Induction of Regulatory T Cells and Dominant Tolerance by Dendritic Cells Incapable of Full Activation[1]

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Transplants tolerated through a process known as infectious tolerance evoke continuous recruitment of regulatory T (Treg) cells that are necessary to maintain the unresponsive state. This state is maintained long-term and requires continuous Ag exposure. It is not known, however, whether infectious tolerance operates through sustained recruitment of pre-existing regulatory cells, induction of regulatory cells, or both. Using mice deficient in natural Treg cells, we show here that quiescent donor dendritic cells (DC) laden with histocompatibility Ag can induce Treg cells de novo that mediate transplantation tolerance. In contrast, fully activated DC fail to do so. These findings suggest that DC incapable of delivering full activation signals to naive T cells may favor their polarization toward a regulatory phenotype. Furthermore, they suggest a role for quiescent endogenous DC in the maintenance of the tolerant state. ***The Journal of Immunology,*** **2007, 179: 967–976.**

In recent years, it has been possible to induce dominant and infectious tolerance to allogeneic transplants by short-term use of mAb blocking either T cell coreceptors or costimulatory molecules (1, 2). Such tolerance is mediated, in part, by Ag-specific $CD4^+$ regulatory T (Treg)[4] cells. In the steady state, Treg cells have specificity for fragments of donor Ags presented by host MHC molecules (3), suggesting that processing by host APC is required. However, within the tolerant host, such Ag-laden APC would be considered quiescent and ignorant of "danger" signals. This suggests that quiescent or resting APC have the intrinsic ability not only to induce tolerance, but to drive uncommitted T cells toward a regulatory function. This possibility has been highlighted by recent studies using A1.RAG-$1^{-/-}$ mice, a TCR-transgenic strain in which all $CD4^+$ T cells are specific for a defined peptide from the male minor histocompatibility Ag, Dby. Although these animals carry no natural Treg cells, "vaccination" with either the antigenic peptide (4) or an appropriate altered peptide ligand (APL) (5) results in tolerance through the induction of adaptive Treg cells. The assumption is that the relevant peptide is presented by quiescent, host APC. In this study, we directly test whether quiescent dendritic cells (DC) can achieve the same result by examining the outcome of vaccination with Ag-presenting DC, modulated to exhibit different degrees of activation. Bone marrow-derived DC (bmDC), activated through TLR4, were not competent to tolerize. By contrast, DC that had not been TLR activated induced both tolerance and regulation. Pharmacological modulation by 1α, 25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), an analog of vitamin D_3 known to constrain DC maturation (6), evoked dominant tolerance irrespective of any additional TLR stimulation. We conclude that DC that are unable to deliver full activation signals may, by default, induce tolerance, T cell unresponsiveness, and Treg cells.

Materials and Methods

Mice

CBA/Ca mice (H-2^k), C57BL/10 (H-2^b), CBA/Ca.RAG-$1^{-/-}$, and A1.RAG-$1^{-/-}$ TCR-transgenic mice (7) were bred and maintained under specific pathogen-free conditions at the Sir William Dunn School of Pathology (University of Oxford, Oxford, U.K.). A1.RAG-$1^{-/-}$ mice are transgenic for a TCR specific for the Dby epitope, REEALHQFRSGRKPI, in the context of H-$2E^k$. All experimental procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 and received local ethical committee approval.

Preparation of DC

Bone marrow cells from CBA/Ca or C57BL/10 mice were cultured in RPMI 1640 (Invitrogen Life Technologies) containing 10% (v/v) FCS, 50 μg/ml penicillin-streptomycin, 2 mM L-glutamine, and 50 μM 2-ME (R10 medium), supplemented with ~5 ng/ml murine GM-CSF (harvested from supernatant of an X63.10 cell line stably transfected with the murine *Gm-csf* gene). On days 3 and 6 of culture, nonadherent cells were removed, the remaining cells being cultured in fresh medium supplemented with GM-CSF and harvested for use on day 7. Where indicated, 1α,25$(OH)_2D_3$ (Sigma-Aldrich) was added to yield a final concentration of 10^{-7} M on days 3 and 6. LPS, derived from *Escherichia coli* serotype 0127:B8 (Sigma-Aldrich), was added at a final concentration of 1 μg/ml on day 6 to stimulate maturation during the final 18–20 h of culture.

mAbs and flow cytometry

For flow cytometric analysis of surface phenotype, cells were suspended in buffer (PBS/1% FCS/0.01% NaN_3) containing the appropriate mAb and incubated at 4°C for 30 min. The following mAb were used: MHC class II (clone Ox-6), CD86 (clone GL1), CD4 (clone H129.19), CD8 (clone 53-6.7), CD25 (clone 7D4), glucocorticoid-induced TNFR (GITR; clone YGITR 860), and CD103 (clone M290). Allophycocyanin-conjugated streptavidin was used as a secondary reagent. Cells were incubated with 10 μg/ml 7-aminoactinomycin D (7-AAD; Sigma-Aldrich) for 15 min before

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[4] Abbreviations used in this paper: Treg, regulatory T; APL, altered peptide ligand; DC, dendritic cell; bmDC, bone marrow-derived DC; 1α,25$(OH)_2D_3$, 1α,25 dihydroxyvitamin D_3; GITR, glucocorticoid-induced TNFR; 7-AAD, 7-aminoactinomycin D.

fixation to enable the exclusion of debris and nonviable cells. Four-color analysis was performed using a FACSCalibur (BD Biosciences) with CellQuest 3.1 software.

ELISA

Day 6 bmDC generated with or without $1\alpha,25(OH)_2D_3$ were plated in equal numbers (10^6/ml) in R10 medium supplemented with 20 ng/ml IFN-$\gamma \pm 1\alpha,25(OH)_2D_3 \pm 1$ μg/ml LPS. After incubation for 24 h, culture supernatants were assayed using an IL-12p70 Duoset ELISA kit (R&D Systems), calibrated against a standard dilution series of recombinant mouse IL-12 p70, according to the manufacturer's instructions. IL-10 secretion was assessed using a bead-based assay and analyzed on a Bio-Plex System machine using Bioplex Manager Software 4.0 (Bio-Rad).

T cell proliferation assay

To assess the capacity of modified male CBA/Ca DC to stimulate primary responses, nylon wool-purified splenocytes from naive, female A1.RAG-$1^{-/-}$ mice were cultured in triplicate with varying numbers of DC. To assess T cell responses from grafted A1.RAG-$1^{-/-}$ mice, nylon wool-purified splenocytes (5×10^4/well) were cocultured with mitomycin C-treated immature female CBA/Ca-derived bmDC (1×10^4/well) and Dby peptide at a final concentration of 1, 10, or 100 nM, as appropriate. Responder spleen cell suspensions were nylon wool-purified and depleted of RBC by Tris-buffered ammonium chloride lysis, washed, and cultured in 96-well round-bottom plates at 5×10^4 cells/well in R10 medium. Stimulators were prepared by treating bmDC with 10 μg/ml mitomycin C for 30 min at 37°C and were titrated into cocultures. After incubating for 72 h, cocultures were pulsed with 0.5 μCi/well [^{3}H]TdR (Amersham International) for a further 18–24 h. Incorporation was measured after 18–24 h using a flat-bed scintillation counter (LBK Wallac).

