Dendritic Cell Type Determines the Mechanism of Bystander Suppression by Adaptive T Regulatory Cells Specific for the Minor Antigen HA-1[1]

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One hallmark of acquired tolerance is bystander suppression, a process whereby Ag-specific (adaptive) T regulatory cells (T_R) inhibit the T effector cell response both to specific Ag and to a colocalized third-party Ag. Using peripheral blood T cells from recipients of HLA-identical kidney transplants as responders in the trans vivo-delayed type hypersensitivity assay, we found that dendritic cells (DC), but not monocyte APCs, could mediate bystander suppression of EBV-specific recall response. When HA-1^H peptide was added to mixtures of plasmacytoid DC (pDC) and T cells, bystander suppression of the response to a colocalized recall Ag occurred primarily via indolamine-2,3-dioxygenase (IDO) production. Similarly, addition of HA-1^H peptide to cocultures of T cells and pDC, but not myeloid DC (mDC), induced IDO activity in vitro. When mDC presented HA-1^H peptide to Ag-specific $CD8^+$ T_R, cytokine release (TGF-β, IL-10, or both) was the primary mode of bystander suppression. Bystander suppression via mDC was reversed not only by Ab to TGF-β and its receptor on T cells, but also by Ab to thrombospondin-1. EBV addition did not induce IDO or thrombospondin-1 in T-DC cocultures, suggesting that these DC products are not induced by T effector cells, but only by T_R cells. These results shed light upon the mechanism of bystander suppression by donor Ag-specific T_R in patients with organ transplant tolerance and underscores the distinct and critical roles of mDC and pDCs in this phenomenon. *The Journal of Immunology,* 2007, 179: 3443–3451.

A prototypical feature of acquired peripheral tolerance is the suppression of a response to colocalized third-party Ag by a donor Ag-specific T regulatory (T_R)[4] cell. The characteristic features of this process were originally described by Bullock et al. (1). Using a guinea pig model, the authors showed that T cells specific for a hapten were able to suppress the development of a contact sensitivity response by T cells specific for a carrier molecule, if the hapten and carrier are linked covalently (1). This same concept was later expanded upon primarily by Streilein and colleagues (2–4), who studied ocular immune privilege, and later by Waldmann and colleagues (5) in mouse skin allograft tolerance models. Due to the requirement that the tolerogen and Ag be physically linked, i.e., expressed by the same APC in order for suppression to occur, the terms "linked suppression" or "linked recognition" were coined. In the field of oral tolerance, the term "bystander suppression" was introduced to describe an inhibition of a T memory response as a result of a regulatory response to an unrelated but colocalized tolerogen (6). In bystander suppression, the tolerogen and third-party Ag need not be presented on the same APC. In this case, it is soluble mediators, triggered by the tolerogen, which serve to induce suppression of response to the third-party Ag (6–8).

Once the concept of multiple lineages of dedicated T_R became widely accepted, it quickly became apparent that without an APC, tolerogen-specific T_R would be unable to affect a third-party Ag-specific T effector cell (T_E) (9). In particular, direct T-T interaction models did not adequately account for T_R-mediated suppression in vivo when natural (i.e., nonadaptive) T_R were the source of T_R (10). These novel findings in mice raised questions regarding the role of the APC in immune regulation of T_E responses during human and rhesus monkey renal transplant tolerance, modeled in the SCID mouse footpad using the trans vivo-DTH (TV-DTH) assay system (11–13). Because the tolerogens and third-party recall Ags are not covalently linked in these studies, we will use the term bystander suppression from this point forward to describe our regulation model.

We envisioned two alternative hypotheses of APC function in bystander suppression: 1) a passive APC model, in which the APC serves to facilitate T-T interactions by presenting MHC-peptide ligands to the T_R, stimulating it to produce IL-10 or TGF-β that binds IL-10- and/or TGF-β receptors on the third-party T_E; or 2) an active APC model, in which the APC propagates regulatory effects from the T_R to the T_E through various APC products. If the second model is correct and the APC is of critical importance in bystander suppression, an additional question follows: can any APC type mediate this process or is a specialized APC needed? Peripheral blood dendritic cells (DC) have been shown to have regulatory properties that differ in mechanism based on subtype (14). Recent work in the field of transplantation tolerance has suggested that plasmacytoid DCs (pDC) may be necessary to induce

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[4] Abbreviations used in this paper: T_R, T regulatory cell; T_E, T effector cell; TV-DTH, trans vivo-delayed type hypersensitivity; DC, dendritic cell; pDC, plasmacytoid DC; mDC, myeloid DC; IDO, indolamine-2,3-dioxygenase; 1MT, 1-methyl-tryptophan; TSP-1, thrombospondin-1; LAP, latency-associated peptide.

tolerance and immune regulation in both human and mouse systems (15, 16); pDC have also been implicated in the generation of human IL-10-producing $CD8^+$ T_R in vitro (12). We wished to determine whether pDC might be required for bystander suppression, or rather, whether myeloid DCs (mDC), thought to be primarily involved in T_E stimulation, could also mediate bystander suppression.

To test these alternative hypotheses, we used a model dependent on $CD8^+$ T_R which recognize the hemopoietic-restricted minor histocompatibility (H) Ag $HA-1^H$ in the context of HLA-A2 (11). We have previously shown in a case of long-term kidney allograft tolerance that $CD8^+$ T_R with low binding of $HLA-A2/HA-1^H$ tetramer were present in PBMC. These cells could suppress both 1) $CD8^+$ T_E that bind to the same tetramer but with higher staining intensity, and 2) $CD4^+$ T_E specific for a recall Ag that is colocalized with $CD8^+$ T_R and $HA-1^H$ Ag. The suppressive response initiated by the $CD8^+$ T_R was exquisitely sensitive to physiologic levels of $HA-1^H$ Ag endogenously expressed by donor cells (11).

We report here that while both monocytes and DC could mediate a positive TV- DTH response to a viral recall Ag, only DC could mediate bystander suppression of this response by $HA-1^H$-specific T_R. Both mDC and pDC were found to share the ability to mediate bystander suppression, but used different mechanisms to silence a T_E response.

Materials and Methods
Source of human PBMC and peptides

All blood donors were EBV seropositive and thus had memory B and T cells specific for EBV Ags. Each study subject had received an HLA-identical kidney transplant from a sibling donor and had excellent graft function at time of blood draw or leukapheresis. PBMC were obtained by leukapheresis and purified over a Ficoll density gradient. Cells were stored in cryopreservation at $-140°C$ until use. Peptides $HA-1^R$ (control; VLRD DLLEA) and $HA-1^H$ (allo-minor H Ag; VLHDDLLEA) were synthesized and purified as previously described (11). Underlined residue indicates amino acid polymorphism.

