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Tolerogenic Dendritic Cells Generated with Different Immunosuppressive Cytokines Induce Antigen-Specific Anergy and Regulatory Properties in Memory CD4+ T Cells

Honorio Torres-Aguilar,* Sergio R. Aguilar-Ruiz,* Gabriela González-Pérez,* Rosario Munguía,† Sandra Bajaña,*1 Marco A. Meraz-Ríos,* and Carmen Sánchez-Torres*

Dendritic cells (DCs) are professional APCs involved in the initiation of both immunity and immunological tolerance. In autoimmune diseases or graft rejections, most reactive lymphocytes are effector/memory cells. It is believed that memory T cells are more resistant to tolerance induction than naive lymphocytes; however, studies on mechanisms for their efficient tolerization are still scarce. In this study, we generated human monocyte-derived DCs by culture with GM-CSF and IL-4 (control DCs), as well as tolerogenic DCs (tDCs) by adding IL-10, IL-10/TGF-β1, or IL-10/IL-6. Cells were matured with TNF-α/PGE2. Compared with control DCs, tDCs had similar expression of HLA-DR, CD80, and CD86, lower expression of CD40, higher levels of macrophage markers, enhanced endocytic ability, increased secretion of IL-6, IL-10 (only tDCs generated with IL-10 and tDCs generated with IL-10/IL-6), and PGE2, and lower secretion of IL-12 and IL-23. In vitro, tDCs had the capacity to induce anergy in tetanus toxoid-specific memory CD4+ T cells, whereas the proliferative response to an unrelated Ag was intact. Anergy could be reverted upon exposure to IL-2. tDC-primed T cells have low suppressive ability. Nevertheless, the generation of both anergic and regulatory T cells was more efficient with tDCs generated with IL-10/TGF-β1. Microarray-based gene expression profiling reflected modulated expression of several transcripts in tDCs. Surface CLIP–HLA-DR complexes and intracellular thrombospondin-1 were increased in the three tDCs. CD39 was highly expressed only in tDC-TGF, which correlated with increased adenosine production.

We propose that these molecules, together with IL-10 and prostanoids, are key factors to induce Ag-specific tolerance in memory T cells. The Journal of Immunology, 2010, 184: 1765–1775.

Abbreviations used in this paper: cDC, control dendritic cell; cT, control T lymphocyte; DC, dendritic cell; DMPS, 3,7-dimethyl-1-propargylxanthine; DX, dextran; FcXp3, forkhead box p3; FTH1, ferritin heavy polypeptide 1; iDC, immature dendritic cell; ILT, Ig-like transcript; iTSP-1, intracellular thrombospondin 1; L-NAME, L-nitro-arginine methyl ester; M1, or IL-10/IL-6; MFI, mean fluorescence intensity; rT, T cell with no prior stimulation in vitro; TDC, tolerogenic dendritic cell; IVTC-IL6, tolerogenic dendritic cell generated with IL-10/IL-6; tDC, tolerogenic dendritic cell generated with IL-10; TGF, tolerogenic dendritic cell generated with IL-10/10/10-TGF/T; Tind, indicator responder T cell; Treg, regulatory T cell; TSP-1, thrombospondin 1; rT, tolerant T lymphocyte; TT, tetanus toxoid; rIL-10, tolerant IL-10 T cell; sTGF, tolerant TGF/T cell; sIL-6, tolerant IL-6 T cell.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00 leading to the promotion of anergy, immune deviation, deletion of self-reactive lymphocytes, or generation of regulatory T cells (Tregs) (2). Tolerogenic DCs (tDCs) are characterized by low constitutive expression of positive costimulatory molecules compared with inhibitory factors (i.e., Ig-like transcripts [ILT] 2, 3, 4, B7-H1), as well as by their ability to suppress a broad range of effector T cell responses (3). Immature DCs (iDCs) and semimature developmental stages of DC differentiation and some subtypes of DCs, such as resting plasmacytoid DC, are prone to induce T cell anergy and Treg development (4–8). Moreover, alternatively activated mature DCs exposed to various suppressive agents, as well as to immunomodulatory drugs, are endowed with tolerogenic functions. Several factors, including IL-10, TGF-β1, hepatocyte growth factor, vasoactive intestinal peptide, vitamin D3, or corticosteroids, among others, and exposure to microbial products or tumor-derived factors, modulate DC function and favor tDC differentiation (9–18). Monocyte-derived tDCs reflect an incomplete or altered status in DC differentiation and are being considered in the design of therapeutic strategies.

Induction of tolerance of self-reactive naive T cells is essential for prevention of unwanted responses against peripheral self-Ag. However, once conditions such as autoimmune diseases develop, tolerization must also be focused on memory cells (19). Furthermore, all individuals consider allreactive T cells within both the naive and memory T cell population that are potentially deleterious in transplantation (20). Naive and memory T lymphocytes respond differently to immune stimuli, and although naive cells can successfully be tolerized, it is believed that memory cells are more resistant to tolerance induction (20). Memory cells far outnumber the precursor frequencies of Ag-specific naive cells, rapidly divide and display immediate effector functions on Ag encounter, and have low costimulatory requirements for full activation, which made them...
refractory to tolerance induction using conventional costimulatory (CD28/CD40L) blockade (21). However, experimental autoimmune encephalomyelitis induced by memory CD4+ T lymphocytes can be ameliorated by blockade of ICOS or OX40 pathways (22). Furthermore, peripheral memory CD4+ and CD8+ T cells become tolerant in response to low doses of Ag and low-avidity agonist-altered peptide ligands (23), and tolerization can also occur by persistent exposure to systemic Ag (24). Additionally, CD4+CD25+ Treg cells can dampen memory T cell responses (25).

