

Inhibitory Feedback Loop Between Tolerogenic Dendritic Cells and Regulatory T Cells in Transplant Tolerance¹

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An active role of T regulatory cells (Treg) and tolerogenic dendritic cells (Tol-DC) is believed important for the induction and maintenance of transplantation tolerance. However, interactions between these cells remain unclear. We induced donor-specific tolerance in a fully MHC-mismatched murine model of cardiac transplantation by simultaneously targeting T cell and DC function using anti-CD45RB mAb and LF 15-0195, a novel analog of the antirejection drug 15-deoxyspergualin, respectively. Increases in splenic Treg and Tol-DC were observed in tolerant recipients as assessed by an increase in CD4⁺CD25⁺ T cells and DC with immature phenotype. Both these cell types exerted suppressive effects in MLR. Tol-DC purified from tolerant recipients incubated with naive T cells induced the generation/expansion of CD4⁺CD25⁺ Treg. Furthermore, incubation of Treg isolated from tolerant recipients with DC progenitors resulted in the generation of DC with Tol-DC phenotype. Treg and Tol-DC generated in vitro were functional based on their suppressive activity in vitro. These results are consistent with the notion that tolerance induction is associated with a self-maintaining regulatory loop in which Tol-DC induce the generation of Treg from naive T cells and Treg programs the generation of Tol-DC from DC progenitors. *The Journal of Immunology*, 2003, 170: 1304–1312.

Donor-specific transplant tolerance involves active suppression of graft-reactive T cells while sparing responses against other Ags. Regulatory T cells (Treg)³ have been described as primary contributors to the state of self tolerance and tolerance to alloantigens (1) due to their ability to specifically suppress activation and function of graft-reactive T cells. The CD4⁺CD25⁺ T cell subset is a Treg population actively involved in transplant tolerance. These cells increase in numbers during tolerance induction (2–4), and can Ag specifically transfer tolerance to secondary recipients (4). CD4⁺CD25⁺ Treg were originally identified by their ability to prevent systemic autoimmunity in neonatally thymectomized mice (1). These cells were found to inhibit T and B cell activation in both an Ag-specific and nonspecific manner, in part through membrane-bound TGF- β and IL-10 secretion (1). The importance of CD4⁺CD25⁺ Treg in maintaining tolerance was illustrated in experiments in which administration of these cells prevented autoimmunity (5) and graft rejection (6). Although Treg are generated intrathymically during development (7), their production also occurs in the periphery due to in-

teraction between naive T cells and subsets of dendritic cells (DC) (8, 9), which we collectively term tolerogenic DC (Tol-DC). Tol-DC possess an immature DC phenotype, as assessed by low expression of MHC class II, CD40, CD80, CD86, and IL-12 (10–12). Tol-DC have previously been used therapeutically to prevent allograft rejection (13–15). Lu and colleagues (16) have shown that inhibition of DC maturation through blockade of NF- κ B activity endowed the DC with tolerogenic ability.

Induction of transplantation tolerance using agents that target T cell function has been well established. Unfortunately, most of these approaches are not robust enough to induce reliable tolerance, and they are also associated with a variety of adverse effects in clinic. Immunosuppressants that act at the level of the APC have also been described (17). Promisingly, a synergistic effect on tolerance induction has been achieved by targeting both T cells and APC. Thomas et al. (17) have demonstrated in macaques that simultaneously targeting T cells using immunotoxin and DC using deoxyspergualin results in synergistic tolerance induction. Recently, we have found that a short-term combined treatment of anti-CD45RB mAb and LF 15-0195 (LF) resulted in permanent graft acceptance in the C57BL/6 > BALB/c cardiac allograft model, while treatment with either agent alone did not induce tolerance. LF is a novel analog of the antirejection drug 15-deoxyspergualin (DSG), which induces donor-specific tolerance in rodent models of fully MHC-mismatched cardiac transplants with lower toxicity than its parent compound (4, 19). The parent compound, DSG, has been demonstrated to act at the level of the APC, specifically inhibiting maturation of DC (17).

A desirable approach inducing tolerance would involve the simultaneous induction of Treg and Tol-DC. In such a scenario, synergistic graft survival would result as a consequence of the ability of these cells to complement each other in inducing tolerance. We proposed that stable tolerance takes place in two phases: 1) an induction phase that targets T cells and/or APC (e.g., DC), and 2) a self-regulatory phase operating as a result of Tol-DC/Treg

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Received for publication July 17, 2002. Accepted for publication November 20, 2002.

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¹ This study is supported by Canadian Institutes of Health Research, Roche Organ Transplantation Research Foundation, Physicians Services Incorporate Foundation, a grant from MultiOrgan Transplant Program at London Health Sciences Center, and Internal Research Fund Grant from Lawson Health Research Institute.

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³ Abbreviations used in this paper: Treg, regulatory T cell; DC, dendritic cell; DSG, 15-deoxyspergualin; Tol-DC, tolerogenic DC.

interactions. This feedback loop would allow the Tol-DC to promote differentiation of naive T cells into graft-protecting Treg, while Treg would stimulate the production of Tol-DC from DC progenitors. It has been reported that CD4⁺CD25⁺ cells can be generated *in vitro* by activation of naive T cells in the presence of Tol-DC (20). The Tol-DC subset possesses low levels of MHC class II, CD80/86, and IL-12, while secreting high levels of IL-10 (10, 20). In contrast, T cells that secrete TGF- β and IL-10 also can promote the generation of Tol-DC with immature phenotype (21, 22). However, these studies are *in vitro*, and demonstrate a one-way regulation of Treg on Tol-DC and Tol-DC on Treg, respectively. Whether or not a two-way interaction between T and DC exists in tolerance remains to be demonstrated.

In this study, we used a short-term treatment of anti-CD45RB mAb and LF to initiate the induction phase of tolerance by simultaneously targeting APC and T cells. The establishment *in vivo* of a putative regulatory feedback loop was tested by examining *in vitro*, Treg cells, and Tol-DC isolated *ex vivo* from long-survived (>100 day) tolerant recipients, for their ability to induce Tol-DC and Treg, respectively. We show that Tol-DC isolated from tolerant recipients promoted the generation or expansion of functional CD4⁺CD25⁺ Treg from naive T cells. Similarly, Treg isolated from tolerant recipients induced the generation of Tol-DC from bone marrow progenitors. These data suggest that a regulatory feedback loop occurs *in vivo* in tolerant recipients. To our knowledge, this is the first report finding a Tol-DC/Treg two-way interaction in transplantation.

