Defect in regulatory B-cell function and development of systemic autoimmunity in T-cell Ig mucin 1 (Tim-1) mucin domain-mutant mice

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Tim-1, a type I transmembrane glycoprotein, consists of an IgV domain and a mucin domain. The IgV domain is essential for binding Tim-1 to its ligands, but little is known about the role of the mucin domain, even though genetic association of TIM-1 with atopy/asthma has been linked to the length of mucin domain. We generated a Tim-1-mutant mouse (Tim-1^{Δmucin}) in which the mucin domain was deleted genetically. The mutant mice showed a profound defect in IL-10 production from regulatory B cells (Bregs). Associated with the loss of IL-10 production in B cells, older Tim-1^{Δmucin} mice developed spontaneous autoimmunity associated with hyperactive T cells, with increased production of IFN-γ and elevated serum levels of Ig and autoantibodies. However, Tim-1^{Δmucin} mice did not develop frank systemic autoimmune disease unless they were crossed onto the Fas-mutant lpr mice on a C57BL/6 background. Tim-1^{Δmucin}lpr mice developed accelerated and fulminant systemic autoimmunity with accumulation of abnormal double-negative T cells and autoantibodies to a number of lupus-associated autoantigens. Thus, Tim-1 plays a critical role in maintaining suppressive Breg function, and our data also demonstrate an unexpected role of the Tim-1 mucin domain in regulating Breg function and maintaining self-tolerance.

inflammation | hepatitis A virus cellular receptor 1 | kidney injury molecule 1

The T-cell Ig mucin (Tim) family of genes consists of eight members in mice and three members in humans (1–4). The three human TIM genes, TIM-1, TIM-3, and TIM-4, are homologs of mouse Tim-1, Tim-3, and Tim-4, respectively. In addition, mouse Tim-2 is considered to be another ortholog of human TIM-1. The Tim proteins are type I cell-surface glycoproteins with common structural features including an N-terminal IgV domain, a stalk region with *N*-glycosylation site(s), a transmembrane domain, and a short cytoplasmic tail (1–5). The Tim gene family is located at chromosome 5q33.2 in humans and 11B1.1 in mice and frequently has been linked to asthma, allergy, and autoimmunity in both mice and humans (1–4, 6). Tim proteins have been reported to be expressed on various immune cells including T cells, macrophages, and dendritic cells (DCs) where they have been implicated in regulating broad immune responses, including asthma and allergy, autoimmunity, transplant tolerance, and responses to tumors and viral infection (1–4).

Tim-1 also has been identified as a cellular receptor for hepatitis A virus (HAVCR1); the IgV domain is crucial for binding to the virus, and the mucin domain is critical for uncoating the viral particles before entry and cellular infection (7–9). Interestingly, exposure/infection to hepatitis A virus (HAV) is associated with a reduced risk of developing asthma, atopy, and allergies in humans, and, similarly, Tim-1 has been linked genetically to Th2-driven murine airway hypersensitivity, leading to the identification of the *Tapr* locus and Tim-1 as an asthma susceptibility gene (6, 10–13). Although there are small allelic variations in the IgV

domain, the genetic linkage to susceptibility to allergy following HAV infection was linked mainly to the length of the mucin domain of TIM-1 (14). An insertion of six amino acids forming a long TIM-1 mucin domain (157insMTTTPV) resulted in protection against asthma and allergy in subjects exposed to HAV (6, 11–13). Similarly, the mucin domain in Tim-1 is longer in BALB/c mice (6, 10, 11), which are susceptible to Th2-driven airway hypersensitivity, than in DBA/2 and C57BL/6 mice, which develop less airway reactivity following antigen challenge in murine airway hyperreactivity models. These data underscore the importance of the mucin domain of Tim-1 in regulating immune responses and in the development of atopic diseases. In addition, human NKT cells expressing the long form of TIM-1 showed greater cytolytic activity against HAV-infected liver cells (14). These data on genetic linkage to allergies, HAV infection, and immune responses demonstrate that the length of the mucin domain of TIM-1 has important functional consequences in human immune and infectious diseases, but the actual mechanism by which the TIM-1 mucin domain regulates immune responses has not been analyzed.

