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*J Clin Invest.* 2008;118(2):742-751. <https://doi.org/10.1172/JCI32451>.

### Research Article

T cell Ig mucin 1 (TIM-1) plays an important role in regulating immune responses in autoimmune and asthma models, and it is expressed on both Th1 and Th2 cells. Using an antagonistic TIM-1-specific antibody, we studied the role of TIM-1 in alloimmunity. A short course of TIM-1-specific antibody monotherapy prolonged survival of fully MHC-mismatched vascularized mouse cardiac allografts. This prolongation was associated with inhibition of alloreactive Th1 responses and preservation of Th2 responses. TIM-1-specific antibody treatment was more effective in Th1-type cytokine-deficient *Stat4*<sup>-/-</sup> recipients as compared with Th2-type cytokine-deficient *Stat6*<sup>-/-</sup> recipients. Subtherapeutic doses of rapamycin plus TIM-1-specific antibody resulted in allograft acceptance and prevented the development of chronic allograft vasculopathy. Allograft survival via this treatment was accompanied by a Th1- to Th2-type cytokine switch. Depletion of natural Tregs abrogated the graft-protecting effect of the TIM-1-specific antibody. Importantly, CD4<sup>+</sup>CD25<sup>+</sup> Tregs obtained from long-term survivors had enhanced regulatory activity as compared with naive CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Consistent with this, TIM-1-specific antibody treatment both preserved Tregs and prevented the expansion of alloreactive effector Th1 cells in an alloreactive TCR transgenic adoptive transfer model. These studies define previously unknown functions of TIM-1 in regulating alloimmune responses in vivo and may provide a novel approach to promoting transplantation tolerance.

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# The emerging role of T cell Ig mucin 1 in alloimmune responses in an experimental mouse transplant model

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**T cell Ig mucin 1 (TIM-1) plays an important role in regulating immune responses in autoimmune and asthma models, and it is expressed on both Th1 and Th2 cells. Using an antagonistic TIM-1-specific antibody, we studied the role of TIM-1 in alloimmunity. A short course of TIM-1-specific antibody monotherapy prolonged survival of fully MHC-mismatched vascularized mouse cardiac allografts. This prolongation was associated with inhibition of alloreactive Th1 responses and preservation of Th2 responses. TIM-1-specific antibody treatment was more effective in Th1-type cytokine-deficient *Stat4*<sup>-/-</sup> recipients as compared with Th2-type cytokine-deficient *Stat6*<sup>-/-</sup> recipients. Subtherapeutic doses of rapamycin plus TIM-1-specific antibody resulted in allograft acceptance and prevented the development of chronic allograft vasculopathy. Allograft survival via this treatment was accompanied by a Th1- to Th2-type cytokine switch. Depletion of natural Tregs abrogated the graft-protecting effect of the TIM-1-specific antibody. Importantly, CD4<sup>+</sup>CD25<sup>+</sup> Tregs obtained from long-term survivors had enhanced regulatory activity as compared with naive CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Consistent with this, TIM-1-specific antibody treatment both preserved Tregs and prevented the expansion of alloreactive effector Th1 cells in an alloreactive TCR transgenic adoptive transfer model. These studies define previously unknown functions of TIM-1 in regulating alloimmune responses in vivo and may provide a novel approach to promoting transplantation tolerance.**

## Introduction

The T cell Ig mucin (TIM) family of genes encodes proteins that are expressed by T cells and contain an IgV-like and a mucin-like domain (1). The TIM family comprises 8 genes on mouse chromosome 11B1.1 and 3 genes on the human chromosome 5q33.2. The 3 human TIM genes are most similar to mouse TIM-1/TIM-2, TIM-3, and TIM-4. The ligands for TIM proteins are TIM-4 for TIM-1; semaphorin 4A for TIM-2; and galectin-9 for TIM-3 (2–4). The role of TIM proteins in T cell differentiation, in T cell effector function, and in autoimmunity and allergy/asthma are just beginning to emerge (5). Genetic and functional data indicate that the TIM family might have an important role in susceptibility to autoimmune diseases and asthma/allergy. Indeed, TIM-1 has been genetically linked to murine airway hypersensitivity (6), and polymorphic forms of TIM-1 have been associated with susceptibility to human asthma, eczema, and rheumatoid arthritis (7, 8). TIM-1 is also expressed on activated T cells and, upon CD4<sup>+</sup> T cell polarization, is expressed at a higher level on Th2 cells than on Th1 cells (9). Initial data have suggested that TIM-1 expressed on the T cells is a positive costimulatory molecule resulting in enhancement of T cell proliferation, cytokine production, and abrogation

of tolerance (9, 10). TIM-4 is expressed on APCs and was recently identified as the natural ligand for TIM-1 (3).

The functions of the TIM-1/TIM-4 pathway in regulating alloimmunity remain unknown. Our group has recently characterized 2 monoclonal anti-TIM-1 antibodies that differentially regulate expansion of antigen-specific T cells, cytokine production, and development of autoimmunity in vivo. We demonstrated that TIM-1 can deliver either a positive or negative/inhibitory signal into T cells, depending on the affinity with which TIM-1 is crosslinked (11).

In the current study, we used a blocking anti-TIM mAb, RMT1-10, to show that TIM-1 blockade prolongs allograft survival by downregulation of Th1 cell-mediated responses while promoting and preserving regulatory networks that include Th2 cells and CD4<sup>+</sup>CD25<sup>+</sup> Tregs. These data, together with those of the accompanying article by Degauque et al. (12) indicate that TIM-1-TIM-4 interaction plays an important role in regulating alloreactive T cells and provide the rationale to develop novel strategies to target TIM-1 to promote transplantation tolerance.

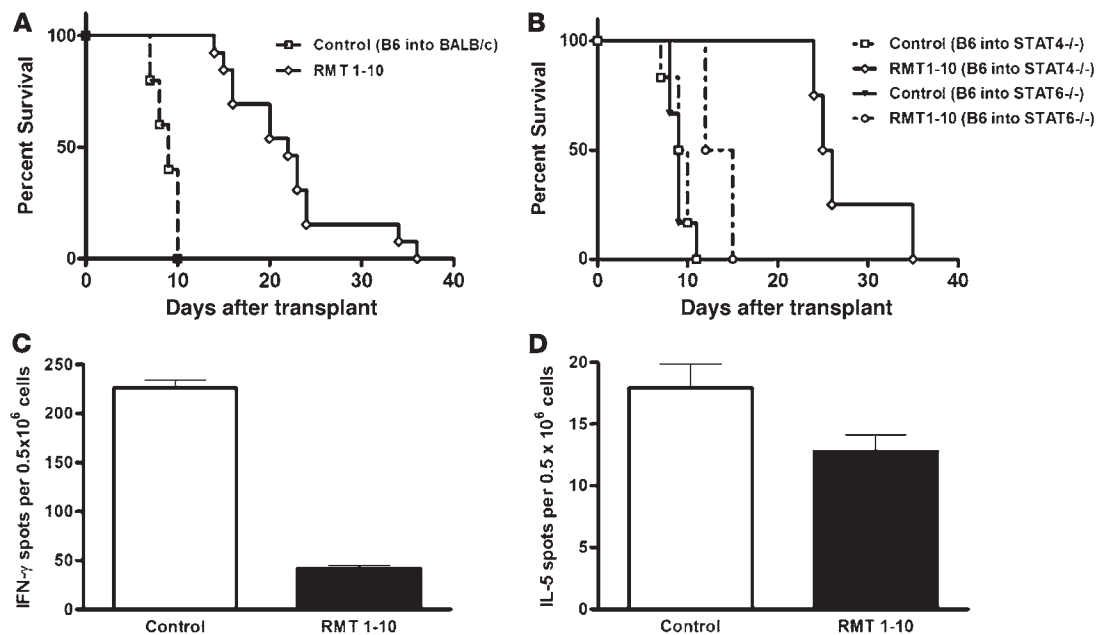
## Results

*RMT1-10 prolongs allograft survival in a fully MHC-mismatched cardiac transplant model.* We have recently characterized 2 different anti-TIM-1 antibodies that differ in T cell activation and proliferation and cytokine production depending on their binding affinities to TIM-1 molecules on T cells (11). The high-affinity agonistic 3B3 anti-TIM-1 mAb was originally generated by Umetsu et al. and was

**Nonstandard abbreviations used:** B6, C57BL/6; dLN, draining lymph node; MST, median survival time; Teff, effector T cell; TIM, T cell Ig mucin.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Citation for this article:** *J. Clin. Invest.* 118:742–751 (2008). doi:10.1172/JCI32451.

