Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice

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T cell Ig domain and mucin domain protein 1 (TIM-1) is a costimulatory molecule that regulates immune responses by modulating CD4+ T cell effector differentiation. However, the function of TIM-1 on other immune cell populations is unknown. Here, we show that in vivo in mice, TIM-1 is predominantly expressed on B rather than T cells. Importantly, TIM-1 was expressed by a large majority of IL-10–expressing regulatory B cells in all major B cell subpopulations, including transitional, marginal zone, and follicular B cells, as well as the B cell population characterized as CD1d−CD5+. A low-affinity TIM-1–specific antibody that normally promotes tolerance in mice, actually accelerated (T cell–mediated) immune responsiveness in the absence of B cells. TIM-1+ B cells were highly enriched for IL-4 and IL-10 expression, promoted Th2 responses, and could directly transfer allograft tolerance. Both cytokine expression and number of TIM-1+ regulatory B cells (Bregs) were induced by TIM-1–specific antibody, and this was dependent on IL-4 signaling. Thus, TIM-1 is an inclusive marker for IL-10+ Bregs that can be induced by TIM-1 ligation. These findings suggest that TIM-1 may be a novel therapeutic target for modulating the immune response and provide insight into the signals involved in the generation and induction of Bregs.

Introduction
T cell Ig domain and mucin domain (TIM) proteins constitute a family of costimulatory molecules that play an important role in effector differentiation of CD4+ cells (1). The 8 murine and 3 human genes encoding the TIM family are clustered in a chromosomal region (5q32.2 in humans and 11B1.1 in mice) closely associated with autoimmune disease. For example, TIM domain protein 1 (TIM-1) polymorphisms are associated with susceptibility to human asthma, eczema, and rheumatoid arthritis (2, 3). TIM-1 is expressed on activated CD4+ cells and Th2 cells after polarization in vitro (4). TIM-4 is a putative TIM-1 ligand; however, these phosphatidylserine receptors may interact indirectly through an exosome bridge (5, 6). The role of TIM-1 has previously been studied using anti–TIM-1 mAbs. For example, TIM-1 ligation with a high-affinity mAb, 3B3, promotes expansion of antigen-specific T cells expressing Th1 and Th17 cytokines while inhibiting Tregs (4, 7, 8). Cordantly, 3B3 treatment exacerbates EAE (7) and prevents allograft tolerance mediated by anti–CD154 (8). In contrast, a low-affinity anti–TIM-1 mAb, RMT1-10, inhibits EAE (7) and, when combined with rapamycin, induces long-term allograft acceptance in mice (9). Prolonged engraftment by RMT1-10 is dependent on Th2-cytokine skewing and Treg activity (9). Thus, TIM-1 is a potent regulator of T cell effector responses in both auto- and alloimmunity.

In addition to humoral immunity, B cells play an increasingly recognized role in shaping T effector cell responses through antigen presentation, costimulation, and cytokine production (10). For example, in both humans and mice, B cell deficiency or depletion can ameliorate autoimmune diseases primarily mediated by T cells, including type 1 diabetes and rheumatoid and collagen-induced arthritis (11–13). However, in various other murine models, such as EAE, inflammatory bowel disease, and contact hypersensitivity, B cell deficiency or depletion worsens disease (14–18), which suggests that B cells can also exhibit immunomodulatory function. Indeed, subpopulations of splenic B cells from naive or autoimmune mice can inhibit inflammation in an IL-10–dependent manner (10, 19–21). However, definitive identification has been challenging because such regulatory B cells (Bregs) are rare, lack a specific marker, and express detectable IL-10 only upon ex vivo stimulation.

Various Breg phenotypes have been described. For example, Bregs have been detected within splenic marginal zone (MZ) populations (22–24) or less-mature transitional 2–MZ precursor (T2-MZ) populations (18, 25). Some studies suggest that Bregs may also reside within the much larger follicular (FO) B cell subset (23, 25, 26). Recently, Yanaba et al. identified a small subset (~2%) of splenic B cells expressing a CD1dhiCD5+ phenotype that partially overlapped with that of MZ, T2-MZ, and B1 B cells (15). CD1dhiCD5+ B cells are more enriched for IL-10–producing cells (9%–15%) than other B cell subsets, and it was suggested that they might account for most Breg activity observed in spleen. However, most IL-10− B cells fall outside of the CD1dhiCD5+ population. A marker that can identify the majority of IL-10− B cells is critical for improved understanding of Breg biology, including the relationships among Bregs exhibiting different phenotypes.

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In the present study we showed that in vivo, TIM-1 was predominantly expressed on B cells, both constitutively and after activation. Surprisingly, the tolerogenic effects of RMT1-10 were completely dependent on TIM-1+B cells. TIM-1 identified a large majority of B cells capable of IL-4 and IL-10 expression regardless of other markers. Finally, TIM-1+Bregs were induced by TIM-1 ligation and could transfer long-term acceptance of islet allografts to otherwise-untreated recipients in an IL-10–dependent fashion and could also inhibit allergic airway disease.