Materials and Methods

Animals and tolerance induction

Eight- to 12-wk-old male C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H (H-2^k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and used as donors, recipients, or third party controls. Recipient mice (BALB/c) were divided into groups and treated with: 1) anti-CD45RB mAb, 3 mg/kg/day *i.v.*, days -1 to 7; 2) LF, 2 mg/kg/day *s.c.*, days 0-7; and 3) anti-CD45RB mAb + LF according to the same regimen as in groups 1 and 2. Untreated recipients and age-matched BALB/c naive mice were used as rejecting and normal controls.

Heterotopic cardiac transplantation

Treated and untreated BALB/c mice were subject to allogeneic cardiac transplantation using C57BL/6 donors. Direct abdominal palpation was used for assessing graft viability. Heterotopic heart transplantation was performed according to routine procedure in this laboratory (23). The pulsation of heart grafts was monitored daily by two independent observers without prior knowledge of the treatment protocol. The degree of pulsation was scored as A, beating strongly; B, noticeable decline in the intensity of pulsation; or C, complete cessation of pulsation. Recipients with grafts surviving >100 days were defined as tolerant and were used for *in vitro* experiments. Untreated recipients (BALB/c) rejected allografts on day 8 and were used as rejecting controls.

Isolation of splenic DC

For each experiment, five mice from the following groups were sacrificed: recipients with long-term (>100-day) allograft survival, rejecting controls, or untreated mice. Mononuclear cells were isolated from spleens by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and labeled with anti-mouse CD11c mAb-conjugated superparamagnetic microBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD11c⁺ cells were isolated by passage through a magnetic column.

Generation of bone marrow-derived DC

The procedure used in our laboratory to generate DC was described elsewhere (24). In brief, bone marrow cells were flushed from the femurs and tibias of tolerant, rejecting, and naive mice, washed, and cultured at 2×10^6 cells/well in 24-well plates (Corning Glass, Corning, NY) in 2 ml RPMI 1640 (Life Technologies, Ontario, Canada) supplemented with 10% FCS (Life Technologies), 100 U/ml of penicillin, 100 μ g/ml of streptomycin, 50 μ M of 2-ME (Life Technologies), 10 ng/ml of murine rGM-CSF (Peprotech, Rocky Hill, NJ), and 10 ng/ml of IL-4 (Peprotech). Nonadher-

ent cells were removed after 48 h of culture, and fresh medium was added every 48 h. DC were used for *in vitro* experiments after 7 days of culture.

Flow cytometry

Phenotypic analysis of isolated or cultured DC was performed on a FACScan (BD Biosciences, San Jose, CA). All Abs were purchased from BD PharMingen (San Diego, CA), unless otherwise indicated. For T cells we used FITC-, PE-, or CyChrome-conjugated anti-mouse CD4, CD152 (CTLA-4), CD45RB mAb, and CD25 (eBiosciences, San Diego, CA). For DC we used FITC- or PE-conjugated anti-mouse I-A^d, CD11c, CD40, and CD86 mAb. CD152 and IL-12 expression was assessed by intracellular staining using a cell permeabilization kit (Caltag Laboratories, Burlingame, CA). T cell and DC subsets were analyzed by two- or three-color staining with various combinations of mAbs. All flow cytometric analyses were performed using appropriate isotype controls (Cedarlane Laboratories, Hornby, Ontario, Canada).

RT-PCR for cytokine determination

Total RNA was purified from T cells using TRIzol (Life Technologies Invitrogen, Burlington, Ontario, Canada), according to the manufacturer's protocol, after MLR stimulation with DC from tolerant or rejecting allograft recipients. First strand cDNA was synthesized using an RNA PCR kit (Life Technologies) with the oligo(dT)₁₆ primer. Each PCR consisted of 1 μ l of cDNA, 100 nM of the primers for GAPDH as a reference gene, and 200 nM of each pair of specific primers for cytokines. The primers used in this study were: IFN- γ (376 bp), sense, 5'-AGCTCTGAGACAATG AACGCTACAC-3' and antisense, 5'-ACCTGTGGGTTGTTGACCTCAA AC-3'; IL-2 (417 bp), sense, 5'-ACATTGACACTTGTGCTCCGT GTC-3' and antisense, 5'-TTGAGGGCTTGTGAGATGATGCT-3'; IL-4 (432 bp), sense, 5'-AGCTAGTTG TCATCTGCTCTTC-3' and antisense, 5'-AGCATGGTGGCTCAGTACTACG-3'; IL-10 (404 bp), sense, 5'-TGCTATGCTGCCTGCTCTTACTGAC-3' and antisense, 5'-AATCA CTCTTCACTGCTCCACTG-3'; GAPDH (249 bp), sense, 5'-TGATGA CATCAAGAAGGTGGTGAA-3' and antisense, 5'-TCCTTGAGGCCA TGTAGGCCAT-3'. The samples were denatured for 1 min at 94°C, annealed for 1 min at 53°C, and extended for 1 min at 72°C, for a total of 35 cycles. The PCR products were subjected to 2% agarose electrophoresis containing ethidium bromide and visualized by UV illumination.

Mixed leukocyte reaction

Splenic DC isolated from tolerant or rejecting recipients (BALB/c) were irradiated at 3000 rad. Varying numbers of DC were seeded in triplicate in a flat-bottom 96-well plate (Corning) for use as stimulator cells. T cells were prepared from spleens and isolated by T cell enrichment columns. T cells ($1-5 \times 10^5$ /well) from C57BL/6 mice were added to the DC cultures, with the final MLR taking place in 200 μ l of RPMI 1640 (Life Technologies) supplemented with 10% FCS (Life Technologies), 100 U/ml of penicillin (Life Technologies), and 100 μ g/ml of streptomycin (Life Technologies). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ for 3 days, and pulsed with 1 μ Ci of [³H]thymidine (Amersham Pharmacia Biotech) for the last 16 h of culture. Cells were harvested onto glass fiber filters, and the radioactivity incorporated was quantitated using a Wallac Betaplate liquid scintillation counter. Results were expressed as the mean cpm of triplicate cultures \pm SEM.

Alternatively, to assess T cell function in MLR, CD4⁺ T cells were isolated from tolerant or rejecting recipients (BALB/c) and used as responders (2×10^5 /well). Irradiated (3000 rad) spleen cells from C57BL/6 (donor-specific stimulation) or C3H (third party control) were added to the cultures as stimulators (5×10^5 /well). The experiment was conducted as described above.

To determine the ability of T cells to inhibit a MLR, T cells isolated from tolerant or rejecting recipients (BALB/c) were added to an MLR using normal BALB/c T cells as responders (2×10^5 /well) and irradiated (3000 rad) C57BL/6 spleen cells as stimulators (2×10^5 /well). The experimental procedure was the same as described above.