**Figure 1**

Prolongation of cardiac allograft survival by RMT1-10 is associated with a Th1-to-Th2 cytokine switch. B6 hearts were transplanted into BALB/c wild-type, STAT4<sup>-/-</sup>, and STAT6-deficient recipients. **(A)** Treatment with RMT1-10 resulted in significant prolongation of allograft survival in wild-type recipients. **(B)** Both treated STAT4<sup>-/-</sup> and STAT6<sup>-/-</sup> recipients showed significant prolongation of graft survival compared with untreated mice. However, anti-TIM-1 mAb was significantly more effective in STAT4<sup>-/-</sup> mice. Using the ELISPOT assay, the frequency of cytokine-producing allo-specific T cells was measured between days 10 and 12 after transplantation by stimulating splenocytes of BALB/c recipients of B6 heart allografts with irradiated B6 splenocytes. Administration of RMT1-10 decreased allospecific Th1 cytokines **(C)** and preserved the frequency of IL-5-producing alloreactive T cells **(D)**. Data are representative of 3 independent experiments and indicate the mean of triplicate results in each experiment.

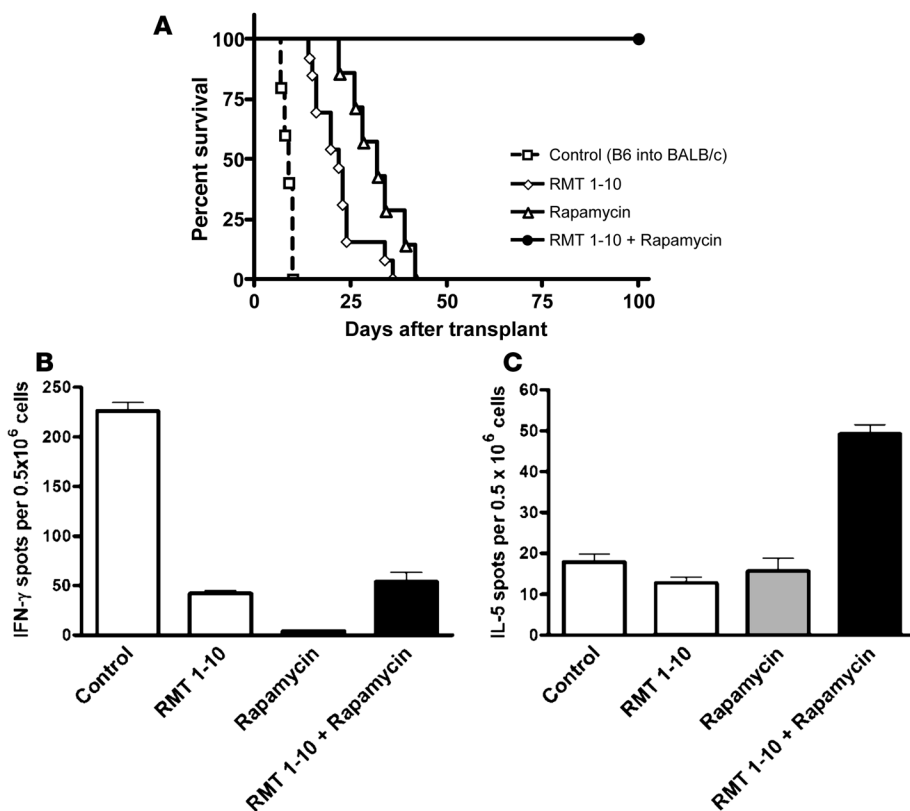
shown to heighten T cell activation and prevent the development of respiratory tract tolerance in a Th2-driven model of asthma (9). Our recent work has demonstrated that 3B3 anti-TIM-1 antibody binds TIM-1 with very high affinity and enhances the severity of EAE due to increasing auto-pathogenic Th1 and Th17 responses. The low-affinity blocking antibody, RMT1-10, inhibits auto-pathogenic Th1 and Th17 responses, induces a strong Th2 response, and protects from EAE (11). Therefore, we tested the effects of RMT1-10 mAb in an acute heterotopic vascularized cardiac allograft rejection model (C57BL/6 [B6] into BALB/c). First, recipients were treated with RMT1-10 mAb alone or isotype control Ig (0.5  $\mu$ g i.p. on day 0 and 0.25  $\mu$ g on days 2, 4, 6, 8, and 10). As shown in Figure 1A, a short-course monotherapy with RMT1-10 resulted in significant prolongation of allograft survival as compared with isotype-treated controls (median survival time [MST] 22 days,  $n = 13$  vs. MST 9,  $n = 5$ ;  $P < 0.0001$ ). A more prolonged therapy (above regimen plus 250  $\mu$ g 2x/week for 4 additional weeks) resulted in similar allograft survival (MST 23 days,  $n = 4$ ).

Analysis of the frequency of cytokine-producing alloreactive T cells by ELISPOT 14 days after cardiac transplantation in wild-type recipients demonstrated that there was significant reduction in the frequency of allospecific IFN- $\gamma$ -producing splenocytes in the treated animals ( $42.20 \pm 12.6$  vs.  $226.2 \pm 49.4$ ;  $P < 0.0001$ ), while the number of allospecific Th2- (IL-5-) producing cells was preserved ( $12.7 \pm 6.5$  vs.  $17.9 \pm 11.8$ ) (Figure 1, C and D).

To further elucidate the role of TIM-1 in alloreactive Th1/Th2 cell differentiation, we transplanted B6 hearts into STAT4<sup>-/-</sup> or STAT6<sup>-/-</sup> mice on the BALB/c background and treated them with

RMT1-10 or isotype control Ig. The STAT4<sup>-/-</sup> mice are deficient in Th1 cytokines, while the STAT6<sup>-/-</sup> mice are unable to produce Th2 cytokines, and both acutely reject fully allogeneic cardiac allografts at the same pace (MST 9.5 days,  $n = 6$  vs. MST 9,  $n = 6$ ) (13). Interestingly, as shown in Figure 1B, RMT1-10 mAb was significantly more effective in prolonging survival of B6 allografts in BALB/c STAT4<sup>-/-</sup> as compared with STAT6<sup>-/-</sup> recipients (MST 25.5,  $n = 4$  vs. MST 13.5,  $n = 6$ ;  $P = 0.008$ ). In addition, ELISPOT analysis of STAT6-deficient recipients demonstrated a more moderate reduction in the frequency of allospecific IFN- $\gamma$ -producing splenocytes ( $132 \pm 25$  vs.  $215 \pm 65$ ;  $P = 0.03$ ) and, as expected, lack of IL-5-producing splenocytes (<5 spots per well).

*Combination therapy with RMT1-10 and rapamycin results in long-term allograft acceptance and prevents development of chronic allograft vasculopathy.* Rapamycin has been reported to synergize with a variety of strategies targeting T cell costimulatory pathways (14, 15). We therefore evaluated the combination of RMT1-10 with a subtherapeutic short course of rapamycin (3 mg/kg on days 0–3), which only resulted in moderate prolongation of allograft survival (MST 32 days,  $n = 7$ ;  $P = 0.0001$ ). Combination of RMT1-10 mAb and rapamycin ( $n = 23$ ) resulted in synergistic prolongation of allograft survival and induction of long-term acceptance (>100 days) not seen with either agent alone (Figure 2A). As above, analysis of the frequency of cytokine-producing cells retrieved from recipients on day 14 after transplantation by ELISPOT demonstrated that there was significant reduction in the frequency of allospecific IFN- $\gamma$ -producing splenocytes in animals treated with combined RMT1-10 and rapamycin ( $53.7 \pm 47.5$  vs.  $226.2 \pm 49.4$ ;  $P < 0.0001$ ), while



**Figure 2**

Combination therapy with RMT1-10 and rapamycin results in allograft acceptance. (A) B6 hearts were transplanted into BALB/c recipients. While a subtherapeutic dose of rapamycin resulted in significant prolongation of cardiac allograft survival, combination of RMT1-10 with rapamycin resulted in allograft acceptance in all recipients. (B and C) ELISPOT assay was performed on day 14 after transplantation. Combination of RMT1-10 with rapamycin resulted in a significant reduction in IFN-γ-producing T cells and enhanced significantly the frequency of IL-5-producing T cells. Data are representative of 3 independent experiments and indicate the mean of triplicate results in each experiment.

the number of allospecific IL-5-producing cells was significantly enhanced ( $49.25 \pm 11$  vs.  $17.9 \pm 11.8$ ;  $P < 0.0001$ ), indicating that the long-term cardiac allograft survival with combined RMT1-10 and rapamycin is associated with a marked Th2 switch in the cytokine profile of alloreactive T cells (Figures 2, B and C).

