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CUTTING EDGE

Cutting Edge: Murine Vascular Endothelium Activates and Induces the Generation of Allogeneic $CD4^+25^+Foxp3^+$ Regulatory T Cells[1]

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***Unlike graft-resident donor-derived hemopoietic APCs, which decrease in number over time after transplantation, vascular endothelial cells are lifelong residents of a vascularized allograft. Endothelial cells are potent APCs for allogeneic $CD8^+$ T lymphocytes but are unable to induce proliferation of allogeneic $CD4^+$ T lymphocytes. Although the reason for this differential response has been poorly understood, here we report that alloantigen presentation by vascular endothelium to $CD4^+$ T lymphocytes activates and induces $CD4^+25^+Foxp3^+$ regulatory T cells, which can inhibit proliferation of alloreactive T cells both in vitro and in vivo. This process occurs independently of B7.1 costimulation but is dependent on programmed death ligand 1 (B7-H1). This finding may have important implications for tolerance induction in transplantation. The Journal of Immunology,* 2005, 175: 6265–6270.**

Several investigators, including our group, have described that vascular endothelium isolated from various sources, such as the murine aorta and pulmonary parenchyma, constitutively express MHC class I as well as costimulatory molecules (1). We have shown that endothelial cells can act as APCs to allogeneic $CD8^+$ T lymphocytes and can trigger allograft rejection via $CD8^+$ direct allorecognition (2, 3). However, despite expression of both inducible MHC class II and costimulatory molecules, vascular endothelium is unable to induce cell division in allogeneic $CD4^+$ T cells in vitro or in vivo (4, 5). More importantly, endothelial cells are unable to initiate acute graft rejection via $CD4^+$ direct allorecognition (6). Based on these observations, the interaction between vascular endothelium and allogeneic $CD4^+$ T cells has been generally viewed as a neutral encounter (4–6). In this study, we have further analyzed the interaction between vascular endothelium and allogeneic $CD4^+$ T lymphocytes and show that activated vascular endothelium generates alloantigen-specific $CD4^+25^+Foxp3^+$ regulatory T cells, which inhibit T cell proliferation both in vitro and in vivo. As vascular endothelium persists for the life of the allograft, the ability to induce regulatory $CD4^+$ T cells may be important for tolerance induction.

Materials and Methods

Animals and cell isolation

CBA/J (H-2^k), C57BL/6 (B6) (H-2^b), BALB/c (H-2^d), B7-deficient, and MHC class II-deficient mice on a B6 background were purchased from The Jackson Laboratory. Endothelial cells and $CD4^+$ T cells were isolated from thoracic aorta and pooled splenocytes, respectively (1–4).

Primary and secondary cultures

$CD4^+$ T lymphocytes were resuspended in RPMI 1640 medium and cultured with endothelium activated with 500 U/ml murine IFN-γ (R&D Systems) for 72°C before coculture (2, 4). For some experiments, CTLA4Ig fusion protein (15 μg/ml), hamster Ig (15 μg/ml), rat IgG2a (1 μg/ml) (BD Pharmingen), anti-programmed death ligand 1 (PDL-1)[4] (1 μg/ml), or anti-PDL-2 (1 μg/ml) blocking Abs (eBioscience) were added during the primary $CD4^+$T cell/endothelial coculture. After 5 days in culture, the $CD4^+$ T cells were reselected by $CD4^+$ magnetic beads and added to a secondary culture as "regulators" of syngeneic CFSE-labeled $CD8^+$ responder cells. $CD4^+$ T cell survival and recovery within these primary cultures ranged between 25 and 40%. Stimulator cells consisted of irradiated (30 Gy) B6 or BALB/c CD90-depleted (midiMACS system; Miltenyi Biotec) splenocytes. In vivo adoptive transfer experiments were performed as described previously (2, 4).

Flow cytometry, RT-PCR, and ELISA

All Abs and reagents for flow cytometric analysis were purchased from BD Pharmingen or eBioscience and were primarily conjugated with either PE, allophycocyanin, or peridinin chlorophyll protein-cyanin 5.5. Intracellular Foxp3 staining was performed using a commercially available kit (eBioscience). Flow cytometric sorting of $CD4^+$ T cells was performed on a MoFlow (DakoCytomation) high-speed sorter based on CD25 expression. Purity of the $CD4^+25^-$ cell fraction was consistently >99.7%, and the purity of the $CD4^+25^+$ fraction ranged between 85 and 95%.