Histopathological examination

Heart grafts were harvested, fixed in 10% buffered formaldehyde, embedded in paraffin, and then stained with hematoxylin-phloxine saffron. The 5- μ m sections were examined for evaluation of rejection. Criteria for rejection included the presence of vasculitis, infarction, lymphocytic infiltration, thrombosis, and hemorrhage. These changes were scored as: 0, no change; 1, minimum change; 2, mild change; 3, moderate change; or 4, marked change (25). Pathology slides were read by a pathologist (B. Garcia) without knowledge of treatment for each case.

Statistical analysis

Graft survival was compared among experimental groups using the rank-log test. The unpaired Student *t* test, assuming equal variances, was used to determine the statistical significance of the difference in mean cell number or mean percentage in flow cytometry. This test was also used analyzing data in which two groups were compared. MLR data were analyzed using one-way ANOVA, followed by the Newman Keuls Test. Differences with *p* values less than 0.05 were considered significant.

Results

LF inhibits DC and anti-CD45RB mAb inhibits T cell responses in vitro

CD45 is a single chain glycoprotein expressed on hemopoietic cells such as T cells. Members of the CD45 family of transmembrane protein tyrosine phosphatases are critically involved in lymphocyte activation. The anti-CD45RB mAb used in this study is directed to the RB cluster of CD45. This Ab can transiently deplete lymphocytes (26) and generate regulatory cells by shifting CD45RB from high to low (27, 28). In our in vitro study, T cells pretreated with anti-CD45RB mAb for 4 h had suppressed proliferative responses in MLR. The inhibition of T cell responses by anti-CD45RB mAb was dose dependent (Fig. 1). The decreased T cell response may be attributed to T cell apoptosis after treatment with anti-CD45RB mAb (29).

DSG was reported to prevent DC maturity and impair APC function. To investigate whether LF, a newly developed analog of DSG, affects DC maturity and function, we treated DC in vitro with LF. Bone marrow progenitors isolated from BALB/c mice were cultured in the presence of recombinant murine GM-CSF (10 ng/ml) and IL-4 (10 ng/ml). LF was added to DC culture at concentrations of 5–10 μ g/ml from days 1 to 5 of culture. Expression of MHC class II and costimulatory molecules was assessed by flow cytometry. After LF treatment, expression of MHC class II, CD40, and CD86 was significantly decreased as compared with nontreated control DC, indicating that LF inhibited DC maturity (Fig. 2A). Using LF-treated DC as stimulators, the allogeneic MLR was significantly inhibited (Fig. 2B).

Synergy of anti-CD45RB mAb and LF in tolerance induction

The C57BL/6 (H-2^b) > BALB/c (H-2^d) strain combination is a stringent murine model of cardiac transplantation. We have pre-

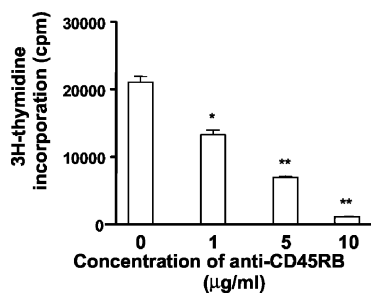


FIGURE 1. Anti-CD45RB mAb inhibits T cell response in vitro. CD4⁺ T cells from BALB/c mice were treated with anti-CD45RB mAb in vitro at indicated concentrations for 4 h. Subsequently, cells were washed to remove Ab and were seeded in triplicate, in a 96-well round-bottom plate as responders (2×10^5 cells/well). C57BL/6 spleen cells were irradiated (3000 rad) and used as stimulators (2×10^5 cells/well). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ for 3 days, and pulsed with 1 μ Ci [³H]thymidine for the last 16 h. Cells were harvested onto glass fiber filters, and the amount of radioactivity was determined in a scintillation counter. The data represent means \pm SEM and are representative of three repeat experiments (*, *p* < 0.05; **, *p* < 0.01 by unpaired Student's *t* test as comparing with untreated control).

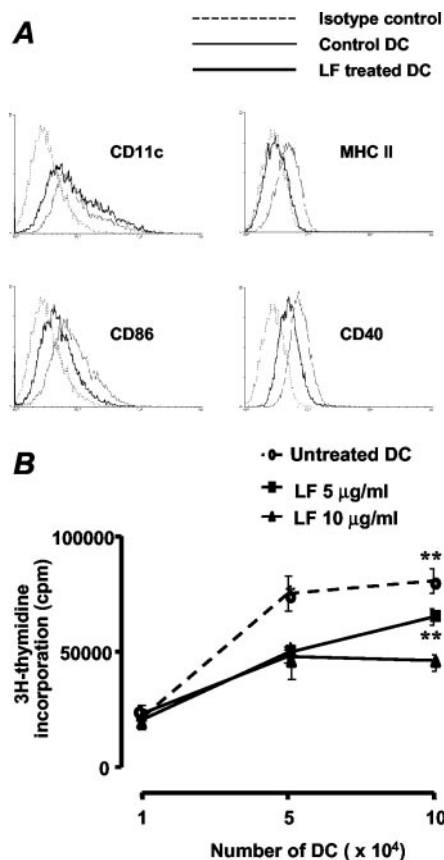
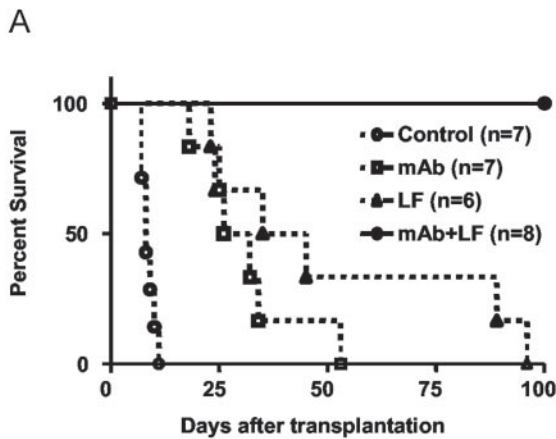


