



TIM-1 signaling in B cells regulates antibody production

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ABSTRACT

Members of the T cell Ig and mucin (TIM) family have recently been implicated in the control of T cell-mediated immune responses. In this study, we found TIM-1 expression on anti-IgM- or anti-CD40-stimulated splenic B cells, which was further up-regulated by the combination of anti-IgM and anti-CD40 Abs. On the other hand, TIM-1 ligand was constitutively expressed on B cells and inducible on anti-CD3⁺ anti-CD28-stimulated CD4⁺ T cells. In vitro stimulation of activated B cells by anti-TIM-1 mAb enhanced proliferation and expression of a plasma cell marker syndecan-1 (CD138). We further examined the effect of TIM-1 signaling on antibody production in vitro and in vivo. Higher levels of IgG2b and IgG3 secretion were detected in the culture supernatants of the anti-TIM-1-stimulated B cells as compared with the control IgG-stimulated B cells. When immunized with T-independent antigen TNP-Ficoll, TNP-specific IgG1, IgG2b, and IgG3 Abs were slightly increased in the anti-TIM-1-treated mice. When immunized with T-dependent antigen OVA, serum levels of OVA-specific IgG2b, IgG3, and IgE Abs were significantly increased in the anti-TIM-1-treated mice as compared with the control IgG-treated mice. These results suggest that TIM-1 signaling in B cells augments antibody production by enhancing B cell proliferation and differentiation.

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1. Introduction

The T cell immunoglobulin and mucin domain (TIM) family has recently been implicated in the regulation of T cell activation and immune responses [1,2]. The genes of this family were found within the *Tapr* (T cell and airway phenotype regulator) locus on mouse chromosome 11B1.1, which is syntenic to human chromosome 5q33.2, a region that has been linked to allergic and autoimmune diseases [3]. To date, four proteins (TIM-1, -2, -3, and -4) have been identified in mice and three proteins (TIM-1, -3, and -4) have been found in humans [2]. In the mouse, TIM-1 and TIM-3 have polymorphism at the protein level, represented by BALB/c-type and B6-type [3]. The polymorphism of mouse TIM-1 is strongly associated with Th2 differentiation and the expression of airway hyperreactivity [3]. No human orthologue for mouse TIM-2 has not been identified although, given its close sequence homology, TIM-1 may share some of functions [1–3]. All proteins are type I transmembrane protein with common structural motifs including extracellular IgV and mucin domains, and intracellular domain.

TIM-1, TIM-2, and TIM-3, but not TIM-4, contain a conserved intracellular tyrosine phosphorylation motif that is involved in transmembrane signaling [2,3].

The expression of TIM-1 has been found on Th2 cells, mast cells, NKT cells, and tubular epithelial cells of kidney [1,3]. Some studies have indicated that mouse TIM-1 can bind to TIM-1, TIM-3, and TIM-4 [4,5]. TIM-1 also induces phagocytosis by recognizing phosphatidylserine (PtdSer) on apoptotic cells [6–8]. It has been shown that TIM-1 and TIM-4 can bind to separate sites on the surface of exosome [8]. Therefore, it is possible that TIM-1 interacts with other TIM molecules through binding to PtdSer on the surface of exosome or membrane fragments. Some studies have indicated that TIM-1 has a costimulatory effect on T cell responses. Cross-linking of TIM-1 with an agonistic anti-TIM-1 mAb increased the proliferation of CD4⁺ T cells and enhanced the production of IL-4 by Th2 cells [9]. Administration of agonistic anti-TIM-1 mAb in vivo increased antigen-specific T cell proliferation and production of IL-4 and IFN- γ [9]. It has been shown that TIM-1 costimulation prevents allogeneic transplant tolerance by reducing Foxp3 expression and thereby preventing regulatory T cell development, while enhancing development of Th1 and Th17 responses [10]. Moreover, administration of agonistic anti-TIM-1 mAb during the induction phase of experimental autoimmune encephalomyelitis

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enhanced pathogenic Th1 and Th17 responses and increased the severity [11].