Tolerance induction and skin grafting

Age-matched female A1.RAG-$1^{-/-}$ mice were given a single i.v. injection of 2×10^6 bmDC in PBS. Control mice received PBS alone. Twenty eight days later, skin grafting was performed by transplanting full thickness tail skin (1 cm^2) from male CBA/Ca.RAG-$1^{-/-}$ mice onto the lateral flank, as previously described (8). Grafts were observed after the removal of the plaster cast at day 7 and considered rejected when no donor skin could be identified.

Isolation of cells from tolerated grafts

T cells were prepared from tolerated skin grafts by cutting them into small pieces, digesting with 0.125% (v/v) trypsin at 37°C for 1 h, and removing dead cells by passage over nylon wool. Viable lymphocytes were enriched by Histopaque-1083 centrifugation (Sigma-Aldrich). Total cell infiltrates, consisting of $\sim 5 \times 10^3$ CD4$^+$ T cells per graft, were characterized either by real-time PCR or flow cytometry.

Real-time quantitative PCR

DNase-I-treated total RNA was prepared from tissues using the SV Total RNA isolation system (Promega). Reverse transcription was performed using the Prostar First Strand Synthesis kit (Stratagene), primed with random hexamers. Multiplex PCR was performed using gene-specific primers, fluorogenic probes, and the Universal MasterMix kit (Applied Biosystems) in triplicate with primers at a concentration of 300 nM and the probe at 200 nM. A two-step PCR procedure of 15 s at 95°C and 60 s at 60°C was applied for 50 cycles. PCR and TaqMan analysis was performed using the ABI/PRISM 7700 Sequence Detector System (Applied Biosystems). Standard curves of appropriate cDNAs were used to calibrate threshold cycles to amounts of test and normalizing cDNAs on each 96-well sample plate. Normalized values for mRNA expression were calculated as test mean/normalizer mean. Forward and reverse primer sequences and TaqMan probes (Eurogentec) were as follows: foxp3, forward: 5′-CCCAGGAAA GACAGCAACCTT-3′, reverse: 5′-TTCTCACAACCAGGCCACTTG-3′; TaqMan probe: FAM-5′-CTACCCACTGCTGGCAAATGGAGTC-3′-TAMRA; CD3γ, forward: 5′-TTACAGAATGTGTGAAAACTGCATTG-3′, reverse: 5′-CACCAAGAGCAAGGAAGAAGATG-3′; TaqMan probe: VIC-5′-ACATAGGCACCATATCCGGCTTTTCTTCG-3′-TAMRA.

Cell separation

Separation of CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells was performed similar to the method described in Graca et al. (9). Briefly, to obtain a population of splenic CD4$^+$ cells, splenocytes were incubated with the MACS CD4$^+$ T cell isolation kit biotin-Ab mixture (Miltenyi Biotec) according to the manufacturer's instructions, and positively selected over two magnetic columns using the "POSSELD" program of AutoMACS (Miltenyi Biotec). CD4$^+$CD25$^-$ cells were sorted from the CD4$^+$CD25$^+$ fraction by incubation with biotinylated anti-CD25 Ab (clone 7D4; BD Pharmingen) for 20 min at 4°C. After washing, the cells were incubated with streptavidin microbeads (Miltenyi Biotec) and were selected using the AutoMACS POSSEL program, according to the manufacturer's instructions. Typically, the purity of CD4$^+$CD25$^-$ T cells exceeded 90% of total CD4$^+$ T cells. The purity of the CD4$^+$CD25$^+$ T cells routinely exceeded 80% of total CD4$^+$ T cells.

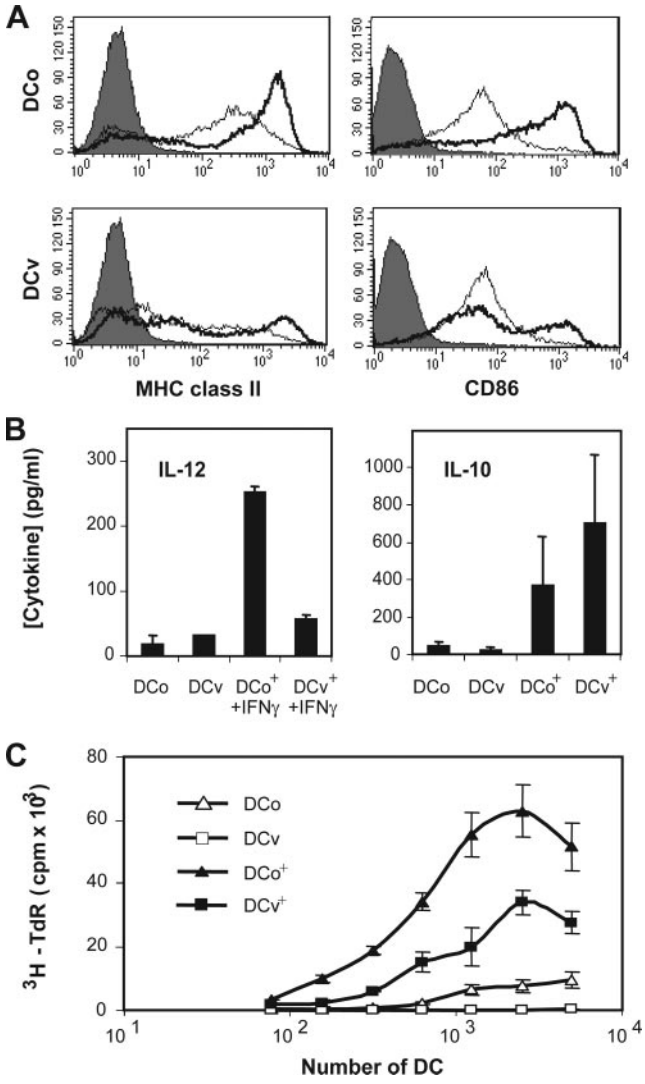

FIGURE 1. Phenotype of DC populations. *A*, DC from day 7 bone marrow cultures with (thick line) or without (thin line) exposure to LPS stained with mAb specific for MHC class II and CD86. The shaded histograms represent the level of background staining obtained using isotype-matched negative control mAb. *B*, Levels of IL-12p70 and IL-10 in the supernatants of untreated (DCo) and $1\alpha,25(OH)_2D_3$-modulated DC (DCv) in the absence or presence of LPS (DCo$^+$ and DCv$^+$), measured by ELISA. Values represent means $\pm$ SD of triplicate cultures. *C*, Proliferative response of female A1.RAG-$1^{-/-}$ T cells to varying numbers of male CBA/Ca-derived DC. Values represent means $\pm$ SD of triplicate cultures.