FIGURE 2. LF inhibits DC maturation and function in vitro. *A*, LF inhibits expression of MHC class and costimulatory molecule expression on DC. Bone marrow progenitors derived from BALB/c mice were cultured for 7 days (see *Materials and Methods*). LF was added in culture at 10 μ g/ml from days 0 to 5. After this, DC were washed to remove LF and were cultured for 2 additional days. LF-treated DC (bold filled lines) and untreated control DC (filled lines) were harvested for FACS analysis. Rat anti-mouse IgG2a was used as isotype controls (broken lines). *B*, LF-treated DC show poor allostimulatory capacity. DC (BALB/c) cultured from bone marrow progenitors were treated with LF, as described above, at 5 μ g/ml (■) or 10 μ g/ml (▲). DC in culture medium were used as controls (○). LF-treated DC were irradiated (3000 rad) and used as stimulators at indicated numbers. T cells from C57BL/6 mice were used as responders (5×10^5 cells/well). MLR was performed as described in Fig. 1. Data represent mean \pm SEM and are representative of three experiments (*, *p* < 0.05; **, *p* < 0.01 by one-way ANOVA and Newman-Keuls test).

viously demonstrated that in this model, neither cyclosporin A (30) nor anti-CD45RB mAb (23) prevented allograft rejection. The untreated recipients rejected allografts on day 8. Monotherapy by administration of anti-CD45RB mAb or LF alone marginally increased graft survival to 29 and 40 days, respectively. However, coadministration of these two agents achieved permanent acceptance (>100 days, Fig. 3A). Furthermore, histological assessment at 100 days posttransplantation showed sign of neither chronic rejection nor arteriosclerosis (Fig. 3B). Tolerance was donor specific since C57BL/6 skin grafts were accepted for >100 days, whereas C3H (H-2^k) third party grafts were rejected in 11 days (not shown). Collectively, these data indicate that the combination of anti-CD45RB mAb and LF induces donor-specific tolerance.

Generation of Treg and Tol-DC in tolerant recipients

CD4⁺ T cells isolated from long-term survivors showed lower response to allogeneic stimulation in MLR. The hyporesponsiveness was donor specific because decreased response was seen in



B

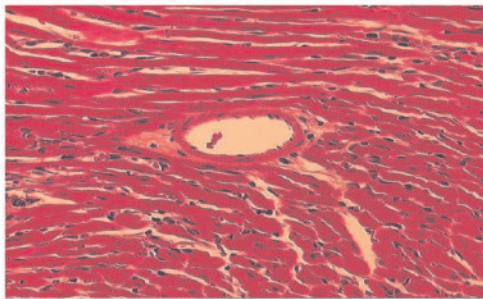
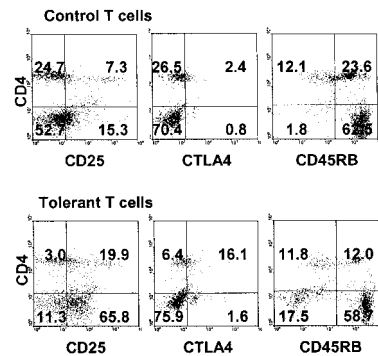


FIGURE 3. Treatment with LF and anti-CD45RB mAb acts synergistically to induce tolerance in allogeneic cardiac recipients. *A*, Survivor proportions. BALB/c recipients were administered with anti-CD45RB mAb (3 mg/kg i.v., days -1 to 7) alone (\square), LF (2 mg/kg s.c., days 0-7) alone (\blacktriangle), or anti-CD45RB mAb (3 mg/kg i.v., days -1 to 7) + LF (2 mg/kg s.c., days 0-7) combination (\bullet). No further immunosuppression was used. Untreated mice served as a control (\circ). MHC fully mismatched heterotopic allogeneic cardiac transplantation was performed from C57BL/6 (H-2^d) to BALB/c (H-2^b) mice. The rejection of cardiac grafts was judged by the cessation of heartbeat. The number of animals in each group is indicated. *B*, Histology. A representative C57BL/6 heart allograft was harvested from an anti-CD45RB mAb + LF-treated BALB/c recipient on day 100 posttransplantation. The graft was fixed in 10% buffered formaldehyde, embedded in paraffin, and then stained with hematoxylin-phloxine saffron. The microscopic sections were examined for evaluation of rejection based on the criteria described in *Materials and Methods*. The microphoto shows normal histology of a transplanted heart without evidence of acute or chronic rejection (magnification $\times 40$).

MLR using donor-derived (C57BL/6) stimulators, but not third party, C3H stimulators (data not shown). This observation prompted us to investigate the possibility of Treg cells being present in the tolerant recipients. Flow cytometric analysis of recipient T cells was performed to identify phenotypic changes associated with tolerance induction. Tolerant recipients (>100 days graft survival) had an increase in the CD4⁺CD25⁺ and CD4⁺CTLA-4⁺ cells, and a decrease of CD4⁺CD45RB^{high} cells compared with control animals (Fig. 4A). To assess overall regulatory cell function in tolerant recipients, CD4⁺ T cells isolated from recipients with long-term surviving allografts were added to an ongoing MLR. CD4⁺ T cells from tolerant recipients and control BALB/c mice were added at various concentrations to a MLR consisting of BALB/c responders and C57BL/6 stimulators. Addition of CD4⁺ T cells from tolerant recipients inhibited ongoing MLR in a dose-dependent manner, whereas CD4⁺ T cells from control mice did not affect MLR (Fig. 4B). Inhibition of MLR was

A



B

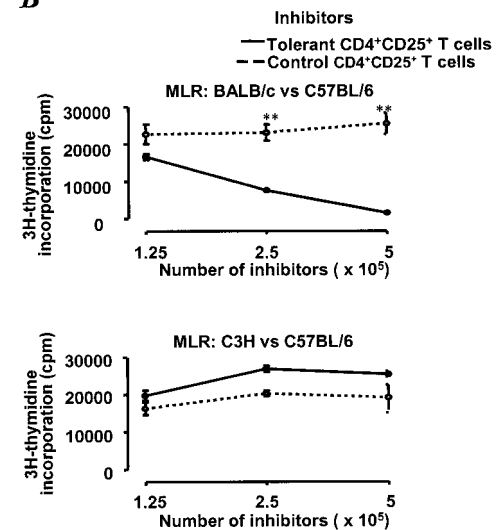


FIGURE 4. Treg are detected in tolerant recipients. *A*, Phenotypic analysis. T cells were isolated from tolerant recipients (*lower panels*) or age-matched normal BALB/c mice (*upper panels*). The cells were double stained with FITC-labeled anti-mouse CD4, PE-labeled anti-mouse CD152 (CTLA4) or anti-mouse CD45RB, and CyChrome-labeled anti-mouse CD25. The cells were analyzed by flow cytometry. *B*, Inhibition of MLR. BALB/c (*upper panel*) or third party C3H (*lower panel*) CD4⁺ T cells were isolated by positive selection and used as responders (2×10^5 cells/well). C57BL/6 spleen cells were irradiated (3000 rad) and seeded in triplicate in a 96-well round-bottom plate and used as stimulators (2×10^5 cells/well). CD4⁺ T cells (2×10^5 cells/well) from tolerant (>100 days) recipients (filled lines) or from control BALB/c mice (broken lines) were added to each well as inhibitors. MLR was performed as described in Fig. 1. The data represent means \pm SEM and are representative of six repeat experiments (**, $p < 0.01$ by unpaired Student's *t* test).

