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ORIGINAL RESEARCH

TIM-4 Regulates Allergic Airway Inflammation and Mast Cell Activation by Binding to CD300b

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Background: Although T cell immunoglobulin and mucin domain-4 (TIM-4) is involved in immune regulation, the function of TIM-4 in allergic responses is not understood. We investigated the effects of anti-TIM-4 monoclonal antibody (mAb) in a murine model of allergic airway inflammation.

Methods: Anti-mouse TIM-4 mAb was administered to various allergic airway inflammatory model mice. A soluble form of TIM-4 (sTIM-4) was detected by newly developed a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) and immunoblotting using anti-mouse or human TIM-4 mAbs. Bone marrow-derived mast cells (BMMCs) were generated from C57BL/6 and CD300b-deficient mice to determine the contribution of sTIM-4 to mast cell activation. The concentrations of serum sTIM-4 in patients with asthma in 124 adult patients were quantified using ELISA.

Results: Accumulation of eosinophils and production of T helper type 2 (Th2) cytokines in the lung were significantly reduced in anti-TIM-4-treated mice. High amounts of sTIM-4 through proteolytic cleavage were detected in bronchoalveolar lavage fluid and sera from allergic airway inflammatory mice. sTIM-4 induced proinflammatory cytokine production in mast cells by interacting with CD300b. We also detected human sTIM-4 on TIM-4 transfected cells, which induced interleukin-6 (IL-6) production in a human mast cell line. Moreover, serum sTIM-4 levels were associated with asthma severity in patients with asthma.

Conclusion: TIM-4 contributes significantly to the effector phase of allergic airway inflammation. TIM-4 may serve as a therapeutic target and sTIM-4 may have the potential to be used as surrogate marker in asthma.

Keywords: TIM-4, animal model, asthma, Inflammation, mast cells, ELISA

Introduction

Mast cells are the central effectors in allergic disease and are present in increased numbers in the airways of patients with asthma.[1] Activation of mast cells by allergens, particularly through the high-affinity IgE receptor (FcεRI), leads to the release of inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), interleukin-6 (IL-6), and IL-13. The early release of these cytokines in airways induces vasodilatation, edema, and bronchoconstriction. In addition, the release of inflammatory factors by mast cells occurs in the absence of IgE and specific allergens. For example, thymic stromal lymphopoietin and IL-33 can directly activate mast cells.[2] It is possible that additional FcεRI-independent molecules act on mast cells to play an important role in the inflammatory response during the late phase of allergic airway inflammation.

To address this notion, we focused on T cell immunoglobulin and mucin domain-4 (TIM-4), which is member of the TIM family and regulates immune responses.[3–6] The genes encoding TIM proteins reside within the T cell and airway phenotype regulator (*Tapr*) locus on mouse chromosome 11B1.1, syntenic to human chromosome 5q33.2, which is linked

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to asthma, allergic diseases, and autoimmune diseases.[7] 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.[8] All proteins are type I transmembrane proteins with common structural motifs, including extracellular immunoglobulin variable region and mucin domains and intracellular domains. TIM-4 is expressed on peritoneal macrophages, marginal zone macrophages, dendritic cells, and peritoneal B-1 B cells.[9–16] Unlike the other TIM family members, the cytoplasmic tail of TIM-4 lacks putative signaling motifs and is therefore unlikely to mediate direct inward signaling.[3,4,7,8,17–19] We have previously demonstrated the critical contribution of TIM-4 to the development of murine arthritis.[20] Administration of anti-TIM-4 monoclonal antibody (mAb) significantly inhibited the development of arthritis with a concomitant decrease in IL-6, TNF-α, and IL-1β in the ankle joints. Notably, these inhibitory effects against the development and progression of arthritis by anti-TIM-4 mAb were observed when anti-TIM-4 mAb was administered after the onset or even after the establishment of arthritis. These effects of anti-TIM-4 mAb on arthritis seemed to be mediated by the inhibition of proinflammatory cytokine production in macrophages by blocking interactions between TIM-4 and CD300b, also called leukocyte mono-immunoglobulin-like receptor 5 (LMIR5); however, the biological significance of this interaction is unknown. CD300b is an activating receptor that interacts with DAP12, an immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor predominantly expressed by myeloid cells.[21] Thus, in this study, we investigated whether TIM-4 regulates mast cell activation and the allergic response by interacting with CD300b.

Material and Methods

Mice

BALB/c (total 224) and C57BL/6 (total 20) mice were purchased from Japan SLC (Hamamatsu, Japan). The generation of *Cd300lb*$^{-/-}$ mice (total 6) was described previously.[22] *Tim-3*$^{-/-}$ mice (total 10) were provided by A. N. J. McKenzie (MRC Laboratory of Molecular Biology, UK).[23] All mice were 6–8 weeks of age at the start of the experiments and were maintained under specific pathogen-free conditions. All animal experiments were approved by the Animal Experimental Ethics Committees of Juntendo University, registered under approval numbers 310006, 2,020,025, 2,021,010, 2,022,228, 2,023,111, 2024238, and 2025215, and were performed in accordance with institutional guidelines.

Induction of Allergic Airway Inflammation and mAb Treatment

Allergic airway inflammation was induced and assessed as described previously.[24,25] Briefly, BALB/c mice (n = 7 to 12 per group) were intraperitoneally (i.p.) immunized by injection of 100 μg Ovalbumin (OVA; Sigma–Aldrich, St. Louis, MO, USA) emulsified with 2 mg alum adjuvant (Thermo Fisher Scientific, Waltham, MA, USA) on days 0 and 14. On days 22, 24, 26, and 28, the mice were challenged with inhalation of aerosolized 1% OVA in Phosphate Buffered Saline (PBS). The aerosol was generated using a nebulizer (NE-U07; Omron Healthcare Kyoto, Japan). The mice were i.p. treated with 300 μg an anti-TIM-4 mAb (clone: RMT4-53) or control IgG (clone: RTG2b) on days 21, 24, and 27 to determine the influence of the former on the effector phase.

Mast Cell-Dependent Airway Inflammation and mAb Treatment

BALB/c mice (n = 5 to 8 per group) were i.p. immunized with 10 μg OVA on days 0, 7, 14, 21, 28, and 35, challenged with intranasal OVA (20 μg) or PBS on days 40, 43, and 46, and sacrificed 24 h after the last challenge.[26–28] Mice were i.p. administered 300 μg anti-TIM-4 mAb or control IgG 3 h before each challenge with OVA. Negative control mice were immunized with OVA, challenged with PBS, and intraperitoneally injected with PBS in a similar manner.

Characterization of Bronchoalveolar Lavage (BAL) Fluid

The trachea was cannulated with a polyethylene tube through which the lungs were gently lavaged four times with 0.5 mL PBS containing 5% fetal bovine serum (FBS; Biosera, Nuaille, France) (2 mL total of BAL fluid). The total number of cells was determined using Turk dye exclusion. Cytospins were stained with Diff-Quik (Sysmex International Reagents, Kobe, Japan) to determine differential cell counts. Cell-free supernatants of BAL fluids were assayed for IL-4,

IL-5, Interferon-γ (IFN-γ) (BD Biosciences, San Jose, CA, USA), and IL-13 (eBioscience, San Diego, CA, USA) using Enzyme-Linked Immunosorbent Assay (ELISA) kits.

Histological Analysis of Lung Sections

Lungs were removed from mice that were not subjected to the bronchial lavage procedure, inflated, and fixed by intratracheal instillation of 10% buffered formalin (pH 7.4) for 24 h. After successive dehydration, the lungs were embedded in paraffin and sectioned in the sagittal plain. Sections 5-mm-thick were stained with hematoxylin and eosin or periodic acid-Schiff (PAS) to measure mucus production. To quantify the infiltration of lung tissue with eosinophils, cell counting was performed in a masked manner using a 5-point scoring system as follows: 0, no eosinophils; 1, few eosinophils; 2, a ring of eosinophils one cell-layer deep; 3, a ring of eosinophils 2–4 cells deep; and 4, a ring of eosinophils >4 cells deep. Scoring of at least 15 different fields was performed for each lung section. Mean scores were obtained from six mice. Airway mucus levels were determined in a masked manner and were expressed as the percentage of PAS-positive cells of total epithelial cells.

ELISA of OVA-Specific Serum Antibodies

Anti-OVA IgG1, IgG2a, and IgE serum antibody levels were determined by ELISA. Immulon 2 HB 96-well microtiter plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 10 μg/mL OVA for IgG1 and IgG2a or with 10 μg/mL anti-mouse IgE mAb (6HD5) for IgE and incubated at 4°C overnight. Wells were then blocked with 1% BSA in PBS for 1 h at room temperature. Serially diluted serum samples were added to each well and then incubated for 2 h at room temperature. After washing with 0.05% Tween 20 in PBS, the wells were incubated with biotin-conjugated anti-mouse IgG (A85-1, BD Biosciences) or IgG2a (R19-15, BD Biosciences), or 1 μg/mL biotin-conjugated OVA for IgE for 1 h and washed, and then the antigen–antibody complexes were detected using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) and *o*-phenylenediamine (Wako Pure Chemical Industries, Osaka, Japan). After terminating the reaction with 2N H_2SO_4, absorbance at 490/595 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Flow Cytometric Analysis

Mouse cells were incubated with unlabeled anti-CD16/32 mAb (2.4G2) to prevent nonspecific binding of antibodies to the FcγR. Fluorochrome-conjugated mAbs specific for mouse CD11b (M1/70), F4/80 (BM8), FceRI (MAR-1), KIT/CD117 mAb (2BB), and human TIM-4 (9F4) were purchased from eBiosciences (San Diego, CA, USA) or BD Biosciences (San Jose, CA, USA). Anti-human and anti-mouse CD300b antibody (Ab) and a control goat IgG were purchased from R&D Systems (Minneapolis, MN, USA). In some experiments, 0.5 μg CD300-Fc or control mouse IgG2a was preincubated with 10 μg anti-TIM-4 mAb (RMT4-53) or control IgG (RTG2b) and then reacted with cells, followed by the addition of a PE-labeled anti-mouse IgG2a mAb in PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The cells (live-gated according to forward and side scatter profiles and propidium iodide exclusion) were analyzed using a FACSCalibur, and data were processed using CellQuest software (BD Biosciences, San Jose, CA, USA).