One of the potential targets to inhibit specific recall responses is the tDC. However, the current knowledge on the modulation of memory lymphocyte function by these APCs is still limited (9) and is mostly based on studies of allogeneic responses (17, 26). In the present work, we sought to identify factors involved in the induction of Ag-specific tolerance of human memory CD4+ T cells by tDCs. tDCs were generated from monocytes with different immunosuppressive cytokines, IL-10, TGF-β1, and IL-6. Collectively, our data demonstrated that IL-10/TGF-β1, and to a lesser extent IL-10 and IL-10/IL-6, generate tDCs capable of inducing anergy in Ag-specific memory CD4+ T lymphocytes and of differentiating them into IL-10-producing regulatory cells. The suppression induced by tDC is achieved through mechanisms involving IL-10, thrombospondin 1 (TSP-1), and PGs. Finally, we describe that tDCs express a high level of cell surface CLIP bound to HLA-DR, which might be associated with reduced presentation of exogenous Ag.

**Materials and Methods**

**Media and reagents**

Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated autologous serum, 2 mM-L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-ME. The following reagents were used: human recombinant GM-CSF (1000 U/ml), Leuconix, Schering-Plough, Kenilworth, NJ); IL-4 (15 ng/ml), TNF-α (40 ng/ml), IL-10 (20 ng/ml), TGF-β1 (20 ng/ml), IL-6 (25 ng/ml), and IL-12 (5 ng/ml) (R&D Systems, Minneapolis, MN); IL-2 (20 U/ml), Life Technologies BRL, Rockville, MD); PGE2 (0.1 µg/ml) (Calbiochem, San Diego, CA); rat neutralizing mAb to IL-10 (10 µg/ml), to IL-6 (10 µg/ml), and Ig isotype controls (BD Biosciences, San Jose, CA); mouse neutralizing mAb to TSP-1 (25 µg/ml) (Neomarkers, Fremont, CA); CFSE and FITC-coupled dextran (FITC-DX, 0.5 µg/ml) (Molecular Probes, Eugene, OR); tetanus toxoid (TT, 0.1 µg/ml) and candidin (0.1 µg/ml), kindly supplied by the National Institute of Hygiene and the Department of Microbiology and Parasitology, Universidad Nacional Autónoma de México, Mexico City, Mexico; respectively; LPS from Escherichia coli 0111:B4 (0.5 µg/10^6 cells/ml), PKH26 red fluorescent cell linker, n-tetroic-arginine methyl ester (0.5 mM), indomethacin (10 µM), ARL67156 (10 µM), and 3,7-dimethyl-1-propargylxanthine (DMPX) (10 µM) (Sigma-Aldrich, St. Louis, MO).

**Cell separation and differentiation of peripheral blood monocytes**

PBMCs were purified from buffy coats of healthy volunteers vaccinated with TT. CD14+ monocytes and autologous or allogeneic memory CD4+CD45RA- T lymphocytes, as well as allogeneic naive CD4+CD45RO+ T lymphocytes, were separated by magnetic cell sorting using MACS isolation kits (Miltenyi Biotec, Auburn, CA). T cells were separated by negative selection using a CD4+ T cell isolation kit and anti-CD45RA Abs (naive). Monocytes were treated with GM-CSF and IL-4 alone to obtain control DCs (cDCs) or simultaneously with IL-10 (tDC-IL10), IL-10/TGF-β1 (tDC-TGF), or IL-10/IL-6 (tDC-IL6) to obtain tDCs. Cultures were fed with fresh medium and cytokines every 2 d. At day 6, TT, candidin (0.1 µg/ml), or no Ag was added to iDCs, and 8 h later, cells were treated with TNF-α and PGE2 for 2 more d to obtain mature DCs (mDCs). mDCs were employed along the study except for endocytosis and cytokine production assays under LPS stimulation, where iDCs were the cells evaluated.

**Flow cytometry analysis and Abs used for staining**

Purified mAbs to CD1a, CD14, CD16, CD32, CD40, CD80, CD83, CD163, CD16, FITC-labeled mAbs to CLIP-loaded HLA-DR molecules, PE-conjugated mAbs to HLA-DR, CCR7, CD25, CD127, CD152, and CD205 (BD Biosciences), purified mAbs to TSP-1 (NeoMarkers, Thermo Fisher Scientific), to CD39 (Santa Cruz Biotechnology, Santa Cruz, CA), and allophycocyanin-labeled mAbs to forkhead box p3 (Foxp3) (eBioscience, San Diego, CA) were used. Purified mAbs were detected with FITC-labeled goat F(ab)2 anti-mouse Ig polyclonal Abs (DakoCytomation, Carpinteria, CA). For detection of intracellular CTLA-4, Foxp3, or TSP-1, cells were fixed and permeabilized before staining. Cell death was evidenced by Annexin V (BD Biosciences) and propidium iodide (50 µg/ml) staining. CLIP expression was defined, taking into account the amount of HLA-DR and CLIP per cell, as follows: relative CLIP amount = (% CLIP+/DR+ cells) × [mean fluorescence intensity (MFI) CLIP/MFI DR]. Samples were analyzed on an FACS caliber (BD Biosciences).

