Regulatory T Cells Induced by 1α,25-Dihydroxyvitamin D_3 and Mycophenolate Mofetil Treatment Mediate Transplantation Tolerance[1]

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1α,25-Dihydroxyvitamin D_3, the active form of vitamin D_3, and mycophenolate mofetil, a selective inhibitor of T and B cell proliferation, modulate APC function and induce dendritic cells (DCs) with a tolerogenic phenotype. Here we show that a short treatment with these agents induces tolerance to fully mismatched mouse islet allografts that is stable to challenge with donor-type spleen cells and allows acceptance of donor-type vascularized heart grafts. Peritransplant macrophages and DCs from tolerant mice express down-regulated CD40, CD80, and CD86 costimulatory molecules. In addition, DCs from the graft area of tolerant mice secrete, upon stimulation with $CD4^+$ cells, 10-fold lower levels of IL-12 compared with DCs from acutely rejecting mice, and induce a $CD4^+$ T cell response characterized by selective abrogation of IFN-γ production. $CD4^+$ but not $CD8^+$ or class II^+ cells from tolerant mice, transferred into naive syngeneic recipients, prevent rejection of donor-type islet grafts. Graft acceptance is associated with impaired development of IFN-γ-producing type 1 $CD4^+$ and $CD8^+$ cells and an increased percentage of $CD4^+CD25^+$ regulatory cells expressing CD152 in the spleen and in the transplant-draining lymph node. Transfer of $CD4^+CD25^+$ cells from tolerant but not naive mice protects 100% of the syngeneic recipients from islet allograft rejection. These results demonstrate that a short treatment with immunosuppressive agents, such as 1α,25-dihydroxyvitamin D_3/mycophenolate mofetil, induces tolerance to islet allografts associated with an increased frequency of $CD4^+CD25^+$ regulatory cells that can adoptively transfer transplantation tolerance. ***The Journal of Immunology,*** **2001, 167: 1945–1953.**

Representing 5–10% of peripheral T cells in normal mice, $CD4^+CD25^+$ T cells display regulatory functions both in vitro and in vivo (1, 2) and can control pathogenic T cells in different autoimmune disease models (3–5). More recently, tolerance to alloantigens in vivo has been found to be maintained by $CD4^+$ cells expressing CD25 or $CD45RB^{low}$ (6). The immunosuppressive activity of $CD4^+CD25^+$ regulatory cells does not appear to depend on soluble factors, but to require cell contact, possibly involving signaling via CD152 (CTLA-4), a CD28 homologue expressed on activated T cells (1).

Interference with the CD80/CD86-CD28 costimulatory pathway by administration of the fusion protein CD152-Ig induces long-term allograft survival in rodents (7), although it is less effective in prolonging islet allograft survival in nonhuman primates (8). Blocking the CD40/CD154 pathway with anti-CD154 mAb has also been demonstrated to prevent rejection of heart, skin (9), and islet (10) allografts in rodents. Moreover, administration of anti-CD154 prevents acute renal allograft rejection (11) and induces long-term survival of islet allografts (12, 13) in nonhuman primates. Simultaneous blockade of the CD28 and CD40 pathways most effectively promotes long-term graft survival and inhibits the development of chronic rejection (9). Activation-induced cell death is an important mechanism of the prolonged allograft survival induced by anti-CD154 mAb (14, 15), but in addition to deletion of alloreactive T cells this treatment also induces infectious transplantation tolerance (16).

1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$),[3] the activated form of vitamin D_3, not only has a vital role in bone and calcium metabolism, but also modulates the immune response via specific receptors expressed in APCs and activated T cells (17). $1,25(OH)_2D_3$ and its analogs have been shown to inhibit autoimmune diseases and graft rejection in several experimental models (18). Intriguingly, renal graft loss has been found decelerated in patients treated with $1,25(OH)_2D_3$ (19). $1,25(OH)_2D_3$ inhibits Ag-induced T cell proliferation (20) and cytokine production (21), and prevents Th1 cell development (22, 23). APCs and, in particular, dendritic cells (DCs), are primary targets for the immunosuppressive activity of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ inhibits the differentiation and maturation of human DCs, leading to down-regulated expression of CD40, CD80, and CD86 costimulatory molecules and to inhibition of alloreactive T cell activation (24–27). In addition, $1,25(OH)_2D_3$ inhibits IL-12 and enhances IL-10 production by DCs, leading to induction of $CD4^+$ cells characterized by up-regulated CD152 expression and hyporesponsiveness to alloantigens (24). Based on these results, we have analyzed the ability of $1,25(OH)_2D_3$, administered alone or in combination with mycophenolate mofetil (MMF), to induce transplantation tolerance. MMF, an immunosuppressive agent clinically used to inhibit allograft rejection (28, 29), inhibits both T and B cell proliferation to mitogenic and allogenic stimulation (30), and also directly affects

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[3] Abbreviations used in this paper: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; MMF, mycophenolate mofetil; DC, dendritic cell.

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DCs by inhibiting costimulatory molecule expression and IL-12 production (31).

Results in this paper demonstrate that $1,25(OH)_2D_3$/MMF treatment induces donor-specific transplantation tolerance to islet allografts. $CD4^+$ cells from tolerant mice transferred into diabetic syngeneic recipients prevent donor-type islet allograft rejection, and tolerant mice display an increased percentage of $CD4^+CD25^+$ regulatory T cells, suggesting an active mechanism of suppression. Active suppression of islet allograft rejection is shown by the capacity of $CD4^+CD25^+$ cells to transfer transplantation tolerance. These results demonstrate that low m.w. immunosuppressive agents, like a combination of $1,25(OH)_2D_3$ and MMF, can induce in vivo DCs with a tolerogenic phenotype, enhance the frequency of $CD4^+CD25^+$ regulatory cells, and promote peripheral tolerance to allografts.

Materials and Methods

Mice

BALB/c, C57BL/6 (B6), and C_3H female mice were purchased from Charles River Laboratories (Calco, Italy), and kept under specific pathogen-free conditions.

Transplantation

Hand-picked B6 islets were transplanted (350/mouse), after overnight culture at 37°C, under the kidney capsule of BALB/c recipient mice rendered diabetic by a single i.v. injection of streptozotocin (250 mg/kg; Sigma, St. Louis, MO), as described (32). Two consecutive nonfasting blood glucose levels of ≥200 mg/dl defined islet graft rejection. Fully vascularized hearts were grafted into the abdomen of recipient mice as described (33). Graft survival was monitored by palpation, and rejection was confirmed by direct graft inspection.

Immunosuppressive treatments

MMF, a prodrug of mycophenolic acid, was dissolved in a vehicle consisting of sodium chloride (0.9%), sodium carboxymethylcellulose (0.5%), polysorbate 80 (0.4%), benzyl alcohol (0.9%) (all obtained from Sigma), and distilled water (97.3%). $1,25(OH)_2D_3$ was dissolved in ethanol (1 mg/ml) and then diluted in miglyol. Mice were dosed orally with MMF (100 mg/kg/day) and/or $1,25(OH)_2D_3$ (5 μg/kg p.o. three times per week), from day –1 to day 30 relative to the time of grafting. Control mice were dosed with vehicles only. Purified anti-CD4 GK1.5 mAb (American Type Culture Collection (ATCC), Manassas, VA) was injected i.p. (10 mg/kg) at days –1, 0, 1, and 2 relative to islet transplantation.

