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

Richard A. Derks, Ewa Jankowska-Gan, Qingyong Xu, and William J. Burlingham

One hallmark of acquired tolerance is bystander suppression, a process whereby Ag-specific (adaptive) T regulatory cells (T<sub>R</sub>) 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<sup>H<sub>1</sub></sup> 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<sup>H<sub>1</sub></sup> peptide to cocultures of T cells and pDC, but not myeloid DC (mDC), induced IDO activity in vitro. When mDC presented HA-1<sup>H<sub>1</sub></sup> peptide to Ag-specific CD<sup>8</sup><sup>T</sup> effector 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<sub>R</sub> cells. These results shed light upon the mechanism of bystander suppression by donor Ag-specific T<sub>R</sub> 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<sub>R</sub>) 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 could be 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<sub>R</sub> became widely accepted, it quickly became apparent that without an APC, tolerogen-specific T<sub>R</sub> would be unable to affect a third-party Ag-specific T effector cell (T<sub>E</sub>) (9). In particular, direct T-T interaction models did not adequately account for T<sub>R</sub>-mediated suppression in vivo when natural (i.e., nonadaptive) T<sub>R</sub> were the source of T<sub>R</sub> (10). These novel findings in mice raised questions regarding the role of the APC in immune regulation of T<sub>R</sub> 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<sub>R</sub>, stimulating it to produce IL-10 or TGF-β that binds IL-10- and/or TGF-β receptors on the third-party T<sub>E</sub>; or 2) an active APC model, in which the APC propagates regulatory effects from the T<sub>R</sub> to the T<sub>E</sub> 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
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+ Treg 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 Treg stimulation, could also mediate bystander suppression.

To test these alternative hypotheses, we used a model dependent on CD8+ Treg which recognize the hemopoietic-restricted minor histocompatibility (H) Ag HA-1H in the context of HLA-A2 (11). We have previously shown in a case of long-term kidney allograft tolerance that CD8+ Treg with low binding of HLA-A2/HA-1H tetramer were present in PBMC. These cells could suppress both 1) CD8+ Treg that bind to the same tetramer but with higher staining intensity, and 2) CD4+ Treg specific for a recall Ag that is colocalized with CD8+ Treg and HA-1H Ag. The suppressive response initiated by the CD8+ Treg was exquisitely sensitive to physiologic levels of HA-1H 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-1H-specific Treg. Both mDC and pDC were found to share the ability to mediate bystander suppression, but used different mechanisms to silence a Treg 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-1H (control; VLHDLEA) and HA-1H (allo-minor H Ag; VLHDDLLEA) were synthesized in cryopreservation at 106 negatively selected, T cell-enriched fraction for footpad injection. 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 co-injected 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); Labvision Neomarkers).

To block the TGF-βR, either DC or T cells were incubated alone in PBS containing 100 μl 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 × 105 DC; 2 × 105) T cells, in a volume of 200 μl of RPMI 1640 complete medium, supplemented with 2 mM tryptophan. HA-1H and HA-1R 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% CO2 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 supernant 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 supernant after spinning down to remove cellular debris at 15,000 × g for 3 min. Active TGF-β in culture supernatants was detected by adding 100 μl of supernant 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 × 105 T cells:1 × 104 mDC in 100 μl of serum-free medium (X-Vivo; Invitrogen Life Technologies), along with either 50 μg/ml HA-1H or HA-1R 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 ILS1K (a gift of Dr. C. Orzos, 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% CO2 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 × 105) were cultured in serum free medium (X-Vivo; Invitrogen Life Technologies), with 1 × 105 mDC or pDC and 50 μg/ml HA-1H peptide for 9 days. Cells were harvested and stained with PE-labeled HA-1H/2 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

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Reference</th>
<th>Recipient HA-1</th>
<th>Donor HA-1</th>
<th>(T_R) Type</th>
<th>Graft Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11</td>
<td>R/R</td>
<td>H/H</td>
<td>TGF-(\beta) plus IL-10</td>
<td>Tolerant</td>
</tr>
<tr>
<td>II</td>
<td>11</td>
<td>R/R</td>
<td>H/R</td>
<td>IL-10</td>
<td>Maintenance</td>
</tr>
<tr>
<td>III</td>
<td>11</td>
<td>R/R</td>
<td>H/R</td>
<td>TGF-(\beta)</td>
<td>Maintenance</td>
</tr>
<tr>
<td>V</td>
<td>This study</td>
<td>R/R</td>
<td>H/R</td>
<td>TGF-(\beta)</td>
<td>Tolerant</td>
</tr>
</tbody>
</table>

*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\(^+\)-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\(^{HR}\) or HA-1\(^{HR}\) 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 \(\sim 1\% - 3\%\) 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., \(1 \times 10^6\) DC to \(7 \times 10^6\) T cells, or 1.5%. When EBV Ag, mDC, and T cells were co-injected 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 \((p < 0.001, \text{ HA-}1^{H}\text{ plus EBV vs EBV alone}\); 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 \(T_R\) and \(T_E\). 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-\(\beta\) (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-\(\beta\)- or IL-10-producing \(T_R\) (Fig. 1B).

