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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**[*]

[*]Center for Neurologic Disease, Harvard Medical School, Boston, Massachusetts, USA 02115

[†]Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA 02115

[‡]Palo Alto Veteran's Administration Health Care System and Department of Pathology, Stanford University School of Medicine, Stanford, California, USA 94304.

Abstract

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 multi-organ 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 (Tr1) cells) and enhance the severity of experimental autoimmune encephalomyelitis (EAE). 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 AC reduces EAE severity in wildtype (WT) 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.

Introduction

B cells are generally considered to act as positive regulators of immune responses by serving as antigen presenting cells (APC) and producing cytokines for optimal T cell activation. In addition to producing antibodies, B cells have also been shown to negatively regulate immune responses (1-6). Lack or loss of IL-10-producing B cells (called Bregs) accelerates and exacerbates many autoimmune and inflammatory diseases, including EAE, chronic colitis, arthritis, type 1 diabetes, lupus, and delayed type contact hypersensitivity. On the other hand, transfer or increase in the number of Bregs reduces autoimmune and inflammatory diseases (1-4, 6). In many models, IL-10 appears to be critical for the regulatory function of Bregs, although other mechanisms in addition to IL-10 production might also be operational for the regulatory function of Bregs (1-4, 6). In spite of their

To whom correspondence should be addressed: Sheng Xiao (sxiao2@rics.bwh.harvard.edu) or Vijay K. Kuchroo (vkuchroo@rics.bwh.harvard.edu).

critical role in regulating immune and autoimmune responses, lack of a universal marker for identifying Bregs has hampered our understanding of the critical biologic functions of Bregs. Furthermore, the processes and mechanisms by which Bregs are generated have not been identified.

Tim-1, a transmembrane glycoprotein, was identified as a member of the Tim family genes that regulates immune responses (7). In the immune system, Tim-1 was first identified to be expressed on T cells and DCs where it plays an important role in regulating important cellular functions (7-10). More recently, Tim-1 has also been shown to be expressed on B cells (11, 12). The vast majority of Tim-1^+ B cells produce IL-10; and transfer of Tim-1^+ Bregs led to long-term acceptance of islet allografts and inhibited allergic airway responses (13). We have also demonstrated that B cell-derived IL-10 is produced mainly by Tim-1^+ B cells (14). We generated a Tim-1 mutant mouse (Tim-$1^{\Delta mucin}$) and demonstrated that the mouse has a profound defect in B cell-derived IL-10 production. Associated with the loss of IL-10 production in B cells, 10-12 month old Tim-$1^{\Delta mucin}$ mice showed increased effector/memory Th1 responses and autoantibody production without any systemic autoimmunity (14). These data supported the idea that Tim-1 may be critical for Breg function.

In this report, we demonstrate that Tim-1 is required for optimal IL-10 production in Bregs. B cells with Tim-1 deficiency or mutation show a defect in IL-10 production with an increase in proinflammatory cytokine production. In vitro, Tim-1 deficient B cells promote IL-17 and IFN-γ production in T cells and inhibit the generation of $Foxp3^+$ Tregs and Tr1 cells. In *in vivo* transfer models of EAE, hosts with Tim-1-deficient B cells developed more severe disease associated with increased generation of pathogenic Th1/Th17 cells and decreased $Foxp3^+$ Treg frequency and IL-10 production in the central nervous system (CNS). In contrast, transfer of Tim-1^+ Bregs but not Tim-1-negative B cells reduced incidence the severity of EAE. As a phosphatidylserine receptor, Tim-1 is essential for binding of apoptotic cells (AC) to Bregs. Co-culturing of B cells with AC increased IL-10 production in WT but not Tim-1-deficient B cells. Further, AC treatment reduces EAE in hosts with WT but not Tim-1 deficient B cells. Tim-$1^{\Delta mucin}$ mice that progressively 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, $Rag1^{-/-}$, $IL10^{GFP}$ reporter (only heterozygous mice were used; also known as Tiger) mice were purchased from The Jackson Laboratory. Tim-$1^{-/-}$ and Tim-$1^{\Delta mucin}$ mice were described (11, 14). Tim-$1^{-/-}$ mice were bred with $IL10^{GFP}$ reporter mice to obtain Tim-$1^{-/-}IL10^{GFP}$ mice. Mice were maintained and all animal experiments were done according to the animal protocol guidelines of Harvard Medical School. MOG35-55 was synthesized by Quality Controlled Biochemicals. Cytokines and antibodies for cell culture, flow cytometry, and cytometric bead array were obtained from BioLegend, eBioscience, BD Biosciences, and R&D Systems. Anti-Tim-1 antibody RMT1-4 (BioLegend) was used for flow cytometry. Anti-Tim1 antibody 5F12 was described previously (14).

Cell Purification and Cultures

Splenic $CD19^+$ B cells from 2-4 month 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-Tim-1 (clone 5F12), $(Fab')_2$ fragment anti-IgM, Anti-CD40, IL-21, or their combinations. After 3 days, IL-10 production in culture supernatants was measured by cytokine bead array (CBA). MACS purified $CD19^+$ B cells were labeled with PE-anti-Tim-1 (RMT1-4) and then separated into $Tim\text{-}1^+$ and $Tim\text{-}1^-$ B cells by fluorescence-activated cell sorting for further uses.