Microarray analysis

Custom immune system-directed microarrays were prepared using 70-mer probes specific for 768 genes. Each probe was printed with four nonadjacent replicates onto Genetix amine slides with a QArray mini microarray printer using 150-μm aQu tungsten pins. A full description of the microarray is available from ArrayExpress (accession number: A-MEXP-239). The integrity of RNA was validated using a 2100 BioAnalyser (Agilent). Total RNA was reverse transcribed using Superscript III and was otherwise

Table I. *Differential gene expression between untreated and 1α,25(OH)$_2$D$_3$-modulated DC populations*[a]

Table Section	DCo	DCv	DCo$^+$	DCv$^+$	Gene Description
Section I					LPS responsive genes induced in mature, immunogenic DCs
	1	0.17	7.43	1	*CCL22*
	1	0.23	5.18	1	*CCR7*
	1	0.28	4.62	1	*IL-1β*
	1	0.37	4.39	1	*CD83*
	1	0.26	4.23	1	*DCIP-1*
	1	0.18	3.76	1	*MIP2*
	1	0.38	3.22	1	*STAT4*
	1	1	3.15	1	*Cyclin D2*
	0.21	1	2.89	1	*IL-6*
	1	1	2.84	1	*MARCO*
	1	0.3	2.83	1	*CXCL16*
	1	0.41	2.73	0.56	*DNA-binding protein inhibitor ID-2*
	1	0.44	2.64	1	*Fructose-2,6-biphosphatase 3*
	0.21	1	2.48	1	*IL-12p40*
	1	1	2.46	1	*Cyclin-dependent kinase 4 inhibitor B (p14-INK4b)*
	1	0.52	2.45	1	*IL-27*
	1	0.48	2.42	1	*NKG2-D type II integral membrane protein*
	1	0.41	2.42	1	*CXCL1*
	1	1	2.41	1	*TNFRSF9 (4-1BB)*
	1	0.44	2.38	1	*PDL1*
	1	0.2	2.32	1	*FMLP-related receptor I (lipoxin A4 receptor)*
	1	0.49	2.3	1	*2′-5′-oligoadenylate synthetase 1A*
	0.19	1	2.24	1	*Interferon regulatory factor 7 (IRF-7)*
	1	0.49	2.19	1	*H-2D^B*
					LPS responsive genes depressed in mature, immunogenic DCs
	1	1	0.15	1	*Integrin β-7 precursor*
Section II					Genes increased in response to modulation
	0.27	6.56	0.14	4.16	*PDGF-1*
	0.28	6.22	1	3.13	*TGF-β3*
					Genes decreased in response to modulation
	4.16	0.06	4.71	1	*TARC*
Section III					Genes increased by LPS in 1α,25(OH)$_2$D$_3$ modulated DC
	1	1	0.06	15.24	*Matrix metalloproteinase-13*
	1	1	1	3.64	*TNFRSF23 (decoy TRAIL receptor 1)*
	0.22	1	1	3.2	*Arginase 1*
	1	1	0.08	3.16	*Matrix metalloproteinase-8*
	1	1	1	3.03	*Heat-shock protein 105 kDa*
	1	1	1	2.93	*Tranglutaminase 2*
					Genes decreased by LPS in 1α,25(OH)$_2$D$_3$ modulated DC
	1	1	1	0.4	*Lysozyme C*
	1	1	1	0.41	*Elongation factor 1-α1 (EF-1-α-1)*
	1	1	1	0.42	*IFN-γ-inducible protein 31*
Section IV					LPS responsive genes induced in mature and modulated DCs
	1	0.08	12.93	3.48	*CCL5*
	0.27	0.16	6.53	3.58	*Serum amyloid A-3 protein precursor*
	0.18	0.36	4.25	3.66	*iNOS*
	1	0.36	2.68	3.09	*Endothelial protein C receptor precursor*
	1	0.24	3.06	3.67	*Lymphocyte antigen Ly-6A.2/Ly-6E.1*
Section V					Genes increased in DCs in response to both LPS and modulation
	0.24	1	1	1	*HO-1*
	0.28	1	1	1	*OX-2*
					Genes decreased in DCs in response to both LPS and to modulation
	19.42	0.16	1	1	*ATP-sensitive inward rectifier potassium channel 10*
	7.61	1	1	1	*CCR2 (MCP-1 receptor)*
	7.58	1	1	0.15	*Calgranulin B*
	5.16	1	1	1	*CD209 (DC-SIGN)*
	5.13	1	0.14	1	*Osteopontin (secreted phosphoprotein 1) (SPP-1)*
	4.86	1	1	0.5	*TNFSF3 (lymphotoxin-beta)*
	4.85	1	1	0.25	*Calgranulin A*
	4.28	1	1	0.33	*Neutrophilic granule protein*
	4.23	1	1	0.51	*PIL type 2 receptor α (FDF03) inhibitory*
	4.21	0.58	1	0.57	*PIL type 2 receptor β (FDFACT) activatory*
	4.17	1	1	0.55	*Placental calcium-binding protein*
	3.61	1	1	1	*Glandular kallikrein K8 precursor*
	3.46	1	1	1	*Early growth response protein 1 (EGR-1)*
	3.42	1	1	0.46	*Allograft inflammatory factor 1*
	3.21	1	1	1	*Integrin α-E precursor (integrin α M290)*
	3.13	1	1	1	*Early growth response protein 2 (EGR-2)*
	3.12	1	1	1	*Macrophage scavenger receptor 2*
	3.11	1	0.14	1	*γ-Parvin*
	2.73	1	1	0.43	*5-Lipoxygenase activating protein (FLAP)*

(*Table continues*)

Table I. (*Continued*)

Table Section	DCo	DCv	DCo$^+$	DCv$^+$	Gene Description
	2.62	0.59	1	0.6	*H-2, A-B α chain*
	2.56	1	1	1	*CD11b (CR3)*
	2.54	1	1	0.66	*Properdin (factor P)*
	2.4	1	1	1	*C-type lectin, member 10*
	2.39	1	1	0.63	*Proto-oncogene tyrosine-protein kinase FGR*
	2.38	1	1	0.53	*DNAX-activation protein 12*
	2.35	1	1	1	*Glucosaminyl N-acetyl transferase 1*
	2.29	1	1	1	*Vitamin K-dependent protein S precursor*
	2.26	1	1	1	*Transferrin receptor protein 1 (TfR1)*
	2.23	1	1	1	*Transmembrane 6 superfamily member 1*
	2.19	1	1	0.57	*Mitotic spindle checkpoint protein MAD2A*
	2.19	1	1	0.49	*LR8 protein*
	2.17	1	1	0.61	*Annexin A1 (annexin I)*
	2.16	1	1	1	*Cyclin D3*
	2.1	1	1	1	*Ganglioside GM2 activator precursor (GM2-AP)*

[a] Numbers indicate the fold-change in gene expression relative to the mean expression of a particular gene across all populations. Numbers above 1.0 indicate an up-regulation and numbers below 1.0 a down-regulation of gene expression. Values within two SD of the mean were not considered significant and are represented by a fold change of 1.0. Genes have been grouped as: section I, genes exhibiting at least a 2-fold change from the overall mean in response to LPS in mature immunogenic, but not 1α,25(OH)$_2$D$_3$-modulated DC; section II, genes altered only in 1α,25(OH)$_2$D$_3$-modulated DC; section III, genes LPS responsive only in 1α,25(OH)$_2$D$_3$-modulated DC; section IV, genes altered in response to LPS in mature and modulated DC; section V, genes altered in response to 1α,25(OH)$_2$D$_3$ modulation that are in common with LPS responsive genes in mature immunogenic DC.

processed and labeled as directed using the Cy3 and Cy5 3DNA 900 Dendrimer Labeling kit (Genisphere) and a two-step hybridization protocol with on-slide mixing on a Slide Booster (Advalytix). Arrays were scanned at 5-μm resolution using autocalibration for a peak target saturation of 95% using a ScanArray Express HT scanner (PerkinElmer). Image analysis was performed using BlueFuse version 2 (BlueGnome) and, following manual exclusion of artifacts, replicate data were combined using the fusion algorithm. Further analysis of the data was performed in Microsoft Excel to correct for channel overlap and globally normalize expression signals. Microarray data are available at the EMBL-EBI ArrayExpress database (accession number: E-MEXP-506).