Cytokine analysis of culture supernatant was performed using commercially available ELISA kits (BioSource International). Quantitative and semiquantitative RT-PCR were performed using previously published primer sequences (7). Quantitative analysis was performed after normalization of the data to 18

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[4] Abbreviations used in paper: PDL-1, programmed death ligand1, GITR, glucocorticoid-induced TNFR.

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sRNA, and relative Foxp3 expression was calculated by setting expression in $CD4^+25^-$ cells to 1. All statistical analysis was performed by Student's *t* test.

Results and Discussion

Coculture of $CD4^+$ T lymphocytes with activated vascular endothelium results in the expansion of activated $CD4^+25^+Foxp3^+$ regulatory T cells, which can inhibit T cell proliferation in an alloantigen-specific fashion

To further evaluate the interaction between T cells and endothelium, we analyzed the supernatants collected from cocultures of activated B6 vascular endothelium and CBA $CD8^+$ and $CD4^+$ T cells, respectively (Fig. 1*A*). $CD8^+$ T cell/endothelial cell coculture supernatants had high levels of IFN-γ but no IL-2. Lack of IL-2 is consistent with previous reports describing production of IL-2 by $CD8^+$ T cells for predominantly autocrine use (8). In the supernatants of $CD4^+$ T/endothelial cell cocultures, we observed low but detectable levels of IL-2 and remarkably high levels of IL-10 and TGF-β compared with $CD8^+$ T/endothelial cell cocultures. Because a similar cytokine pattern has been described previously during the generation of

