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Islet allograft tolerance in the absence of invariant natural killer T cells

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Abstract

The invariant NKT cells are involved in both immunity and immune tolerance. However, their roles in transplant models remain controversial. We studied the role of NKT cells in the allograft response using two different strains of NKT deficient mice (CD1d^{-/-} and Jα18^{-/-} mice), and found that CD1d^{-/-} and Jα18^{-/-} mice rejected islet allografts with a similar kinetics as wild type B6 mice. Treatment of CD1d^{-/-} and Jα18^{-/-} mice with donor specific transfusion and anti-CD154 induced donor specific tolerance, which was identical to similarly treated wt B6 mice. The islet allograft tolerance requires Foxp3⁺ Tregs. In the periphery, Foxp3⁺ Tregs in CD1d^{-/-}, Jα18^{-/-}, and wt B6 mice were comparable both phenotypically and functionally. In addition, CD1d^{-/-} and Jα18^{-/-} CD4⁺ T cells (non-Tregs) could be readily converted to Foxp3⁺ Tregs by TGF-β in vitro. Our data suggest that islet allograft tolerance can be successfully established without invariant NKT cells.

Keywords

NK cells; Islets; Transplantation; Tolerance; Tregs

INTRODUCTION

The natural killer T cells (NKT) are an unusual cell type in the immune system that exhibits features of both innate and adaptive immune cells; they express an invariant TCR encoded by Vα14Jα18 and Vβ8 (also referred to as invariant NKT or iNKT) and the C-type lectin NK1.1, which typically defines the innate NK cells (1). iNKT cells are selected by the MHC class I-like molecule CD1d in the thymus, and in the periphery, their invariant TCR recognizes glycolipids presented in the context of the CD1d molecules. Similar to NK cells, iNKT cells preferentially reside in the liver and respond to cytokines that normally regulate

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NK cells (2). Current data support the notion that iNKT cells are one of the key players in viral immunity, tumor immunity, and in certain autoimmune diseases (3, 4). In transplant models, however, iNKT cells appear to have divergent roles in graft survival. Earlier studies demonstrated the requirement of iNKT cells in the induction of allograft tolerance, as prolonged heart allograft survival was induced in wt mice, but not in CD1d^{-/-} deficient mice, by blocking LFA-1 and CD154 costimulation (5). Furthermore, tolerance to corneal allografts in the eye also requires iNKT cells, and this is mediated by NKT-derived IL-10 (6). Similar requirement for NKT cells was reported in spontaneous liver allograft tolerance in mice as well as in prevention of GVHD response following bone marrow transplantation. In stark contrast, iNKT cells are also shown as key effector cells in allograft rejection impeding transplant survival in other models. For example, rejection of islet allografts is mediated primarily by iNKT cells after intra-portal transplantation and depletion of iNKT cells is a prerequisite for islet allograft survival (7, 8). Additionally, activation of NKT cells hinders the induction of mixed chimerism and the induction of donor specific tolerance using the bone marrow chimeric approach (9). A key message from these reports is that iNKT cells are clearly involved in the allograft response. But depending on the context, iNKT cells may employ different mechanisms in regulating transplant outcomes.

Given the potential regulatory role of iNKT cells in transplant tolerance, we revisited the issue of iNKT cells in transplant models using two different iNKT deficient mouse strains as allograft recipients (i.e., CD1d^{-/-} and J α 18^{-/-} mice). We found that islet allograft tolerance can be successfully established without iNKT cells and induction of Foxp3⁺ Tregs was not impaired in the iNKT deficient transplant recipients.

MATERIALS AND METHODS

Animals

C57BL/6 (H-2^b), DBA/2 (H-2^d), and C3H (H-2^k) mice were purchased from Taconic Farms (Germantown, NY). CD1d^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). J α 18^{-/-} mice were provided by Dr. Mark Exley at Beth Israel Deaconess Medical Center in Boston, MA. Both CD1d^{-/-} and J α 18^{-/-} mice are on the B6 background.

