of the two $CD4^+$ T cell subsets as described in *A*. Data represent the mean $\pm$ SD of three replicate samples. *D*, Dose-response analysis of regulation by the two $CD4^+$ T cell subsets. The regulatory cocultures were conducted with different CD8:CD4 ratios, and the expansion of the epitope-specific CTLs was evaluated by tetramer staining. The percent change (amplification or suppression) was calculated by using the CTL number in the absence of $CD4^+$ T cells as 0.

lower quadrant) did not increase in coculture with the $CD4^+CD25^-$ subset. As such, the low number of MART-1 tetramer-positive $CD8^+$ T cells (*right upper quadrant*) was not due to preferential proliferation of epitope-negative $CD8^+$ subset. There was, however, some increase in the number of cells in the *left lower quadrant* (i.e., CD8 negative and tetramer negative). When these cells were separately analyzed, most of them were $CD4^+$ T cells (data not shown). Although the $CD4^+$ T cells added to the coculture did undergo some degree of proliferation (contributing to the cells in the *left lower quadrant*), their expansion does not explain the low number of epitope-specific $CD8^+$ T cells in the presence of the $CD4^+CD25^-$ subset (1.4 of 79.4 $CD8^+$ cells in coculture with the $CD4^+CD25^-$ subset vs 8.5 of 96.5 $CD8^+$ cells in coculture with the $CD4^+CD25^+$ subset).

In addition, because $CD4^+CD25^-$ T cells at times proliferated at varied degrees in the regulatory cocultures, the suppression of CTL expansion could be explained by growth factor being used up by the proliferating $CD4^+$ T cells. We ruled out the possibility of quenching of growth factor by adding a 10-fold excess of IL-2 and IL-15, which did not alter the outcome (collective data not shown).

Fig. 3*A* shows that these Tregs were MHC class II restricted, because the suppression of CTL expansion by $CD4^+CD25^-$ T