Ag specific because CD4⁺ T cells from tolerant recipients did not inhibit MLR between third party C3H responders and C57BL/6 stimulators (Fig. 4B). Furthermore, we have found that the inhibitory activity resides in the CD4⁺CD25⁺ T cell fraction, but not the CD4⁺CD25⁻ fraction (data not shown).

Because Tol-DC are considered to be involved in tolerance, we assessed whether such cells were expanded *in vivo* as a result of treatment. DC were purified from the spleens of tolerant recipients (>100 days graft survival), and from untreated recipients rejecting their grafts on day 8. A decrease in MHC class II expression, CD40, and intracellular IL-12 was seen in DC from tolerant mice

compared with age-matched controls and those rejecting their grafts (Fig. 5A). Decreased expression of these molecules is consistent with the Tol-DC phenotype. To assess whether DC from tolerant mice functionally act as Tol-DC, their ability to stimulate an MLR was investigated using C57BL/6 cells as responders. Splenic DC from the BALB/c mice undergoing rejection stimulated proliferation of C57BL/6 responders in a dose-dependent manner. In contrast, DC from tolerant recipients failed to stimulate C57BL/6 proliferation (Fig. 5B). The poor allostimulatory response was not strain specific because DC from tolerant recipients also did not stimulate third party (C3H) T cells (not shown). Tol-DC isolated from tolerant mice seemed to be maturation resistant, as treatment with anti-CD40 did not induce phenotypic maturation, but was successful at inducing maturation in control DC (not shown). Furthermore, DC isolated from tolerant recipients stimulated Th2 differentiation of allogeneic T cells, as demonstrated by increased expression of IL-4 and IL-10 mRNA and suppressed expression of the Th1 cytokines IFN- γ and IL-2 mRNA (Fig. 5C).

Therefore, it appears that tolerance induced by our combined treatment protocol of anti-CD45RB mAb and LF increased the number and function of Treg and Tol-DC. The fact that both Treg and Tol-DC are present 100 days after transplantation suggests that graft rejection is prevented by an immunoregulatory loop between these two cell types.

Inhibitory feedback loop between Tol-DC and Treg in transplant tolerance in vitro

Because functional Treg and Tol-DC were identified in the tolerant mice for >100 days posttransplantation, we postulated that a regulatory feedback loop exists between Tol-DC and Treg. It has been reported that CD4⁺CD25⁺ cells can be generated in vitro by activation of naive T cells in the presence of immature DC that possess low levels of MHC class II, CD80/86, and IL-12, but secrete high levels of IL-10 (10, 20). In contrast, anergized T cells secreting TGF- β and IL-10 also can promote the generation of Tol-DC with immature phenotype (21, 22). However, the mutual relationship between Tol-DC and Treg has never been investigated in transplant tolerance. To test the possibility that Tol-DC from tolerant recipients generate Treg, or vice versa, in vitro priming experiments were conducted. Naive CD4⁺ T cells from untreated BALB/c mice were activated with anti-CD3 mAb and cocultured with DC from tolerant mice, rejecting mice, or naive mice. After 5 days, there was an increased number of CD4⁺CD25⁺ cells in the coculture with DC from the tolerant mice (20.1%) compared with the cocultures with rejecting DC (13%), and naive DC (14.2%) (Fig. 6A). Although the differences in CD4⁺CD25⁺ cell numbers were small, they were consistently reproducible. Assessment of Treg function was performed by FACS sorting. Purified CD4⁺CD25⁺ cells generated from coculture with the various DC (control, tolerant, or rejecting mice) were added into an ongoing MLR consisting of BALB/c responders and C57BL/6 stimulators. CD4⁺CD25⁺ T cells generated by coculture with DC from tolerant recipients strongly suppressed the ongoing MLR, while CD4⁺CD25⁺ T cells from coculture with rejecting DC had no inhibitory effect on MLR (Fig. 6B). The CD4⁺CD25⁻ cells generated in vitro by coculture with DC from tolerant or rejecting mice had no effect on the ongoing MLR (data not shown). These data suggest that Tol-DC isolated from tolerant recipients can generate suppressive CD4⁺CD25⁺ Treg from naive T cells in vitro.

The inhibitory feedback loop model that we are proposing implies that Treg cells contribute to the generation of Tol-DC. To assess this hypothesis, in vitro, bone marrow-derived DC were generated in the presence of CD4⁺ T cells purified from tolerant,

