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Anti-CD45RB/anti-TIM-1-induced tolerance requires regulatory B cells

Kang Mi Lee, PhD^{*,1}, James I. Kim, PhD^{*,1}, Ryan Stott^{*}, Julie Soohoo^{*}, Matthew R O'Connor^{*}, Heidi Yeh, MD^{*}, Gaoping Zhao, MD^{*,†}, Philip Eliades^{*}, Courtney Fox^{*}, Nan Cheng^{*}, Shaoping Deng, MD^{*,†}, and James F. Markmann, MD, PhD^{*}

^{*}Division of Transplantation, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

[†]Department of Surgery, Sichuan Provincial People's Hospital and Sichuan Academy of Medical Sciences, Chengdu 610072, Sichuan Province, P. R. China

Abstract

The role of B cells in transplant tolerance remains unclear. Although B cell depletion often prolongs graft survival, sometimes it results in more rapid rejection, suggesting that B cells may have regulatory activity. We previously demonstrated that tolerance induction by anti-CD45RB antibody requires recipient B cells. Here we show that anti-CD45RB in combination with anti-TIM-1 antibody has a synergistic effect, inducing tolerance in all recipients in a mouse islet allograft model. This effect depends on the presence of recipient B cells, requires B cell IL-10 activity, and is antigen-specific. These data suggest the existence of a regulatory B cell population that promotes tolerance via an IL-10-dependent pathway.

Keywords

regulatory B; TIM-1; anti-CD45RB; tolerance; allograft

Introduction

B cells have a well-established role in allograft rejection, both in hyperacute and antibody-mediated rejection (1), as well as in promoting cellular immunity (2, 3). However, there has been increasing evidence for a subset of B cells that is capable of suppressing, rather than enhancing immunity. In the experimental autoimmune encephalitis (EAE) mouse model, adoptive transfer of a specific subset of CD1d^{hi} CD5⁺ B cells negatively regulates disease induction in B cell-depleted mice in an IL-10-, CD40-, and B7-dependent manner (4, 5). IL-10-producing B cells are also associated with increased tumor growth and impaired T cell responses (4, 5). In a collagen-induced arthritis model, CD19⁺CD21^{hi}CD23^{hi}CD24^{hi} transitional B cells have suppressive activity (6).

Small, resting B cells have been associated with prolonged islet allograft survival in mice (7), although it is unclear whether this is due to true, regulatory activity, or simply induction

Address Correspondence to either: James Markmann, MD, PhD, 55 Fruit Street – 5 White, Massachusetts General Hospital, Boston, MA 02114, Phone: 617-643-4533, FAX: 617-643-4579, jmarkmann@partners.org. James I Kim, PhD, 807 Thier Building, Massachusetts General Hospital, Boston, MA 02114, Phone: 617-643-0373, FAX: 617-724-7165, jkim35@partners.org.

¹These authors contributed equally to this manuscript.

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of anergy resulting from aberrant antigen presentation by the B cells (8–10). However, we previously reported more compelling results that transplant tolerance induced by anti-CD45RB is dependent on the presence of recipient B cells. In our model, tolerance required both CD40 and B7 expression on the B cells, suggesting that lack of co-stimulatory molecules and subsequent anergy did not play a major role in the pathway to long-term graft acceptance (11).

In that model, tolerance occurred in only a fraction of recipients, so we investigated the effect of combining anti-CD45RB treatment with an antibody that could be expected to promote tolerance via T cell effects. The T cell immunoglobulin and mucin domain (TIM) family proteins are potent costimulatory molecules in T cell activation (12). RMT1-10, a monoclonal antibody that blocks TIM-1 signaling, prolongs graft survival and promotes costimulatory blockade-induced tolerance (13). Here, we report that the effects of anti-CD45RB in combination with anti-TIM-1 are not only additive, but synergistic. Their combined effect is dependent on the presence of B cells, regulatory T cells, and B cell IL-10.

