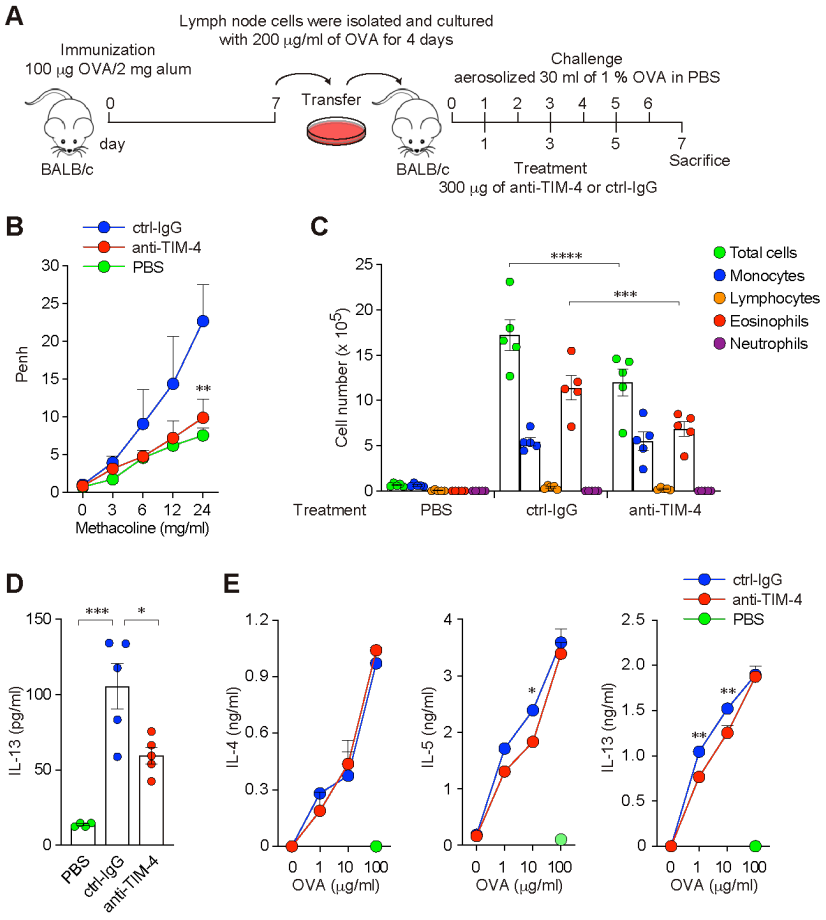
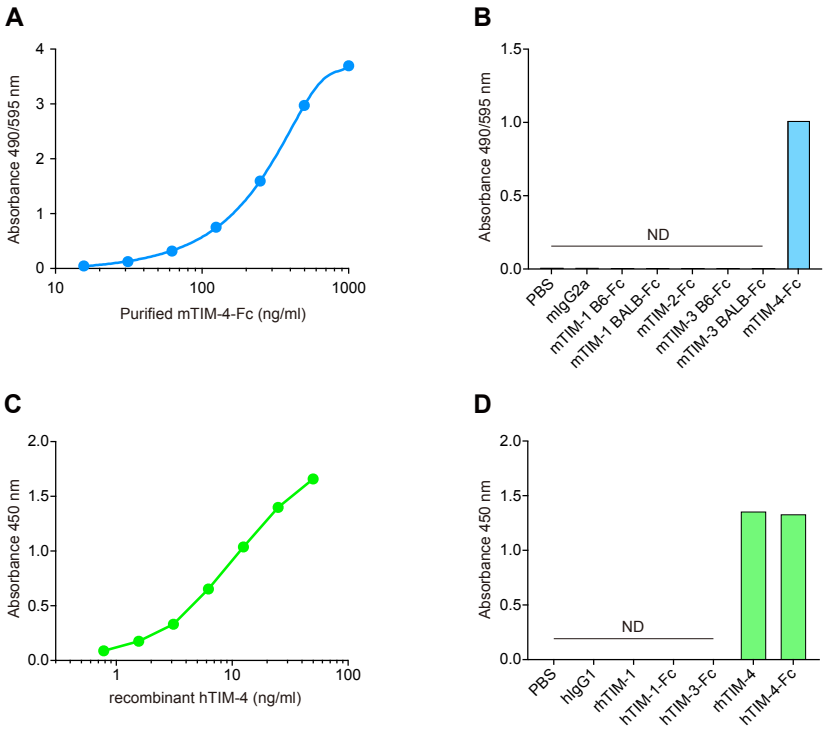