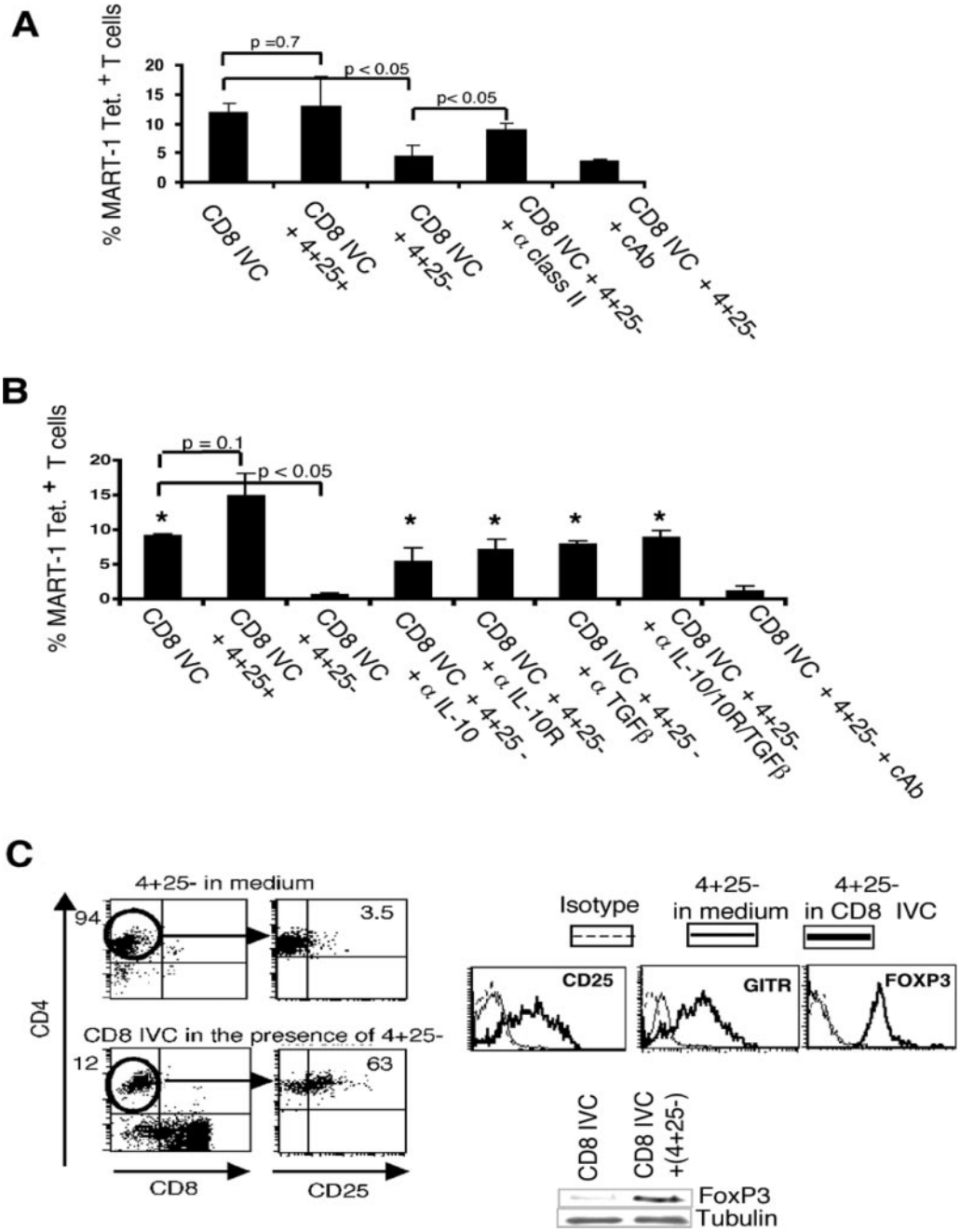

FIGURE 3. Mechanism of suppression by the $CD4^+CD25^-$ subsets. *A*, MHC class II restriction analysis of suppression by $CD4^+CD25^-$ subsets. *B*, Effects of anti-IL-10, anti-IL-10R, and anti-TGF-β Abs on suppression (cAb, control Ab). Data represent the mean $\pm$ SD value of three replicates (*, not significant compared with the number of epitope-specific CTLs in control culture). *C*, Phenotype of the freshly purified $CD4^+25^-$ T cells maintained in medium (4 + 25- in medium) and in the regulatory coculture with $CD8^+$ T cells and $CD4^+CD25^-$ T cells. CD25, GITR, and FoxP3 expressions were analyzed by flow cytometry with the CD4-gated population, and FoxP3 expression was also assessed by Western blot analysis (CD8 IVC = CTL generation culture with $CD8^+$T cells; CD8 IVC + 4+25- = CTL generation culture in the presence of $CD4^+CD25^-$ T cells).