$CD4^+CD62L^{hi}CD25^-$ naive $CD4^+$ T cells were purified by fluorescence-activated cell sorting after a MACS bead isolation of $CD4^+$ cells as previously described (15, 16). Naive $CD4^+$ cells were activated with either plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) or B cells plus soluble anti-CD3 (1 μg/ml) under Th0 (no cytokine), Th1 (IL-12 + anti-IL-4), Th2 (IL-4 + anti-IL-12/anti-IFN-γ), Th17 (TGF-β1 + IL-6), Tr1 (TGF-β1 + IL-27), and iTreg (TGF-β1) conditions. After 96 h, cells were collected for further experiments.

To isolation of 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) 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) for 6 h. After extensive wash, AC were used for in vivo injection. For the preparation of labeled AC for in vitro experiments, thymocytes were first labeled with 0.2 μg/mL CellTracker Green 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 (LN) cells (pooled superfacial cervical, axillary, brachial, and inguinal LN cells) from mice were treated with ACK lysis buffer (Lonza), and then cell numbers were determined. Frequencies of immune cell subsets of splenocytes and LN cells were determined by flow cytometry using antibodies to cell surface molecules. Purified B cells were incubated with CMFDA-labeled AC for 2 h. After fixation and extensive wash to remove non-associated AC, cells were analyzed by flow cytometry and confocal microscopy.

For intracellular cytokine staining, cells were stimulated in culture medium containing phorbol 12-myristate 13-acetate (30 ng/ml, Sigma-Aldrich), ionomycin (500 ng/ml, Sigma-

Aldrich), and GolgiStop (1 μl/ml, BD Biosciences) in a cell incubator with 10% CO_2 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-AAD (BD Biosciences) was also included to gate out the dead cells. All data were collected on a FACSCalibur or an LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

EAE

Total $CD4^+$ T cells were co-transferred together with $CD19^+$ B cells into $Rag1^{-/-}$ mice. Mice were immunized subcutaneously in the flanks with an emulsion containing MOG35–55 (100 μg/mouse) and *M. tuberculosis* H37Ra extract (3 mg/ml, Difco Laboratories) in CFA (100 μl/mouse). Pertussis toxin (100 ng/mouse, List Biological Laboratories) was administered intraperitoneally on days 0 and 2. For AC treatment, AC were intravenously injected one day before immunization. Mice were monitored and assigned grades for clinical signs of EAE as previously described (10, 17).

RNA isolation, Real-time PCR, and Histology

RNA was extracted with RNeasy Plus kits (Qiagen) and cDNA was made by Iscript (BioRad). All of the real-time PCR probes were purchased from Applied Biosystems. Quantitative PCR were performed using ViiA™ 7 Real-Time PCR System (Applied Biosystems).

Tissues and organs from mice were fixed in 10% neutral buffered formalin for 12 hours, processed, embedded in paraffin wax, sectioned, and stained with H&E using standard procedures. Evaluations were made in a blinded fashion.

Statistics

The clinical score and incidence of EAE were analyzed by Fisher's exact test, and comparisons for CBA and real-time PCR results were analyzed by Student's t test. $P < 0.05$ was considered significant.

Results Tim-$1^{\Delta mucin}$ mice spontaneously develop multi-organ 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-$1^{\Delta mucin}$ mice, which express a loss of function form of Tim-1, because of deletion of the mucin domain (14). We demonstrated that the major defect in young (< 6-month old) Tim-$1^{\Delta mucin}$ mice is impaired Breg IL-10 production. Associated with the progressive loss of IL-10 production in B cells, 10-12 month-old Tim-$1^{\Delta mucin}$ mice showed increased effector/memory Th1 responses and autoantibody production; however, these mice did not develop frank systemic autoimmune disease (14). Interestingly, Tim-$1^{\Delta mucin}$ mice at 16-18^+ months of age developed splenomegaly and lymphadenopathy with hyperactivated IFN-γ- and IL-17-producing T cells (**Figure 1A&B**). In addition, 3 out of 10 16-18^+ month old Tim-$1^{\Delta mucin}$ mice also showed enlarged livers that

were necrotic and hemorrhagic. There were massive mononuclear cell infiltrates in multiple organs composed of macrophages/monocytes, T and B cells, particularly in livers and lungs (**Figure 1A&C**). Histopathologic analysis demonstrated that WT liver showed few aggregates of mononuclear cells confined to the periportal region, whereas Tim-1$^{\Delta mucin}$ liver had massive periportal and diffuse parenchymal mononuclear cell infiltrates. Similarly, in lungs of WT mice there were small aggregates of mononuclear cells confined to the periarterial and peribronchial regions and there was minimal interstitial infiltration, whereas lungs in age-matched Tim-1$^{\Delta mucin}$ mice showed massive peribronchial and diffuse interstitial mononuclear cell infiltrates (**Figure 1D**). Tim-1$^{\Delta mucin}$ mice that develop progressive loss of IL-10 production from Bregs develop severe autoimmune disease with multi-organ/tissue inflammation which may lead to end-organ damage, especially in liver and lungs. The disease pattern in Tim-1$^{\Delta mucin}$ mice is very different from that in the hosts with impaired Foxp3^{+} Tregs, which develop very severe tissue inflammation and die within few months after birth (Josefowicz et al., 2012).

Tim-1 defects in B cells reduce Breg IL-10 production upon various stimuli

B cell receptor (BCR) and CD40 signaling has been shown to be required for the generation of IL-10^{+} Breg (2), and to increase Tim-1 expression (11, 18). We have previously reported that treatment with an anti-Tim-1 mAb promotes IL-10 production in WT but not Tim-1$^{\Delta mucin}$ 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-1$^{-/-}$, (11)) or Tim-1$^{\Delta mucin}$ 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 (**Figure 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-1$^{-/-}$ and Tim-1$^{\Delta mucin}$ B cell cultures, when compared to the WT B cells (**Figure 2A**). Similar observation was obtained when anti-IgM was replaced with antibodies 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-1$^{-/-}$ or Tim-1$^{\Delta mucin}$ B cells (**Figure S1**).

