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SYNERGISTIC TOLERANCE INDUCED BY LF15-0195 AND ANTI-CD45RB MONOCLONAL ANTIBODY THROUGH SUPPRESSIVE DENDRITIC CELLS[1]

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Background. **LF 15-0195 (LF), a novel analogue of 15-deoxyspergualin (DSG), inhibits maturation of dendritic cells (DC). Anti-CD45RB is a monoclonal antibody (mAb) that blocks activation of T-helper (Th) 1 cells and generates T-regulatory cells. This study addressed whether these two reagents act synergistically to inducing tolerance, and investigated associated cellular mechanisms.**

Methods. **BALB/c recipients were treated by a short course of mAb alone, LF alone, or the combination of both agents. Mice that accepted a C57BL/6 cardiac allograft for more than 100 days were considered tolerant. Splenic DC were purified using positive selection for CD11c. Bone marrow DC were generated by culture with interleukin-4 and granulocyte-macrophage colony-stimulating factor. Surface marker expression was determined by fluorescence-activated cell sorter analysis. DC function was assessed by the ability to stimulate or inhibit T cells in vitro.**

Results. **Although monotherapy with LF or mAb failed to induce tolerance, combination therapy resulted in long-lasting acceptance of allogeneic hearts (>200 days) and secondary donor skin grafts (>100 days). DC from tolerant recipients possessed lower major histocompatibility complex class II and CD40 expression, and were poorer co-stimulators for T-cell proliferation than control DC. Furthermore, DC from tolerant mice induced Th2 differentiation, suppressed overall T-cell proliferation, and were poor presenters of T cells specific for antigen to pigeon cytochrome *c* 81–104.**

Conclusions. **The combination of LF and anti-CD45RB mAb induced stable tolerance. The synergy of these two approaches appears to be mediated through formation of tolerogenic DC and T-regulatory cells.**

Agents that temporarily deplete T cells such as anti-CD45RB monoclonal antibodies (mAb), when administered before organ transplantation, have been shown to induce transplant tolerance (*1*, *2*). In addition, agents such as 15-deoxyspergualin (DSG) that inhibit antigen-presenting cell (APC) function are also capable of inducing tolerance (*3*). In the context of transplantation, the antigen-presentation step of T-cell activation determines whether the T cell will reject the graft, ignore the graft, or inhibit other T cells from attacking the graft. The T cell that blocks other T cells from attacking the graft is termed a T-regulatory (Treg) cell. By modulating the recipient's APC, it may be possible to cause expansion of graft protecting Treg cells without suppressing immune responses to other antigens.

Dendritic cells (DC) are the most potent APC that are capable of activating naive T cells (*4*). DC in the periphery capture and process antigens and, on activation, migrate to lymphoid organs where they express co-stimulatory molecules and secrete cytokines to initiate T-cell responses. DC are believed to play a critical role in the process of both central and peripheral tolerance (*5*). Although DC in the thymus are responsible for negative selection (*6*), some investigators have reported that immature DC in the periphery cause deletion of autoreactive cells (*7*, *8*) or lead to generation of self-reactive Treg cells, or both (*9*). The subsets of DC responsible for tolerance induction are not fully characterized yet and are different with respect to anatomic location and function. However, DC with an immature phenotype (low expression of major histocompatibility complex [MHC] class II, CD40, CD80, CD86, interleukin [IL]-12) are generally considered to possess tolerance-promoting properties and are termed tolerogenic DC (Tol-DC) (*10*). The role of Tol-DC in transplantation tolerance has been demonstrated in several animal models. For example, prolonged cardiac allograft survival was achieved in both mice and rats when donor strain immature DC were administered to the recipient before transplantation (*11–13*). The inherent tolerogenicity of liver allografts has been attributed, in part, to the large number of donor Tol-DC residing within the transplanted liver (*14*, *15*).

In light of the potential for Tol-DC to induce tolerance, the authors targeted DC function in transplant recipients using LF 15-0195 (LF), an analogue of DSG. The authors have previously shown that LF monotherapy (20-day course) induces permanent allograft tolerance (*16*); however, 20% of recipients died because of LF-induced toxicity. Because of the importance of the DC–T-cell interaction during graft rejection, the authors tested whether concurrent modulation of T-cell function by means of anti-CD45RB mAb treatment

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along with a safe, short-course, low-dose treatment of LF could result in enhanced graft survival.