Cell separation

Both mDC and pDC were separated on an immunogenetic bead column (autoMACS; Miltenyi Biotec) per the manufacturer's instructions. Briefly, $CD19^+$ and $CD14^+$ cells were removed first. If monocytes were to be used, $CD14^+$ cells were removed by positive selection, before the removal of $CD19^+$ cells; otherwise both subsets were removed in a single step. Next, $CD1c^+$ ($BDCA-1^+$) and $BDCA-3^+$-positive cells were removed to isolate the mDC. Finally, neuropilin-1^+ (BCDA-4) pDC were selected, leaving a negatively selected T cell-enriched population. Purity was determined by flow cytometry and judged to be over 95% for each APC population, based on CD14 (monocytes), CD11c (mDC), and CD123 (pDC) immunostaining. Purity of the T cell-enriched population was 97% by CD3 immunostaining.

TV-DTH assay

The TV-DTH assay was used as a readout for bystander suppression (17–19). Patient PBMC were injected into the footpad of a SCID mouse with EBV Ags prepared from lysate or cells infected with EBV (Viral Ag). EBV induces a swelling response due to antiviral memory T_E, primarily of the $CD4^+$ subset (11). This response can be inhibited by bystander suppression, when the donor Ag (or tolerogen) is added before injection of a T cell plus APC plus EBV. The use of the footpad for this assay is critical, because bystander suppression cannot be detected by TV-DTH using the ear as the injection site (20). Bystander suppression could be detected when unfractionated PBMC ($7–9 \times 10^6$) were injected into the footpads of SCID mice, along with recall Ag (EBV) and donor Ag (1 μg of $HA-1^H$ peptide). By comparing net swelling response to recall Ag in the presence or absence of allopeptide, a percent inhibition may be determined: $1 - ((recall + donor Ag mixture)/(recall Ag alone)) \times 100 = percent of inhibition$. Inhibition of 50% or more of the recall response is considered significant. Alternatively, to determine the effect of each cell type as the sole APC, 1×10^5 positively selected pDC, mDC, or monocytes were added individually to 8×10^6 negatively selected, T cell-enriched fraction for footpad injec-

tion. DTH response, measured by change in footpad thickness, was determined after 18–24 h using a dial thickness gauge (18).

Reversal of bystander suppression assays

Indolamine-2,3-dioxygenase (IDO) activity was inhibited by incubating each DC/T cell mixture in PBS containing 10 mM 1-methyl-tryptophan (1MT; Sigma-Aldrich) for 1 h and washing with PBS before injection. In some experiments, aliquots of 10 μl of the 10 mM solution were also coinjected with cells and Ag into each footpad, or added to the in vitro culture without the 1-h preincubation and wash step.

TGF-β and thrombospondin-1 (TSP-1) were neutralized by either 25 μg of rabbit anti-TGF-β Ab (R&D Systems) which recognizes the active site common to TGF-β1, -β2, -β3 or 5 μg of an anti-TSP-1 Ab which recognizes the collagen V-binding domain of TSP-1 (TSP-1 Ab 4 (A6.1); Lab-vision Neomarkers).

To block the TGF-βR, either DC or T cells were incubated alone in PBS containing 10 μg/ml anti-TGF-βRII Ab (BD Biosciences) for 1 h. Cells were then washed once and added to their reciprocal cell type (unmanipulated DC or T cells) and injected into the mouse footpad.

Induction of IDO and enzyme assay system

Either mDC or pDC were incubated separately with the T cell-enriched fraction at a ratio of 1:20 (1×10^5 DC: 2×10^6) T cells, in a volume of 200 μl of RPMI 1640 complete medium, supplemented with 2 mM tryptophan. $HA-1^H$ and $HA-1^R$ peptides were added at a final concentration of 50 μg/ml or alternatively self or donor cell lysates were added at a concentration of 8–10 μg and left to incubate at $37°C$ in 5% CO_2 for 72 h. To detect IDO activity, 100 μl of supernatants were harvested and kynurenine was detected using the absorbance method, as previously described (21).

ELISA tests for TSP-1 and TGF-β

Cell cultures were set up as described above for kynurenine detection. After 48 h of culture, TSP-1 was detected by adding 100 μl of supernatant to the TSP-1 ELISA kit (Chemicon International). After 48 h of culture, cell-associated TGF-β was detected by washing the cell pellets in PBS, followed by incubation in RPMI 1640 at a pH of 2.0 for 15 min to remove latency-associated peptide (LAP), and assaying the resulting supernatant after spinning down to remove cellular debris at $15,000 \times g$ for 3 min. Active TGF-β in culture supernatants was detected by adding 100 μl of supernatant to the TGF-β ELISA kit (R&D Systems) without prior acid activation. Our experience was that all of the released TGF-β was already active, because activation did not increase the ELISA readings.

TSP-1 inhibition assay

For short-term cultures to analyze the role of TSP-1 in release of active TGF-β from cell-bound latent forms, a 10:1 ratio of T cells-mDC (1×10^6 T cells:1×10^5 mDC) was incubated in 100 μl of serum-free medium (X-Vivo; Invitrogen Life Technologies), along with either 50 μg/ml $HA-1^H$ or $HA-1^R$ peptide. mAb 133 (a gift from Dr. D. Mosher, Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI) was added at a final concentration of 50 μg/ml, or alternatively, TSP-1 inhibitor peptide LSLK (a gift of Dr. C. Orosz, Department of Surgery, Columbus, Ohio State University, OH) was added at a final concentration of 10 μM. Cells were cultured for 6 h at $37°C$ in 5% CO_2 and then spun down. Supernatants and cell pellets were collected and processed as described above for TGF-β detection.

In vitro T-DC coculture

T cells (8×10^5) were cultured in serum free medium (X-Vivo; Invitrogen Life Technologies), with 1×10^5 mDC or pDC and 50 μg/ml $HA-1^H$ peptide for 9 days. Cells were harvested and stained with PE-labeled HA-$1^H/A2$ tetramers (Beckman Coulter), CD8-FITC (BD Pharmingen), and biotinylated chicken anti-human TGF-β1 (R&D Systems), followed by streptavidin-allophycocyanin (BD Pharmingen). Samples were analyzed by flow cytometry.

Statistical analysis

Prism Graphpad software version 4 for Macintosh was used for paired t tests comparing data of various APC-T cell cultures for each experimental condition.

Table I.   *Summary of HLA-A2[+] recipients of an HLA-identical, HA-1-mismatched kidney transplant[a]*

Patient Number	Reference	Recipient HA-1	Donor HA-1	T_R Type	Graft Status
I	11	R/R	H/H	TGF-β plus IL-10	Tolerant
II	11	R/R	H/R	IL-10	Maintenance
III	11	R/R	H/R	TGF-β	Maintenance
V	This study	R/R	H/R	TGF-β	Tolerant

[a] Shown are four patients who donated cells for this study. Listed is the patient number, donor, and recipient HA-1 type. Patient IV, a control HA-1-compatible patient, was not included in this study. Regulation type refers to which cytokine each patient was found to regulate by as determined by the TV-DTH assay (Ref. 11; Fig. 1B).