**Endocytosis assay**

IFN-γ, IL-10, and PGE2 for 2 more d to obtain mDCs were rested in fresh medium for 4 h at 37°C. Then supernatant was removed, and cells were fixed with methanol for 3 min and analyzed by light microscopy.

**Morphological analysis**

IfDCs were incubated with FITC-DX (DX, M = 40,000) for 1 h at 37°C. Uptake was stopped by rinsing the cells with ice-cold PBS containing BSA and sodium azide. Controls were set by incubating the cells with FITC-DX at 4°C. Samples were analyzed by flow cytometry.

**Allogeneic and autologous CD4+ T cell proliferation assay**

CD4+CD45RA- lymphocytes were labeled with CFSE. Then T cells were cocultured with allogeneic DCs or with autologous TT-loaded or unloaded DCs at a 10:1 T cell/DC ratio for 5 d. Subsequently, lymphocytes were harvested and stained with mAbs to CD25, CD39, CD127, CD152, or Foxp3. In some assays, lymphocytes were collected on day 5 and rested in fresh medium for up to 8 d. Then they were stained with the above mAbs. Lymphocyte proliferation was assessed by the CFSE dilution method along the study.

**Anergy assay**

To detect Ag-specific T cell unresponsiveness, unlabeled CD4+CD45RA- lymphocytes were cocultured with autologous TT-loaded cDCs or tDCs at a 10:1 T cell/DC ratio for 5 d. On day 5, lymphocytes were harvested and rested in fresh medium for 4 additional d. Lymphocytes primed with cDCs were referred as control T lymphocyte cells (cT) and those primed with tDC-IL10, tDC-TGF, or DC-IL6 as tolerant T-IL10, TGF, or IL-6 T cells (tT-IL10, tT-TGF, and tT-IL6), respectively. As a control, autologous CD4+CD45RA- T cells with no prior stimulation in vitro (tT) were used. After the rest period, each lymphocyte population was labeled with CFSE. Then, T cells were rechallenged in a second proliferation assay with autologous TT-loaded, candidin-loaded, or unloaded cDCs at a 10:1 T cell/DC ratio for 5 d. Occasionally, exogenous IL-2 was added to the cocultures. On day 5, lymphocytes were harvested and stained with the Ab mentioned above.

**Cytokine detection in culture supernatants**

iDCs were incubated for 24 h with LPS or without additional stimulus as a control. Production of soluble factors in these supernatants was quantified in supernatants of lymphocytes harvested after 5 d of culture with ELISA kits: IL-6, IL-10, IL-12 p75 (BD Biosciences), IL-23 p19/p40 (eBioscience), active TGF-β1, and PGE2 (R&D Systems). Production of IL-2, IFN-γ, IL-10, and PGE2 in supernatants collected at 1, 3, and 5 d of cocultures of memory CD4+ T cells with mDC was measured with ELISA kits (BD Biosciences and R&D Systems). Cytokine secretion was also quantified in supernatants of lymphocytes harvested after 5 d of stimulation, rested for 4 d, and cultured for 24 h in fresh medium with immobilized anti-CD3 mAb (5 µg/ml, BD Biosciences).

**Analysis of regulatory T cell function**

For suppression assays on memory T cells, lymphocytes previously stimulated with TT-pulsed cDCs (cT) were used as indicator responder T cells (cT). TregA were labeled with CFSE and cultured at different ratios with PKH26-labeled tolerant T cells (tT-IL10, tT-TGF, or tT-IL6) and TT-loaded or unloaded cDCs (10:1 T cell/DC ratio) for 5 d. Controls were established by adding PKH26-labeled cT or rT. For suppression assays on naive T lymphocytes, memory rT and cT cells were generated from donor A as mentioned previously. CD4+ naive T cells were purified from donor B and labeled with CFSE. TregA were cocultured with TT- or unloaded donor A in the presence or absence of PKH26-labeled memory cT or rT cells at a 10:1 T cell/DC ratio. In both assays, lymphocytes were collected on day 5 and CFSE dilution of Tind analyzed by flow cytometry.
Microarray assays and data analysis

RNA was isolated from cell lysates of 12-h maturated cDCs and tDCs from four different healthy donors using Trizol reagent (Life Technologies BRL). Equal amounts of total RNA from the four donors was pooled, and 30 μg was used for cDNA synthesis with CyScribe First-Strand cDNA labeling kit (Amersham Biosciences, Piscataway, NJ). cDNA synthesis from cDCs was carried out by incorporating dUTP-Cy3 and dUTP-Cy5 for tDC. Equal quantities of labeled cDCs and tDC cDNA were hybridized to the Human arrays (Institute of Cellular Physiology, DNA Microarray Unit, Universidad Nacional Autónoma de México, Mexico City, Mexico) for 14 h at 42˚C. Human array was designed with the Human 10,000 50-mer oligo
library from MWG Biotech Oligo sets (Huntsville, AL; www.mwgbiotech.com). Acquisition and quantification of array images were performed in a ScanArray 4000 scanner (Packard BioChips, PerkinElmer, Waltham, MA). For each spot, the Cy3 and Cy5 density mean and background mean values were calculated with the ArrayPro Analyzer software (Media Cybernetics, Bethesda, MD). Data were normalized and analyzed with the GenArise software (Institute of Cellular Physiology, Universidad Nacional Autónoma de México, Mexico City, Mexico; www.ifc.unam.mx/genarise/). The software identifies differentially expressed genes by calculating an intensity-dependent z-score, which measures the number of SD of a data point from the mean according to the following formula: $z_i = \frac{R_i - \text{mean}(R)}{\text{sd}(R)}$, where $z_i$ is the z-score, $R_i$ the log-ratio for each element, and $\text{sd}(R)$ the SD of the log-ratio. With these criteria, the elements with a z-score $\geq 2$ SD would be significantly differentially expressed genes.