MATERIALS AND METHODS

Animals and Tolerance Induction

Male C57BL/6, BALB/c, and C3H mice were purchased from Jackson Laboratories (Bar Harbour, Maine) and were used as donors, recipients, and third-party controls, respectively. Eight mice (BALB/c) of each group were treated with anti-CD45RB mAb 3 mg/kg/day, day −1 to 7 intravenously (group 1); LF 2 mg/kg/day, day 0 to 7 subcutaneously (group 2); and combination LF plus mAb using the same regimen as in groups 1 and 2 (group 3). Untreated allograft recipients were used as controls.

Heterotopic Cardiac Transplantation

Treated and untreated BALB/c mice were subjected 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 the authors' laboratory (*17*). Rejection of graft was judged by the cessation of heartbeat. Recipients with grafts surviving longer than 100 days were defined as tolerant and were used for in vitro experiments.

Generation of Bone Marrow-Derived Dendritic Cells

The procedure used in the authors' laboratory to generate DC was described elsewhere (*18*). In brief, bone marrow cells were flushed from the femurs and tibias of tolerant, rejective, naive mice, and washed and cultured at a concentration of 2×10^6 cells per well in 24-well plates (Corning, Corning, NY) in 2 mL RPMI 1640 (GIBCO Life Technologies, Burlington, Ontario, Canada) supplemented with 10% fetal calf serum (FCS) (GIBCO), 100 U/mL of penicillin, 100 μg/mL of streptomycin, 50 μM of 2-mercaptoethanol (GIBCO), 10 ng/mL of recombinant murine granulocyte-macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ), and 10 ng/mL of IL-4 (Peprotech). Nonadherant cells were removed after 48 hr of culture, and fresh medium was added every 48 hr. DC were used for in vitro experiments after 9 days of culture.

Isolation of Splenic DC from Allograft Recipients

Splenic mononuclear cells were isolated from recipient spleens by gradient centrifugation over Ficoll-Paque (Amersham, Pharmacia Biotech, Uppsala, Sweden) and labeled with anti-mouse CD11c mAb conjugated MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, the $CD11c^+$ cells were positively separated by a magnetic column. The $CD11c^+$ DC were used directly for further experiments.

Flow Cytometry

Phenotypic analysis of isolated or cultured DC was performed on a FACScan (Becton Dickinson, San Jose, CA). The following fluorescein isothiocyanate- or phycoerythrin-conjugated anti-mouse mAb were purchased from BD PharMingen (San Diego, CA): $I\text{-}A^d$, CD11c, CD40, CD86, and IL-12. IL-12 expression was assessed by intracellular staining using a cell permeabilization kit (Cedarlane Laboratories, Hornby, Ontario, Canada). All flow cytometric analyses were performed using appropriate isotype controls (Cedarlane).

Mixed Leukocyte Reaction

Splenic DC isolated from tolerant or rejecting recipients (BALB/c) were irradiated at 3,000 rad. Varying numbers of DC were seeded in triplicate in a flat-bottomed 96-well plate (Corning) for use as stimulator cells. T cells were prepared from spleens and isolated by T-cell enrichment columns. T cells ($1\text{–}5\times10^5$ cells/well) from C57BL/6 mice were added to the DC cultures, with the final mixed leukocyte reaction (MLR) reaction taking place in 200 μL of RPMI 1640 (GIBCO) supplemented with 10% FCS (GIBCO), 100 U/mL of penicillin (GIBCO), and 100 μg/mL of streptomycin (GIBCO). Cells were cultured for 3 days and pulsed with 1 μCi of ^{3}H-thymidine (Amersham Pharmacia Biotech) for the last 16 hr of culture. Cells were harvested onto glass fiber filters, and the radioactivity incorporated was quantitated using a Wallac Betaplate liquid scintillation counter (Beckman, Fullerton, CA). Results were expressed as the mean counts per minute of triplicate cultures±SEM.

T-Cell Proliferation Assays

To test co-stimulatory function of DC, purified BALB/c $CD4^+$ T cells (1×10^6 cells/well) were incubated with syngeneic DC (1×10^5 cells/well) from rejecting, tolerant, or normal mice in the presence of soluble anti-CD3 mAb (1 μg/mL). The cells were cultured in 96-well plates (Corning) in a total volume of 200 μL. After 3-day culture, cells were pulsed with 1 μCi of ^{3}H-thymidine (Amersham) for the last 16 hr. Cultures were harvested, and the incorporated ^{3}H-thymidine was determined with a Wallac Betaplate liquid scintillation counter.