Surprisingly mice with either complete Tim-1 deficiency (Tim-1^{−/−}) or with overexpression of the full-length Tim-1 molecule showed no defects in cellular phenotype, nor did they show any significant differences in Th2 responses and Th2-mediated airway inflammation (15, 16), again raising the question whether the mucin domain has critical biological functions in immune regulation.

All Tim-1 ligands identified thus far require the Tim-1 IgV domain for their ligand binding (3, 4, 17). For example, Tim-4 expressed on antigen-presenting cells (APCs) has been reported to costimulate T-cell responses by phosphorylating Tim-1 expressed on activated T cells (18, 19). The Tim-1 IgV domain also binds phosphatidylserine exposed on the surface of apoptotic cells and has been shown to clear apoptotic cells when expressed on kidney epithelial cells or when Tim-1 was overexpressed artificially on transfectants (20–23). The IgV domain therefore serves as the ligand-binding domain for Tim-1. Given that loss of full-length Tim-1 in the knockout mice did not show any phenotype and that genetic linkage to infection and allergies is associated with the length of the TIM-1 mucin domain, we generated a mutant mouse in which the Tim-1 was expressed at normal levels but did not contain the mucin domain (Tim-1^{Δmucin} mice). Because the Tim-1-mutant mice expressed an intact ligand-binding IgV domain, we were able to analyze the role of Tim-1 in the immune system in the absence of the mucin domain.

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For the most part, Tim-1^{Δmucin} mice appeared normal at <6 mo of age, but as the mice aged (>10 mo), there was an impairment in IL-10 production by regulatory B cells (Bregs). Associated with the loss of Breg IL-10 production, Tim-1^{Δmucin} mice developed features of systemic autoimmune disease including hyperactivated T cells with increased IFN-γ production and autoantibody formation. When introduced into Fas-mutant lpr mice on the C57BL/6 background, Tim-1^{Δmucin} remarkably accelerated and worsened autoimmunity with increased accumulation of normal and abnormal double-negative T cells and an increase in autoantibodies to a number of lupus antigens including antibodies to dsDNA. These data suggest that the Tim-1 mucin domain is critical for IL-10 production by B cells and that in the absence of this domain mutant mice develop severe systemic autoimmunity when Tim-1^{Δmucin} is expressed on a susceptible genetic background.

Results

Generation and Characterization of Tim-1^{Δmucin} Mice. The mucin domain of Tim-1 is a highly O-glycosylated threonine-, serine-, and proline-rich region encoded by exon 3 of the *Tim-1* gene in mice on the C57BL/6 background. To study the role of the mucin domain for Tim-1 function in vivo, we replaced the *Tim-1* exon 3 with a PGK promoter-driven neomycin-resistance cassette (Fig. 1A) and generated a mutant mouse on the C57BL/6 background in which the Tim-1 proteins do not contain the mucin domain (Tim-1^{Δmucin} mice). Tim-1^{Δmucin} mice were produced in normal Mendelian ratios and showed no obvious abnormalities before the age of 6 mo. As expected, RT-PCR combined with sequencing assays showed that B cells from WT mice expressed the full-length 849-bp Tim-1 mRNA, whereas B cells from Tim-1^{Δmucin}

mice expressed a 696-bp Tim-1 mRNA, which lacks the 159-bp *Tim-1* exon 3 (Fig. 1B). Consistent with the loss of 53 amino acids in Tim-1^{Δmucin} proteins as predicted based on the sequencing data (Fig. 1C and D), Western blot data showed that Tim-1^{Δmucin} cells expressed shortened Tim-1 proteins (mainly an ~25-kDa unglycosylated band and an ~60-kDa glycosylated band), whereas WT Tim-1⁺ cells expressed primarily ~35-kDa unglycosylated and ~75-kDa glycosylated Tim-1 proteins (Fig. 1B). Furthermore, flow cytometric data showed that Tim-1^{Δmucin} proteins, like the WT Tim-1 proteins, were expressed normally on the surface of B cells (Fig. 1E). These data confirmed that we generated mice that expressed a mutant mucinless Tim-1 protein. The extracellular portion of this Tim-1 mutant was composed of the IgV domain and the stalk domain containing two N-glycosylation sites but completely lacked O-glycosylated mucin domain (Fig. 1F).

Tim-1^{Δmucin} Mice Develop an Activated Immune Phenotype with Age.