Results

B lymphocytes express relatively high levels of TIM-1. (A) Representative flow cytometry plots showing TIM-1 expression on splenic CD4+ and CD8+ T cells and CD19+ B cells in naive BALB/c mice or at 14 days after immunization with either allogeneic (B6) islet transplantation (Txpl) or OVA (20 μg with 4 mg alum i.p. on days 0 and 7). Cell staining was performed in the presence of anti-CD16/CD32 to block FcR binding, and isotype- and fluorochrome-matched negative controls were used to set the cursors. Isotype control staining for CD19+ cells is shown at right. N ≥ 3 per group. (B) Representative flow cytometry plots showing TIM-1 expression on CD19+ B cells from naive mice assessed by indirect staining with anti–TIM-1 mAbs RMT1-10, RMT1-4, and 3B3 followed by PE-conjugated anti-rat Ig secondary mAb. N = 3 per group. (C) Anti–TIM-1 immunoblot of cell lysates from sort-purified T cells, B cells, TIM-1+B cells, and TIM-1−B cells. Representative of 2 independent experiments. Numbers denote percent TIM-1+ cells within each population.

Figure 1

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ment with 3 doses of anti–TIM-1 significantly prolonged islet allograft survival (MST, 28 days), with approximately 30% of mice achieving long-term engraftment (>100 days). Depletion of B cells in recipients prior to transplantation using anti-CD20 slightly shortened allograft survival compared with B cell–intact mice (MST, 10 days). Surprisingly, rather than prolonging allograft survival, anti–TIM-1 treatment significantly accelerated allograft rejection in B cell–depleted recipients (MST, 6 days). This was confirmed in chemically diabetic B cell–deficient JHD (BALB/c) recipients of B6 islets. Untreated JHD recipients, or those treated with isotype control mAb, rejected islet allografts with a MST of acute rejection (MST, 7 days). Reconstitution of JHD mice with WT B cells, followed by anti–TIM-1 treatment, significantly enhanced IFN-γ expression and augmented IL-4 and IL-10 expression by CD4+ T cells (Figure 3, A and C). B cell depletion alone (anti-CD20) modestly increased IFN-γ expression in allograft recipients. However, treatment of B cell–depleted recipients with anti–TIM-1 significantly enhanced IFN-γ and completely prevented the normally observed increase in Th2 cytokines. Thus, B cells are required for the Th2 shift observed after anti–TIM-1 treatment. Previous findings also suggest that anti–TIM-1 can enhance Treg function (9).

This may be explained in part by the enhanced IL-10 expression by Foxp3+ Tregs we observed after anti–TIM-1 treatment, averaging 1.6-fold (Figure 3B and data not shown). However, in the setting of B cell depletion, anti–TIM-1 reduced IL-10 expression by Tregs by an average of 40%, which suggests that B cells may also support Treg function after TIM-1 ligation.

B cell IL-4 and IL-10 are primarily expressed by the TIM-1+ subset and are induced by TIM-1 ligation. Anti–TIM-1 enhances Th2 cytokine expression in the presence of B cells, yet promotes expression of Th1 cytokines in the absence of B cells. Cytokines produced by B cells can influence T cell polarization (28, 29). This raised the possibility that TIM-1 ligation augments IL-4 production by B cells, which could then promote a Th2 response. Using IL-4 EGFP reporter mice (BALB/c) and WT littermates as negative controls, we detected very low but reproducible IL-4 expression by splenic B cells from naive mice (Figure 4, A and B). Immunization by islet transplantation increased IL-4 expression from 0.25% to 0.7% of B cells, and this doubled to 1.4% when recipients received anti–TIM-1. Compared with the overall B cell population, TIM-1+ B cells were highly enriched for IL-4 expression, which was localized almost entirely within this subset (Figure 4, A and C). Moreover, the percentage of TIM-1+ B cells expressing IL-4 increased when allograft recipients were treated with anti–TIM-1, reaching 4.5% (Figure 4, A and C).

In addition to promoting a Th2 response, B cells might support TIM-1–mediated engraftment through regulatory activity. Breg activity corresponds closely with IL-10 expression (19, 20). B cells expressing IL-10 were infrequent (~1%) among total splenic B cells (Figure 4, A and B). While islet transplantation increased IL-10 expression on B cells from 0.8% to 1.5%, treatment of recipients with anti–TIM-1 increased IL-10 expression on B cells almost 5-fold (3.8%; Figure 4, A and B). As with IL-4, TIM-1+ B cells were highly enriched for IL-10 expression. In naive mice and in untreated allograft recipients, roughly 5% of TIM-1+ B cells expressed IL-10, a 25-fold enrichment compared with TIM-1– B cells and a greater than 5-fold enrichment compared with total splenic B cells (Figure 4A). Treatment of allograft recipients with anti–TIM-1 increased TIM-1+ B cells expressing IL-10 more than 2-fold (~13%; Figure 4, A and C).