Patients

Consecutive patients (n = 124) with mild to severe asthma who were aged 20 years or older were recruited with informed consent from our outpatient clinic at Juntendo University Hospital (Tokyo, Japan). The baseline characteristics of this study population are presented in Supplementary Table 1. Asthma was diagnosed based on a clinical history of episodic symptoms with airflow limitation and by variations in pulmonary function monitored by Forced Expiratory Volume in one second (FEV_1) or Peak Expiratory Flow (PEF) in accordance with the Global Initiative for Asthma (GINA) guidelines. Disease severity was also determined according to the GINA guidelines, mild (steps 1–2), moderate (step 3), and severe (steps 4–5). The present study was reviewed and approved by the Juntendo University Research Ethics Committee (approval numbers H12-0178 and E25-0069) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient prior to study participation. Patients were excluded if they had a diagnosis of chronic obstructive pulmonary disease, as defined by the Global Initiative for Chronic Obstructive Lung Disease guidelines, or any current respiratory disorder other than asthma. Pulmonary function tests

and venous blood sampling were performed on the same day, and serum samples were collected for subsequent analyses. Inhaled Corticosteroids (ICS) were administered, with 67 patients receiving dose of ≥500 μg/day and 57 patients receiving <500 μg/day. In addition, 20 patients were treated with omalizumab, a recombinant humanized anti-IgE monoclonal antibody.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 10 Software (GraphPad Software, San Diego, CA, USA). Unless specified, data are presented as mean ± SEM with each symbol representing an individual donor or mouse. Sample normality was examined using the D'Agostino–Pearson test. Mann–Whitney *U*-test or unpaired Student's *t*-test were used for single comparisons between two groups. For more than two groups, data were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA, followed by Tukey's or Bonferroni's multiple comparisons test. For correlations between variables, the Pearson's correlation coefficient and Spearman's rank correlation coefficient, which is denoted as r for a sample statistic, were used where appropriate.

Additional methods are described in the Supplementary Methods.

Results

Anti-TIM-4 mAb Treatment at the Effector Phase Ameliorates Allergic Airway Inflammation in Mice

To determine whether TIM-4 contributes to the pathogenesis of allergic airway inflammation, we administered anti-TIM-4 mAb or control IgG during the effector phase (Figure 1A). On day 30, control IgG-treated mice developed a typical asthmatic phenotype characterized by airway hyperreactivity (AHR) to methacholine (Figure 1B) and accumulation of eosinophils and production of IL-13 in BAL fluid (Figure 1C and 1D), respectively. There was massive infiltration of eosinophils around the bronchioles and overproduction of mucus in the bronchioles (Figure 1E and 1F). Administration of anti-TIM-4 mAb significantly inhibited the development of AHR ($p < 0.0001$; Figure 1B), the accumulation of eosinophils ($p = 0.0438$) and production of IL-13 ($p = 0.0486$) in BAL fluid (Figure 1C and 1D) respectively, and eosinophilia and overproduction of mucus in the lung ($p = 0.007$; Figure 1E and 1F), as compared with control IgG. In contrast, the serum levels of OVA-specific IgG1, IgG2a, and IgE Abs did not differ between anti-TIM-4-treated mice and the controls (Figure 1G). Moreover, treatment with anti-TIM-4 mAb during the effector phase did not affect OVA-specific production of IL-4, IL-5, and IL-13 by lymph node cells compared with the controls (Figure 1H). Thus, it is likely that T or B cells did not mediate the suppression at the effector phase of allergic airway inflammation by anti-TIM-4 mAb.

To address this possibility, we examined the effect of anti-TIM-4 mAb in an adoptive transfer model of allergic airway inflammation (Supplementary Figure 1A).[24,25] The development of AHR ($p = 0.0026$; Supplementary Figure 1B), the accumulation of eosinophils ($p = 0.0003$; Supplementary Figure 1C), and the production of IL-13 in BAL fluid ($p = 0.0155$; Supplementary Figure 1D), but not OVA-specific Th2 cytokines (IL-4, IL-5, and IL-13) in cells (Supplementary Figure 1E), were significantly inhibited in the presence of anti-TIM-4 mAb as compared with control IgG. Taken together, these results indicate that TIM-4 contributes significantly to the effector phase of allergic airway inflammation without affecting T or B cell responses.

Anti-TIM-4 mAb Inhibits Mast Cell-Dependent Airway Inflammation

To further evaluate the contribution of TIM-4 to the pathogenesis of the effector phase of allergic airway inflammation, we administered anti-TIM-4 mAb to mice that served as a model of mast cell-dependent airway inflammation (Figure 2A).[26,28] On day 47, eosinophils accumulated in the BAL fluid of control IgG-treated mice (Figure 2B) massively infiltrated the tissue surrounding the bronchioles (Figure 2C and 2D), and the bronchioles overproduced mucus (Figure 2C and 2E). The levels of Th2 cytokines (IL-4, IL-5, and IL-13) but not that of the Th1 cytokine IFN-γ were increased in the BAL fluid of control IgG-treated mice (Figure 2F). In contrast, treatment with anti-TIM-4 mAb significantly inhibited eosinophil accumulation ($p = 0.0046$; Figure 2B), eosinophilia ($p < 0.0001$; Figure 2C and 2D), mucus overproduction ($p < 0.0001$; Figure 2C and 2E), and Th2 cytokine levels in BAL fluid, including IL-4 ($p = 0.0052$), IL-5 ($p = 0.0205$), and IL-13 ($p = 0.0054$) (Figure 2F). However, the levels of OVA-specific IgG1, IgG2a, and