We first evaluated whether the mucin deletion affected the development and phenotype of immune cells. Up to 6 mo of age, Tim-1^{Δmucin} mice were apparently healthy and did not show any significant abnormality in cellularity of immune compartments or any defect in kidneys or other organs. For example, Tim-1^{Δmucin} mice showed normal T-cell development in thymi, normal distribution of T and B cells, DCs, and macrophages, and normal activation status of T cells and APCs in peripheral immune compartments (Fig. 2A).

Because Tim-1 has been reported to be expressed on T cells upon activation and to regulate T-cell responses (24, 25), we assessed the responses of T cells from Tim-1^{Δmucin} mice. After activation, Tim-1^{Δmucin} CD4⁺ cells showed slightly increased proliferation and IFN-γ production but slightly decreased levels of IL-17 compared with WT CD4⁺ cells (Fig. 2B). No obvious differences in IL-4 or IL-10 production were observed between WT and Tim-1^{Δmucin} CD4⁺ cells (Fig. 2B). These data suggest that the net effect of Tim-1^{Δmucin} in CD4⁺ cells is mainly an enhanced Th1 response, as indicated by IFN-γ production.

Similar to the younger mice, WT and Tim-1^{Δmucin} mice at 10–12 mo of age did not exhibit any dramatic differences in the percentage or number of CD4⁺ or CD8⁺ T cells or B cells in spleen or lymph nodes. However, the percentage and number of activated/memory (CD44^{hi}) T cells in Tim-1^{Δmucin} mice were much higher than in WT mice (Fig. 2C). Also, the frequency and number of both IFN-γ⁺CD4⁺ and IFN-γ⁺CD8⁺ T cells were much higher in the older Tim-1^{Δmucin} mice than in WT mice, although the number of IL-17⁺ T cells remained similar between the two groups of mice, and IL-4⁺ T cells were not detectable (Fig. 2D). CD4⁺ T cells from the older Tim-1^{Δmucin} mice also showed slightly higher IL-10 production, probably as a result of ongoing activation and inflammation (Fig. 2E). More interestingly, when Tim-1^{Δmucin} mice were more than 18 mo old, many developed enlarged spleens, and almost all their T cells were CD44^{hi}. Of interest, there was an increase in CD3⁺CD4[−]CD8[−] double-negative (normal DN) T cells in Tim-1^{Δmucin} mice that were more than 18 mo old (Fig. 2F). This subset is reminiscent of a T-cell subset expanded in systemic autoimmunity, such as lupus, in both human and mouse (26, 27).

APCs, such as DCs, from 10- to 12-mo-old Tim-1^{Δmucin} mice showed increased expression of CD80, CD86, and MHC class II molecules, compared with WT mice, that was not seen in younger Tim-1^{Δmucin} mice (Fig. 2G). We then determined the effects of Tim-1^{Δmucin} DCs on T-cell responses. WT and Tim-1^{Δmucin} splenic DCs were cocultured with WT CD4⁺ T cells in the presence of anti-CD3. Tim-1^{Δmucin} DCs slightly increased T-cell proliferation and variably increased the production of cytokines including IFN-γ, IL-17, IL-10, and IL-4 (Fig. 2H). These data suggest that Tim-1^{Δmucin} DCs can increase effector T-cell responses modestly. Taken together, the data showed that Tim-1^{Δmucin} mice did not have any obvious defects when young (<6 mo old); however, when these mice become older (typically >10 mo old), they begin to show signs of autoimmunity, mainly characterized by hyperactivated effector T-cell responses.

Fig. 1. Generation and characterization of Tim-1^{Δmucin} mice. (A) The gene structures of the WT *Tim-1* allele, the *Tim-1*^{Δmucin} targeting construct, and the targeted *Tim-1* allele. Colored boxes represent coding sequences; Roman numerals represent exons. E, EcoRI sites. (B) (Left) CD19⁺ B cells isolated from WT and Tim-1^{Δmucin} mice were used to determine *Tim-1* mRNA expression by RT-PCR. (Right) 293T cells were overexpressed with WT Tim-1 and Tim-1^{Δmucin} and then were used to determine Tim-1 protein expression by Western blot. (C and D) RT-PCR products were sequenced, and parts of cDNA and predicted encoding amino acid sequences of WT Tim-1 (C) and Tim-1^{Δmucin} (D) are shown. (E) Isolated WT and Tim-1^{Δmucin} B cells were examined for Tim-1 expression by flow cytometry. (F) Schematic representation of Tim-1 and Tim-1^{Δmucin} protein structures.