Materials and Methods

Mice

BALB/c, C57BL/6, B6 μ MT^{-/-}, and IL-10^{-/-} mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed under specific pathogen-free barrier conditions. All procedures detailed below were performed under the principles of laboratory animal care and approved by the IACUC committee at Massachusetts General Hospital.

Allografts

Diabetes in C57BL/6 mice or B6 μ MT^{-/-} mice was induced by a single intraperitoneal injection of 200 mg/kg streptozotocin (STZ, Sigma-Aldrich). Diabetes was defined as blood glucose levels > 300 mg/dL for at least 3 consecutive days. Islets from BALB/c donors were isolated by the standard technique of collagenase digestion and Ficoll density gradient purification. 500 fresh islets were transplanted under the kidney capsule of diabetic mice. Euglycemia was defined as a non-fasting blood glucose level < 200mg/dL. Rejection was diagnosed when animals became hyperglycemic again, with blood glucose > 200 mg/dL for at least two consecutive days. Allograft function was confirmed by nephrectomy of the kidney containing the transplanted islets. All recipients with long term grafts became hyperglycemic within 48 hours of nephrectomy.

Antibody Therapies

100 μ g of anti-CD45RB mAb was administered on days 0, 1, 3, 5, and 7 following transplant, 500 μ g of antagonistic anti-TIM-1 mAb (RMT1-10) on day -1, 300 μ g on days 0 and 5, 250 μ g of anti-CD25 mAb was on days -6 and -1. Antibodies were purchased from Bio Express, Inc. (West Lebanon, NH). For IL-10 neutralization, 200 μ g rat anti-mouse IL-10 antibody (clone JES5-2A5 from Bio Express, NH) was administered i.p. every other day post-transplantation for a total of 5 doses. 250 μ g anti-CD20 mAb (provided by Biogen IDEC) was administered on day 9 i.p.

Cell Sorting and Transfer

B cells from C57BL/6 mice bearing long term islet allografts (BALB/c) after combined antibody treatment were selected using Miltenyi anti-CD19 microbeads (Germany). Purity of the resulting B cell population exceeded 95%. 5×10^6 B cells were then injected into either C57BL/6 or B6 μ MT^{-/-} that received simultaneous BALB/c islet transplants without antibody treatment.

Diabetic B6.RAG recipients received 5×10^6 sorted TIM-1+ or TIM-1- B cells along with 5×10^6 B cell-depleted naive C57BL/6 splenocytes intravenously. Recipients received islet allografts on the day of adoptive transfer.

Flow cytometry

Lymphocytes were prepared from spleen and peripheral lymph nodes of mice that were transplanted and treated with combined antibody therapy. The following antibodies were used for staining: CD4-PECy7 (eBioscience), B220 Pacific Blue, CD1d PE, CD5 Pacific Blue, IgM FITC, CD21 APC, CD24 Pacific Blue, RMT1- 4-biotin (all from eBioscience), and streptavidin-A750 (Invitrogen). Cells were analyzed on the LSRII cytometer (Becton Dickinson) and the results were analyzed with FlowJo (Treestar) software.

Statistical Analysis

For survival studies, Kaplan-Meier survival curves were generated and statistical analysis performed by use of the log-rank test. All data were analyzed by the Prism 5 Program (Graph Pad, San Diego, CA). A value of $p < 0.05$ was considered significant.

Results

100% long-term graft survival with combined antibody treatment

Anti-TIM-1 treatment alone did not prolong islet allograft survival. About half of allografts survive long-term in anti-CD45RB treated recipients, consistent with previous reports (14). However, anti-CD45RB and anti-TIM-1 treatments together resulted in long-term survival of grafts in all recipients (Figure 1).

Significant TIM-1 upregulation on B cells upon anti-CD45RB and anti-TIM-1 treatment

While TIM-1 was originally described as a costimulatory molecule for T cells, it is more strongly expressed on B cells than on CD4+ T cells (15). TIM-1 expression on splenic B cells is significantly upregulated in islet recipients treated with anti-CD45RB and anti-TIM-1 (Figure 2). The absolute number of B cells or total splenocytes is not significantly altered in any of the groups (data not shown). There is no increase in TIM-1 expression following antibody injection alone, of either anti-TIM-1 or anti-TIM-1/anti-CD45RB, in the absence of transplant (data not shown).