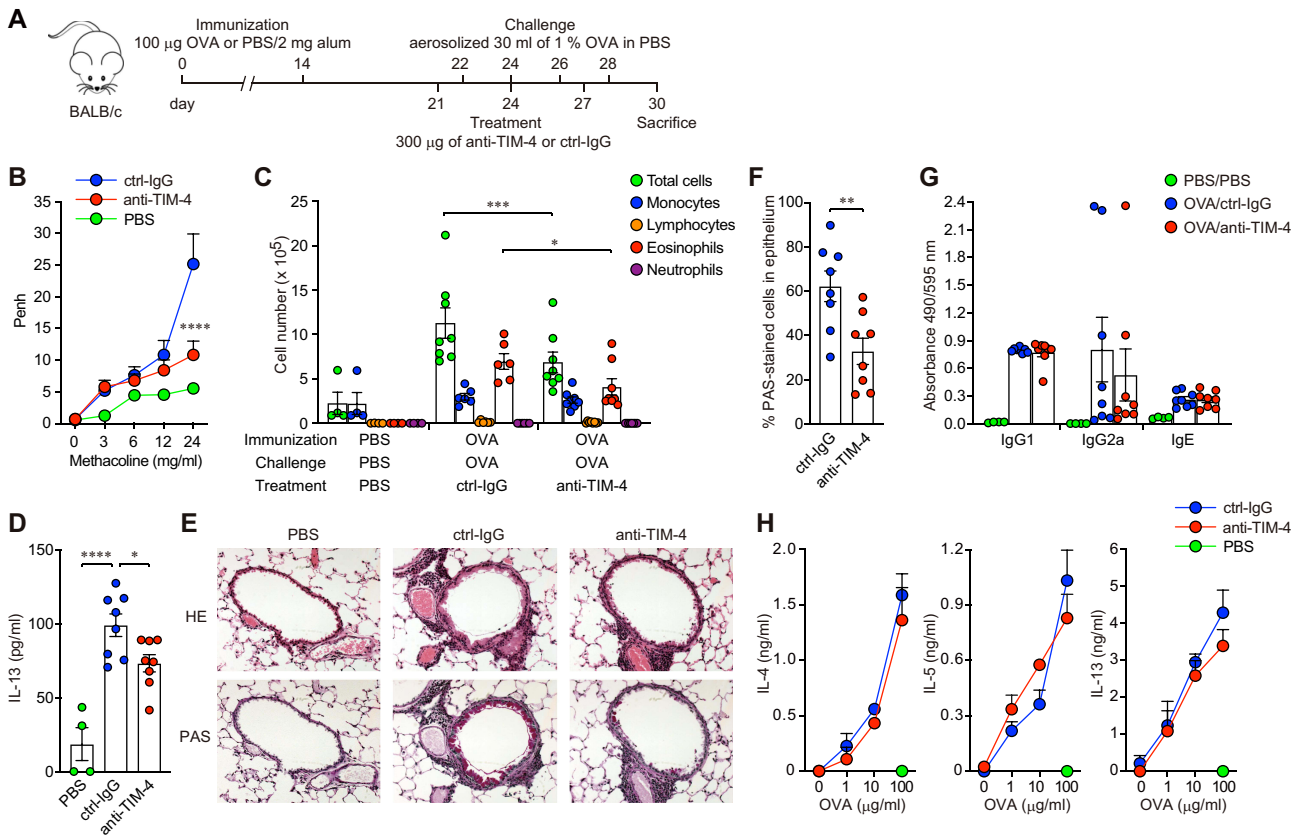

Figure 1 Effect of anti-TIM-4 monoclonal antibody (mAb) treatment during the effector phase of allergic airway inflammation. (**A**) Experimental protocol. (**B**) Airway hyperreactivity to methacholine in individual mice. (**C**) Bronchoalveolar lavage (BAL) fluid cell composition. (**D**) Interleukin (IL)-13 levels in BAL fluid. (**E**) Representative lung sections stained with hematoxylin and eosin (HE) and periodic acid–Schiff (PAS). (**F**) Quantification of PAS-positive epithelial cells. (**G**) Serum anti-ovalbumin (OVA) IgG1, IgG2a, and IgE levels. (**H**) Cytokine production by bronchial lymph node cells stimulated with OVA. Data are means ± SEM of three independent experiments. Statistics: two-way ANOVA with Bonferroni's multiple comparisons test (**B** and **H**) and with Tukey's multiple comparison test (**C** and **G**), one-way ANOVA with Tukey's multiple comparison test (**D**), and Mann–Whitney test (**F**). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

IgE in serum were comparable between mice treated with anti-TIM-4 mAb or control IgG-treated mice (Figure 2G). These results suggest that TIM-4 participates in the development of mast cell-dependent allergic diseases.

TIM-4-Transfected Cells and Macrophages Secrete a Soluble Form of TIM-4

We previously revealed that TIM-4 binds CD300b; however, the biological significance of this interaction is unknown. TIM-4 expression is restricted on antigen-presenting cells. TIM-1-designated kidney injury molecule-1 (KIM-1)[29] and a soluble form of KIM-1, which is generated by membrane-proximal cleavage of its precursor, is shed into the urine of patients with kidney disease.[30,31] We therefore hypothesized that binding of a soluble form of the extracellular domain of TIM-4 (sTIM-4) to CD300b activates mast cells.

To detect sTIM-4, we developed a sandwich ELISA using anti-TIM-4 mAbs (Supplementary Figure 2A). The ELISA specifically detected TIM-4-Fc because mouse IgG2a and Fc fusion proteins of the other TIM molecules were not recognized (Supplementary Figure 2B). Flow cytometric and ELISA analyses detected sTIM-4 in the culture supernatants of murine T lymphoma L5178Y cells and normal rat kidney (NRK) cells transfected with a TIM-4 expression vector (TIM-4/L5178Y and TIM-4/NRK, respectively) but not in supernatants harvested from untransfected cells (Figure 3A and 3B; $p < 0.0001$). Treatment of the transfectants with BB-94, a broad-spectrum inhibitor of matrix metalloproteinases (MMPs), significantly reduced the concentration of sTIM-4 in culture supernatants ($p < 0.0001$; Figure 3C). Immunoblot analysis detected a band corresponding to full-length TIM-4 (approximately 70 kDa) in lysates prepared from TIM-4/L5178Y cells, as well as a short form of TIM-4 (approximately 50 kDa) in culture supernatants (Figure 3D). Neither band was detected in the medium or lysates of untransfected cells (Figure 3E) nor in the lysates or culture supernatants of TIM-4/L5178Y cells immunoprecipitated with control IgG

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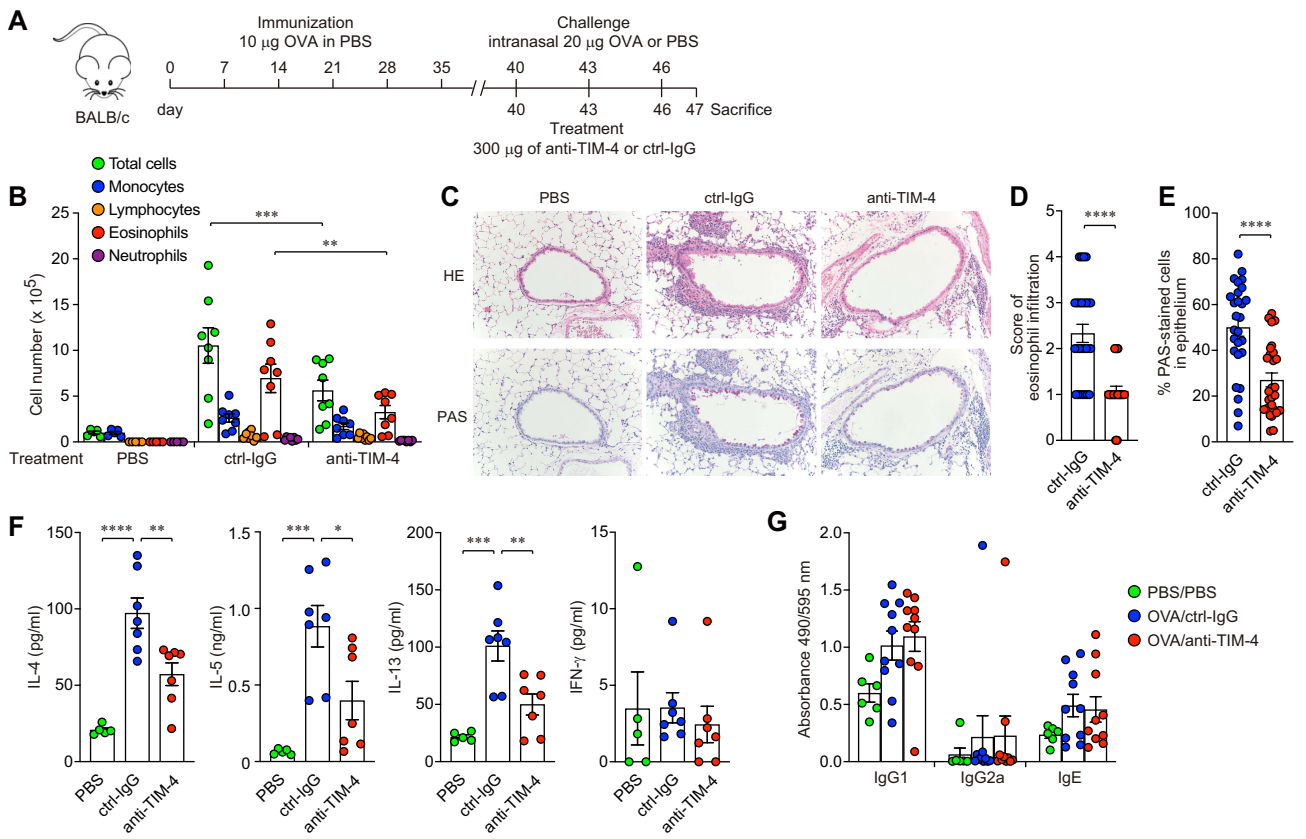

Figure 2 Anti-TIM-4 mAb inhibits mast cell-dependent airway inflammation. (**A**) Experimental protocol. (**B**) Bronchoalveolar lavage (BAL) fluid cell composition. (**C**) Representative lung sections stained with hematoxylin and eosin (HE) and periodic acid–Schiff (PAS). (**D**) Eosinophil infiltration scores. (**E**) Quantification of PAS-positive epithelial cells. (**F**) Cytokine levels in BAL fluid. (**G**) Serum anti-ovalbumin (OVA) IgG1, IgG2a, and IgE levels. Data are means ± SEM of three independent experiments. Statistics: two-way ANOVA with Tukey's multiple comparison test (**B**), unpaired Student's *t*-test (**D** and **E**), and one-way ANOVA with Tukey's multiple comparison test (**F** and **G**). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

(Figure 3D). The short form was undetectable when cells were treated with BB-94 (Figure 3E). These results indicate that sTIM-4 was generated by proteolysis of the extracellular domain of TIM-4 expressed on the surface of TIM-4-transfected cells.

Cell-surface expression of TIM-4 on mouse peritoneal $CD11b^+$ $F4/80^+$ macrophages was decreased (Figure 3F) and the levels of sTIM-4 in culture supernatants were increased after culture for 24 h ($p < 0.0001$; Figure 3G). Addition of BB-94 to the cultures significantly decreased the concentration of sTIM-4 from macrophages ($p < 0.0001$; Figure 3G). These results indicate that MMPs cleaved TIM-4 on the surface of transfected cells and primary macrophages to generate sTIM-4. Moreover, higher levels of sTIM-4 were detected in the BAL fluids ($p = 0.0037$) and sera ($p = 0.0352$) of mice with allergic airway inflammation compared with levels in the BAL fluids and sera of naïve mice (Figure 3H). These results suggest that the levels of sTIM-4 are enhanced locally and systemically during allergic inflammation.

sTIM-4 Enhances Inflammatory Cytokine Production by Mast Cells

To determine whether sTIM-4 influences the phenotype of mast cells, BMMCs were cultured with supernatants harvested from untransfected L5178Y cells or TIM-4-transfected L5178Y cells. Although the production of IL-6 and IL-13 was not affected when BMMCs were cultured with only the supernatants of TIM-4/L5178Y or L5178Y cultures, their levels were increased significantly when BMMCs were stimulated with FcεRI and culture supernatants of TIM-4/L5178Y cells but with not those of L5178Y cells ($p < 0.0001$; Figure 4A). Furthermore, IL-6 and IL-13 production was significantly inhibited when an anti-TIM-4 mAb was added to the cultures ($p < 0.0001$; Figure 4A).