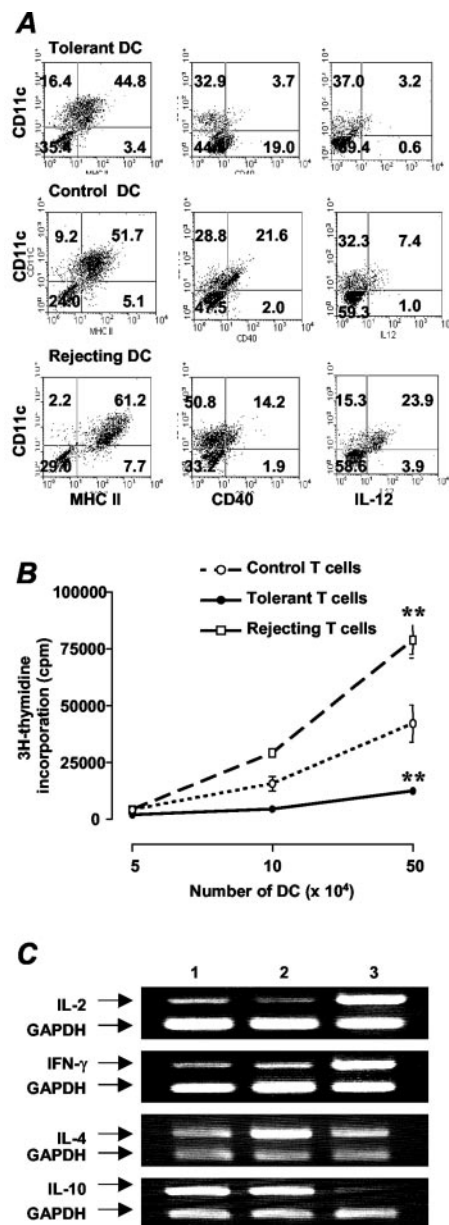


FIGURE 5. Tol-DC identified in tolerant recipients. **A**, Immature and DC2-like phenotypes in splenic DC isolated from tolerant recipients. Splenic DC were separated by CD11c⁺ MACS beads from tolerant recipients on day 100 (upper panel), from age-matched controls (middle panel), or from nontreated rejecting recipients on day 8 (lower panel). The cells were double stained with FITC-labeled anti-mouse CD11c mAb and PE-labeled anti-mouse I-A^d, CD40, and IL-12 (p70), respectively. The cells were analyzed on flow cytometry. **B**, MLR using DC isolated from tolerant recipients. Splenic CD11c⁺ DC were isolated from tolerant, age-matched control or rejecting recipients. DC were irradiated (3000 rad) and used as stimulators at indicated cell numbers. T cells from allogeneic C57BL/6 and third party C3H mice were used as responders (5×10^5 cells/well). Cells were cultured at 37°C with 5% CO₂ in a humidified atmosphere for 3 days and pulsed with 1 μ Ci of [³H]thymidine in the last 16 h. Cells were harvested onto glass fiber filters, and the radioactivity incorporated was quantitated in a scintillation counter. Data represent mean \pm SEM and are representative of five experiments (**, $p < 0.01$ by unpaired Student's t test). **C**, Tolerant DC promote Th2 differentiation. Allogeneic T cells were isolated from C57BL/6 spleens and incubated with CD11c⁺ DC derived from normal mice (lane 1), long-term survival (>100 days) recipients (lane 2), and untreated allograft-rejected mice (lane 3). After 48-h incubation, T cells were isolated by a T cell enrichment column, and mRNA of T cells was extracted from each culture. The expression of IFN- γ , IL-10, and IL-4 was assessed by RT-PCR, as described in *Materials and Methods*. Each lane shown here is a representative of three mice.

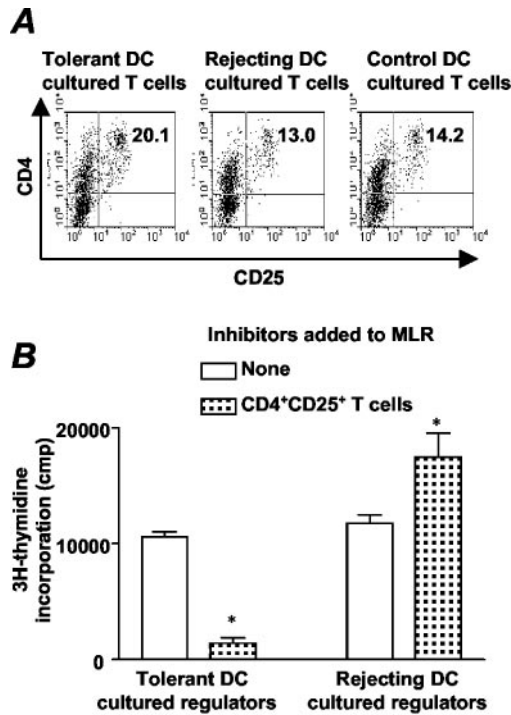


FIGURE 6. Tolerant DC generate Treg in vitro. *A*, Phenotypic analysis. DC were isolated from spleens of tolerant recipients (>100 days), rejecting recipients (day 8), or age-matched normal BALB/c mice. DC (1×10^6) were incubated with T cells (5×10^6) from normal BALB/c mice in the presence of $1 \mu\text{g/ml}$ of anti-mouse CD3 ϵ mAb. After a 5-day coculture, T cells were double stained with FITC-labeled anti-CD4 mAb and Cy-Chrome-labeled anti-CD25 mAb. Cells were sorted and analyzed on flow cytometry. *B*, CD4⁺CD25⁺ T cells generated by tolerant DC inhibit MLR. BALB/c T cells were used as responders (5×10^5 cells/well), and C57BL/6 spleen cells (irradiated at 3000 rad) were used as stimulators (5×10^5 cells/well). CD4⁺CD25⁺ T cells from the cocultures in above (Fig. 6A) were isolated by FACS sorting, and were added to each well as inhibitors (5×10^5 cells/well). MLRs with CD4⁺CD25⁺ inhibitors (▨) or without inhibitors (□) were performed as described in Fig. 1. Data represent mean \pm SEM and are representative of three experiments (*, $p < 0.05$ by unpaired Student's *t* test).

rejecting, or naive recipients. MHC class II expression was 21.4% on DC raised in the presence of T cells from tolerant mice, 35.5% on DC raised with T cells from naive mice, and 43.6% on T cells from rejecting mice. A similar pattern was seen for CD86 expression (6.2, 8.1, and 11.2% after coculture with tolerant, control, and rejecting CD4⁺ T cells, respectively) and CD40 expression (18.9, 27.6, and 40.9% after coculture with tolerant, control, and rejecting CD4⁺ T cells, respectively) (Fig. 7A). DC raised in the presence of CD4⁺ T cells from tolerant mice were the weakest allostimulators of MLR, whereas DC raised in the presence of CD4⁺ T cells from rejecting mice possessed the strongest allostimulatory ability. DC raised in the presence of naive CD4⁺ T cells had intermediate allostimulatory capacity (Fig. 7B). Collectively, these data suggest that tolerance induced by anti-CD45RB mAb + LF is associated with induction of a self-maintaining regulatory feedback loop between Tol-DC and Treg that maintains graft survival.

Discussion

In this study, we have shown that both Treg and Tol-DC can be isolated from allograft recipients made tolerant by a short course treatment of anti-CD45RB mAb and LF. To our knowledge, this is the first report that Treg isolated from an in vivo state of tolerance