IL-21 has recently been shown to be required for IL-10 production not only in T cells but also critical for Breg development and expansion (19). Indeed, IL-21 treatment alone or together with anti-IgM or anti-CD40 increased IL10 production in WT B cell cultures (**Figure 2B** and data not shown). IL-21 treatment also significantly increased the frequency of Tim-1^{+} B cells (**Figure 2C**). Interestingly, IL-21 and anti-Tim-1 together dramatically promoted IL-10 production in WT B cell cultures, with or without addition of anti-IgM or anti-CD40. In contrast, IL-21-induced IL-10 production was dramatically reduced in Tim-1$^{-/-}$ and Tim-1$^{\Delta mucin}$ B cells under all these conditions (**Figure 2B** and data not shown).

Altogether, these data suggest that Tim-1 expression and signaling are essential for the maintenance and promotion of IL-10 production in Bregs. Defect in Tim-1 expression/

signaling severely impairs Breg derived IL-10 production, which cannot be rescued by BCR, CD40 or IL-21 signaling. These data also confirm that Tim-1$^{\Delta mucin}$ is a loss of function form of Tim-1 mutant, since Tim-1$^{\Delta mucin}$ can be normally expressed on cell surface in the mutant mice but does not act normally to maintain/induce IL-10 production from Bregs (14). Tim-1$^{\Delta mucin}$ mice, therefore, provide a valuable tool for studying the effect of loss of Tim-1 signaling on Breg function and also provide a tool by which Bregs can be isolated from Tim-1$^{\Delta mucin+}$ cells.

Regulatory and proinflammatory cytokines are differentially expressed between Tim-1-positive and -negative B cells and a Tim-1 defect in B cells alters the balance between regulatory and proinflammatory cytokines

Because Tim-1 defects in Bregs impair their IL-10 production, we next studied whether Tim-1 defects would alter proinflammatory cytokine expression in B cells. WT or Tim-1$^{-/-}$ splenic B cells were stimulated with BCR ligation, and expression of Tim-1, IL10, IL12, IL6, IL23, and IL1b mRNA was measured by real-time PCR analysis. The results showed that there was no detectable Tim-1 mRNA expression in Tim-1$^{-/-}$ B cells due to Tim-1 deficiency (**Figure 3A** and data not shown). Compared to WT B cells, Tim-1$^{-/-}$ B cells had > 50% of reduction in IL10 mRNA expression, consistent with reduced IL-10 cytokine production (**Figure 2**). Interestingly, expression of IL12, IL6, and IL1b mRNA in Tim-1$^{-/-}$ B cells was increased, while IL23 mRNA was not detected in either WT or Tim-1$^{-/-}$ B cells (**Figure 3A**). These data suggest that Tim-1 deficiency in B cells alters the balance between regulatory and proinflammatory cytokines towards a pro-inflammatory response.

Since Tim-1$^{-/-}$ B cells produce less IL-10 but more IL-6, IL-1β, and IL-12 than WT B cells, we then analyzed whether Tim-1-positive (Tim-1^{+}) and -negative (Tim-1^{-}) B cells differentially express these proinflammatory factors, and if so, how Tim-1 mutation in B cells affects Tim-1^{+} and Tim-1^{-} B cell responses. For this purpose, we chose an in vivo setting by co-transferring WT T cells together with WT or Tim-1$^{\Delta mucin}$ B cells into Rag1$^{-/-}$ 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^{+} and Tim-1^{-} B cells between WT and Tim-1$^{\Delta mucin}$ groups. The results showed that Tim-1^{-} B cells from both WT and Tim-1$^{\Delta mucin}$ groups had no detectable Tim-1 and little IL10 mRNA while Tim-1^{+} B cells from both groups expressed Tim-1 mRNA. However, WT Tim-1^{+} B cells had much higher IL10 mRNA than Tim-1$^{\Delta mucin}$ Tim-1^{+} B cells (**Figure 3B**). These data are consistent with the notion that Tim-1 identifies IL-10^{+} Bregs and Tim-1 defect impairs Breg derived IL-10 production. Interestingly, Tim-1^{-} B cells from both groups had much higher IL6, IL1b, and IL12 mRNA than Tim-1^{+} B cells. More interestingly, both Tim-1^{+} and Tim-1^{-} B cells from Tim-1$^{\Delta mucin}$ mice had much higher IL6, IL1b, and IL12 mRNA than Tim-1^{+} and Tim-1^{-} B cells, respectively (**Figure 3B**). Because only ~10% of B cells are Tim-1^{+}, these data indicate that these proinflammatory cytokines are largely produced by Tim-1^{-} cells, which are proinflammatory. These data further support a critical and essential role of Tim-1^{+} 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 towards a proinflammatory response.