Histology

Kidney poles containing islet grafts were snap-frozen in Tissue Tek (Miles Laboratories, Elkhart, IN) and stored at −70°C. Frozen sections, 5 μm thick, were stained with biotinylated mAb against CD4, CD8, B220, CD11b (all purchased from PharMingen, San Diego, CA), or CD11c (N418; ATCC), followed by streptavidin-peroxidase conjugate. 3-amino-9-ethylcarbazole (DAKO, Carpenteria, CA) was used as chromogen, and hematoxylin as a counterstain. Cryostat sections were also stained with anti-porcine insulin (Sigma) followed by peroxidase-anti-peroxidase (Sigma).

Intracellular staining for cytokine production

Cells were stained for IFN-γ, IL-4, IL-10, and IL-2 as previously described (34). Reagents for intracytoplasmic staining contained 1% FCS, 0.5% saponin (Sigma), and 0.1% sodium azide. All incubations were performed at room temperature. Cells were washed, preincubated for 10 min with PBS/FCS/saponin, and then incubated with FITC-labeled rat anti-mouse IFN-γ (XMG1.2) and PE-labeled rat anti-mouse IL-4 (11B11), PE-labeled rat

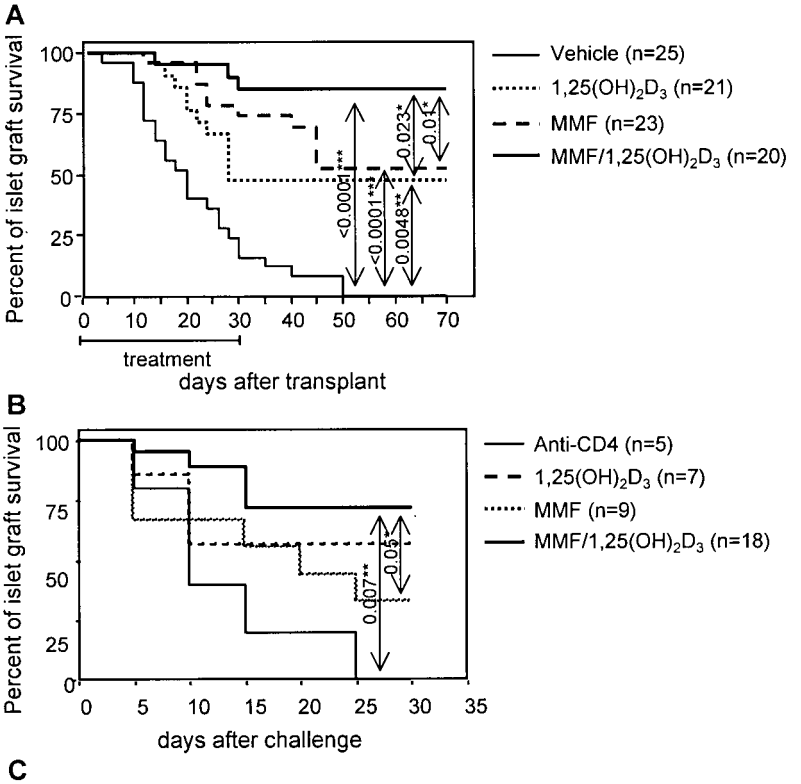

FIGURE 1. Tolerance induction by combined treatment with MMF and $1,25(OH)_2D_3$. *A*, Long-term islet allograft survival induced by MMF and $1,25(OH)_2D_3$ treatment. BALB/c mice were rendered diabetic by a single injection of streptozotocin (250 mg/kg i.v.) and transplanted with 350 B6 islets. Recipient mice were treated with MMF (100 mg/kg p.o. daily) and/or $1,25(OH)_2D_3$ (5 μg/kg p.o. three times per week) from day −1 to day 30. The function of islet allografts was monitored two times per week by blood glucose measurement. Values of *p* were determined by Fisher's exact test. *B*, Percent islet graft survival after B6 spleen cell challenge. Recipient mice were treated with MMF (100 mg/kg p.o. daily) and/or $1,25(OH)_2D_3$ (5 μg/kg p.o. three times per week) from day −1 to day 30. Alternatively, recipient mice were treated at days −1, 0, 1, and 2 with anti-CD4 mAb i.p. (10 mg/kg/day). Mice with functioning islet grafts 70 days after transplantation were injected i.p. with 10^6 B6 spleen cells. The function of islet allografts was monitored two times per week by blood glucose measurement. Values of *p* were determined by Fisher's exact test. *C*, Vascularized heart graft survival. Mice still normoglycemic 4 wk after spleen cell challenge were transplanted with B6 (donor-type) or C_3H (third party) vascularized heart grafts. As controls, naive BALB/c mice were transplanted with B6 (allograft) hearts. Heart function was monitored daily by palpation, and islet graft function was monitored two times per week by blood glucose measurement. The *p* value was calculated by Fisher's exact test.

C

Strain combination		*n*	% of heart graft survival, days after challenge								
Donor	Recipient		5	10	15	20	25	30	35	40	
BALB/c	naïve BALB/c	3	100	100	100	100	100	100	100	100	
C57BL/6	naïve BALB/c	6	100	0	0	0	0	0	0	0	0.0043**
C57BL/6	tolerant BALB/c	5	100	100	100	100	80	80	80	80	
C_3H	tolerant BALB/c	4	100	25	0	0	0	0	0	0	

anti-mouse IL-10 (JES5-16E3), or PE-labeled rat anti-IL-2 (JE56-5H4), all obtained from PharMingen. Isotype controls were FITC- and PE-labeled rat IgG1 (R3-34). After 30 min, cells were washed twice with PBS/FCS/saponin and then with PBS containing 5% FCS without saponin to allow membrane closure. The cell surface was then stained with CyChrome-labeled anti-CD4 (L3T4) for 15 min at room temperature. Analysis was performed with a FACScan flow cytometer equipped with CellQuest software (BD Biosciences, Mountain View, CA).

Flow cytometric analysis

Flow cytometric analysis was performed on single-cell suspensions of graft-associated cells, isolated by mechanical disruption. Stainings were performed in the presence of 100 μg/ml mouse IgG using the following mAbs, all obtained from PharMingen: CyChrome-labeled anti-CD45 (30-F11), FITC-labeled anti-CD11b (M1/70), FITC-labeled anti-CD40 (HM40-3), FITC-labeled anti-CD80 (16-10A1), PE-labeled anti-CD11c (HL-3), FITC-labeled anti-CD25 (7D4), and FITC-labeled anti-CD38 (H1.2F3). Cells were analyzed with a FACScan flow cytometer equipped with CellQuest software (BD Biosciences).