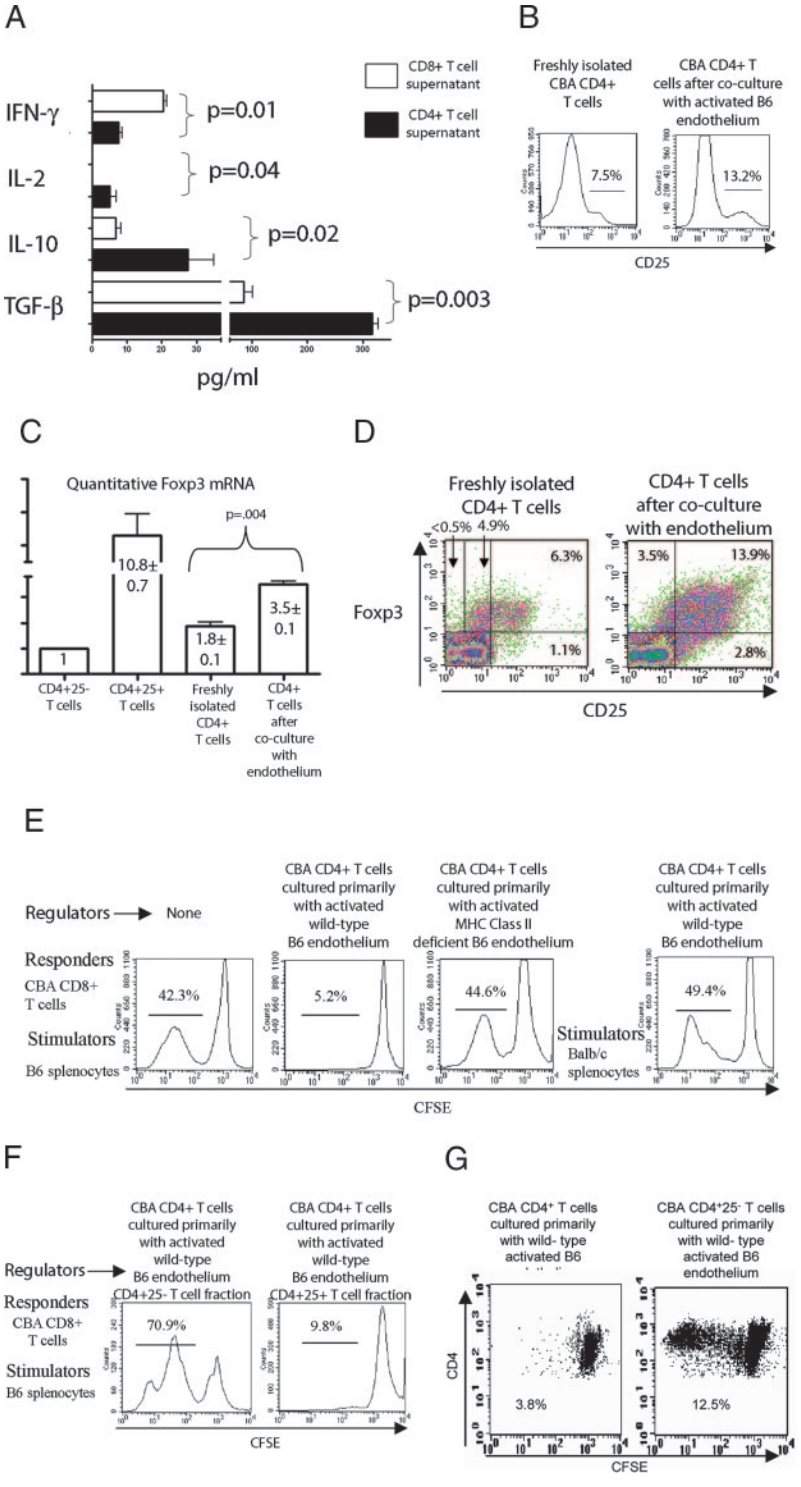

FIGURE 1. Coculture of $CD4^+$ T cells with activated vascular endothelium results in the expansion of $CD4^+25^+Foxp3^+$ regulatory T cells, which can inhibit T cell proliferation in an alloantigen-specific fashion. Cocultures of CBA $CD4^+$ T cells with B6 vascular endothelium demonstrate higher levels of TGF-β and IL-10 than $CD8^+$ T cell cocultures (*A*). Coculture of CBA $CD4^+$ T cells with B6 vascular endothelium results in an increase in the proportion of $CD4^+25^+$ T cells (*B*), as well as an increase in Foxp3 mRNA by quantitative RT-PCR (*C*). Freshly isolated $CD4^+$ T cells contain $CD25^-Foxp3^-$, $CD25^{low}Foxp3^+$, and $CD25^+Foxp3^+$ cells. However, after coculture with allogeneic vascular endothelium, the majority of $Foxp3^+$ cells are $CD25^+$ (*D*). CBA $CD8^+$ T lymphocytes proliferate after coculture with T cell-depleted, irradiated B6 splenocytes, but the addition of CBA $CD4^+$ T cells that have been cocultured with activated B6 endothelium in primary culture leads to inhibition of $CD8^+$ T cell proliferation (*E*). Such inhibition is abrogated in the absence of TCR engagement during primary culture with MHC class II-deficient B6 endothelium or whether third-party (BALB/c) splenocytes are used as stimulators during secondary culture (*E*). Suppression is limited to the $CD4^+25^+$ T cell fraction (*F*). Unlike unfractionated $CD4^+$ T cells, $CD4^+25^-$ T cells do proliferate when cultured with activated allogeneic vascular endothelium (*G*). All plots are representative of at least three independent experiments.

regulatory T lymphocytes, we decided to evaluate whether coculturing vascular endothelium with allogeneic $CD4^+$ T cells results in the generation of regulatory T cells (9, 10).

We noticed an increase in the percentage of $CD25^+$ cells after 5 days of coculturing CBA $CD4^+$ T cells with activated B6 endothelium (Fig. 1*B*). As surface expression of CD25 in $CD4^+$ T cells is characteristic of both regulatory and activated alloreactive responder cells, we documented increased expression of Foxp3 mRNA by quantitative RT-PCR in CBA $CD4^+$ T lymphocytes after coculture with activated B6 vascular endothelium suggesting the expansion of regulatory T cells (Fig. 1*C*). Flow cytometrically we were able to determine that the vast majority of $CD4^+25^+$ T cells express Foxp3 after coculture with allogeneic vascular endothelium, while only a small fraction of the $CD25^+$ population did not express Foxp3 (Fig. 1*D*). The $CD4^+25^+Foxp3^+$ T cells also demonstrated up-regulation of surface glucocorticoid-induced TNFR (GITR) and intracellular CTLA4 when compared with freshly isolated, resting $CD4^+25^+Foxp3^+$ naturally occurring T cells, suggesting activation of this cell population by activated allogeneic vascular endothelium.

