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Tim-1 Is Essential for Induction and Maintenance of IL-10 in Regulatory B Cells and Their Regulation of Tissue Inflammation

Sheng Xiao,* Craig R. Brooks,† Raymond A. Sobel,‡§ and Vijay K. Kuchroo*

T cell Ig and mucin domain (Tim)-1 identifies IL-10–producing regulatory B cells (Bregs). Mice on the C57BL/6 background harboring a loss-of-function Tim-1 mutant showed progressive loss of IL-10 production in B cells and with age developed severe multiorgan tissue inflammation. We demonstrate that Tim-1 expression and signaling in Bregs are required for optimal production of IL-10. B cells with Tim-1 defects have impaired IL-10 production but increased proinflammatory cytokine production, including IL-1 and IL-6. Tim-1–deficient B cells promote Th1 and Th17 responses but inhibit the generation of regulatory T cells (Foxp3+ and IL-10–producing type 1 regulatory T cells) and enhance the severity of experimental autoimmune encephalomyelitis. Mechanistically, Tim-1 on Bregs is required for apoptotic cell (AC) binding to Bregs and for AC-induced IL-10 production in Bregs. Treatment with ACs reduces the severity of experimental autoimmune encephalomyelitis in hosts with wild-type but not Tim-1–deficient Bregs. Collectively, these findings suggest that in addition to serving as a marker for identifying IL-10–producing Bregs, Tim-1 is also critical for maintaining self-tolerance by regulating IL-10 production in Bregs. The Journal of Immunology, 2015, 194: 1602–1608.

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Abbreviations used in this article: AC, apoptotic cell; Breg, regulatory B cell; CMFDA, 5-chloromethylfluorescein diacetate; EAE, experimental autoimmune encephalomyelitis; iTreg, inducible regulatory T cell; MOG, myelin oligodendrocyte glycoprotein; PS, phosphatidylserine; Tim, T cell Ig and mucin domain; Tim-1Δmucin, Tim-1 mutant mouse; Treg, regulatory T cell; T1, type 1 regulatory T; WT, wild-type.

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gressively lose IL-10 in Bregs develop severe spontaneous inflammation in multiple organs with massive inflammatory cell infiltration at 16–18+ months of age.

Materials and Methods

Mice and reagents

C57BL/6 mice, Rag1Δ−−, and IL-10FPP reporter mice (only heterozygous mice were used; also known as Tiger) were purchased from The Jackson Laboratory. Tim-1Δ−− and Tim-1Δmucin mice were described previously (11, 14). Tim-1Δ−− mice were bred with IL-10FPP reporter mice to obtain Tim-1Δ−−/IL-10FPP mice. Mice were maintained and all animal experiments were done according to the animal protocol guidelines of Harvard Medical School. Myelin oligodendrocyte glycoprotein (MOG)35–55 was synthesized by Quantum controlled Biochemicals. Cytokines and Abs for cell culture, flow cytometry, and cytometric bead array were obtained from BioLegend, eBioscience, BD Biosciences, and R&D Systems. Anti–Tim-1 Ab RMT1-4 (BioLegend) was used for flow cytometry. Anti-Tim-1 Ab 5F12 was described previously (14).

Cell purification and cultures

Splenic CD19+ B cells from 2- to 4-mo-old mice were purified using MACS columns following staining with anti-mouse CD19 MACS beads. Cells were cultured in round-bottom 96-well plates in the presence of anti–CD28 (2 mg/ml) and anti-CD3 (1 mg/ml) and anti-CD28 (2 mg/ml) or B cells plus soluble anti-CD3 (1 mg/ml) under Th0 (no cytokine), Th1 (IL-12 plus anti–IL-4), Th2 (IL-4 plus anti–IL-12/anti–IFN-γ), Th17 (TGF-β1 plus IL-6), Tr1 (TGF-β1 plus IL-27), and inducible Treg (iTreg; TGF-β1) conditions. After 96 h, cells were collected for further experiments. To isolated CNS-infiltrating mononuclear cells, mice were first perfused through the left cardiac ventricle with cold PBS. The forebrain and cerebellum were dissected and spinal cords flushed out with PBS by hydrostatic pressure. CNS tissue was cut into pieces and digested with collagenase D (2.5 mg/ml Roche Diagnostics) and DNase I (1 mg/ml; Sigma-Aldrich) at 37°C for 30 min. Mononuclear cells were isolated by passing the tissue through a 70-μm cell strainer, followed by a 70/37% Percoll gradient centrifugation. Mononuclear cells were removed from the interphase, washed, and resuspended in culture medium for further analysis.