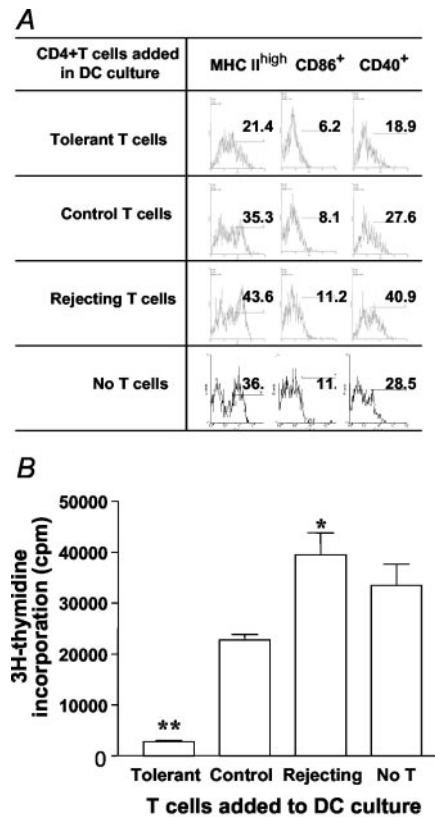


FIGURE 7. Treg generate Tol-DC in vitro. *A*, Phenotypic analysis of DC after incubation with T cells isolated from tolerant recipients. CD4⁺ T cells were isolated from spleens of tolerant recipients (>100 days), rejecting recipients (8 days), or age-matched normal BALB/c mice, respectively. Syngeneic (BALB/c) bone marrow progenitors (8×10^6) were cultured in six-well plate in 4 ml complete RPMI medium in the presence of GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) in the presence or absence of T cells. CD4⁺ T cells (1×10^6) were added to DC cultures on day 0. On day 7, DC were harvested. Ab staining and flow cytometric analysis was performed as described in Fig. 2. *B*, Poor allostimulatory capacity of DC after incubation with T cells from tolerant recipients. DC were incubated alone, or with T cells isolated from tolerant recipients, age-matched normal BALB/c mice, and rejecting recipients, as described above (A). T cell-cocultured DC (1×10^5 cells/well) were used as stimulators at indicated cell numbers. C57BL/6 T cells were used as responders (5×10^5 cells/well). MLR was performed as described in Fig. 1. Data represent mean \pm SEM and are representative of three experiments (*, $p < 0.05$; **, $p < 0.01$ by unpaired Student's *t* test).

can guide the in vitro differentiation of DC into Tol-DC, and that in vivo isolated Tol-DC can stimulate the in vitro production of Treg from naive T cells. This suggests that tolerance is associated with a self-maintaining feedback loop between Treg and DC progenitors, and Tol-DC with naive T cells (Fig. 8).

Others have demonstrated that Treg, consistent with the phenotype we described in this study, play a critical role in tolerance induction. Kingsley et al. (3, 18) showed that tolerance to allogeneic skin grafts induced by donor-specific transfusion plus anti-CD4 mAb can be transferred to naive mice by CD4⁺CD25⁺ cells, but not by CD4⁺CD25⁻ cells. Furthermore, tolerance to allogeneic islet cells induced by 1 α ,25-dihydroxyvitamin D3 and mycophenolate mofetil was also transferred to naive recipients by the CD4⁺CD25⁺, but not by CD4⁺CD25⁻ compartment (31). In both studies, the CD4⁺CD25⁺ cells secreted IL-10 and expressed CTLA-4. In our study, the phenotype of Treg in tolerant recipients was CD4⁺CD25⁺CTLA4⁺, and RT-PCR revealed expression of

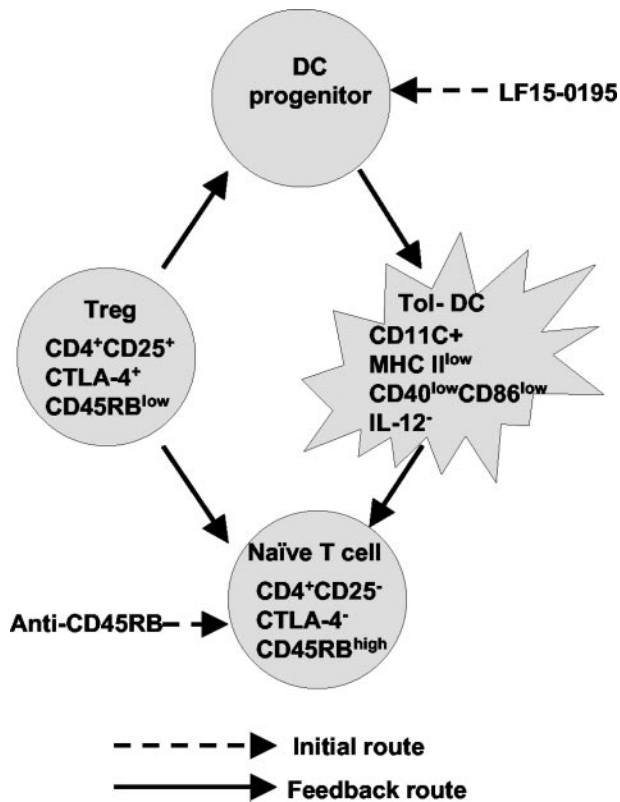


FIGURE 8. Proposed regulatory feedback loop in transplantation. LF targets DC and facilitates generation of MHC II^{low}, CD40^{low}, CD86^{low}, and IL-12⁻ Tol-DC. Anti-CD45RB Ab targets T cells and promotes augmentation of CD4⁺CD25⁺CTLA4⁺CD45RB^{low} Treg. Tol-DC instructs the differentiation of naive T cells into Treg and vice versa, thus forming a regulatory feedback loop that maintains the tolerant state.

IL-10. Controversy exists surrounding Ag specificity of T cell suppression by CD4⁺CD25⁺ Treg. Earlier studies by Shevach (32) suggested that these Treg are activated in an Ag-specific manner, but suppression is Ag nonspecific. Yet, in the above-mentioned examples of tolerance transfer by CD4⁺CD25⁺ Treg, suppression was specific to donor Ag (3, 31). Treg isolated from tolerant mice in our study possessed the ability to mediate Ag-specific suppression, as shown by inhibition of recipient-donor MLR, but not MLR using a third party instead of the recipient strain. We observed increases in CD4⁺CD25⁺ Treg in the spleen. However, others have reported that the anatomical location from where these cells are derived is also important. Green et al. (33) showed that only CD4⁺CD25⁺ Treg isolated from pancreatic lymph nodes of a diabetes-protected mouse, but not Treg isolated from inguinal lymph nodes, could transfer protection. CD4⁺CD25⁺ cells localized in the microenvironment of the lung maintain an immunosuppressive environment in lung cancer patients, whereas increases in the number of these cells are not seen systemically (34). We are presently investigating the importance of Treg in the draining lymph nodes of mice made tolerant using our protocol.