We next used a two-stage culture system to test the inhibitory properties of our cells. First, CBA $CD4^+$ T cells were cocultured with activated B6 vascular endothelium for 5 days (primary culture), and subsequently, these CBA $CD4^+$ T cells were mixed as "regulators" with responder CBA $CD8^+$ T cells and irradiated T cell-depleted B6 splenocyte stimulators (secondary culture). CBA $CD8^+$ T cells proliferated vigorously when stimulated by irradiated, T cell-depleted B6 splenocytes (Fig. 1*E*). The addition of CBA $CD4^+$ T cells that had been cocultured with activated B6 endothelium during the primary culture resulted in inhibition of CBA $CD8^+$ T cell proliferation (Fig. 1*E*). Near complete inhibition of $CD8^+$ T cell proliferation could be achieved with a 3:1 "regulator":responder cell ratio in secondary cultures. The importance of $CD4^+$ T cell TCR engagement by inducible MHC class II on vascular endothelium during primary culture was demonstrated by abrogation of suppression when CBA $CD4^+$ T cells were cocultured with MHC class II-deficient B6 endothelium before transfer to secondary cultures as "regulators" (Fig. 1*E*). Suppression was alloantigen specific as CBA $CD4^+$ T cells, stimulated primarily by B6 endothelium, did not inhibit CBA $CD8^+$ T cell proliferation induced by stimulators derived from a third party (BALB/c splenocytes) during the secondary culture (Fig. 1E). Of note, CBA $CD4^+$ T cells stimulated primarily by B6 endothelium did not undergo proliferation and remained anergic upon secondary culture with B6 splenocytes as well (data not shown).

We next set out to evaluate whether $CD4^+$ T cells, cocultured with activated vascular endothelium, are able to inhibit proliferative responses in vivo. We have described previously that CBA $CD8^+$ T cells proliferate vigorously after adoptive transfer into a supralethally irradiated B6 mouse (2). However, when CBA $CD4^+$ T cells that had been cocultured previously with activated B6 vascular endothelium in primary culture were cotransferred along with CBA $CD8^+$ T cells, the proliferative responses of the CBA $CD8^+$ T cells were markedly inhibited. Analogous to the in vitro observation, this inhibition was alloantigen specific as CBA $CD4^+$ T cells cocultured primarily with activated B6 vascular endothelium were unable to affect CBA $CD8^+$ T cell proliferation after adoptive transfer into a supralethally irradiated BALB/c mouse (data not shown).

Although a large body of literature supports the role of $CD4^+25^+$ T cells in regulating immune responses, some investigators have suggested that the $CD4^+25^-$ T cell fraction can also inhibit T cell proliferation and mediate transplantation tolerance (11). Based on the Foxp3 expression in $CD4^+25^+$ T cells after coculture with endothelium (Fig. 1*D*), we hypothesized that this population would have suppressive function. To this end, CBA $CD4^+$ T cells were flow cytometrically sorted based on CD25 expression after 5 days of coculture with activated B6 vascular endothelium. Suppression was limited to the $CD4^+25^+$ T cell fraction, and the $CD4^+25^-$ T cells were unable to suppress proliferation of $CD8^+$ T cell responders in secondary cultures (Fig. 1*F*).

To evaluate the role of naturally occurring $CD4^+25^+$ regulatory T cells in inhibition of $CD4^+$ T cell proliferation in primary T cell/endothelial cell cultures, we depleted the $CD4^+25^+$ T cell fraction from freshly isolated CBA $CD4^+$ T cells by flow cytometric sorting. Unlike the case for unfractionated $CD4^+$ T cells, $CD4^+25^-$ T cells did proliferate when cultured with activated B6 vascular endothelium (Fig. 1*G*). Taken together with the up-regulation of CTLA4 and GITR in the $CD4^+25^+$ T cell fraction, these findings support the notion that the coculture of $CD4^+$ T cells with allogeneic vascular endothelium results in the activation of naturally occurring $CD4^+25^+Foxp3^+$ regulatory T cells as well as expansion of the $CD4^+25^+Foxp3^+$ regulatory T cell pool.