Results

DC are critical for inhibition of TV-DTH response by HA-1[H]-specific T_R

Table I describes the four patients used as a source of PBMC for these studies. Each had received an HLA-matched kidney transplant from a sibling. All four were HLA-A2/HA-1[R/R] genotype with HLA-A2/HA-1[H/R] or HA-1[H/H] donors. Two were off all immunosuppressive drugs with excellent renal function, i.e., functionally tolerant, at the time of PBMC donation (I and V), while the other two were still taking immunosuppressive medication (II and III). Table II provides information on the ratios of mDC and pDC for each patient. Lineage-negative, HLA-DR-positive DCs were quantitated by flow cytometry, and the relative numbers of mDC (CD11c^{+}) and pDC (CD123^{+}) were determined.
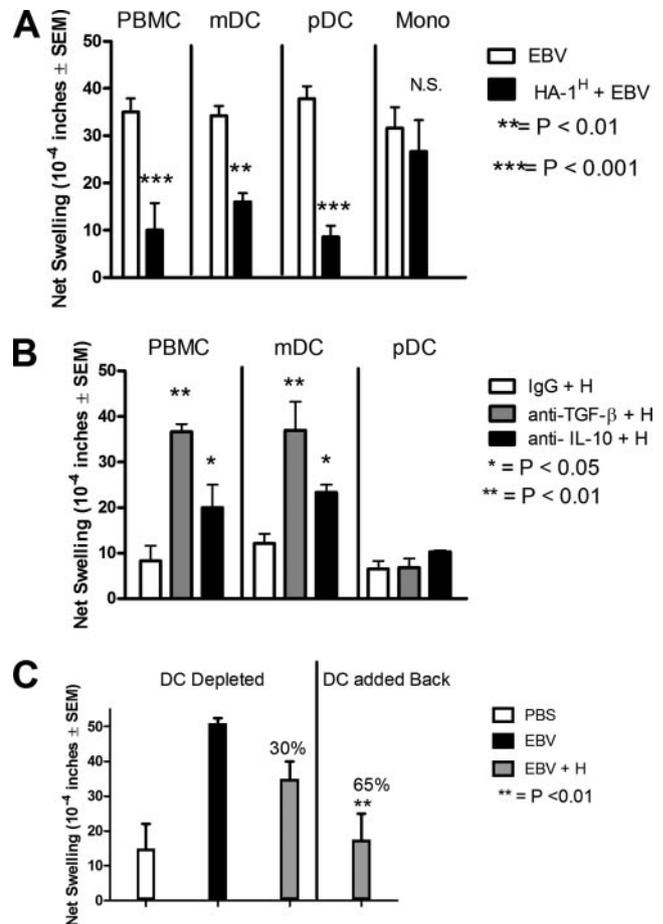
Fig. 1A summarizes bystander suppression data from unseparated PBMC as well as T cells coinjected with purified monocytes, mDC, or pDC. All four types of challenge caused an equivalent footpad swelling response to EBV recall Ags. The bystander suppression seen with T-DC mixtures was similar to the bystander suppression seen with unseparated PBMC, which contains ∼1–3% DC. In the TV-DTH system, we kept the ratio of T cells-DC the same as what is seen physiologically in whole PBMC, i.e., 1×10^5 DC to 7×10^6 T cells, or 1.5%. When EBV Ag, mDC, and T cells were coinjected along with HA-1[H] peptide, a significant ($p < 0.01$) inhibition of anti-EBV response occurred. A more profound bystander suppression was observed in the T cell-pDC mixtures ($p < 0.001$, HA-1[H] plus EBV vs EBV alone; Fig. 1A). Surprisingly, when purified monocytes were used as the sole APC, no significant inhibition of the recall response occurred in the presence of HA-1[H] peptide as compared with DC, CD14^{+} monocytes were not involved in bystander suppression.

Fig. 1B examines the role of DC type and cytokines in the balance between HA-1[H]-specific T_E and T_R. As shown, control IgG-treated PBMC were unresponsive to HA-1[H] Ag in TV-DTH. Swelling responsiveness mediated by HA-1[H]-specific T_E present in whole PBMC could be restored by either anti-TGF-β (patients I and III; $p < 0.01$) or anti-IL-10 (patients I and II; $p < 0.05$) indicating the presence of both HA-1[H]-specific T_E cells and TGF-β- or IL-10-producing T_R (Fig. 1B).

FIGURE 1. TV-DTH responses in the presence of donor-type minor H Ag HA-1. A, Bystander-suppression of recall response tested with whole PBMC or mixtures of T cells and APC as indicated. APC fractions were separated and added to enriched T cells from the same individual along with EBV alone, or EBV plus HA-1[H] peptide (denoted H) and injected into the footpad of a SCID mouse. Values of p were determined by paired t test comparing EBV to EBV plus H. Values are presented as mean ± SEM × 10^{-4} inches and results are a composite of results using cells from patients I to V ($n = 2$ independent experiments/patient). B, Attempts to reveal cryptic T_E responses to HA-1 by neutralization of IL-10 or TGF-β. mDC and pDC were separated from patients I–III and added to purified T cells. Cells were then injected in the DTH footpad assay along with donor Ag and either control IgG, anti-TGF-β, or anti-IL-10. Values of p were determined by paired t test comparing the TGF-β or IL-10 conditions to the IgG conditions. Results are the mean ± SEM of net swelling (test-PBMC alone control) responses in $n = 3$ patients. C, Bystander suppression of recall response tested with PBMC from which DC had been depleted (left). The right side depicts DC-depleted PBMC that have had the DC added back. The percent inhibition is listed above each condition. All results taken from cells from patient III ($n = 2$ independent experiments).

Table II.   *Analysis of DC percentages from each patient[a]*

Patient Number	Total DC, %	mDC, %	pDC, %	pDC:mDC
I	0.65	0.55	0.1	0.18
II	2.69	1.9	0.79	0.41
III	2.0	1.7	0.3	0.17
V	1.28	1.0	0.18	0.18

[a] Each value is calculated as a percentage of gated live cell events, i.e., total PBLs as determined from flow cytometry. mDC were defined as CD3,CD14,CD19^{−}, HLA-DR^{+}, CD11c^{+}; pDC were defined as CD3,CD14,CD19^{−}, HLA-DR^{+}, CD123^{+}.
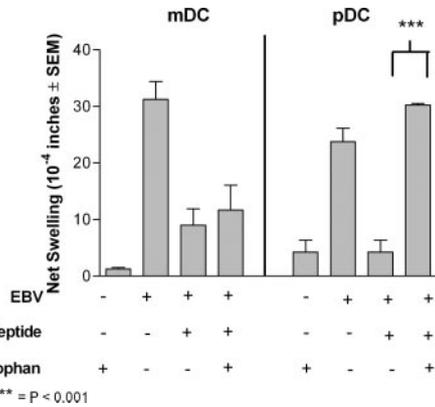
FIGURE 2. 1-MT reverses HA-1-specific suppression of recall response to EBV Ags. DC (mDC or pDC) were isolated and added to T cells into the DTH assay along with EBV, H peptide, or 1MT. Footpad swelling was measured, as indicated on the y-axis. TV-DTH values are presented as mean $\pm$ SEM $\times 10^{-4}$ inches and are a composite of results using cells from patients II and III ($n = 2$ independent experiments/patient). Values of p were determined by paired t test comparing EBV plus H to EBV plus H plus 1MT for both mDC and pDC.