Statistical analysis

Statistical analysis of graft survival was assessed by means of the log-rank method. Differences in median graft survival were considered significant for $p < 0.05$ (one-tailed test). For microarray analysis, fold changes were considered significant if they exceeded 2-fold and were >2.0 SD from the geometric mean (geometric mean fold changes were 0.77, 1.76, 0.52, and 1.42 for DCo, DCv, DCo$^+$, and DCv$^+$, respectively; $n = 766$).

Results

Phenotypic and functional characterization of DC populations

We wished to determine whether DC incapacitated for full activation are able to induce tolerance and regulation in vivo. We used bmDC as a source of immature DC (DCo), and cells treated with 1 μg/ml LPS for the final 18 h of culture, as a source of fully activated DC (DCo$^+$) with the ability to present peptide to naive T cells in an optimal fashion. Because immature DC might, in theory, mature when injected into recipients, we also preincubated these cells with 1α,25(OH)$_2$D$_3$, known to impair their full maturation. Immunostaining for MHC class II and costimulatory molecules, in vitro functional assays, and transcriptional gene profiling were used to validate the effects of LPS and the pharmacological efficacy of 1α,25(OH)$_2$D$_3$ treatment.

Consistent with previous findings (6, 10, 11), the surface expression of MHC class II was significantly reduced in cultures supplemented with 10^{-7} M 1α,25(OH)$_2$D$_3$ (DCv) compared with untreated controls (DCo) (Fig. 1*A*). Whereas DCo$^+$ were able to up-regulate MHC class II and CD86 upon TLR4 stimulation with LPS, DCv$^+$ were substantially impaired in this respect (Fig. 1*A*). The restraining effect of 1α,25(OH)$_2$D$_3$ was further evident from the inability of DCv to secrete significant levels of IL-12p70 in response to a combination of LPS and IFN-γ, compared with control DC, cultured without further stimulation (Fig. 1*B*). In contrast, DCv$^+$ were found to secrete elevated levels of IL-10 compared with other populations, as previously documented (11) (Fig. 1*B*). These phenotypic and functional changes were accompanied by the reduced capacity of 1α,25(OH)$_2$D$_3$-treated male CBA/Ca DC to stimulate proliferation of naive female A1.RAG-1$^{-/-}$ T cells when compared with untreated controls (Fig. 1*C*). Indeed, DCv were unable to elicit any detectable proliferative response among naive T cells, confirming previous reports of the immunomodulatory effects of vitamin D$_3$ and its analogs (6, 10, 11) and drawing close parallels with DC modulated by exposure to IL-10 and TGF-β (A. Paterson, C. Farquhar, S. Cobbold, H. Garcia Rueda, S. Yates, P. Fairchild, N. Saunders, H. Waldmann, and K. Nolan, manuscript in preparation) (12).

Validation of DC populations by gene expression profiling

Differences in the LPS responsiveness of untreated DC and DC modulated with 1α,25(OH)$_2$D$_3$ were reflected in their patterns of gene expression, as evidenced by microarray. Total RNA from DCo, DCv, DCo$^+$, and DCv$^+$ populations was used to analyze transcriptional changes in 786 immunologically relevant genes represented on a custom-designed oligoprobe microarray. Data are presented in Table I as fold changes from the overall mean of the four populations. In keeping with the differential response to LPS observed by flow cytometry, 24 of the 786 genes exhibited at least a 2-fold increase from the geometric mean only among DC treated with LPS in the absence of exposure to 1α,25(OH)$_2$D$_3$ (Table I, section I). These included CCR7, known to facilitate full maturation and homing of licensed DC (13), and the chemokines CCL22 and CXCL16, which attract recently activated and memory T cells. Other examples included a number of classic proinflammatory genes, such as those encoding the chemokines DCIP-1, CXCL1, and the cytokine, IL-1, the LPS responsiveness of which was retained in IL-10-modulated DC that have been implicated in tolerance induction (14). MHC class II, CD83, 4-1BB, MARCO, PD-L1, and CD40 are all surface molecules well-documented to increase following TLR-induced DC maturation. Nevertheless, these were not up-regulated following pretreatment with 1α,25(OH)$_2$D$_3$; other genes implicated in Th1 induction, such as STAT4 (15), IL-12p40 (16, 17), and IL-27 (18, 19) were likewise not up-regulated, as were IRF-5 and -7, important in MyD88-dependent TLR signaling (20, 21).

Twelve gene changes were observed that were unique to the $1\alpha,25(OH)_2D_3$-modulated DC (Tables I, sections II and III), confirming the biological activity of the reagent used and its impact on gene expression. Interestingly, only 1 of the 768 genes analyzed, that encoding the chemokine TARC (CCL17), was specifically down-regulated in response to treatment with $1\alpha,25(OH)_2D_3$ (Table I, section II), consistent with previous observations of the effect of this agent on human myeloid DC (22). Although $1\alpha,25(OH)_2D_3$ modulation prevented many transcriptional changes classically associated with the maturation of DC following exposure to TLR agonists, it did not fully inhibit all responsiveness, suggesting that exposure to $1\alpha,25(OH)_2D_3$ does not simply maintain DC in an immature state. Instead, $1\alpha,25(OH)_2D_3$ altered the nature of the response to LPS, at least part of the normal transcription program invoked by LPS being retained (Table I, section IV). Interestingly, 36 of the 786 genes represented on the microarray that varied in response to $1\alpha,25(OH)_2D_3$ alone also overlapped with the normal LPS response (Table I, section V), findings consistent with our previous observations of DC modulated with IL-10 (14).

Both immature and pharmacologically modified DC induce transplantation tolerance

We investigated the tolerogenic potential of the above populations of DC by assessing their capacity to modulate the rejection of male CBA.RAG-$1^{-/-}$ skin grafts by female A1.RAG-$1^{-/-}$ mice. These mice contain neither B cells nor $CD8^+$ T cells and, like most other RAG-$1^{-/-}$ mice, lack any naturally occurring $CD4^+CD25^+$ $foxp3^+$ Treg cells. All female A1.RAG-$1^{-/-}$ control mice treated with PBS alone ($n = 16$) rejected their male grafts with a median survival time of 14 days (Fig. 2*A*). Preliminary studies indicated that as few as 2×10^6 immature male DC per mouse were capable of achieving durable engraftment of male skin, transplanted 28 days later. Using this protocol, 100% of mice treated with either of the immature populations of DC (DCo or DCv) accepted their grafts indefinitely ($n = 19$ in each case). In contrast, mice that had received LPS-matured DC (DCo^+) ($n = 16$) did not become tolerant but rejected male skin grafts with a median survival time of 13 days. Remarkably, DC that had been cultured with $1\alpha,25(OH)_2D_3$ before LPS exposure (DCv^+) induced 100% acceptance of male skin grafts ($n = 15$). By comparison, all control mice receiving treated populations of syngeneic DC derived from female CBA/Ca donors rejected their grafts (Fig. 2*B*), indicating the Ag-dependent nature of tolerance induction. In all cases, mice that accepted their skin grafts, also accepted a second graft 100 days later, confirming that a form of tolerance had been established that persisted long after the demise of the DC responsible for its induction.

Transplantation tolerance induced by DC is dominant

One of the cardinal features of dominant tolerance mediated by Treg cells is resistance to the rejection of established or fresh grafts by newly infused immunocompetent lymphocytes. To investigate the nature of the tolerant state induced by DC, 10^7 splenocytes from naive female A1.RAG-$1^{-/-}$ mice were administered to mice that had accepted male skin grafts. All mice previously rendered tolerant still retained both of their existing grafts and accepted fresh skin grafts indefinitely. In contrast, all control female CBA/Ca.RAG-$1^{-/-}$ mice, receiving the same number of naive splenocytes, rapidly rejected male skin grafts ($n = 5$) (Fig. 2*C*).