In addition to increasing the percentage of TIM-1+ B cells expressing IL-4 and IL-10, anti–TIM-1 treatment also increased the percentage of B cells expressing TIM-1, from 7% to 25% of splenic B cells (Figure 4D). Since neither transplantation nor anti–TIM-1 affected the total number of splenic B cells (Figure 4E), these percentages directly reflect differences in cell number. Thus, RMT1-10 treatment increases both the number of TIM-1+ B cells and the percentage of TIM-1+ B cells expressing cytokines, resulting in a 5-fold increase in the number of B cells expressing IL-4 and IL-10. These increases are likely to promote Th2 cytokine expression and allograft survival. Consistent with this finding, treatment of allograft recipients with
anti–TIM-1 mAb 3B3, which promotes rejection and a Th1 response (7, 8), did not augment either TIM-1 or expression of IL-4 or IL-10 by B cells (data not shown). Taken together, these observations suggest that anti–TIM-1 (specifically RMT1-10) induces a subset of TIM-1+ and IL-10+B cells with potential regulatory activity.

To determine whether anti–TIM-1 can induce TIM-1 expression de novo, sort-purified TIM-1–B cells were transferred into naive or transplanted JHD mice with or without anti–TIM-1 treatment. In the absence of an allograft, transferred TIM-1–B cells remained essentially TIM-1–, regardless of whether mice received anti–TIM-1 (Figure 4F). In the presence of an alloantigen (with or without control Ig), approximately 2% of TIM-1–B cells expressed TIM-1. However, treatment of allograft recipients with anti–TIM-1 induced TIM-1 expression on approximately 20% of B cells that were originally TIM-1–. Similar findings were obtained when JHD mice were exposed to alloantigen as i.p. splenocytes (2 × 10⁷; mito- mycin C–treated) rather than an allograft (data not shown).

In this setting, anti–TIM-1 could act directly on the small percentage of B cells induced to express TIM-1 by antigen exposure. Alternatively, TIM-1 induction on B cells could occur indirectly through mAb binding to antigen-activated T cells, which expressed similar levels of TIM-1. To address this issue, TIM-1–B cells were transferred into RAG2-KO mice rather than JHD mice (Figure 4G). In naive RAG2-KO mice (with or without anti–TIM-1), TIM-1–B cells remained essentially TIM-1–. When RAG2-KO recipients were exposed to alloantigen plus control Ig, approximately 3% of B cells expressed TIM-1. However, when RAG2-KO mice received alloantigen in combination with anti–TIM-1, TIM-1 was induced on 20% of the B cells. Thus, induction of TIM-1 on B cells by anti–TIM-1 is T cell independent.

Anti–TIM-1–mediated graft survival is associated with B cell TIM-1 expression and with IL-10 and IL-4 signaling. Next we assessed the role of IL-4 and IL-10 expression by B cells on their immunomodulatory function. Anti–TIM-1 shortened allograft survival in B cell–deficient (JHD) allograft recipients, but significantly enhanced allograft survival when recipients were reconstituted with WT B cells (MST, 46 days; Figure 2B and Figure 5A). In contrast, reconstitution of anti–TIM-1–treated JHD recipients with Il4−/− or Il10−/− B cells only modestly prolonged allograft survival (MST, 23 days and 26 days, respectively; Figure 5A). This suggests that IL-10 and IL-4 are both important for the immunomodulatory function of B cells mediated through TIM-1. However, whereas Il10−/− mice had normal B cell TIM-1 expression, Il4−/− mice exhibited a marked reduction in TIM-1+ B cells both constitutively and after transplantation.
(Supplemental Figure 2A). Indeed, after adoptive transfer into anti–TIM-1–treated JHD allograft recipients, over 23% of WT B cells expressed TIM-1 and greater than 6% expressed IL-10, whereas only 6% of Il4–/– B cells expressed TIM-1 and 3% expressed IL-10 (Figure 5, B and C). The defects in TIM-1 and IL-10 expression were even more severe in B cells from Il4ra–/– mice. After adoptive transfer into treated JHD recipients, only 3% of Il4ra–/– B cells expressed TIM-1, and 1.3% expressed IL-10 (Figure 5, B and C). Concordantly, Il4ra–/– B cells were unable to protect JHD recipients from accelerated rejection after anti–TIM-1 treatment, as seen in mice lacking B cells altogether (Figure 2 and Figure SA). Thus, prolongation of anti–TIM-1–mediated graft survival by B cells correlates directly with TIM-1 and IL-10 expression. Moreover, normal TIM-1 and IL-10 expression on B cells is critically dependent on IL-4 signaling.