To assess the ability of DC to stimulate antigen-specific T-cell responses, lymph node T cells were isolated from BALB/c mice immunized with pigeon cytochrome *c* peptide (PCC) 81–104. PCC-specific T cells (2×10^5 cells/well) were incubated with splenic DC (5×10^4 cells/well) isolated from tolerant, rejective, or naive mice in the presence of $PCC_{81\text{–}104}$ peptide (1–100 μg/mL) for 3 days. T-cell proliferation was assessed by ^{3}H-thymidine uptake assay as described above.

Enzyme-Linked Immunosorbent Assay

Bone marrow-derived DC (10^5, BALB/C origin) were cultured with the C57B/L6 T cells (1×10^6) for 48 hr. The supernatants were harvested and assessed for interferon (IFN)-γ and IL-4 by enzyme-linked immunosorbent assay (ELISA). Cytokine-specific ELISA (Endogen, Rockford, IL) was used for detecting protein concentrations in culture supernatants according to the manufacturer's instructions using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA). The amounts of IFN-γ and IL-4 produced by the T cells were determined from standard curves generated with recombinant murine IFN-γ and IL-4 (Endogen).

RESULTS

Combination of Anti-CD45RB mAb and LF Act Synergistically to Induce Tolerance

Untreated C57BL/6 cardiac allografts were rapidly rejected, with median survival of 7.5 days. Monotherapy with mAb or LF significantly prolonged C57BL/6 allograft survival to 42 and 40 days, respectively, but both agents alone failed to induce tolerance. In contrast, a short course of LF plus mAb resulted in indefinite survival (>200 days) (Table 1). The long-term survivors accepted skin grafts from the donor strain permanently (>100 days) and rejected third-party skin grafts (11 days, data not shown). These data indicate that LF and mAb act synergistically to induce donor-specific tolerance.

Pathologic examination at necropsy showed that allografts from untreated recipients, and those treated with LF or mAb alone, developed moderate to severe cellular and vascular rejection, characterized by massive cell infiltration, vasculitis, hemorrhage, infarction, and thrombosis (Fig. 1A–C). In contrast, allografts from long-term survivors (>200 days) treated with a short course of LF plus mAb showed normal histopathology of heart grafts and no evidence of chronic rejection (Fig. 1D). Therefore, targeting both T cells and APC

TABLE 1. Survival of C57BL/6 heart allografts in BALB/c mice

Group	Treatment	Survival (d×No.)	Median	P value[a]
1	Untreated	7×4, 8×4	7.5	
2	mAb 3 mg/kg, IV, d–1–7	35×2, 39, 42×2, 43×3	42	<0.001
3	LF 2 mg/kg, SC, d 0–7	24[b], 29, 35×2, 45×2, 89, 96	40	<0.001
4	mAb 3 mg/kg plus LF 2 mg/kg	>200×8	>200	<0.001

IV, intravenously; SC, subcutaneously.
[a] Compared with untreated control (log-rank test).
[b] The mouse died with a beating heart.

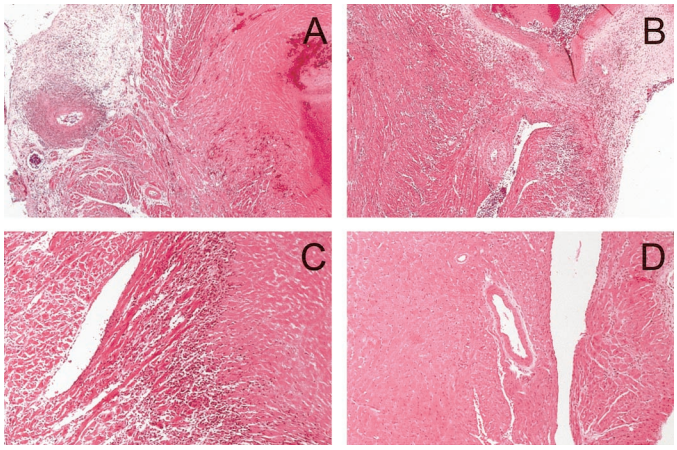


FIGURE 1. Heart grafts were harvested from (A) untreated control at day 7, (B) mAb treated on day 40 (end of rejection), (C) LF treated on day 40 (end of rejection), and (D) LF plus mAb on day 200. Note severe vascular and cellular rejection including the presence of vasculitis, infarction, lymphocytic infiltration, thrombosis, and hemorrhage in untreated, LF monotherapy, and mAb CD45RB monotherapy groups (A, B, and C). In contrast, an allograft on day 200 treated with LF plus mAb shows a normal histology of heart graft (D).