Tolerance induced by DC does not require reprocessing of donor Ags

The induction of transplantation tolerance by donor-specific transfusion of spleen cells has been shown to be mediated through the reprocessing of donor Ag by host DC (23), as has that mediated by uptake of apoptotic cells (24, 25). To investigate the possibility of host reprocessing in our studies, we examined the tolerizing

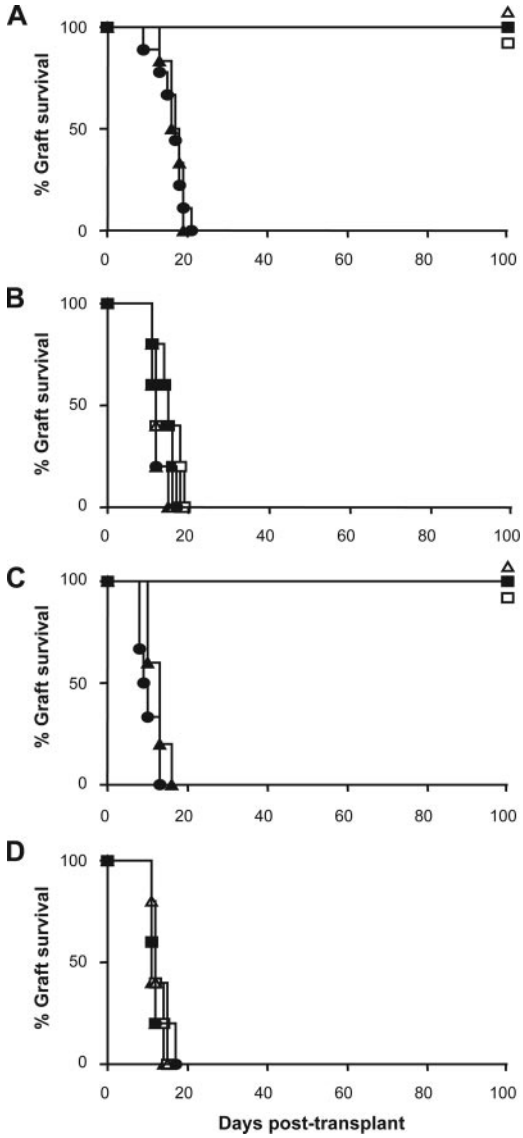

FIGURE 2. Induction of tolerance to skin grafts by prior administration of immature and $1\alpha,25(OH)_2D_3$-modulated DC. *A*, Female A1.RAG-$1^{-/-}$ mice received one dose of either PBS alone (●) ($n = 16$) or 2×10^6 male CBA/Ca DC (△, DCo ($n = 19$); □, DCv ($n = 19$); ▲, DCo^+ ($n = 16$); ■, DCv^+ ($n = 15$)). After 28 days, recipient mice were grafted with male CBA/Ca.RAG-$1^{-/-}$ skin grafts and were monitored for a further 100 days. *B*, Tolerance induction is Ag specific. Female A1.RAG-$1^{-/-}$ mice were treated with one dose of PBS alone (●) ($n = 5$) or 2×10^6 female CBA/Ca DC (△, DCo,; □, DCv; ▲, DCo^+; ■, DCv^+) and were grafted 28 days later with male CBA/Ca.RAG-$1^{-/-}$ skin (for all groups $n = 5$). *C*, The form of tolerance induced by DC is dominant. After 100 days, each group of female A1.RAG-$1^{-/-}$ mice which had received male CBA/Ca DC was infused with 10^7 naive A1.RAG-$1^{-/-}$ splenocytes and a fresh male CBA/Ca.RAG-$1^{-/-}$ skin graft was applied a day later (△, DCo ($n = 6$); □, DCv ($n = 6$); ▲, DCo^+ ($n = 5$); ■, DCv^+ ($n = 5$)). Positive rejection controls consisted of age-matched female CBA/Ca.RAG-$1^{-/-}$ mice infused with 10^7 naive A1.RAG-$1^{-/-}$ splenocytes and given a fresh male CBA/Ca.RAG-$1^{-/-}$ skin graft a day later (●) ($n = 6$). *D*, Tolerance induction does not require processing of Dby by host DC. Female A1.RAG-$1^{-/-}$ mice were treated with one dose of PBS alone (●) ($n = 5$) or 2×10^6 male C57BL/10 DC (△, DCo; □, DCv; ▲, DCo^+; ■, DCv^+). Mice were grafted 28 days later with male CBA/Ca.RAG-$1^{-/-}$ skin ($n = 5$ for all groups).

Table II. *Comparison of numbers of CD4$^+$ T cells in the spleen and thymus of PBS- or DC-treated female A1.RAG-1$^{-/-}$ mice at 100 days postgrafting*[a]

Treatment	PBS	DCo	DCv	DCo$^+$	DCv$^+$
Spleen					
Total splenocytes ($\times 10^7$)	1.28 ± 0.29	2.05 ± 0.35	3.58 ± 0.07	1.56 ± 0.25	3.29 ± 0.30
% CD4$^+$ T cells	13.62 ± 1.49	21.65 ± 3.46	11.83 ± 1.66	15.25 ± 6.72	12.89 ± 3.64
Absolute CD4$^+$ T cells ($\times 10^6$)	1.74 ± 0.24	4.38 ± 0.06	4.23 ± 0.51	2.29 ± 0.67	4.29 ± 1.59
% CD4$^+$CD25$^+$ T cells	0.62 ± 0.40	2.72 ± 0.54	1.75 ± 0.35	0.64 ± 0.16	2.66 ± 0.80
Absolute CD4$^+$CD25$^+$ T cells ($\times 10^5$)	0.80 ± 0.53	5.66 ± 2.08	6.28 ± 1.39	0.98 ± 0.40	8.84 ± 3.43
% of CD4$^+$ cells expressing CD25	4.6	12.9	14.8	4.3	20.6
Thymus					
Total thymocytes ($\times 10^7$)	10.40 ± 2.05	8.10 ± 1.27	9.15 ± 1.91	9.45 ± 0.78	9.00 ± 0.71
% of CD4$^+$CD8$^-$ thymocytes	19.84 ± 1.22	21.83 ± 4.48	20.56 ± 3.46	20.38 ± 1.15	18.10 ± 1.68
Absolute CD4$^+$CD8$^-$ thymocytes ($\times 10^7$)	2.04 ± 0.28	1.80 ± 0.64	1.85 ± 0.76	1.92 ± 0.05	1.62 ± 0.03

[a] Total viable splenocytes were enumerated by trypan blue exclusion and percentages were obtained by FACS staining using mAb specific for CD4 and CD25 (means ± SD of three samples).

potential of male DC from C57BL/10 mice. Such DC express the H-2^b haplotype and are, therefore, unable to present the Dby epitope directly to TCR-transgenic, H-2E^k-restricted A1.RAG-1$^{-/-}$ T cells. Recipient A1.RAG-1$^{-/-}$ mice remained unchanged in their ability to reject male CBA/Ca.RAG-1$^{-/-}$ skin grafts following infusion of male C57BL/10 DC, whether or not they had been pharmacologically conditioned with $1\alpha,25(OH)_2D_3$. These results confirm the need for cognate interactions between administered DC and alloreactive T cells and exclude the possibility that administered DC serve solely as a source of the Dby Ag, presented to naive T cells by endogenous DC (Fig. 2*D*).