An identical pattern of recovery of HA-1^H-specific TV-DTH response was noted when mDC were used as the sole APC to purified T cells. In contrast, no response to HA-1^H could be revealed with either cytokine-neutralizing Abs when pDC were used as the sole APC source. This could not be due to a general failure of pDC to support a TV-DTH response, because as shown in Fig. 1A, a strong ($\geq 30 \times 10^{-4}$ inches swelling) response to EBV could be detected after pDC-T cell coinjection.

To further show that DC are necessary for bystander suppression to occur in the presence of donor allopeptide, we depleted them from whole PBMC and then used the remaining DC-negative fraction in the DTH assay with recall and donor Ags (Fig. 1C). The DC-depleted fraction failed to induce significant suppression of recall response. When DC were added back to the DC-depleted population, strong suppression (65% inhibition) was restored.

Role for IDO in pDC-mediated suppression of recall response by HA-1-specific T_R

The data in Fig. 1 indicate that DC, but not monocytes, can mediate the inhibition of recall response in the presence of donor Ag, and that pDC, unlike mDC, can mediate suppression by a cytokine-independent mechanism. Previous reports have attributed the regulatory effects of pDCs to the enzyme IDO (22, 23). To see whether this was true of bystander suppression, we used 1-methyltryptophan (1-MT) to block IDO activity. As shown in Fig. 2, when mDC-T cell mixtures were coinjected with 1-MT, EBV, and HA-1^H, no reversal of bystander suppression was observed. In contrast, when pDC-T cell mixtures were coinjected with EBV plus HA-1^H in the presence of 1-MT, full reversal of bystander suppression was seen ($p < 0.001$). Control injections of T-DC mixtures with 1-MT showed no response to the 1-MT alone (Fig. 2).

Induction of IDO activity in T-pDC cultures in response to cross-presentation of donor minor H Ag

The above results indicate that bystander suppression of DTH mediated via pDC was due to the inhibitory effects on T_E cells caused by IDO production. To confirm that IDO was induced by cognate interaction between pDC and minor H Ag-specific T_R, we used an in vitro assay for kynurenine, a specific by-product of the IDO-
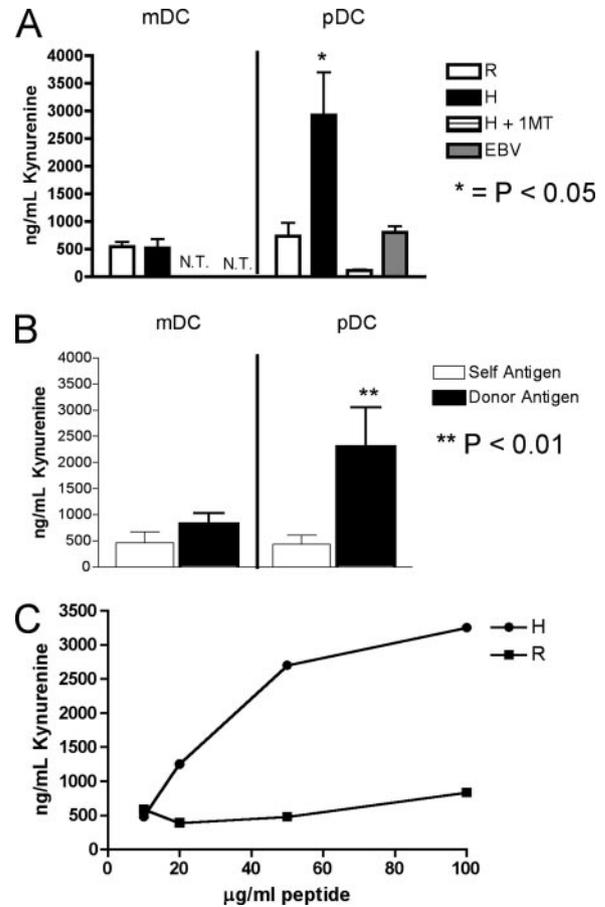
FIGURE 3. Kynurenine production of pDC and not mDC correlates with inhibition of recall DTH in response to indirect presentation of donor minor Ag. DC and T cells were incubated in an in vitro culture along with recipient or donor Ags, or EBV, for 48 h. Culture supernatants were then analyzed for the presence of kynurenine, a product of the breakdown of tryptophan catalyzed by IDO. Values are presented as mean $\pm$ SEM. A, Recipient DC and T cells incubated with H or R peptides, H peptide with 1MT, or EBV. Results are composite of tests using cells from patients I and II ($n = 2$ independent experiments/patient). Values of p were determined by the paired t test comparing R and H peptides. B, Recipient DC and T cells incubated with self or donor whole cell lysates. Results are taken from tests using cells from patient III ($n = 2$ independent experiments/patient). Values of p were determined by paired t test comparing self and donor lysates. C, Recipient pDC and T cells were incubated with self or donor peptides at various concentrations, and kynurenine levels were assayed. Results taken from cells of patient III.

catalyzed catabolism of tryptophan (21). Patients' mDC or pDC were incubated with autologous T cells in the presence of self HA-1^R or donor-type HA-1^H peptide, or cell lysates of self or donor origin. As shown in Fig. 3, self Ag HA-1^R (Fig. 3A) and self PBMC lysates (Fig. 3B) both induced baseline levels of kynurenine (500–700 ng/ml) when pDC were present, whereas donor HA-1^H peptide ($p < 0.05$; Fig. 3A) or lysate ($p < 0.01$; Fig. 3B) each induced a 4- to 5-fold increase in IDO activity. As expected, the elevated kynurenine release in pDC-T cell cultures with HA-1^H was completely blocked by the specific IDO inhibitor 1-MT (Fig. 3A). In contrast, low amounts of kynurenine were not induced at significant levels in supernatants of T-mDC cultures upon addition of donor Ag. Importantly, stimulation of pDC-T cell cocultures with EBV Ag did not lead to IDO induction. Kynurenine production was dependent on the concentration of the donor peptide added, as shown in Fig. 3C with doses of $\geq 50 \ \mu$g/ml yielding
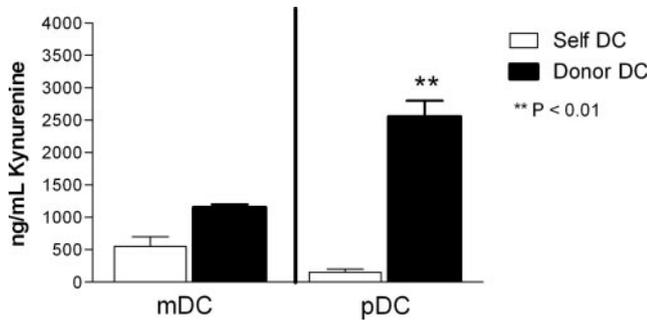
FIGURE 4. Kynurenine production of pDC and not mDC correlates with inhibition of recall DTH in response to direct presentation of donor minor Ag. Recipient T cells were incubated with donor or self DCs and incubated in in vitro culture as described in Fig. 3. Supernatants were analyzed for kynurenine. Results are taken from tests using cells from patient III ($n = 2$ independent experiments). Values of p were determined by paired t test comparing self or donor DC.