by treatment with anti-CD45RB mAb and LF, respectively, is an effective and practical strategy for inducing tolerance.

Bone Marrow DC Isolated from Tolerant Recipients Show Immature Phenotype and Promote T-Helper Cell Type 2 Polarization

Because a short term (8- to 9-day) treatment with the combination of anti-CD45RB mAb and LF resulted in indefinite tolerance to allografts (Table 1), the authors postulated that in addition to T-cell–mediated tolerance, tolerogenic DC were simultaneously generated in tolerant recipients. Indeed, the authors have previously reported that splenic DC isolated from tolerant recipients expressed tolerogenic phenotypes and function (*16*). The authors investigated whether bone marrow-derived DC possess tolerogenic properties. Bone marrow progenitors were isolated from tolerant and age-matched naive mice. After 9 days of in vitro culture, DC generated from bone marrow of control mice and tolerant mice expressed similar levels of CD11c and DEC-205, indicating that the authors' protocol for generating DC was efficient. DC generated from naive mice showed high expression of MHC class II and the co-stimulatory molecules CD40 and CD86. In contrast, DC cultured from bone marrow of tolerant recipients showed lower expression of MHC class II and CD40 (Fig. 2A). In addition, DC generated from bone marrow of tolerant mice did not produce IL-12 protein or transcript as detected by flow cytometry and reverse-transcriptase polymerase chain reaction (data not shown). To investigate whether the lack of IL-12 expression on the DC altered ability to polarize T-helper (Th) cell differentiation, cytokines produced by allogeneic T cells responding to these DC were assessed by ELISA. In agreement with the potential tolerogenic properties of these cells, when DC generated from the bone marrow of tolerant recipients were used as stimulators in MLR, the responding T cells acquired a Th2 cytokine profile as evidenced by increased expression of IL-4 and decreased expression of IFN-γ (Fig. 2B).

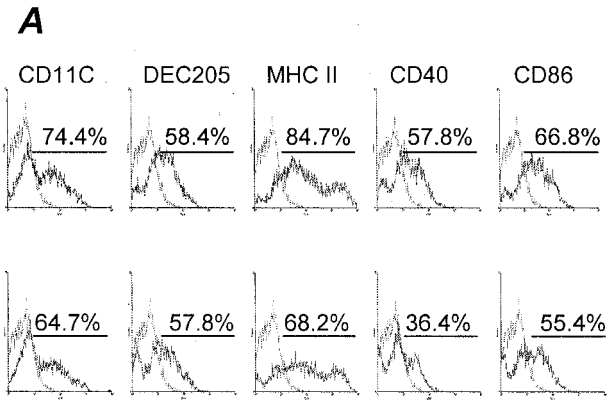
Splenic DC Isolated from Tolerant Recipients Inhibit MLR and Antigen-Specific T-Cell Response

Recipient DC may play a role in the induction of transplantation tolerance by presenting donor antigens to T cells and causing their differentiation to Treg cells (*19*). For this to occur, the recipient DC must be immature or possess Tol-DC function. The data in Figure 2(A) showed that bone marrow-derived DC isolated from tolerant recipients exhibited immature phenotype. Because a major proportion of bone marrow-derived DC are found in the spleen, the authors assessed the function of splenic DC for rejecting and tolerant recipients for their ability to stimulate allogeneic T-cell responses. In an MLR assay, DC isolated from tolerant recipients failed to stimulate allogeneic T-cell responses (Fig. 3A).