Tim-1$^{-/-}$ B cells promote Th17 differentiation but inhibit the generation of regulatory T cells

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 nTreg function and iTreg generation (20). Since Tim-1$^{-/-}$ B cells produced less IL-10 but more IL-12, IL-6 and IL-1β, we next studied whether Tim-1$^{-/-}$ B cells would affect T cell differentiation. We co-cultured WT naïve T cells with either WT or Tim-1$^{-/-}$ B cells in the presence of anti-CD3 under various T cell polarizing conditions. Interestingly, compared to WT B cells, Tim-1$^{-/-}$ B cells enhanced IFN-γ production under unbiased neutral setting (Th0), which is most likely due to increased IL-12 in Tim-1$^{-/-}$ B cells. The increased IFN-γ in neutral cultures with Tim-1$^{-/-}$ B cells was not observed in Th1 cultures since large amount of exogenous IL-12 was added (**Figure 3C**). Tim-1$^{-/-}$ B cells also promoted IL-17 production in Th17 cultures and inhibited induction of Foxp3^{+} in the presence of TGF-β1. More interestingly, Tim-1$^{-/-}$ B cells also have reduced differentiation of IL-10-producing Tr1 cells. Tim-1$^{-/-}$ B cells did not affect IL-4 production in Th2 cultures, however (**Figure 3C**).

We also measured IL-10 production from B cells in these T/B cell co-cultures. Interestingly, in all the T cell polarizing cultures, compared to WT B cells, Tim-1$^{-/-}$ B cells produced much less IL-10 (**Figure 3C**), further indicating that Tim-1 is critical and essential for Breg IL-10 production.

We also compared Tim-1^{+} Bregs and Tim-1^{-} B cells isolated from WT and Tim-1$^{\Delta mucin}$ mice for their ability to induce differentiation of Th17, Foxp3^{+} iTreg, and Tr1 cells. Compared to Tim-1^{-} B cells, WT Tim-1^{+} Bregs dramatically inhibited Th17 differentiation but promoted Foxp3^{+} Treg and Tr1 generation. In contrast, these differences in T cells differentiation were largely lost when using Tim-1^{+} B cells from Tim-1$^{\Delta mucin}$ mice (**Figure 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$^{-/-}$ B cells promote EAE associated with an increase in pro-inflammatory cytokine production

EAE is an animal model of multiple sclerosis (MS) and is considered to be a T cell-mediated autoimmune disease in the CNS. Th1 and Th17 cells are pathogenic while IL-10 and Foxp3^{+} 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^{+} Treg and Tr1 cells. Thus, WT T cells together with WT or Tim-1$^{-/-}$ B cells were co-transferred into Rag1$^{-/-}$ mice. After immunization with MOG35-55/CFA to induce EAE, Rag1$^{-/-}$ hosts co-transferred with WT T cells and Tim-1$^{-/-}$ B cells developed more severe clinical disease

than the hosts co-transferred with WT T cells and WT B cells (**Figure 4A**). The recipients that received Tim-1$^{-/-}$ B cells showed increased pathogenic Th1/Th17 responses but decreased Foxp3^{+} Treg frequency and IL-10 expression in T cells obtained from the CNS (**Figure 4A**). We then studied the effect of transfer of Tim-1^{+} B cells on EAE development. Our data showed that transfer of Tim-1^{+} B cells not only reduced EAE severity in WT mice (**Figure S2**) but also decreased the severity of EAE in a Tim-1$^{-/-}$ B cell-mediated transfer model (**Figure 4B**). The data further emphasize that Tim-1 indeed identifies Bregs and is functionally critical for Bregs in modulating EAE severity by regulating the balance between pathogenic and protective regulatory T cells.

Apoptotic cells (AC) promote WT but not Tim-1$^{-/-}$ B cell IL-10 production by binding to Tim-1, and AC treatment reduces EAE in the recipients with WT but not Tim-1$^{-/-}$ B cells

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

Administration of AC has been reported to reduce EAE severity through a Breg-dependent manner (26). Therefore, we next asked whether administration of AC would alter the development of EAE in hosts with Tim-1$^{-/-}$ B cells. WT T cells together with WT or Tim-1$^{-/-}$ B cells were co-transferred into Rag1$^{-/-}$ mice. AC were administrated one day before immunization with MOG35-55/CFA for EAE induction. As shown in Figure 4A, Rag1$^{-/-}$ hosts co-transferred with WT T cells and Tim-1$^{-/-}$ B cells developed more severe clinical disease than the hosts co-transferred with WT T cells and WT B cells. AC treatment dramatically reduced EAE severity in hosts with WT B cells but not in hosts with Tim-1$^{-/-}$ B cells (**Figure 5C**). These data indicate that Breg expressing Tim-1 is almost completely required for AC-mediated Breg-dependent inhibition of EAE.

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 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-1^{+} but not Tim-1^{-} B cells (Figure 3B). In addition to serving as a Breg marker, Tim-1 is functionally required for Breg-derived IL-10 production, as both Tim-1$^{-/-}$ and Tim-1$^{\Delta mucin}$ 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-1$^{-/-}$ or Tim-1$^{\Delta mucin}$ 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-1$^{\Delta mucin}$ is a loss of function form of Tim-1 mutant, at least in terms of Breg IL-10 production. Since Tim-1$^{\Delta mucin}$ is still expressed on cell surfaces and can be identified by anti-Tim-1 staining, Tim-1$^{\Delta mucin}$ 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). Since Tim-1 identified IL-10^{+} Bregs, it was reassuring to see that Tim-1^{+} 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 (Figures 2, S1, and 3B), as well as in various T/B cell co-cultures (Figure 3C), Bregs with Tim-1 defects (Tim-1$^{-/-}$ or Tim-1$^{\Delta mucin}$) consistently showed about 50% loss in IL-10 production. This suggests that there are also Tim-1-independent mechanisms by which Bregs produce IL-10. Nevertheless, Tim-1 ligation with anti-Tim-1 antibody 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 IL10 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-1^{+} Bregs mainly produce regulatory cytokines (e.g., IL-10) with low levels of proinflammatory cytokines, while Tim-1^{-} B cells, presumably are a part of effector B cells and mainly produce proinflammatory cytokines with little IL-10. Thus, in contrast to Tim-1^{-} “effector” B cells, Tim-1^{+} Bregs regulate the balance between proinflammatory Th1/Th17 cells and regulatory Foxp3^{+} Tregs and Tr1 cells towards a regulatory response. In addition to regulating T cell responses directly, Tim-1^{+} 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-1^{-} “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 towards a dominant proinflammatory response.