Cell cultures

To obtain graft-associated CD11c$^+$ cells, kidney poles containing the islet grafts were injected with 1 mg/ml collagenase D (Boehringer Mannheim, Mannheim, Germany) and 0.02 mg/ml DNase I (Sigma), and incubated at 37°C for 15 min. Single cell suspensions were collected after diluting the enzyme with ice-cold HBSS containing 2% FCS, 2.5 mM HEPES, and 5 mM EDTA and removing the aggregates by settling for 2 min on ice. Aggregates were further digested with collagenase D and DNase I for 5 min. Single cell suspensions were washed three times, and CD11c$^+$ cells were purified by positive selection on MiniMACS (Miltenyi Biotec, Auburn, CA). CD11c$^+$ cells (4×10^3 cells/well) from tolerant or acutely rejecting BALB/c mice were cultured in 96-well plates with splenic CD4$^+$ cells (2×10^5 cells/well) from donor-type B6 mice, positively selected by MiniMACS as described (35), in synthetic HL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin (Sigma). After 2 days, IL-12p75, IL-12p40, IFN-γ, IL-2, and IL-4 were quantified in culture supernatants.

Quantification of secreted cytokines

Secreted IL-12p75, IL-12p40, IFN-γ, IL-4, and IL-2 were quantified by two-site sandwich ELISA, as described (35–37). For IL-12p40 and IFN-γ, polyvinyl microtiter plates (Falcon 3012) were coated with 100 μl of 10F6 anti-mouse IL-12p40 (IL-12p40) and AN-18.17.24 (IFN-γ) in carbonate buffer. Samples were titrated in test solution (PBS containing 5% FCS and 1 g/L phenol) and incubated overnight at 4°C. To detect bound cytokines, plates were then incubated with biotinylated goat anti-mouse IL-12 (IL-12p40) or XMG1.2 mAb (IFN-γ) in test solution. After washing, the bound biotinylated Abs were revealed by an additional 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Avondale, PA) diluted 1/5000. The plates were washed again and incubated with the developing substrate *p*-nitrophenylphosphate disodium (Sigma) in diethanolamine buffer (pH 9.6; 100 μl/well). For IL-12p75 determination, plates were coated with 100 μl of the rat anti-mouse IL-12 heterodimer 9A5 mAb in carbonate buffer. After blocking, samples were incubated with 50 ml peroxidase-conjugated 5C3 mAb (rat anti-mouse IL-12p40 subunit). Anti-IL-12 Abs were provided by Dr. M. K. Gately (Hoffmann-LaRoche, Nutley, NJ). After overnight incubation at room temperature, bound peroxidase was detected by 3,3′,5,5′-tetramethylbenzidine (Fluka Chemicals, Ronkonkoma, NY), and absorbance was read at 450 nm with an automated microplate ELISA reader (MR5000; Dynatech Laboratories, Chantilly, VA). For IL-2 and IL-4 determination, two-site ELISAs were performed with paired mAbs obtained from PharMingen. For capture, JES6-1A12 (anti-IL-2) and BVD4-1D11 or 11B11 (anti-IL-4) mAbs were used. Samples were titrated in test solution and incubated overnight at 4°C. To detect bound cytokines, plates were then incubated with the biotinylated mAb JES6-5H4 (anti-IL-2) or BVD6-24G2 (anti-IL-4) in test solution. After washing, the bound biotinylated mAbs were revealed by an additional 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) diluted 1/5000. The plates were washed again and incubated with the developing substrate *p*-nitrophenylphosphate disodium (Sigma) in diethanolamine buffer (pH 9.6, 100 μl/well). The reaction was stopped by adding 50 μl/well NaOH 3N, and absorbance was read at 405 nm. Cytokines were quantified from two to three titration points using standard curves generated by purified recombinant mouse cytokines, and results were expressed as cytokine concentration in picograms per milliliter. Detection limits were 15 pg/ml for IFN-γ, IL-2, and IL-4, and 5 pg/ml for IL-12p40 and IL-12p75.

Results

Transplantation tolerance induced by combined treatment with 1,25(OH)$_2$D$_3$ and MMF

We first analyzed the ability of 1,25(OH)$_2$D$_3$ and MMF, administered alone or in combination, to inhibit islet allograft rejection. Pancreatic islets isolated from B6 mice were transplanted under the kidney capsule of BALB/c mice rendered diabetic by a single injection of streptozotocin. Recipient mice were treated from day –1 to day 30 with a nonhypercalcemic dose of 1,25(OH)$_2$D$_3$ (5 μg/kg p.o. three times per week) and/or MMF (100 mg/kg p.o. daily). The mean rejection time in vehicle-treated recipients was 23 $\pm$ 3 days. 1,25(OH)$_2$D$_3$ and MMF administered alone prolonged islet graft survival, but only in ~50% of the recipients. Conversely, over 80% of the mice treated with both drugs showed long-term (>70 days) islet graft acceptance (Fig. 1*A*). Next, we challenged BALB/c recipients showing long-term (>70 days) allograft acceptance with i.p. injection of 10^6 donor-type B6 spleen cells (Fig. 1*B*). Recipient mice treated with peritransplant administration of anti-CD4 mAb accommodated the islet graft but were not tolerant, because all mice rejected the graft after challenge with a mean survival time of 14 $\pm$ 2.4 days. Indeed, induction of transplantation tolerance in fully mismatched combinations by anti-CD4 mAb has been found to require additional treatments, such as CD152-Ig (38) or donor-specific transfusion (39).

Forty percent of allografts accepted under the cover of MMF alone were resistant to rejection upon challenge, confirming the tolerogenic properties of MMF in this model (40), and

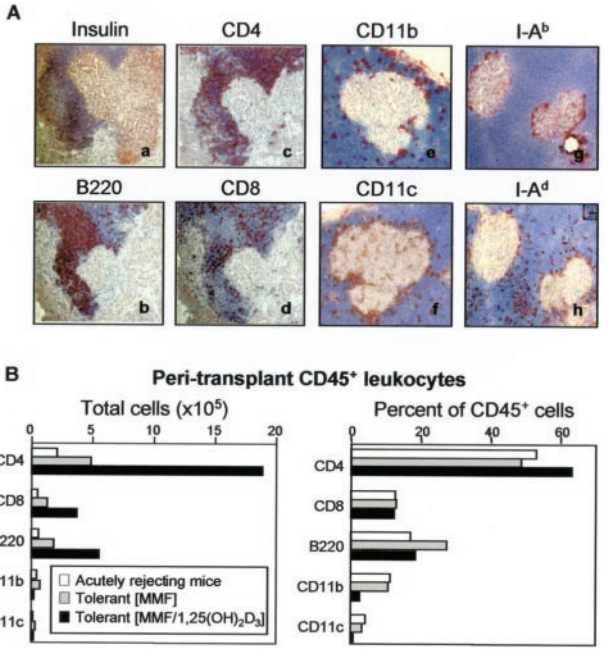

FIGURE 2. Tolerant mice display a massive peritransplant infiltrate characterized by reduced graft-infiltrating APCs. *A*, Histological analysis of the islet graft from a representative tolerant BALB/c mouse (of five tested) performed 140 days after islet transplantation. Frozen sections were stained for expression of insulin (*a*), B220 (*b*), CD4 (*c*), CD8 (*d*), CD11b (*e*), CD11c (*f*), I-A^b (*g*), and I-A^d (*h*). *B*, Cytofluorometric analysis of graft-infiltrating cells. Graft-associated cells isolated from untreated, acutely rejecting (30 days after transplantation) and tolerant (140 days after transplantation) mice treated from day −1 to day 30 with MMF alone or in combination with 1,25(OH)$_2$D$_3$ were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry. Acquisition was performed on CD45$^+$ cells. Results are from one representative mouse of 10 tested.