De novo generation of CD4$^+$CD25$^+$ cells in the periphery of tolerant mice

CD4$^+$ T cells in the spleens of tolerant mice remained numerically unchanged when compared with naive, age-matched mice, indicating that tolerance was not accompanied by the gross deletion of T cells (Table II). In fact, in tolerized mice, the total number of splenocytes ranged from 2.05×10^7 to 3.58×10^7, which was barely different from controls.

The A1.RAG-1$^{-/-}$ strain cannot produce natural CD4$^+$CD25$^+$foxp3$^+$ Treg cells centrally within the thymus. Any T cells expressing this phenotype that arise following administration of DC must, therefore, have been induced in the periphery de novo. In all tolerant mice, irrespective of the DC population administered, the absolute number of CD4$^+$CD25$^+$ cells was significantly increased, ranging from 12.9 to 20.6% of the total CD4$^+$ population, compared with 4.6% among PBS-treated control mice and 4.3% among mice which had received DCo$^+$ (Table II). Quantitative real-time PCR analysis of foxp3 mRNA expression by splenocytes from tolerant, rejecting, and control mice, normalized to CD3γ to correct for T cell content, showed elevated levels in tolerant, compared with control or rejecting, splenocytes (Fig. 3*A*). In subsequent studies, mRNA levels have been shown to correlate precisely with expression of the foxp3 protein, as determined by flow cytometry, suggesting a positive correlation between the appearance of a CD4$^+$CD25$^+$foxp3$^+$ population of Treg cells and the induction of dominant tolerance in our model (26, 27). These conclusions are further supported by flow cytometric analysis of the CD4$^+$CD25$^+$ population from tolerant mice which showed increased expression of GITR and CD103 compared with CD4$^+$CD25$^-$ splenocytes (Fig. 3*B*), consistent with a regulatory phenotype (28–30). Importantly, these T cells must have been derived de novo from the naive CD4$^+$CD25$^-$GITR$^-$CD103$^-$foxp3$^-$ population of recipient T cells, because all skin grafts were derived from T cell-deficient CBA.RAG-1$^{-/-}$ donors. The above findings are in marked contrast to the thymus where we observed no increase in either total thymocyte numbers or CD4$^+$CD25$^+$ cells, indicating no effect on central tolerance processes (Table II).

CD4$^+$CD25$^+$foxp3$^+$ cells infiltrate tolerated skin grafts

Previous data from our laboratory have documented how CD4$^+$CD25$^+$foxp3$^+$ Treg cells may be isolated from skin grafts accepted through dominant tolerance (5, 31). Consistent with this finding, we observed an increase in relative *foxp3* mRNA expression among graft-infiltrating cells from tolerant mice (Fig. 4*A*), while analysis of cells infiltrating the grafts of mice tolerized with either DCo or DCv revealed the appearance of CD25$^+$GITR$^+$ cells which represented ~30–40% of CD4$^+$ cells (Fig. 4*B*).

Both CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells isolated from tolerant mice are hyporesponsive to Ag stimulation

On a functional level, Treg cells can inhibit the proliferation of the whole T cell population in response to cognate Ag (32). To assess the impact on proliferation of CD4$^+$CD25$^+$ T cells induced by DC administration, splenocytes from tolerant, rejecting, and control mice were cocultured with bmDC derived from female CBA/Ca mice pulsed with various concentrations of the H-2E^k-restricted Dby peptide (1–100 nM). The splenocytes from tolerant groups appeared profoundly inhibited in their proliferative response to Ag compared with control mice (Fig. 5*A*).

To clarify which subpopulation of T cells expresses regulatory activity, CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were purified from tolerant mice by magnetic separation. Both populations were stimulated with mitomycin C-treated female CBA/Ca DC, pulsed with 100 nM Dby peptide. Although CD4$^+$CD25$^+$ T cells were refractory to Ag stimulation, removal of the CD4$^+$CD25$^+$ cells from the CD4$^+$ population relieved suppression, albeit only partially (Fig. 5*B*). The proliferative response of the CD4$^+$CD25$^-$ population was ~20–50% of that observed among naive T cells, suggesting that administration of DC may induce some level of anergy among Ag-specific T cells, as well as the de novo induction of Treg cells.

Hyporesponsiveness to Ag is TGF-β dependent

Finally, we assessed the extent to which certain regulatory cytokines contribute to maintenance of the hyporesponsive state, by using mAb specific for the IL-10R (clone 1B1.2) and TGF-β (clone 11D11.16.8). Both IL-10 and TGF-β have, in some instances, been implicated in the activity of CD4$^+$CD25$^+$ Treg cells (33, 34). Addition of anti-TGF-β, but not anti-IL-10R, to a Dby

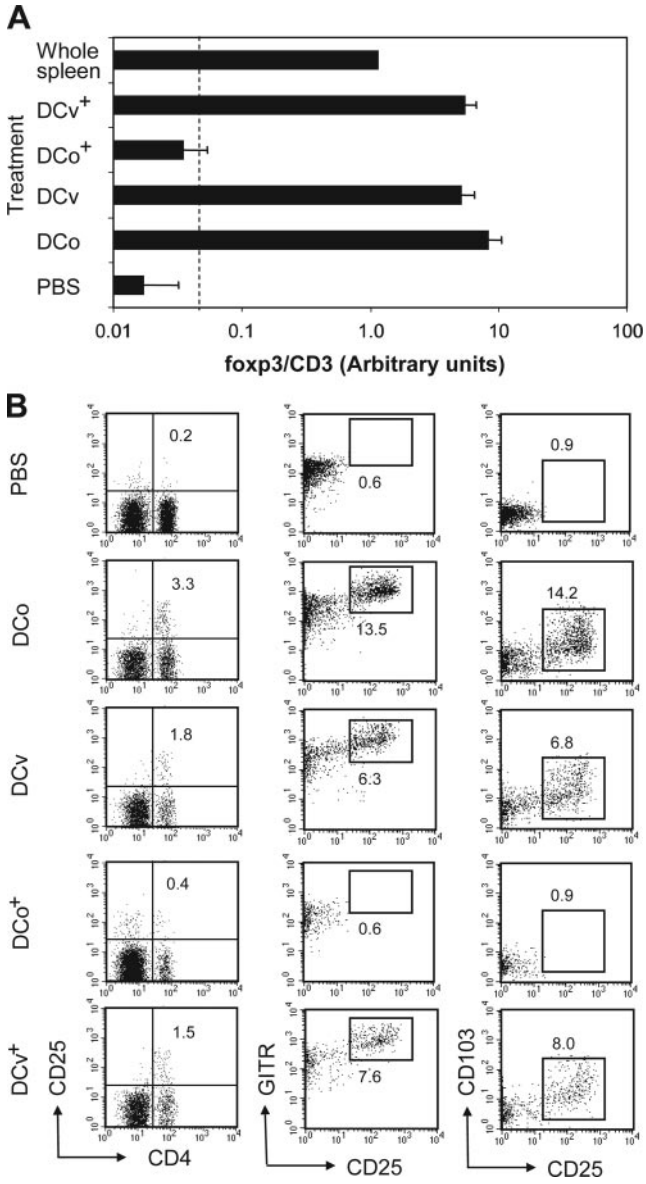

FIGURE 3. Characterization of splenocytes from tolerant, rejecting, or control mice 100 days after grafting. *A*, Expression of *foxp3* transcripts in splenocytes from tolerant mice compared with splenocytes from mice actively rejecting their grafts. Real-time quantitative PCR analysis of *foxp3* mRNA expression, normalized to levels of CD3γ expression. A value of 1.0 represents the relative expression from a population of total CBA/Ca splenocytes (of which 10% are $CD4^+CD25^+$ natural regulatory cells). *B*, Phenotypic analysis of $CD4^+$ splenocytes from tolerant compared with rejecting mice. Dot plots show staining for CD4 and CD25 (numbers indicate percentages of $CD4^+CD25^+$ cells). Cells gated according to CD4 expression were analyzed for expression of GITR and CD103 which is plotted against CD25.