Fig. 2. Immune phenotypes in $\text{Tim-1}^{\Delta\text{mucrin}}$ mice. (A) Spleen, lymph nodes, and thymus were analyzed for the expression of indicated markers by flow cytometry. Data shown are representative of $n = 8$ –10 per group of 3- to 6-mo-old mice. (B) CD4^+ T cells isolated from 3- to 6-mo-old mice were treated with anti-CD3 and anti-CD28. Cytokines in 48-h culture supernatants were measured by cytometric bead array. Proliferation was measured by [^3H]-thymidine incorporation. (C) Splenocytes from 10- to 12-mo-old mice were examined for CD62L and CD44 expression on CD3^+ cells by flow cytometry. (D and E) Splenocytes from 10- to 12-mo-old mice also were stained for IFN- γ and IL-17 (D) and IL-10 (E) expression after stimulation with phosphomolybdic acid (PMA)/ionomycin. (F) Splenocytes from 20-mo-old WT and $\text{Tim-1}^{\Delta\text{mucrin}}$ mice were analyzed for CD3, CD4, and CD8 expression by flow cytometry (gated on CD3^+ cells). (G) Splenocytes from 10- to 12-mo-old mice were examined for CD80, CD86, and MHC class II expression on CD11c^+ cells by flow cytometry. (H) DCs isolated from WT and $\text{Tim-1}^{\Delta\text{mucrin}}$ mice were cocultured with WT CD4^+ T cells in the presence of anti-CD3. Cytokines in 48-h culture supernatants were measured by cytometric bead array. Proliferation was measured by [^3H]-thymidine incorporation. $^*P < 0.05$.

$\text{Tim-1}^{\Delta\text{mucrin}}$ Mice Have Normal Foxp3 $^+$ Regulatory T Cells but Impaired Breg IL-10 Production. Regulatory T cells (Tregs) have been shown to play a critical and essential role in the maintenance of immune tolerance (28). Therefore, we next asked whether the T-cell hyperactivity and DC activation developed in $\text{Tim-1}^{\Delta\text{mucrin}}$ mice was caused by a Treg defect. To study the frequency and function of Foxp3 $^+$ Treg cells, we generated $\text{Tim-1}^{\Delta\text{mucrin}}$ Foxp3/GFP-knockin (KI) mice by breeding $\text{Tim-1}^{\Delta\text{mucrin}}$ mice with Foxp3/GFP-KI mice (29). We did not observe any obvious differences between WT and $\text{Tim-1}^{\Delta\text{mucrin}}$ mice in the number or frequency of Foxp3 $^+$ Tregs or in their suppressive capacity (Fig. 3A and B). Recent studies also have suggested that IL-10-producing Bregs are essential in immune tolerance and that loss of IL-10-producing Bregs leads to the development of autoimmune

disease (30). We asked whether the autoimmunity developed in $\text{Tim-1}^{\Delta\text{mucrin}}$ mice with age was caused by a defect in Bregs. Although there is not a clear marker for Bregs, at this stage they are defined best by a combination of cell-surface markers that include $\text{CD19}^+\text{CD5}^+\text{CD1d}^{\text{hi}}$ cells. Because a portion of B cells express Tim-1 (16), and because $\text{CD19}^+\text{CD5}^+\text{CD1d}^{\text{hi}}$ B cells are the most competent B-cell subset for IL-10 production (30), we first determined whether the $\text{CD19}^+\text{CD5}^+\text{CD1d}^{\text{hi}}$ B-cell subset expressed Tim-1. We found that $\sim 10\%$ of splenic CD19^+ B cells from naive mice expressed Tim-1, and Tim-1^+ cells were enriched mainly in CD5^+ B cells: $\sim 50\%$ of $\text{CD5}^+\text{CD1d}^{\text{hi}}$ and $\sim 30\%$ of $\text{CD5}^+\text{CD1d}^{\text{low}}$ B cells expressed Tim-1, whereas $<5\%$ of CD5^- B cells were Tim-1^+ regardless their CD1d levels (Fig. 3C). We then examined whether Tim-1^+ B cells produced IL-10 using the IL-10/Thy1.1 reporter mice (31). Interestingly, we found that IL-10 production in B cells was almost exclusively