To facilitate the characterization of sTIM-4, we generated TIM-4-Fc fusion protein as a surrogate for sTIM-4 and programmed cell death 1 ligand 2-Fc (PD-L2-Fc) fusion protein to exclude nonspecific binding of Fc portion. BMMCs did not express PD-1, which is a receptor of PD-L2.[32–34] We determined the levels of cytokines produced by BMMCs cultured with

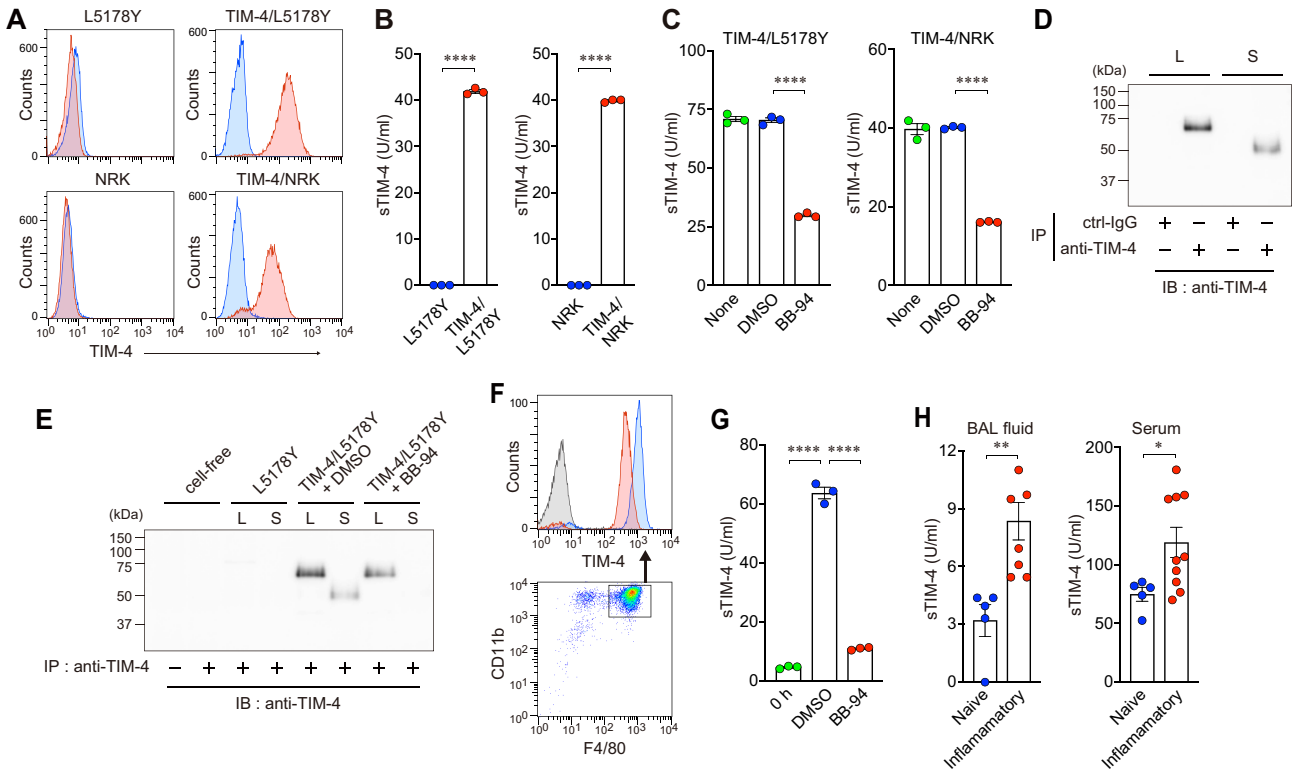

Figure 3 Soluble TIM-4 (sTIM-4) is released from TIM-4-transfected cells and macrophages. (**A**) TIM-4 surface expression on TIM-4/L5178Y, TIM-4/NRK, or untransfected cells analyzed by flow cytometry. Red histograms indicate staining with anti-TIM-4 mAb, and blue histograms indicate background staining using control IgG (ctrl-IgG). (**B**) and (**C**), sTIM-4 levels in culture supernatants measured by ELISA. (**D**) and (**E**), Immunoprecipitation (IP) and immunoblotting (IB) analyses of cell lysates **L** and supernatants S from L5178Y or TIM-4/L5178Y cells. (**F**) TIM-4 expression on peritoneal macrophages gated on CD11b+ F4/80+ cells. The gray histogram indicates background staining and the blue and red histograms indicate staining with anti-TIM-4 mAb at 0 h and 24 h, respectively. (**G**) sTIM-4 levels in macrophage culture supernatants. (**H**) sTIM-4 levels in bronchoalveolar lavage (BAL) fluid and sera from naïve or ovalbumin-challenged mice. Data are means ± SEM from representative experiments. Statistics: unpaired Student's *t* test (**B** and **H**) and one-way ANOVA with Tukey's multiple comparison test (**C** and **G**). *P < 0.05, **P < 0.01, ****P < 0.0001.

TIM-4-Fc in the presence or absence of IgE, which binds to and activates FcεRI. TIM-4-Fc, but not mouse IgG2a or PD-L2-Fc, induced the production of IL-6 ($p < 0.0001$) and IL-13 ($p < 0.0001$) in the absence of IgE (Figure 4B). Moreover, TIM-4-Fc significantly enhanced the production of IL-6 ($p < 0.0001$) and IL-13 ($p < 0.0227$) by FcεRI-stimulated BMMCs (Figure 4C). Similar results were obtained when BMMCs were stimulated with the combination of TIM-4-Fc and LPS ($p < 0.0001$; Supplementary Figure 3). IL-4, IL-5, and IL-1β were not detectable.

To assess the contribution of sTIM-4 to mast cell degranulation, we measured the release of β-hexosaminidase, which is stored in preformed mast cell granules.[35] Treating cells with TIM-4-Fc in the absence or presence of IgE did not increase the release of β-hexosaminidase (Figure 4D). FcεRI-mediated passive cutaneous anaphylaxis (PCA) in mice depends on the degranulation of mast cells.[36] Although the amounts of extravasated dye were increased in anti-2,4-dinitrophenyl IgE-injected ears compared with that in PBS-injected ears, pretreatment with anti-TIM-4 mAb did not inhibit PCA (Figure 4E). Collectively, these results indicated that sTIM-4 regulates cytokine production without inducing the degranulation of mast cells.

sTIM-4–CD300b Interactions Upregulate Cytokine Production by BMMCs

We next determined whether sTIM-4 bound BMMCs. $FcεRI^+$ KIT^+ BMMCs did not bind IgG2a or PD-L2-Fc (Figure 5A upper). The BMMCs bound TIM-4-Fc, which was blocked by preincubation with anti-TIM-4 mAb (Figure 5A upper). BMMCs expressed CD300b and TIM-3 but not TIM-1, TIM-2, or TIM-4 on BMMCs (Figure 5A lower).

We found that TIM-4/NRK cells bound an CD300b-Fc fusion protein. Furthermore, this interaction was substantially blocked by anti-TIM-4 mAb (Figure 5B). We therefore examined the effect of an siRNA targeting *Cd300lb* mRNA on the cytokine production of BMMCs treated with TIM-4-Fc. The levels of *Cd300lb* mRNA as well as those of cell-surface-localized CD300b were reduced in BMMCs transfected with *Cd300lb* siRNA ($p < 0.0008$; Figure 5C and D). Cytokine production