The data obtained in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE18921 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18921).

Semiquantitative RT-PCR

RNA was isolated from cell lysates of 12-h matured cDCs and tDCs from four different healthy donors, and cDNA was synthesized by reverse transcription with oligo(dT)$_{15}$ and Superscript RNase H-reverse transcriptase (Life Technologies BRL). PCR was performed with 10 pmol of 5' and 3' specific primers for the selected genes (Supplemental Table I). GAPDH mRNA was amplified as a control of RNA integrity. PCR products were separated on agarose gels. Ratios of mRNA/GAPDH band densities were determined by densitometry.

Statistical analysis

The Student two-tailed paired $t$ test was used to assess statistical differences between two experimental groups, assuming equal variances. Probability levels for correlation were calculated using the Spearman’s rank test (correlation coefficient, $r$).

Results

tDCs have mixed characteristics of both DCs and macrophages

Mature cDCs and tDCs were generated in vitro as described in Materials and Methods. cDCs had typical DC morphology, with large cell bodies and sharp cytoplasmic projections; they were nonadherent to plastic and promoted few cluster formations. Immunosuppressive cytokines affected tDC morphology: cells were bigger (only tDC-IL10 and tDC-IL6), with spread cytoplasmic projections, more adherent, and frequently aggregated in clusters (Fig. 1A, upper panel). FACS analysis revealed a similar profile of forward and side light scatter of cDCs and tDC-TGF, whereas tDC-IL10 and tDC-IL6 were bigger and more complex (Fig. 1A, lower panel). cDCs displayed characteristic phenotypes of mDCs, expressing CD83, CD1a, CD205, HLA-DR, CD40, CD80, and CD86, but with null or low expression of monocyte/macrophage (Mφ) markers. In contrast, although tDCs had similar expression of CD1a, CD83, CD205, HLA-DR, CD40, CD80, and CD86, they...
showed significantly higher expression of CD14, CD16, CD32, CD163, CD206, and CCR7 (always greater in tDC-IL10 and tDC-IL6 than in tDC-TGF) and lower levels of CD40 (Table I). These phenotypic similarities and differences were already present in immature cDCs and tDCs (Supplemental Table II). Thus, tDCs display a mixed phenotype, bearing monocyte/MΦ and DC markers. Functionally, tDCs had higher endocytic ability than cDCs (Fig. 1B). They secreted reduced levels of IL-12 p75 and IL-23 and enhanced amounts of PGE2 in response to LPS; production of IL-6 and IL-10 was also increased in tDC-IL10 and tDC-IL6. TGF-β1 levels were very low in all DCs, but significantly higher in tDC-TGF and tDC-IL10 (Fig. 1C).

**Lymphocyte proliferation induced by DCs**

A key feature of immunogenic DCs is their high capacity for priming T cells. Thus, we analyzed the ability of DCs to present TT as a soluble Ag to autologous CD4+ memory T lymphocytes. As shown in Fig. 2A, tDCs had reduced APC capacity compared with cDC. We also evaluated the direct pathway of alloantigen presentation (Fig. 2B). Similarly, tDCs were poorer stimulators of lymphocyte proliferation than cDCs in this experimental model. Cell death did not seem to influence the decreased proliferation of tDC-stimulated lymphocytes, as there was no enhanced apoptosis in lymphocytes upon activation with tDC compared with cDC (Supplemental Fig. 1).

Proliferating cDC-stimulated memory T cells (cT) presented an activated phenotype in TT-specific responses (Fig. 2C). They up-regulated CD25 and CTLA-4 and downregulated CD127. We also tested Foxp3 and CD39 expression as Ag related with Treg cells (27, 28). Both markers were expressed in substantial numbers of dividing lymphocytes. The phenotype of tDC-activated lymphocytes (tT) did not exhibit significant differences among the three tDCs (Fig. 2C). However, the percentage of cycling iTs expressing CD25, CTLA-4, Foxp3, and CD39 was lower than that for cTs and positively correlated with the ability of DCs to induce lymphoproliferation (r = 0.90), whereas CD127 expression was higher and inversely correlated (r = −0.88) (data not shown). To determine the phenotype stability of proliferating lymphocytes, cells were harvested after 5 d of priming and rested for 8 d (Fig. 2D). At that time, the expression of CD25 and CD39 notably diminished, paralleled with an increase of CD127. Overall, there were no significant differences between lymphocytes stimulated with cDC or tDC, although (tT-TGF lymphocytes maintained significant high levels of CTLA-4 (Fig. 2D). The percentage of Foxp3+ cells remained high (40–50% of the cells), but very similar among conditions (Fig. 2D).