As shown in Figure 5B, TIM-1 expression remained low on Il4ra–/– B cells transferred into anti–TIM-1–treated JHD allograft recipi-
ents. This indicates that IL-4 produced by B cells themselves is crucial. While this effect could be direct (B cell IL-4 acts in an autocrine fashion to induce TIM-1+ B cells), it could also be indirect. For example, B cell IL-4 (induced by anti–TIM-1; Figure 4) could promote Th2 cytokines (which are B cell dependent; Figure 3), and these could induce TIM-1+ B cells. Indeed, in the absence of B cell IL-4 signaling, treatment of allograft recipients with anti–TIM-1 promoted Th1 rather than Th2 cytokine expression by CD4+ T cells (Figure 5D).

To determine whether IL-4 from B cells or T cells can induce TIM-1 expression, TIM-1– B cells were transferred into alloantigen-exposed RAG2-KO mice. As before (Figure 4G), alloantigen induced TIM-1 expression on approximately 3% of transferred B cells (Figure 5E). However, cotransfer of congeneric (CD45.1) TIM-1+ B cells from WT allograft recipients (enriched for IL-4 expression) increased TIM-1 expression in initially TIM-1+ B cells to 10%. In contrast, cotransfer of TIM-1+ B cells from the same allograft recipients, or TIM-1+ B cells from Ili4−/− allograft recipients, had no effect on expression of TIM-1 on initially TIM-1+ B cells (3%). This suggests that IL-4 from TIM-1+ B cells can indeed augment TIM-1 expression on TIM-1+ B cells.

Next, CD4+ T cells were examined. Cotransfer of CD4+ T cells from anti–TIM-1–treated WT allograft recipients (Th2 enriched) increased TIM-1 expression in initially TIM-1+ B cells by almost 2-fold. In contrast, CD4+ T cells from anti–TIM-1–treated Ili4−/− allograft recipients or CD4+ T cells from untreated allograft recipients (Th1 enriched) had no effect on TIM-1 expression by B cells. Thus, although IL-4 from CD4+ T cells is not essential, it can augment B cell TIM-1 expression. IL-10 expression on B cells directly paralleled TIM-1 expression (Supplemental Figure 2B).

To further confirm the role of IL-4 signaling on TIM-1 induction, sort-purified TIM-1+ B cells from WT, Ili4−/+ and Ili4ra−/− mice were stimulated in vitro for 48 hours in the presence or absence of exogenous IL-4. TIM-1 expression was readily induced on WT
TIM-1+ B cells by B cell receptor (BCR) ligation with anti-IgM, increasing from 2%–3% (media alone) to greater than 15% (Supplemental Figure 2, C and D). Addition of exogenous IL-4 to anti-IgM markedly enhanced TIM-1 expression, reaching 35% of WT B cells. In the absence of anti-IgM, IL-4 had only a minor effect compared with media alone (Supplemental Figure 2D). In contrast, TIM-1 induction by anti-IgM was markedly reduced in \( \text{Il4}^{--} \) (TIM-1–) B cells (Supplemental Figure 2C). However, when exogenous IL-4 was added, TIM-1 expression by \( \text{Il4}^{--} \) and WT B cells was comparable. Thus, \( \text{Il4}^{--} \) B cells are capable of TIM-1 induction if IL-4 signaling occurs. In contrast, \( \text{Il4ra}^{--} \) B cells had defective TIM-1 induction after anti-IgM, and, as expected, this was not restored by exogenous IL-4. These results correlated closely with the in vivo effects of B cells from \( \text{Il4}^{--} \) and \( \text{Il4ra}^{--} \) mice (Figure 5); together, these findings indicate that induction of TIM-1 expression requires BCR and IL-4 signaling.

TIM-1+ B cells are regulatory and transfer donor-specific long-term graft survival. Next, TIM-1+ B cells were directly tested for regulatory activity. BALB/c allograft recipients treated with anti–TIM-1 were sacrificed on day 14. Splenic B cells from these mice were sorted into TIM-1+ and TIM-1− populations and transferred into otherwise untreated JHD recipients of B6 islets. Whereas transfer of TIM-1− B cells had no effect (MST, 15 days), transfer of TIM-1+ B cells markedly prolonged allograft survival, with 50% surviving long-term (Figure 6A). In contrast, TIM-1+ B cells obtained from \( \text{Il10}^{--} \) islet allograft recipients were defective in their regulatory capacity (MST, 22 days) compared with WT TIM-1+ B cells. Similarly, TIM-1+ B cells from \( \text{Il4ra}^{--} \) allograft recipients lacked regulatory activity, consistent with their significantly reduced IL-10 expression.

