Blockade of LIGHT/HVEM and B7/CD28 Signaling Facilitates Long-Term Islet Graft Survival With Development of Allospecific Tolerance

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Background. Previous studies have shown that blockade of LIGHT, a T-cell costimulatory molecule belonging to the tumor necrosis factor (TNF) superfamily, by soluble lymphotoxin β receptor-Ig (LTβR-Ig) inhibited the development of graft-versus-host disease. The cardiac allografts were significantly prolonged in LIGHT deficient mice. No data are yet available regarding the role of the LIGHT/HVEM pathway in more stringent fully allogeneic models such as skin and islet transplantation models.

Methods. Streptozotocin-induced chemical diabetic BALB/C mice underwent transplantation with allogeneic C57BL/6 islets and were treated with LTβR-Ig, CTLA4-Ig or a combination of both in the early peritransplant period.

Results. Administration of CTLA4-Ig or LTβ R-Ig alone only increased graft survival to 55 days and 27 days respectively, whereas simultaneous blockade of both pathways significantly prolonged the islet allograft survival for more than 100 days. Long-term survivors were retransplanted with donor-specific (C57BL/6) islets and the grafted islets remained functional for more than 100 days. All of islet allografts were protected against rejection when the mixtures of 1×10^6 $CD4^+$ T cells from tolerant mice and islet allografts were cotransplanted under the renal capsule of the naïve BALB/c recipients.

Conclusions. These data indicate that: 1) a synergistic effect for prolonged graft survival can be obtained by simultaneously blocking LIGHT and CD28 signaling in the stringent model of islet allotransplantation; 2) development of donor-specific immunological tolerance is associated with the presence of regulatory T-cell activity; and 3) local cotransplantation of the allografts with the regulatory T cells can effectively prevent allograft rejection and induce donor-specific tolerance in lymphocytes-sufficient recipients.

Keywords: Islet, Allograft, Allospecific, Tolerance, Costimulatory, Pathway.

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Recently, transplantation of pancreatic islet cells has become the most specific replacement therapy for treatment of type I insulin-dependent diabetes because transplantation of purified pancreatic islet cells overcomes the associated limitations encountered with transplantation of the whole pancreas (*1*). Although nonspecific immunosuppressive agents have been instrumental in controlling alloreactivity to transplanted islet cells, graft rejection and immune suppression still occur. Therefore, strategies that favor minimal toxicity and ultimately towards development of tolerance protocols remain a primary focus as islet transplantation evolves.

One promising strategy to prevent graft rejection and to facilitate tolerance induction is the blockade of costimulatory signals to inhibit T-cell activation. Due to T cells that perceive antigen in the absence of costimulation become anergic and may undergo apoptosis (*2–4*), blocking costimulation at the time of transplantation may facilitate graft acceptance through inactivation or deletion of alloreactive T cells.

LIGHT, a novel member of the tumor necrosis factor (TNF) superfamily, has three receptors, HVEM, LTβR, and a soluble form DcR3 (TR6). LTβR is expressed on nonlymphoid cells including monocytes. The biological activities of LIGHT–LTβR signaling include developmental roles such as mesenteric lymph node organogenesis and the restoration of secondary lymphoid structure and function (*5–7*). Infusion of LTβR–Ig inhibits the lymph node organogenesis and prevents the development of normal spleen architecture (*8*), as

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well as decreases the number of follicular dendritic cells, leading to impaired humoral immune responses (*9*). HVEM is mainly expressed on T cells and is the primary receptor for T cell costimulation by LIGHT (*10–12*). LIGHT-HVEM interaction provides potent costimulatory activity for T cells and enhances proliferation and the production of Th1 cytokines independently of the B7/CD28 pathway (*10–12*). Blockade of LIGHT–HVEM signaling by the application of soluble HVEM–Fc or LTβR-Fc fusion proteins or antibodies to HVEM significantly reduces allogeneic T cell immune responses in vitro (*10*, *11*). Furthermore, in vivo administration of LTβR-Fc or anti-LIGHT antibody (Ab) inhibits anti-host cytotoxic T cell (CTL) responses in a murine acute graft-versus-host disease (GVHD) model, leading to improved survival of recipients (*12*). Gene targeting of LIGHT results in impaired T cell immunity and a compromised allograft rejection (*13*). No data are yet available concerning the role of the LIGHT/HVEM pathway in more stringent fully allogeneic models, such as skin and islet transplantation models.

Previous studies have demonstrated that blockade of CD28 signaling with CTLA4-Ig inhibits T cell response and prolongs allograft survival in several rodent models and, in some organ transplantation models, leads to tolerance (*14–18*). However, conventional T cell costimulatory blockade directed at the CD28/B7 pathways is not effective in inducing reproducible tolerance in some stringent murine transplantation models and in primates (*19–21*), in which rejection is thought to be due to T-cell activation through alternative costimulatory pathways (*22–24*). Moreover, the efficacy of costimulation blockade in regulating alloimmune responses is organ/tissue dependent and has a limited success in more stringent fully allogeneic models, such as skin and islet transplantation (*25*). These observations suggest more robust strategies that combine two or more agents toward different signaling pathways may be required to induce tolerance in islet transplantation.

In a fully major histocompatibility complex (MHC)-mismatched mouse islet transplantation model, we developed an effective approach for induction of immunological tolerance with long-term survival of islet graft by administration of LTβR-Ig in combination with CTLA4-Ig. The mechanisms by which immunological tolerance was induced were further explored.