AC preparation

Thymocytes from C57BL/6 mice were treated with 1 μM dexamethasone (Sigma-Aldrich) for 6 h. After an extensive wash, ACs were used for in vivo injection. For the preparation of labeled ACs for in vitro experiments, thymocytes were first labeled with 0.2 μg/ml CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) for 30 min at room temperature and then treated with 1 μM dexamethasone for 6 h. Annexin V and propidium iodide (BD Biosciences) staining was used to confirm apoptosis of thymocytes.

Flow cytometry

Splenocytes and lymph node cells (pooled superficial cervical, axillary, brachial, and inguinal lymph node cells) from mice were treated with ACK lysis buffer (Lonza) and then cell numbers were determined. Frequencies of immune cell subsets of splenocytes and lymph node cells were determined by flow cytometry using Abs to cell surface molecules. Purified B cells were incubated with CMFDA-labeled ACs for 2 h. After fixation and extensive wash to remove nonassociated ACs, cells were analyzed by flow cytometry and confocal microscopy.

For intracellular cytokine staining, cells were stimulated in culture medium containing PMA (30 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and GolgiStop (1 μl/ml; BD Biosciences) in a cell incubator with 10% CO2 at 37°C for 4 h. After surface markers were stained, cells were fixed and permeabilized with Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences) according to the manufacturer’s instructions. Then, cells were stained with fluorescence-conjugated cytokine Abs at 25°C for 30 min before analysis. 7-Aminoactinomycin D (BD Biosciences) was also included to gate out the dead cells. All data were collected on a FACS Calibur or an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Experimental autoimmune encephalomyelitis

Total CD4+ T cells were cotransferred together with CD19+ B cells into Rag1Δ−− mice. Mice were immunized s.c. in the flanks with an emulsion containing MOG35–55 (100 μg/mouse) and Mycobacterium tuberculosis H37Ra extract (3 mg/ml; Difco Laboratories) in CFA (100 μg/mouse). Pertussis toxin (100 ng/mouse; List Biological Laboratories) was administered i.p. on days 0 and 2. For AC treatment, ACs were i.v. injected 1 d before immunization. Mice were monitored and assigned grades for clinical signs of EAE as previously described (10, 17).

Statistical analysis

The clinical score and incidence of EAE were analyzed by a Fisher exact test, and comparisons for cytokine bead array and real-time PCR results were analyzed by a Student t test. A p value <0.05 was considered significant.

Results

Tim-1Δmucin mice spontaneously develop multorgan and tissue inflammation

Tim-1 has been shown to identify most of IL-10–producing Bregs (13, 14). We have previously reported generation of Tim-1Δmucin mice, which express a loss-of-function form of Tim-1, because of deletion of mucin mice also showed enlarged livers that infiltrated with mononuclear cells confined to the periportal region, whereas Tim-1Δmucin mice also showed enlarged livers that were necrotic and hemorrhagic. There were massive mononuclear cell infiltrates in multiple organs composed of macrophages/monocytes and T and B cells, particularly in livers and lungs. The disease progressed over time and mice died within a few months after birth.

Histopathologic analysis demonstrated that WT liver showed few aggregates of mononuclear cells confined to the periportal regions, whereas lungs in age-matched Tim-1Δmucin mice showed few aggregates of mononuclear cells confined to the peribronchial regions and there was minimal interstitial infiltration, whereas lungs in age-matched Tim-1Δmucin mice also showed greater liver damage. In lungs of WT mice there were small aggregates of mononuclear cells confined to the peribronchial regions. Histopathologic analysis demonstrated that WT liver showed few aggregates of mononuclear cells confined to the periportal regions, whereas lungs in age-matched Tim-1Δmucin mice showed massive peribronchial and diffuse parenchymal mononuclear cell infiltrates. Similarly, in lungs of WT mice there were small aggregates of mononuclear cells confined to the peribronchial and peribronchovascular regions and there was minimal interstitial infiltration, whereas lungs in age-matched Tim-1Δmucin mice showed massive peribronchial and diffuse interstitial mononuclear cell infiltrates (Fig. 1D).