Pancreatic islet transplantation

Islet transplantation was performed as previously reported (10). Briefly, pancreatic islets were prepared from donor mice and transplanted under the renal capsule into recipients rendered diabetic by single i.p. injection of streptozotocin. Groups of recipient mice were treated with 10 $\times$ 10⁶ donor spleen cells (donor specific transfusion or DST) and 0.25 mg anti-CD154 (MR1) 10 days prior to islet grafting, and another dose of MR1 (0.25mg) was given at the time of transplantation. This protocol has been shown to induce allograft tolerance in this model (11). To deplete Foxp3⁺ Tregs in transplant recipients, a depleting anti-CD25 mAb (clone PC61) was given at 0.25 mg i.p. on days -2, 0, and +2; and day 0 was the time of islet grafting. Recipient mice treated with rat IgG were included as controls.

Cell sorting

Spleen and lymph nodes were harvested from host mice and single cell suspension prepared in complete RPMI-1640 medium (East Rutherford, NJ). The cells were labeled with FITC-anti-mouse CD4 and PE-anti-mouse CD25 (eBioscience, San Diego, CA), and then sorted into CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ T effector cells using the MoFlo high speed cell sorter (Dako-Cytomation, Ft Collins, CO). The purity of cells sorted using this method is consistently >96%, as we previously reported (12).

T cell suppression assay

Spleen and lymph node cells were prepared from wt B6, CD1d^{-/-}, and J α 18^{-/-} mice, and FACS sorted into CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ T effector cells. The sorted cells were resuspended at 2 $\times$ 10⁶/ml in complete RPMI 1640 medium and Tregs and T effector cells were mixed to achieve different Treg to T effector ratios. The cell mixture was plated into 96-well tissue culture plates and stimulated with anti-CD3 (2 μ g/ml, clone 2C11) plus syngeneic APCs at 37°C for four days. For the last 16 hrs of culture, cells were pulsed with 1 μ Ci ³H-TdR/well (Amersham, Boston, MA), and ³H-TdR uptakes were determined using a scintillation counter (Perkin-Elmer, Wellesley, MA). Data were presented as mean CPM of triplicate assays. Suppression of T effector cell proliferation was calculated based on proliferation of T effector cells in the absence of Tregs.

Induction of Foxp3⁺ Tregs *in vitro*

Conversion of CD4⁺CD25⁻ T effector cells to Foxp3⁺ Tregs *in vitro* was performed as we previously reported (12). Briefly, the sorted CD4⁺CD25⁻ T effector cells from wt B6, CD1d^{-/-}, and J α 18^{-/-} mice were stimulated with anti-CD3 (clone 2C11, 3 μ g/mL) plus equal number of syngeneic APCs with or without TGF- β 1 (2 ng/ml) for 3 to 5 days. Cells were permeabilized and stained with FITC-anti-Foxp3 mAb (clone FIK-16s, eBioscience) and induction of new Foxp3⁺ T cells was analyzed by FACS. All samples were acquired using the FACScan (BD Biosciences, Mountain View, CA). Data analysis was performed using the FlowJoTM software (TreeStar, Ashland, OR).

Statistics

Graft survival was compared using the log-rank test and a p value < 0.05 was considered significant.

RESULTS AND DISCUSSION

We transplanted the fully MHC mismatched DBA/2 islet allografts into wt C57BL/6, CD1d^{-/-}, and J α 18^{-/-} mice and monitored their survival. As shown in Fig 1A, wt C57BL/6 mice promptly rejected the DBA/2 islets; the mean survival time (MST) for the islet allografts was only 16 days (n=5). CD1d^{-/-} and J α 18^{-/-} recipients rejected the DBA/2 islet allografts with a similar kinetics, and as compared to the wt B6 recipients, there was no marked differences in graft survival (MST=17 days, n=5 to 8). To determine whether long-term graft survival could be achieved in the absence of iNKT cells, we treated recipient mice with a tolerizing protocol consisting of DST and anti-CD154 mAb (11). Treatment of wt B6 recipients with the DST/anti-CD154 protocol uniformly induced long-term allograft survival (>100 days, n=7). Interestingly, this protocol also produced indefinite islet allograft survival in either CD1d^{-/-} recipients or in J α 18^{-/-} recipients; all islet allografts survived for >100 days in the treated recipients (n=7) (Fig 1A).