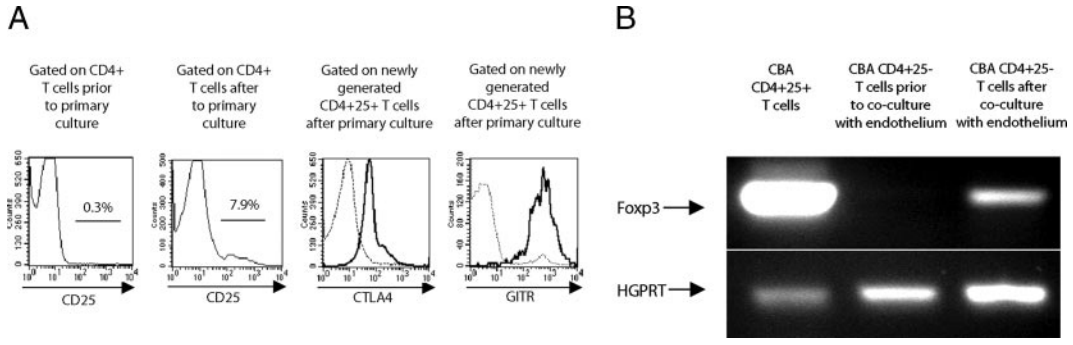

FIGURE 2. Expansion of regulatory T cells results from the conversion of $CD4^+25^-$ to $CD4^+25^+Foxp3^+$ cells. CBA $CD4^+25^-$ T cells express CD25 after 5 days of coculture with activated B6 vascular endothelium. These newly generated $CD4^+25^+$ T cells express intracellular CTLA4 and surface GITR (solid line = protein expression; dotted line = isotype control) (*A*). Expression of Foxp3 mRNA was also detectable in the initially $CD25^-$ CBA $CD4^+$ T cells after coculture with activated B6 endothelium by semiquantitative RT-PCR (*B*).