**Cytokine production by DC-stimulated memory lymphocytes**

Given the inferior APC capacity of tDC, we asked if this event would be reflected in the extent of cytokine production by memory T cells. To this end, we measured their cytokine production during priming with TT-loaded DCs. Differences between cTs and iTs in IL-2 and IL-10 secretion were evident at day 1, when IL-2 production was significantly lower, and IL-10 higher, in supernatants of cultures containing tDCs (Fig. 3A). IFN-γ production by iT was significantly lower from day 3 onwards. iT secreted inferior amounts of IL-2 compared with cT throughout the culture, whereas only tT-IL10 and tT-IL6 maintained significantly greater production of IL-10. Furthermore, we measured cytokine production in supernatants of T cells harvested from the primary culture and restimulated with immobilized anti-CD3 Ab (Fig. 3B). iT secreted significantly higher levels of IL-10 and lower amounts of IL-2 and IFN-γ than cT. These data confirmed that at least part of the IL-10 response detected during priming was driven by lymphocytes.

**IL-10/TGF-β1-treated DCs induced strong Ag-specific anergy in memory T cells**

To further determine whether tDCs induced tolerance of memory T cells, we tested the proliferative response of these lymphocytes in a two-step culture system. cTs cocultured with TT-loaded cDCs in the first coculture responded vigorously to rechallenge with the same stimulators (Fig. 4A, top panel). In contrast, iTs were hypersensitive to further stimulation with cDCs, thus confirming the tolerogenic profile of these DCs. As a control, we used CD4+ memory T cells, which did not receive the first stimulation but were challenged with TT-pulsed cDCs (rT), and corroborated that these cells proliferated robustly. When lymphocytes were rechallenged with the unrelated Ag candidin, cTs and iTs responded similarly, which suggests that tolerant lymphocytes were rendered anergic specifically to TT (Fig. 4A, lower middle panel). Moreover, anergy can be circumvented by adding exogenous IL-2 (Fig. 4A, bottom panel). Addition of IL-12 at priming (26) did not significantly reverse the anergy induced by any tDC (Supplemental Fig. 2). The weakest responses among tolerant lymphocytes were detected in iT-TGF (Fig. 4B), pointing out that the combination of IL-10 and TGF-β1 generated DCs with substantial tolerogenic abilities for memory T cells. In terms of cytokine production, the three iTs had an IL-2loIFN-γhiIL-10hi profile (Fig. 4C); which is analogous to what was found during priming.

**Memory iT cells have low suppressor capacity**

According to numerous studies, anergy and suppression are key characteristics of Tregs (10, 12). Thus, we wondered whether memory iTs have suppressive abilities on conventional memory T cells. To this purpose, CFSE-labeled cTs (referred to as Tmad) were rested for 4 d. Then, cells were counted, and equal numbers were restimulated for 24 h with immobilized anti-CD3 mAb. Analysis of IL-2, IFN-γ, and IL-10 secretion on culture supernatants was performed by ELISA. Data are the mean ± SD of three (IL-2) or four (IFN-γ, IL-10) different donors. Statistical analysis (tT versus cT).

**FIGURE 3.** Cytokine production by memory CD4+ T cells stimulated with TT-pulsed cDCs and tDCs. A, Kinetics of cytokine production by primed T lymphocytes cultured as in Fig. 2A. Supernatants of DC/T cell cocultures were harvested at days 1, 3, and 5 of the primary stimulation, and the amount of IL-2, IFN-γ, and IL-10 was evaluated by ELISA. B, Lymphocytes activated as outlined in A were rested for 4 d. Then, cells were counted, and equal numbers were restimulated for 24 h with immobilized anti-CD3 mAb. Analysis of IL-2, IFN-γ, and IL-10 secretion on culture supernatants was performed by ELISA. Data are the mean ± SD of three (IL-2) or four (IFN-γ, IL-10) different donors. Statistical analysis (tT versus cT). *p < 0.05.
were stimulated with TT-pulsed cDCs, either alone or in presence of different ratios of PKH26-labeled tTs (Fig. 5A, upper panel). In addition, we included controls with different ratios of PKH26-labeled cTs or autologous memory T cells with no prior in vitro stimulation (rT) (Fig. 5A, lower panel). We detected dose-dependent inhibition of T cell proliferation only with tTs, but at ratios equal to or higher than 1:1 (tT:T cell). tT-TGFs were the most efficient suppressor cells. Likewise, tT-TGFs were able to suppress, to some extent, naive CD4+ T cell alloresponses, whereas other tTs were ineffective (Fig. 5B).

**Global gene expression profile of DCs by oligonucleotide array**

We next investigated the mechanisms underlying the induction of the anergic state and/or regulatory function of tT cells. To define the molecular changes induced in tDCs in the presence of tTs or autoaggressive memory T cells with no prior in vitro stimulation (rT) (Fig. 5A, lower panel). We detected dose-dependent inhibition of T cell proliferation only with tTs, but at ratios equal to or higher than 1:1 (tT:T cell). tT-TGFs were the most efficient suppressor cells. Likewise, tT-TGFs were able to suppress, to some extent, naive CD4+ T cell alloresponses, whereas other tTs were ineffective (Fig. 5B).