To further characterize the expression and the role of TIM-1 in the immune response, we newly generated an mAb against mouse TIM-1 and found that TIM-1 was highly expressed on splenic B cells after anti-IgM and anti-CD40 stimulation. On the other hand, TIM-1 ligand (TIM-1L) was constitutively expressed on B cells and also inducible on anti-CD3⁺ anti-CD28-stimulated CD4⁺ T cells. In vitro stimulation of activated B cells by the anti-TIM-1 mAb induced expression of syndecan-1 (CD138), which is a plasma cell marker. Moreover, in vivo administration of the anti-TIM-1 mAb increased serum levels of antigen-specific antibodies in response to T-independent 2 (TI-2) and T-dependent (TD) antigens. These results suggest that the TIM-1 signaling in B cells may be a novel target for manipulating antibody responses.

2. Materials and methods

2.1. Animals and cells

Female BALB/c mice and Sprague Dawley rats were purchased from Charles River Laboratories (Kanagawa, Japan). FcγR-deficient mice were kindly supplied by Y. Suzuki (Department of Nephrology, Juntendo University, Tokyo, Japan) [12]. All mice were 6–8 weeks old at the start of experiments and kept under specific pathogen-free conditions during the experiments. All animal experiments were approved by Juntendo University Animal Experimental Ethics Committee.

2.2. Generation of anti-mouse TIM-1 mAb

The anti-mouse TIM-1 mAb was generated by immunizing Sprague Dawley rats with TIM-1-Ig, consisting of the extracellular domains (aa 1–236 of TIM-1 BALB) [3] and the Fc portion of mouse IgG2a, emulsified in CFA (Difco Laboratories, Detroit, MI, USA). Three days after the final immunization, lymph node (LN) cells were fused with P3U1 myeloma cells (American Type Culture Collection, Manassas, VA, USA). After hypoxanthine–aminopterin–thymidine selection, a hybridoma producing anti-TIM-1 mAb (RMT1-17, rat IgG1/κ) was selected by its reactivity to mouse TIM-1-transfected cells, but not to parental cells by flow cytometry and then cloned by limiting dilution. Anti-TIM-2 (RMT2-14), anti-TIM-3 (RMT3-23), and anti-TIM-4 (RMT4-54) mAbs were also generated in our laboratory [13].

2.3. Preparation of activated CD4 T cells and B cells

CD4 T cells were purified from the spleen of BALB/c mice by passage through nylon wool column (Wako Biochemicals, Tokyo, Japan) and by using an auto-MACS column with CD4 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purified CD4 T cells (3×10^6 /ml; >95% CD4⁺) were stimulated with immobilized anti-CD3 mAb (145-2C11; 5 μg/ml) in the presence or absence of anti-CD28 mAb (PV-1; 5 μg/ml) for 24–72 h. Small resting B cells were purified from the spleen of BALB/c mice as previously described [14]. Purified B cells (3×10^6 /ml; >95% B220⁺) were stimulated with goat anti-mouse IgM F(ab')₂ Ab (5 μg/ml) and/or anti-CD40 mAb (HM40-3; 5 μg/ml), or LPS (5 μg/ml) for 24–72 h.

2.4. Flow cytometric analysis

Spleen cells were digested with 400 U/ml collagenase (Wako Biochemicals) and 5 mM EDTA. Cells were first preincubated with unlabeled anti-CD16/32 mAb to avoid non-specific binding of Abs

to FcγR and then incubated with FITC- or APC-labeled mAb, or biotinylated mAb followed by PE-labeled streptavidin. The stained cells (live gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed on a FACSCalibur (BD Biosciences), and data were processed using the CellQuest program (BD Biosciences).

2.5. In vitro B cell proliferation and Ig production assays

Purified B cells (1×10^5 /well) from BALB/c or FcγR-deficient mice were cultured with anti-IgM Ab (5 μg/ml), anti-CD40 mAb (5 μg/ml), and/or recombinant mouse IL-4 (20 ng/ml; BD Biosciences) in 96-well flat-bottomed plates. Anti-CD16/32 mAb (5 μg/ml) and 10 μg/ml of anti-TIM-1 mAb or control rat IgG were also added at the start of culture. To assess proliferative responses, the cultures were pulsed with ³H-thymidine (0.5 μCi/well; Perkin-Elmer, MA, USA) for the last 6 h of a 48 h culture and harvested on a Micro96 Cell Harvester (Molecular Devices, Sunnyvale, CA USA). Incorporated radioactivity was measured on a microplate beta counter (Micro β Plus; PerkinElmer). For analysis of Ig secretion, 50 μl of day 7 culture supernatants were subjected to the cytometric bead array (CBA) using Mouse Immunoglobulin Isotyping Kit (BD Biosciences) according to the manufacturer's instructions.