Statistical analysis

The clinical score and incidence of EAE were analyzed by a Fisher exact test, and comparisons for cytokine bead array and real-time PCR results were analyzed by a Student t test. A p value <0.05 was considered significant.

Results

Tim-1Δmucin mice spontaneously develop multorgan and tissue inflammation

Tim-1 has been shown to identify most of IL-10–producing Bregs (13, 14). We have previously reported generation of Tim-1Δmucin mice, which express a loss-of-function form of Tim-1, because of deletion of mucin mice also showed increased splenomegaly and lymphadenopathy with hyperactivated IFN-γ and IL-17-producing T cells (Fig. 1A, 1B). Additionally, three of ten 16 to 18+–month old Tim-1Δmucin mice also showed enlarged livers that were necrotic and hemorrhagic. There were massive mononuclear cell infiltrates in multiple organs composed of macrophages/monocytes and T and B cells, particularly in livers and lungs (Fig. 1A, 1C). Histopathologic analysis demonstrated that WT liver showed few aggregates of mononuclear cells confined to the periportal regions, whereas lungs in age-matched Tim-1Δmucin mice also showed enlarged livers that were necrotic and hemorrhagic. There were massive mononuclear cell infiltrates in multiple organs composed of macrophages/monocytes and T and B cells, particularly in livers and lungs (Fig. 1A, 1C). Histopathologic analysis demonstrated that WT liver showed few aggregates of mononuclear cells confined to the periportal regions, whereas lungs in age-matched Tim-1Δmucin mice also showed enlarged livers that were necrotic and hemorrhagic. There were massive mononuclear cell infiltrates in multiple organs composed of macrophages/monocytes and T and B cells, particularly in livers and lungs (Fig. 1A, 1C). Histopathologic analysis demonstrated that WT liver showed few aggregates of mononuclear cells confined to the periportal regions, whereas lungs in age-matched Tim-1Δmucin mice also showed enlarged livers that were necrotic and hemorrhagic.
Tim-1 mAb promotes IL-10 production in WT but not Tim-1Δmucin B cells (14). Thus, we studied whether BCR and CD40 signaling-mediated IL-10 production was affected in B cells from Tim-1-deficient (Tim-1Δ/) mice. Indeed, anti-IgM treatment in in vitro cultures increased B cell Tim-1 expression. Both anti-IgM and anti-Tim-1 treatment alone modestly but significantly enhanced IL-10 production from WT B cells (Fig. 2A). Strikingly, treatment with anti-IgM and anti-Tim-1 together strongly promoted IL-10 production in WT B cells, which is much higher than either treatment alone. However, IL-10 production induced by all these treatment conditions was significantly reduced in Tim-1Δ mice and Tim-1Δ B cell cultures, when compared with the WT B cells (Fig. 2A). A similar observation was obtained when anti-IgM was replaced with Abs against CD40, which is also required for Breg IL-10 production. Anti-CD40 treatment also increased Tim-1 expression on B cells, and CD40 and Tim-1 signaling together synergistically promoted IL-10 production from WT but not Tim-1Δ or Tim-1Δ mice (Supplemental Fig. 1).

Because Tim-1Δ B cells produce less IL-10 but more IL-6, IL-1β, and IL-12 than do WT B cells, we then analyzed whether Tim-1Δ and Tim-1Δ B cells differentially express these proinflammatory cytokines and, if so, how Tim-1 mutation in B cells affects Tim-1Δ and Tim-1Δ B cell responses. For this purpose, we chose an in vivo