$1,25(OH)_2D_3$ had even superior activity. Combined treatment with $1,25(OH)_2D_3$ and MMF resulted in resistance to rejection upon challenge in 73% of allografts (Fig. 1*B*). Mice that continued to show islet graft function for 4 wk after challenge were transplanted, 100 days after the initial islet graft, with a vascularized heart from B6 (donor-type) or C_3H (third party) mice. Results in Fig. 1*C* show that naive BALB/c mice rejected B6 heart grafts in 10 days, whereas only one tolerant mouse of five rejected the heart graft 25 days after transplant. In contrast, tolerant BALB/c mice rejected a third-party heart in 10–12 days. These results demonstrate that a short-term treatment with $1,25(OH)_2D_3$ and MMF can induce donor-specific tolerance to allografts.

Tolerant mice display a massive peritransplant infiltrate

Islet grafts were analyzed, 140 days after the initial transplant, in BALB/c mice accepting B6 heart grafts. Immunohistochemical analysis in mice rendered tolerant by MMF and $1,25(OH)_2D_3$ treatment (Fig. 2*A*) showed a massive infiltrate around the islet grafts comprising B cells (*b*), CD4$^+$ (*c*) and CD8$^+$ (*d*) T cells, macrophages (*e*), and DCs (*f*). Only a few scattered cells were observed within the islet grafts. Thus, graft function, as demonstrated by the positive staining for insulin (*a*), persisted despite substantial peritransplant infiltration. Few I-A^b positive cells were found only within the B6 islets (*g*), whereas graft-surrounding lymphomononuclear cells expressed the recipient I-A^d molecule (*h*), indicating the recruitment of recipient cells. This implies that recipient-derived APCs could interact with recipient T cells via the indirect pathway of Ag presentation. The cells surrounding the tolerated grafts were analyzed by flow cytometry in mice rendered tolerant with MMF alone or $1,25(OH)_2D_3$/MMF treatment and compared with acutely rejected islet grafts 30 days after transplantation (Fig. 2*B*). CD45$^+$ cells recovered from the graft area of BALB/c mice rendered tolerant with $1,25(OH)_2D_3$/MMF treatment (3×10^6/mouse) were 10-fold higher compared with mice acutely rejecting B6 islet grafts (0.4×10^6/mouse). In contrast, 10^6 CD45$^+$ cells/mouse were recovered from the graft area of mice rendered tolerant with MMF alone. This increase was mostly due to perigraft accumulation of CD4$^+$ cells and, in part, of B and

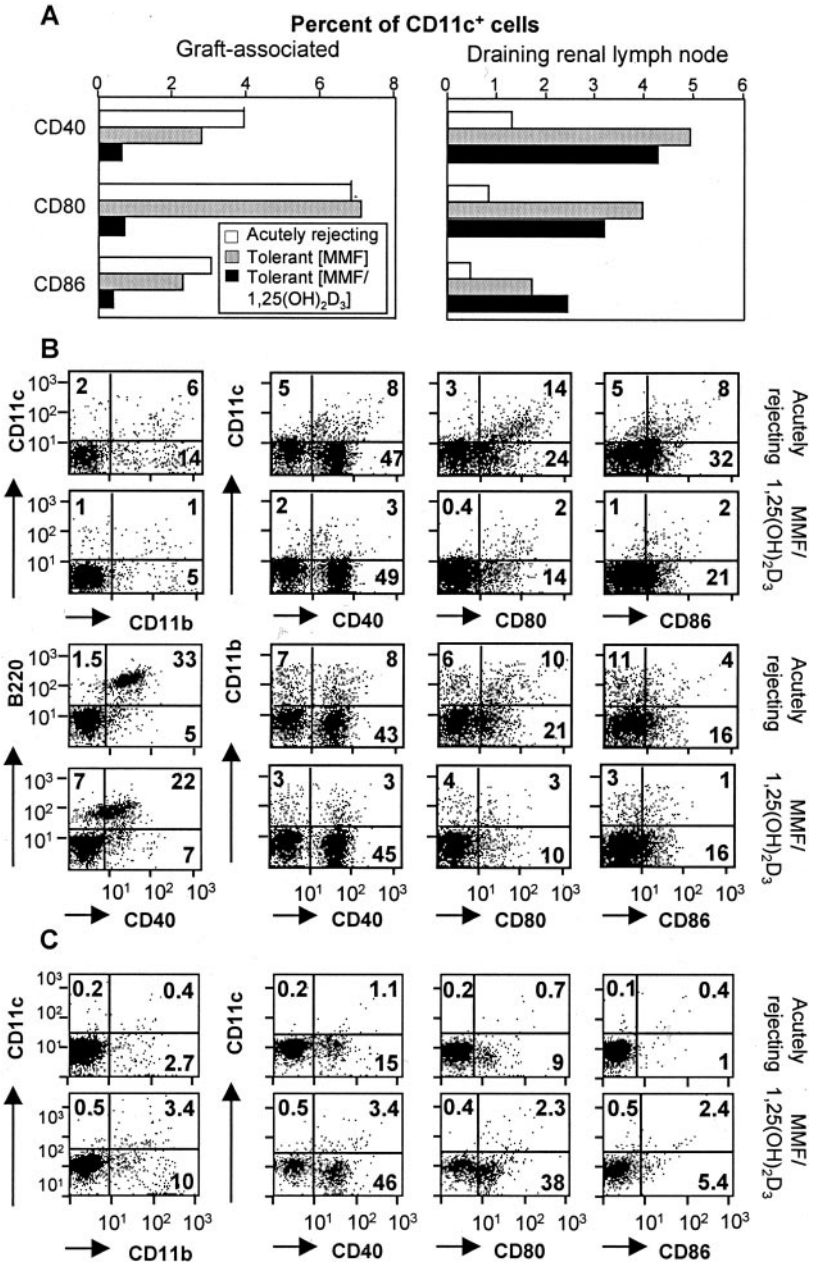

FIGURE 3. Cytofluorometric analysis of graft-associated and draining lymph node cells. *A*, Graft-associated and kidney lymph node cells pooled from three untreated, acutely rejecting (30 days after transplantation) and tolerant (140 days after transplantation) mice treated from day −1 to day 30 with MMF alone or combined with $1,25(OH)_2D_3$ were stained with mAbs specific for CD45, CD11c, and the indicated surface molecules. The cells were analyzed by flow cytometry, and acquisition was performed on CD45$^+$ cells. *B*, Expression of costimulatory molecules on graft-surrounding APCs. Graft-associated cells isolated from untreated, acutely rejecting (30 days after transplantation) and tolerant (140 days after transplantation) mice treated from day −1 to day 30 with MMF and $1,25(OH)_2D_3$ were stained with mAbs specific for CD45 and the indicated surface molecules, and analyzed by flow cytometry. Acquisition was performed on CD45$^+$ cells. Percentages of positive cells, set according to the isotype controls, are shown in the relative quadrants. *C*, Costimulatory molecule expression on draining lymph node APCs. Kidney lymph node cells isolated from untreated, acutely rejecting (30 days after transplantation) and tolerant (140 days after transplantation) mice treated from day −1 to day 30 with MMF and $1,25(OH)_2D_3$ were stained with mAbs specific for CD45 and the indicated surface molecules, and analyzed by flow cytometry. Acquisition was performed on CD45$^+$ cells. Percentages of positive cells, set according to the isotype controls, are shown in the relative quadrants. Results are from a representative experiment of two to four performed.