2.6. ELISA for antigen-specific serum Abs

To measure the production of Abs against a T-independent 2 (TI-2) antigen, BALB/c mice ($n = 8$) were i.p. immunized with 10 μg of TNP-Ficoll (Biosearch technologies, Novato, CA, USA) on days 0 and administrated with 300 μg of anti-TIM-1 mAb or control rat IgG (Sigma–Aldrich) on days 1 and 3. Sera were collected on day 7 and the titers of anti-TNP Abs were measured by ELISA against TNP-BSA (Biosearch technologies). To examine the response against a T-dependent (TD) antigen, BALB/c mice ($n = 8$) were i.p. immunized with 50 μg of OVA (Sigma–Aldrich) in 4 mg of alum (Thermo Scientific, Rockford, IL, USA) on days 0 and 14 and administrated with 300 μg of anti-TIM-1 mAb or control rat IgG every 3 days from day 0 to day 18. Sera were collected on day 21 and the titers of anti-OVA Abs were measured by ELISA.

2.7. Statistical analysis

Significant differences between two experimental groups were analyzed by the unpaired Student's *t*-test. The results are expressed as the mean ± SEM. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Expression of TIM-1 and TIM-1L on spleen cells

Newly generated an anti-TIM-1 mAb (RMT1-17) reacted with TIM-1 transfectants (TIM-1 BALB/NRK, TIM-1 B6/NRK, TIM-1 BALB/L5178Y, and TIM-1 B6/L5178Y) but not with parental NRK and L5178Y cells (Fig. 1A). Anti-TIM-1 mAb did not bind to the other TIM family-transfected cells (TIM2/NRK, TIM-3 BALB/NRK, TIM-3 B6/NRK, and TIM-4/NRK) (Fig. 1B), indicating that anti-TIM-1 mAb is specific for TIM-1. We examined the expression of TIM-1 and TIM-1L on freshly isolated spleen cells by flow cytometric analysis using this anti-TIM-1 mAb and TIM-1-Ig. As shown in Fig. 1C, a marginal expression of TIM-1 was detected on B220⁺ CD19⁺ B cells. On the other hand, a substantial level of TIM-1L expression was found on antigen-presenting cells including B cells, CD11b⁺ F4/80⁺ macrophages, and both CD8[−] CD11c⁺ and CD8⁺