optimal response to H; no reaction to R was seen at the same dose of peptide. These in vitro data were consistent with the results seen in the TV-DTH system, and show that indirect or cross-presentation of donor minor H Ag by recipient pDC to T_R induced bystander suppression via IDO.

Induction of IDO activity in T-pDC cultures in response to direct presentation of donor minor H Ag

HA-1^H expression is generally limited to cells of hemopoietic origin, thus a relevant target of CD8$^+$ T_R specific for HA-1 would be donor-derived T cells and DC that persist long after transplantation (11, 24). We questioned whether the kynurenine assay might detect regulation induced by the "direct pathway," i.e., minor HA-1 Ag naturally presented on an intact donor DC. We cocultured T cells from patient III with patient or donor pDC or mDC as the sole source of Ag. As shown in Fig. 4, donor pDC were able to induce a significant production of kynurenine. Specifically, there was a 17-fold increase in IDO activity in donor pDC vs autologous pDC ($p < 0.01$) compared with only a 2-fold increase in donor vs autologous mDC. These results are consistent with the higher IDO

competence of pDC vs mDC. It is possible that the low number of pDC contaminating the donor mDC preparation ($<1\%$) are responsible for the weak IDO signal in the T-mDC cultures.

TGF-βR on the T cells is required for suppression of recall DTH in T-mDC cultures

To further characterize the TGF-β pathway of DC-mediated inhibition of the recall TV-DTH response, DC or T cells from patient III, a TGF-β regulator (Table I), were pretreated separately with anti-TGF-βRII blocking Ab and then washed and tested in TV-DTH assay (Fig. 5A). When TGF-βRII was blocked on the T cells, with unmanipulated mDC as the sole APC, recall TV-DTH response was restored (Fig. 5B; $p < 0.05$). When TGF-βRII was blocked on the mDC, no effect was seen, i.e., recall response inhibition still occurred (Fig. 5B), indicating a dominant role of T cell-expressed TGF-βRII in recall suppression. When pDC served as the sole APC, no reversal of bystander suppression was observed when either the T cell or pDC were pretreated with the anti-TGF-βRII Ab.