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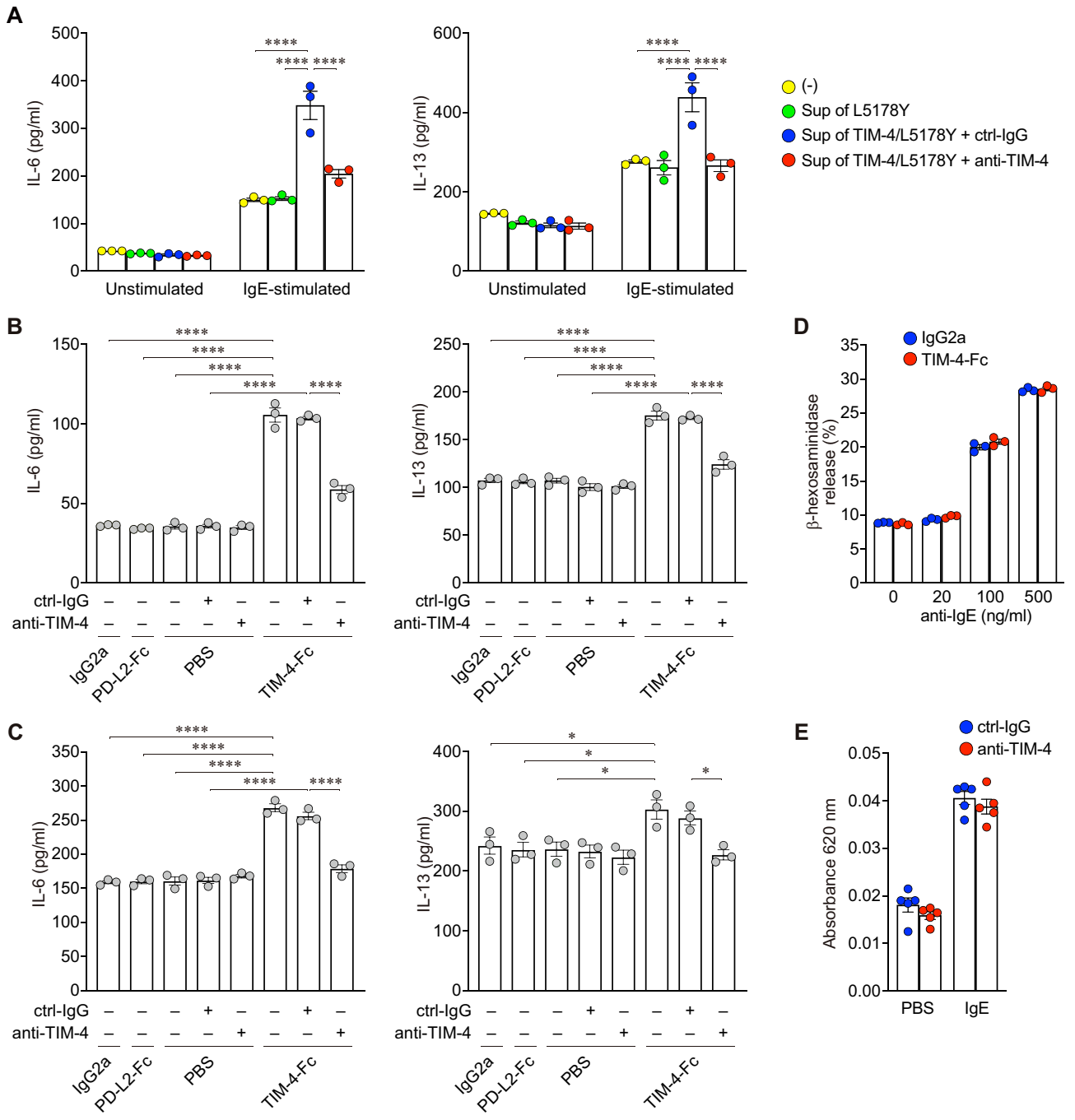

Figure 4 Soluble TIM-4 (sTIM-4) enhances cytokine production but not degranulation in mast cells. (**A**) Interleukin (IL)-6 and IL-13 levels in culture supernatants of bone marrow–derived mast cells (BMMCs) cocultured with supernatants (Sup) from L5178Y or TIM-4/L5178Y cells in the presence of anti-TIM-4 mAb or control IgG (ctrl-IgG). (**B**) and (**C**) IL-6 and IL-13 production by BMMCs treated with PBS, mouse IgG2a, PD-L2-Fc, or TIM-4-Fc, with or without anti-TIM-4 mAb. (**D**) β-hexosaminidase release from IgE-sensitized BMMCs after stimulation with IgG2a or TIM-4-Fc. (**E**) Passive cutaneous anaphylaxis (PCA) responses in mice. Data are means ± SEM from representative experiments. Statistics: two-way ANOVA with Tukey's multiple comparison test (**A**) and with Bonferroni's multiple comparisons test (**D** and **E**), and one-way ANOVA with Tukey's multiple comparison test (**B** and **C**). *P < 0.05, ****P < 0.0001.

induced by TIM-4-Fc was reduced in BMMCs transfected with *Cd300lb* siRNA compared with that in BMMCs transfected with a control siRNA ($p < 0.0001$; Figure 5E). Furthermore, *Cd300lb*$^{-/-}$ BMMCs stimulated with TIM-4-Fc produced lower levels of cytokines compared with wild-type (WT) BMMCs ($p < 0.0001$; Figure 5F). The differences were not due to aberrant development because the percentages of FcεRI^{+} KIT^{+} cells were closely comparable between WT and *Cd300lb*$^{-/-}$ BMMCs (Figure 5G). These data indicate that sTIM-4–CD300b interactions upregulate cytokine production by BMMCs.

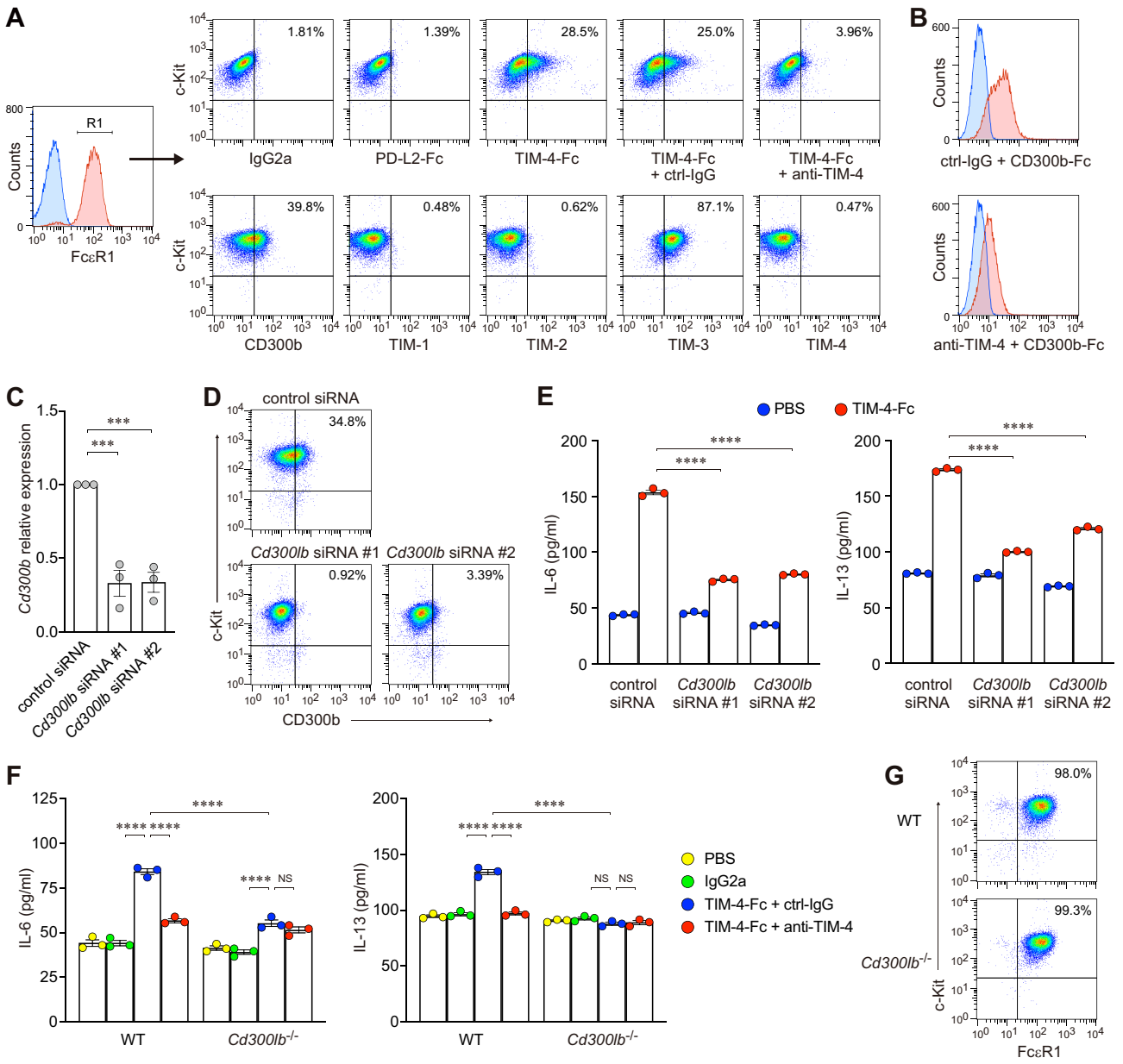

Figure 5 Interaction of TIM-4 with CD300b induces cytokine production by mast cells. (**A**) Binding of TIM-4-Fc and expression of CD300b and TIM family proteins on bone marrow–derived mast cells (BMMCs) analyzed by flow cytometry. The percentage of specifically stained cells is shown in the top right quadrant of each panel. (**B**) Inhibition of CD300b-Fc binding to TIM-4–transfected TIM-4/NRK cells by anti-TIM-4 monoclonal antibody. (**C**) *Cd300lb* mRNA expression in siRNA-transfected BMMCs assessed by quantitative PCR. (**D**) CD300b protein expression in BMMCs treated with control or *Cd300lb* siRNA. (**E**) Interleukin (IL)-6 and IL-13 production in BMMCs treated with *Cd300lb* siRNA and stimulated with TIM-4-Fc. (**F**) IL-6 and IL-13 production in wild-type (WT) or *Cd300lb*$^{-/-}$ BMMCs stimulated with TIM-4-Fc. (**G**) Normal development of *Cd300lb*$^{-/-}$ BMMCs confirmed by c-KIT and FcεRI expression. Data are means ± SEM from representative experiments. Statistics: one-way ANOVA with Tukey's multiple comparison test (**C**) and two-way ANOVA with Tukey's multiple comparison test (**E** and **F**). ***P < 0.001, ****P < 0.0001. NS, not significant.