To test whether a state of donor specific tolerance is induced NKT-deficient mice, we performed nephrectomy on a cohort of DST/anti-CD154 treated CD1d^{-/-} and J α 18^{-/-} recipients 100 days after transplantation to remove the primary islet allografts (transplanted under the renal capsule). This led to a sharp rise in blood sugar levels exceeding 400 mg/dl 3 days later (data not shown), demonstrating that the euglycemia is maintained by the transplanted islets. The nephrectomized recipients were then grafted with a second DBA/2 islet allograft and a third-party C3H/He islet allograft under the renal capsule of the other kidney without further treatment. We observed that such recipient mice readily accepted the second DBA/2 islet allografts (>60 days, n=3) but promptly rejected the third party ones (MST=19 days, n=3), suggesting that a state of donor specific tolerance is indeed achieved.

These data demonstrate that tolerance can be successfully established in the absence of iNKT cells in the model.

It has been shown that tolerance induced by the DST/anti-CD154 protocol depends on Foxp3⁺ Tregs (11). It is possible that tolerance in CD1d^{-/-} and J α 18^{-/-} mice may involve other types of regulatory cells or may similarly require Foxp3⁺ Tregs (13). To address this question, we depleted CD4⁺CD25⁺ T cells, which are highly enriched for the Foxp3⁺ Tregs (14), from DST/MR1 treated mice at the time islet transplantation, and graft survival was determined and compared to the non-depleted controls. As shown in Fig 1B, depletion of Foxp3⁺ Tregs rendered the DST/MR1 protocol completely ineffective at inducing islet allograft tolerance in CD1d^{-/-} and J α 18^{-/-} hosts (Fig 1B), suggesting that, similar to wt B6 mice, CD1d^{-/-} and J α 18^{-/-} mice also require Foxp3⁺ Tregs to achieve islet allograft tolerance following DST/MR1 treatment.

We further analyzed the CD4⁺Foxp3⁺ Tregs in the spleen and lymph nodes of CD1d^{-/-} and J α 18^{-/-} mice, and compared those in wt B6 mice. We did not find noticeable differences in Foxp3⁺ Tregs among these mice, neither in relative percentage to total CD4⁺ T cells nor in absolute cell numbers (Fig 2). Furthermore, Foxp3⁺ Tregs sorted from wt B6, CD1d^{-/-}, and J α 18^{-/-} mice exhibited comparable efficacy in suppressing proliferation of CD4⁺CD25⁻ T effector cells in vitro (Fig 2). Thus, genetic deficiency of iNKT cells did not seem to impact the genesis and the function of naturally arising Foxp3⁺ Tregs. Additionally, CD4⁺CD25⁻ T effector cells sorted from CD1d^{-/-} and J α 18^{-/-} mice could be readily converted to Foxp3⁺ cells by TGF- β in vitro, and the extent of conversion was also comparable to that of wt B6 CD4⁺CD25⁻ T cells (Fig 3).