MATERIALS AND METHODS

Mice

Adult C57BL/6 (H-2^b), BALB/c (H-2^d) and C3H (H-2^k) female mice were purchased from the Shanghai Experimental Center, Chinese Academy of Sciences. All animals in this study were housed in pathogen-free conditions and were maintained in accordance with guideline of the committee on animals of the Second Military Medical University, Shanghai, PR China.

Induction of Diabetes and Islet Transplantation

BALB/c recipient mice were rendered chemically diabetic by a single intraperitoneal injection of streptozotocin (200 mg/kg; Sigma, St. Louis, MO). Diabetes occurrence was defined as two consecutive nonfasting blood glucose readings >300 mg/dl. Fully MHC-mismatched donor C57BL/6 by 1.5 mg/ml type V collagenase digestion (Sigma) followed by Ficoll purification (*26*, *27*). Approximately 500 islets were then transplanted under the left renal capsule of diabetic recipient mice. Allograft function was monitored by serial blood glucose measurements. Successful engraftment was defined by correction of the serum glucose level to <200 mg/dl by the third day posttransplantation and graft rejection was defined as a rise in serum glucose >300 mg/dl for two consecutive days.

Reagents and Treatment Protocols

Murine CTLA4-human immunoglobulin (Ig)-G1 Fc, murine LTβR-human IgG1 Fc, and anti-mouse LTβR monoclonal antibody (mAb) were produced and characterized in our laboratory as described previously (*12*, *17*). To evaluate the role of these agents on islet allograft rejection, recipient mice were treated either with CTLA4-Ig, LTβR-Ig or anti-mouse LTβR mAb. Control groups were treated with human IgG1 (Sigma) or rat IgG1 individually (Sigma). The day of transplantation was defined as day 0. All reagents were administered intraperitoneally (200 μg, days −1, 1, 3, 5, 7, and 9).

Confirmation of the Graft Function and Retransplantation

Long-term graft function of normoglycemic mice after 100 days was confirmed by returning them into hyperglycemia after nephrectomy of the kidney bearing the islet grafts. To test immunological tolerance, nephrectomized mice underwent retransplantation of the same donor-strain islets into the remaining contralateral kidney. No immunosuppressive therapy was given and blood glucose was serially monitored to detect graft rejection.

Skin Allograft Transplantation

A round full-thickness skin graft measuring 8 mm is obtained by slicing the skin with a number-12 scalpel blade from the thorax of a C57BL/6 mouse. The panniculus carnosus was scraped away using a scalpel blade. Then, the skin graft was placed over a reciprocal defect on an anesthetized BALB/c recipient. The edges were bonded together with Histoacryl. The graft was covered with a square of tulle gras and several turns of wetted plaster of Paris bandage were applied around the thorax over the graft. This was kept in place for 7 days. Rejection was defined as destruction of >90% of the graft surface area (*28*).

Magnetic Cell Separation

Spleen and lymph node mononuclear leukocytes (MNLs) were prepared by mincing the spleen and lymph node tissues through a 60-gauge stainless steel mesh. After washing, red blood cells were lysed by exposure to Tris/ammonium chloride buffer for 5 min at room temperature; the mixture was then washed. Magnetic beads coated with mAbs (Dynal Biotech, Oslo, Norway) were used for separating $CD4^+$ and $CD4^-$ T cell subsets. Briefly, MNLs were incubated with anti-CD4 mAb-coated magnetic beads at a 10:1 bead to cell ratio for 30 min at 4°C with gentle rotation. The selected $CD4^+$ T cells were isolated from the bead/cell mixture by exposure to a magnetic field using a magnetic particle concentrator (Dynal Biotech, Oslo, Norway) according to the

manufacturer's instructions. Typical purity of $CD4^+$ cells was >95%, which was determined by fluorescence-activated cell sorting (FACS) analysis. The remaining cells in which the proportion of $CD4^+$ T cells was <2% determined by FACS analysis were collected and used as $CD4^+$ depleted MNLs. The viability of the cells exceeded 95% in all of experiments determined by trypan blue staining.

In Vitro Mixed Lymphocyte Reactions (MLRs)

Single-cell suspensions were prepared from spleen and lymph node as described previously. Various dilutions of irradiated (20Gy; ^{60}Co source) allogeneic (C57BL/6), third-party (C3H), or control BALB/c MNLs were used as stimulator cells. Then 2×10^5 MNLs from recipient mice 100 days after transplantation or from naïve mice were used as responders. In some experiments, exogenous interleukin (IL)-2 (100 U/ml) was added into the above tests. Cultures were maintained in 96-well plates for 72 hr and ^{3}H-thymidine (1μCi per well) was added for the final 18 h of culture. The uptake of radioactivity was measured by liquid scintillation counting.

To assess the presence of regulatory T cells in tolerant mice, MNLs from tolerant and naïve mice were co-cultured with donor antigen or third-party antigen. Naïve BALB/c MNLs (2×10^5) were cultured with irradiated MNLs (20Gy; ^{60}Co source) from allogeneic (C57BL/6), third-party (C3H), or control BALB/c mice in the presence of various dilutions of MNLs from tolerant or control naïve BALB/c mice. In some cultures, positively selected $CD4^+$ T cells or $CD4^+$-depleted MNLs from tolerant or naïve mice were co-cultured with donor antigen for mixed lymphocyte reaction (MLR) assay.