Combined antibody-induced graft survival requires B cells

Due to the significant upregulation of TIM-1 on B cells upon transplant with antibody treatment, we investigated graft survival in B cell-deficient mice. BALB/c islets transplanted into $\mu\text{MT}^{-/-}$ (C57BL/6 background) recipients are rejected consistently and with a tempo comparable to wild-type (WT) recipients (Figure 3A). Graft survival in $\mu\text{MT}^{-/-}$ mice treated with anti-TIM-1 antibody alone or with anti-CD45RB antibody alone is prolonged compared to wild-type recipients. In contrast, combined antibody treatment in $\mu\text{MT}^{-/-}$ recipients resulted in no extension of graft survival over wild-type recipients (Figure 3A). We confirmed the role of B cells by reconstituting the B cell-deficient recipients with mature syngeneic B cells by iv injection from normal C57BL/6 on the day of transplant. Dual antibody treatment restores tolerance induction to islet allografts in these mice (data not shown and Figure 4).

To further demonstrate B cell dependence, we performed antibody based B cell depletion in WT recipients. C57BL/6 recipients were given a standard course of anti-TIM-1 and anti-CD45RB and additionally treated with a single dose of anti-CD20 on day 9 post-transplant. Anti-CD20 treatment results in sustained B cell depletion more than one week post-injection

(data not shown). 60% of these mice rapidly rejected their islet grafts following anti-CD20 administration (Figure 3B). Thus we conclude that graft acceptance following combined antibody treatment is B cell-dependent.

Regulatory B cells have been demonstrated to be IL-10-dependent (4, 15, 16), thus, we tested whether the combined antibody treatment was also IL-10-dependent. On the day of transplant, μ MT^{-/-} recipients received standard dual antibody treatment plus either IL-10 competent or deficient purified B cells. Transfer of wild-type B cells results in significant prolongation of graft survival, while in contrast, IL-10-deficient B cells are unable to significantly prolong graft survival (Figure 4). We conclude that in this model graft survival is dependent on IL-10 production by B cells.

Tolerant B cells transfer antigen-specific tolerance

Given the requirement for B cells, we FACS purified B cells from recipients bearing long-term surviving BALB/c donor grafts (> 100 days, LTS) and adoptively transferred them to μ MT^{-/-} and WT mice that received BALB/c transplants without getting antibody treatment. Indefinite graft survival was seen in these recipients, although the effect was more pronounced in μ MT^{-/-} mice than in WT mice (Figures 5A&B). Adoptive transfer of naive B cells does not prolong graft survival (Figure 5A). LTS B cells cannot prolong graft survival of third party C3H grafts, which suggests that these tolerant B cells are antigen-specific. Adoptive transfer of B cells from long-term survivor anti-CD45RB-treated recipients does not prolong graft survival (Figure 5B).

We then sorted TIM-1⁺ and TIM-1⁻ B220 B cells from C57BL/6 mice 14 days after injecting 20×10^6 irradiated BALB/c splenocytes and the standard course of anti-CD45RB/anti-TIM-1 antibodies, as an alternate immunizing/tolerizing stimulus. B cell subpopulations were then adoptively transferred with an equal number of B cell-depleted naive C57BL/6 splenocytes to diabetic B6.RAG recipients simultaneously transplanted with BALB/c islets. Compared to TIM-1⁻ B cells, co-transfer of TIM-1⁺ B cells results in significant prolongation of BALB/c islet grafts (Figure 5C). From this we conclude that TIM-1⁺ B cells contain a regulatory population and that TIM-1 may be a marker for regulatory B cells.

Tregs are also required for long-term graft acceptance

Regulatory T cells (Tregs) have previously been demonstrated to be involved in anti-CD45RB and anti-TIM-1-mediated allograft survival (13). We found that the percentage of Tregs significantly increases following combined antibody treatment (Figures 6A and B). Depletion of Tregs by anti-CD25 PC61 treatment pre-transplant resulted in prompt rejection in nearly all such treated recipients (Figure 6C).