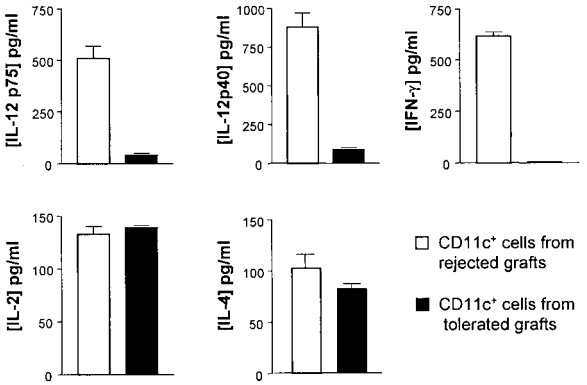

FIGURE 4. Peritransplant DCs from tolerant mice fail to produce IL-12 and induce a response in donor-type $CD4^+$ cells characterized by lack of IFN-γ secretion. $CD4^+$ spleen cells (2×10^5 cells/well) from a pool of three 8-wk-old B6 mice were cultured with $CD11c^+$ cells (4×10^3 cells/well) isolated from the graft area of three acutely rejecting (30 days after transplantation) and three tolerant (140 days after transplantation) BALB/c mice treated from day −1 to day 30 with MMF and $1,25(OH)_2D_3$. After 48 h of culture, IL-12p40, IL-12p75, IFN-γ, IL-2, and IL-4 were quantified in culture supernatants by two-site ELISA. Bars represent the mean ± SE of cytokine production by duplicate cultures.

$CD8^+$ cells. Conversely, the total numbers of macrophages and DCs were not increased (Fig. 2*B, left panel*). No difference in the percentage of $CD4^+$, $CD8^+$, and B cells was found between the two groups (Fig. 2*B, right panel*). Strikingly, the percentages of $CD11b^+$ macrophages and $CD11c^+$ DCs were reduced 5-fold in mice rendered tolerant under the cover of MMF and $1,25(OH)_2D_3$ compared with infiltrates in acutely rejecting mice or in mice rendered tolerant under the cover of MMF alone (Fig. 2*B, right panel*).

Selective down-regulation of T cell costimulatory molecules in peritransplant APCs from tolerant mice

Costimulatory molecule expression on macrophages and DCs was further analyzed in tolerant and acutely rejecting mice. Down-regulated CD40, CD80, and CD86 costimulatory molecules were observed on perigraft $CD11c^+$ DCs from mice rendered tolerant under the cover of MMF and $1,25(OH)_2D_3$ treatment, as demonstrated by the representative experiment shown in Fig. 3, *A, left panel,* and *B*. The percentage of both macrophages and DCs was reduced in grafts from tolerant ($CD11b^+/CD11c^-$ 5%, $CD11c^+$ 2%) compared with acutely rejecting ($CD11b^+/CD11c^-$ 14%, $CD11c^+$ 8%) mice (Fig. 3*B*). CD40, CD80, and CD86 expression was 3- to 7-fold reduced on DCs and macrophages surrounding tolerated islet grafts compared with acutely rejecting ones. Results (means ± SE) from four experiments showed a significant reduction from 4.4 ± 0.7 to 0.7 ± 0.3 in the percentage of $CD11c^+$ DCs expressing CD40 ($p < 0.05$ by Mann-Whitney *U* test). Similarly, DCs expressing CD80 were reduced from 9.2 ± 1.5 to 0.9 ± 0.4 ($p < 0.05$), and DCs expressing CD86 molecules from 4.1 ± 0.5 to 0.8 ± 0.4 ($p < 0.05$). The percentage of graft-associated macrophages and DCs, as well as their expression of costimulatory molecules, in rejected grafts from untreated mice or from the 27% of $1,25(OH)_2D_3$/MMF-treated mice that failed to become tolerant, was indistinguishable (data not shown). Intriguingly, the expression of CD40 on B cells was not significantly down-regulated (Fig. 3*B*). Results (means ± SE) from four experiments showed a reduction from 25.0 ± 4.7 to 19.5 ± 1.7 ($p = 0.7$) in the percentage of $CD40^+$ B cells surrounding acutely rejected vs tolerated islet grafts.

Conversely, mice rendered tolerant under the cover of MMF alone did not show a reduced DC percentage or a reduced expression of costimulatory molecules (Fig. 3*A*). Thus, $1,25(OH)_2D_3$ treatment is required to induce long-lasting down-regulation of costimulatory molecules. Interestingly, tolerant mice displayed an increased percentage of $CD11c^+$ DCs (3.9 vs 0.6% in acutely rejecting mice) and $CD11b^+$ macrophages (10 vs 2.7%) in the kidney lymph nodes draining the islet graft (Fig. 3*C*). CD40, CD80, and CD86 costimulatory molecules were up-regulated in the kidney lymph node cells (Fig. 3, *A, right panel,* and *C*) and in the spleen or mesenteric lymph node cells of tolerant compared with acutely rejecting mice (data not shown). This indicates a selective effect of $1,25(OH)_2D_3$ treatment on APCs recruited to the graft area. The tolerogenic phenotype, as defined by down-regulated costimulatory molecules, expressed by perigraft DCs from tolerant mice, prompted us to test their function in terms of IL-12 production and capacity to activate donor-type $CD4^+$ cells. $CD11c^+$ cells surrounding the islet grafts of tolerant and acutely rejecting BALB/c mice were purified and cultured with $CD4^+$ spleen cells from donor-type B6 mice. Results in Fig. 4 demonstrate that DCs from the graft area of tolerant mice secrete, upon stimulation with $CD4^+$ cells, 10-fold lower levels of IL-12p75 and IL-12p40 compared with DCs from acutely rejecting mice. In the same culture supernatants, the T cell-derived cytokines IFN-γ, IL-2, and IL-4 were also measured. Consistent with the profound decrease of IL-12 secretion by DCs, IFN-γ production by $CD4^+$ cells was selectively abrogated. Thus, perigraft DCs from tolerant mice, as predicted by their reduced expression of CD40 molecules, are less sensitive to IL-12-inducing signals from $CD4^+$ cells. In turn, they induce only partial activation of $CD4^+$ cells, characterized by lack of IFN-γ production.