Next, we addressed whether TIM-1+ B cells present in the absence of TIM-1 ligation (which augments TIM-1, IL-10, and IL-4 expression) are also regulatory. Adoptive transfer of TIM-1+ B cells from untreated BALB/c recipients of a B6 allograft were able to markedly prolong graft survival in JHD recipients of B6 islets (Figure 6B). In contrast, TIM-1+ B cells obtained from untreated BALB/c recipients of a C3H allograft were unable to prolong graft survival when transferred into JHD recipients. These data were consistent with previous reports indicating that Bregs must be activated to be effective (19). Moreover, consistent with other Bregs (15, 16, 30, 31), TIM-1+ Bregs were antigen specific, since TIM-1+ B cells from mice receiving an unrelated (C3H) allograft were unable to prolong graft survival when transferred into JHD recipients of B6 islets (Figure 6B). Likewise, transfer of TIM-1+ B cells from BALB/c mice exposed to B6 alloantigen protected JHD recipients from rejecting B6 islets,
TIM-1⁺ B cells are highly enriched for IL-10 expression across a wide spectrum of phenotypes. BALB/c splenocytes were assessed for expression of various cell surface markers and IL-10 by multicolor flow cytometry. \( n = 3–6 \) mice/group in at least 3 independent experiments. (A) Left: Representative expression of CD5 versus CD1d on CD19⁺ B cells in naive mice with the CD1d⁺CD5⁺ population (2.3% of total B cells; rectangular gate). Right: IL-10 and TIM-1 expression on CD19⁺ (total B) and on TIM-1⁺ and TIM-1⁻ B cells within the CD1d⁺CD5⁺ and non-CD1d⁺CD5⁺ B cell populations. (B) Representative expression of markers on CD19⁺IL-10⁺ B cells. Cells within the IL-10⁺ B cell gate were assessed for CD1d and CD5 or TIM-1 expression. (C) Percent (mean + SD) B cells expressing TIM-1 within FO, MZ, T2-MZ, T1, CD1d⁺CD5⁺, and B1 B cells from naive and transplanted mice with or without anti–TIM-1 or control Ig treatment. \( * \ P < 0.05 \) vs. naive; \( + \ P < 0.05 \) vs. other allograft recipients. (D) IL-10 expression (mean + SD) on B cell subpopulations and mice as in C. \( ^* \ P < 0.05 \) vs. other total B cell groups; \( ^\# \ P < 0.05 \) vs. other TIM-1⁺ B cell groups. \( P < 0.05 \), TIM-1⁺ vs. TIM-1⁻ (all groups). Numbers denote percent cells within the designated areas.
but not C3H islets (Supplemental Figure 3A). When JHD mice received both B6 and C3H islets (right and left kidney capsules, respectively), acute rejection did not occur. However, histological assessment of allografts revealed heavy CD3+ infiltration of C3H grafts on day 14 (data not shown) and complete disappearance by day 25, whereas B6 allografts were preserved (Supplemental Figure 3B). This demonstrated that activation of antigen-specific Bregs was insufficient to prevent an immune response against a different antigen in the same host. Thus, TIM-1+ B cells exhibited potent antigen-specific regulatory function and were capable of augmenting IL-4 and IL-10 expression while inhibiting IFN-γ expression by endogenous CD4+ T cells (Figure 6C). Transferred TIM-1+ Bregs also augmented the frequency of Foxp3+ Tregs.

Bregs have previously been implicated in amelioration of allergic airway disease (31–33). Adoptive transfer of TIM-1+; but not TIM-1–, B cells from mice with OVA-induced local inhalational tolerance, markedly reduced OVA-induced allergic airway disease in JHD recipients, as determined by reduced BAL leukocytosis, tissue inflammation, and bronchiolar goblet cell hyperplasia (Supplemental Figure 4), thus confirming our results in an independent model.

**Discussion**

TIM-1 has previously been identified as a T cell costimulatory molecule that regulates both auto- and alloimmunity through its effects on CD4+ effector responses (1). We now show, for the first time to our knowledge, that TIM-1 not only broadly identifies Bregs, but plays an important functional role on B cells that is critical for anti–TIM-1–mediated tolerance. TIM-1 ligation with the low-affinity anti–TIM-1 mAb RMT1-10, that normally promotes a Th2 cell within other B cell subpopulations previously shown to contain Bregs. Within the CD1d+CD5+ B cell population discussed above, TIM-1+ cells were even more highly enriched for IL-10 (33%). In contrast, TIM-1+ cells within the CD1d+CD5+ subset exhibited an approximate 8-fold decrease in frequency of IL-10 expression (Figure 7, A and D).