Flow Cytometry Analysis

Phycoerythrin (PE)-conjugated monoclonal antibodies to CD4 (GK1.5), allophycocyanin-conjugated monoclonal antibodies to CD25 (7D4), fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies to Foxp3 (FJK-16s) and isotype control rat IgG1 (R3–34) were purchased from eBioscience (San Diego, CA). To analyze the phenotype of spleen MNLs, FACS analysis was performed.

Measurement of Cytokines

For cytokine measurement, the supernatants from cocultured MLR cultures in vitro on days 3 were harvested and subjected to the detection of interferon (IFN)-γ, IL-4, IL-2, IL-10, and transforming growth factor (TGF)-β concentrations with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). For TGF-β ELISA, the culture supernatant was first treated with acid to reduce the pH to 2.0 to denature latency-associated peptide to allow the detection of active TGF-β. The supernatant was neutralized to pH 7.0 before ELISA.

Adoptive Transfer of Naïve and Tolerant MNLs Into Irradiated Recipients

BALB/c mice were irradiated with 1000 rad to completely eliminate their immune system and then various mixtures of MNLs from naïve and/or tolerant recipients were transferred i.v. into the irradiated recipients at day 2 after irradiation. These treated BALB/c mice were transplanted with C57BL/6 or third-party strain (C3H) islets at the day after cell transfer.

Local Cotransplantation of Islets and Lymphocytes Into Nonimmunocompromised Recipients

Various numbers of $CD4^+$ T cells from tolerant mice and 500 islets from naïve C57BL/6 mice were mixed in vitro and cotransplanted under the renal capsule of naive diabetic BALB/c recipients. No immunosuppressive therapy was given after the transplantation and blood glucose was serially monitored to detect graft rejection.

Statistical Analyses

Comparisons between the groups were analyzed by two-sided *t* test or by two-way analysis of variance for experiments with more than two subgroups. Results are presented as mean±SEM. Graft survival was analyzed using Kaplan-Meier cumulative plots and comparisons between groups were performed using a log-rank test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Administration of LTβ R-Ig in Combination With CTLA4-Ig Facilitates Long-Term Islet Allograft Survival

We first investigated the effect of LTβ R-Ig or CTLA4-Ig as a single agent therapy on islet allograft rejection. As shown in Figure 1, the mice treated with LTβ R-Ig demonstrated significantly prolonged islet allograft survival compared with control mice ($P<0.05$). Administration of CTLA4-Ig alone also prolonged the survival of islet allograft, but all recipients eventually rejected the islet allografts. In contrast, mice treated with both LTβ R-Ig and CTLA4-Ig significantly prolonged the islet allograft survival and 80% of grafts survived more than 100 days (mean survival time [MST]>100 days). Thus, these results indicated that blockade of a single pathway for T cell activation was ineffective in completely preventing islet graft rejection and the combined administration of LTβ R-Ig facilitated CTLA4-Ig treatment in inducing stable and long-term islet engraftment.

To further determine whether the benefit in graft survival is due to blockade of LIGHT-HVEM or LIGHT-LTβ R interactions by LTβR-Ig, we generated antagonistic mAbs to LTβR. The resulting anti-LTβR mAb could effectively blocked LIGHT-LTβR interaction and would serve a control for LTβ R-Ig, which interfered with both LIGHT-LTβR and LIGHT-HVEM interactions. As shown in Figure 1, BALB/c recipient mice treated with anti-mouse LTβR mAb readily rejected the fully MHC-mismatched C57BL/6 islet allografts (MST=11 days), suggesting no benefit on allograft survival compared with control mice. Thus, these results demonstrated that the benefit of prolonged allograft survival achieved by administration of LTβ R-Ig was mainly due to blockade of LIGHT-HVEM interaction, which indicated that LIGHT-HVEM pathway, not LIGHT-LTβR pathway, was responsible for LIGHT-mediated transplantation rejection.

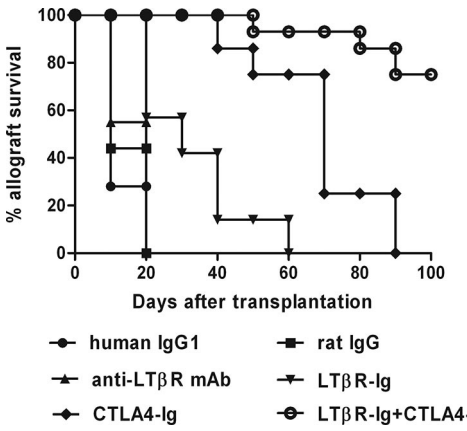

FIGURE 1. Treatment of recipient mice with LTβ R-Ig and CTLA4-Ig produced long-term islet allograft survival. C57BL/6 islets were transplanted into streptozotocin-induced diabetic BALB/c mice. The survival of islet allografts was merely prolonged in BALB/c recipients treated with LTβ R-Ig alone (▼: MST=27 days, n=12, $P<0.05$ vs. control mice). Injection of CTLA4-Ig also prolonged the survival of islet allograft (◆: MST=55 days, n=12, $P<0.01$ vs. control mice), but all recipients eventually rejected the islet allografts. However, administration of LTβ R-Ig in combination with CTLA4-Ig facilitated long-term islet allograft survival in 80% of recipients (○: MST>100 days, n=20, $P<0.001$ vs. any other groups). The islet grafts were readily rejected in the mice treated with anti-mouse LTβR mAb and two control groups and the median survival time was 11 days (▲: n=11), 9 days (■: n=11) and 10 days (●: n=9), respectively.