So, all together, our data suggest that the iNKT cells are dispensable for the induction of islet allograft tolerance in a model that requires Foxp3⁺ Tregs. Clearly, both natural Tregs and induced Tregs are normal, both phenotypically and functionally, in the absence of iNKT cells. The loss of tolerance in Foxp3⁺ Treg-depleted hosts suggests that other types of regulatory cells unlikely play a significant role in tolerance induction in CD1d^{-/-} and J α 18^{-/-} recipients. The use of two NKT deficient strains (CD1d^{-/-} and J α 18^{-/-}) in the present study should be complementary and informative. Clearly, the question remains, why are iNKT cells required for allograft survival in some models, but not in others (15)? We consider the following possibilities. First, tolerizing protocols that stimulate a prominent activation of Foxp3⁺ Tregs, like the DST and anti-CD154 one used in the present study, may not rely on iNKT cells for tolerance induction. There is no question that iNKT cells are regulatory cells in some settings, but the catastrophic autoimmune diseases in mice (and in humans) deficient for Foxp3⁺ Tregs, a feature that is not seen in NKT deficient mice, suggest that it is the Foxp3⁺ Tregs that dominate the regulatory network in the immune system (16). Thus, in tolerant models where Foxp3⁺ Tregs are predominant, iNKT cells are likely to be dispensable. Second, iNKT cells are not a uniform population but rather consist of many different subsets; such subsets exhibit fine differences in functions and locations in vivo, those that are involved in heart and liver allograft survival may not be necessarily critical to islet allograft survival (7). Finally, islets grafted under the renal capsule may have limited access to iNKT cells compared to those transplanted in the liver where iNKT cells usually reside in large numbers (8), this different accessibility may also reflect their different roles in graft survival. Central to those questions is whether and how NKT cells respond to allotransplants. In contrast to classical T cells, the invariant TCR on NKT cells recognizes glycolipids, which are not known to have allospecificity (17). Hopefully, our data will stimulate continued interests in further uncovering the exact mechanisms of iNKT cells in regulating transplant outcomes.

Highlights

>Allograft tolerance can be established in the absence of NKT cells. > Tolerance in this model is dependent on Foxp3+ Tregs. > The role of NKT cells may be highly conditional in transplant settings.

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ABBREVIATIONS

MST	median survival time
KO	knockout
NKT	natural killer T cells

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FIGURE 1. Islet allograft survival in wt B6, CD1d^{-/-}, and Ja18^{-/-} mice treated with DST/anti-CD154 mAb

(A). DBA/2 islets were transplanted under the renal capsule into B6, CD1d^{-/-}, and Ja18^{-/-} mice (~400 islets per recipients), and groups of recipients were also treated with DST/anti-CD154. Graft survival was shown in a Kaplan-Meier plot. (B). Islet allograft survival in DST/anti-CD154 treated recipients with or without depletion of CD25⁺CD4⁺ Tregs. Treg depletion was accomplished by treating recipient mice with anti-CD25 (clone PC61, 0.25mg i.p.) on days -2, 0, +2, and day 0 was the time of transplantation.

FIGURE 2. Comparison of CD4⁺Foxp3⁺ Tregs in wt B6, CD1d^{-/-}, and Ja18^{-/-} mice
 (A). Cells were prepared from the spleen and lymph nodes of wt B6, CD1d^{-/-}, and Ja18^{-/-} mice, and stained with FITC-anti-mouse CD4 and PE-anti-mouse Foxp3. Tregs were identified as CD4⁺Foxp3⁺, and the Tregs among total CD4⁺ T cells were plotted and shown (mean $\pm$ SD of 3 experiments). (B). Comparison of wt B6, CD1d^{-/-}, and Ja18^{-/-} CD4⁺CD25⁺ Tregs in suppressing T effector cell proliferation in vitro. Suppression was calculated based on proliferation of T effector cells in the absence of Tregs. One of 3 individual experiments is shown.

FIGURE 3. Conversion of CD4⁺CD25⁻ T cells to Foxp3⁺ Tregs in vitro by TGF- β

The CD4⁺ CD25⁻ T effector cells were FACS sorted from wt B6, CD1d^{-/-}, and Ja18^{-/-} mice and stimulated with anti-CD3/APCs with or without TGF- β for 3 days. Induction of Foxp3⁺ T cells was assessed by FACS and shown. Representative data of 1 of 3 experiments are shown.