**Immunosuppressive factors expressed by tDCs**

Based on the microarray results, we subsequently evaluated at the protein level some of the molecules increased on tDCs with potential immunosuppressive effects. We first assessed the levels of intracellular (i)TSP-1, which promotes the generation of human...
peripheral Tregs through its ligation with CD47 (28). Our data demonstrate that tDCs had significant enhanced expression of iTSP-1 compared with cDCs (Fig. 6A, left panel) and that IL-10 is its major inducer (Fig. 6A, right panel).

The ecto-ATPase CD39, together with CD73, has been associated with the suppressive machinery of Tregs, acting through the modulation of pericellular levels of adenosine, which has potent antiproliferative effects (29, 30). Among the DCs, tDC-TGF had the highest expression of CD39, whereas tDC-IL10 and tDC-IL6 expressed significantly lower levels than cDCs (Fig. 6B, upper and left lower panels). TGF-β1 was the major inducer of CD39 expression, whereas IL-10 or IL-6 reduced its expression (not depicted). Adenosine production correlated with the levels of surface CD39, but differences among DCs were not significant (Fig. 6B, right lower panel). Finally, we noticed that several genes involved in Ag processing and presentation were modulated on tDCs, such as the invariant chain (CD74), the cysteine proteases cathepsins B and L, and HLA-DOB (Fig. 6C, upper panel, and Supplemental Fig. 3). We hypothesized that these changes could affect the Ag processing machinery of tDC and therefore their ability to present soluble Ag. Particularly, the enhanced levels of HLA-DOB mRNA in tDCs led us to evaluate the amount of surface CLIP peptides bound to HLA-DR molecules. The three tDCs showed a significant increase in the relative amount of surface CLIP compared with cDCs (Fig. 6C, left lower panel), and this enhancement was induced by IL-10 (Fig. 6C, right lower panel).

Identification of factors involved in tDC suppression

We further investigated the mechanisms by which tDCs inhibit T cell proliferation. As tDCs secreted immunosuppressive IL-10 and IL-6 and expressed increased levels of TSP-1, we added neutralizing Abs against these factors to the cultures of TT-loaded tDCs and memory lymphocytes (Fig. 7A). IL-10 and TSP-1 were involved in the inhibitory function of all tDCs, and their participation was especially determinant in tDC-IL10 and tDC-IL6. Blockade of IL-6 also augments T cell division with tDC-IL6. The involvement of PGE2 in the diminished lymphoproliferation induced by tDCs was also investigated. The PGE2 synthesis inhibitor indomethacin significantly increased the T cell proliferation induced by the three tDCs (Fig. 7B). Indeed, PGE2 is secreted at high levels in tDC/T cell cocultures, whereas it is almost absent in cDC/T cell cocultures (Supplemental Fig. 4). Another inhibitor of T cell proliferation, NO (31), did not have any influence in our system (Fig. 7C). None of the factors tested above seemed to be involved in cDC-induced T cell proliferation (Fig. 7A–C). Lastly, we evaluated the role of adenosine. The addition of the ecto-ATPase inhibitor ARL67156 or the A2 adenosine receptor antagonist DMPX fully restored T cell proliferation (Fig. 7A–C). Consequently, PGs are common factors responsible for memory T cell hyporesponsiveness mediated by the three tDCs; additionally, IL-10 and TSP-1 appear to be decisive in tDC-IL10 and tDC-IL6, whereas adenosine seems to have a unique role in tDC-TGF.

Discussion

Regulatory DCs are promising tools for clinical application in transplantation, autoimmunity, or allergies (8). However, T cells of the memory subset are key players in those diseases (19), and their resistance to immune suppression has been stated previously (20, 21). Increased understanding of what makes DCs tolerogenic for memory T cells is, therefore, important for therapeutic purposes. In this study, we evaluated the tolerogenic abilities of three types of DCs, generated with IL-10, IL-10/TGF-β1, or IL-10/IL-6. The two former DCs have been previously described (9, 10, 12). For the third type, we used IL-6 to enforce the differentiation of monocytes from the DC toward the Mφ pathway (32), with the rationale that Mφ are more inferior APCs than DCs (33). Nevertheless, we found almost no differences in phenotype or function between tDC-IL10 and tDC-IL6, perhaps owing to the overlapping signaling of IL-10 and IL-6 through STAT-3.

Phenotypically, mature tDCs express HLA-DR, CD80, CD86, and CD83 at levels comparable to cDCs, which differs from that reported by others (9, 12). We attribute those disparities to the use of different maturation stimuli and/or different timing in the addition of the suppressive cytokines during DC differentiation. However, and in agreement with other studies (9, 12, 33), tDCs also express low