Although uncommon, IL-10+ cells within the non-CD1d+CD5+ population represented a numerical majority of IL-10–expressing B cells in spleen (see above). Unfortunately, to our knowledge there has been no consistent marker allowing identification of rare IL-10+ cells within this vast B cell population. Here, we found that TIM-1 was present on 8% of non-CD1d+CD5+ B cells, and in this population, TIM-1+ B cells were enriched greater than 15-fold for IL-10 expression compared with their TIM-1– counterparts (Figure 7A). Similar findings were observed using IL-10 reporter mice (B6 background; Supplemental Figure 5A), confirming these findings in a second strain. Moreover, after TIM-1 ligation, TIM-1 expression was increased, reaching 45% of CD1d+CD5+ B cells, of which approximately 45% expressed IL-10 (Figure 7C and Supplemental Figure 5B). After TIM-1 ligation, TIM-1 expression on non-CD1d+CD5+ B cells increased to 15%, of which approximately 10% expressed IL-10. Thus, TIM-1 identified most IL-10+ B cells within the CD1d+CD5+ subset and also identified a large number of IL-10+ B cells excluded by the CD1d+CD5+ phenotype.

We also examined the distribution of TIM-1 on more typical splenic B cell subsets in naive mice (35). TIM-1 was preferentially expressed on MZ (IgM+ IgD+ CD21hiCD23–) and B1 (CD5–) B cells (~15%), but was also expressed on approximately 5% of immature transitional 1 (T1; IgM+ IgD- CD21hiCD23-), T2-MZ (IgM+ IgD- CD21hiCD23hi), and FO (IgM+ IgD- CD21hiCD23hi) B cells (Figure 7C). Within each subset, TIM-1 identified B cells that were 8- to 20-fold enriched for IL-10 expression compared with their TIM-1– counterparts (Figure 7D). Moreover, TIM-1 ligation in the presence of alloantigen significantly enhanced the percentage of cells expressing TIM-1 (Figure 7C). Thus, in naive mice, 33% of TIM-1– MZ B cells expressed IL-10. However, anti–TIM-1 treatment of allograft recipients resulted in a 2.8-fold increase in the number of TIM-1– MZ B cells (Figure 7C) and a 40% increase in the proportion of these TIM-1+ cells expressing IL-10 (Figure 7D), resulting in a 3.5-fold increase in number of TIM-1– IL-10–MZ B cells. FO B cells accounted for approximately 40% of splenic B cells, of which less than 1% expressed IL-10 (Figure 7D). However, approximately 6% of TIM-1– FO B cells expressed IL-10, a more than 20-fold increase over TIM-1– FO B cells. Moreover, TIM-1 ligation in transplant recipients increased the number of TIM-1– FO B cells 2.2-fold and increased IL-10 expression on TIM-1– B cells by 45%, resulting in a greater than 3-fold increase in TIM-1– IL-10– B cells in this large subset (Figure 7, C and D). Taken together, these data suggest that TIM-1 identifies an inducible subset of B cells that are highly enriched for IL-10 production, regardless of other phenotypic markers.

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Although uncommon, IL-10+ cells within the non-CD1d+CD5+ population represented a numerical majority of IL-10–expressing B cells in spleen (see above). Unfortunately, to our knowledge there has been no consistent marker allowing identification of rare IL-10+ cells within this vast B cell population. Here, we found that TIM-1 was present on 8% of non-CD1d+CD5+ B cells, and in this population, TIM-1+ B cells were enriched greater than 15-fold for IL-10 expression compared with their TIM-1– counterparts (Figure 7A). Similar findings were observed using IL-10 reporter mice (B6 background; Supplemental Figure 5A), confirming these findings in a second strain. Moreover, after TIM-1 ligation, TIM-1 expression was increased, reaching 45% of CD1d+CD5+ B cells, of which approximately 45% expressed IL-10 (Figure 7C and Supplemental Figure 5B). After TIM-1 ligation, TIM-1 expression on non-CD1d+CD5+ B cells increased to 15%, of which approximately 10% expressed IL-10. Thus, TIM-1 identified most IL-10+ B cells within the CD1d+CD5+ subset and also identified a large number of IL-10+ B cells excluded by the CD1d+CD5+ phenotype.