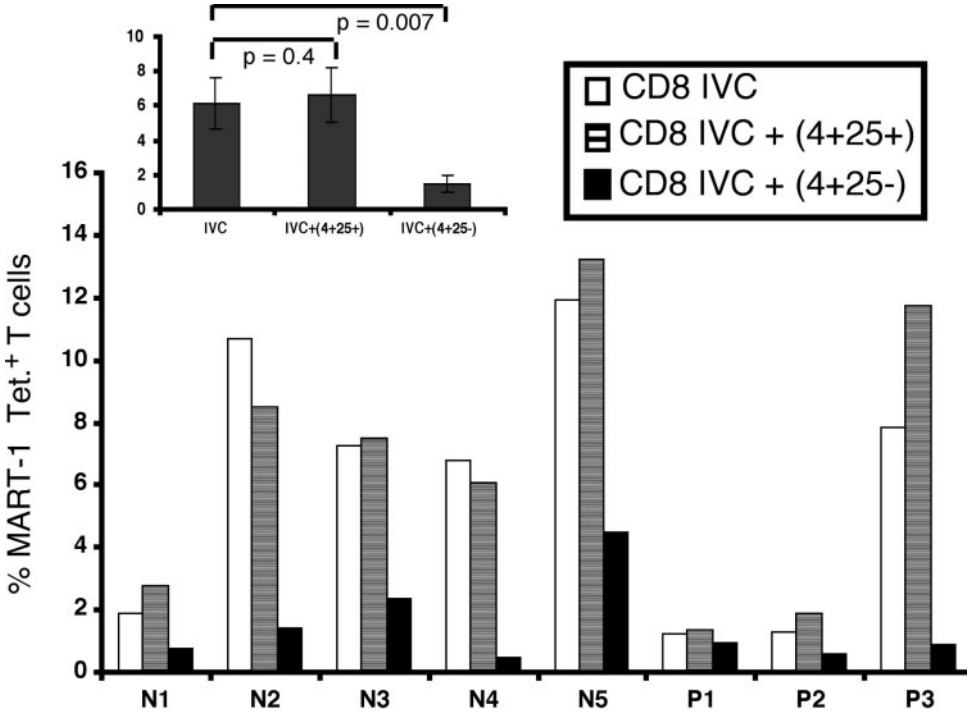

FIGURE 4. Collective effects of two CD4 subsets on the CTL response against MART-$1_{27–35}$ epitope-specific system in eight cases (N, normal donors; P, melanoma patients). The *inset* graph shows the composite analysis of the eight cases (mean ± SD).

cells was blocked by an anti-MHC class II Ab. Fig. 3*B* shows that the suppression of CTL expansion by $CD4^+CD25^-$ T cells could also be blocked by Abs to IL-10, IL-10R, and TGF-β. These experiments were repeated three times using more than one donor with similar results. Fig. 3*C* shows the phenotypic characteristics of the $CD4^+$ T cells emerging from $CD4^+CD25^-$ precursors in a regulatory assay. A large number of $CD4^+CD25^+$ expressing GITR and FoxP3 was generated in the regulatory coculture with $CD4^+CD25^-$ T cells. In addition, supernatants from cocultures of $CD8^+$ T cells and $CD4^+CD25^-$ subsets contained large amounts of IL-10 measurable in an ELISA (data not shown).

Fig. 4 shows a composite analysis of eight separate case studies (five HLA-A2 and normal donors and three melanoma patients). The precursor frequencies of the epitope-specific CTL in these donors varied from 0.15 to 0.6% of the $CD8^+$ populations, and the degree of expansion of the epitope-specific CTL also varied between cases. However, CTL generation was suppressed only by the $CD4^+CD25^-$ subsets and not by the $CD4^+CD25^+$ subsets. In a few cases, $CD4^+CD25^+$ T cells modestly amplified CTL expansion. We did not address whether there were differences in the level of suppression exhibited by the $CD4^+CD25^-$ subsets derived from normal donors vs melanoma patients. A carefully controlled, large case study will be needed to answer this question.

We finally examined the effect of $CD4^+CD25^+$ T cells on the $CD4^+CD25^-$ subsets in the CTL generation assay. Regulatory cocultures between $CD8^+$ T cells as effector cells and $CD4^+CD25^-$ subsets as precursors for iTregs (as shown in Fig. 2) were set up in the presence or the absence of autologous $CD4^+CD25^+$ T cells. As shown in Fig. 5, the $CD4^+CD25^-$ subset exhibited a marked suppressor activity, but the addition of $CD4^+CD25^+$ T cells did not substantially affect the outcome. Of note, the CTL generation assay conducted in the presence of the unfractionated whole $CD4^+$ population showed marked diminution of expansion of the epitope-specific CTL.