Anti-TSP-1 and anti-TGF-β reverse suppression of recall DTH mediated via mDC

Having established that TGF-β produced in T-mDC interaction must bind to its receptor on the T cell to induce the inhibition of recall response in the presence of donor Ag, the question remains: by what mechanism do mDC enable T_R to mobilize and release TGF-β for suppression? Previous studies in tolerant monkey and mouse kidney allograft recipients and in mouse models of anterior chamber-associated immune deviation have shown that T_R-mediated regulation of anti-donor DTH responses required both TGF-β and TSP-1 (13, 25). Because TSP-1 is known to be an autocrine-negative regulator produced by DCs (26) and because it is known to regulate the conversion of TGF-β from latent to active form (27), we wished to determine whether mDC or pDC could induce their effects through TSP-1. Both mDC and pDC from patients III or V (both TGF-β regulators, Table I) were separated and added to the T cells of the same patient in the DTH assay along with either anti-TGF-β or anti-TSP-1 Ab. When Ab to the active form of
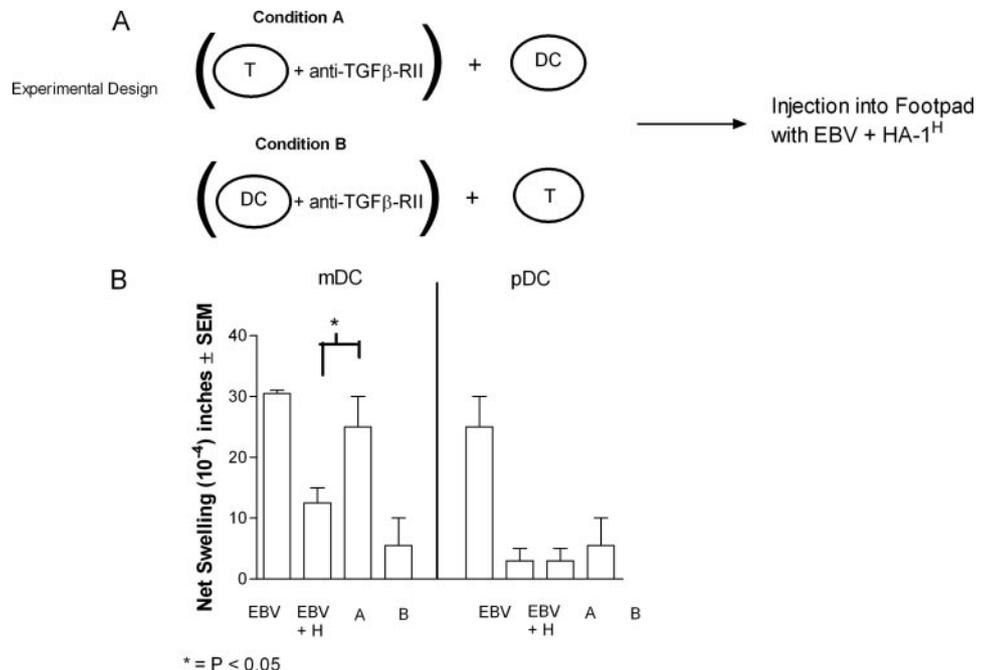
FIGURE 5. The TGF-βR on the T cell is required for the suppression of recall DTH. Either T cells or DCs were reacted with anti-TGF-βRII Ab and washed (*top*, shown diagrammatically). Values are presented as mean $\pm$ SEM $\times$ 10^{-4} inches and are a composite results using cells from patients II and III. Value of p was determined by paired t test comparing EBV plus H with each condition, *A* or *B* (*bottom*).
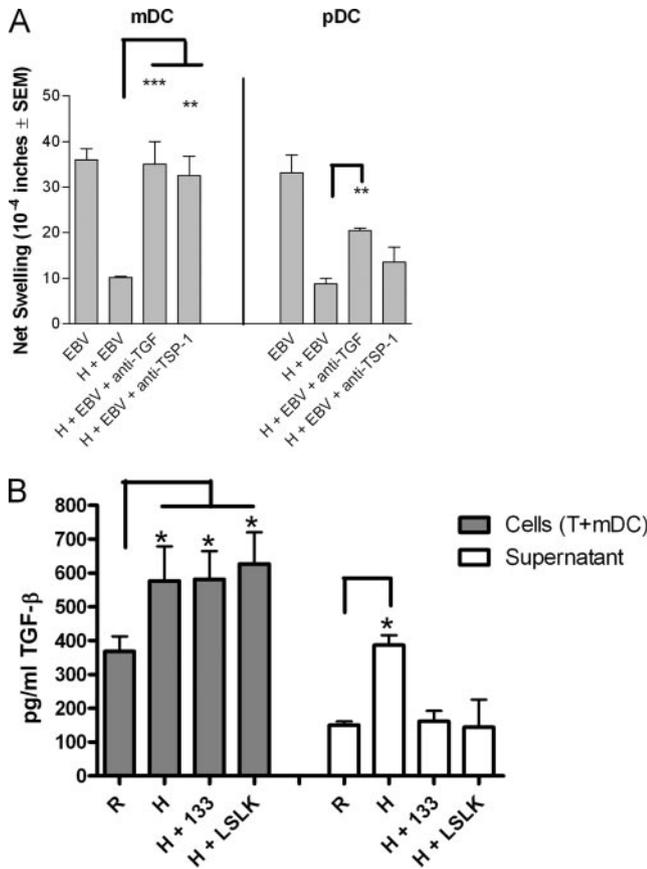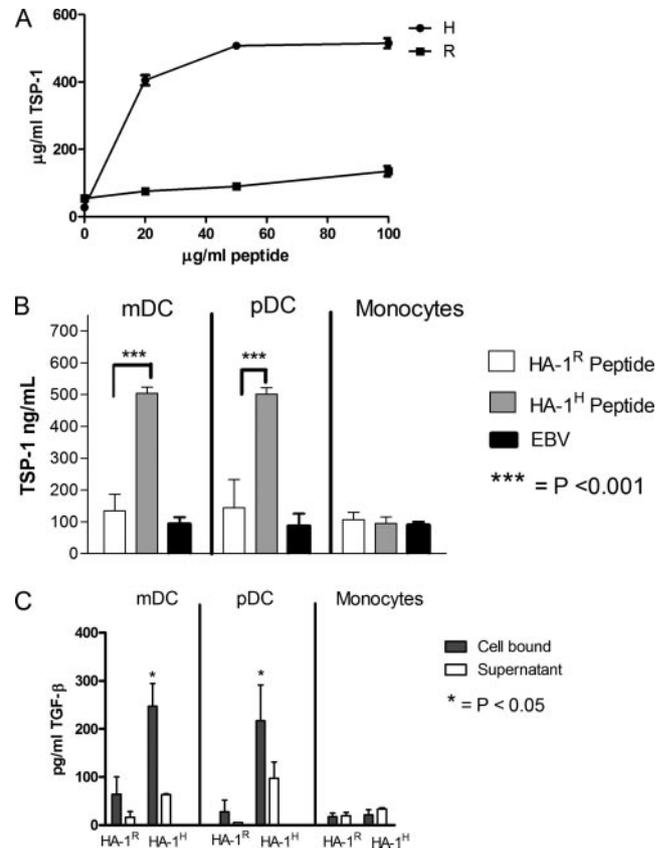
FIGURE 6. Anti-TSP-1 reversed TGF-β-mediated suppression of the recall response. DC and T cells were isolated and added to the following assays. *A*, TV-DTH assay. Results from patients III and V. Values of *p* determined from the paired *t* test comparing H plus EBV with either H plus EBV plus anti-TGF-β or H plus EBV plus anti-TSP-1 condition. A6.1 (Ab4) was the anti-TSP Ab used in this experiment. *B*, TGF-β assay 6-h cell lysates or supernatants were taken from cultures to which R or H peptides have been added with two inhibitors of TSP-1: mAb 133, or peptide LSLK. Results from patient V. Values of *p* determined from paired *t* test comparing each H condition to the R condition. Values are presented as mean ± SEM. *, $p < 0.5$; **, $p < 0.01$; ***, $p < 0.001$.


FIGURE 7. TGF-β and TSP-1 are both produced by T-mDC and T-pDC coculture, but not T-monocyte coculture. *A*, TSP-1 ELISA dose response titration. Cell culture supernatants from mDC-T cell cultures to which different doses of H or R peptide were added for 48 h. *B*, TSP-1 ELISA from 48-h cell culture supernatants of cells to which R, H, or EBV have been added. Results from cells taken from patients I, III, and V. Values of *p* were determined by paired *t* test comparing the H condition to the R condition. *C*, TGF-β ELISA of 48 h cultures of cells to which R or H peptides have been added. Results are from patient III and V. Values of *p* determined by paired *t* test.

TGF-β was added, the bystander suppression response was abrogated fully in the case of mDC ($p < 0.001$), and partially in the case of pDC ($p < 0.01$; Fig. 6*A*). When anti-TSP-1 was added, HA-1^H-triggered inhibition of recall DTH was fully reversed in mDC-T cell coinjections ($p < 0.01$), but was only slightly affected in pDC-T cell coinjections ($p = NS$; Fig. 6*A*).

To determine how TSP-1 works to facilitate TGF-β-type regulation in the T-mDC culture system, HA-1^R or HA-1^H peptide was added to in vitro T-DC cultures for 6 h along with inhibitors of the catalytic activity of TSP-1. These inhibitors were the mAb 133 (28) or the antagonist peptide LSLK (27), both of which interfere with the active site of TSP-1 responsible for LAP removal from the LAP:TGF-β1 latent complex.

The supernatants and cell lysates were tested for TGF-β levels by ELISA to determine the amount of bound and free TGF-β. When H peptide was added to the cell culture, TGF-β levels in the supernatant and cell pellet rose at 6 h, compared with cultures incubated with R peptide ($p < 0.001$ H vs R for the supernatants; $p < 0.05$ H vs R for the cell pellets) (Fig. 6*B*). However, when the TSP-1 inhibitors were added, TGF-β was no longer detectable in the supernatant, while the levels remained unchanged in the pellet (Fig. 6*B*). This result suggests that TSP-1 is responsible for the

conversion of latent TGF-β into secreted active TGF-β that can bind to TGF-βRII on the recall Ag-specific T$_E$ cell.