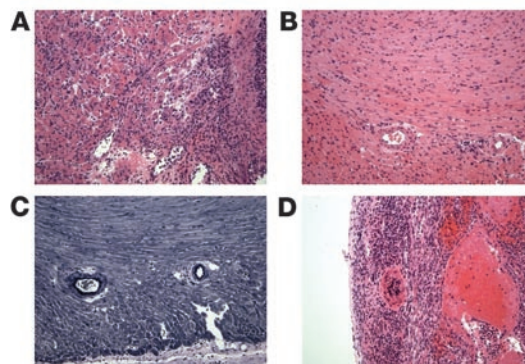
Histological assessment of harvested grafts mirrored the survival data. In untreated control recipients (Figure 3A), diffuse mononuclear cell infiltration was seen. In the RMT1-10 plus rapamycin-treated recipients (MST >100 days), little inflammatory infiltrate was seen, and myocytes were undamaged (Figure 3B). For assessment of chronic rejection and allograft vasculopathy, grafts were analyzed in sections stained with elastin van Gieson and showed no luminal occlusion (Figure 3C). There was also no perivascular fibrosis by Masson trichrome staining (data not shown). This finding is in contrast to the effect of other T cell costimulatory

blockade strategies, such as those targeting B7 and/or CD154, which can lead to prolonged allograft survival (>100 days) but do not necessarily prevent the development of chronic allograft vasculopathy (15–17). Furthermore, these data are in agreement with previously published data demonstrating attenuated chronic rejection and allograft vasculopathy in IFN-γ- (18) and STAT4-deficient (Th1-deficient) mice (19).

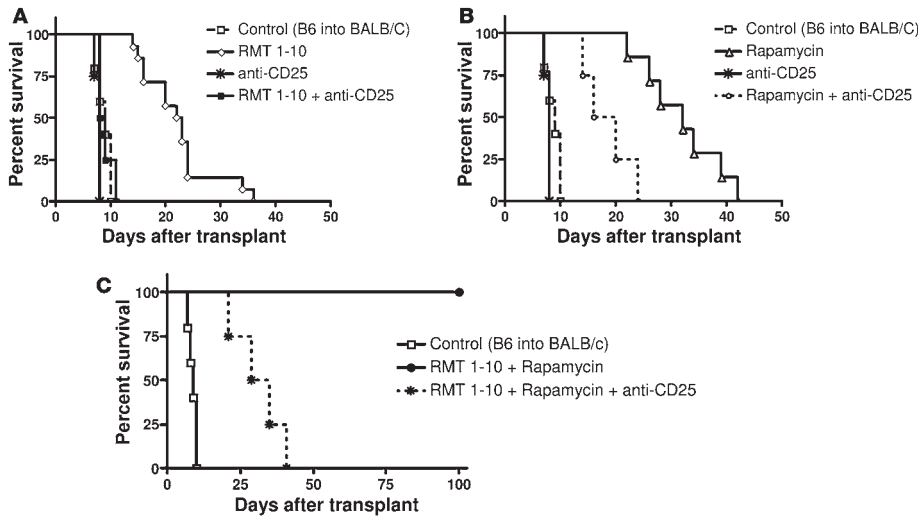
*Natural Tregs are essential for promotion of long-term allograft acceptance.* Tolerance induction is believed to involve, at least in part, a T cell-mediated process of immune regulation (20). Rapamycin was found to favor CD4<sup>+</sup>CD25<sup>+</sup> T cell-dependent immunoregulation in vitro and in vivo (21, 22). Furthermore, Th2 cytokines have been recently directly linked to the conversion of Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> Tregs from CD4<sup>+</sup>CD25<sup>-</sup> precursors in the periphery (23). To evaluate the role of CD4<sup>+</sup>CD25<sup>+</sup> Tregs in our model,

**Figure 3**

Histology of murine cardiac allografts from animals treated with RMT1-10 plus rapamycin. (A) Rejected B6 grafts were harvested from BALB/c recipients 10–14 days after transplantation in animals receiving no treatments and demonstrated diffuse mononuclear cell infiltrates. (B) B6 grafts harvested from BALB/c recipients more than 100 days after treatment with RMT1-10 plus rapamycin demonstrate little inflammatory cells and well-preserved cardiomyocytes. (C) Elastin van Gieson staining of cardiac allografts more than 100 days after treatment with RMT1-10 plus rapamycin showed intact blood vessels with minimal or no occlusion. (D) Rejected B6 grafts harvested from BALB/c recipients treated with RMT1-10 plus rapamycin after removal of natural Tregs demonstrated diffuse mononuclear cell infiltrates, myocyte destruction, and interstitial hemorrhage. Original magnification, ×10.







**Figure 4** Natural Tregs are essential for allograft prolongation based on RMT1-10–based strategies. Prolongation of allograft survival by RMT1-10 (A) and rapamycin (B) is clearly abrogated by depletion of CD25 prior to transplantation. (C) More importantly, administration of anti-CD25 mAb abrogated tolerance-inducing effects of combination treatment of RMT1-10 with rapamycin.

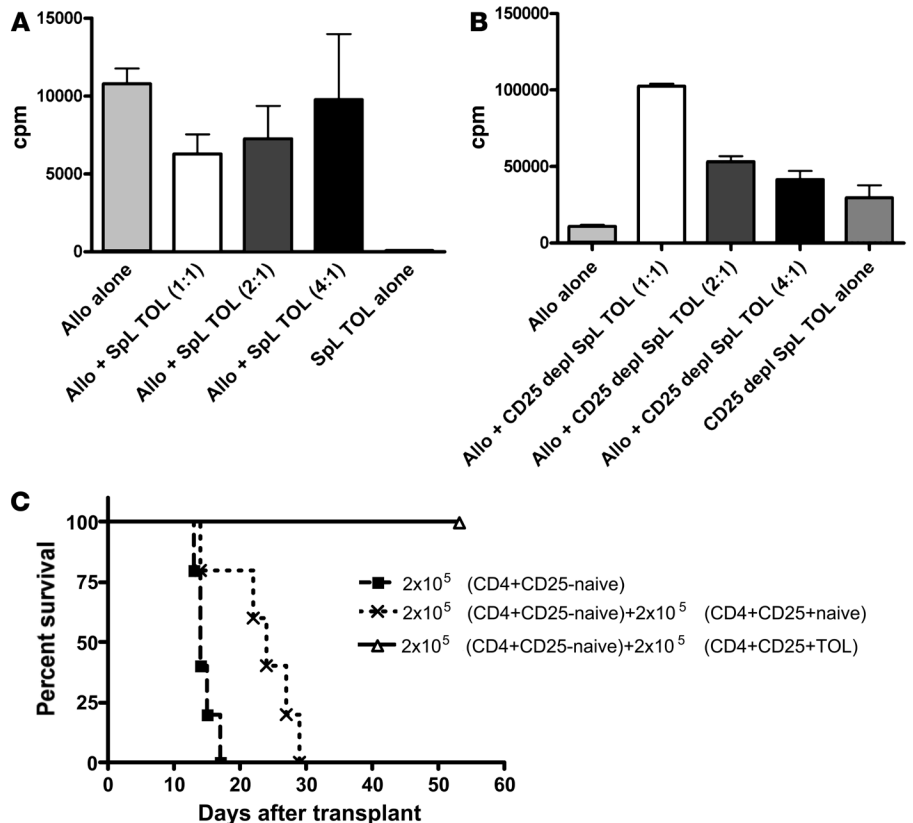
we depleted CD25<sup>+</sup> T cells by administering anti-CD25 mAb as previously described (24, 25). This regimen of anti-CD25 mAb (250 µg on days 6 and 1 before transplantation) resulted in acute rejection similar to that in untreated recipients (MST 8 days, *n* = 4; *P* = NS) (Figure 4A). The effect of RMT1-10 mAb was clearly dependent on the presence of CD4<sup>+</sup>CD25<sup>+</sup> T cells, as their prior depletion abrogated the protective effect of RMT1-10 (Figure 4A) (MST 8.5 days, *n* = 4; *P* < 0.0001 vs. RMT1-10 alone). CD25 depletion also abrogated the beneficial effect of rapamycin alone (Figure 4B) (MST 19 days, *n* = 4; *P* = 0.002). More importantly, RMT1-10 plus rapamycin therapy led to long-term allograft survival only in the presence of natural Tregs (Figure 4C) (MST 32 days, *n* = 4; *P* = 0.0001). The histological examination of rejected grafts demonstrated diffuse mononuclear cell infiltration with associated myocyte necrosis and interstitial hemorrhage (Figure 3D). Overall, the prolongation