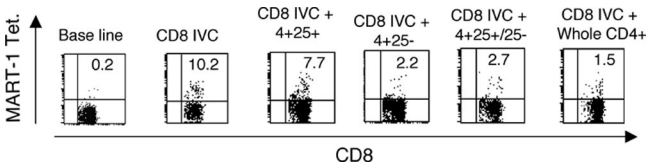

FIGURE 5. Effect of autologous $CD4^+CD25^+$ T cells on suppression of CTL expansion induced by $CD4^+CD25^-$ T cells. The numbers of MART-$1_{27–35}$ tetramer cells were analyzed with $CD8^+$-gated populations.

Discussion

To our knowledge, this is the first study that has compared the regulatory properties of nTregs and iTregs on the generation of CTL responses to a bona fide human tumor-associated Ag in a strictly autologous setting. We show in this study that $CD4^+CD25^+$ T cells, although fully capable of suppressing the proliferation of $CD4^+CD25^-$ effector T cells in the now standard Treg assay (31), do not prevent the activation and expansion of self, but melanoma epitope-specific, autologous $CD8^+$ CTL precursors stimulated by the relevant peptide-loaded mDC in vitro. In contrast, the $CD4^+CD25^-$ T cell subsets from the same individual give rise to $CD4^+CD25^+$, $GITR^+$, and $FoxP3^+$ populations and suppress the activation and expansion of tumor epitope-specific CTL. Our studies do not prove that the suppression was conducted by $CD4^+GITR^+FoxP3^+$ cells generated from $CD4^+CD25$-precursors. However, it has been shown that $FoxP3^+$ Tregs can be generated from $CD4^+CD25^-$ precursors (34–38), although this type of conversion has been questioned (39). Regardless of how the $CD4^+CD25^-$ T cells become suppressor cells, the fact remains that in our system, they are the major constraints in activating and expanding tumor epitope-specific CTLs.

It is now widely believed that antitumor immunity is primarily controlled by $CD25^+CD4^+$ T cells (reviewed in Refs. 27 and 28). Unfortunately, most of these studies of the suppression of antitumor CTL responses by $CD4^+CD25^+$ T cells have been conducted in Ag-nonspecific assays. Furthermore, as pointed out previously, a careful comparison of the two $CD4^+$ Tregs has not been performed in a relevant human tumor Ag model. It is, therefore, by no means clear that the generation of antitumor immunity is solely

controlled by nTregs. Curiel et al. (40) have reported that the accumulation of $CD4^+/CD25^+/GITR^+/FoxP3^+$ T cells in ovarian tumors correlates with poor outcome. Viguier et al. (41) have found both IL-10-producing Tr1-type Tregs as well as IL-10-negative Tregs in tumor-infiltrated nodes and have suggested that both nTregs and iTregs contribute to the local immunosuppressive milieu. These two studies were conducted in two different tumor types. Although this could explain the difference in the observations and interpretations (although one would like to see a basic rule on Treg-mediated suppression of antitumor immunity), it should be pointed out that all $CD4^+CD25^+$ T cells found at tumor sites may not be nTregs; some of them might be iTregs. Additionally, it should also be pointed out that all $CD4^+CD25^+$ T cells are not functionally suppressor-type regulatory cells, because helper cells are also $CD4^+CD25^+$ T cells.

It might be argued that our in vitro CTL generation assay might not have provided the necessary condition(s) needed to activate nTregs. The CTL generation assay in which we looked for the regulatory effect of the two $CD4^+$ T cell subsets contained autologous DC (the same DC that were used in the proliferation-based Treg assays) and IL-2, two critical requirements (TCR signal and IL-2) for Treg activation. Similarly, it can be argued that the failure of nTregs to suppress the expansion of CTL in our system might have resulted from the use of mDC in the cultures. However, the precise conditions (nature of the DC, cytokine, immunogenic vs subimmunogenic milieu, microenvironment, etc.) that lead to the induction of a Treg response remain unresolved and somewhat confusing. For example, although nTregs are believed to be anergic, it has been shown that mDC can expand nTregs (42). The mDCs have also been shown to be capable of blocking nTreg function (43). Similarly, although it has been shown that the generation of Tregs is facilitated under subimmunogenic conditions (44–46), mDCs have also been found to activate Tregs (47–51). Clearly, the Tregs in our system were generated under conditions that could not be described as subimmunogenic.