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**FIGURE 2.** Tim-1 and BCR or IL-21 signaling together strongly promoted B cell IL-10 production whereas a defect in Tim-1 signaling in B cells reduced IL-10 production. Purified splenic CD19+ B cells from 2- to 3-mo-old WT, Tim-1Δ, or Tim-1Δ mice were cultured in the presence of anti-Tim-1 (clone 5F12), F(ab')2 fragment anti-IgM, or both without (A) or with IL-21 (B). After 3 d, IL-10 production in culture supernatants was measured by cytometric bead array. *p < 0.01. (C) Representative flow cytometry plots showing Tim-1 expression by splenic CD19+ B cells from WT and Tim-1Δ mice after 3 d of culture in the presence of IL-21. n ≥ 3/group.
FIGURE 3. Tim-1 expression or defects affect the balance between regulatory and inflammatory cytokines in B cells that subsequently alter T cell responses. (A) Purified splenic CD19+ B cells from WT or Tim-1 Δmucin mice were cultured in the presence of anti-IgM [F(ab')2 fragment] for 24 h. Total RNA was isolated, and relative expression (mean ± SEM; n = 5) of Tim-1, IL-10, IL-12, IL-6, and IL-1b mRNA was measured by real-time PCR. *p < 0.01. (B) WT total CD4+ T cells (10^6/mouse) were cotransferred together with WT or Tim-1 Δmucin CD19+ B cells (20 * 10^6) into Rag1 Δ/Δ mice. One day after, mice were immunized with MOG(35–55)/CFA to induce EAE. At the peak of disease, splenic Tim-1+ and Tim-1 Δmucin B cells were purified from WT and Tim-1 Δmucin groups of mice. Total RNA was isolated, and relative expression (mean ± SEM; n = 5) of Tim-1, IL-10, IL-12, IL-6, and IL-1b mRNA was measured by real-time PCR. *p < 0.01. (C) WT naive CD4+ T cells were cultured with splenic CD19+ B cells purified from WT or Tim-1 Δmucin mice in the presence of anti-CD3 under Th0 (no cytokine), Th1 (IL-12 plus anti–IL-4), Th2 (IL-4 plus anti–IL-12/anti–IFN-γ), Th17 (TGF-β1 plus IL-6), Tr1 (TGF-β1 plus IL-27), and iTreg (TGF-β1) conditions. After culture for 4 d, production of indicated cytokines in T cells and IL-10 (GFP+) in B cells was measured by flow cytometry after intracellular cytokine staining. A representative of five independent experiments is shown. (D) WT CD4+ naive T cells were cultured with Tim-1+ or Tim-1 Δmucin B cells purified from WT mice in the presence of anti-CD3 under Th17, Tr1, and iTreg conditions. After culture for 4 d, production of indicated cytokines in T cells was measured by flow cytometry after intracellular cytokine staining. Representative data from three independent experiments are shown.
setting by cotransferring WT T cells together with WT or Tim-1<sup>Δmucin</sup> B cells into Rag1<sup>−/−</sup> mice that were then immunized for the induction of EAE. At the peak of disease, we examined expression of these proinflammatory cytokines in Tim-1<sup>+</sup> and Tim-1<sup>−/−</sup> B cells between WT and Tim-1<sup>Δmucin</sup> groups. The results showed that Tim-1<sup>+</sup> B cells from both WT and Tim-1<sup>Δmucin</sup> groups had no detectable Tim-1 and little IL-10 mRNA whereas Tim-1<sup>−/−</sup> B cells from both groups expressed Tim-1 mRNA. However, WT Tim-1<sup>+</sup> B cells had much higher IL-10 mRNA than did Tim-1<sup>Δmucin</sup> Tim-1<sup>+</sup> B cells (Fig. 3B). These data are consistent with the notion that Tim-1 identifies IL-10<sup>+</sup> Bregs and a Tim-1 defect impairs Breg-derived IL-10 production. Interestingly, Tim-1<sup>−/−</sup> B cells from both groups had much higher IL-6, IL-1b, and IL-12 mRNA than did Tim-1<sup>+</sup> B cells. More interestingly, both Tim-1<sup>−/−</sup> and Tim-1<sup>−/−</sup> B cells from Tim-1<sup>Δmucin</sup> mice had much higher IL-6, IL-1b, and IL-12 mRNA than did Tim-1<sup>−/−</sup> and Tim-1<sup>−/−</sup> B cells from WT mice (Fig. 3B). Because only ~10% of B cells are Tim-1<sup>+</sup>, these data indicate that these proinflammatory cytokines are largely produced by Tim-1<sup>−/−</sup> cells, which are proinflammatory. These data further support a critical and essential role of Tim-1<sup>+</sup> Bregs in limiting inflammatory responses of effector B cells; a Tim-1 defect in Bregs alters the balance between regulatory and proinflammatory activities in B cells toward a proinflammatory response. *Tim-1<sup>−/−</sup> B cells promote Th17 differentiation but inhibit the generation of Tregs*