FIGURE 5. tDCs induce differentiation of memory T cells with suppressive abilities. A, Memory CD4+ Tind were labeled with CFSE and seeded with or without different rations of PKH26-labeled (T-IL10, T-TGF, or T-IL6) (upper panel). Lymphocytes were stimulated with TT-loaded or unloaded cDCs for up to 5 d. On day 5, lymphocytes were harvested, and CFSE dilution was analyzed by flow cytometry. Shown is a graphical representation of the data (mean ± SD) obtained from four separate donors, where the percentage of cycling Tind was set to 100%. Proliferation of Tind alone is shown as dotted lines, and solid lines represent the proliferation of Tind in presence of PKH26-labeled T cells. Controls of the above assays were established with PKH26-labeled cTs or rTs, cultured under the same conditions (lower panel). Statistical analysis (tT versus Tind) 0.05. B, Naive CD4+ Tind were labeled with CFSE and cultured with allogeneic TT-loaded cDCs in the presence or absence of different rations of allogeneic memory PKH26-labeled cTs, T-IL10, T-TGF, or T-IL6 (memory lymphocytes and cDCs were from the same donor). On day 5, lymphocytes were harvested, and CFSE dilution was analyzed by flow cytometry. The percentage of cycling Tind was set to 100%. Shown is the result of a representative experiment.
levels of CD40, which could be involved in their reduced APC ability, and markers tightly associated with the Mφ lineage, such as CD14, CD16, CD32, CD163, and the macrophage mannose receptor (CD206). This could, in fact, partially mediate their increased ability to endocytose FITC-DX, which is also characteristic of Mφ (33). Of note, tDC-TGFs were more similar to cDCs both morphologically and phenotypically, and they expressed Mφ markers at lower levels than the two other tDCs, suggesting that TGF-β1 and IL-10, in addition to their synergistic immunosuppressive effect, could exert some opposite roles (34). tDCs also have a strikingly high expression of CCR7 that might be an advantage for therapeutic purposes (35). We have previously detected higher expression of CCR7 in Mφ differentiated from monocytes, either with GM-CSF or M-CSF, than in DCs from the same donors differentiated with GM-CSF plus IL-4 and matured with LPS (data not shown). Taking this into account, we do not discount that the high levels of CCR7 in tDCs reflect their partial Mφ phenotype.

In terms of cytokine secretion, the three tDCs generated in this study had in common an IL-12p40/IL-23p19/PGE2hi phenotype. However, only tDC-IL10 and tDC-IL6 secreted high amounts of IL-10 and IL-6, unlike tDC-TGF, again showing a closer relationship between cDC and tDC-TGF. The presence of TGF-β1 in tDC-

FIGURE 6. Expression of intracellular TSP-1, CD39, and surface CLIP in mature DCs. A, Expression of iTSP-1 in cDCs and tDCs. MFI of iTSP-1 of three independent donors is depicted as the mean ± SD (left panel). Percentage of iTSP-1+ mDC cultured in absence (none) or presence of the immunosuppressive cytokines alone (IL-10, TGF-β1, IL-6) (right panel). B, Expression of CD39 and adenosine production in culture media. Expression of CD39 in a representative donor (upper panel). Solid histograms represent the fluorescence with anti-CD39 mAb and dotted histograms the isotype control mAb. Numbers above the histograms are the MFI of CD39 in this particular individual. MFI of CD39, given as the mean ± SD of seven independent donors (left lower panel), and adenosine production by DCs (right lower panel), depicted as the mean ± SD of three different experiments. Adenosine was measured by HPLC in supernatants of mDC incubated in fresh medium containing 5 mM ATP for 1 h at 37°C. C, Expression of molecules involved in Ag processing and presentation in DC. RT-PCR of CD74, HLA-DOB, and GAPDH from a representative donor (upper panel). Relative surface amount of CLIP bound to HLA-DR on DCs (left lower panel) and on DCs cultured in absence (none) or presence of the immunosuppressive cytokines alone (right lower panel). Statistical analysis (tDC versus cDC). *p < 0.05.
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FIGURE 7. IL-10, TSP-1, PGE2, and adenosine inhibit T cell proliferation induced by tDCs. TT-loaded DCs were cocultured with CFSE-labeled CD4+ memory T cells in the presence of neutralizing mAb to IL-10, IL-6, TSP-1, or their respective Ig isotype controls (A), the PG synthesis inhibitor indomethacin (B), the NO synthesis inhibitor 1-nitro-arginine methyl ester (L-NAME) (C), the ecto-ATPase inhibitor ARL67156, the A2 adenosine receptor antagonist DMPX (D), or vehicle (B–D). After 5 d, the number of proliferating cells was evaluated by the CFSE dilution method. Statistical analysis (inhibitors versus control conditions for each DC). *p < 0.05.

TGF cultures may account for their low IL-10 secretion (13, 36). In addition, all DCs synthesize very low levels of TGF-β1, which is what it takes to suggest that TGF-β1 secreted by tDCs would not have a determinant role in their inhibitory ability.

The low APC capacity of the three tDCs was exhibited in allogeneic and autologous memory responses against soluble Ag, which suggests their potential use for a variety of conditions. Moreover, many of the tDC-stimulated T cells became anergic. Their hyporesponsiveness was Ag-specific, as responses to an unrelated Ag were not impaired, and based on an IL-2 deficiency. Despite the fact that all tDCs similarly stimulated memory lymphocytes at priming (Fig. 2A), tDC-TGFs induce a more robust anergic state (Fig. 4B), thus they must deliver unique anergic signals to T cells. Moreover, they generate T cells with regulatory capacities (Fig. 5).