In our system, the regulatory cell function in MHC class II-restricted fashion and by elaborating IL-10, TGF-β, or both. They, therefore, bear the characteristic features of $CD4^+$ Tr1-type suppressor T cells that others have described (29, 30) and we have reported (9–11). The mechanism underlying their activation and specificity for iTregs remain obscure. It is conceivable that iTregs were activated by the presentation of some endogenous class II-determined epitope as has been the case for a $CD4^+$ Treg line (52), and the Tregs suppressed the activation of the CTLs in a bystander manner.

Shevach et al. (31) applied their in vitro Treg assay to study the regulation of OVA and influenza nuclear peptide-specific $CD8^+$ T cells by $CD4^+CD25^+$ T cells in mice (53). Interestingly, they found no suppression of activation of Ag-specific transgenic T cells by fresh $CD4^+CD25^+$ cells, but found suppression only by the preactivated $CD4^+CD25^+$ T cells. Others have reported regulatory function by $CD4^+CD25^+$ subsets on $CD8^+$ T cells, in one way or another, in Ag-nonspecific as well as in Ag-specific systems and in vitro and in vivo assays (54–56). Thus, our results are in apparent conflict with these reports. In this context, it should be pointed out that a head-to-head comparison of the $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets in a robust CTL generation assay (i.e., stimulation of CTL precursors by fully activated DCs presenting the peptide) in a relevant self, but human tumor-associated, Ag has not been reported. Camara et al. (54) examined the regulation of $CD8^+$ human T cells in a polyclonal and alloantigen-stimulated system. Osada et al. (57) have, however, recently reported that $CD4^+$ T cells do not regulate the generation of MART-$1_{27–35}$ epitope-specific CTL, but $CD4^+$NKT cells do. We have not examined whether the iTregs activated in our system might have been $CD4^+$NKT cells. We believe that this is unlikely, because the iTregs in our system are MHC class II restricted.

The failure of nTregs to suppress the activation and expansion of melanoma epitope-specific CTLs when optimally stimulated is consistent with what is known about the efficiency of nTregs in in vitro assays. The nTregs are not highly effective in in vitro Treg assays when the activation signal for the effector cells is strong (58). Yet, a role for $CD4^+CD25^+$ Tregs in maintaining self-tolerance in the animal model is indisputable (3), and a role of nTregs in antitumor immunity, at least a qualified one, cannot be ruled out. We have recently argued (28) that nTregs may be able to regulate an antitumor response at a steady state or when the activation signal for the CTL effector response is suboptimal. Tumor-associated, but self-Ag, presentation in the steady state is unlikely to be optimum and rarely would be viewed as dangerous (growth of and invasion by tumor cells seldom present with the same danger as do invading pathogens). A suboptimal stimulation may lead to a low level effector T cell activation that is likely to contract on its own or controlled by nTregs when needed. The nTregs, however, may not be able to abort an effector T cell response orchestrated by optimal stimulation with all the right ingredients (Ag presentation by fully activated APCs, inflammatory backdrop, etc.). The iTregs are likely to be more efficient by virtue of their mode of action. IL-10 and TGF-β are two formidable immunosuppressive cytokines. When taken in this overall context, our data beg the question of how relevant and central nTregs act physiologically when an immune response is called for in the defense against infectious agents or transplantation Ags or when the generation of an antitumor T cell response is deliberately attempted with the full force of Ag presentation and costimulation. Our data show that although the regulatory control of nTregs can be overcome by optimum signaling to the CTL precursors, iTregs pose a more formidable constraint against generating a robust and long-lived antitumor CTL response. Considering the current interest in the question of how to deal with Tregs in antitumor immunity and immunotherapy, our data have significant implications.

Disclosures

The authors have no financial conflict of interest.

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