Administration of LTβ R-Ig in Combination With CTLA4-Ig Induced Specific Transplantation Tolerance

To determine whether long-term allograft survival is due to development of immunological tolerance, long-term survivors (>100 days) in the group treated with the combinatory therapy underwent left nephrectomy to remove the islet grafts. After operation, the mice promptly became hyperglycemic, which indicated that euglycemia in these animals was dependent on the presence of the grafts and was irrelevant to endogenous insulin secretion. When these animals were retransplanted with either donor-specific (C57BL/6) or third-party (C3H) islets, the third-party islets were rejected at a median of 12 days, whereas donor-specific islets remained functional for more than 100 days, which clearly demonstrated that the recipients had developed donor-specific tolerance to islet graft (Table 1).

In a separate set of experiments to test the organ specificity of tolerance, C57BL/6 skin was transplanted onto long-term survivors. Skin graft survival was significantly prolonged and the median skin graft survival was 33 days in long-term survivors compared to 12 days in naïve BALB/c mice in which the same skin grafts were transplanted (data not shown). Thus, the data suggested that the tolerance induced by this approach was organ specific.

Regulatory T-Cell Activity in Mice Treated With LTβ R-Ig and CTLA4-Ig

To investigate the mechanisms by which long-term allograft survival is successfully induced with this approach, we first sought to evaluate the robustness of the tolerances induced by LTβ R-Ig and CTLA4-Ig. Lymphocyte responses to donor (C57BL/6) or third-party (C3H) antigens were analyzed by MLRs. In this assay, MNLs from long-term engrafted mice had lower proliferative response to donor C57BL/6 stimulators in MLRs than MNLs from naïve animals did. Furthermore, the hyporesponsiveness could not be reversed through provision of exogenous IL-2. In contrast, these MNLs still maintained a similar alloreactivity to third-party C3H stimulators (Fig. 2A). To evaluate whether the presence of regulatory T cells contribute to donor-specific peripheral hyporesponsiveness, MNLs cells from tolerant mice in different proportions were cocultured with naïve MNLs and proliferation was evaluated in response to donor antigen in MLRs. Our results showed that MNLs from tolerant mice had a significantly suppressive function. With increasing numbers of tolerant lymphocytes in the tested MLRs, suppression of the MLRs in response to alloantigen was enhanced. In contrast, MNLs from tolerant mice were unable to inhibit the proliferative response of naïve lymphocytes against third-party C3H stimulators (Fig. 2B). In addition, high concentrations of IL-10, TGF-β were observed in T cells from tolerant mice in the co-culture MLR assay (Supplementary Table 1, available online at www.transplantjournal.com). Thus, the data indicated that the presence of regulatory T cells in mice treated with both LTβ R-Ig and CTLA4-Ig. IL-10 and TGF-β were probably required to confer this suppression.

When purified $CD4^+$ T cells from tolerant mice were added to the wells containing MNLs from naïve donor-strain and recipient mice, the proliferation of naïve T cells in response to donor antigen was also significantly inhibited. However, in the coculture MLR assay, removal of $CD4^+$ T cells from tolerant mice restored the response of MNLs obtained from BALB/c tolerant hosts to the cells from donor, indicating $CD4^+$ regulatory T cells were necessary for the suppressive effect of the lymphocytes from tolerant hosts (Fig. 2C).

TABLE 1. Survival of secondary islet allografts in animals bearing primary islet allografts

Recipient	First graft	Second graft	Graft survival (days)
BALB/c	C57BL/6	C57BL/6	$>100\times5^a$
BALB/c	C57BL/6	C3H	10, 11, 12, 12, 15

In animals bearing long-term functioning C57BL/6 islet allografts after treatment with LTβR-Ig and CTLA4-Ig, the primary islet allograft was removed by nephrectomy of the left kidney and a secondary islet allograft from C57BL/6 or C3H was grafted into the renal subcapsular space of the right kidney. The second islet allografts survive for more than 100 days after treatment with LTβR-Ig in combination with CTLA4-Ig.

[a] $P < 0.01$ vs. C3H groups.

Phenotypic Analysis of Regulatory Cells

Because $CD4^+CD25^+$ T cells have been well-established as critical mediators in the maintenance of peripheral allograft tolerance. Foxp3, a key molecule in the development of regulatory T cells, is considered as a specific marker for these regulatory T cells (*29*, *30*). We determined the phenotypes of regulatory T cells in spleen MNLs from the tolerant mice by flow cytometry. As shown in Figure 3, a higher proportion of