To assess whether Tol-DC are poor antigen presenters to T cells, an antigen-specific recall experiment was performed. Lymph node T cells were isolated from BALB/c mice immunized with $PCC_{81–104}$. PCC-specific T-cell responses were assessed in the presence of splenic DC isolated from normal mice and from rejecting and tolerant recipients. In the presence of $PCC_{81–104}$ peptide, rejective DC evoked a vigorous antigen-specific T-cell proliferative response, whereas DC isolated from tolerant recipients failed to elicit an antigen-specific T-cell response (Fig. 3B). These data support the concept that tolerant recipients possess increased numbers of Tol-DC.

Splenic DC Isolated from Tolerant Recipients Fail to Provide Co-stimulatory Signals to T Cells

T-cell activation needs both antigenic stimuli from MHC–T-cell receptor (signal 1) and co-stimulatory signals (signal 2). Without co-stimulation, T cells become anergic, undergo apoptosis, or differentiate into Treg cells. To assess whether splenic DC from tolerant mice provide weaker co-stimulatory signals than DC from rejecting and control mice, an in vitro model of co-stimulation was used. Purified $CD4^+$ T cells from naive BALB/c mice were stimulated with soluble anti-CD3 mAb as a source of signal 1. DC from the three groups were



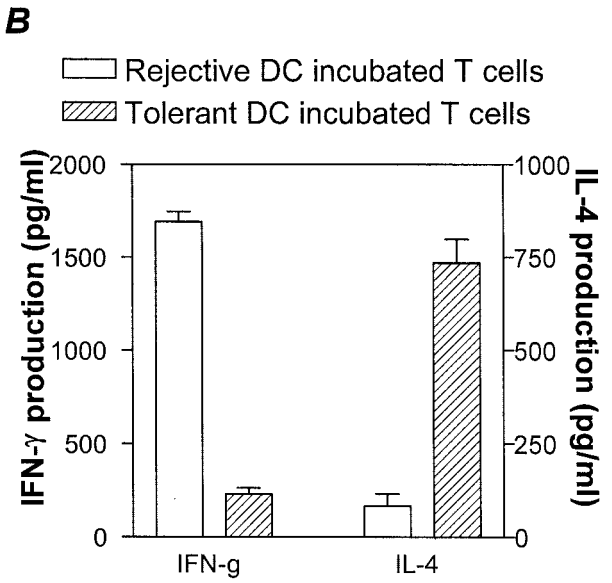
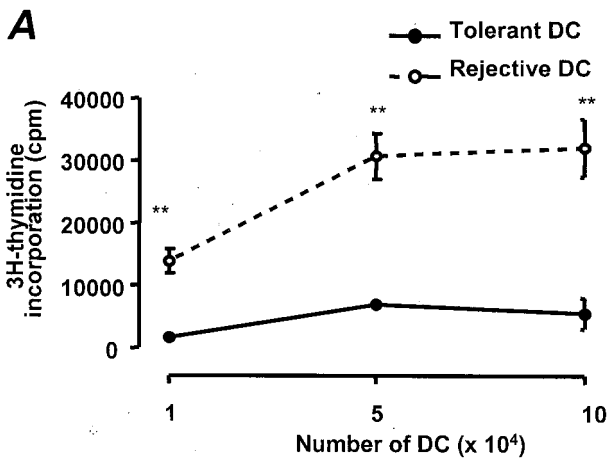


FIGURE 2. **(A) Phenotypic analysis of DC. Bone marrow progenitor cells isolated from tolerant allogeneic mice that underwent heart transplantation (*lower*) or control mice that did not undergo transplantation (*upper*) were cultured with recombinant murine granulocyte-macrophage colony-stimulating factor and IL-4 for 9 days. Cells were stained with the indicated antibodies (*solid lines*) or isotype control mAb (*broken lines*) and analyzed by flow cytometry. (B) Cytokine expression by T cells stimulated with DC from tolerant or rejective recipients. $CD4^+$ T cells (5×10^6) isolated from normal C57BL/6 mice were incubated with bone marrow-derived DC (5×10^5) from tolerant recipients or rejective recipients. After 48 hr of incubation, the culture supernatants were harvested and IFN-γ (Th1 cytokine) and IL-4 (Th2 cytokine) production was assessed by ELISA (see *Materials and Methods*). Data showed a representative of three experiments.**

added as a source of signal 2. DC isolated from rejective recipients showed the strongest co-stimulatory activity for anti-CD3–induced T-cell proliferation (Fig. 4). DC isolated from naive mice provided weaker co-stimulation, and DC from tolerant mice actually inhibited proliferation. These data suggest that tolerance induction causes a functional