Fig. 3. Foxp3 $^+$ Tregs are normal, but IL-10-producing Bregs are impaired in $\text{Tim-1}^{\Delta\text{mucrin}}$ mice. (A) Frequency of Foxp3/GFP $^+$ Tregs in spleens of 4- to 6-mo-old WT Foxp3/GFP-KI and $\text{Tim-1}^{\Delta\text{mucrin}}$ Foxp3/GFP-KI mice was analyzed by flow cytometry. (B) Effector T cells (Teff) and DCs from WT Foxp3/GFP-KI mice were cocultured with different ratios of Tregs in the presence of anti-CD3, and cell proliferation was measured by [^3H]-thymidine incorporation. (C) Splenocytes from 3-mo-old WT mice were stained with CD19, CD5, CD1d, and Tim-1 mAb, and Tim-1 expression on different CD19^+ B-cell subsets was analyzed by flow cytometry. (D) Splenocytes from 10-mo-old reporter mice were activated with LPS/PMA/ionomycin for 5 h, and Tim-1 expression and IL-10 production (Thy1.1 $^+$) in CD19^+ B cells were analyzed by flow cytometry. (E) Splenocytes from WT and $\text{Tim-1}^{\Delta\text{mucrin}}$ mice were activated with LPS/PMA/ionomycin for 5 h, and IL-10 production in CD19^+ B cells was analyzed by intracellular cytokine staining (Left and Middle). Isolated splenic CD19^+ cells were activated with LPS for 24 h, and IL-10 production in culture supernatants was measured by cytokine bead array (Right). $^*P < 0.05$.

from Tim-1⁺ cells (Fig. 3D), consistent with a recent report suggesting that Tim-1 identifies IL-10-producing B cells regardless of other markers (32). In fact, it was shown that Tim-1 ligation could induce Tim-1⁺ Bregs, which could transfer long-term acceptance of islet allografts and inhibit allergic airway disease (32).

We then compared IL-10 production from WT and Tim-1^{Δmucin} B cells. When mice were <6 mo old, Tim-1^{Δmucin} B cells clearly produced less IL-10 than WT B cells, as determined by both intracellular cytokine staining and ELISA (Fig. 3E). When mice were >10 mo old, the difference became even more dramatic. Although the frequency of IL-10⁺ B cells increased in aged WT mice, as reported previously (33), the frequency of IL-10⁺ B cells decreased markedly in Tim-1^{Δmucin} mice (Fig. 3E).

Taken together, these data suggest that Tim-1⁺ B cells are the major B cells for IL-10 production. Lack of the mucin domain in Tim-1 protein impairs IL-10-producing Bregs, and the difference in IL-10 production becomes even more dramatic with age. Also, the defect in IL-10 production is CD19⁺ B-cell specific in Tim-1^{Δmucin} mice, because there was no obvious difference in IL-10 production between WT and Tim-1^{Δmucin} CD19⁺ cells from young mice. Furthermore, Tim-1^{Δmucin} CD19⁺ cells from old mice produced slightly higher IL-10 levels than did WT cells (Fig. S1). In fact, Tim-1^{Δmucin} CD4⁺ cells from old mice produced higher IL-10 than did WT T cells (Fig. 2E).