$CD4^+$ T cells from tolerant mice inhibit donor-type allograft rejection

The tolerant state of recipients with long-term accepted grafts following $1,25(OH)_2D_3$/MMF treatment was characterized by a reduced percentage of IFN-γ-producing $CD4^+$ and $CD8^+$ cells in the kidney lymph nodes draining the islet allografts. In contrast, IL-2-producing cells were reduced only among $CD8^+$ cells. IL-4- and IL-10-producing cells were comparably low in acutely rejecting and tolerant mice (Fig. 5*A*). To assess whether transplantation tolerance could be transferred to naive syngeneic recipients, total spleen cells from tolerant BALB/c mice were injected into diabetic BALB/c mice (20×10^6/recipient) 2 days before transplantation of either B6 (donor-type) or C_3H (third party) islets. Transfer of unfractionated spleen cells from tolerant BALB/c mice inhibited donor-type (B6) allograft rejection and induced in 50% of the recipients long-term (>70 days) graft survival. In contrast, third party (C_3H) islets were rejected with a mean graft survival time of 16 ± 2 days, similar to that of B6 islets transplanted in naive diabetic BALB/c mice (Fig. 5*B*). Following transfer of spleen cells from tolerant mice, the percentage of IFN-γ-producing $CD4^+$ and $CD8^+$ cells in the kidney lymph nodes draining the islet graft of recipients with long-term accepted allograft was 3- to 6-fold reduced compared with recipients that rejected the islet graft (Fig. 5*C*). Conversely, the percentage of IL-4- or IL-10-producing cells was very low in both groups. Similar results were obtained with spleen cells (data not shown). Thus, the patterns of cytokine production by $CD4^+$ and $CD8^+$ cells from primarily tolerant mice and recipients rendered tolerant by adoptive transfer of spleen cells were essentially superimposable.

Next, we analyzed whether transplantation tolerance could be transferred by T cells or by APCs. $CD4^+$ plus $CD8^+$ or class II^+ cells, equivalent in number to those contained in 20×10^6 total

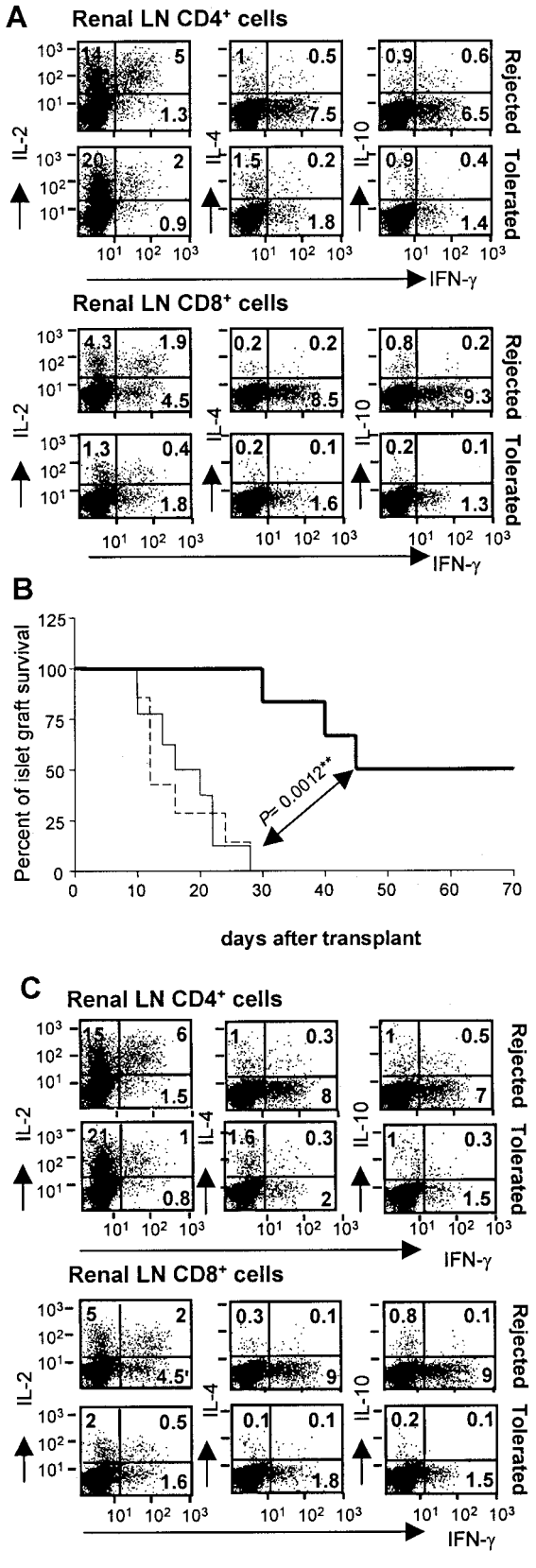

FIGURE 5. Spleen cells from tolerant mice inhibit donor-type allograft rejection. *A*, Intracytoplasmic cytokine production by draining renal lymph node $CD4^+$ and $CD8^+$ T cells from tolerant mice. $CD4^+$ or $CD8^+$ T cells, isolated from kidney lymph nodes of acutely rejecting or tolerant mice as described in Fig. 3, were stimulated with PMA/ionomycin, stained for intracytoplasmic IL-4, IL-10, IL-2, and IFN-γ as well as for surface CD4 or CD8 expression, and analyzed by flow cytometry. Percentages of cytokine-positive cells, set according to the isotype controls, are shown in the relative quadrants. Results are from one of two experiments performed with similar results. *B*, Islet graft survival following transfer of spleen cells from tolerant mice. BALB/c mice rendered diabetic by a single injection of streptozotocin were transferred with 20×10^6 spleen cells isolated from tolerant BALB/c mice. Two days later, they were transplanted with either B6 (thick line) or C_3H (solid thin line) islets. As controls, naive BALB/c mice (broken line) were transplanted with B6 islets. The function of islet allografts was monitored two times per week by blood glucose measurement. The *p* value was determined by Fisher's exact test. *C*, Intracytoplasmic cytokine production by draining renal lymph node $CD4^+$ and $CD8^+$ T cells from transferred recipients. $CD4^+$ or $CD8^+$ T cells isolated from kidney lymph nodes of the recipients with accepted or rejected islet grafts were analyzed as in *A*.

spleen cells, isolated from the recipients with tolerated grafts, were transferred into diabetic BALB/c mice 2 days before transplantation of donor-type B6 islets. Transfer of 14×10^6 class II^+ cells (85% B cells, 10% macrophages, and 3% DCs according to the expression of B220, CD11b, and CD11c, respectively) did not significantly extend allograft survival over controls. Conversely, over 80% of the mice transferred with a total of 6×10^6 $CD4^+$ and $CD8^+$ cells showed functioning islet grafts 60 days after transplantation (Table I).

To evaluate whether $CD4^+$ or $CD8^+$ T cells were responsible for adoptive transfer of tolerance, $CD4^+$ and $CD8^+$ T cells were purified from spleens of mice tolerized by T cell transfer and injected into naive diabetic BALB/c mice 2 days before transplantation of donor-type B6 islets. Results in Table I demonstrate that $CD4^+$ spleen cells from tolerant mice, equivalent in number to those contained in 20×10^6 spleen cells, prevented donor-type islet allograft rejection in all recipients, indicating an active and effective tolerogenic mechanism. Interestingly, mice transferred with $CD8^+$ spleen cells isolated from tolerant mice displayed a primary nonfunction of the islet grafts and never returned normoglycemic.