Different subsets of DC are involved in the activation or suppression of T cell responses. DC suppression of T cell responses can occur through weak costimulation (14, 35, 36), or through the activation of Treg (37, 38). We use the term Tol-DC to encompass DC subsets that possess an immature phenotype (low expression of MHC class II, CD40, CD80, CD86, and IL-12, and high expression of IL-10). Tol-DC include lymphoid, CD8α⁺ DC that direct Th2 differentiation (39), immature myeloid DC that activate Treg (20), and hepatic DC that possess weak costimulatory molecules

and are believed to be responsible for the low immunogenicity of hepatic allografts (40). In the context of transplantation, generation of Tol-DC has been thought to occur as a secondary mechanism for commonly used antirejection drugs (41). An active antirejection effect of Tol-DC has been seen in studies in which the administration of immature DC promotes tolerance induction (42). In our study, we found increased numbers of immature splenic DC that are poor allostimulators in vitro. Confirmation of Tol-DC function in our model was provided by the ability of the purified splenic DC to induce secretion of the Th2 cytokines IL-4 and IL-10, while suppressing expression of Th1 cytokines IFN-γ and IL-2 in MLR. DC maturation was previously shown to be dependent on NF-κB activation. LF mediates its immune-suppressive function by blocking NF-κB activation.⁴ Recently, it was shown that Tol-DC possess suppressed NF-κB activity (43); therefore, LF generation of Tol-DC may occur through inhibition of this pathway.

In this study, Treg and Tol-DC were isolated from tolerant recipients 100 days after transplantation, even though the immune-suppressive treatment with anti-CD45RB mAb and LF was only administered for 8–9 days posttransplantation. The observation that Treg and Tol-DC still possessed ex vivo suppressive activity on MLR such a long time after cessation of treatment suggested that either permanent changes occurred in these cells, or that a self-renewal mechanism existed for de novo production of regulatory cells. Incubation of Tol-DC from tolerant mice with naive T cells resulted in the in vitro production of CD4⁺CD25⁺CTLA-4⁺ Treg, which were able to suppress ongoing MLR. Ex vivo generation of Treg has previously been reported after cocultivation with Tol-DC (20). An active role for Tol-DC in generation of Treg is important for providing Ag specificity, as well as costimulation. Because the expression of costimulatory molecules CD80/86 on Tol-DC is lower than on DC from normal or rejecting mice, the generation of Treg by Tol-DC may need a lower level of costimulation than that received for the production of effector T cells. The complete absence of costimulatory signals on DC may be connected with the induction of T cell anergy. Active suppression of T cell responses by DC coinhibitory molecules such as PD-1 ligand (44) and CD47 (45) has been described. Although coinhibitory molecules on Tol-DC such as the Ig-like transcript-3 and -4 are important for generation of CD8⁺ Treg (46), little is known about such molecules that facilitate CD4⁺CD25⁺ Treg formation. In our model system, Tol-DC were purified from tolerant recipients. Our recent data show that in vitro treatment of DC progenitors with LF gives rise to Tol-DC with the same phenotype as the ex vivo isolated Tol-DC described in this study (unpublished data). Furthermore, in vitro generated Tol-DC are also capable of inducing the expansion of CD4⁺CD25⁺ Treg in vitro (unpublished data). Tol-DC isolated from tolerant recipients, or generated in vitro by LF treatment, may offer a practical method of generating Treg for Ag-specific suppression of pathological immune responses.

In this study, generation of Tol-DC from DC progenitors was also observed by incubation with CD4⁺CD25⁺ Treg from tolerant recipients. This type of Treg has been reported to possess surface-bound TGF-β, and to secrete IL-10 (47), both of which can induce generation of Tol-DC (48, 49). The ability of Treg to inhibit DC maturation was previously demonstrated by coculture of CD4⁺CD25⁺ cells with bone marrow DC progenitors. Cederbom

⁴ J. Yang, S. M. Bernier, T. E. Ichim, X. Xia, D. Zhou, X. Huang, G. H. Strejan, R. Zhong, and W.-P. Min. LF15-0195 generating tolerogenic dendritic cells is associated with suppression of NF-κB signaling through inhibition of IKK activity. *Submitted for publication.*

et al. (21) reported that DC incubated with CD4⁺CD25⁻ cells possessed high levels of CD80 and CD86, whereas cocultivation with CD4⁺CD25⁺ cells resulted in DC with low levels of CD80 and CD86 as well as poor allostimulatory ability. It should be noted, however, that the CD4⁺CD25⁺ cells were isolated from naive mice. Because, in our study, CD4⁺CD25⁺ cells from tolerant recipients were more potent suppressors of MLR than CD4⁺CD25⁺ cells from naive and rejecting recipients, it is possible that CD4⁺CD25⁺ cells may vary in their tolerogenic ability depending on their previous *in vivo* environment. When CD4⁺CD25⁺ cells from tolerant recipients were incubated with naive DC progenitors, Tol-DC were generated. This was in contrast to DC progenitors incubated with CD4⁺CD25⁺ T cells from naive or rejecting animals. Similarly, Tol-DC isolated from tolerant, but not naive or rejecting mice induced production of CD4⁺CD25⁺ Treg that blocked MLR in a donor Ag-specific manner.

We propose that tolerance induction in the C57BL/6 > BALB/c cardiac allograft model by anti-CD45RB mAb and LF occurs through the initiation of a self-maintaining regulatory loop, which we have reproduced *in vitro* (Figs. 6 and 7). The role of anti-CD45RB mAb is to provide a period of Th1 cell suppression during which LF can give rise to Tol-DC. We speculate that LF alone is capable of inducing formation of the tolerogenic loop, but suppression of T cell responses for a window of time is needed. Once the tolerogenic loop is established, Ag-specific self maintenance occurs. Ag specificity in this study was demonstrated by rejection of third party skin graft on tolerant mice, and on the inability of Treg to inhibit an MLR with third party responders. Using LF monotherapy in a rat model of cardiac allotransplantation, Chiffolleau et al. (4) suggested that Treg generation in LF-treated recipients is dependent on donor APC; however, direct involvement of DC was not assessed. Allostimulatory APC have been demonstrated to give rise to Treg in other model systems (50). Cardiac allografts have previously been shown to possess passenger DC (51). However, in our model system, Tol-DC were of recipient origin, as they possessed BALB/c (H-2^d), but not C57BL/6 (H-2^b) MHC class II. The importance of recipient APC in transplantation tolerance was recently shown in experiments with MHC class II-deficient recipients, but not donor APC. In this experiment, induction of transplantation tolerance failed (52).

This study suggests the existence of a bidirectional interaction in which Tol-DC stimulate naive T cells to become Treg, and Treg stimulate differentiation of DC progenitors into Tol-DC. Understanding the mechanisms by which such a tolerogenic loop can be initiated in a clinical setting will offer potential treatments for diseases in which Ag-specific immune suppression is required.

Acknowledgments

We thank Christine Ichim for statistical analysis, and Drs. Reginald Gorczynski (University of Toronto, Toronto, Canada) and Peta J. O'Connell (University of Western Ontario) for critically reading the manuscript. We also thank Xiaotao Yan for excellent technical assistance, and Sharon Mutch for secretarial assistance.

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