Immune Phenotypes in Tim-1^{Δmucin}lpr Mice. Our data thus far have shown that the major defect in naive Tim-1^{Δmucin} mice at age <6 mo was impaired IL-10 production by Bregs. When Tim-1^{Δmucin} mice were >10 mo old, these Bregs lost their IL-10 production almost completely. Associated with the defect in IL-10-producing Bregs, Tim-1^{Δmucin} mice showed signs of systemic inflammation with age. Because *Fas*-deficient *lpr* mice on the C57BL/6 background develop only a mild systemic autoimmunity (34), we asked whether introducing Tim-1^{Δmucin} into *lpr* mice on the C57BL/6 background would promote more severe autoimmunity. By 14 wk of age, Tim-1^{Δmucin}lpr mice already had a much greater accumulation of B220⁺CD3⁺ (abnormal) T cells in the peripheral lymphoid compartments than did *lpr* mice (Fig. 4A). By 10⁺ mo of age they developed much more severe splenomegaly and lymphadenopathy (Fig. 4B) with massive accumulation of T cells (both CD3⁺B220⁺CD4⁺ and CD8⁺ T cells), B cells (CD3⁺B220⁺), CD3⁺B220⁺CD4⁺CD8⁺ double-negative T cells, and the abnormal CD3⁺B220⁺CD4⁺CD8⁺ T cells (Fig. 4C and D). Furthermore, the majority of T cells in these Tim-1^{Δmucin}lpr mice had an activated/memory-like (CD44^{hi}) phenotype, compared with those in *lpr*, WT, or Tim-1^{Δmucin} mice (Fig. 4D). The frequency and number of IFN-γ⁺CD4⁺ and IFN-γ⁺CD8⁺ T cells in Tim-1^{Δmucin}lpr mice were much higher than in *lpr* mice, whereas the number of IL-17⁺CD4⁺ T cells was similar in the two groups (Fig. 4E); IL-17⁺CD8⁺ T cells were not detectable. Interestingly, compared with *lpr* mice, Tim-1^{Δmucin}lpr mice also had a massive accumulation of CD3⁺B220⁺CD4⁺CD8⁺ (normal DN) T cells and many of them produced IFN-γ and/or IL-17 (Fig. 4E); the number of normal DN T cells was very low and was similar in 10- to 12-mo-old WT and Tim-1^{Δmucin} mice (Fig. 4C). However, the frequency and number of the normal DN T cells increased dramatically in Tim-1^{Δmucin} mice that were >18 mo old (Fig. 2F), indicating that this cell population eventually does accumulate in Tim-1^{Δmucin} mice even without the *lpr* mutation.

Tim-1^{Δmucin}lpr mice also had a greater accumulation of B cells than *lpr* mice, whereas *lpr* mice had more B cells than Tim-1^{Δmucin} mice (Fig. 4C). *Lpr* mice had higher serum levels of IgM, IgG1, IgG2a, and IgG3 than either WT or Tim-1^{Δmucin} mice, whereas Tim-1^{Δmucin}lpr mice produced higher levels of IgG1, IgG2a, IgG2b, and IgG3 but lower levels of IgM than *lpr* mice (Fig. 5A). Using a lupus-associated autoantigen microarray (35), we found that serum levels of autoantibodies to three antigens (cytochrome C, histone III, and histone 2b) out of 39

Fig. 4. Immune phenotypes in Tim-1^{Δmucin}lpr double-mutant mice. (A) Increased accumulation of abnormal CD3⁺B220⁺CD4⁺CD8⁺ cells in Tim-1^{Δmucin}lpr mice. CD3 and B220 expression in splenocytes of 14-wk-old mice was determined by flow cytometry. (B) Increased splenic and lymph node sizes in Tim-1^{Δmucin}lpr mice. (Left) Representative images of spleens and lymph nodes from 10- to 12-mo-old WT, Tim-1^{Δmucin}, *lpr*, and Tim-1^{Δmucin}lpr mice. (Right) Splenic and lymph node cells from 10- to 12-mo-old WT (*n* = 8), Tim-1^{Δmucin} (*n* = 12), *lpr* (*n* = 10), and Tim-1^{Δmucin}lpr (*n* = 15) mice were counted. (C–E) Cells from *B* were examined for indicated surface markers (C and D) or for IFN-γ and IL-17 production (E) by flow cytometry. Different immune cell populations then were enumerated based on the percentage. **P* < 0.05.

lupus-associated autoantigens were increased significantly in all Tim-1^{Δmucin}lpr mice at the age of 14 wk but not in any of the age-matched *lpr*, Tim-1^{Δmucin}, or WT mice (Fig. 5B). In fact, some of the Tim-1^{Δmucin}lpr mice at 10 wk of age already showed increased autoantibodies to cytochrome C (Fig. 5B). At 10⁺ months of age, Tim-1^{Δmucin}lpr mice had the highest levels of anti-dsDNA autoantibodies, followed by *lpr* mice, Tim-1^{Δmucin} mice, and then WT mice (Fig. 5C). In addition to increased anti-dsDNA autoantibodies, Tim-1^{Δmucin} mice also developed anti-bodies against other lupus-associated autoantigens including anti-Sm and anti-histone, among others (Fig. S2).