Table II. Analysis of DC percentages from each patient

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Total DC, %</th>
<th>mDC, %</th>
<th>pDC, %</th>
<th>pDC:mDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.65</td>
<td>0.55</td>
<td>0.1</td>
<td>0.18</td>
</tr>
<tr>
<td>II</td>
<td>2.69</td>
<td>1.9</td>
<td>0.79</td>
<td>0.41</td>
</tr>
<tr>
<td>III</td>
<td>2.0</td>
<td>1.7</td>
<td>0.3</td>
<td>0.17</td>
</tr>
<tr>
<td>V</td>
<td>2.12</td>
<td>1.0</td>
<td>0.18</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*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 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 donor Ag and either control IgG, anti-TGF-\(\beta\), or anti-IL-10 conditions to the IgG conditions. Results are the mean ± SEM of net swelling (test-PBMC alone control) responses in 3 patients. B. Attempts to reveal cryptic \(T_E\) responses to HA-1 by neutralization of IL-10 or TGF-\(\beta\). 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-\(\beta\), or anti-IL-10. Values of \(p\) were determined by paired \(t\) test comparing EBV to EBV plus H. Values are presented as mean ± SEM \(\times 10^{-4}\) inches and results are a composite of results using cells from patients I to V (\(n = 2\) independent experiments/patient). C. Brisk suppression of recall response tested with pure 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).
suppression was seen (in vitro assay for kynurenine, a specific by-product of the IDO- plus HA-1H in the presence of 1-MT, full reversal of bystander contrast, when pDC-T cell mixtures were coinjected with EBV by HA-1-specific TR. Role for IDO in pDC-mediated suppression of recall response population, strong suppression (65% inhibition) was restored. When DC were added back to the DC-depleted fraction failed to induce significant suppression of them from whole PBMC and then used the remaining DC-negative population, strong suppression (65% inhibition) was restored.

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 ± SEM × 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-1H-specific TV-DTH response was noted when mDC were used as the sole APC to purified T cells. In contrast, no response to HA-1H 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 (>30 × 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 alloepitope, 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

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-1H, no reversal of bystander suppression was observed. In contrast, when pDC-T cell mixtures were coinjected with EBV plus HA-1H in the presence of 1-MT, full reversal of bystander suppression was observed (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<sub>reg</sub> cells caused by IDO production. To confirm that IDO was induced by cognate interaction between pDC and minor H Ag-specific T<sub>reg</sub>, we used an in vitro assay for kynurenine, a specific by-product of the IDO- catalyzed catabolism of tryptophan (21). Patients’ mDC or pDC were incubated with autologous T cells in the presence of self HA-1<sup>H</sup> or donor-type HA-1<sup>H</sup> peptide, or cell lysates of self or donor origin. As shown in Fig. 3, self Ag HA-1<sup>H</sup> (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<sup>H</sup> 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<sup>H</sup> 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-pDC 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 ≥50 µg/ml yielding...
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 TR induced bystander suppression via IDO.

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

HA-1H expression is generally limited to cells of hemopoietic origin, thus a relevant target of CD8+ TR 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 TR 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 TR-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 TGF-β was added to the T cells, the recall response to HA-1 was restored (Fig. 5B, p < 0.05).
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. 6A). When anti-TSP-1 was added, HA-1H-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. 6A).

To determine how TSP-1 works to facilitate TGF-β-type regulation in the T-mDC culture system, HA-1H or HA-1R 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 cell-bound and secreted 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. 6B). 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. 6B). 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<sub>T</sub> cell.

**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 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<sup>R</sup> or HA-1<sup>H</sup> peptide in vitro culture for 24 h. As shown in Fig. 7A, HA-1<sup>H</sup> induced TSP-1 production in mDC-T cell cultures in a dose-dependent, Ag-specific manner (Fig. 7A). Using an optimal concentration determined by the previous experiment (≥50 μg/ml), cultures 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 co-cultures with Ag failed to release TSP-1 (Fig. 7B). 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<sub>T</sub> 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<sup>H</sup>, but not HA-1<sup>R</sup>, in both mDC-T and pDC-T cocultures.

The difference between HA-1<sup>H</sup> and HA-1<sup>R</sup> stimulation was significant for cell-bound TGF-β (p = 0.043) and approached significance for
monocytes plus T, nor DC-depleted PBMC could mediate HA-1H-β1 antihapten recall response. Our findings support this hypothesis in the fol-

cate this TR phenotype in an in vitro system, we incubated T cells a mixed population of APC (11). To test whether we could repli-

minor H Ag to TR are the critical component mediating inhibition involving both TSP-1 and IDO, we also found that mDC are ca-

FIGURE 8. H peptide induces surface TGF-β on CD8⁺. HA-1H tet-

mDC+T pDC+T

rramer-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-1H-specific T cells with tetramer and cell surface TGF-β. The x-axis represents HA-1H tetramer staining, and the y-axis represents cell sur-

face 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-1H peptide induces cell-surface TGF-β1 on CD8⁺, tetramer low, minor Ag-specific TR in T-DC cultures