We also examined the distribution of TIM-1 on more typical splenic B cell subsets in naive mice (35). TIM-1 was preferentially expressed on MZ (IgM+ IgD+ CD21hiCD23–) and B1 (CD5–) B cells (~15%), but was also expressed on approximately 5% of immature transitional 1 (T1; IgM+ IgD- CD21hiCD23-), T2-MZ (IgM+ IgD- CD21hiCD23hi), and FO (IgM+ IgD- CD21hiCD23hi) B cells (Figure 7C). Within each subset, TIM-1 identified B cells that were 8- to 20-fold enriched for IL-10 expression compared with their TIM-1– counterparts (Figure 7D). Moreover, TIM-1 ligation in the presence of alloantigen significantly enhanced the percentage of cells expressing TIM-1 (Figure 7C). Thus, in naive mice, 33% of TIM-1– MZ B cells expressed IL-10. However, anti–TIM-1 treatment of allograft recipients resulted in a 2.8-fold increase in the number of TIM-1– MZ B cells (Figure 7C) and a 40% increase in the proportion of these TIM-1+ cells expressing IL-10 (Figure 7D), resulting in a 3.5-fold increase in number of TIM-1– IL-10–MZ B cells. FO B cells accounted for approximately 40% of splenic B cells, of which less than 1% expressed IL-10 (Figure 7D). However, approximately 6% of TIM-1– FO B cells expressed IL-10, a more than 20-fold increase over TIM-1– FO B cells. Moreover, TIM-1 ligation in transplant recipients increased the number of TIM-1– FO B cells 2.2-fold and increased IL-10 expression on TIM-1– B cells by 45%, resulting in a greater than 3-fold increase in TIM-1– IL-10– B cells in this large subset (Figure 7, C and D). Taken together, these data suggest that TIM-1 identifies an inducible subset of B cells that are highly enriched for IL-10 production, regardless of other phenotypic markers.
response and prolongs allograft survival, paradoxically induced a Th1 response and accelerated rejection in the absence of B cells. TIM-1 ligation on B cells enhanced their IL-4 and IL-10 expression, and both were required for Th2 responses in the allograft setting (Figure 3A, Figure 5D, and data not shown). Although very few B cells expressed IL-4, we demonstrated that these were greatly enriched in the fraction bearing TIM-1. Of note, overexpression of TIM-1 in T cells augments IL-4 transcription (36), and a similar signaling pathway could operate in B cells. In addition to promoting Th2 deviation, TIM-1 ligation also enhances IL-10 expression on Tregs in a B cell–dependent manner. Both Tregs and Th2 cytokines are required for prolonged allograft survival mediated by anti–TIM-1 (9). However, these data do not preclude the possibility that anti–TIM-1 has important effects on T cells that either are independent or amplify the effects on B cells.

It is now recognized that Bregs promote tolerance in a number of autoimmune models, including EAE, inflammatory bowel disease, collagen-induced arthritis, allergic airway disease, and diabetes mellitus (15, 17–21, 30, 31, 37). Lacking a specific marker, such Breg activity appears to reside within uncommon IL-10–expressing B cells scattered within various B cell subpopulations. Moreover, IL-10 production by B cells capable of regulatory activity does not occur in situ, but can be detected only after brief stimulation ex vivo (10). Even then, only 1%–2% of all B cells in spleen, and even less in LN or bone marrow, are IL-10 competent (10). These factors have complicated the identification and study of Bregs.

The recent identification of CD1dhiCD5– B cells, which express IL-10 with relatively high frequency, was an important step forward. This subset was thought to identify most IL-10–producing Bregs, including many previously attributed to the MZ or T2-MZ subsets (10, 15). Although strongly enriched for IL-10 expression, the CD1dhiCD5– population is very small and actually accounts for less than 25% of all IL-10–B cells in spleen. An approach for identifying infrequent IL-10–B cells scattered within or larger B cell subpopulations has been lacking. We showed here that within multiple B cell subpopulations, TIM-1hi B cells were enriched 8 to 20-fold for IL-10 expression compared with their TIM-1– counterparts. Indeed, when splenic B cells were first gated on IL-10 expression, TIM-1 encompassed more than 70% of the IL-10– population. Thus, TIM-1 not only markedly enhanced identification of IL-10–expressing B cells within the CD1dhiCD5– and MZ populations, but also substantially enriched for IL-10hi B cells within the large FO B cell population (~40% of splenic B cells). Moreover, unlike other markers (19), TIM-1 also identified IL-10hi B cells in LNs and peritoneal cavity. For example, in naive mice, TIM-1 identified 3%–5% of LN B cells, and these were 6-fold enriched for IL-10 expression compared with TIM-1– cells (Supplemental Figure SC). TIM-1 also identified approximately 15% of peritoneal B1-b and B1-a cells, of which 18%–25% expressed IL-10 (Supplemental Figure SD). We therefore believe that TIM-1 represents the broadest and most specific marker for Bregs identified thus far.

The finding that TIM-1 ligation on B cells induced TIM-1hi B cells with regulatory activity indicates that TIM-1 signaling is involved in this process. Thus far, anti–TIM-1 appears unique in its ability to induce Bregs across a wide spectrum of phenotypes. In comparison, BAFF and anti-CD40 are reported to induce Bregs specifically within the splenic MZ and T2 populations, respectively (38, 39). Moreover, both of these agents exhibit dual agonist and antagonist functions, raising the concern that they may not uniformly inhibit the immune response in all disease settings.

In addition to increasing the number of TIM-1hi B cells, anti–TIM-1 also augmented their ability to express immunomodulatory cytokines. Thus, TIM-1 ligation in allograft recipients resulted in TIM-1 expression in 35%–40% of CD1dhiCD5– B cells as well as in the MZ and B1 populations, and IL-10 was expressed by 35%–45% of these cells. Even within the major FO B cell population, anti–TIM-1 treatment increased TIM-1 and IL-10 expression, resulting in a 3-fold increase in number of IL-10hi cells. Since TIM-1 identifies IL-10hi cells within each B cell subset, it may allow the relative regulatory activity and lineal relationship between such cells to be studied.