TIM-3 Downregulates sTIM-4-Induced Cytokine Production by BMMCs

BMMCs express high levels of TIM-3 but not TIM-1 or TIM-2 (Figure 5A lower). Therefore, we determined whether TIM-4 bound TIM-3. Mouse TIM-3 is polymorphic and is represented by the C57BL/6 type and the BALB/c type.[7] Notably, we found that NRK cells transfected with vectors that expressed either TIM-3 C57BL/6 or TIM-3 BALB/c bound TIM-4-Fc and that the binding of TIM-4-Fc to these transfectants was inhibited by anti-TIM-4 mAb (Figure 6A). These results suggest that TIM-3 is a TIM-4 receptor.

To evaluate the contribution of TIM-4–TIM-3 interactions to cytokine production by BMMCs treated with TIM-4-Fc, we determined TIM-4-Fc binding to BMMCs transfected with *Tim3* siRNA as well as their levels of cytokine production.

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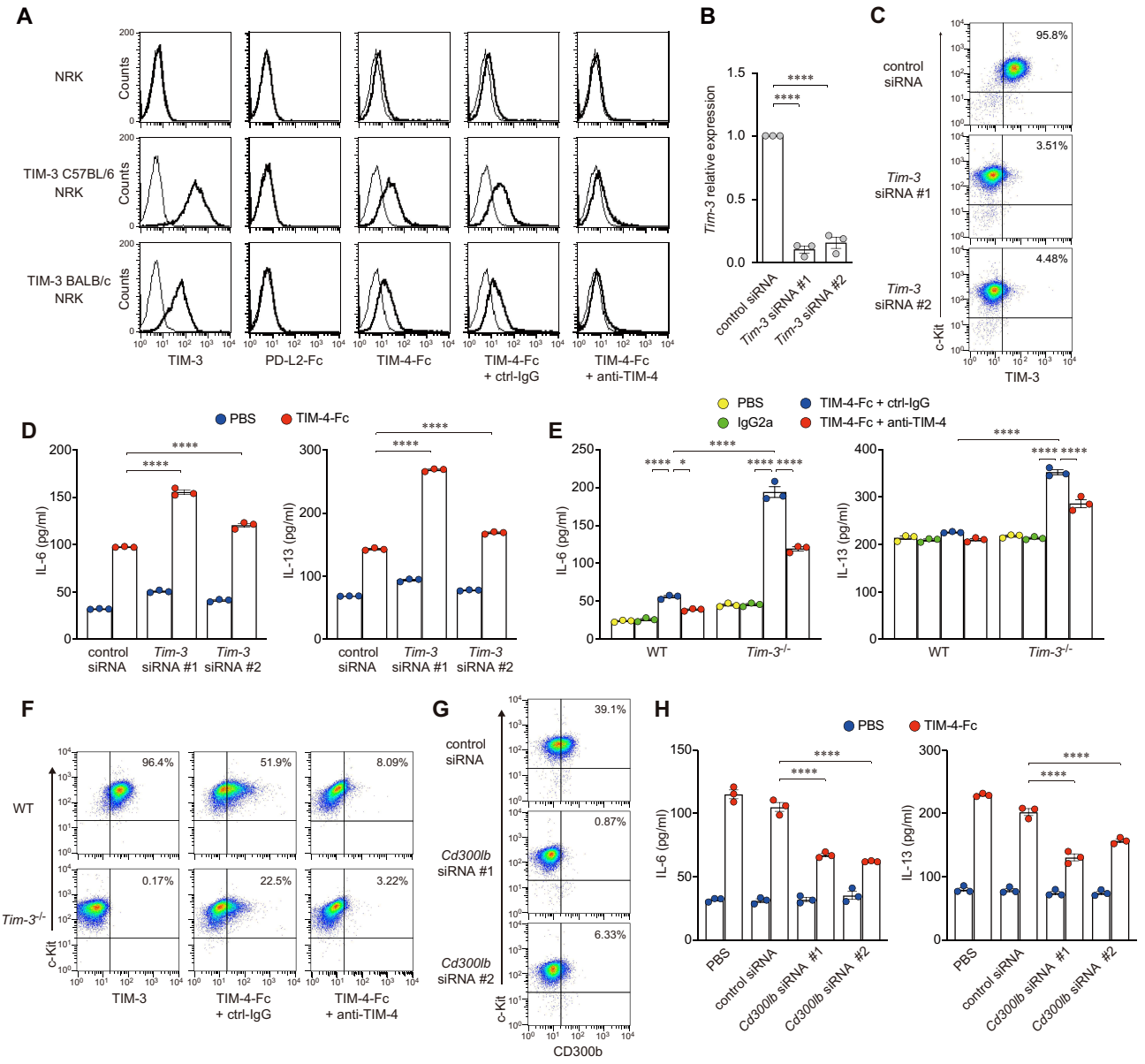

Figure 6 TIM-3 inhibits soluble TIM-4 (sTIM-4)-induced cytokine production by mast cells. (**A**) TIM-3 expression and TIM-4-Fc reactivity in NRK, TIM-3 C57BL/6/NRK, or TIM-3 BALB/c/NRK cells analyzed by flow cytometry. Thick lines indicate staining with anti-TIM-3 mAb, PD-L2-Fc, or TIM-4-Fc and thin lines indicate background staining with rat IgG2a or mouse IgG2a. (**B**) *Tim3* mRNA expression in siRNA-transfected bone marrow–derived mast cells (BMMCs) assessed by quantitative PCR. (**C**) TIM-3 protein expression in BMMCs treated with control or *Tim3* siRNA. (**D**) Interleukin (IL)-6 and IL-13 production in BMMCs treated with *Tim3* siRNA and stimulated with TIM-4-Fc. (**E**) IL-6 and IL-13 production in wild-type (WT) or *Tim3*$^{-/-}$ BMMCs treated with TIM-4-Fc. (**F**) TIM-3 expression and TIM-4-Fc binding in WT or *Tim3*$^{-/-}$ BMMCs. (**G**) CD300b expression in *Tim3*$^{-/-}$ BMMCs treated with control or *Cd300lb* siRNA. (**H**) IL-6 and IL-13 production in *Tim3*$^{-/-}$ BMMCs treated with *Cd300lb* siRNA and stimulated with TIM-4-Fc. Data are means ± SEM from representative experiments. Statistics: one-way ANOVA with Tukey's multiple comparison test (**B**) and two-way ANOVA with Tukey's multiple comparison test (**D, E**, and **H**). *P < 0.05, ****P < 0.0001.

We also performed the same analyses using *Tim3*$^{-/-}$ BMMCs. The levels of *Tim3* mRNA and the cell-surface expression of TIM-3 by BMMCs transfected with *Tim3* siRNA were reduced ($p < 0.0001$; Figure 6B and 6C), and TIM-4-Fc-induced cytokine production was significantly increased after transfection with *Tim3* siRNA compared with the levels in the controls ($p < 0.0001$; Figure 6D). Furthermore, there was a significant increase in TIM-4-Fc-induced cytokine production by *Tim3*$^{-/-}$ BMMCs ($p < 0.0001$; Figure 6E). Anti-TIM-4 mAb markedly inhibited TIM-4-Fc-induced cytokine production as well as the binding of TIM-4-Fc to *Tim3*$^{-/-}$ BMMCs ($p < 0.0001$; Figure 6E and 6F).

We hypothesized that CD300b signaling contributes to the TIM-4-Fc-induced cytokine production by *Tim3*$^{-/-}$ BMMCs. To test this possibility, we transfected *Tim3*$^{-/-}$ BMMCs with *Cd300lb* siRNA (Figure 6G). The enhanced

cytokine production by *Tim3*$^{-/-}$ BMMCs stimulated with TIM-4-Fc was significantly reduced by transfection with *Cd300lb* siRNA ($p < 0.0001$; Figure 6H). These results indicate that the interaction between TIM-4 and TIM-3 inhibited CD300b-mediated cytokine production by BMMCs; however, TIM-4–CD300b appeared to be the dominant signaling pathway of cytokine production by BMMCs.

Detection of Human sTIM-4, Which Induces IL-6 Production in a Human Mast Cell Line

We next examined whether sTIM-4 is also present in humans and induces cytokine production in mast cells. To detect human sTIM-4, we established a sandwich ELISA using anti-TIM-4 mAbs (Supplementary Figure 2C). The ELISA detected recombinant human TIM-4 and human TIM-4-Fc specifically because recombinant human TIM-1 and Fc fusion proteins of the other human TIM molecules were not recognized (Supplementary Figure 2D). An anti-human TIM-4 mAb bound to human leukemic TIM-4/K562 cells but not to mock/K562 cells (Figure 7A). Western blot analysis detected a band corresponding to full-length TIM-4 (approximately 75 kDa) in lysates prepared from TIM-4/K562 as well as a short form of human TIM-4 (approximately 60 kDa) in culture supernatants (Figure 7B). The production of the short form was suppressed when cells were treated with BB-94 (Figure 7C). Addition of BB-94 to the culture medium significantly decreased the concentration of sTIM-4 ($p = 0.0001$; Figure 7D).