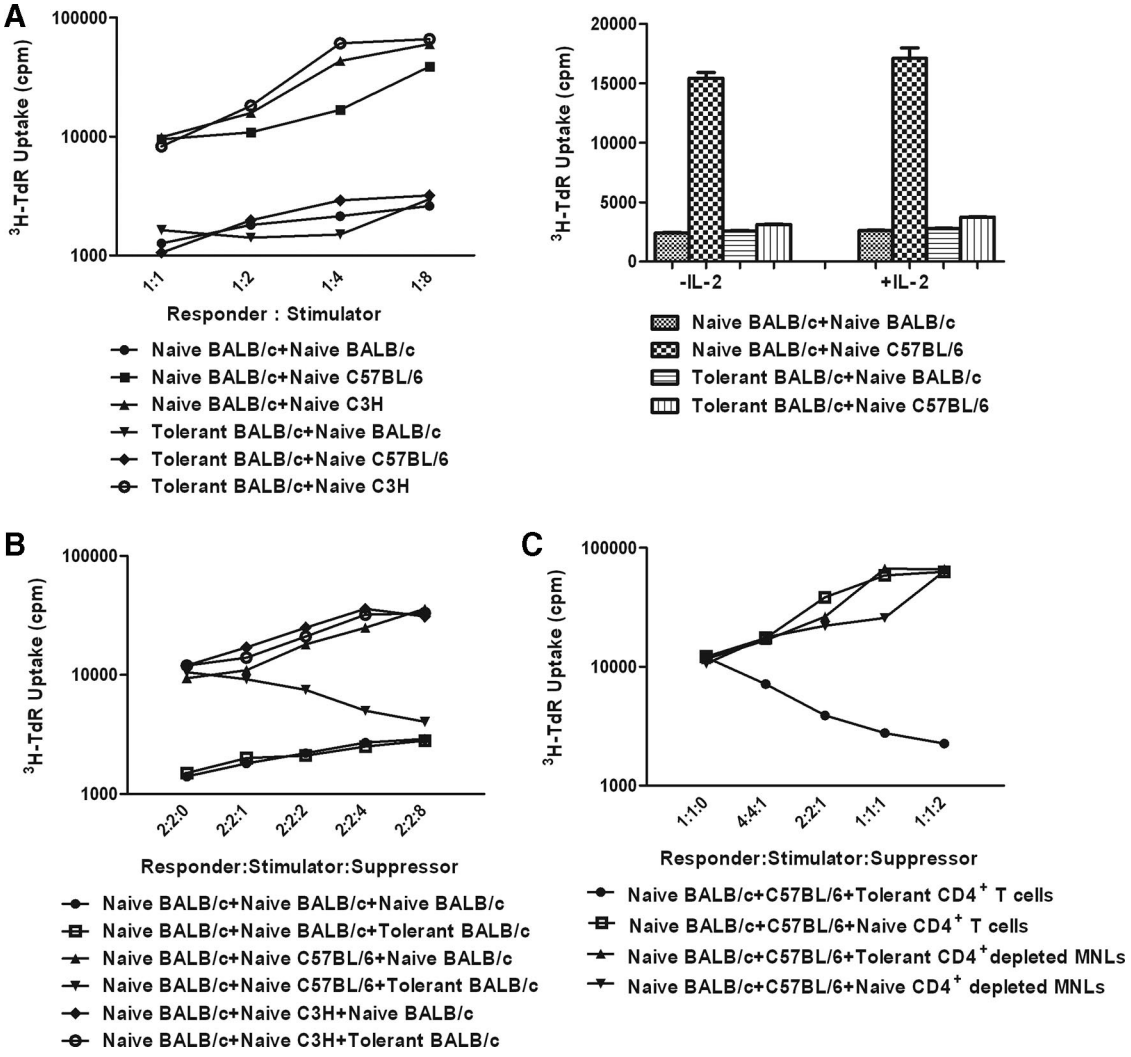

FIGURE 2. Regulatory T-cell activity in mice treated with LTβ R-Ig and CTLA4-Ig. (A) Lymphocytes harvested from tolerant hosts were hyporesponsive to donor-spleen stimulator cells in the MLR, but reacted normally to third-party stimulator cells. Various dilutions of allogeneic (C57BL/6), third-party (C3H), or control BALB/c MNLs were used as stimulator cells. MNLs from tolerant or naïve mice were used as responders. (B) Lymphocytes harvested from tolerant hosts inhibit the proliferation of naïve lymphocytes in response to donor alloantigen in the coculture MLR. Responders (naïve BALB/c lymphocytes) and irradiated stimulator cells (naïve BALB/C, naïve C57BL/6, and naïve C3H lymphocytes) were cultured at a concentration 2×10^5 cells each in 96-well microculture plates. Suppressor cells from tolerant mice or naïve mice were added at various concentrations to the MLR. (C) $CD4^+$ T lymphocytes harvested from tolerant hosts inhibited the proliferation of naïve lymphocytes in response to donor alloantigen in the co-culture MLR. Increasing numbers of $CD4^+$ T cells or $CD4^+$ depleted MNLs from tolerant mice or naïve mice were used as suppressor cells. Data are expressed as the mean cpm of triplicate cultures of one representative experiment. These experiments were repeated three times with similar results.

$CD4^+CD25^+$ T cells in MNLs from the spleen of tolerant mice was observed, compared with that from naïve mice (6.3% vs. 3.2%, $P<0.01$). The population of $CD4^+CD25^+$ T cells constituted ~17% of $CD4^+$ T cells from the spleen of the tolerant mice and constituted only ~10% of $CD4^+$ T cells from the spleen of naive mice. Foxp3 expression on $CD4^+CD25^+$ T cells from tolerant mice was also significantly higher than that from naïve mice (62.2% vs. 49.5%, $P<0.05$), which suggested immunoregulatory function of T cells from tolerant mice was associated with the presence of $Foxp3^+$ $CD4^+CD25^+$ regulatory T cells.