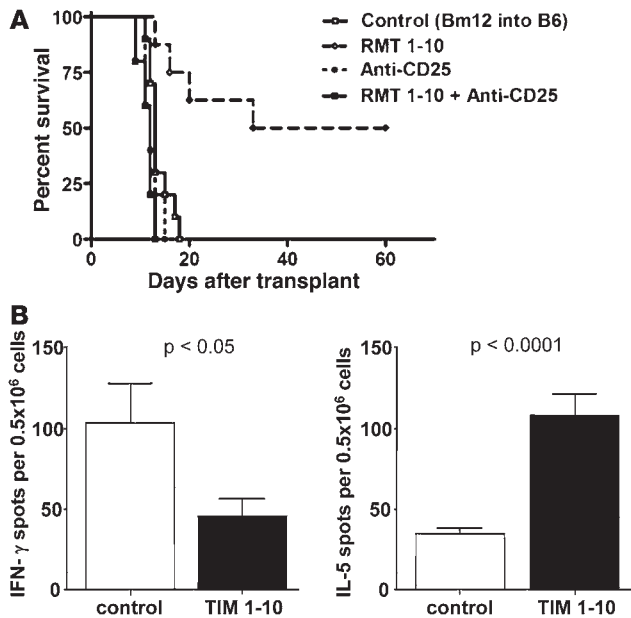
of allograft survival by RMT1-10 seems to be dependent on the presence of natural Tregs.

*Functions of in vivo-generated Tregs by RMT1-10 and rapamycin.* To establish the regulatory functions of CD4<sup>+</sup>CD25<sup>+</sup> Tregs after treatment with RMT1-10 and rapamycin, we first added splenocytes of tolerant animals with graft survival of more than 100 days before and after ex vivo CD25 depletion to a mixed lymphocyte reaction (MLR) at various ratios (Figure 5, A and B). While splenocytes from long-term surviving animals clearly suppressed the MLR in a dose-dependent manner (1:1, 5,079 ± 913.9 vs. 1,0783.8 ± 2,409 cpm;

**Figure 5**

RMT1-10 and rapamycin treatment leads to generation of Tregs with enhanced antigen-specific suppressive activity. (A and B) Modulatory cells from tolerant mice (SpL TOL) treated with RMT1-10 and rapamycin could suppress the mixed lymphocyte reaction (MLR) before but not after ex vivo depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells (CD25 depl). (C) Administration of 2 × 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells derived from naive BALB/c splenocytes into RAG-deficient recipients of B6 heart grafts resulted in prompt rejection. Transfer of 2 × 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>-</sup> naive splenocytes with CD4<sup>+</sup>CD25<sup>+</sup> naive Tregs resulted in moderate prolongation of cardiac allografts. In contrast, recipients of 2 × 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>-</sup> naive T cells with CD4<sup>+</sup>CD25<sup>+</sup> Tregs from tolerant mice experienced long-term graft survival.





**Figure 6**

Prolongation of skin allograft survival in a bm12 into B6 MHC class II-mismatched model by RMT1-10 is associated with a Th1-to-Th2 cytokine switch. (A) B6 recipients of bm12 skin grafts were left untreated ( $n = 9$ ) or treated with anti-TIM-1 mAb (RMT1-10) ( $n = 8$ ) as described in Methods. Kaplan-Meier plots demonstrate prolonged skin graft rejection in anti-TIM-1 mAb-treated recipients compared with untreated recipients. Depletion of CD4<sup>+</sup>CD25<sup>+</sup> Tregs with a depleting anti-CD25 mAb prior to transplantation abrogated the graft-protective effects of anti-TIM-1 mAb. (B) We explored the effect of anti-TIM-1 antibody on alloantigen-specific cytokine generation 14 days following engraftment. Splenocytes from anti-TIM-1-treated skin graft recipients or untreated recipients were isolated and stimulated with irradiated donor splenocytes from bm12 mice, and the frequency of Th1 (IFN- $\gamma$ ) and Th2 (IL-5) alloreactive T cells was enumerated using the ELISPOT assay. As shown in B, anti-TIM-1 treatment was associated with a dramatic reduction in the precursor frequency of IFN- $\gamma$ -secreting alloreactive T cells as compared with untreated recipients. Simultaneously, the frequency of alloreactive IL-5-secreting T cells was significantly increased. Data are representative of 3 independent experiments and indicate the mean of triplicate results in each experiment.

$P = 0.0063$ ), CD25-depleted splenocytes did not have any regulatory activity, establishing that CD4<sup>+</sup>CD25<sup>+</sup> T cell subset plays a key role in the observed suppression.

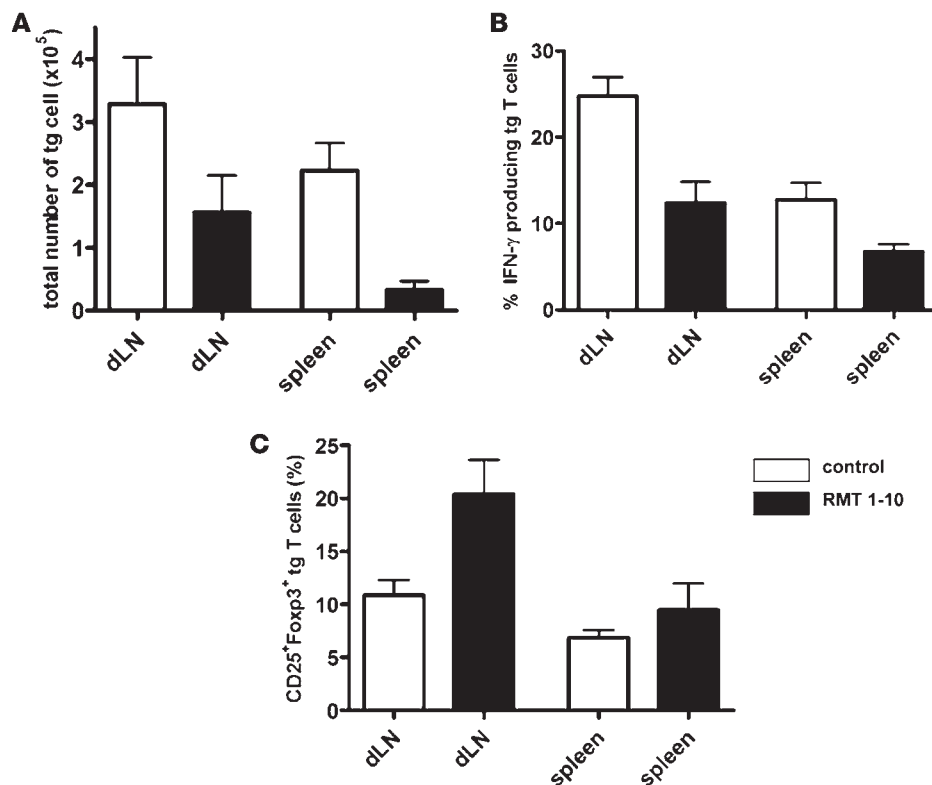
Next, to further confirm the *in vivo* regulatory functions of CD4<sup>+</sup>CD25<sup>+</sup> Tregs generated in the mice exhibiting long-term allograft survival by combination therapy with RMT1-10 and rapamycin, we adoptively transferred  $2 \times 10^5$  CD4<sup>+</sup>CD25<sup>+</sup> responder T cells from naive BALB/c mice into BALB/c Rag2<sup>-/-</sup> recipients of a B6 cardiac allograft, with or without cotransfer of an equal number of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells from either naive mice or animals with long-term allograft survival (treated with RMT1-10 plus rapamycin, >100 days). Rag2<sup>-/-</sup> recipients that received CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells from naive mice along with CD4<sup>+</sup>CD25<sup>+</sup> responders had slight but significant prolongation of allograft survival (MST 24 days,  $n = 5$  vs. 14 days,  $n = 5$ ;  $P = 0.015$ ) as compared with mice that received CD4<sup>+</sup>CD25<sup>+</sup> responders alone (Figure 5C). However, Rag2<sup>-/-</sup> recipients that received a similar number of CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells from long-term surviving mice along with naive CD4<sup>+</sup>CD25<sup>+</sup> responders all exhibited long-term graft survival (MST >100 days,  $n = 4$ ;  $P = 0.001$  vs. control mice that received responder cells only and  $P = 0.003$  vs. mice that received responder cells plus Tregs from naive mice). Thus, on a per cell basis, Tregs from RMT1-10 plus rapamycin-treated mice are more potent in transferring inhibition of alloreactive T cell responses *in vivo* in our model.