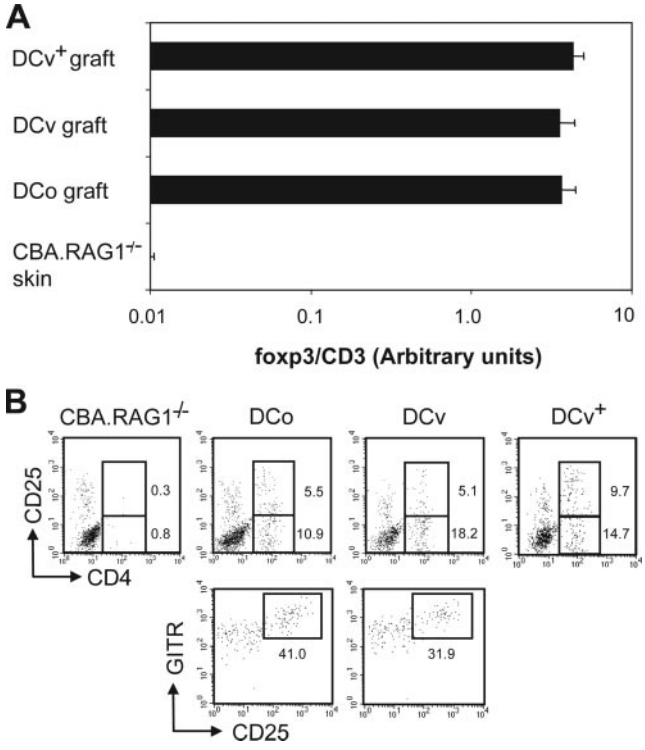

FIGURE 4. Characterization of cells infiltrating tolerated grafts 100 days after grafting. *A*, Expression of *foxp3* transcripts by lymphocytes extracted from tolerated grafts compared with control CBA.RAG-$1^{-/-}$ skin. Real-time quantitative PCR analysis of *foxp3* mRNA expression was normalized to levels of CD3γ expression. A value of 1.0 represents the relative expression from a population of total CBA/Ca splenocytes (of which 10% are $CD4^+CD25^+$ natural regulatory T cells). *B*, Appearance of $CD4^+$ $CD25^+GITR^+$ cells within the infiltrates of tolerated grafts. Total cell infiltrates were gated on forward scatter vs side scatter and dead cells excluded by their uptake of 7-AAD. The yield was $\sim 5 \times 10^3$ $CD4^+$ cells/graft. Flow cytometric dot plots are shown for CD4 and CD25 staining, the numbers representing the percentages of $CD25^+$ and $CD25^-$ cells. $CD4^+$ cells isolated from the grafts of mice tolerized with DCo or DCv were also gated for analysis of GITR and CD25.

specific in vitro proliferation assay significantly reversed the suppression (Fig. 5*C*), indicating a role for TGF-β in maintaining the hyporesponsive state, perhaps by increasing the activation threshold of TCR triggering (35).

Discussion

Since our first description of dominant and infectious tolerance in 1993 (36), we have sought to explain how the tolerated graft is able to induce and sustain Treg cells throughout its tenure. Treg cells were induced in the face of saturating levels of blockading CD4 mAb and the form of Ag to sustain this regulation could only have been presented by APC which had experienced neither danger nor maturation signals. The recent finding that sustained adjuvant-free Ag (4) or danger-free APL (5) can induce $foxp3^+$ Treg cells de novo, led us to propose that tolerated grafts provide a continuous source of danger-free Ag which, when processed by APC, is capable of delivering persistent, partial signals to T cells, so as to induce tolerance, anergy, and regulation, as well as providing sufficient signals to sustain active Treg cells. The experiments described here represent a direct test of this hypothesis by examining whether DC, incapacitated for their ability to activate naive T cells, are able to induce tolerance by driving regulation.

We used a TCR-transgenic mouse model as a platform to focus on the mechanisms underlying peripheral tolerance. Intravenous administration of 2×10^6 $1\alpha,25(OH)_2D_3$-conditioned male DC into female A1.RAG-$1^{-/-}$ mice resulted in dominant tolerance among peripheral T cells, as evidenced both by acceptance of male skin grafts and the failure of nontolerant, naive splenocytes to abrogate the tolerant state upon adoptive transfer. Tolerance arising from DC administration was associated with the appearance of a population of profoundly hyporesponsive Treg cells in the spleen and tolerated graft, but not within the host thymus.