It has been well demonstrated that IL-12 is essential for the development of IFN-γ—producing Th1 responses and that IL-6 and IL-1β are critical in the development of IL-17—producing Th17 responses (20). IL-6 also inhibits natural Treg function and iTreg generation (20). Because Tim-1<sup>−/−</sup> B cells produced less IL-10 but more IL-12, IL-6, and IL-1β, we next studied whether Tim-1<sup>−/−</sup> B cells would affect T cell differentiation. We cocultured WT naive T cells with either WT or Tim-1<sup>−/−</sup> B cells in the presence of anti-CD3 under various T cell—polarizing conditions. Interestingly, compared with WT B cells, Tim-1<sup>−/−</sup> B cells enhanced IFN-γ production under an unbiased neutral setting (Th0), which is most likely due to increased IL-12 in Tim-1<sup>−/−</sup> B cells. The increased IFN-γ in neutral cultures with Tim-1<sup>−/−</sup> B cells was not observed in Th1 cultures because a large amount of exogenous IL-12 was added (Fig. 3C). Tim-1<sup>−/−</sup> B cells also promoted IL-17 production in Th17 cultures and inhibited induction of Foxp3<sup>+</sup> in the presence of TGF-B1. More interestingly, Tim-1<sup>−/−</sup> B cells also have reduced differentiation of IL-10—producing Tr1 cells. Tim-1<sup>−/−</sup> B cells did not affect IL-4 production in Th2 cultures, however (Fig. 3C).

We also measured IL-10 production from B cells in these T/B cell cocultures. Interestingly, in all the T cell—polarizing cultures, compared with WT B cells, Tim-1<sup>−/−</sup> B cells produced much less IL-10 (Fig. 3C), further indicating that Tim-1 is critical and essential for Breg IL-10 production.

We also compared Tim-1<sup>+</sup> Bregs and Tim-1<sup>−/−</sup> B cells isolated from WT and Tim-1<sup>Δmucin</sup> mice for their ability to induce differentiation of Th17 cells, Foxp3<sup>+</sup>iTregs, and Tr1 cells. Compared to Tim-1<sup>−/−</sup> B cells, WT Tim-1<sup>+</sup> Bregs dramatically inhibited Th17 differentiation but promoted Foxp3<sup>+</sup> Treg and Tr1 generation. In contrast, these differences in T cell differentiation were largely lost when using Tim-1<sup>−/−</sup> B cells from Tim-1<sup>Δmucin</sup> mice (Fig. 3D).

These data suggest that B cells with defects in Tim-1 differentially regulate the generation of regulatory and proinflammatory T cells at least partly because of the difference in their regulatory and proinflammatory cytokine production.

*Tim-1<sup>−/−</sup> B cells promote EAE associated with an increase in proinflammatory cytokine production*

EAE is an animal model of multiple sclerosis and is considered to be a T cell—mediated autoimmune disease in the CNS. Th1 and Th17 cells are pathogenic whereas IL-10 and Foxp3<sup>+</sup> Tregs are beneficial in the disease (21). Our data thus far showed that Tim-1 is required for optimal Breg IL-10 production. Furthermore, Tim-1 defects in B cells alter the balance between regulatory and proinflammatory cytokines in B cells, under both in vitro and in vivo settings. We then asked whether Tim-1 defects in B cells would alter the incidence and severity of EAE by enhancing Th1/Th17 responses and inhibiting Foxp3<sup>+</sup> Treg and Tr1 cells. Thus, WT T cells together with WT or Tim-1<sup>−/−</sup> B cells were cotransferred into Rag1<sup>−/−</sup> mice. After immunization with MOG<sub>35-55</sub>/CFA to induce EAE, Rag1<sup>−/−</sup> hosts cotransferred with WT T cells and Tim-1<sup>−/−</sup> B cells developed more severe clinical disease than did the hosts cotransferred with WT T cells and WT B cells (Fig. 4A). The recipients that received Tim-1<sup>−/−</sup> B cells showed increased pathogenic Th1/Th17 responses but decreased Foxp3<sup>+</sup> Treg frequency and IL-10 expression in T cells obtained from the CNS (Fig. 4A). We then studied the effect of transfer of Tim-1<sup>−/−</sup> B cells on EAE development. Our data showed that transfer of Tim-1<sup>−/−</sup> B cells not only reduced EAE severity in WT mice (Supplemental Fig. 2) but they also decreased the severity of EAE in a Tim-1<sup>−/−</sup> background.**
ACs promote WT but not Tim-1Δmucin B cell IL-10 production by binding to Tim-1, and AC treatment reduces EAE in the recipients with WT but not Tim-1Δmucin B cells