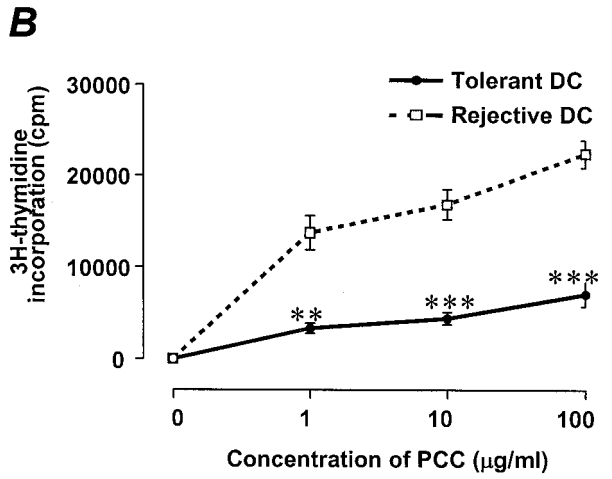


FIGURE 3. **Impaired antigen-presenting function of splenic DC isolated from tolerant recipients. (A) MLR using DC isolated from tolerant recipients. Splenic $CD11C^+$ DC were isolated from tolerant or rejective recipients. DC were irradiated (3,000 rad) and used as stimulators at indicated cell numbers. 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 (98.6°F) with 5% carbon dioxide in a humidified atmosphere for 3 days and pulsed with 1 μCi of ^{3}H-thymidine in the last 16 hr. Cells were harvested onto glass fiber filters, and the radioactivity incorporated was quantitated in a scintillation counter. Data represent means±SEM and are representative of five experiments (**$P<0.01$ by unpaired Student t test). (B) Antigen-specific response using DC isolated from tolerant recipients. BALB/C mice were immunized with $PCC_{81–104}$ peptide in complete Freund adjuvant. After 14 days, lymph node cells were isolated and used as responders (1×10^5 cells/well) and seeded in triplicate in 96-well, round-bottom plate. DC isolated from tolerant or rejective recipients were irradiated (3,000 rad) and added at 5×10^4 cells/well to the T cells in the presence of $PCC_{81–104}$ peptide at the indicated concentrations and used as stimulators (5×10^4 cells/well). Data represent means±SEM and are representative of three experiments. **$P<0.01$ and **$P<0.001$ (vs. rejective DC) by one-way analysis of variance and Newman-Keuls test.**

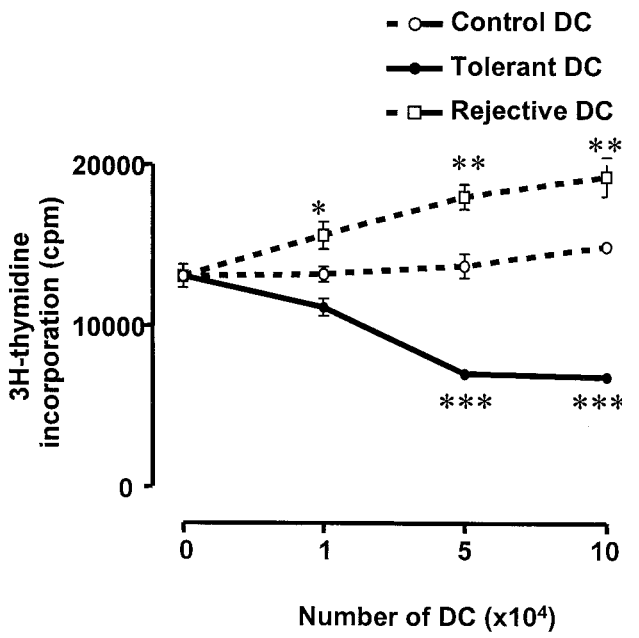


FIGURE 4. Tolerant DC inhibit anti-CD3 mAb-stimulated T-cell response. Splenic lymphocytes were isolated by gradient centrifugation on Ficoll-Paque. $CD4^+$ T cells (1×10^6) were isolated from naive BALB/c mice and stimulated with anti-CD3 mAb (1 μg/mL). Splenic DC isolated from tolerant recipients, rejective recipients, and naive mice were added to the culture at indicated cell numbers. Data represent means±SEM and are representative of three experiments. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ by one-way analysis of variance and Newman-Keuls test (vs. DC from naive mice).

alteration in DC co-stimulatory activity that is present even 100 days after transplantation.