Taken together, these data suggested that the combination of Tim-1^{Δmucin} and *Fas* mutation strongly promoted autoimmunity

Fig. 5. Increased serum levels of Ig and anti-dsDNA autoantibodies in Tim-1^{Δmucin}lpr mice. (A) Levels of different Igs in sera from >10-mo-old WT ($n = 8$), Tim-1^{Δmucin} ($n = 12$), lpr ($n = 10$), and Tim-1^{Δmucin}lpr ($n = 15$) mice were determined by ELISA. (B) Heat map of lupus-associated autoantigen microarray. Serum IgG reactivity to 5 of 39 lupus-associated autoantigens is shown. Other lupus-associated autoantigens showed no significant difference among the groups of mice. (C) Levels of anti-dsDNA IgG in sera from A were determined by ELISA. * $P < 0.05$.

which was much more severe than that caused by Tim-1^{Δmucin} or Fas mutation alone.

Discussion

In the present study, we generated Tim-1^{Δmucin} mice and studied the effect of the loss of the mucin domain in Tim-1-regulated immune responses. We found that the major defect in Tim-1^{Δmucin} mice was impaired IL-10 production in B cells and that this impairment became more severe with age. Associated with the loss of IL-10 production from B cells, Tim-1^{Δmucin} mice older than 10 mo of age developed autoimmune disease characterized mainly by hyperactivated T cells, increased Th1 responses, activated DCs, and elevated serum Ig and many lupus-associated autoantibodies including anti-dsDNA autoantibodies. When introduced into Fas-mutant lpr mice, Tim-1^{Δmucin} accelerated and worsened systemic autoimmunity as observed in lpr mice.

Although Tim-1 expression has been reported on activated T cells, where it regulates T-cell activation (24), and on DCs, where it affects DC function (36), Tim-1 also is expressed on B cells (16). A recent study has suggested that Tim-1 may be a better marker for IL-10-producing Bregs (32). Indeed, we confirmed that ~10% of B cells in naive mice expressed Tim-1, and IL-10 was produced almost exclusively by Tim-1⁺ B cells. CD5⁺CD1d^{hi} have been shown to be the markers for IL-10-producing Bregs (30), and indeed Tim-1 is coexpressed with these markers on B cells, raising the question of whether Tim-1 is functionally important for Bregs. Our data support this notion, because the expression of mucinless Tim-1 in Tim-1^{Δmucin} mice affected IL-10 production from B cells as the mice aged. Also, we found that Rag1^{-/-} hosts cotransferred with T cells and Tim-1^{Δmucin} B cells developed more severe experimental autoimmune encephalitis (EAE) associated with enhanced pathogenic Th1 and Th17 responses than mice cotransferred with T cells and WT B cells (Fig. S3). This result further supports the notion that Tim-1 is functionally important for Bregs.

B cells generally are considered to act as positive regulators of immune responses by promoting antigen presentation for optimal T-cell activation and by producing antibodies, but now it is clear that Bregs are essential for inducing immune tolerance by negatively regulating immune responses via IL-10 production (30). Lack or loss of Bregs accelerates and exacerbates many autoimmune and inflammatory diseases, including EAE, chronic colitis, arthritis, type 1 diabetes, lupus, and contact hypersensitivity. In many models, IL-10 appears to be critical for Breg activity, although other mechanisms in addition to IL-10 production also might be operational for the regulatory function of Bregs. Impaired IL-10 production in Bregs is the major defect in Tim-1^{Δmucin} mice, and the appearance of systemic autoimmunity correlates with the progressive loss of IL-10 production only in B cells, suggesting that deficient IL-10 production by Bregs is most likely the cause for the development of systemic autoimmune disease in these mice. It is also possible, however, that IL-10 is a functional marker defining the Bregs and that there are additional defects in the Tim-1-mutant Bregs that are regulated by expression of Tim-1 and are responsible for suppressive activity. It will be of interest to determine whether transfer of WT Tim-1⁺ B cells into Tim-1^{Δmucin} mice reverses the systemic autoimmunity observed in the Tim-1^{Δmucin} mice. In fact, a recent study has shown that transfer of Tim-1⁺ B cells led to long-term acceptance of islet allografts and inhibited allergic airway disease (32), supporting previous reports that transfer of Bregs inhibited autoimmunity in animal models of many autoimmune diseases, including lupus (30, 37).