Tim-1 is a PS receptor for binding ACs (22–24). ACs have previously been shown to promote IL-10 production from Bregs (25, 26). Thus, we determined whether ACs would bind to Tim-1Δmucin Bregs and promote IL-10 production. Indeed, ACs bound to Tim-1Δmucin B cells at a much higher level than did Tim-1+ B cells from WT mice, and this binding of Tim-1Δmucin B cells was lost in Tim-1Δmucin mice (Fig. 5A). Interestingly however, unlike Tim-1+ epithelial cells (14, 24), Tim-1Δmucin B cells did not phagocytize ACs (data not shown). Furthermore, ACs binding to Tim-1 promoted IL-10 in WT but not Tim-1Δmucin B cell cultures (Fig. 5B). These data suggest that both AC binding to Tim-1Δmucin Bregs and AC-mediated induction of IL-10 production in Bregs depend on Tim-1 expression on Bregs.

Administration of ACs has been reported to reduce EAE severity through a Bregs-dependent manner (26). Therefore, we next asked whether administration of ACs would alter the development of EAE in hosts with Tim-1Δmucin B cells. WT T cells together with WT or Tim-1Δmucin B cells were cotransferred into Rag1Δmucin mice (data not shown). Furthermore, ACs binding to Tim-1Δmucin B cells promoted IL-10 in WT but not Tim-1Δmucin B cell cultures (Fig. 5B). These data suggest that both AC binding to Tim-1Δmucin Bregs and AC-mediated induction of IL-10 production in Bregs depend on Tim-1 expression on Bregs.

FIGURE 5. Effect of apoptotic cells on WT and Tim-1Δmucin B cells and the development of EAE. (A) WT, Tim-1Δmucin, and Tim-1Δmucin B cells from 2- to 4-month-old mice were incubated with CMFDA-labeled apoptotic WT thymocytes (ACs) for 30 min and analyzed by flow cytometry. WT and Tim-1Δmucin B cells had comparable Tim-1 expression. Gating strategy for Tim-1 staining is shown in the left panel (n = 3–5/group). (B) WT and Tim-1Δmucin B cells were cultured with unlabeled ACs for 3 d, and IL-10 production in culture supernatants was then measured by cytometric bead array. *p < 0.001; n = 5. (C) WT total CD4+ T cells (10 × 10^5/mouse) were cotransferred together with either WT or Tim-1Δmucin CD19+ B cells (20 × 10^5) into Rag1Δmucin mice. Apoptotic WT thymocytes (30 × 10^5/mouse) were injected 1 d before immunization with MOG35-55/CFA for EAE induction. Mice (n = 8/group) were scored daily for clinical signs of EAE. *p < 0.05.

Discussion

In the present study, we determined the role of Tim-1 in Bregs and their effect on T cell responses and development of autoimmune diseases. Our data indicate that Tim-1 not only identifies IL-10+ Bregs, but also that it is required for Breg regulatory function in the inhibition of the development of autoimmune diseases.

Our data in the present study further support the notion that Tim-1 identifies IL-10+ Bregs, as IL-10 is detected predominantly in Tim-1Δmucin B cells (Fig. 3B). In addition to serving as a Breg marker, Tim-1 is functionally required for Breg-derived IL-10 production, as both Tim-1Δmucin and Tim-1Δmucin B cells show impairment in IL-10 production. Further support for the role of Tim-1 in regulating Breg functions comes from the observation that treatment with anti–Tim-1 mAb promotes IL-10 only in WT but not Tim-1Δmucin B cells. These data also emphasize the importance of the Tim-1 mucin domain for Tim-1–mediated signaling and function and indicate that Tim-1Δmucin is a loss-of-function form of Tim-1 mutant, at least in terms of Breg IL-10 production. Because Tim-1Δmucin is still expressed on cell surfaces and can be identified by anti–Tim-1 staining, Tim-1Δmucin mice provide a valuable tool for studying the effect of loss of Tim-1 signaling in Bregs.