To explore whether the enhanced Treg activity is simply due to enrichment of allospecific Tregs in transplanted animals, we compared the suppressive functions of Tregs from naive versus rejecting mice using 2 different strategies. First, we isolated CD4<sup>+</sup>CD25<sup>+</sup> Tregs from naive BALB/c mice and BALB/c mice after rejection of B6 allografts (days 12–14) and added these purified cells at different ratios (Treg/effector T cell [Treg/Teff] ratios of 1:1 to 1:256) to ELISPOT assays, which measured the ability of BALB/c responder cells to produce IFN- $\gamma$  when stimulated by irradiated B6 stimulator cells. Using this *in vitro* suppression assay, we found no statistically significant difference in the suppressive function of naive and rejecting Tregs at any of the ratios tested (Supplemental

Figure 1; supplemental material available online with this article; doi:10.1172/JCI32451DS1). Intriguingly, the natural Tregs performed consistently better than rejecting Tregs at any given ratio, although the differences were not statistically significant.

Second, in order to compare the suppressive function of naive versus rejecting Tregs *in vivo*, we utilized a well-defined ABM TCR-Tg system: ABM mice are on B6 background in which greater than 90% of CD4<sup>+</sup> T cells (CD4<sup>+</sup>V $\alpha$ 2<sup>+</sup>V $\beta$ 8<sup>+</sup>) carry a transgenic TCR that directly recognizes the mutated MHC class II molecule I-A<sup>bm12</sup> (26, 27). Since ABM CD4<sup>+</sup>V $\alpha$ 2<sup>+</sup>V $\beta$ 8<sup>+</sup>CD25<sup>+</sup> T cells express Foxp3 and exhibit regulatory functions *in vitro* and *in vivo* that are specific for I-A<sup>bm12</sup> (24), we used these cells to investigate the activity of a clonal and defined allospecific population of Tregs. To this end, ABM Tregs (CD4<sup>+</sup>V $\alpha$ 2<sup>+</sup>V $\beta$ 8<sup>+</sup>CD25<sup>+</sup>) were isolated and sorted from lymph nodes and spleens of either unprimed or primed ABM mice that rejected bm12 skin grafts 60 days after skin transplantation to insure adequate expansion of Tregs. Naive ABM CD4<sup>+</sup> T cells ( $1.0 \times 10^5$ , CD4<sup>+</sup>V $\alpha$ 2<sup>+</sup>V $\beta$ 8<sup>+</sup>CD25<sup>-</sup>CD44<sup>lo</sup>) were adoptively transferred to B6 RAG-1-knockout mice alone or with the same number of naive or primed ABM Tregs. The bm12 skin transplantations were performed 1 day after adoptive transfer. As shown in Supplemental Figure 2, there was no difference in the graft-prolonging effect of naive Tregs (MST 32 days,  $n = 9$ , vs. MST 11 days in controls,  $n = 7$ ;  $P = 0.01$ ) versus Tregs exposed to alloantigen for about 60 days (MST 34 days,  $n = 11$ ;  $P = 0.03$ ). These *in vitro* and *in vivo* data clearly establish that rejecting Tregs (in spite of enhanced allospecificity) do not perform better than polyclonal naive Tregs. This finding is in stark contrast to the markedly superior suppressive function of Tregs from animals rendered tolerant by RMT1-10 plus rapamycin (Figure 5C), although it should be kept in mind that induction of a Treg as a result of antigen exposure in the context of anti-TIM-1 plus rapamycin therapy may differ from the emergence of Tregs as a result of prolonged antigen exposure in the context of graft rejection.

*RMT1-10 prolongs allograft survival by altering the balance between Tregs and Teffs.* To gain more insight into the mechanism of action of RMT1-10, we first evaluated the role of the TIM-1 pathway in

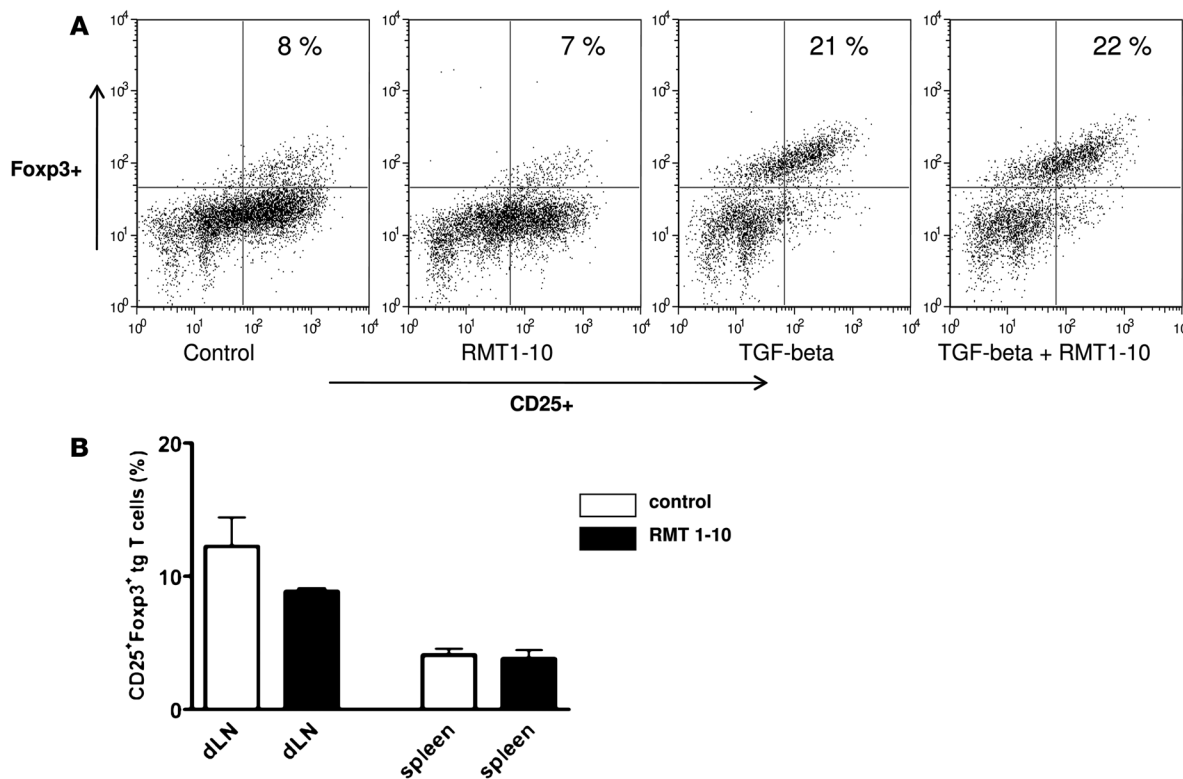
**Figure 7**

Anti-TIM-1 inhibits the expansion of ABM TCR-Tg T cells, tipping the balance from effector to regulatory T cells. (A) Following adoptive transfer of  $2 \times 10^6$  ABM TCR-Tg T cells into nude B6 mice, recipients were transplanted with bm12 skin grafts and received anti-TIM-1 mAb or were left untreated. After 7 days, the dLNs and the spleen were harvested, and the total leukocyte number in the different compartments was determined. Leukocytes were stained for expression of V $\alpha$ 2.1 and V $\beta$ 8.1, and the percentage of these cells was determined. The percentage and total leukocyte count were then used to calculate the absolute number of ABM TCR-Tg T cells present in the dLNs and spleen. Anti-TIM-1 mAb significantly decreased the number of ABM TCR-Tg T cells present in the dLNs and spleen compared with untreated controls. (B) Cells harvested from dLNs and spleen were cultured with PMA/ionomycin and brefeldin A for 4 hours and stained with mAbs against IFN- $\gamma$ . Cytokine production was determined by flow cytometry after gating on V $\alpha$ 2.1+V $\beta$ 8.1+ cells. Anti-TIM-1 mAb significantly decreased the frequency of allospecific IFN- $\gamma$ -producing T cells present in the dLNs and spleen compared with untreated controls. Values represent the mean  $\pm$  SEM of data obtained from 5 separate experiments. (C) Using the adoptive transfer model on day 7 after engraftment, allospecific Tg T cells were harvested from lymphoid compartments. The percentage of Tg cells expressing the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory phenotype was then enumerated using flow cytometry. As demonstrated in C, anti-TIM-1 triggered a significant increase in the percentage of Tg cells expressing a regulatory phenotype in the dLNs. However, given the overall decrease in the size of the Tg cell compartment (A), there was no significant change in the absolute number of Tregs after anti-TIM-1 treatment in this model.