Establishment of Immune Tolerance by Adoptive Transfer of T Cells From the Mice Treated With LTβ R-Ig in Combination With CTLA4-Ig

The mechanism by which tolerance was maintained in long-term survivors was further investigated by adoptively transfer experiments. As shown in Figure 4, adoptive transfer of 5×10^7 MNLs from tolerant BALB/c hosts did not lead to rejection in 80% of syngeneic recipients. Moreover, this suppressive effect was donor antigen specific, as the irradiated recipients adoptively transferred with MNLs from tolerant BALB/c mice rejected third-party (C3H) islet grafts. In accor-

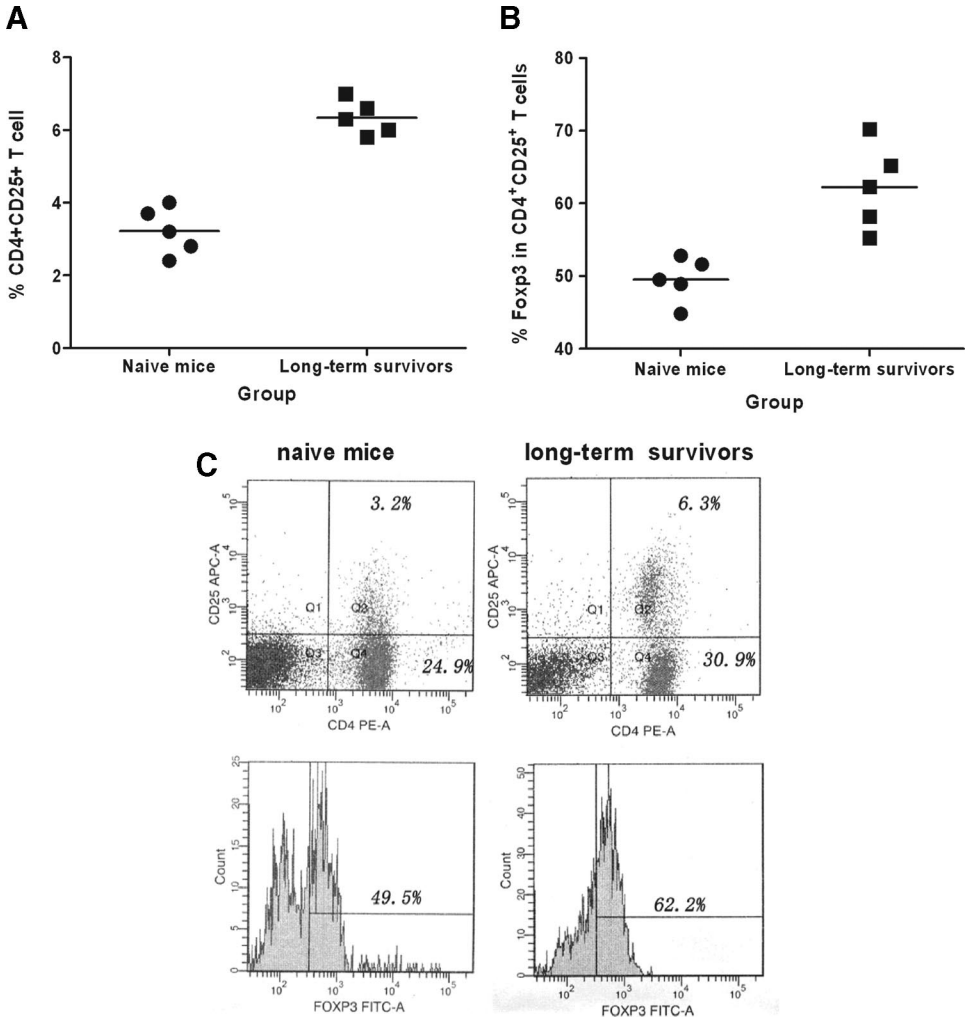

FIGURE 3. The phenotypic analysis of regulatory T cells. The spleen MNLs from tolerant or naïve mice were stained with PE-anti-CD4, APC-anti-CD25, and FITC-anti-Foxp3 antibodies followed by FACS analysis. (A) The percentage of $CD4^+CD25^+$ T cells in the spleen MNLs from long-term survivors was significantly higher than that from naïve mice (6.3% vs. 3.2%, n=5, $P<0.01$). (B) The percentage of $Foxp3^+$ T cells in $CD4^+CD25^+$ T cells from long-term survivors was also significantly higher than that from naïve mice (62.2% vs. 49.5%, n=5, $P<0.05$). (C) One representative of FACS plots was shown.

dance with the experimental results in vitro, it was clearly shown that the ability of MNLs from tolerant BALB/c mice to constrain rejection was cell number dependent. When the number of MNLs from naïve mice increased or the number of MNLs from tolerant hosts decreased, MNLs from tolerant animals were unable to inhibit allograft rejection effectively. In addition, removal of $CD4^+$ T cell subpopulation from tolerant hosts abolished the capability of MNLs to delay graft rejection.