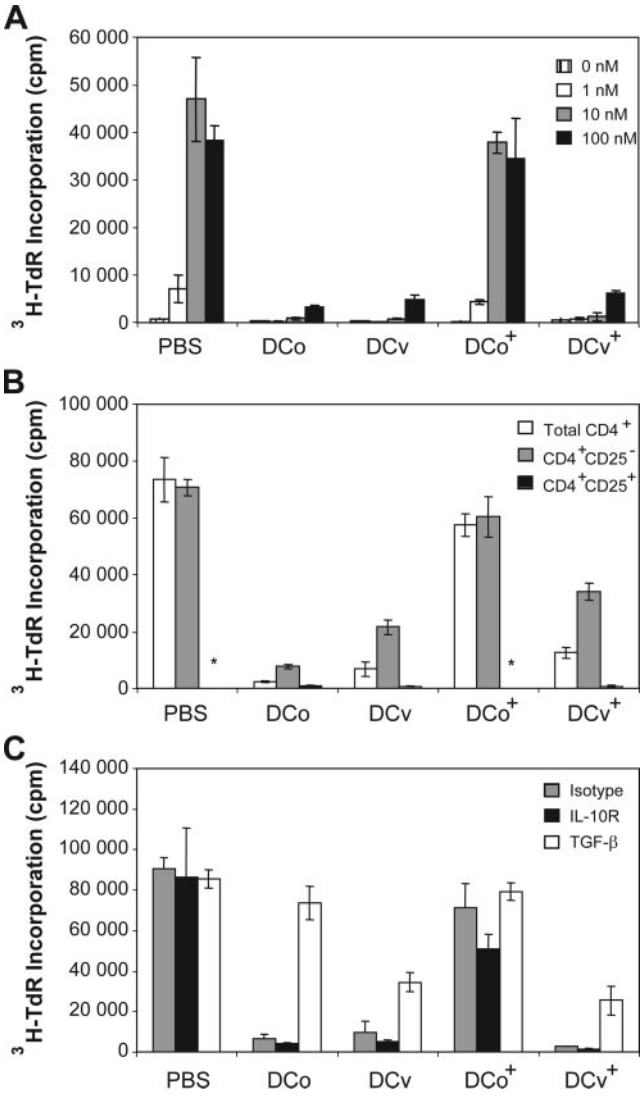

FIGURE 5. Functional characterization of T cells from mice tolerized by administration of DC. *A*, Profoundly reduced proliferation of nylon wool-purified T cells from tolerant mice compared with mice actively rejecting their grafts. T cells (5×10^4 cells/well) were stimulated with female DC (10^4/well) and varying concentrations of Dby peptide (0–100 nM). T cell proliferation was measured by [^{3}H]TdR incorporation for the final 18–24 h of a 4 day coculture (graphs are plotted as mean ± SD of triplicate cultures). *B*, Both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ splenocytes from tolerant mice are hyporesponsive to Ag stimulation. CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells purified from tolerant and rejecting female A1.RAG-1$^{-/-}$ mice were cultured with female CBA/Ca-derived immature DC prepulsed with 100 nM Dby peptide. Proliferation was assessed by [^{3}H]TdR incorporation for 24 h after 3 days of culture (* represents condition not tested). *C*, Reduced T cell proliferation is partially reversed by addition of mAb specific for TGF-β, as measured by [^{3}H]TdR incorporation (plotted as mean ± SD). Nylon wool-purified T cells were stimulated with female DC pulsed with 100 nM Dby peptide, in the presence of various neutralizing mAb at a final concentration of 100 μg/ml (IgG1 isotype control, clone YCATE55.9; IL-10R, clone 1B1.2; TGF-β, clone 11D11.16.8). T cell proliferation was measured by [^{3}H]TdR incorporation for the final 18–24 h of a 4-day culture (plotted as mean ± SD).

These CD4$^+$ Treg cells were characterized by their expression of foxp3 and the cell surface markers CD25, CD103, and GITR. Not surprisingly, tolerance could not be induced with female DC, indicating that tolerance was entirely Ag dependent. In addition, tolerance could not be achieved when using male DC of a different MHC haplotype, indicating that reprocessing of the male Dby Ag by host DC was not sufficient to account for the induction of tolerance in this model, although it is presumably responsible for its ongoing maintenance following the demise of the donor DC.

Although not formally the subject of this study, our microarray analysis of the DC populations used provides a starting point for the identification of genes that contribute to a protolerogenic phenotype. That DCv retain the same capacity as DCo to induce tolerance, irrespective of their exposure to LPS, is particularly significant in this respect because genes whose expression is modulated differentially compared with DCo$^+$ may be considered likely candidates for tipping the balance between tolerance and immunity (Table I, section I). Furthermore, those genes universally modulated by LPS are evidently insufficient to override the tolerogenic potential of DC (Table I, section II). Equally illuminating are genes anticipated to be involved in tolerance, which do not appear to be modulated by 1α,25(OH)$_2$D$_3$. Both programmed death ligand 1 and paired Ig-like receptor type 2 receptor α, known to act as inhibitory receptors, are specifically down-regulated following exposure to 1α,25(OH)$_2$D$_3$ (Table I, sections I and V). Because paired Ig-like receptor-B has only recently been designated the ortholog of the human inhibitory receptors Ig-like transcript 3/4, the corresponding gene was not included during the design of our custom microarray. Nevertheless, Serial Analysis of Gene Expression analysis of DCo and DCv in our laboratory likewise reveals that 1α,25(OH)$_2$D$_3$ has no apparent impact on expression of this gene, unlike exposure to IL-10, which strongly induces its up-regulation. These findings are consistent with those of Penna and colleagues (37) whose studies of human 1α,25(OH)$_2$D$_3$-treated DC reveal that the expression of ILT3 is dispensable for the induction of Treg cells.

The findings of the current work extend previous observations of tolerance in mice systemically treated with vitamin D$_3$ and mycophenolate mofetil (38). In this study, tolerance was associated with increased numbers of CD4$^+$CD25$^+$ T cells, together with DC displaying reduced costimulatory signals. It is possible that the tolerogenic phenotype displayed by the DC arose as a result of an inhibitory feedback loop between Treg cells and DC (39), the Treg cells being responsible for down-regulating costimulatory molecules on the APC (40). We have shown that both immature DC, as well as DC conditioned with 1α,25(OH)$_2$D$_3$, are able to induce CD4$^+$CD25$^+$ T cells, as postulated by Adorini et al. (41), based on studies in the human. Nevertheless, DCv may differ from human myeloid DC treated with 1α,25(OH)$_2$D$_3$ in terms of their capacity to recruit pre-existing natural Treg cells to sites of inflammation, because 1α,25(OH)$_2$D$_3$ not only down-modulated CCL17, as previously described (22), but also failed to augment expression of the chemokine CCL22 (Table I, section I), which, like CCL17, has been shown to contribute to the recruitment of foxp3$^+$ Treg cells through ligation of CCR4.

Tolerized mice contained normal numbers of T cells, indicating that the administration of Ag-laden DC had not resulted in systemic T cell deletion. Nevertheless, tolerant T cells exhibited a profound hyporesponsiveness in vitro that was partially reversible upon addition of mAb specific for TGF-β, but not IL-10R. Interestingly, administration of neutralizing mAb specific for TGF-β failed to inhibit the induction of tolerance with DCo and DCv even though the same treatment regime abrogated tolerance induced using mAb specific for CD4 and CD8 (31) (data not shown). Although these experiments argue against a role for TGF-β in the induction phase, we cannot

exclude its involvement in the ongoing maintenance of the tolerant state, although, given that suppression is only partially reversible in vitro (Fig. 5*C*), other mechanisms are likely to be involved. Irrespective of the role of TGF-β in their physiology, $CD4^+CD25^+$ T cells induced in these mice appear to be suppressive for the $CD4^+CD25^-$ population, since the latter, when isolated from $CD4^+CD25^+$ T cells, exhibit some capacity to proliferate in response to Ag, albeit reduced by comparison with naive T cells.

Our gene expression data lend credence to the hypothesis that vitamin D_3 acts by retaining DC in a state which, while clearly distinct from immaturity, is incompatible with full signaling potential, because a number of key immunogenic molecules are not up-regulated by DCv^+. These findings embrace other studies which have documented the induction of tolerance upon administration of APL (5), apoptotic material (25), and infectious tolerance due to the continuous release of graft Ags (2). Each of these scenarios may be anticipated to involve the presentation of Ag by steady state, quiescent DC. It should be noted, however, that immature and $1\alpha,25(OH)_2D_3$-treated DC did not display identical gene expression profiles, suggesting that $1\alpha,25(OH)_2D_3$ does not simply maintain the DC in a frozen state (14). Clearly, any effector function may be the result of subtle combinatorial changes in gene expression, which individually may not appear significant, but together act synergistically to greatly affect outcome. In addition, we recognize that although $1\alpha,25(OH)_2D_3$ ensures a reduced expression of activation signals (42, 43), its action may also be due, in part, to concomitant up-regulation of those inhibitory molecules not represented on the custom chip (43).

In conclusion, the current study demonstrates unequivocally that DC, incapacitated so as to avoid full signaling potential, either through their immaturity or through pharmacological modulation, are capable of inducing tolerance and regulation among naive $CD4^+$ T cells. These findings support the notion that immature host DC acquiring alloantigens under the noninflammatory conditions that prevail following healing of an allograft may sustain dominant tolerance by the steady state induction of Treg cells. Infectious tolerance may, therefore, involve a continuous process of conversion of naive T cells to regulatory function, resulting from exposure to donor graft Ags processed by quiescent host DC. Such insights into DC-mediated generation of $CD4^+CD25^+$ Treg cells in vivo may spawn therapeutic strategies to ensure selective vaccination for the generation of Treg cells.

Disclosures

The authors have no financial conflict of interest.

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