skin allograft rejection using the bm12 into B6 MHC class II-mismatched model, in which allograft rejection is primarily mediated by CD4<sup>+</sup> T cells. In keeping with previously published data, the MST of bm12 (I-A<sup>bm12</sup>) skin grafts in untreated wild-type B6 (I-A<sup>b</sup>) mice was 13 days ( $n = 10$ ) (Figure 6A). Administration of anti-TIM-1 mAb to recipients led to a significant prolongation of allograft survival (MST 46.5,  $n = 8$ ;  $P = 0.0007$ ; Figure 6A). Notably, this effect was also dependent on the presence of CD4<sup>+</sup>CD25<sup>+</sup> Tregs, as their prior depletion with anti-CD25 mAb abrogated the graft-protective effects of RMT1-10 (MST 12 days,  $n = 5$ ; Figure 6A). Next, we explored the effect of anti-TIM-1 antibody on alloantigen-specific cytokine production 14 days following engraftment. Splenocytes

from anti-TIM-1-treated skin graft recipients or untreated recipients were isolated and stimulated with irradiated donor splenocytes from bm12 mice, and the frequency of Th1 (IFN- $\gamma$ ) and Th2 (IL-5) alloreactive T cells was enumerated using the ELISPOT assay. As shown in Figure 6B, anti-TIM-1 treatment was associated with a dramatic reduction in the precursor frequency of IFN- $\gamma$ -secreting alloreactive T cells as compared with untreated recipients. Simultaneously, the frequency of alloreactive IL-5-secreting T cells was significantly increased. These data are consistent with our findings in the fully allogeneic heart transplant model (Figure 1, C and D).

To further study the mechanisms of action of anti-TIM-1 in vivo, we next used the previously published ABM TCR-Tg system (26) described above. We adoptively transferred  $2 \times 10^6$  ABM TCR-Tg T cells into syngeneic T cell-deficient nude B6 mice that subsequently received bm12 skin grafts. On day 7 following transplantation, we harvested the draining lymph nodes (dLNs) and spleen. The ABM TCR-Tg T cells were identified thereafter by TCR V $\alpha$ 2.1 and TCR V $\beta$ 8.1 expression on flow cytometry, as previously described (26, 27). Enumeration of alloantigen-specific ABM TCR-Tg T cells in the dLN and spleen of skin graft recipients demonstrated a significant decrease in the number of ABM TCR-Tg T cells in anti-TIM-1-treated recipients as compared with untreated controls, indicating that anti-TIM-1 mAb interrupted alloantigen-driven T cell expansion in vivo (Figure 7A). Indeed, in recipients treated with anti-TIM-1,  $1.5 \times 10^5 \pm 1.3 \times 10^5$  ABM TCR-Tg T cells were present in the dLNs compared with  $3.2 \times 10^5 \pm 1.6 \times 10^5$  ABM TCR-Tg T cells in untreated controls ( $P = 0.001$ ). Similarly, anti-TIM-1 mAb significantly diminished the number of ABM TCR-Tg T cells present in the spleen ( $0.3 \times 10^5 \pm 0.3 \times 10^5$  vs.  $2.2 \times 10^5 \pm 0.8 \times 10^5$  ABM TCR-Tg T cells;  $P = 0.02$ ) as compared with untreated recipients (Figure 7A). We then examined the effect of anti-TIM-1 on alloreactive Th1 cell differentiation in vivo by examining the intracellular expression of the IFN- $\gamma$  by alloantigen-specific ABM TCR-Tg T cells. In keeping with the finding that anti-TIM-1 triggers a reduction in alloreactive T cell expansion in vivo, treatment with anti-TIM-1 mAb significantly decreased the percentage of allospecific cells producing IFN- $\gamma$  as compared with untreated controls in the 2 lymphoid compartments ( $12.4\% \pm 5.4\%$  vs.



**Figure 8**

Anti-TIM-1 does not convert naive CD4<sup>+</sup>CD25<sup>-</sup> to CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells. (A) Freshly isolated ABM TCR-Tg CD4<sup>+</sup> CD25<sup>-</sup> T cells were stimulated with allospecific irradiated bm12 splenocytes in the absence and presence of TGF-β (3 ng/ml) and increasing concentrations of anti-TIM-1 mAb (1 μg/ml, 10 μg/ml, and 100 μg/ml) for 4 days. Viable Vα2.1<sup>+</sup>Vβ8.1<sup>+</sup> cells were stained for the expression of CD25 and Foxp3. TGF-β induced the conversion of CD4<sup>+</sup>CD25<sup>-</sup> to CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells; however, the addition of anti-TIM-1 (here shown for the concentration of 100 μg/ml) had no additional effect. The dot plots shown are representative of 3 separate experiments. (B) Following adoptive transfer of 2 × 10<sup>6</sup> CD4<sup>+</sup>CD25<sup>-</sup> ABM TCR-Tg T cells into nude B6 mice, recipients were transplanted with bm12 skin grafts and received mAbs against TIM-1. Cells from the dLNs and the spleen were again harvested after 7 days. The frequency Foxp3<sup>+</sup> ABM TCR-Tg T cells was determined as described in Methods. Anti-TIM-1 mAb did not enhance the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> ABM TCR-Tg T cells present in the dLNs when only ABM TCR-Tg CD4<sup>+</sup>CD25<sup>-</sup> cells were transferred. Similarly, no effect was observed in the spleen.

24.7% ± 4.3% in dLNs, *P* < 0.0001; and 6.7% ± 1.5% vs. 12.7% ± 4.3% in spleen, *P* = 0.01) (Figure 7B). These findings indicate that anti-TIM-1 may enhance allograft survival at least in part by reducing the alloreactive Th1 cell clone size.

Given the above data suggesting a requisite role for natural CD4<sup>+</sup>CD25<sup>+</sup> T cells in mediating the immunomodulatory effects of anti-TIM-1, we asked whether anti-TIM-1 enhanced the generation of allospecific Tregs in vivo. Using the adoptive transfer model on day 7 after engraftment, allospecific Tg T cells were harvested from the lymphoid compartments. The percentage of Tg cells expressing the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory phenotype was then enumerated using flow cytometry. As demonstrated in Figure 7C, anti-TIM-1 triggered a significant increase in the percentage of Tg T cells expressing a regulatory phenotype in the dLNs (20.3% ± 6.4% vs. 10.8% ± 3.1%; *P* = 0.008). However, given the overall decrease in the size of the Tg cell compartment (Figure 7A), there was no significant change in the absolute number of Tregs after anti-TIM-1 treatment in this model.

These data indicate that anti-TIM-1 mAb, while inhibiting expansion of alloreactive Th1 cells, preserves the frequency of Tregs. We next explored whether this may be the result of conver-

sion of naive CD4<sup>+</sup>CD25<sup>-</sup>FoxP3<sup>-</sup> T cells into Tregs in the periphery. To address this issue, we evaluated whether anti-TIM-1 mAb could trigger *Foxp3* expression in CD4<sup>+</sup>CD25<sup>-</sup> naive T cells using 2 strategies. First, TGF-β has been shown to induce *Foxp3* gene expression in TCR-stimulated CD4<sup>+</sup>CD25<sup>-</sup> T cells in vitro (28). Freshly isolated ABM TCR-Tg CD4<sup>+</sup>CD25<sup>-</sup> responder T cells were cultured with irradiated alloreactive bm12 splenocytes in the populations in the absence and presence of TGF-β (3 ng/ml) and increasing concentrations of anti-TIM-1 mAb (1 μg/ml, 10 μg/ml, and 100 μg/ml). After 4 days, viable Vα2.1<sup>+</sup>Vβ8.1<sup>+</sup> TCR-Tg T cells were stained for the expression of CD25 and Foxp3. When naive responder T cells were stimulated with allogeneic APCs in the presence or absence of anti-TIM 1 mAb, Foxp3 expression was not upregulated (5.8% ± 1.9%) (Figure 8A). As expected, stimulation of CD4<sup>+</sup>CD25<sup>-</sup> TCR-Tg T cells with bm12 APCs in the presence of TGF-β significantly increased the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells (18.5% ± 6.5%). However, the addition of various concentrations of anti-TIM-1 mAb (1–100 μg/ml) had no additional effect (17.1% ± 8.7% at 100 μg/ml), suggesting that anti-TIM-1 does not convert CD4<sup>+</sup>CD25<sup>-</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in vitro, at least in the model system explored (Figure 8A).