We have previously shown that Breg IL-10 production in young (e.g. < 6-month old) Tim-1$^{\Delta mucin}$ mice is not as profoundly impaired as in old (10-12^{+}-month old) Tim-1$^{\Delta mucin}$ mice (14). Tim-1$^{\Delta mucin}$ mice are overall normal at a young age and develop spontaneous systemic autoimmune disease only as they get old (16-18^{+}-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. 2-3 month-old) mice is severe enough to alter the phenotype and enhance the severity of EAE. Th1 and Th17 cells are pathogenic while IL-10 and Foxp3^{+} Tregs are beneficial in the disease (21). Since Tim-1^{+} Bregs involve in regulating the balance between Th1/Th17 cells and Foxp3^{+} Tregs and Tr1 cells, this begins to explain why Tim-1^{+} Bregs inhibit EAE while 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 decrease in Breg population but rather due to impaired Breg function resulting from Tim-1 defects, as the percentage of Tim-1^+ Bregs in Tim-$1^{\Delta mucin}$ mice is not decreased but rather increased as the mice age (Figure S3). However, these Bregs do not make appropriate levels of IL-10, when compared to 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 exposed on AC (22-24, 27). However, we found that treatment with Tim-4-Ig does not significantly alter IL-10 production in B cells from WT, Tim-$1^{-/-}$ or Tim-$1^{\Delta mucin}$ B cells (data not shown), indicating that Tim-4 may not be the endogenous Tim-1 ligand for maintaining optimal function of Tim-1^+ Bregs. AC have been shown to play a critical role in immunological tolerance and suppress autoimmune disease via promoting an anti-inflammatory response in terms of IL-10 production (25, 26, 28). Interestingly, we demonstrate that as a PS receptor, crosslinking of Tim-1 by PS exposed on the surface of AC is required for Breg function. Thus, maintenance of optimal Breg function in the hosts apparently depends on the interaction of Tim-1 with AC, which mediates persistent Tim-1 signaling to maintain and/or induce Breg function (e.g., IL-10 production). Due to loss of AC sensing, Bregs from Tim-1 mutant mice have defects in regulatory functions, which shifts the immune balance towards a proinflammatory T cell response. This partly explains why Tim-$1^{\Delta mucin}$ mice develop spontaneous multi-organ autoimmunity with age. The spontaneous multi-organ/tissue inflammation is not unique to Tim-$1^{\Delta mucin}$ mice, since we have also observed that Tim-$1^{-/-}$ mice at 12^+ months of age start to develop inflammation with increased infiltration of mononuclear cells in livers (Figure S4). Further investigation is needed to determine whether Tim-$1^{-/-}$ mice will finally develop spontaneous multi-organ inflammation in multiple organs as seen in 16-18^+-month old Tim-$1^{\Delta mucin}$ mice.

In summary, we demonstrate that in addition to serving as a Breg marker, Tim-1 as a PS receptor is critical and essential for optimal Breg regulatory function in maintaining immune tolerance by sensing apoptotic cells. Thus, Tim-1 may be a valuable therapeutic target for B cell-targeted therapies of autoimmune inflammatory diseases in which Bregs play a critical regulatory role.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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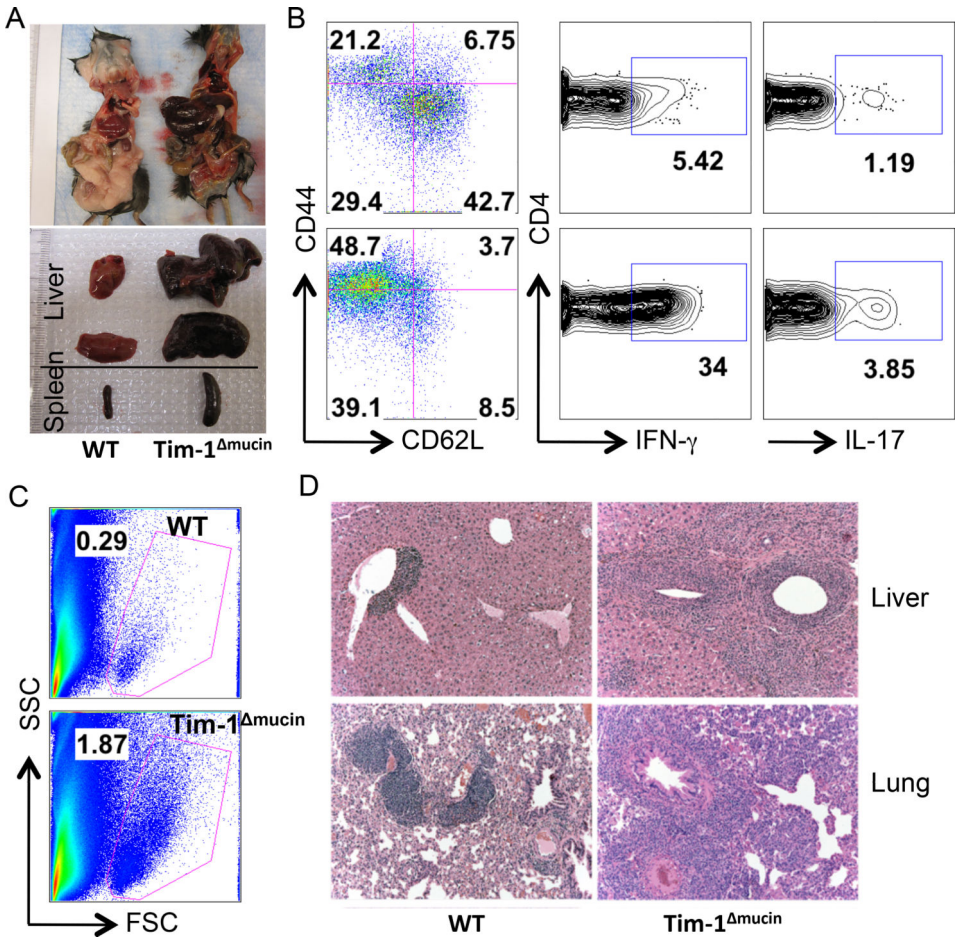