Discussion

Anti-TIM-1 alone did not prolong survival of islet allografts and anti-CD45RB alone promoted long-term survival in only 40% of recipients, but the two antibodies combined resulted in 100% long-term graft survival. This synergistic effect was completely dependent on the presence of recipient B cells. Although the number of Tregs increased, and Treg depletion led to rejection, isolated B cells, in particular, TIM-1⁺ B cells from antibody-treated recipients were sufficient to transfer tolerance, without the transfer of Tregs.

Each antibody alone prolongs graft survival in the absence of B cells, which is not unexpected as the antibodies do not target B cells exclusively (13, 18, 19), and adoptive transfer of B cells from mice treated with either antibody alone into μ MT or WT recipient is not capable of increasing graft survival (Figure 5B and data not shown). The synergistic effect of the two antibodies suggests that each antibody is acting on an independent

signaling pathway, and that the two pathways interact to generate an effective regulatory, TIM-1+ B cell population. It is interesting to note that anti-CD45RB alone can induce tolerance in μ MT mice, but combined anti-CD45RB/anti-TIM-1 cannot. Although this could be explained by Ding and Rothstein's finding that anti-TIM-1 treatment actually accelerates rejection in B cell-deficient mice, in our strain combination, anti-TIM-1 minimally prolongs graft survival. This suggests that either anti-CD45RB and anti-TIM-1 function in a single pathway or that the dependence of anti-TIM-1 on B cells is dominant to the B cell-independent effects of anti-CD45RB.

Ding *et al.* recently published studies demonstrating regulatory activity by TIM-1+ B cells in the reverse strain combination (C57BL/6 to BALB/c), using anti-TIM-1 treatment in an islet transplant model (15). Using C57BL/6 recipients, with anti-TIM-1 monotherapy we do not observe significant graft prolongation (Figure 1) while we do observe prolongation in the BALB/c background consistent with Ding *et al.* This may be due to strain-dependent response variation (20). For example, in response to the same immune stimulus, BALB/c are more likely to develop a Th2 response while C57BL/6 are more likely to develop a Th1 response.

Differences in vascularization, expression of costimulatory markers, adhesion molecules, cytokines, chemokines, and receptors/ligands on leukocytes as well as on endothelial cells have been proposed for understanding organ-specific differences in tolerance and rejection (24–27). Thus, specific organs may produce a unique set of cytokines and/or respond differently to cytokines produced by the immune system. For example, recipients deficient in CCR2, the receptor for chemokine MCP-1, reject heart allografts but mount a limited response against islet allografts. Similarly, we have previously shown that anti-CD45RB cannot induce tolerance to cardiac allografts, but find in this study that it does induce tolerance to islet allografts. IL-10 is not required for tolerance to heart grafts induced by anti-CD45RB, but IL-10 production by B cells is required for tolerance to islets. Furthermore, anti-CD45RB-induced cardiac tolerance requires B cell expression of B7 and CD40, but does not require expression of antibody (or IL-10) (11, 17), suggesting that in islet tolerance, Bregs interact directly with T cells rather than promoting tolerance by using cytokine expression to influence T cell function or differentiation. Thus, anti-CD45RB-induced tolerance could serve as an additional model to study organ-specific rejection.

B cells are known to be important as antigen presenting cells (28), providing costimulatory signals (29), and driving Th1 or Th2 differentiation (30). Considering the simultaneous requirement for Tregs in combined antibody induced tolerance and the fact that antigen is required for the development of TIM-1+ B cells, it is tempting to speculate that TIM-1+ B cells act as Treg inducing antigen presenting cells. This will require further work to elucidate. While regarded as an antagonistic Ab, the mechanism of RMT1-10 action remains unclear, including its ability to increase the percentage of TIM-1+ B cells. Potentially the TIM-1-TIM-4 interaction downmodulates TIM-1 expression and by blocking this interaction, TIM-1 expression is increased.