Local Transplantation of the Mixture of Donor Islets and Lymphocytes From Tolerant Mice Facilitates Long-Term Islet Allograft Survival

Previous studies have shown that $Foxp3^+$ regulatory T cells can be detected in the local site of islet allografts in several animal models and the local regulatory T cells may play a role in induction of tolerance. Therefore, we further investigated whether the cotransplantation of mixture of islet allografts and $CD4^+$ regulatory T cells could induce allospecific tolerance. As shown in Figure 5, when the mixtures of 5×10^4 $CD4^+$ T cells from the tolerant mice and islet allografts were cotransplanted under the renal capsule of the naïve diabetic BALB/c recipients, all recipient mice rejected the islet allografts (MST=24 days). However, increasing numbers of tolerant $CD4^+$ lymphocytes in the mixture, the survival of islet allografts was significantly prolonged. All of islet allografts were protected against rejection when 1×10^6 $CD4^+$ T cells from tolerant mice were added into the mixture. These results indicated that regulatory T-cell therapy could be used successfully in lymphocyte-sufficient animals when the mixture of islet allografts and $CD4^+$ regulatory T cells were cotransplanted. Moreover, a certain ratio of regulatory T cells to islet allografts was required.

DISCUSSION

In this study, we have developed a new approach to completely prevent rejection of islet allograft in MHC-

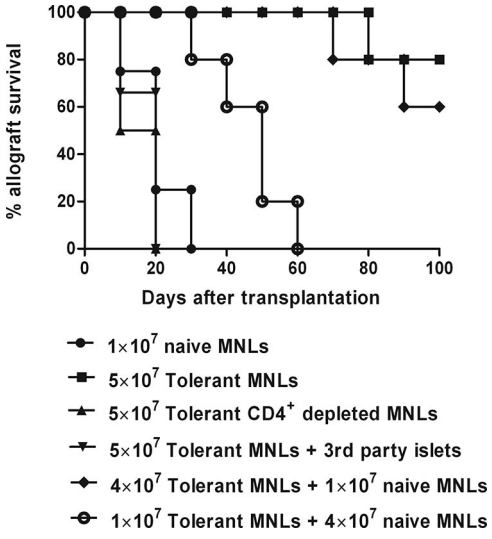

FIGURE 4. Adoptive transfer of tolerant MNLs resulted in indefinite C57BL/6 islet graft survival in irradiated recipients. When naive MNLs were adoptively transferred in the absence of cells from tolerant mice, the grafts were readily rejected (●: MST=15 days, n=5). A similar rate of rejection was observed when 5×10^7 $CD4^+$ depleted MNLs from tolerant mice were transferred (▲: MST=12 days, n=5). Whereas when 1×10^7 tolerant MNLs plus 4×10^7 naïve MNLs were transferred, graft rejection was significantly delayed (○: MST=46 days, n=5). When the number of tolerant MNLs was increased to 4×10^7, survival of islet allografts was further prolonged and 60% of animals accepted the grafts indefinitely (♦: MST>100 days, n=5). Eighty percent of grafts were long-term survival in the groups injected with 5×10^7 tolerant MNLs (■: MST>100 days, n=5). Recipients receiving the third party (C3H) grafts with tolerant MNLs (▼: n=5) rejected their grafts at a median of 18 days.

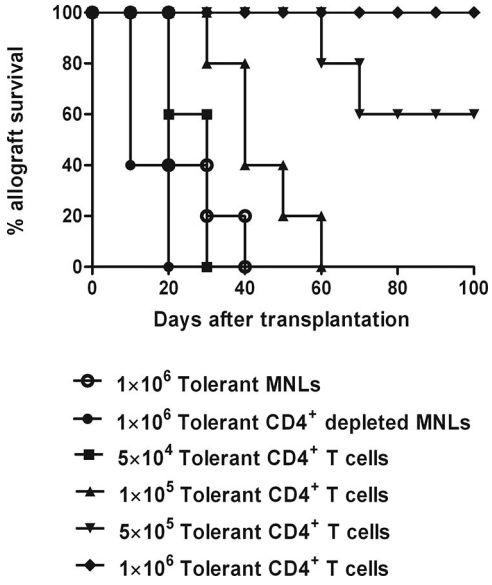

FIGURE 5. Survival of islet allografts in nonlymphopenic BALB/c recipients with local cotransplantation of the mixtures of 500 islet grafts with various numbers of MNLs from tolerant mice. When 1×10^6 $CD4^+$ depleted tolerant MNLs were co-transplanted, the grafts were readily rejected (●: MST=16 days, n=5). The rejection was not significantly delayed in the group where 5×10^4 tolerant $CD4^+$ cells were cotransplanted into each mouse (■: MST=24 days, n=5). With the number of tolerant $CD4^+$ cells increased to 1×10^5, rejection was significantly delayed (▲: MST=58 days, n=5). However, when 5×10^5 (▼: MST>100 days, n=5) or 1×10^6 tolerant $CD4^+$ cells (♦: MST>100 days, n=5) were added, the grafts were accepted indefinitely. Recipients receiving 1×10^6 tolerant MNLs (○: n=5) rejected their grafts at a median of 33 days.

mismatched islet transplantation model. Administration of LTβ R-Ig in combination with CTLA4-Ig induced long-term survival of islet allografts (more than 100 days). In contrast, the monotherapy using either CTLA-4Ig or LTβ R-Ig leads to eventual allograft rejection. Moreover, organ-specific tolerance was achieved by the blockade of both signaling pathways, as rechallenging with a donor-specific skin graft resulted in prolonged survival, but not indefinite acceptance. Our results indicate that mechanisms underlying this protection are associated with the induction of $Foxp3^+$ $CD4^+CD25^+$ regulatory T cells. Furthermore, local cotransplantation of the islet allografts together with the $CD4^+$ regulatory cells can effectively prevent allograft rejection in lymphocyte-sufficient animals. Our findings suggest a novel strategy to induce allospecific tolerance in mouse islet transplantation model.