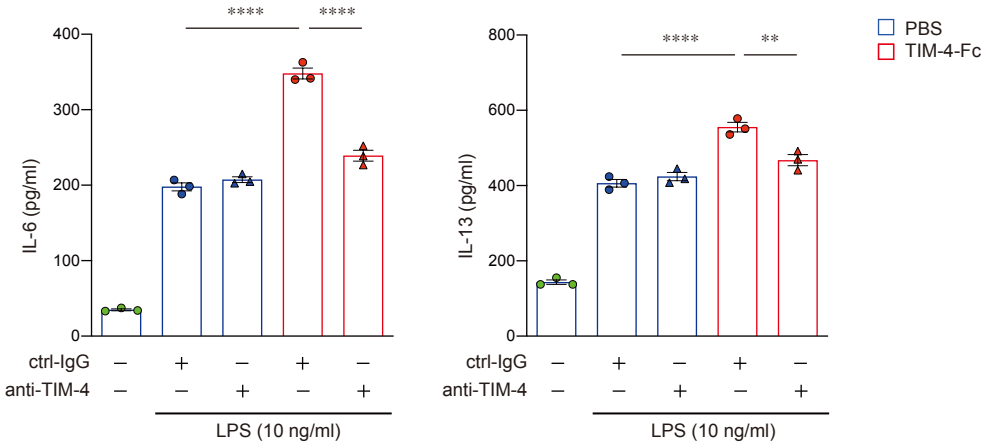
Supplementary Figure 1. Inhibitory effect of anti-TIM-4 monoclonal antibody (mAb) on adoptively transferred asthma.

A, Lymph node cells from Ovalbumin (OVA)-immunized mice were cultured with OVA in vitro, and then 2×10^6 cells were intravenously injected into naïve mice and challenged with an OVA/PBS aerosol. Groups of mice were intraperitoneally injected with anti-TIM-4 mAb (RMT4-53), ctrl-IgG (RTG2b), or PBS ($n = 5$). **B,** Individual mice were assessed for airway hyperreactivity. **C,** Bronchoalveolar lavage (BAL) fluid was collected from individual mice, and the cellular composition of the airway infiltrates is shown. **D,** IL-13 levels in BAL fluid were determined by ELISA. **E,** Bronchial lymph node cells were isolated and cultured with the OVA. Cytokine levels in the culture supernatants at 72 h were determined by ELISA. Data are presented as the means ± SEM. Statistics: two-way ANOVA with Bonferroni's multiple comparisons test (**B** and **E**) and with Tukey's multiple comparison test (**C**), and one-way ANOVA with Tukey's multiple comparison test (**D**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.