The current study also provides what we believe to be new insight into signals involved in Breg generation (Figure 8). The increase in TIM-1lihi B cells induced by anti–TIM-1 is caused, at least in part, by de novo induction of TIM-1 expression on TIM-1hi B cells, and this does not require T cells. This apparent paradox may be resolved by the finding that BCR ligation induced TIM-1 expression on a subset of B cells, providing a target for anti–TIM-1 and further enhancing TIM-1 expression. IL-4 signaling in B cells played a key role in both constitutive and induced TIM-1 as well as in IL-10 expression and Breg activity. Thus, both Il4–/– and Il4ra–/– mice were defective in TIM-1, IL-10, and Breg activity. Conversely, TIM-1 ligation augmented B cell IL-4 expression, and this could act in an autocrine fashion to significantly enhance TIM-1 and IL-10 expression. IL-4, provided by either B cells or CD4hi T cells, could also promote TIM-1 (and IL-10) expression by activated B cells. However, as previously noted, in the absence of B cell IL-4 signaling, few T cells expressed IL-4, at least in the allograft setting.

To our knowledge, this is the first report to identify a requisite role for IL-4 signaling in B cell IL-10 expression and Breg activity, and our findings have a number of implications. First, in contrast to Harris et al. (28), we found that Th2 cells were not required for induction of IL-4 expression by B cells. IL-4 and IL-10 expressed by B cells is known to augment Th2 expression (17, 28, 29, 40). Although B cell IL-10 may act by inhibiting IL-12 expression by DCs (40), IL-4 was thought to directly promote Th2 skewing. However, IL-4 may also enhance CD4hi T cell IL-4 expression indirectly by promoting B cell IL-10, forming another positive regulatory loop. Thus, B cells and T cells engage in reciprocal regulation of IL-4 on several levels, promoting TIM-1, IL-10, and Breg function.

In agreement with previous studies, IL-10 played a critical role in Breg activity. Neither TIM-1hi nor Il4ra–/– B cells (defective in IL-10 expression) could transfer tolerance to otherwise-untreated allograft recipients. However, Il10–/– B cells modestly prolonged allograft survival in anti–TIM-1–treated B cell–deficient recipients, which suggested that in this setting, additional regulatory mechanisms may play a role.

TIM-1 plays an important role in regulating the immune response. We showed here that many of the effects on T cells resulting from TIM-1 ligation with a low-affinity mAb were actually B cell dependent. TIM-1 represented an inclusive marker for Bregs highly enriched for IL-10 expression in spleen and LN of both naive mice and those undergoing active immune responses. Transfer of TIM-1hi, but not TIM-1–, B cells after antigen challenge markedly prolonged survival of fully MHC-mismatched allografts in otherwise-untreated recipients. To our knowledge, RMT1-10 is the first agent to selectively induce Bregs in vivo and the first tolerogenic agent to promote allograft tolerance through Bregs. These findings may represent a fresh therapeutic approach and provide insight into the signals involved in the generation and induction of Bregs.
Intracellular staining was conducted using intracellular staining kits from BD Biosciences or eBioscience.

**Western blotting**. Cell lysates were prepared from sort-purified CD3+, CD19+, and CD19+TIM-1+ cells using Sigma Celllytic reagent containing leupeptin (10 μg/ml), aprotinin (10 μg/ml), DTT (1 mM), and PMSF (1 mM). 40 μg protein was loaded per lane. After SDS-PAGE, proteins were transferred to a PVDF membrane, incubated with anti-TIM-1 (clone 222414; R&D), and HRP-conjugated anti-IgG (Sigma-Aldrich), as described previously (27).

**Cell preparation and adoptive transfer**. CD19+ B cells were enriched by negative selection (EasySep; StemCell Technologies). Purity of the B cell subset was greater than 95%. CD19+TIM-1+ and CD19+TIM-1− B cells were subsequently isolated by FACS using BD FACSAria. For adoptive transfer studies, 1 × 10^7 sort-purified CD19+ TIM-1+ B cells (i.e., index cells) from naive BALB/c mice were adoptively transferred into syngeneic Rag2−/− recipients. In some experiments, CD19+ cells were treated with anti-IgM or anti-CD16+CD5+ B cells from spleens of naïve BALB/c mice or BALB/c recipients of B6 islets (day 14) were injected into otherwise untreated JHD allograft recipients. In some experiments, naive BALB/c mice received 10^6 sort-purified CD19− or TIM-1− and/or CD19− and TIM-1− T cells. After 14 days, TIM-1+ or IL-10 expression on the index cells was determined by flow cytometry.

**Statistics**. Statistical analyses used unpaired 2-tailed Student’s t test and log-rank (Mantel-Cox) test. Differences were considered to be significant at P values less than 0.05.

**Study approval**. Animal studies were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh (Pittsburgh, Pennsylvania, USA).

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