The results from the study showed that infusion of LTβR–Ig alone ameliorated rejection of islet transplantation in MHC-mismatched recipient mice. Although the result can be interpreted as a consequence of blockade of LIGHT-HVEM by LTβR–Ig, the possibility that blockade of LTβ-LTβR or LIGHT-LTβR may also occur in this process must be excluded. The previous reports have clearly showed that transfer of LTα-/- donor cells, in which both LTα and membrane-bound LTβ are deficient, still generate GVHD in vivo, indicating that a complex of LTα or LT$\alpha\beta$ may be not required in the transplant model (*31*). Moreover, an alloreactive CTL response can be normally generated from LTα-deficient mice in vitro (*32*), again indicating that lymphotoxins are not required in this system. In addition, our results showed that infusion of anti-LTβR mAb can not prolong the survival of islet allograft, suggesting that LIGHT-LTβR pathway also was not involved in this model. Therefore, these results demonstrated that the benefit of prolonged allograft survival achieved by administration of LTβ R-Ig was mainly due to blockade of LIGHT-HVEM interaction, not other signal pathway.

Induction of regulatory T cells is a unique consequence achieved by the combined treatment with LTβR-Ig and CTLA4-Ig. T cells from mice treated with the combined therapy fail to respond to donor-antigen and addition of exogenous IL-2 can not reverse T cell responsiveness in MLR. The data from the co-culture MLR assay showed that the induction of this unresponsiveness was an active immune process, because the lymphocytes from tolerant mice were capable of significantly inhibiting allogeneic T cell proliferation in vitro. However, anergic T cells were incapable of inhibiting immune cells to proliferate and differentiate (*3*). Moreover, this was further confirmed by adoptive transfer experiments, in which tolerant lymphocytes prevented the graft rejection induced by the transferred naïve lymphocytes.

Although a high proportion of CD4+CD25+Foxp3+ T cells from tolerant mice was observed in this study, other regulatory cell populations, such as the Th3-like or Tr1-like regulatory T cells, which also function through secretion of IL-10 and/or TGF-β (*33–35*), may be involved in the immunoregulation process in this model system. It has been suggested that Th3-like or Tr1-like regulatory T cells may be prominent in the gut and in other mucosal tissues, where they play key roles in the maintenance of self-tolerance and the prevention of autoimmune diseases (*33*). However, whether Th3-like or Tr1-like regulatory cells were directly involved in this process still needs further investigation. In addition, it has been shown that CTLA4-Ig can induce indoleamin 2,3 dioxygenase (IDO) in dendritic cells (*36*). The dendritic cells expressing IDO catabolize tryptophan and can suppress T cell responses (*37–39*). Thus, the dendritic cells expressing IDO may be required for induction of tolerance to allogeneic antigens, which is mediated by B7/CD28 and the LIGHT/HVEM pathway. Our results showed that addition of 1-MT, an inhibitor of IDO (*40*), in the MLRs could not reverse the T-cell hyporesponsiveness, suggesting that IDO+ dendritic cells might be not involved in the maintenance of T cell hyporesponsiveness in this model system (data not shown).

It has been well-documented that regulatory T cells suppress allograft rejection and induce infectious tolerance when adoptively transferred into other naïve recipients (*41*, *42*), which is consistent with the results obtained from this study. By this in vivo adoptive transfer experiment, we clearly demonstrated that systemically adoptive transfer of regulatory T cells from tolerant animals can induce tolerance in recipient mice. But, this approach needs a large number of T cells and a certain ratio of regulatory T cells to alloaggressive lymphocytes is required to produce effective donor-specific suppression of allograft rejection.

Although immunoregulatory activity of regulatory T cell was demonstrated in many model systems, most of the studies were based on adoptive transfer models that took advantage of lymphopenic mice to enhance regulatory T cell proliferation (*43–45*). Therefore, we examined the ability of the lymphocytes from tolerant mice together with islet allografts to prevent rejection in nonimmunocompromised syngeneic mice (naïve diabetic BALB/c recipients). Our data clearly show that cotransplantation of regulatory T cells together with islet allografts can lead to the long-term survival of islet graft in lymphocyte-sufficient recipients. Importantly, a very small number of CD4+ T cells from long-term survivors are effective in induction of allograft tolerance which is similar with that induced by systemically transfer of a large number of T cells from tolerant animals. As reported previously, regulatory T-cell proliferation and expansion requires antigen stimulation (*46*, *47*). The cotransplantation of islet allografts might be essential for amplification and maintenance of the grafted regulatory T cell locally, which contribute to induction of tolerance. Taken together, our results indicate that combined treatment with LTβ R-Ig and CTLA4-Ig can facilitate long-term islet graft survival by induction of allospecific tolerance in a mouse model. This novel strategy may have a potential for clinical application because of its effectiveness and simplicity.

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