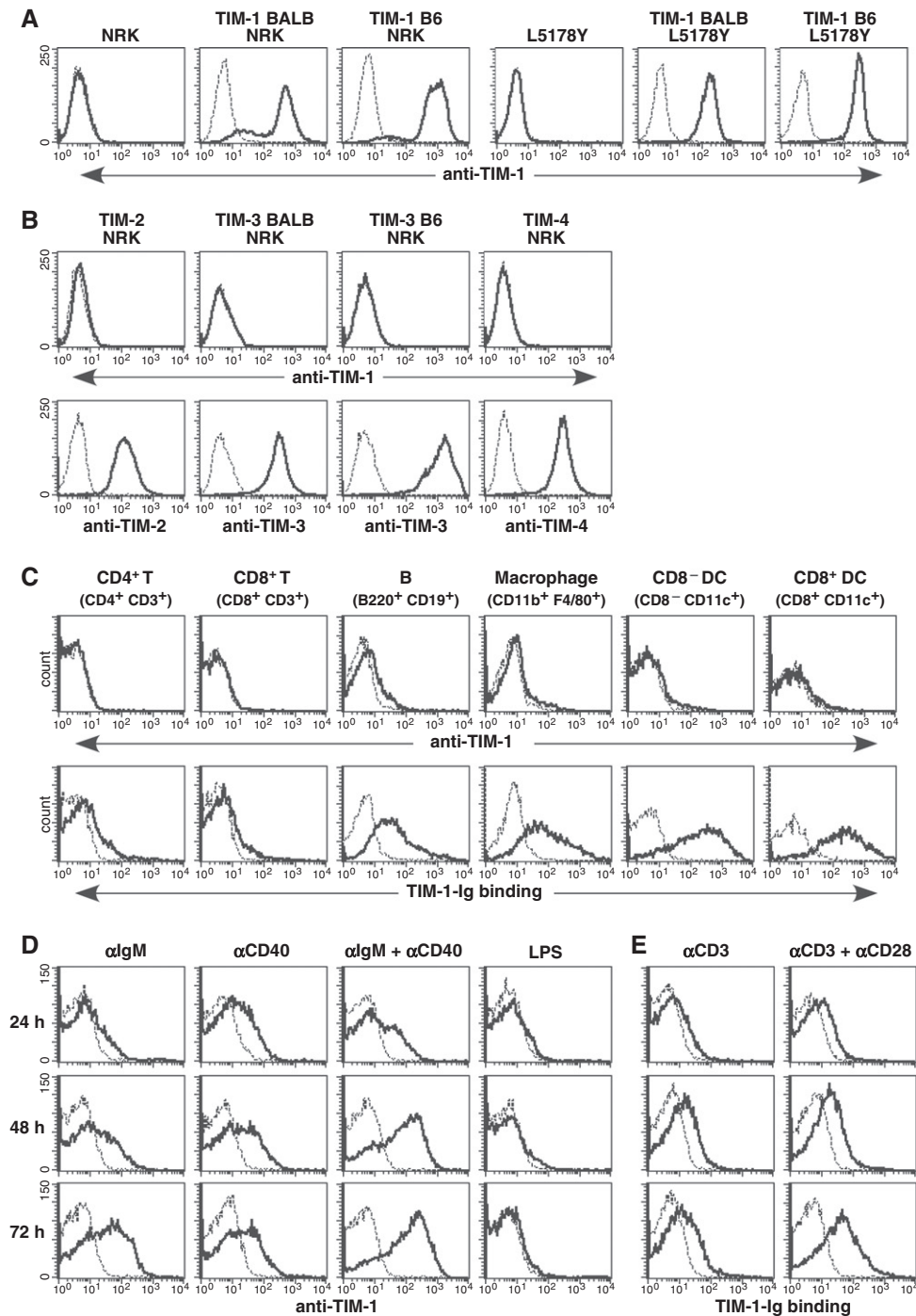


Fig. 1. (A) Reactivity of anti-TIM-1 mAb to mouse TIM-1 transfectants. NRK-52E- and L5178Y-derived TIM-1 transfectants and parental cells were stained with anti-TIM-1 mAb or control rat IgG. (B) Anti-TIM-1 mAb dose not react with the other TIM family molecules. NRK-derived transfectants were stained with anti-TIM-1 mAb, specific mAbs against TIM-2, TIM-3, TIM-4, or control rat IgG. (C) Reactivity of anti-TIM-1 mAb and TIM-1-Ig to spleen cells. Freshly isolated spleen cells were triple-stained with the indicated FITC- and APC-labeled mAbs with biotinylated anti-TIM-1 mAb (upper) or TIM-1-Ig (lower) followed by biotinylated anti-mouse IgG2a mAb and/or PE-labeled streptavidin. Histograms shown are staining of electronically gated cells. (D) Expression of TIM-1 on activated B cells. Purified splenic B cells were stimulated with anti-IgM Ab, anti-CD40 mAb, anti-IgM Ab + anti-CD40 mAb, or LPS. Cells were harvested at the indicated periods and stained with anti-TIM-1 mAb or control rat IgG. (E) Expression of TIM-1L on activated CD4⁺ T cells. Purified splenic CD4⁺ T cells were stimulated by immobilized anti-CD3 mAb with or without anti-CD28 mAb and harvested at the indicated periods. Cells were stained with TIM-1-Ig or control mouse IgG2a. Thick lines indicate the staining with the specific mAbs or TIM-1-Ig, and the dotted lines indicate background staining with control rat IgG or mouse IgG2a.

CD11c⁺ dendritic cells. Unexpectedly, a marginal expression of TIM-1L was found on both CD4⁺ T and CD8⁺ T cells (Fig. 1C).

To further determine the expression of TIM-1 upon B cell activation, splenic B cells were stimulated with the combination of anti-IgM and anti-CD40 or LPS for 24–72 h. The stimulation with anti-IgM or anti-CD40 alone up-regulated TIM-1 expression on B

cells (Fig. 1D). The combination of anti-IgM + anti-CD40 markedly enhanced TIM-1 expression at 48–72 h (Fig. 1D). In contrast, the stimulation with LPS did not affect the expression of TIM-1 on B cells (Fig. 1D). We also examined the expression of TIM-1L on activated CD4⁺ T cells. Purified splenic CD4⁺ T cells were stimulated with immobilized anti-CD3 mAb for 24–72 h. TIM-1L expression

was slightly up-regulated after the stimulation with anti-CD3 mAb at 48–72 h (Fig. 1E). The addition of anti-CD28 mAb markedly enhanced TIM-1L expression on anti-CD3-stimulated CD4⁺ T cells