Figure 1. Tim-1$^{\Delta mucin}$ mice at 16-18^{+} months of age develop splenomegaly and multi-organ inflammation

A) Representative images of organs from 16-18^{+}-month old WT and Tim-1$^{\Delta mucin}$ mice. **B&C**) Representative flow cytometry plots showing CD4^{+} T cell phenotypes in spleens (**B**) and livers (**C**) isolated from 16-18^{+}-month old WT and Tim-1$^{\Delta mucin}$ mice (n = 10). **D**) Representative histopathology of livers and lungs from 16-18^{+}-month-old WT and Tim-1$^{\Delta mucin}$ mice. There are massive mononuclear cell infiltrates in the Tim-1$^{\Delta mucin}$ mice. Hematoxylin and eosin stain, 15x.

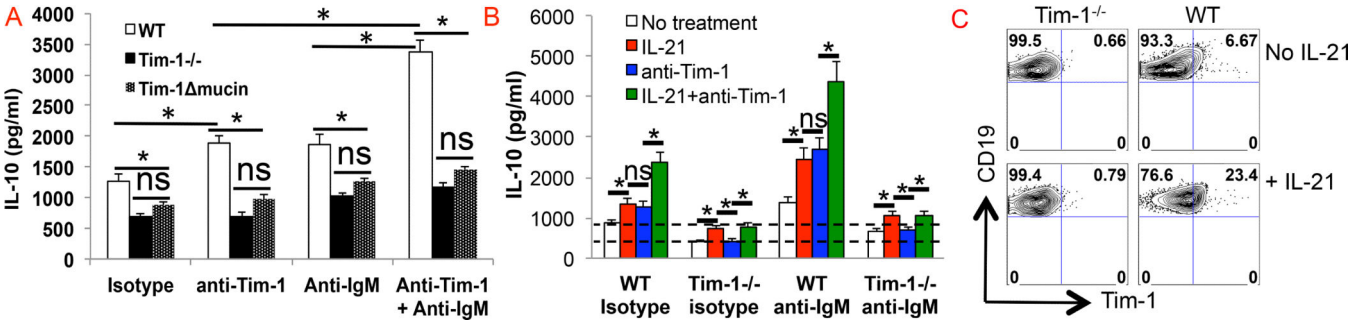

Figure 2. Tim-1 and BCR or IL-21 signaling together strongly promoted B cell IL-10 production while a defect in Tim-1 signaling in B cells reduced IL-10 production

Purified splenic $CD19^+$ B cells from 2-3 month-old WT, Tim-$1^{\Delta mucin}$ or Tim-$1^{-/-}$ mice were cultured in the presence of anti-Tim-1 (clone 5F12), $(Fab')_2$ fragment anti-IgM or both without (**A**) or with IL-21 (**B**). After 3 days, IL-10 production in culture supernatants was measured by ELISA. * $P < 0.01$; ns, not significant. **C**) Representative flow cytometry plots showing Tim-1 expression by splenic $CD19^+$ B cells from WT and Tim-$1^{-/-}$ mice after 3-day culture in the presence of IL-21. $n \geq 3$ per group.