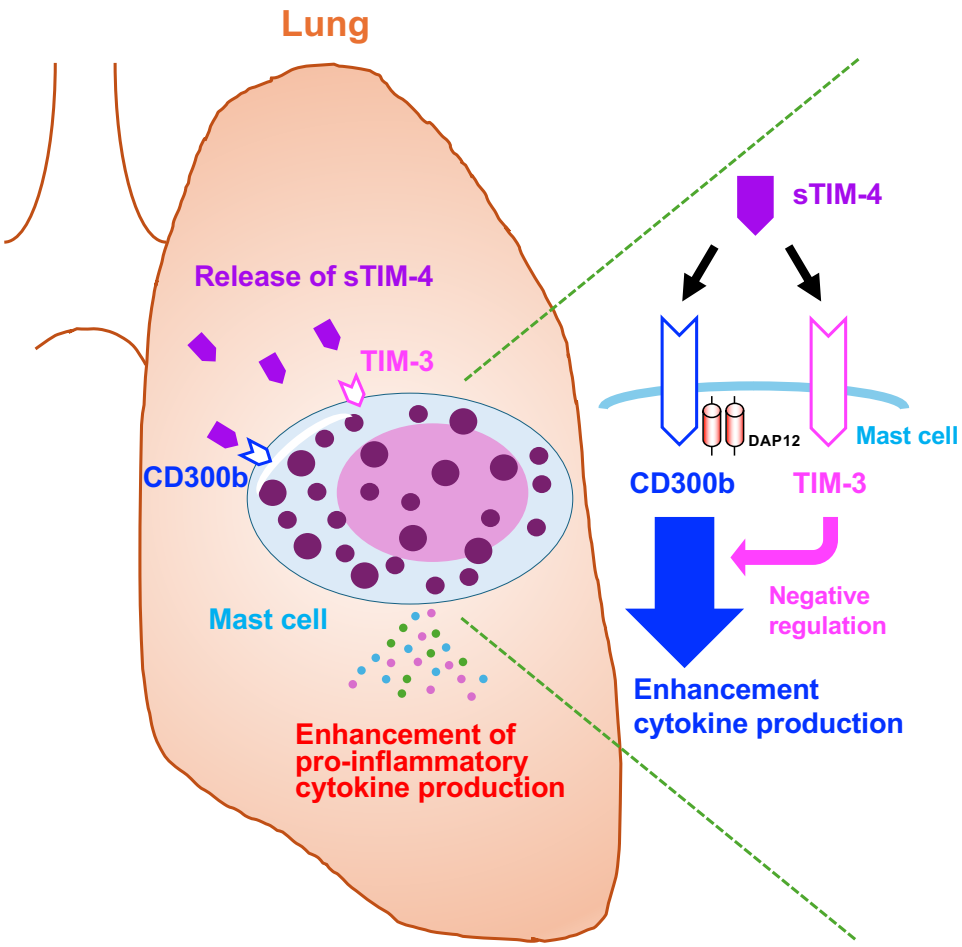
Supplementary Figure 2. Establishment of ELISA for sTIM-4.

A and **B,** ELISA plates were coated with 10 μg/ml anti-mTIM-4 mAb (RMT4-26) and then blocked with 1% BSA in PBS. Serially diluted purified mTIM-4-Fc (**A**) or 100 ng/ml mouse IgG2a (mIgG2a), mTIM-1 B6-Fc, mTIM-1 BALB-Fc, mTIM-2-Fc, mTIM-3 B6-Fc, mTIM-3 BALB-Fc, or mTIM-4-Fc (**B**) were added to each well. After washing, wells were incubated with biotin-conjugated anti-mTIM-4 mAb (RMT4-30), washed, and then developed using a Vectastain ABC kit and *o*-phenylenediamine. Absorbance at 490/595 nm was measured using a microplate reader. **C** and **D,** ELISA plates were coated with 10 μg/ml anti-hTIM-4 mAb (RHT4-89) and then blocked with 1% BSA in PBS. Serially diluted purified recombinant hTIM-4 (**C**) or 50 ng/ml human IgG1 (hIgG1), rhTIM-1, hTIM-1-Fc, hTIM-3-Fc, rhTIM-4, or hTIM-4-Fc (**D**) were added to each well. After washing, wells were incubated with HRP-labeled anti-hTIM-4 mAb (RHT4-62), washed, and then developed using tetramethylbenzidine. Absorbance at 450 nm was measured using a microplate reader. We defined 1 ng of mTIM-4-Fc or rhTIM-4 as 1 unit of sTIM-4. ND, not detected.



Supplementary Figure 3. TIM-4-Fc enhances cytokine production by Lipopolysaccharide (LPS)-stimulated Bone marrow-derived mast cells (BMMCs).

Purified BMMCs were cultured with LPS (O111:B4 *E. coli*) and TIM-4-Fc in the presence of anti-TIM-4 mAb (RMT4-53) or ctrl-IgG (RTG2b). IL-6 and IL-13 in culture supernatants at 6 h were measured by ELISA. Data are representative of two independent experiments. Data are presented as the means ± SEM. Statistics: one-way ANOVA with Tukey's multiple comparison test. $^{**}P < 0.01$ and $^{****}P < 0.0001$.



Supplementary Figure 4. Graphic summary of the study.

TIM-4 promotes allergic inflammation, and its soluble form (sTIM-4) correlates with disease severity in airway allergy. The interaction between TIM-4 and TIM-3 inhibited CD300b-mediated cytokine production by mast cells; however, TIM-4–CD300b appeared to be the dominant signaling pathway of cytokine production by mast cells.