Second,  $2 \times 10^6$  ABM TCR-Tg CD4<sup>+</sup> CD25<sup>-</sup> T cells were injected into nude B6 recipients that had received bm12 skin grafts. On day 7 following transplantation, the frequency of ABM TCR-Tg T cells expressing the regulatory phenotype were assessed in the dLNs and the spleen. Anti-TIM-1 mAb did not enhance the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> ABM TCR-Tg T cells present in the dLNs ( $12.2\% \pm 4.3\%$  vs.  $8.6\% \pm 0.0\%$  after treatment), when only ABM TCR-Tg CD4<sup>+</sup>CD25<sup>-</sup> cells were transferred. Similarly, no effect was observed in the spleen ( $4\% \pm 0.9\%$  vs.  $3.8\% \pm 1.2\%$ ) (Figure 8B). Taken together, these data demonstrate that anti-TIM-1 mAb can prolong allograft survival and this is associated with a reduced Th1 and enhanced Th2 alloreactive T cell response. Importantly, while this results in a significant decrease in the total number of Teffs, the absolute number of Tregs is unchanged, resulting in a 2-fold relative increase in Tregs (compared with untreated allograft recipients).

## Discussion

We have recently discovered the intriguing finding that TIM-1 regulates T cell responses; however, depending on the avidity of its crosslinking, it can either enhance or inhibit T cell proliferation and regulate production of cytokines. While the high-avidity anti-TIM-1 antibody (clone 3B3) enhances proinflammatory Th1 and Th17 responses and the severity of EAE, the low-avidity anti-TIM-1 antibody (clone RMT1-10) inhibited the generation of antigen-specific T cells, the production of Th1 and Th17 responses, and the development of autoimmunity (11). This and the companion article (12) are the first studies to our knowledge describing the role of the TIM-1 molecule in the context of alloimmunity. We now demonstrate that the antagonistic anti-TIM-1 mAb (clone RMT1-10) protects the cardiac allograft from rejection and enables peripheral tolerance in combination with a subtherapeutic course of rapamycin. Our data also demonstrate that anti-TIM-1 mAb can prolong cardiac and skin allograft survival and that this is associated with a reduced Th1 and enhanced Th2 alloreactive T cell responses. This latter finding is consistent with a recent article from our group using the EAE model clearly establishing that addition of RMT1-10 to primed T cells in vitro not only reduced T cell proliferation and production of INF- $\gamma$  but in fact induced the production of the Th2 cytokines (11). These findings are also in agreement with several studies in transplant models showing that tolerance induction by T cell costimulatory blockade is sometimes associated with a state of “immune deviation” toward Th2 cell function, with inhibition of Th1 and upregulation of Th2 cytokines in the target organ (29). Whether a Th2 switch per se can mediate transplant tolerance remains controversial, however (30). Indeed, there are data to indicate that animals that lack Th2 cytokines, for example, IL-4, can be tolerized (30), although this is not universal (31). Indeed, we have previously suggested that the effect of Th2 deviation in promoting graft survival may depend on the alloreactive T cell clone size and degree of donor-recipient histoincompatibility (32, 33). Importantly, the prolongation of allograft survival by RMT1-10 seems to be dependent on both the presence and function of natural Tregs. In the ABM TCR-Tg model, RMT1-10 treatment results in a significant decrease in the total number of Teffs, but the absolute number of Tregs is unchanged, resulting in a 2-fold relative increase in Tregs (compared with untreated allograft recipients). Several groups have shown that the ratio of Tregs/Teffs plays an important role in achieving tolerance (34). While we cannot find evidence of enhanced expansion or induction of allospecific Tregs by RMT1-10 in the model systems tested in vitro and in vivo, it is

possible that it can still enhance the suppressive function of Tregs on a per cell basis. Indeed, on a per cell basis, Tregs from RMT1-10 plus rapamycin-treated but not rejecting mice were more potent in transferring inhibition of alloreactive T cell responses in vivo and in vitro as compared with natural Tregs. These findings are in keeping with previous data that some tolerogenic therapies may either augment the responses of natural Tregs or actually induce Tregs (35). The enhanced suppressive function may be a direct effect of RMT1-10 on Tregs or an indirect effect due to its ability (particularly in combination with rapamycin) to decrease the inflammatory cytokines in treated animals. In fact, a recent study using the EAE model demonstrated that autoantigen-specific Tregs not only expand but also accumulate in the target organ and can only exert their suppressive function when the local inflammatory cytokines are controlled (36). It is also possible that there is slow and progressive expansion and/or induction of allospecific Tregs in long-term tolerant animals in the presence of a Th2 environment, alloantigen, and rapamycin. Recent evidence does indicate a potentially important relationship between Th2 cytokines and Tregs; Th2 cytokines are directly linked to the conversion of Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> Tregs from CD4<sup>+</sup>CD25<sup>-</sup> precursors in the periphery (23). Furthermore, several groups have demonstrated the ability of rapamycin to expand both human and murine Tregs (21, 37, 38). In addition to its ability to expand Tregs, rapamycin may also be contributing by its ability to promote activation-induced cell death and further reduction in the alloreactive T cell pool (39).

Exactly how TIM-1 alters T cell activation remains unclear. Ectopic expression of TIM-1 in murine and human T cell lines augments Th2 differentiation and augments TCR-mediated expression of IL-4, IFN- $\gamma$ , and NFAT/AP-1 response elements (40). Most recently, TIM-1 was shown to coassociate with the TCR complex and augment ZAP-70 and ITK tyrosine phosphorylation and complex formation between PI3K and ITK (10). Taken together, these data imply that TIM-1 can enhance TCR-proximal signals promoting induction of transcription factors including those regulating cytokine genes. While it is unclear how TIM-1 mediates these effects, phosphorylation of tyrosine 276 in its cytoplasmic tail is essential for TIM-1-mediated costimulation (40). We speculate that low-affinity anti-TIM-1 interferes with TIM-1, which normally provides a key costimulatory signal for development of Th1 Teffs. Our data imply that Tregs have a different requirement for TIM-1 signaling, the nature of which will require further study.

Taken together with data from Degauque et al. (12), our findings support a key role for the TIM-1 molecule in regulation of alloimmunity and may help develop novel tolerance-inducing strategies in transplantation. The high-affinity anti-TIM-1 antibody (3B3) enhances the expansion and survival of Th1 effector cells while inhibiting the Treg function, whereas the low-affinity antibody (RMT1-10) inhibits the expansion of alloreactive Th1 responses, tipping the balance toward regulation. These findings provide insights into the unique functions of the TIM-1/TIM-4 pathway, which can both positively and negatively costimulate T cell responses depending on how it is engaged during T cell activation.

## Methods

**Mice.** B6 (H-2<sup>b</sup>), BALB/c (H-2<sup>d</sup>), B6.C-H2<sup>bm12</sup>/KhEg (bm12), and B6 nude mice were obtained from The Jackson Laboratory. STAT4<sup>-/-</sup> and STAT6<sup>-/-</sup> mice on a BALB/c background were also purchased from The Jackson Laboratory. The BALB/c RAG-2-deficient mice were purchased from Taconic. ABM TCR-Tg mice have been described previously (26, 27) and were main-



tained as a breeding colony in our animal facility. All animals were used at 6–10 weeks of age (20–25 g) and were housed in accordance with institutional and NIH guidelines. All animal experiments were approved by the Harvard Medical School Animal Management Committee.

**Antibodies and *in vivo* treatment protocol.** Anti-mouse TIM-1 mAb RMT1-10 (rat IgG2a, $\kappa$ ) was generated by immunizing Sprague-Dawley rats with full-length TIM-1-Ig, which contains both IgV and mucin domains of TIM-1 (41). Lymph node cells were then fused with P3U1 myeloma cells and cloned. All mAbs were manufactured and purified by Bioexpress Cell Culture Inc. RMT1-10 was given *i.p.* according to the following protocol: 0.5 mg mAb on the day of transplantation and 0.25 mg mAb on days 2, 4, 6, 8, and 10 after transplantation. Rapamycin was a generous gift of Joren Madsen (TBRC, Massachusetts General Hospital, Boston, Massachusetts, USA) and was administered at a subtherapeutic regimen (3 mg/kg for days 0–3) in combination therapy with RMT1-10 in some wild-type recipients. CD25<sup>+</sup> T cell depletion was achieved by treating mice preoperatively with 250  $\mu$ g of anti-CD25 mAb (PC61) on days 6 and 1 before transplantation.

**Murine cardiac and skin transplantation.** The vascularized intraabdominal heterotopic transplantation of cardiac allografts was performed using microsurgical techniques. The survival of cardiac allografts was assessed by daily palpation. Rejection was defined as complete cessation of cardiac contractility as determined by direct visualization. Full-thickness trunk skin grafts harvested from bm12 donors (~1 cm<sup>2</sup>) were transplanted onto the dorsal thorax of recipient mice, sutured with 4-0 silk, and secured with dry gauze and a bandage for 7 days. Skin graft survival was monitored daily thereafter, and rejection was defined as complete graft necrosis. In the adoptive transfer experiments, bilateral skin grafts (bm12) were placed onto the dorsal thorax of recipient nude mice so as to maximize recovery of alloantigen-specific T cells from lymph nodes and spleen. Graft survival is shown as the MST in days.