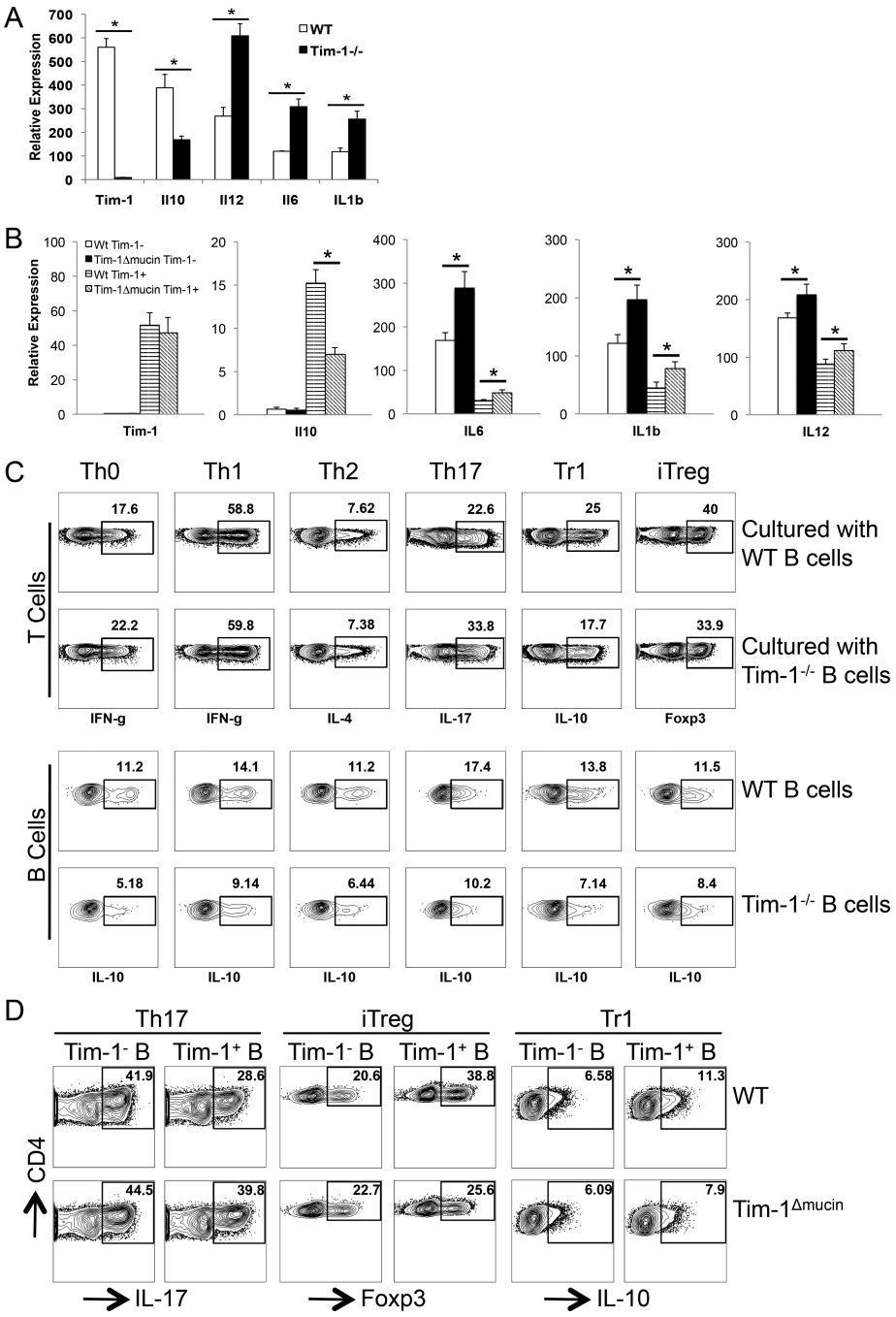

Figure 3. Tim-1 expression or defects affects the balance between regulatory and inflammatory cytokines in B cells that subsequently alter T cell responses

A) Purified splenic $CD19^+$ B cells from WT or Tim-$1^{-/-}$ mice were cultured in the presence of anti-IgM ($(Fab')_2$ fragment) for 24 h. Total RNA was isolated, and relative expression (mean ± SEM; n = 5) of Tim-1, IL10, IL12, IL6, and IL1b mRNA was measured by realtime PCR. * $P < 0.01$. **B**) WT total $CD4^+$ T cells (10 x 10^6/mouse) were co-transferred together with WT or Tim-$1^{\Delta mucin}$ $CD19^+$ B cells (20 x 10^6) into $Rag1^{-/-}$ mice. One day after, mice were immunized with MOG35-55/CFA to induce EAE. At the peak of disease, splenic

Tim-1^{+} and Tim-1^{-} CD19^{+} B cells were purified from WT and Tim-1$^{\Delta mucin}$ groups of mice. Total RNA was isolated, and relative expression (mean + SEM; n = 5) of Tim-1, IL10, IL12, IL6, and IL1b mRNA was measured by realtime PCR. * $P < 0.01$. **C**) WT naïve CD4^{+} T cells were cultured with splenic CD19^{+} B cells purified from WT or Tim-1$^{-/-}$ IL-10$^{GFP/+}$ mice in the presence of anti-CD3 under Th0 (no cytokine), Th1 (IL-12 + anti-IL-4), Th2 (IL-4 + anti-IL-12/anti-IFN-γ), Th17 (TGF-β1 + IL-6), Tr1 (TGF-β1 + IL-27), and iTreg (TGF-β1) conditions. After culture for 4 days, production of indicated cytokines in T cells and IL-10 (GFP^{+}) in B cells was measured by flow cytometry after intracellular cytokine staining. Representative of 5 independent experiments was shown. **D**) WT CD4^{+} naïve T cells were cultured with Tim-1^{+} or Tim-1^{-} B cells purified from WT in the presence of anti-CD3 under Th17, Tr1, and iTreg conditions. After culture for 4 days, production of indicated cytokines in T cells was measured by flow cytometry after intracellular cytokine staining. Representative data from 3 independent experiments are shown.