$CD4^+CD25^+$ cells from tolerant mice transfer transplantation tolerance

Tolerant mice displayed an increased percentage of $CD4^+CD25^+$ regulatory cells both in draining lymph nodes (13.3 vs 6.7%) and in the spleen (32.8 vs 16.4%) compared with acutely rejecting mice, without a corresponding increase of $CD4^+CD69^+$ cells. $CD4^+CD38^+$ cells were also higher in tolerant mice, in particular in the spleen. Moreover, tolerant mice presented a lower percentage of $CD45RB^{high}$ cells and an increased percentage of $CD45RB^{low}$ cells compared with acutely rejecting mice (Table II). An increased expression of CD152 was found in $CD4^+CD25^+$ T cells isolated from tolerant mice compared with acutely rejecting mice (Fig. 6), both in draining lymph nodes (37 vs 17%) and spleens (35.5 vs 20%). To evaluate the ability of $CD4^+CD25^+$ T cells to prevent allograft rejection, $CD4^+CD25^-$ (4×10^6/mouse) or $CD4^+CD25^+$ (5×10^5/mouse) T cells, equivalent in number to those contained in 20×10^6 spleen cells isolated from naive or tolerant mice, were transferred into naive diabetic BALB/c mice. Two days later, these recipients were transplanted with donor-type (B6) islets. Results in Fig. 7*A* show that neither $C4^+CD25^-$ nor $CD4^+CD25^+$ cells from naive BALB/c mice could protect, at least at the dose transferred, from islet allograft rejection. Conversely, results in Fig. 7*B* show that $CD4^+CD25^+$ cells prevented islet allograft rejection, whereas $CD4^+CD25^-$ cells did not, indicating an active mechanism of tolerance induction mediated by $CD4^+CD25^+$ regulatory T cells.

Table I. *Transferable tolerance is mediated by CD4$^+$ spleen cells*[a]

Expt.	Donors	BALB/c Recipients	Graft Survival Time (days)	Mean Graft Survival (days)	*p* Value
1	C57BL/6	Naive	6, 14, 21, 21, 24, 24, 25	19	
		Transferred with class II$^+$ cells (14×10^6 cells/mouse)	6, 6, 8, 22, 36, 36, 36	21	
		Transferred with T cells (6×10^6 cells/mouse)	28, >60, >60, >60, >60, >60, >60	>60	0.0006
2	C57BL/6	Naive	10, 12, 12, 12, 16, 24, 28	16	
		Transferred with CD8$^+$ cells (1.5×10^6 cells/mouse)	Primary nonfunction	0	
		Transferred with CD4$^+$ cells (4.5×10^6 cells/mouse)	>190, >190, >190, >190, >190, >190, >190	>190	0.0006

[a] Naive BALB/c mice rendered diabetic by a single injection of streptozotocin were transferred with 6×10^6 T cells (75% CD4$^+$, 25% CD8$^+$) or with 14×10^6 class II$^+$ APCs (85% B cells, 10% macrophages, and 3% DCs according to the expression of B220, CD11b, and CD11c, respectively), isolated from the spleen of tolerant mice 70 days after spleen cell transfer (Expt. 1). Alternatively, diabetic BALB/c mice were transferred with 4.5×10^6 CD4$^+$ or with 1.5×10^6 CD8$^+$ T cells isolated from the spleen of mice rendered tolerant by T cell transfer, 70 days after T cell transfer (Expt. 2). All BALB/c recipients were transplanted with B6 islets 24 days after cell transfer. As controls, naive BALB/c mice were transplanted with B6 islets. The function of islet allografts was monitored two times per week by blood glucose measurement. The *p* values were determined by Mann-Whitney *U* test.

Discussion

In this study we have demonstrated that a short-term treatment of recipient mice with $1,25(OH)_2D_3$/MMF induces tolerance to islet allografts and enhances CD4$^+$CD25$^+$ regulatory T cells that can adoptively transfer transplantation tolerance. $1,25(OH)_2D_3$/MMF treatment selectively inhibits the perigraft recruitment of DCs and macrophages, and these cells still express down-regulated costimulatory molecules over 100 days after treatment withdrawal. Perigraft DCs display a tolerogenic phenotype characterized by down-regulated CD40, CD80, and CD86 costimulatory molecules and by a profoundly reduced IL-12 production. CD4$^+$ T cells from tolerant mice transfer long-term protection to islet grafts in naive syngeneic recipients, associated with a reduced frequency of IFN-γ-producing type 1 CD4$^+$ and CD8$^+$ cells. Recipients transferred with CD4$^+$ cells from mice rendered tolerant with $1,25(OH)_2D_3$/MMF treatment show an increased percentage of CD4$^+$CD25$^+$ regulatory T cells expressing CD152, and these cells can transfer tolerance to islet allografts.

The rationale of this study was to induce tolerance to allografts by a combined short-term targeting of APCs and T cells. $1,25(OH)_2D_3$ is an immunomodulator inhibiting, in different models, autoimmune diseases and allograft rejection (18). $1,25(OH)_2D_3$ inhibits alloreactive T cell activation by targeting APCs, and in particular DCs (24–27). Notably, $1,25(OH)_2D_3$ inhibits the differentiation, maturation, and activation of DCs in vitro while promoting their apoptosis (24). DCs matured in the presence of $1,25(OH)_2D_3$ display reduced levels of MHC class II and CD40, CD80, and CD86 costimulatory molecules, and induce hyporesponsiveness in alloreactive T cells (24). DC modulation by $1,25(OH)_2D_3$ has recently been shown to require a vitamin D receptor-dependent pathway that could promote a persistent inhibition of DC maturation in vitro and in vivo (41). MMF has also been shown to affect DCs, reducing the expression of costimulatory molecules and inhibiting the production of IL-12 (31). In addition, MMF inhibits the proliferation of activated T and B cells. Orally administered MMF is hydrolyzed by esterases in the intestine and blood to release mycophenolic acid, a potent, selective, noncompetitive inhibitor of the type 2 isoform of inosine monophosphate dehydrogenase, and therefore inhibits the de novo pathway of guanosine nucleotide synthesis (30). Because T and B lymphocytes are critically dependent for their proliferation on de novo purine synthesis, whereas other cell types can use salvage pathways, MMF is a selective lymphocyte inhibitor.

Table II. *Surface markers of CD4$^+$ T cells*[a]

	CD4$^+$ Cells from Renal Lymph Node		CD4$^+$ Cells from Spleen	
	Acutely rejecting	Tolerant upon CD4$^+$ transfer	Acutely rejecting	Tolerant upon CD4$^+$ transfer
CD25	6.7	**13.3**	16.4	**32.8**
CD38	13.7	18.4	15.4	**37.3**
CD45RB^{low}	15.1	20.1	40.7	57.8
CD45RB^{high}	35.2	30.1	57.6	42.2
CD69	11.9	6.5	17.4	20.4

[a] CD4$^+$ T from untreated acutely rejecting (30 days after transplantation) and tolerant mice upon CD4$^+$ transfer (190 days after transplantation) were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry. Acquisition was performed on CD4$^+$ cells. Values in bold represent ≥2-fold increase in marker expression.