These observations also raise the issue of whether Tim-1 is required for the expansion/maintenance of Bregs or simply regulates IL-10 and other effector functions of Bregs. Our data suggest that impaired IL-10 production in Tim-1^{Δmucin} Bregs is not caused by a decrease in the number or percentage of Bregs, because the total number or frequency of Tim-1⁺ B cells in Tim-1^{Δmucin} mice did not decrease but rather increased with age. This result suggests that Tim-1 regulates IL-10 production and other effector functions but not their overall frequency and number. Consistent with these data, we observed that addition of one of our monoclonal anti-Tim-1 Ab increased IL-10 production from B cells isolated from naive WT mice but not from the Tim-1^{Δmucin} B cells (Fig. S4). Furthermore, loss of IL-10 production seems to be an inherent defect in Tim-1^{Δmucin} B cells, because Tim-1^{Δmucin} B cells produced significantly less IL-10 than WT B cells in the same Rag1^{-/-} hosts reconstituted with 1:1 mixed WT and Tim-1^{Δmucin} bone marrow cells (Fig. S5). These data would suggest that Tim-1 signaling is required for the induction and/or maintenance of IL-10 and possibly for other regulatory functions in Bregs.

Tim-1 has been shown to be a receptor of Tim-4 (18) and phosphatidylserine exposed on apoptotic cells (4). Interestingly, although WT and Tim-1^{Δmucin} B cells had similar levels of binding for Tim-4 (Fig. S6A), Tim-1^{Δmucin}-derived cells showed a defect in binding and uptake of apoptotic cells (Fig. S6B), suggesting that deletion of the mucin domain affects Tim-1 binding to phosphatidylserine-positive apoptotic cells but does not affect Tim-1 binding to Tim-4. Because binding and engulfment of apoptotic cells during monocyte activation increased their IL-10 production (38), one possible mechanism by which Tim-1 maintains Breg IL-10 production in the hosts may be through the interaction of Tim-1 with apoptotic cells, which may mediate persistent signaling through Tim-1 to maintain or induce IL-10 production. In this regard, apoptotic cells also have been shown to promote Bregs, which inhibited autoimmune inflammation in an IL-10-dependent manner (39). Tim-1 is expressed on IL-10-producing B cells in the naive state but is expressed on T cells only after activation, and this fact correlates with the defect in IL-10 production only in B cells in Tim-1^{Δmucin} mice.

We recently have found that Tim-1 is expressed on DCs and that DCs activated by Tim-1 signaling enhance T-cell responses (36). Consistently, Tim-1^{Δmucin} DCs became more activated in

aged mice, and Tim-1^{Δmucin} DCs also promoted effector T-cell responses. These results may suggest that mutant Tim-1 in Tim-1^{Δmucin} DCs may have a cell-intrinsic role in activating DCs, but it also is possible that the DC activation observed in the Tim-1^{Δmucin} mice is secondary to the defect in Bregs.

Tim-1^{Δmucin} mice showed impaired Breg IL-10 production and development of systemic autoimmunity with age; however, no significant defects have been reported in Tim-1^{-/-} mice in which the full-length molecule was deleted (16). Because Tim-2, expressed in mouse, is homologous to Tim-1, we hypothesize that Tim-2 may have compensated for the biological function in the complete absence of Tim-1. Tim-2 also is expressed in both T and B cells and has been shown to regulate Th2 responses (40). However, in Tim-1^{Δmucin} mice, the IgV domain for ligand binding is preserved, and Tim-2 expression is not affected (Fig. S7). This difference may contribute to the dramatic effects observed in the Tim-1^{Δmucin} mice, which are not seen in the Tim-1-knockout mice.

When these results are taken together, expression of Tim-1 appears to be crucial in maintaining effector functions in IL-10-producing B cells. Breg functions are compromised in the pres-

ence of mutant Tim-1 that does not contain the mucin domain, and this compromise is associated with the development of autoimmunity. Consistent with the genetic linkage data, our data also underscore the value of the mucin domain in Tim-1 function and in regulating immune responses. Manipulation of the Tim-1 mucin domain without altering ligand binding to the IgV domain may provide a valuable target for regulating autoimmunity and transplant rejection.

Materials and Methods

The Tim-1^{Δmucin} mouse was generated on C57BL/6 background. All mouse experiments were performed according to the animal protocol guidelines of Harvard Medical School. Detailed materials and methods can be found in *SI Materials and Methods*.

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