TGF-β and TSP-1 are induced by addition of allopeptide to T-mDC and T-pDC coculture, but not to T-monocyte coculture

To further analyze the mechanism of bystander suppression via TSP-1 and TGF-β and to clarify the functional deficiency of monocytes, purified T cells along with either purified mDC, pDC, or monocytes were incubated in the presence of HA-1^R or HA-1^H peptide in in vitro culture for 24 h. As shown in Fig. 7*A*, HA-1^H induced TSP-1 production in mDC-T cell cultures in a dose-dependent, Ag-specific manner (Fig. 7*A*). Using an optimal concentration determined by the previous experiment (≥ 50 μg/ml), cocultures of T cells with both mDC and pDC resulted in a 5-fold increase in TSP-1 secretion relative to R peptide control in T cell-monocyte cocultures with Ag failed to release TSP-1 (Fig. 7*B*). Furthermore, EBV did not induce TSP-1 secretion from either mDC or pDC. This implies that the induction of TSP-1 is the result of cognate interaction of DC with T$_R$ cells, and not a general consequence of any cognate T cell-DC interaction. Similarly, both cell-bound and secreted TGF-β were induced in response to HA-1^H, but not HA-1^R, in both mDC-T and pDC-T cocultures. The difference between HA-1^H and HA-1^R stimulation was significant for cell-bound TGF-β ($p = 0.043$) and approached significance for
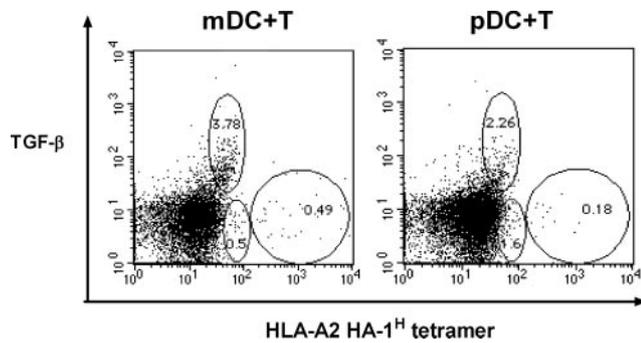
FIGURE 8. H peptide induces surface TGF-β on CD8^{+}, HA-1[H] tetramer-low T cells in T-DC cocultures. DC and T cells were incubated together in an in vitro culture along with H peptide for 9 days and analyzed for HA-1[H]-specific T cells with tetramer and cell surface TGF-β. The x-axis represents HA-1[H] tetramer staining, and the y-axis represents cell surface TGF-β staining. Percentages are given in the corner of each quadrant. *Left plot*, mDC-T cell cultures; *right plot*, pDC-T cell cultures. Results drawn from cells taken from patient III.

secreted TGF-β ($p = 0.067$). Neither form of TGF-β was induced in monocyte-T cell cocultures (Fig. 7C).

HA-1[H] peptide induces cell-surface TGF-β1 on CD8^{+}, tetramer low, minor Ag-specific T_R in T-DC cultures

We have previously reported that in recipients of a kidney transplant from an HLA identical/HA-1[H]-mismatched donor, tolerance was associated with tetramer-dim, minor H Ag-specific CD8^{+} T_R that could be induced to express TGF-β upon Ag challenge using a mixed population of APC (11). To test whether we could replicate this T_R phenotype in an in vitro system, we incubated T cells from patient III with either mDC or pDC and cultured with HA-1[H] peptide for 9 days. We reasoned that 9 days of culture was necessary for TCR expression to return to sufficient levels after the initial down-regulation in response to Ag exposure. In this way, we could simultaneously measure HA-1[H]/HLA-A2-specific tetramer binding and surface TGF-β expression. Using HA-1[H]/HLA-A2-specific tetramers and gating on CD8^{+} cells, we found that when mDC were used to present the HA-1[H] peptide, an increased number of tetramer-dim TGF-β-positive T cells were present (3.78% of total CD8^{+}), as compared with T- pDC cultures, (2.26%; Fig. 8). A significant number of tetramer-bright CD8^{+} T_E (mean fluorescence intensity >100) were present in HA-1[H]-stimulated cultures with mDC, but none were strongly TGF-β1 positive as expected (11). When pDC were the sole APC, the total number of tetramer-bright T cells were reduced, and the relative number of TGF-β-negative, tetramer-dim cells were increased relative to mDC-T cell cultures (1.6 vs 0.5%).

Discussion

DC have been shown to be key to peripheral tolerance in a variety of experimental models (15, 23, 29, 30). We have previously described a model of bystander suppression in which tetramer-dim, minor H Ag HA-1[H]-specific, CD8^{+} T_R from a tolerant transplant recipient mediate inhibition of a DTH response driven by the CD4^{+} memory T_E to a colocalized viral recall Ag (11). We have used this model to test the hypothesis that the DCs presenting minor H Ag to T_R are the critical component mediating inhibition of recall response. Our findings support this hypothesis in the following ways: 1) both mDC plus T and pDC plus T, but neither monocytes plus T, nor DC-depleted PBMC could mediate HA-1[H]-dependent suppression of a recall (EBV) DTH response, 2) the mechanism of recall inhibition bears the imprint of the DC subset

used for Ag presentation, and 3) neither IDO (R. A. Derks, unpublished data), nor TSP-1 (Fig. 7) were induced by HA-1[H] Ag in T-monocyte cocultures.

Recent imaging data in the mouse clearly show that the T_R interacts not with the T_E directly, but with a CD11^{+} DC, altering the latter and diminishing subsequent DC-T_E cell conjugate formation in vivo (10, 31). Some investigators have gone further, proposing that not only are DC required for tolerance mediated by T_R, but that pDC are perhaps the only DC type capable of mediating tolerance to an organ allograft (15, 16). Although we found that pDC do indeed mediate a more robust form of bystander suppression involving both TSP-1 and IDO, we also found that mDC are capable of suppressing a recall DTH response in a TSP-1- and TGF-β-dependent manner. Although we did not explore the IL-10 regulation pathway in depth, it is clear from the analysis of the regulated response to HA-1[H] in patient II (IL-10-only regulator) that both mDC and pDC can support this pathway of bystander suppression. Recent data suggest that pDC carry a special costimulator molecule, ICOS-L, that can induce the T_R-1/IL-10 type of adaptive T_R (32).

The stronger intensity of bystander suppression in T-pDC vs T-mDC was correlated with the induction of IDO during cognate T_R-pDC interaction. IDO has been implicated in the suppressive abilities of pDC in animal models, and is thought to either "starve" the T_E of tryptophan, or produce metabolites that inhibit T_E functions (33, 34). In either case, the catalytic activity of IDO was required for bystander suppression via pDC because 1-MT caused full reversal of recall inhibition under the T-pDC conditions, while the mDC-mediated inhibition of recall Ag response remained unaffected (Fig. 2). The fact that intact donor pDC in the absence of exogenous Ags could trigger an IDO response in allogeneic T-pDC cocultures (Fig. 4) reveals the sensitivity of this system and shows that the amount of minor H Ag naturally presented by the donor DC is enough to activate T_R to induce the IDO response. The induction of an IDO response in donor pDC by recipient T cells might be physiologically relevant, because viable intact donor pDC could be present during tolerance under conditions of microchimerism (11, 24).