We have previously reported that in recipients of a kidney trans-

plant from an HLA identical/HA-1H-mismatched donor, tolerance was associated with tetramer-dim, minor H Ag-specific CD8⁺ T cells 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 TR phenotype in an in vitro system, we incubated T cells from patient III with either mDC or pDC and cultured with HA-1H peptide for 9 days. We reasoned that 9 days of culture was nec-

essary 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-1H/HLA-A2-specific tetramer binding and surface TGF-β expression. Using HA-1H/HLA-A2-specific tetramers and gating on CD8⁺ cells, we found that when mDC were used to present the HA-1H peptide, an increased number of tetramer-dim TGF-β-positive T cells were present (3.78% of total CD8⁺), as compared with T- pDC cul-

tures, (2.26%; Fig. 8). A significant number of tetramer-bright CD8⁺ TR (mean fluorescence intensity >100) were present in HA-1H-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 de-

scribed a model of bystander suppression in which tetramer-dim, minor H Ag HA-1H-specific, CD8⁺ T cells from a tolerant transplant recipient mediate inhibition of a DTH response driven by the CD4⁺ memory T cells to a colocalized viral recall Ag (11). We have used this model to test the hypothesis that the DCs presenting minor H Ag to TR are the critical component mediating inhibition of recall response. Our findings support this hypothesis in the fol-

lowing ways: 1) both mDC plus T and pDC plus T, but neither monocytes plus T, nor DC-depleted PBMC could mediate HA-1H-

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-1H Ag in T-monocyte cocultures.

Recent imaging data in the mouse clearly show that the TR in-


teracts not with the TR directly, but with a CD11c⁺ DC, altering the latter and diminishing subsequent DC-T cell conjugate formation in vivo (10, 31). Some investigators have gone further, proposing that not only are DC required for tolerance mediated by TR, but that pDC are perhaps the only DC type capable of mediating toler-

ance 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 ca-

pable of suppressing a recall DTH response in a TSP-1- and TGF-

β-dependent manner. Although we did not explore the IL-10 reg-

ulation pathway in depth, it is clear from the analysis of the reg-

ulated response to HA-1H 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 costimu-

lator molecule, ICOS-L, that can induce the TR-1/IL-10 type of adaptive T cells (32).

The stronger intensity of bystander suppression in T-pDC vs T-mDC was correlated with the induction of IDO during cognate T-pDC interaction. IDO has been implicated in the suppressive abilities of pDC in animal models, and is thought to either “starve” the TR of tryptophan, or produce metabolites that inhibit TR 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 un-

affected (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 TR 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 micro-

chimerism (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 pro-
duced 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 TR (12, 35, 36). The TGF-β induced in both natural and adaptive TR cells appears to be pri-

arily expressed as a large latent complex of TGF-β, LAP, and latent TGF-β-binding protein (13, 37–39). TSP-1 is the major mol-

ecule 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-bind-
ing 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 TR. TSP-1 also may have suppressive properties independent of TGF-β by its interaction with CD47 on T 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-β (Ref. 28 and R. A. Derks, unpublished observations). Re-

versal of bystander suppression by A6.1 means that either the cat-

alytic role of TSP-1 in conversion of latent-to-active TGF-β is not critical for bystander suppression, or, that A6.1 interferes with other functions of TSP-1, like CD47 binding, that may be impor-

tant in bystander suppression.
Both mDC and pDC were equally capable of producing soluble TSP-1 in vitro cultures with HA-1H 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-1H-specific T cell 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 cell, latent TGF-β is induced on the T cell and TSP-1 is induced in the DC. TSP-1 then binds to surface receptors on both T cell (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 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 cell 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 cell-DC interaction promote IDO and TSP-1 induction, while T cell-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 up-regulated in DC during in vitro culture by the action of ATP binding to the purinergic receptor P2Y1R expressed on these DC (44). One might speculate that interactions between the T cell and APC induce bursts of extracellular ATP that bind P2Y1R, and allow the induction of TSP-1 or IDO. In contrast, the effect of ATP signaling through P2XR-Rs expressed on monocytes is completely different, inducing proinflammatory signals such as IL-1β (45). Preliminary data indicate that the purinergic receptor inhibitor sumatriptan was able to reverse recall DTH inhibition in both T-DC 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 P2Y1R 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 cell as the source for this signal.

In the 9-day culture of DC with T 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 1H) CD8+ T cells (Fig. 8), a subset previously shown to contain TGF-β-producing T cell (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 cell survival and sustained TGF-β expression by CD8+ activated T cell. However, the mDC tend to promote both TGF-β+ tetramer-dim T cell, and TGF-β+ tetramer-bright T cell, maintaining a metastable form of allotolerance.

Besides interacting with donor and recipient DC, it is possible that T cell interacts 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 cell in the presence of colocalized donor Ag-specific T cell. 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-1Ag 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 cell (12).

Acknowledgments
We thank Dr. Els Goulmy for reading and critical comments and Dr. David Wilkes for helpful suggestions regarding the inhibition of IDO.

Disclosures
The authors have no financial conflict of interest.

References