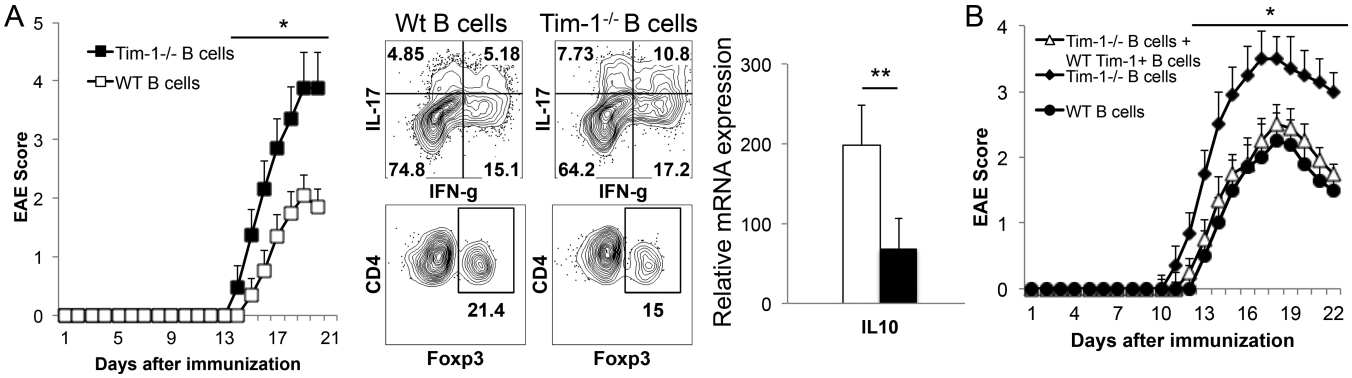

Figure 4. Effect of Tim-1 expression or defects in B cells on EAE and T cell responses

A) WT total $CD4^+$ T cells (10×10^6/mouse) were co-transferred together with either WT or Tim-$1^{-/-}$ $CD19^+$ B cells (20×10^6) into $Rag1^{-/-}$ mice; the recipients were then immunized with MOG35-55/CFA to induce EAE. Mice were scored daily for clinical signs of EAE (left panel; n =10 per group). * $P < 0.05$. On day 20, CNS-infiltrating mononuclear cells were isolated and examined for the frequencies of IFN-γ^+, IL-17^+, and $Foxp3^+$ cells in $CD4^+$ T cell gates by flow cytometry after intracellular staining (middle panel; n = 5). CNS-infiltrating $CD4^+$ T cells were also isolated and measured for their IL10 mRNA expression by realtime PCR (right panel; ** $P < 0.01$; n = 4 per group). **B**) WT total $CD4^+$ T cells (10×10^6) were co-transferred together with WT (20×10^6), Tim-$1^{-/-}$ (20×10^6), or Tim-$1^{-/-}$ (20×10^6) plus WT Tim-1^+ (2×10^6) B cells into $Rag1^{-/-}$ mice; the recipients were then immunized with MOG35-55/CFA to induce EAE. Mice (n = 8-10 per group) were scored daily for clinical signs of EAE. * $P < 0.05$.

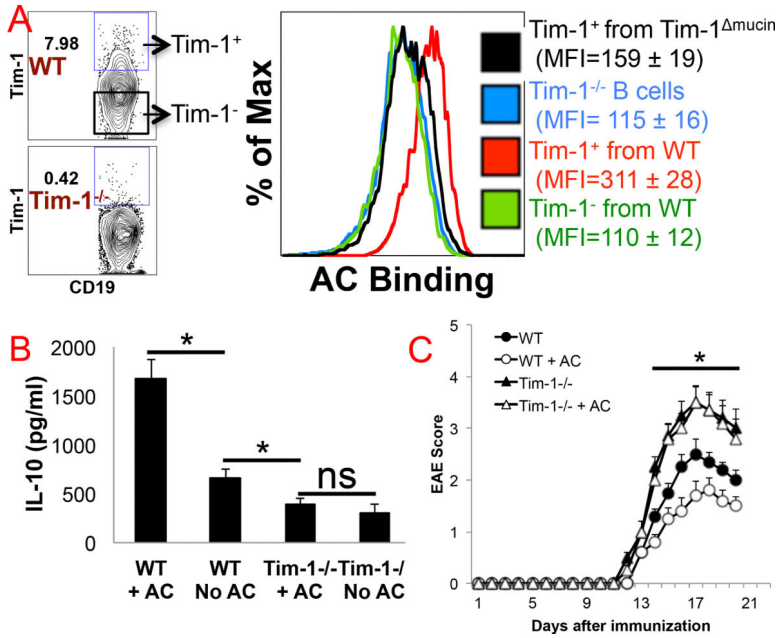

Figure 5. Effect of apoptotic cells on WT and Tim-1$^{-/-}$ B cells and the development of EAE

A) WT, Tim-1$^{\Delta mucin}$ and Tim-1$^{-/-}$ B cells from 2-4 month-old mice were incubated with CMFDA-labeled apoptotic WT thymocytes (AC) for 30 min, and analyzed by flow cytometry. WT and Tim-1$^{\Delta mucin}$ B cells had comparable Tim-1 expression. Gating strategy for Tim-1 staining is shown in the left panel. n = 3-5 per group. **B**) WT and Tim-1$^{-/-}$ B cells were cultured with unlabeled AC for 3 days, and IL-10 production in culture supernatants was then measured by ELISA. * $P < 0.001$; ns, not significant; n = 5. **C**) WT total CD4^{+} T cells (10×10^6/mouse) were co-transferred together with either WT or Tim-1$^{-/-}$ CD19^{+} B cells (20×10^6) into Rag1$^{-/-}$ mice. Apoptotic WT thymocytes (30×10^6/mouse) were injected one day before immunization with MOG35-55/CFA for EAE induction. Mice (n = 8 per group) were scored daily for clinical signs of EAE. * $P < 0.05$.