DISCUSSION

In this study, the authors have demonstrated that the combination treatment of recipients with LF and anti-CD45RB mAb resulted in the induction of synergistic tolerance, whereas nontoxic doses of monotherapy with either reagent could not induce tolerance in the C57B/L6 to BALB/c heart transplantation model (*16*, *17*). This combination protocol is promising from a clinical perspective because the immune-modulating agents were administered for only 8 to 9 days. In agreement with the authors' findings, other studies have successfully prolonged graft survival by simultaneously blocking DC maturation and T-cell function (*20–24*).

LF is a novel antirejection drug developed through the chemical modification of DSG to increase immunosuppressive activity and to decrease toxicity. As compared with conventional immunosuppressants, the uniqueness of LF and DSG as antirejection drugs is that both block rejection responses through inhibition of APC function (*3*). However, the mechanism of action may be different between DSG and LF. DSG inhibits hsc 70 translocation to the nucleus, which contributes to inhibition of nuclear factor (NF)-κB (*25*). In contrast, LF directly binds IκB kinase and blocks phosphorylation of IκB, this in turn, does not allow for release and subsequent nuclear translocation of NF-κB (J. Yang, Ph.D., et al., unpublished data, 2003).

Antigen presentation is primarily performed by DC, which possess both a stimulatory and inhibitory role in T-cell activation, depending in part on whether they are mature or immature. In previous studies, the authors have also demonstrated that tolerance induced by LF is associated with generation of tolerogenic DC *16*. The term Tol-DC defines an immature DC population that possesses low levels of co-stimulatory molecules and is a poor stimulator of MLR. The fact that addition of DC from tolerant recipients actually inhibited T-cell proliferation induced by anti-CD3 mAb (Fig. 4) suggested to the authors that the Tol-DC were actively inhibiting the T cells, either directly by means of the co-inhibitory signals such as immunoglobulin-like transcript (ILT)-3 and ILT-4 (*19*), or indirectly by means of Tol-DC generating Treg cells (*26*), which in turn inhibits proliferation of T cells through inhibitory factors such as transforming growth factor-β and IL-10.

Interestingly and unexpectedly, the DC progenitors in the bone marrow were able to maintain their tolerogenic phenotype after 9 days of in vitro culture. Shurin et al. (*27*) reported that bone marrow progenitors derived from tolerogenic situations such as tumor-bearing mice possess a predisposition toward developing into immature cells ($CD40^{low}$, $CD86^{low}$) similar to the DC observed in this study, even though the cells were allowed to differentiate in vitro in the absence of immunosuppressive agents. This concept of progenitor "programming" by the tolerogenic environment requires further study.

Treg cells are believed to be critical for the induction of donor-specific tolerance because of their ability to suppress activated T cells in an antigen-specific manner. For Treg to be generated and expanded, an antigen-specific signal needs to be provided by an APC. Because APC also activate conventional T cells that cause graft rejection, it is essential that only the APC that stimulate Treg cells are activated in the recipient. Tol-DC possess an immature phenotype and inhibit proliferation of conventional T cells but activate and expand Treg cells. Because LF induces Tol-DC formation, administration of LF should induce Treg cells. In agreement with this, it has recently been demonstrated by Chiffoleau et al. (*28*) that LF blocks graft rejection in a rat cardiac allograft model through generation of Treg cells that were capable of adoptively transferring tolerance. The work by Chiffoleau et al. indicated that Treg cell generation was dependent on donor-derived DC because depleting donor APC resulted in an incapability to generate Treg cells (*28*). In contrast, the authors' previous study demonstrated that recipient-derived Tol-DC contribute to the generation of Treg cells (*26*). It is possible that both the donor and recipient DC play important roles in tolerance induction, depending on the transplant model used. In the current study, the authors further demonstrated that DC from tolerant recipients could neither evoke antigen-specific T-cell responses nor provide co-stimulation to T cells. Furthermore, these DC possessed low phenotypic expression of co-stimulatory molecules. On the basis of these properties, the authors conclude that an expanded Tol-DC population is present in tolerant mice and contributes to maintenance of graft survival.

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