Memory cTs and tTs were phenotypically very similar. Upon stimulation, they increased CD25, CTLA-4, CD39, and Foxp3 expression and downregulated IL-7Rα. The augment was less pronounced in tTs than in cTs, but we found a strong correlation between the percentage of cycling cells and the percentage of cells expressing these markers. Therefore, it is likely that the differences could lie in disparities in the proliferation rate. tDCs did not induce a preferential expansion of Foxp3+ cells compared with cDCs, corroborating that in humans, Foxp3 is not restricted to Treg cells, but is also expressed by activated lymphocytes that may not possess regulatory activity (26, 37). The observed phenotype was not stable, and, after a resting period, lymphocytes downregulated CD25 and CD39, whereas IL-7Rα was upregulated. The percentage of cells expressing CTLA-4 or Foxp3, two negative regulators of lymphocyte activation (27), remained relatively high in all conditions, suggesting that they are necessary for longer periods to return memory lymphocytes to quiescence. Of note, the expression of high levels of CTLA-4 seemed to be a signature of tT-TGFs, which could influence their hyporesponsiveness. Overall, the phenotype of cT and tT (CD25hiCD127hiCD39hiFoxp3int) differs from typical natural or adaptive Tregs (27, 29, 38). It has been established that TDCs generate classical Tregs (CD4+CD25Foxp3hi) from naive CD4+CD25− lymphocytes (14); however, our data suggest a distinctive scenario in Ag-driven memory responses, IFI lymphocytes showed low suppressive ability on memory and naive responses (only significant with tT-TGF, but at high IFI:Ti ratios). The low suppressive ability of memory tTs could be a general feature of these cells, and it has been reported by other authors (26). Furthermore, TTs secrete low but detectable levels of IL-2 and IFN-γ during activation, which may be explained in part by their lower proliferative capacity. However, a hallmark of TIs is their increased secretion of IL-10 (Figs. 3, 4C), which could control their inefficient proliferation and effector cytokine production (14, 17). It seems unlikely that the suppression observed in our system was mediated by classical CD4+CD25+ Treg lymphocytes activated or expanded by tDCs (see above). Given their characteristic high IL-10 secretion and their differentiation in a setting of IL-10–treated DCs and elevated IL-10, we postulate that TIs are closer to the adaptive type 1 Tregs (39).

In an attempt to identify molecules involved in tDC suppression, we found differential gene expression of several factors related with immune tolerance (TSP-1, CD39, ILT5, FTH1, FcγRIIB) (28, 29, 40, 41) and assessed some of them at the protein level. The three tDCs showed significant increased expression of TSP-1 and of CD39 only in tDC-TGF. Regarding CD39, we detected no statistically significant differences in adenosine release among cDCs and tDCs; however, tDC-TGFs have a propensity to generate more adenosine than the other DC populations (Fig. 6B). Notably, almost full abrogation of T cell hyporesponsiveness occurred in cultures of tDC-IL10 and tDC-IL6 after blockade of IL-10 or TSP-1, indicating that production of these factors is a mechanism responsible for the downregulation of T cell proliferation. Conversely, none of these inhibitors noticeably abrogate T cell hyporesponsiveness induced by tDC-TGF, which correlates with their low secretion of IL-10. Thus, we tested the influence of additional factors known to downregulate T cell responses, such as PGs, NO, and adenosine. Whereas NO has no involvement in tDC suppression, the presence of an inhibitor of cyclooxygenase activity fully recovers T cell proliferation with every tDC. Noteworthy is the fact that adenosine boosts the tolerogenic potential only of tDC-TGF. According to these data, we postulate that adenosine plays a central role in tDC-TGF tolerogenicity. This nucleoside could control directly on tDC-TGF or T cells (30, 42) and might also operate indirectly through PGE2 induction (43). In addition, PGE2 and IL-10 may be the primary mediators involved in tDC-IL10 and tDC-IL6–induced T cell hyporesponsiveness. Both PGE2 and IL-10 might in turn induce TSP-1 production, reinforcing tDC regulatory activity (44–46).
Because the molecules involved in T cell suppression are soluble factors, but their action seemed to be Ag-specific, we hypothesized that the anergy and/or the regulatory properties induced in T lymphocytes could be the result of a combination of these factors and specific signals received through the TCR. We describe in this paper for the first time the increase of surface CLIP bound to HLA-DR molecules in tDCs that depend on the presence of IL-10. This could represent a mechanism of tolerance induction due to a lower representation of the antigenic peptides on the cell surface (47), given that engagement of TCR below its threshold of activation induces tolerance in memory T cells (23). The reason why tDCs express increased surface CLIP is currently unknown. In APCs, the nonclassical class II molecule HLA-DM catalyzes CLIP dissociation from class II αβ dimers. Another nonclassical class II Ag, HLA-DO, downmodulates DM function. The amount of cell surface CLIP:HLA-DR seems to correlate with DO:DM ratio and the efficacy of Ag presentation (48). Although we observed an increase of HLA-DOB mRNA in the three tDCs, the presence of HLA-DOB in monocyte-derived DCs has not been demonstrated at the protein level (49); however, it was detected in primary DCs, including immature Langerhans cells, certain subtypes of blood DCs, and tonilar interrogating DCs (50). Thus, although the role of HLA-DOB in our system is unclear, the data provided here indicate that IL-10 is involved in the enhancement of surface CLIP and could represent a novel mechanism of tolerance induction by APCs.

In summary, IL-10 alone or in combination with TGF-β1 induces the generation of tolerogenic DCs and is able to induce an anergic state in CD4+ memory T cells. An altered pattern of immunosuppressive factor production (adenosine, PGs, IL-10, TSP-1, and potentially ILT5 or FTH1), and perhaps a defective presentation of exogenous soluble Ag, may all contribute to confer antigen-specific memory T lymphocytes.