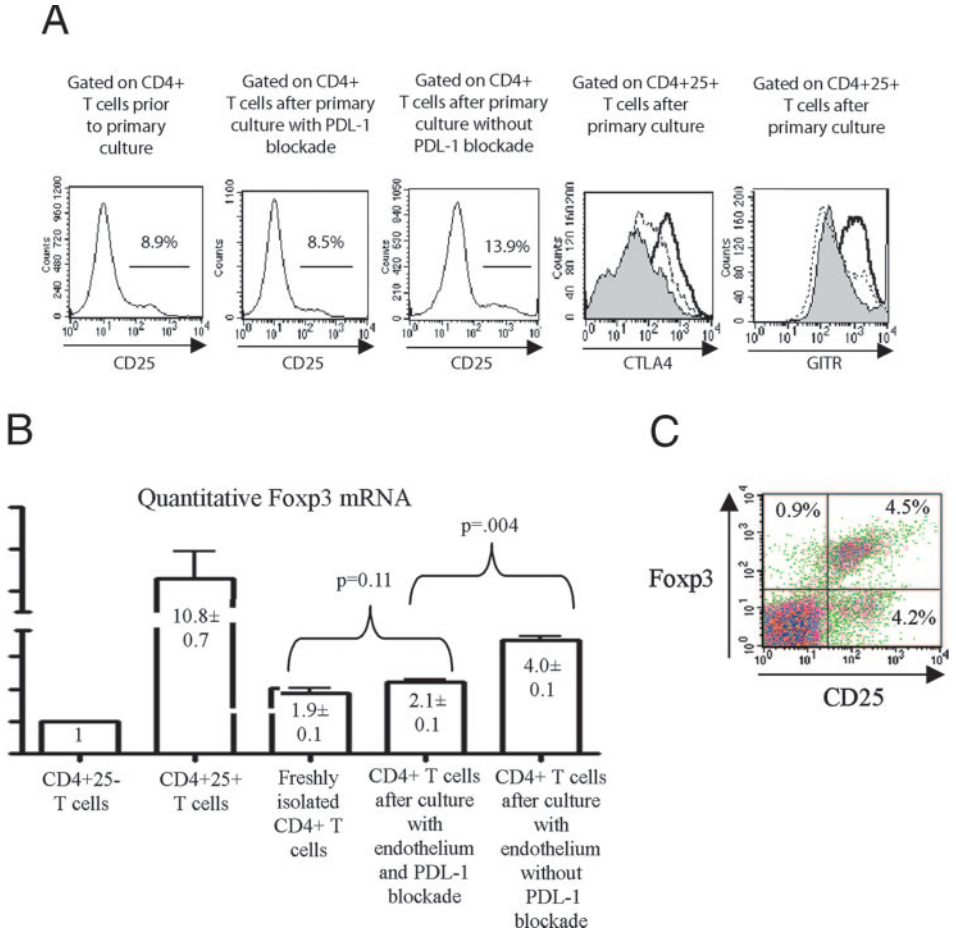

FIGURE 3. The generation of $CD4^+25^+$ regulatory T cells depends on PDL-1 costimulation. PDL-1 blockade during the primary CBA $CD4^+$ T cell/B6 endothelial cell coculture abrogates the increase in $CD25^+$ T cell fraction and the up-regulation of CTLA4 and GITR in the $CD4^+CD25^+$ cells compared with cocultures without PDL-1 blockade (control rat IgG) (shaded = expression in resting $CD4^+25^+$ cells, solid line = expression in $CD4^+25^+$ cells after coculture without PDL-1 blockade, and dotted line = expression in $CD4^+25^+$ cells after coculture with PDL-1 blockade) (*A*). Foxp3 mRNA levels remain statistically identical to those of freshly isolated CBA $CD4^+$ T cells after coculture of CBA $CD4^+$ T cells with activated B6 vascular endothelium and PDL-1 blockade while an increase of Foxp3 mRNA is documented in CBA $CD4^+$ T cells cultured with activated B6 endothelium in the absence of PDL-1 blockade (rat IgG) (*B*). The addition of anti-PDL-1 Ab prevented expansion of the $CD4^+25^+Foxp3^+$ T cell population and also resulted in the generation of $CD25^+Foxp3^-$ $CD4^+$ T cells (*C*). All plots are representative of at least three independent experiments.

Expansion of regulatory T cells results from the conversion of $CD4^+25^-$ to $CD4^+25^+Foxp3^+$ cells

Although we described activation of naturally occurring $CD4^+25^+Foxp3^+$ regulatory T cells by vascular endothelium, consistent with previous reports (12), no proliferation of this cell population was evident in our cultures by CFSE staining. This raised the possibility that the increase in the $CD4^+25^+Foxp3^+$ cells after coculture with cytokine-activated endothelium was due to the conversion of $CD4^+25^-$ T cells into $CD4^+25^+$ regulatory cells.

We further tested this possibility by culturing sorted CBA $CD4^+25^-$ T cells with activated B6 vascular endothelium. After 5 days of coculture, as many as 10% of the initially >99.7% pure $CD4^+25^-$ T cells expressed surface CD25. The newly generated CBA $CD4^+25^+$ cells had surface GITR and intracellular CTLA4 similar to naturally occurring $CD4^+25^+$ regulatory cells (Figs. 2*A*). Expression of Foxp3, which was undetectable by semiquantitative RT-PCR in freshly isolated CBA $CD4^+25^-$ T cells, was documented after 5 days of coculture with activated B6 vascular endothelium indicating the generation of regulatory $Foxp3^+$ T cells (Fig. 2*B*).

Consistent with previous reports, we were able to detect a population of $CD4^+CD25^{low}Foxp3^+$ T cells within freshly isolated $CD4^+$ T cells (Fig. 1*D*) (13). In fact, a recent report suggests that $CD4^+CD25^+Foxp3^+$ regulatory T cells arise from $CD25^{low}Foxp3^+$ cells (13). For our sorting experiments described above, we discarded $CD25^{low}$ as well as $CD25^+$ cells and isolated $CD25^-$ cells only. These cells do not express Foxp3 before coculture with vascular endothelium but after coculture Foxp3 was induced (Fig. 2*B*). Therefore, we conclude that the coculture of $CD4^+$ T cells with allogeneic vascular endothelium results in the generation of $CD4^+25^+Foxp3^+$ regulatory T cells from $CD4^+25^-$ cells. Although our data indicate that our original $CD25^-$ T cells do not express Foxp3, we cannot rule out the possibility that a small population of $CD25^{low}Foxp3^+$ cells with Foxp3 present below the levels of detection by PCR contribute to the generation of $CD25^+$ $Foxp3^+$ regulatory cells.

The generation of $CD4^+25^+$ regulatory T cells depends on PDL-1 costimulation

Although some reports have shown B7.1 to play a critical role in the generation and homeostasis of regulatory T cells, others have suggested that these processes can occur independently of B7.1 (14–16). Based on the expression of B7.1 on endothelial cells (1, 2), we hypothesized that B7.1 is important to the activation and/or generation of regulatory T cells in our system. Interestingly, CBA $CD4^+$ T cells cocultured in the absence of B7.1 stimulation through the addition of soluble CTLA4Ig or use of vascular endothelium isolated from B7-deficient mice during primary culture retained the same phenotype with expansion of the $CD4^+25^+$ fraction as well as up-regulation of CTLA4 and GITR similar to $CD4^+25^+$ T cells generated in the presence of B7.1 costimulation (data not shown).