**ELISPOT assay.** The technique for IFN- $\gamma$  and IL-5 ELISPOT analysis has been described recently by our group and others (15). The resulting spots were counted on a computer-assisted enzyme-linked immunospot image analyzer (Cellular Technology Ltd.), and frequencies are expressed as the number of cytokine-producing spots per  $0.5 \times 10^6$  splenocytes.

**Adoptive transfer of TCR-Tg T cells.** Adoptive transfer of ABM TCR-Tg T cells was performed as previously described (27). Briefly, spleens and lymph nodes were harvested from ABM TCR-Tg mice, and pooled single-cell leukocyte suspensions were prepared. CD4<sup>+</sup> T cells were purified by magnetic bead negative selection (Miltenyi Biotec). Typically, CD4<sup>+</sup> T cells were isolated to greater than 90% purity. An aliquot of cells was stained with anti-CD4, anti-TCR V $\alpha$ 2.1, and anti-TCR V $\beta$ 8.1, 8.2 and analyzed by flow cytometry to determine the percentage of ABM TCR-Tg CD4<sup>+</sup> T cells. Typically, greater than 90% of CD4<sup>+</sup> T cells expressed the Tg TCR. ABM TCR-Tg T cells ( $2 \times 10^6$ ) were then injected intravenously into nude B6 mice 1 day before skin transplantation (day -1). On day 7 following transplantation draining (axillary, lateral axillary) and nondraining (pooled mesenteric and neck) lymph nodes and spleens were subsequently collected, single-cell leukocyte suspensions were prepared, and ABM TCR-Tg T cells were identified by flow cytometry.

**Flow cytometry.** In order to study the frequency of B cell and T cell subsets before and after treatment, cells were stained with CD8-FITC, CD4-FITC, CD19-FITC, and PE-conjugated mAb against CD25. An anti-mouse Foxp3 staining set (eBioscience) was used to assess intracellular expression of Foxp3. To identify the adoptively transferred Tg cells, they ( $1 \times 10^6$ ) were stained with FITC-conjugated anti-TCR V $\alpha$ 2.1 (B20.1) and biotinylated anti-TCR V $\beta$ 8.1 (MR5-2) followed by PerCP-conjugated streptavidin.

All anti-mouse antibodies were obtained from BD Biosciences. Flow cytometry was performed using a FACSCalibur flow cytometer system (BD) and analyzed using CellQuest software (BD).

**Intracellular cytokine staining.** Cells ( $1 \times 10^6$ ) were resuspended in HL-1 medium (BioWhittaker); supplemented with 1% L-glutamine (BioWhittaker), 1% penicillin (BioWhittaker), and 10% FCS (BioWhittaker); and restimulated with PMA (5 ng/ml) (Sigma-Aldrich) plus ionomycin (500 ng/ml) (Sigma-Aldrich); and brefeldin A (10  $\mu$ g/ml) (Sigma-Aldrich) was added. Cells were incubated for 4 hours at 37° C. After staining for the surface markers (CD4, V $\alpha$ 2.1, and V $\beta$ 8.1), cells were fixed and permeabilized with Cytotfix/Cytoperm solution (BD Biosciences), according to the manufacturer's instructions, and incubated with PE-conjugated IFN- $\gamma$  (XMG1.2) or isotype control mAbs for 30 minutes at 4° C. A gate was set on CD4<sup>+</sup>V $\alpha$ 2<sup>+</sup>V $\beta$ 8<sup>+</sup>, and the percentage of IFN- $\gamma$  cells was determined by flow cytometric analysis.

**Histological analysis.** Cardiac allografts were harvested from cardiac allograft recipients 14 or in some cases more than 100 days after operation. Specimens were fixed in 10% buffered formalin and embedded in paraffin. Ventricular short-axis sections were cut and stained with H&E. The sections were also stained with elastin van Gieson (elastic fibers) for arteriosclerosis scoring and with Masson trichrome (collagen) for fibrosis and infiltrate scoring. Arteriosclerosis was assessed by light microscopy. The severity of arteriosclerosis was graded according to the percentage of luminal occlusion by intimal thickening using a previously described scoring system (15).

***In vitro* suppression assays.** To test the regulatory activity of cells *in vitro*, an MLR assay or ELISPOT assay was set up: splenocytes from BALB/c wild-type recipients of B6 allografts were used as responder cells to irradiated B6 splenocytes. Splenocytes from BALB/c recipients that accepted B6 cardiac allografts more than 100 days after treatment with RMT1-10 and rapamycin were then added to each well before and after *ex vivo* depletion of CD25 at various ratios (1:1, 1:2, and 1:4). To this end, splenocytes from tolerant animals were stained with CD25 mAb (eBioscience) and depleted of cells expressing CD25 (<1%) using MACS columns (LS Columns; Miltenyi Biotec) before adoptive transfer. In other experiments, we isolated CD4<sup>+</sup>CD25<sup>+</sup> Tregs from naive BALB/c mice and BALB/c mice after rejection of B6 allografts (days 12–14) (see below) and added these purified cells at different ratios (Treg/Teff ratios of 1:1 to 1:256) to ELISPOT assays measuring IFN- $\gamma$ -secreted by BALB/c responder cells stimulated by irradiated B6 cells. The proliferation of responder cells (cpm) was then estimated by the degree of thymidine incorporation in responder cells. In the case of ELISPOT suppression assays, frequencies of IFN- $\gamma$ -producing responder cells were determined as described above.

**CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cell purification for adoptive transfer experiments.** To obtain 100% purified CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells for adoptive transfer studies, we first prepared a single-cell suspension from spleens of naive wild-type BALB/c or wild-type BALB/c recipients of B6 cardiac allografts undergoing treatment with anti-TIM 1-10 mAb and rapamycin 100 days after transplantation. Mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells were isolated from splenocytes by magnetic activated sorting by a CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cell Isolation Kit (130-091-041; Miltenyi Biotec) that was used in a 2-step procedure based on depleting samples of non-CD4<sup>+</sup> T cells followed by positive selection of CD4<sup>+</sup>CD25<sup>+</sup> cells, and CD4<sup>+</sup>CD25<sup>-</sup> unbound cells were collected. The purity of T cells was estimated to be greater than 90% by FACS.

In other experiments, CD4<sup>+</sup> T cells from ABM TCR-Tg mice were purified by magnetic bead negative selection as described above. To purify CD4<sup>+</sup>CD25<sup>-</sup> T cells, the enriched CD4<sup>+</sup> T cells were incubated with PE-conjugated anti-CD25 for 10 minutes at 4° C, washed, and then incubated with anti-PE microbeads (Miltenyi Biotec) for 15 minutes at 4° C. Magnetic separation was performed using an MS-positive selection column according to the suggested protocol (Miltenyi Biotec). The purity was consistently greater than 95% for CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cell preparations. Again the percentage of ABM TCR-Tg T cells was analyzed by flow cytometry.



etry, and  $2 \times 10^6$  CD4<sup>+</sup>CD25<sup>-</sup> T cells were injected intravenously into nude B6 mice 1 day before skin transplantation.

**Treg conversion in vitro.** Isolated ABM TCR-Tg CD4<sup>+</sup>CD25<sup>-</sup> T cells ( $1 \times 10^5$ ) were cultured with  $2 \times 10^5$  irradiated (30 Gy) allogeneic splenocytes from bm12 mice with or without TGF- $\beta$  (3 ng/ml) (R&D Systems). RMT1-10 mAb was added to the culture in increasing concentrations (1  $\mu$ g/ml, 10  $\mu$ g/ml, 100  $\mu$ g/ml). After 4 days in culture, the percentage of  $\text{V}\alpha 2^+\text{V}\beta 8^+\text{CD}25^+\text{Foxp}3^+$  cells was assessed by flow cytometry.

**Statistics.** Kaplan-Meier survival graphs were constructed, and a log-rank comparison of the groups was used to calculate *P* values. Two-tailed Student's *t* test was used for comparison of means between experimental groups examined by ELISPOT assay. Differences were considered to be significant when *P* was less than 0.05.

## Acknowledgments

This work was supported by NIH grants R01 AI070820, R01 AI037691, and P01 AI041521. Nader Najafian is a recipient of NIH

grant KO8 AI064335 and an American Heart Association Scientist Development Grant.

Received for publication May 2, 2007, and accepted in revised form November 19, 2007.

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