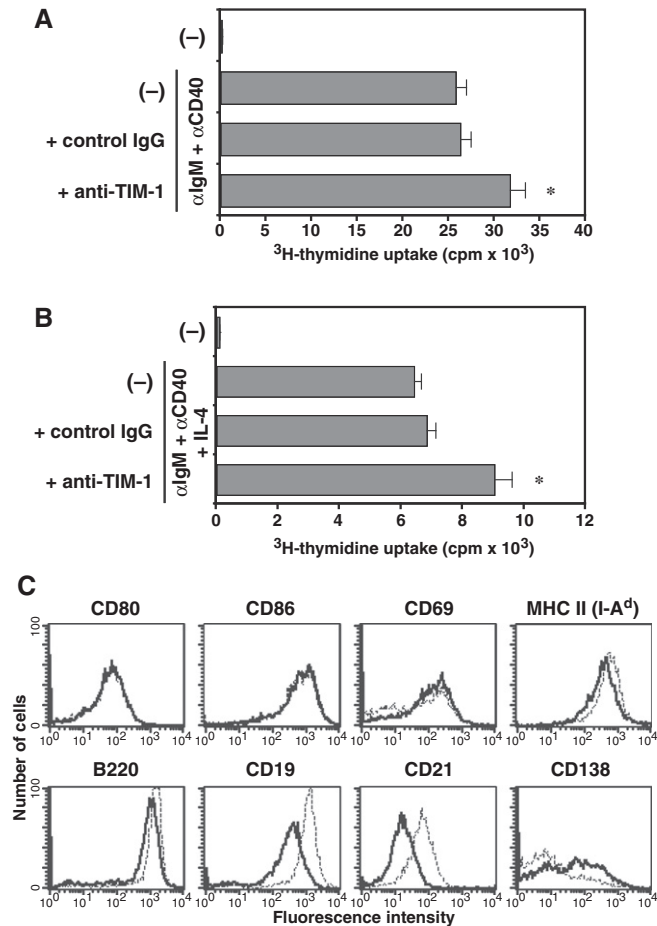


Fig. 2. Effect of anti-TIM-1 mAb on the proliferation and phenotype of B cells. Purified splenic B cells from Fcγ-deficient mice were stimulated with anti-IgM Ab + anti-CD40 mAb without (A) or with (B) IL-4 in the presence of anti-TIM-1 mAb or control IgG for 48 h. Proliferative response was assessed and data was expressed as the mean ± SEM of triplicate wells. **p* < 0.05 as compared to control IgG. Similar results were obtained in two independent experiments. (C) Purified splenic B cells from BALB/c mouse were stimulated with anti-IgM Ab in the presence of anti-TIM-1 mAb or control IgG. Cells were harvested at 48 h and stained with the indicated mAbs. Thick lines indicate the culture with anti-TIM-1 mAb and dotted lines indicate the culture with control IgG. Data are representative of three experiments.

at 72 h (Fig. 1E). Collectively, these results indicate that mouse TIM-1 is expressed on surface Ig- and CD40-stimulated B cells and TIM-1L is expressed not only on antigen-presenting cells but also on TCR/CD3⁺ CD28-stimulated CD4⁺ T cells.

3.2. Effect of anti-TIM-1 mAb on B cell proliferation and differentiation in vitro

The dynamic expression of TIM-1 on B cells implies that TIM-1 may regulate B cell activation or function, and anti-TIM-1 mAb can suppress or stimulate B cells by signaling through TIM-1. To address this possibility, splenic B cells were stimulated with anti-IgM + anti-CD40 or anti-IgM + anti-CD40 + IL-4 in the presence of anti-TIM-1 mAb or control IgG for 48 h, and then the proliferative response was assessed. To avoid positive or negative signaling through FcγRs [15], B cells were purified from Fcγ-deficient mice and anti-CD16/32 mAb was added into the culture. As shown in Fig. 2A and B, proliferation of either anti-IgM/anti-CD40- or anti-IgM/anti-CD40/IL-4-stimulated B cells were significantly enhanced by the addition of anti-TIM-1 mAb as compared to control IgG.