In contrast to pDC, mDC mediated the inhibition of response to a recall Ag primarily through TSP-1 and TGF-β. TSP-1 is produced by a number of cell types, including platelets and DCs (26), while TGF-β is up-regulated after Ag stimulation on the surface of both CD4^{+} and CD8^{+} Ag-specific T_R (12, 35, 36). The TGF-β induced in both natural and adaptive T_R cells appears to be primarily expressed as a large latent complex of TGF-β, LAP, and latent TGF-β-binding protein (13, 37–39). TSP-1 is the major molecule which liberates active TGF-β from its cell surface or matrix-bound, LAP complex form (27). Indeed, while most of the induced TGF-β remained cell-bound, specific inhibitors of the LAP-binding site of TSP-1 could block release of TGF-β into T-DC culture supernatants when minor H Ag was present (Fig. 6B). Together, these results suggest that TSP-1 induction in the mDC is necessary for the activation of latent complex TGF-β and its release from the T_R. TSP-1 also may have suppressive properties independent of TGF-β by its interaction with CD47 on T_E cells (40). A6.1 (Ab-4) is a mAb to TSP-1 that does not affect the LAP binding and does not interfere with TSP-1-mediated conversion of latent to active TGF-β1 (Ref. 28 and R. A. Derks, unpublished observations). Reversal of bystander suppression by A6.1 means that either the catalytic role of TSP-1 in conversion of latent-to-active TGF-β1 is not critical for bystander suppression, or, that A6.1 interferes with other functions of TSP- 1, like CD47 binding, that may be important in bystander suppression.

Both mDC and pDC were equally capable of producing soluble TSP-1 in in vitro cultures with HA-1^H and T cells. TSP-1 was essential for inhibition via mDC, whereas there was only a partial reversal of bystander suppression by either anti-TGF-β or TSP-1 Ab in T-pDC coinjections in the SCID mouse footpad. In our hands, monocytes were not induced to secrete TSP-1 in response to cognate interaction with HA-1^H-specific T_R offering one explanation as to why monocytes do not mediate efferent suppression. In contrast to these results, thioglycolate-elicited macrophages from mouse peritoneal exudates were induced to express TSP-1 following overnight incubation with Ag and active TGF-β1 (25). It is possible that blood monocytes, used in the present study, require differentiation to macrophages to become "TSP-1 competent" cells.

We further defined the target of TGF-β suppression by blocking the TGF-βRII on responder T cells and the mDC. These results indicate that the target of active TGF-β produced during T-DC interaction with minor H peptide is the T cell and not the DC, consistent with recent evidence that a T cell-expressing TGF-βR is required for tolerance (41, 42). Taken together, the data suggest the following sequence: when the DC presents cognate MHC/peptide Ag to the T_R, latent TGF-β is induced on the T_R and TSP-1 is induced in the DC. TSP-1 then binds to surface receptors on both T_R (CD47) and DC (CD36) where it can activate the latent TGF-β1 (43). This active TGF-β1 then binds to the TGF-βR on the bystander T_E cell to generate suppression. One difficulty that could impede the interpretation of this data is what the source of TGF-β is in this system. The data in Fig. 8 suggest that a critical source of TGF-β is the T_R itself. Tetramer-low T cells cocultured for 9 days with mDC and allopeptide were TGF-β positive; these T cells have previously been characterized as the ones in which regulatory function resides (11). This experiment was also done in serum-free medium, eliminating any potential TGF-β contamination from the medium itself binding to the cells.

A key question raised by the data is: why does the T_R-DC interaction promote IDO and TSP-1 induction, while T_R-monocyte interaction does not? We can reasonably assume that all kynurenine measured in T-DC cocultures is from the DC, as IDO competency has been identified in APC only, and not T cells (Figs. 3 and 4) (23). Both IDO and TSP-1 are DC products that are upregulated in DC during in vitro culture by the action of ATP binding to the purinergic receptor $P2Y_{11}$ expressed on these DC (44). One might speculate that interactions between the T_R and APC induce bursts of extracellular ATP that bind $P2Y_{11}$ and allow the induction of TSP-1 or IDO. In contrast, the effect of ATP signaling through $P2X_7Rs$ expressed on monocytes is completely different, inducing proinflammatory signals such as IL-1β (45). Preliminary data indicate that the purinergic receptor inhibitor sumarin was able to reverse recall DTH inhibition in both T-mDC and T-pDC coinjection assays, suggesting that purinoceptors indeed are important in both IDO and TSP-1/TGF-β pathways of suppression (R. A. Derks, E. Jankowska-Gan, and W. J. Burlingham, unpublished data). This $P2Y_{11}R$ ligation may represent an early step common to both mDC and pDC pathways of bystander suppression. The source of extracellular ATP is still also unclear; at this point, we cannot rule out either the APC or the T_R as the source for this signal.

In the 9-day culture of DC with T cells and donor minor H peptide, mDC were able to induce expression of surface TGF-β in a significant portion of low tetramer-binding (HLA-A2/HA 1^H) CD8$^+$ cells (Fig. 8), a subset previously shown to contain TGF-β-producing T_R (11). The pDC-T cocultures generated fewer TGF-β^+ cells in the tetramer-low subset, and fewer tetramer-high cells known to contain the classical CD8$^+$ CTL (11, 46). These

results suggest that, in addition to mediating a more profound form of efferent suppression, pDC-produced IDO may limit T_E survival and sustained TGF-β expression by CD8$^+$ activated T_R. However, the mDC tend to promote both TGF-β^+ tetramer-dim T_R, and TGF-β^- tetramer-bright T_E, maintaining a metastable form of allotolerance.

Besides interacting with donor and recipient DC, it is possible that T_R cells interact with vascular endothelial cells in the kidney transplant inducing yet another pathway of bystander suppression, for example, via induction of IDO (47). This pathway of efferent suppression would not be relevant for minor Ags such as HA-1, which is generally restricted in its expression to cells of hemopoietic origin (46). However, several minor Ags such as HY and HA-8 are "ubiquitous" in nature and thus could be suited to endothelial cell-mediated IDO-based suppression (48).

In conclusion, the present study supports the hypothesis that DC are required for bystander suppression of third-party T_E in the presence of colocalized donor Ag-specific T_R. Surprisingly, each DC precursor subtype used a similar TSP-1 pathway to harness TGF-β1 for bystander suppression, while pDC add an additional IDO pathway to achieve this result. In contrast, monocytes do not appear capable of mediating bystander suppression to minor HA-1^H Ag because they lack TSP-1 and IDO competence in short-term assays. These findings offer insight as to the previously undefined mechanism of bystander suppression in tolerance to minor H-mismatched, HLA-matched renal transplant, insights that may also be relevant to HLA-mismatched transplantation tolerance mediated by allopeptide-specific adaptive T_R (12).

Disclosures

The authors have no financial conflict of interest.

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