Many studies have shown that the BCR and CD40 signaling pathways are required for IL-10–producing Breg development and induction; IL-21 also promotes IL-10+ Bregs (19). Because Tim-1 identified IL-10+ Bregs, it was reassuring to find that Tim-1Δmucin B cells increased when B cells were stimulated via BCR, CD40, and IL-21 signaling pathways. However, in all the in vitro and in vivo conditions (Figs. 2, 3B, Supplemental Fig. 1), as well as in various T/B cell cocultures (Fig. 3C), Bregs with Tim-1 defects (Tim-1Δmucin or Tim-1Δmucin) consistently showed ∼50% loss in IL-10 production. This suggests that there are Tim-1–independent mechanisms by which Bregs produce IL-10. Nevertheless, Tim-1 ligation with anti–Tim-1 Ab synergizes with BCR, CD40, and/or IL-21 signaling pathways to promote Breg IL-10 production. All of these data strongly suggest that in addition to serving as a Breg marker, Tim-1 is required for optimal Breg-derived IL-10 production.

In addition for optimal Breg IL-10 production (and also possibly expression of other factors responsible for Breg suppressive activity), Tim-1 signaling is also required for suppressing proinflammatory cytokine production in Bregs. Tim-1Δmucin Bregs mainly produce regulatory cytokines (e.g., IL-10) with low levels of proinflammatory cytokines, whereas Tim-1Δmucin B cells presumably are a part of effector B cells and mainly produce proinflammatory cytokines with little IL-10. Thus, in contrast to Tim-1Δmucin “effector” B cells, Tim-1Δmucin Bregs regulate the balance between proinflammatory Th1/Th17 cells and regulatory Foxp3+ Tregs and Tr1 cells toward a regulatory response. In addition to regulating T cell responses directly, Tim-1Δmucin Bregs can also regulate the balance between the proinflammatory and regulatory T cell responses indirectly by affecting function and cytokine profile of other immune populations such as Tim-1Δmucin effector B cells. Therefore, Tim-1 defects in Bregs affect both Bregs and effector B cells to regulate the balance between proinflammatory and regulatory responses, pushing them toward a dominant proinflammatory response.

We have previously shown that Breg IL-10 production in young (e.g., < 6 mo old) Tim-1Δmucin mice is not as profoundly impaired
as in old (10- to 12-mo-old) Tim-1Δmucin mice (14). Tim-1Δmucin mice are overall normal at a young age and develop spontaneous systemic autoimmune disease only as they get old (16- to 18+ month-old), which correlates with progressive loss of regulatory function (e.g., IL-10) of Bregs in the mice as they age. However, the impairment in Bregs in young (i.e., 2- to 3-mo-old) mice is severe enough to alter the phenotype and enhance the severity of EAE. Th1 and Th17 cells are pathogenic whereas IL-10 and Foxp3+ Tregs are beneficial in the disease (21). Because Tim-1+ Bregs are involved in regulating the balance between Th1/Th17 cells and Foxp3+ Tregs and T1r cells, this begins to explain why Tim-1+ Bregs inhibit EAE whereas B cells with Tim-1 defects promote EAE.

The progressive loss of Breg IL-10 in mice with Tim-1 defects with age is apparently not due to a decrease in the Breg population but rather is due to impaired Breg function resulting from Tim-1 defects, as the percentage of Tim-1+ Bregs in Tim-1Δmucin mice is not decreased but rather increased as the mice age (Supplemental Fig. 3). However, these Bregs do not make appropriate levels of IL-10, when compared with the WT Tim-1+ Bregs. This further supports the conclusion that Tim-1 expression and signaling are required for maintaining Breg function and their optimal IL-10 production to promote induction of tolerance. The question that still remains is how Tim-1 signaling is triggered and maintained in Bregs for their optimal regulatory function under physiological conditions. Tim-1 has been shown to be a receptor for Tim-4 and PS-

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References


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Disclosures

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