We further investigated whether anti-TIM-1 stimulation influenced expression of some cell surface molecules. Purified splenic B cells were stimulated with anti-IgM in the presence of anti-TIM-1 mAb or control IgG for 48 h. As shown in Fig. 2C, anti-TIM-1 stimulation did not affect the expression of CD80, CD86, and CD69 on anti-IgM-stimulated B cells. Unexpectedly, MHC class II (I-A^d) and B220 were slightly down-regulated, and CD19 and CD21 were greatly down-regulated by anti-TIM-1 stimulation. In contrast, the expression of CD138 (syndecan-1), which is a marker of plasma cells [16,17], was up-regulated by anti-TIM-1 stimulation.

3.3. Anti-TIM-1 mAb enhances production of IgG2b and IgG3 in vitro

We next examined whether anti-TIM-1 stimulation influenced secretion of each Ig isotype. Purified small resting B cells were stimulated with the combination of anti-IgM, anti-CD40, and IL-4 in the presence of anti-TIM-1 mAb or control rat IgG. Culture supernatants were harvested at day 7 and subjected to a cytometric bead array (CBA) assay for the seven mouse Ig isotypes [18]. Ig secretion was not changed when B cells were only treated with anti-TIM-1 mAb or control IgG (data not shown). As shown in Fig. 3, supernatants from the anti-TIM-1 mAb culture with anti-IgM, anti-CD40, or anti-IgM + anti-CD40 stimulation showed higher IgG2b levels as compared to the control IgG cultures. In addition, supernatants from the anti-TIM-1 mAb cultures with

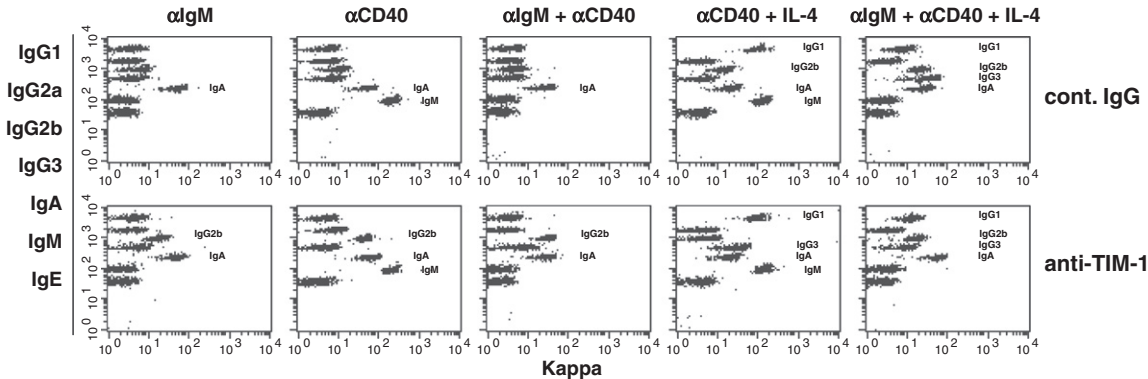


Fig. 3. Effect of anti-TIM-1 mAb on Ig production in vitro. Purified B cells from BALB/c mice were stimulated with the indicated combinations of anti-IgM Ab, anti-CD40 mAb, and IL-4 in the presence of anti-TIM-1 mAb (lower) or control IgG (upper). Culture supernatants were harvested at day 7 and analyzed using a CBA assay that could identify all seven mouse Ig isotypes in a single sample. Presence of a particular isotype is indicated by kappa fluorescence (with labels provided to indicate positive cultures). Data are representative of three experiments.

anti-CD40 + IL-4 stimulation showed higher IgG3 levels as compared to the control IgG culture.

3.4. Effect of anti-TIM-1 mAb treatment on TI-2 and TD antigen-specific Ab production

We further investigated the role of TIM-1 in the production of Ab in vivo. To measure B cell responses against a T-independent 2 (TI-2) antigen, mice were i.p. immunized with TNP-Ficoll and administrated with anti-TIM-1 mAb or control rat IgG on days 1 and 3. Serum levels of anti-TNP Abs were determined on day 7 by isotype-specific ELISA. Serum levels of TNP-specific IgG1, IgG2b, and IgG3 Abs were slightly, but not significantly, increased in the anti-TIM-1-treated mice as compared to the control IgG-treated mice (Fig. 4A).