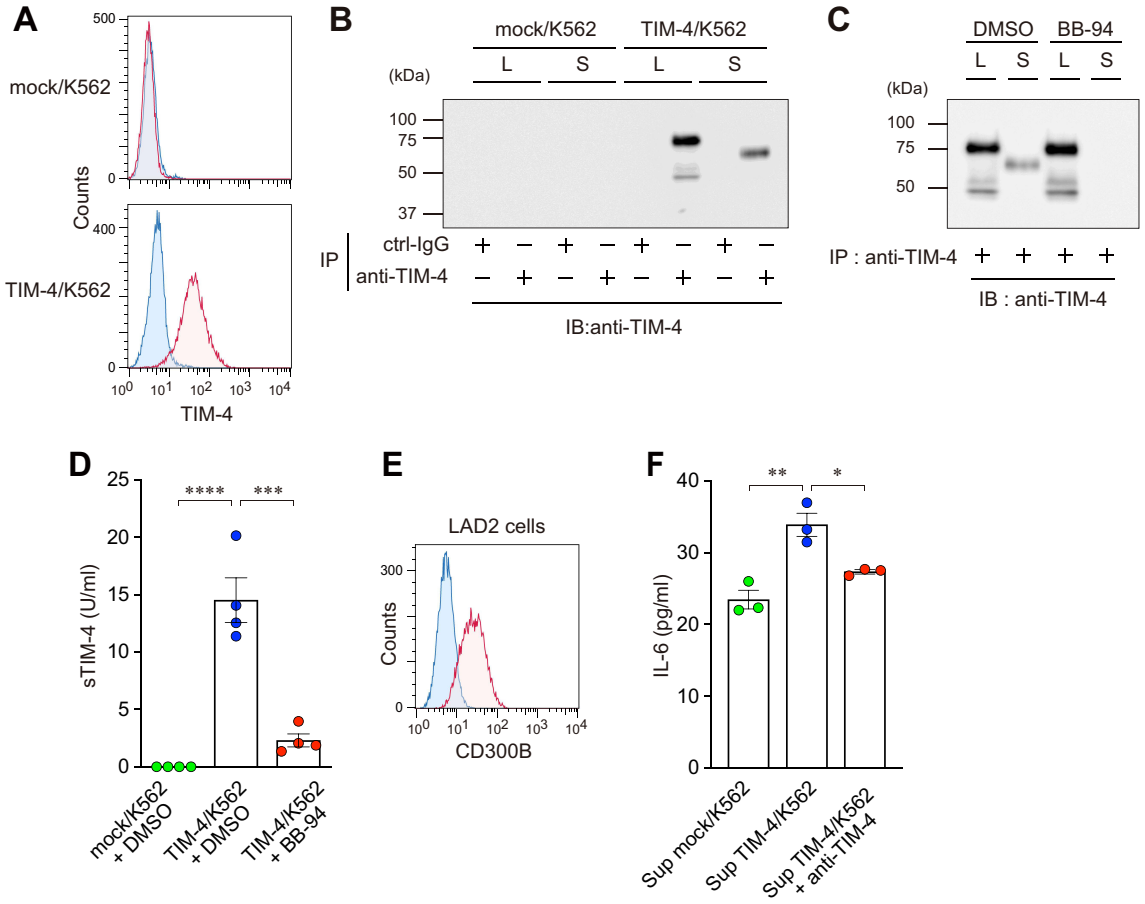

Figure 7 Human soluble TIM-4 (sTIM-4) induces Interleukin-6 (IL-6) production in a human mast cell line. (**A**) TIM-4 expression on mock/K562 and TIM-4/K562 cells analyzed by flow cytometry. Red histograms indicate staining with anti-TIM-4 mAb and blue histograms indicate background staining using control IgG. (**B**) and (**C**) Immunoprecipitation (IP) and immunoblotting (IB) analyses of cell lysates L and supernatants (**S**) from mock/K562 or TIM-4/K562 cells. (**D**) sTIM-4 levels in culture supernatants of mock/K562 and TIM-4/K562 cells measured by ELISA. (**E**) CD300b expression on human LAD2 mast cells. (**F**) IL-6 production in LAD2 cells treated with supernatants (Sup) from mock/K562 or TIM-4/K562 cells in the presence of anti-TIM-4 mAb. Data are means ± SEM from representative experiments. Data are presented as the means ± SEM. Statistics: one-way ANOVA with Tukey's multiple comparison test (**D** and **F**). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

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We further investigated whether sTIM-4 stimulates human mast cells. Human LAD2 mast cells expressed CD300b (Figure 7E), which were cultured with supernatants harvested from mock/K562 cells or TIM-4/K562 cells. Although low levels of IL-6 were detected when LAD2 cells were cultured with the supernatants of mock/K562 cell cultures, their levels were significantly increased when LAD2 cells were cultured with the supernatants of TIM-4/K562 cell cultures ($p = 0.0021$; Figure 7F). Furthermore, IL-6 production was significantly inhibited when anti-TIM-4 mAb was added to the cultures ($p = 0.0195$; Figure 7F). These results indicate that human sTIM-4 was also generated by proteolysis of the extracellular domain of TIM-4 expressed on the surface of TIM-4-transfected cells, and that human sTIM-4 induces IL-6 production by mast cells.

Serum sTIM-4 Level Is Associated with Disease Severity and Airflow Limitation in Patients with Asthma

sTIM-4 induced proinflammatory cytokine production in a human mast cell line. Moreover, the detection of high levels of sTIM-4 in the bronchoalveolar lavage fluid and sera of allergic airway inflammation in mice suggested that sTIM-4 may serve as surrogate marker for allergic diseases. Thus, we measured the concentrations of serum sTIM-4 in patients with asthma in 124 adult patients with mild to severe asthma. Serum sTIM-4 levels were positive, although weak, correlated with age ($p = 0.0015$), age at asthma onset ($p = 0.019$), and the ratio of maximal expiratory flow at 50% to that at 25% of vital capacity (V50/V25; $p = 0.0016$) in asthmatic patients, and negatively correlated with airflow limitation, including forced vital capacity (FVC; $p = 0.0013$), FEV_1 ($p < 0.001$), PEF ($p = 0.038$), V50 ($p = 0.006$), V25 ($p < 0.001$), and maximal mid-expiratory flow (MMF; $p = 0.0017$) (Figure 8A). Moreover, serum sTIM-4 levels were significantly higher in asthmatic patients with severe asthma (GINA step 4–5) compared with moderate asthma (GINA step 3) ($p = 0.048$; Figure 8B). However, there was no significant difference in serum sTIM-4 levels between patients with severe asthma (GINA step 4–5) and those with mild asthma (GINA step 1–2) (Figure 8B). Serum sTIM-4 levels were also significantly higher in patients receiving high doses of Inhaled Corticosteroids (ICS) compared with those receiving low doses of ICS ($p = 0.027$; Figure 8C). Twenty asthmatic patients (16%) had been treated with omalizumab, a recombinant humanized anti-IgE monoclonal antibody for severe asthma, prior to enrollment in this study. Serum sTIM-4 levels in omalizumab-treated patients were significantly higher than in those not treated with omalizumab ($p = 0.015$; Figure 8C). Collectively, these findings suggest that serum sTIM-4 may be associated with asthma severity and airflow limitation and may have the potential utility as a surrogate marker in asthma.

Discussion

In the present study, we used a mouse model to provide compelling evidence that TIM-4 regulates allergic airway inflammation. Specifically, we show here that a soluble form of the extracellular domain of TIM-4 was generated by proteolytic cleavage of TIM-4 expressed on the surface of TIM-4-transfected cells. Furthermore, we show that primary cultures of mouse peritoneal macrophages expressed sTIM-4. sTIM-4 or TIM-4-Fc induced proinflammatory cytokine production by naïve and IgE-stimulated BMMCs independent of degranulation and a human mast cell line. Because TIM-4 binds CD300b, we investigated whether the ability of sTIM-4 to induce proinflammatory cytokine production required CD300b. We showed that this was the case by inhibiting the expression of *Cd300lb* mRNA by BMMCs or using *Cd300lb*$^{-/-}$ BMMCs. Moreover, an anti-TIM-4 mAb prevented the binding of an CD300b-Fc fusion protein to TIM-4-transfected cells. Mice treated with control IgG developed a typical asthmatic phenotype characterized by AHR to methacholine, had accumulation of eosinophils, and exhibited production of IL-13 in BAL fluid. Moreover, there was massive infiltration of eosinophils around the bronchioles and overproduction of mucus in the bronchioles. All these effects were diminished dramatically in mice administered anti-TIM-4 mAb, leading us to conclude that TIM-4 regulates allergic airway inflammation, which was further supported by the detection of high levels of sTIM-4 in the BAL fluid and sera of these mice (Supplementary Figure 4).