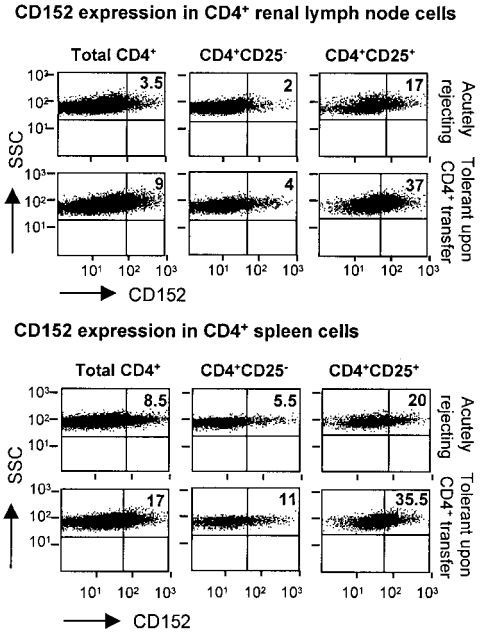

FIGURE 6. CD4$^+$CD152$^+$ cells are increased in tolerant mice. CD152 expression was evaluated in permeabilized CD4$^+$, CD4$^+$CD25$^-$, and CD4$^+$CD25$^+$ T cells isolated from draining lymph node and spleen of the untreated, acutely rejecting and tolerant mice described in Table I, Expt. 2. The analysis was performed 190 days after islet transplantation. Acquisition was performed on CD4$^+$ cells. Percentages of positive cells, set according to the isotype controls, are shown in the relative quadrants.

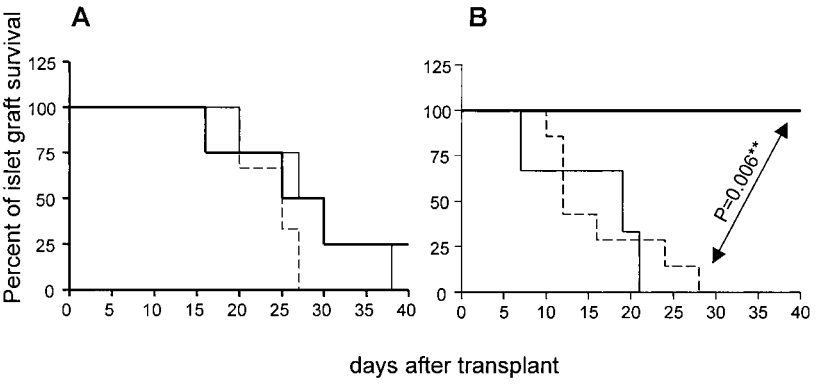

FIGURE 7. CD4$^+$CD25$^+$ T cells transfer tolerance to islet allografts. Naive BALB/c mice rendered diabetic by a single injection of streptozotocin were transferred with 0.5×10^6 CD4$^+$CD25$^+$ (thick line) or 4×10^6 CD4$^+$CD25$^-$ (thin line) T cells isolated from naive BALB/c mice (*A*) or from mice rendered tolerant by transfer of CD4$^+$ T cells 190 days earlier (*B*). Two days later, all recipients were transplanted with B6 islets. As controls, naive BALB/c mice were transplanted with B6 islets (broken line). The function of islet allografts was monitored two times per week by blood glucose measurement. The *p* value was determined by Fisher's exact test.

As shown here, 1,25(OH)$_2$D$_3$/MMF treatment induces similar effects in vivo, leading to a reduction in the percentage of macrophages and DCs surrounding the tolerated graft, both showing down-regulated expression of CD40, CD80, and CD86 costimulatory molecules. 1,25(OH)$_2$D$_3$ has also been shown to inhibit IL-12 production in vitro (42), via inhibition of NF-κB (43), and in vivo (23). The long-lasting down-modulation of costimulatory molecules and the profoundly reduced IL-12 production by DCs recruited to the graft area of tolerant mice, clearly seen over 100 days after treatment withdrawal, cannot be the direct effect of 1,25(OH)$_2$D$_3$/MMF treatment. Rather, this reflects the early disruption of APC-T cell interactions with long-lasting effects, consistent with the observation that both T cell and DC persistence in vivo is dependent on CD40-CD154 interactions (44). It is likely that the inhibition of alloreactive T cell activation fails, in turn, to provide APC-activating signals (45), thus explaining the persistent tolerogenic phenotype of macrophages and DCs recruited to the graft area. Perigraft DCs from tolerant mice induce only partial activation of alloreactive CD4$^+$ cells, characterized by the absence of IFN-γ but conserved IL-2 and IL-4 secretion. The partial activation of CD4$^+$ cells lacking IFN-γ production can be easily explained by the low levels of IL-12 produced by DCs, and provides an example of disruption of APC-activating signals induced by 1,25(OH)$_2$D$_3$/MMF treatment.

Our results highlight the important role of APCs with tolerogenic properties in transplantation tolerance (46–48). Notably, they indicate that a tolerogenic phenotype, characterized by down-regulated costimulatory molecules and profoundly reduced IL-12 production, can be induced in graft-associated macrophages and DCs by a short course of treatment with low m.w. immunomodulatory agents. Importantly, the immature-like tolerogenic phenotype of DCs is maintained for over 100 days after drug withdrawal. A long-term reduction in mature DCs, associated with transplantation tolerance, could also be induced by peritransplant administration of other immunosuppressive agents, like deoxyspergualin combined with anti-CD3 immunotoxin (49).

Mechanistically, immature DCs could favor peripheral tolerance not only by decreasing the activation of effector T cells but also by inducing the differentiation of T regulatory cells (50, 51). Different types of T regulatory cells have been described, all with the ability to regulate both in vitro and in vivo T cell activation (1, 52). CD4$^+$ cells defined by constitutive expression of CD25 (53, 54) represent the most characterized subset of regulatory T cells. CD4$^+$CD25$^+$ peripheral regulatory T cells derive from thymic precursors (55), and their functional development involves an extrathymic phase dependent on the presence of relevant Ags (56, 57). Although the Ag specificity of CD4$^+$CD25$^+$ regulatory T cells is still unclear, our results indicate that priming by alloantigen is required for their induction, because cells from tolerant but not from naive mice could transfer protection from allograft rejection. CD4$^+$CD25$^+$ cells suppress immune responses via non-Ag specific mechanisms that appear to be independent from the production of immunoregulatory cytokines (4, 58), although the alloantigen-specific regulatory T cells contained within the CD45RB^{low} population have been found to require IL-10 for functional activity (6). Rather, the suppressive function of CD4$^+$CD25$^+$ regulatory T cells has been associated with up-regulated expression of CD152 (5, 59). Signaling via CD152 is required for immune suppression in vitro (59), and blockade of CD152 signaling increases T cell-mediated responses in several models (60). In our study, the percentage of CD4$^+$CD25$^+$ regulatory T cells expressing CD152 was increased in tolerant mice and they could transfer very effectively transplantation tolerance, suggesting their relevance in tolerance to allografts. Indeed, regulatory T cells have been documented in patients with long-term surviving allografts (61, 62), and isolated examples of operational tolerance have been noted in graft recipients treated with immunosuppressive drugs (63).

In conclusion, our results demonstrate that a short treatment with 1,25(OH)$_2$D$_3$/MMF can induce transferable tolerance to islet grafts. Tolerance is associated with the induction of macrophages and DCs with a tolerogenic phenotype and with an increase of CD4$^+$CD25$^+$ regulatory T cells. The enhancement of CD4$^+$CD25$^+$ regulatory T cells by low m.w. immunosuppressive agents, such as a combination of 1,25(OH)$_2$D$_3$ and MMF, may provide a new paradigm, applicable to the prevention of allograft rejection in humans and to the treatment of autoimmune diseases.

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