Similar to previously published reports, murine vascular endothelium in our cultures expresses surface PDL-1 (17) but

does not express PDL-2 (data not shown). We next sought to investigate the role of PDL-1 in the generation of regulatory T cells. Interestingly, we found that PDL-1 blockade during the primary $CD4^+$ T cell/endothelial coculture resulted in inhibition of $CD4^+25^+$ T cell expansion and a decrease in markers of $CD4^+25^+$ T cell activation with levels of CTLA4 and GITR comparable to those found in freshly isolated $CD4^+25^+$ regulatory T cells (Fig. 3*A*). No up-regulation of Foxp3 mRNA was seen in $CD4^+$ T cells in the presence of PDL-1 blocking Ab by quantitative RT-PCR (Fig. 3*B*). Intracellular staining with Foxp3 extended our RT-PCR data as we found that the addition of anti-PDL-1 Ab prevented the expansion of the $CD4^+25^+Foxp3^+$ T cell population seen in cocultures without PDL-1 blockade (Figs. 1*D* and 3*C*). Interestingly, unlike the case for cocultures of endothelium with allogeneic $CD4^+$ T cells without PDL-1 blockade, $CD4^+CD25^+Foxp3^-$ T cells were also present when PDL-1-blocking Ab was added. Moreover, CBA $CD4^+$ T cells primarily cultured with activated B6 vascular endothelium in the presence of PDL-1 blockade were unable to inhibit CBA $CD8^+$ T cell proliferation in secondary cultures (data not shown). Thus, our data indicates that PDL-1 blockade prevents both the activation and expansion of $CD4^+25^+Foxp3^+$ regulatory T cells. This observation is supported by other reports that have also shown PDL-1 to be critical to the induction of regulatory T cells (18–20).

Because of progressive diminution of donor-derived hemopoietic APCs, it is generally believed that indirect, self-restricted Ag presentation is the predominant allorecognition pathway with increasing duration after solid organ engraftment. Therefore, it has been postulated that indirect allorecognition is the predominant mode of late Ag presentation to both regulatory as well as CTL (21, 22). Our recent observation that nonhemopoietic cells, which are present for the life of the allograft, can mediate rejection via $CD8^+$ direct allorecognition has opened the possibility of an alternative pathway mediating late allorecognition (3). To our knowledge, this report is the first demonstration of $CD4^+25^+Foxp3^+$ regulatory T cell generation via $CD4^+$ direct alloantigen presentation by nonhemopoietic cells. Our findings are consistent with and extend recent reports describing the peripheral conversion of $CD4^+25^-$ nonregulatory T cells into $CD4^+25^+Foxp3^+$ regulatory T cells in other models (23, 24).

Several observations in transplantation biology may be explained by our finding. Direct $CD4^+$ alloantigen presentation by graft-resident hemopoietic APCs is an important mechanism of alloreactivity and initiation of allograft rejection early after organ transplantation. As these cells progressively decrease in number over time, this pathway of allorecognition is blunted by regimens such as temporary recipient lymphocyte depletion or transient costimulatory blockade. Interestingly, such treatments can also induce regulatory T cells (25, 26). The underlying mechanism for the induction of regulatory T cells by these regimens is currently unknown, but based on our data, we can speculate that it may result from delayed allorecognition. This may allow donor-derived hemopoietic APCs to decrease in number, thus increasing the importance of direct alloantigen presentation to $CD4^+$ T cells by vascular endothelium. Because transplantation of a larger mass of allogeneic tissue has similarly been shown to lead to allograft acceptance, it is possible that delivering a larger mass of endothelium or other parenchymal cells may favor tolerogenic Ag presentation over alloreactivity induced by professional hemopoietic APCs (27). Thus, our finding that alloantigen presentation by vascular endothelium to $CD4^+$ T cells leads to the induction and activation of alloantigen-specific $CD4^+25^+Foxp3^+$ regulatory T cells could have important implications for the design of tolerance induction strategies without global recipient immunosuppression.

Disclosures

The authors have no financial conflict of interest.

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