To assess Ab production against a T-dependent (TD) antigen, mice were i.p. immunized with OVA/alum on days 0 and 14 and administrated with anti-TIM-1 mAb or control IgG every 3 days from day 0 to day 18. Serum levels of anti-OVA Abs were determined on day 21 by ELISA. All isotypes of OVA-specific Abs were increased in the anti-TIM-1-treated mice as compared to the control IgG-treated mice (Fig. 4B). Especially, OVA-specific IgG2b, IgG3, and IgE Abs were significantly increased in the anti-TIM-1-treated mice. Thus, anti-TIM-1 mAb treatment increased the Ab production in response to TI-2 and TD antigens, suggesting that TIM-1 signaling regulates antibody production by B cells autonomously and through T-B cell interaction.

4. Discussion

Here we have characterized the functional expression of TIM-1 on B cells. In vitro stimulation of activated B cells with anti-TIM-1 mAb enhanced proliferation and Ig production. The expression of B220, CD19, CD21, and MHC class II was down-regulated by anti-TIM-1 mAb stimulation. In contrast, the expression of syndecan-1 (CD138) was up-regulated. It has been reported that some cell surface molecules on B cells are down-regulated upon plasma cell differentiation, including B220, CD19, CD21, and MHC class II [17]. On the other hand, syndecan-1 is induced on antibody-secreting B cells and is often used as a plasma cell marker [16]. Thus, our results suggest that TIM-1 may be involved in the differentiation of B cells to plasma cells. B cells receive multiple signals during their differentiation into antibody-secreting cells. These include signals delivered by antigen through the B-cell receptor that drive proliferation, signals delivered by cytokines that initiate Ig class switching, and signals through CD40 or Toll-like receptors, which synergize with cytokine signals to cause class-switch recombination and antibody secretion. Class-switch recombination involves activation of the gene for activation-induced cytidine deaminase (AID), followed by deletional switch recombination and expression of mature transcripts of the switched Ig isotype [19,20]. To confirm the contribution of TIM-1 signal to the induction of class-switch recombination, therefore, further studies are required whether TIM-1 signaling can induce AID activation.

T-dependent humoral immune responses are characterized by the development of germinal center in B-cell follicles of the