In summary, we have shown that combined anti-CD45RB and anti-TIM-1 treatment results in 100% long term islet allograft survival that is B cell-dependent, dependent on B cell production of IL-10, and is associated with upregulation of TIM-1 on B cells. Our data support other's findings that transplant tolerance may depend on a novel TIM-1+ regulatory B cell subset.

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Figure 1. Anti-TIM-1 cooperates with anti-CD45RB to promote long-term graft survival of islet allografts

BALB/c islets were transplanted into diabetic C57BL/6 recipients. Anti-CD45RB treatment results in significant prolongation of islet allograft survival ($p < 0.0001$ *** versus no treatment). While anti-TIM-1 treatment alone provides no graft survival prolongation, anti-TIM-1 plus anti-CD45RB treatment resulted in long-term survival in all recipients ($p < 0.002$ ** versus no treatment; $p < 0.05$ * versus anti-CD45RB alone).

Figure 2. TIM-1 is upregulated on B cells upon dual antibody treatment

TIM-1 expression on B cells increases after islet transplantation and anti-CD45RB/anti-TIM-1 antibody treatment. FACS plots are representative of at least three independent experiments examined at day 14. Dotted line, isotype control. Increased expression in grafted dual antibody treated recipients is statistically significant, $p < 0.01$ versus naive, and $p < 0.02$ versus grafted alone.

Figure 3. Prolonged graft survival is B cell-dependent

(A) B cell-deficient recipients rapidly reject islet allografts. Islet allografts in wild-type recipients treated with anti-TIM-1 plus anti-CD45RB treatment survive indefinitely. In contrast, B cell-deficient recipients treated with anti-TIM-1 plus anti-CD45RB reject promptly. (B) B cell depletion 9 days after transplant results in rejection of islet allografts. B cells were depleted by a single dose of anti-CD20 Ab (250 μ g).

Figure 4. Graft survival by regulatory B cells is IL-10-dependent

Dual antibody-treated B cell-deficient recipients rapidly reject islet allografts. Adoptive transfer of 5×10^6 wild-type purified B cells to $\mu\text{MT}^{-/-}$ recipients restores graft survival in antibody-treated recipients. In contrast, adoptive transfer of IL-10-deficient B cells are unable to restore graft survival.

A. μ MT B6 recipients **B. B6 recipients** **Figure 5C** **Figure 5. B cells confer increased graft survival upon adoptive transfer**

Total B cells from recipients with long-term survivor (LTS) grafts were purified and 5×10^6 were adoptively transferred to B cell-deficient (A) or to wild-type (B) recipients receiving BALB/c islets and no antibody treatment. In contrast to B cells from naive mice, adoptive transfer of LTS B cells significantly prolonged islet allograft acceptance. In contrast to survival of BALB/c islets, third party C3H islets are rapidly rejected demonstrating antigen-specificity of B cells. In C57BL/6 recipients, adoptive transfer of LTS B cell from anti-CD45RB antibody-treated recipients does not prolong graft survival. (C) C57BL/6 mice were given anti-TIM-1, anti-CD45RB, and irradiated BALB/c splenocytes. On day 14, TIM-1+ and TIM-1- B cells were sorted and adoptively transferred to diabetic B6.RAG along with naive C57BL/6 splenocytes. On the day of adoptive transfer, B6.RAG also received BALB/c islet allografts. Relative to TIM-1- B cells, sorted TIM-1+ B cells significantly prolongs islet allograft survival.

Figure 6. Prolonged graft survival is also Treg-dependent

(A and B) Splenocytes were analyzed 2 weeks post-transplant, revealing a significant increase in the percentage of Fop3+ CD4+ T cells following combined antibody treatment. Analysis of lymph node showed similar results (data not shown). n=4 mice per group. FACS plots are representative of at least three independent experiments. (C) Treg depletion with anti-CD25 PC61 prior to transplant (days -6 and -1) and combined antibody treatment results in prompt rejection of islet allografts (median survival time = 10 days).