Whether sTIM-4 is encoded by a unique transcript is unknown; however, we show here that sTIM-4 was secreted by TIM-4-transfected cell lines. The most likely mechanism that generates sTIM-4 is a posttranslational modification (eg, proteolytic cleavage of sTIM-4), because a wide-spectrum MMP inhibitor dramatically reduced the levels of sTIM-4 in the supernatants of murine primary resident peritoneal macrophage cultures. Moreover, it has been reported that human

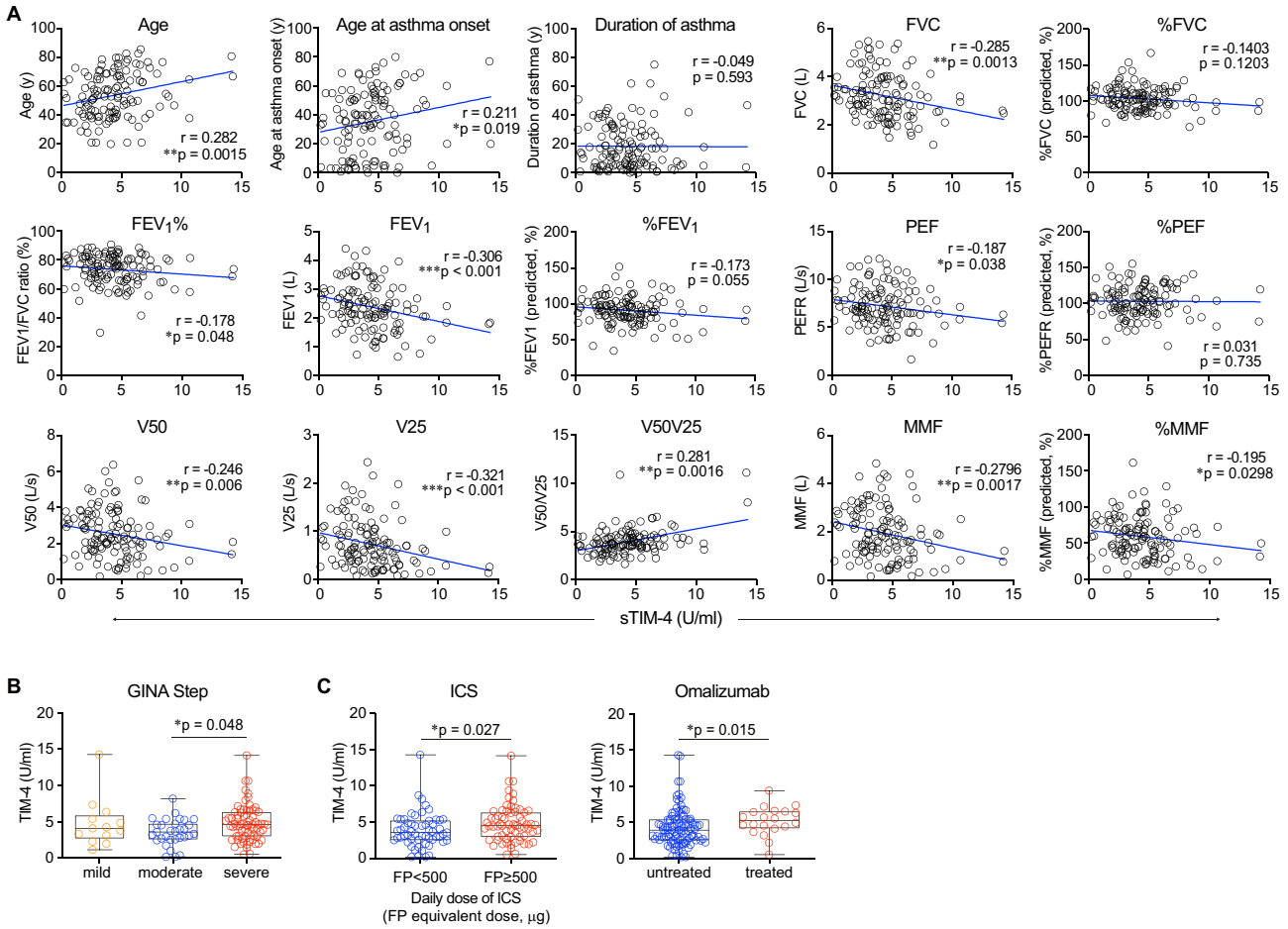

Figure 8 Relationship between serum soluble TIM-4 (sTIM-4) levels and pulmonary function in patients with asthma. (**A**) Correlations between serum sTIM-4 concentrations and clinical parameters, including age, age at onset, duration of asthma, forced vital capacity (FVC), %FVC, forced expiratory volume in one second (FEV_1), %FEV1, FEV1/FVC ratio (FEV1%), peak expiratory flow (PEF), %PEF, maximal expiratory flow at 50% of vital capacity (V50), at 25% (V25), V50/V25 ratio, maximal mid-expiratory flow (MMF), and %MMF, assessed by Spearman correlation. (**B**) Serum sTIM-4 levels according to asthma severity. (**C**) Serum sTIM-4 concentrations in patients receiving different medications. Patients with asthma were treated with corticosteroids (ICS) or omalizumab. Statistics: Spearman correlation (**A**), one-way ANOVA with Tukey's multiple comparison test (**B**), and Mann–Whitney *U*-test (**C**). *P < 0.05, **P < 0.01, and ***P < 0.001.

TIM-4 could be sheared into sTIM-4 by a disintegrin and metalloprotease 10 (ADAM10) and ADAM17.[37] However, the digestion site is not yet clear, and it was not possible from our experiment to show whether sTIM-4 directly binds to CD300b *in vivo* and enhances allergic inflammation. To clarify whether sTIM-4 has an in vivo pathological role, we are currently identifying the cleavage site of TIM-4 and creating non-secreting sTIM-4 mice with mutated amino acids at the cleavage site. In addition, it is necessary to demonstrate that TIM-4-mediated mast cell activation in vivo in allergic inflammation. Thus, we are further investigating using mast cell-deficient mice reconstituted with *Cd300lb*$^{-/-}$ or wild-type BMMCs.

We previously indicated that anti-TIM-3 mAb treatment exacerbates lung inflammation and fibrosis.[38] In this study, we demonstrated that TIM-4 binds TIM-3 and that this interaction suppresses CD300b-mediated cytokine production by BMMCs. TIM-3 has emerged as the next candidate immune checkpoint molecule for antitumor immunotherapy,[39–42] and is expressed on T cells in cancer patients with advanced melanoma and non-small-cell lung carcinoma.[41] To date, four TIM-3 ligands have been identified: galectin-9, phosphatidylserine (PtdSer), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and high mobility group protein B1 (HMGB1).[16,43–46] It is not clear whether TIM-4 contributes to TIM-3-mediated T cell exhaustion. Further investigations are needed to understand the function of TIM-4–TIM-3 interactions in immune-suppressive responses and tumor-promoting roles.

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It has been shown that sTIM-4 could be detected in the plasma of ischemic stroke patients and ankylosing spondylitis patients.[47,48] In addition, the plasma levels of sTIM-4 were significantly correlated with disease severity. We found that serum sTIM-4 level was associated with disease severity and airflow limitation in patients with asthma. These results suggest that sTIM-4 may have the potential to be used as surrogate marker in inflammation and asthma. These findings also contribute towards a better understanding of the pathogenesis and treatment of inflammation in humans. For example, it will be important to determine whether sTIM-4 is present in the BAL of human patients as well as whether CD300b is expressed by inflammatory cells in the airways. Approaches to therapy using sTIM-4 decoys will likely require the identification of the sites of interaction among CD300b. Therapeutic mAbs must be designed to take into account the essential roles of TIM-4 as a PtdSer receptor that maintains the homeostatic function of peritoneal macrophages[49] and as a regulator of adaptive immunity that mediates the clearance of antigen-specific T cells.[50] Moreover, the interaction of TIM-4 with its putative receptor(s) promotes Th2 responses.[51,52] If such antibodies could be designed to react specifically with the CD300b binding site of sTIM-4 generated by the action of the sheddases MMPs, adverse effects on homeostasis might be minimized. Moreover, MMP inhibitors that prevent the generation of sTIM-4 should be considered as potential anti-allergy drugs. Alternatively, it may be possible to use anti-TIM-4 mAbs for this purpose, because we demonstrated that an anti-TIM-4 mAb administered from just before the onset of collagen-induced arthritis (CIA) of mice significantly suppresses the development of CIA by reducing proinflammatory cytokines in the ankle joints without affecting T or B cell responses.[20]

It remains to be determined whether our present findings can be generalized to allergic airway inflammation of humans. The relevance of mouse model systems to human allergic diseases has been the subject of excellent reviews. However, there are major differences between the allergies in humans and mice. For example, long-term exposure to mainly airborne allergens induces asthma in humans, which is characterized by chronic inflammation, unlike mice. Because mice do not develop spontaneous asthma, unlike humans, in the laboratory, disease in mice is induced acutely, typically using OVA, as in the present study. Furthermore, OVA induces severe airway inflammation in mice but only rarely in humans. Nevertheless, valuable insights into the mechanism of the pathogenesis of asthma have been gained using such acute mouse models because they mimic many of the symptoms of clinical asthma, such as increased production of IgE and inflammation of the airways. In contrast, the chronic inflammation of the airway wall and the airway remodeling that occur in humans are not reflected by acute mouse models. More importantly, acute mouse models have contributed to our understanding of human respiratory allergies by indicating that Th2 cells mediate asthma and that eosinophils contribute to the development of AHR. Our present and future research will focus on the role of the TIM-4–CD300b axis in human allergies.

Conclusions

TIM-4 promotes allergic inflammation, and its soluble form (sTIM-4) correlates with disease severity in airway allergy, suggesting TIM-4 as a therapeutic target and sTIM-4 as a potential surrogate marker.

Abbreviations

TIM-4, T cell immunoglobulin and mucin domain-4; sTIM-4, soluble form of TIM-4; mAb, monoclonal antibody; ELISA, Enzyme-Linked Immunosorbent Assay; BMMCs, Bone marrow-derived mast cells; Th2, T helper type 2; FcεRI, high-affinity IgE receptor; TNF-α, Tumor Necrosis Factor-α; IL, interleukin; OVA, Ovalbumin; AHR, airway hyper-reactivity; BAL, bronchoalveolar lavage; MMPs, matrix metalloproteinases; HE, hematoxylin and eosin; PAS, periodic acid-Schiff; ICS, inhaled corticosteroid.

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Disclosure

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