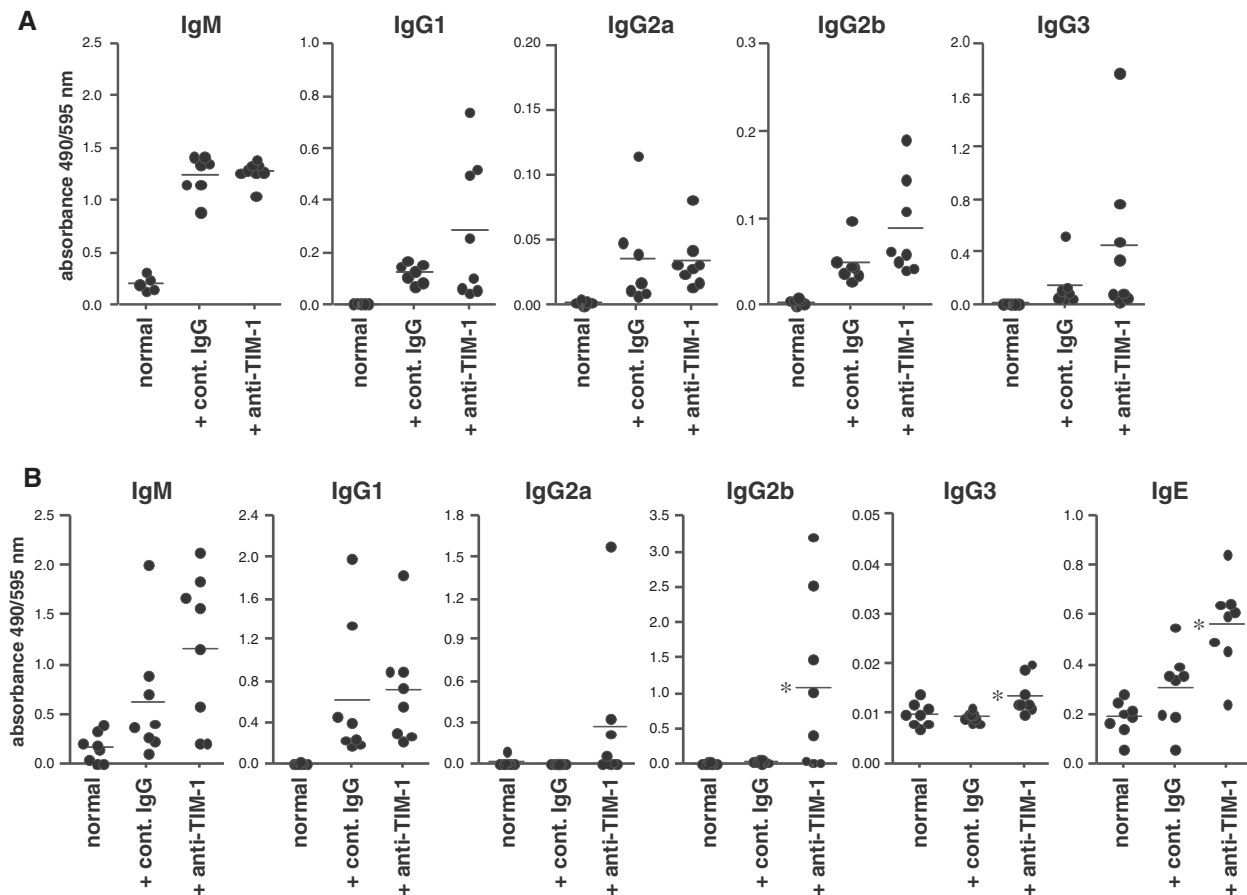


Fig. 4. Effect of anti-TIM-1 mAb on TNP- and OVA-specific Ab production in vivo. (A) BALB/c mice were i.p. immunized with TNP-Ficoll on day 0 and administrated with anti-TIM-1 mAb or control IgG on days 1 and 3. Serum levels of TNP-specific Abs were determined by isotype-specific ELISA against TNP-BSA on day 7. (B) Mice were i.p. immunized with OVA/alum on days 0 and 14, and administrated with anti-TIM-1 mAb or control IgG every 3 days from day 0 to day 18. Serum levels of OVA-specific Abs were determined by isotype-specific ELISA on day 21 and expressed as absorbance values at 490/595 nm. Symbols represent levels in individual animals and bar represents the mean value. * $p < 0.05$ as compared to control IgG. Similar results were obtained in two independent experiments.

secondary lymphoid organs. Isotype switching and affinity maturation of the antibodies produced by B cells, and the development of plasma cells or memory B cells occur within the germinal center. These B cell maturational processes require cognate helps provided by CD4⁺ T cells [21]. It is well known that CD28/B7 (CD80 and CD86) and CD40/CD40L (CD154) interactions are required for optimal activation of CD4⁺ T cells and B cells to support Ab production [22,23]. In this study, we have found that TIM-1 is highly expressed on anti-IgM + anti-CD40-stimulated B cells and TIM-1L is detected on anti-CD3⁺ anti-CD28-stimulated CD4⁺ T cells. Therefore, these data suggest that the TIM1/TIM-1L interaction can participate in the B–T cell interaction. Consistent with this observation, Wong et al. indicated that TIM-1 expression was inducible on B cells through B-cell receptor signaling [24]. TIM-1 expression was also found on germinal center B cells in vivo. They also injected *Schistosoma mansoni* eggs (SE) into TIM-1-transgenic or TIM-1-deficient mice, whereas no difference in the serum levels of SE-specific IgM, IgG, and IgE was observed as compared with wild-type mice. Thus, TIM-1 on B cells may not be essential for physiological antibody responses. However, it is still possible that TIM-1 may contribute to the acceleration of Ab-mediated inflammatory diseases, such as atopy, asthma, and rheumatoid arthritis. We recently demonstrated that administration of anti-TIM-2 mAbs in a collagen-induced arthritis significantly exacerbated disease by enhancement of B cell activation and Ab production (manuscript submitted for publication). Mouse TIM-2 and TIM-1 are highly homologous, but it is unclear whether they have similar functions [1–3]. We have observed that mouse TIM-2 is also highly expressed on splenic B cells after stimulation with anti-IgM and anti-CD40 in vitro. Therefore, it is possible that TIM-1 may contribute to B cell activation and Ab production in humans, which lack TIM-2. Further studies are now under way to examine the effect of anti-TIM-1 mAb treatment on the development of collagen-induced arthritis and asthma in mouse models and the expression and function of TIM-1 on human B cells.

Acknowledgments

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