Supplementary Table 1. Characteristics of patients with asthma

	n = 124
Sex (M/F), n (%)	42 (33.9)/ 82(66.1)
Age (y)	53.95 ± 15.99
Age at asthma onset (y)	35.54 ± 21.92
Duration of asthma (y)	18.41 ± 15.71
BMI (kg/m^2)	24.02 ± 4.91
GINA step 1/2/3/4/5, n (%)	3(2.4)/10(8.0)/32(25.8)/56(45.2)/23(18.6)
Smoking history (never/ex/current), n (%)	77(62.1)/42(33.9)/5(4)
Pack year smoking history (pack year)	5.71 ± 10.85
AERD, n (%)	12 (9.7)
Atopic dermatitis, n (%)	26 (21.0)
Allergic rhinitis, n (%)	64 (51.6)
Chronic sinusitis, n (%)	38 (30.6)
Daily dose of ICS (FP equivalent dose, μg)	577.4 ± 383.88
Daily dose of OCS (PSL equivalent dose, mg)	0.35 ± 1.42
ACT score, n = 123	23.19 ± 2.84
FeNO (ppb)	55.22 ± 44.01
Peripheral neutrophils (cells/μL)	4020.26 ± 1504.34
Peripheral eosinophils (cells/μL)	261.84 ± 237.57
Serum IgE (IU/ mL)	608.81 ± 1698.66
FVC (L)	3.21 ± 0.95
%FVC (predicted, %)	102.97 ± 16.26
FEV_1 (L)	2.38 ± 0.79
%FEV_1 (predicted, %)	91.02 ± 18.51
FEV_1/FVC ratio (%)	73.53 ± 10.33
PEF (L/sec)	7.22 ± 2.05
%PEF (predicted, %)	103.32 ± 21.25
V50 (L/sec)	2.53 ± 1.31
V25 (L/sec)	0.73 ± 0.50
V50/V25	3.99 ± 1.38
MMF (L)	1.95 ± 1.07
%MMF (predicted, %)	59.12 ± 27.63

Data are presented as the mean ± standard deviation unless otherwise indicated.

Abbreviations for all tables: ACT, asthma control test; AERD, aspirin-exacerbated respiratory disease; BMI, body mass index; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; FP, fluticasone propionate; FVC, forced vital capacity; GINA, Global Initiative for Asthma; ICS, inhaled corticosteroid; IgE, immunoglobulin E; MMF, mid-maximal flow rate; OCS, oral corticosteroids; PEF, peak expiratory flow; PSL, predonisolone; V25 and V50 = maximal flow rate of expiration at 25% and 50% of forced vital capacity .

Supplementary Methods

Cells

Bone marrow-derived mast cells (BMMCs) were generated from femoral bone marrow cells of C57BL/6, *Cd300lb*$^{-/-}$, or *Tim3*$^{-/-}$ mice, as described previously.[1] Bone marrow cells were incubated for 4–6 weeks in RPMI1640 (Sigma–Aldrich, St. Louis, USA) containing 10% fetal bovine serum (FBS; Biosera, Nuaille, France), 0.1 mg/ml penicillin and streptomycin, 100 μM 2-mercaptoethanol, 10 mM sodium pyruvate, 10 μM minimum essential medium nonessential amino acids solution (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 10 mM HEPES, 10 ng/ml recombinant murine IL-3, and 10 ng/ml recombinant stem cell factor (Wako Pure Chemical Industries, Osaka, Japan). Flow cytometric analysis identified more than 95% of cells as mast cells (KIT^{+} FcεRI^{+}). Murine T lymphoma cell line L5178Y was purchased from American Type Culture Collection (Manassas, VA, USA). A normal rat kidney cell line NRK-52E was provided by Dr. T. Otsuka (Institute of Cytosignal Research, Tokyo, Japan). Mouse TIM-4-transfected cells (TIM-4/L5178Y and TIM-4/NRK) were previously generated.[2] A cDNA fragment encoding the entire open reading frame of human *TIM4* molecule was prepared by RT-PCR using a cDNA clone (AK301337) from the NITE Biological Resource Center (National Institute of Technology and Evaluation, Kisarazu, Japan). The PCR product was cloned into pMKITneo vector and transfected into human leukemic K562 cells (TIM-4/K562) by electroporation. Mock-transfected cells (mock/K562) were also produced by electroporation of the pMKITneo vector without an insert. Cells were maintained in RPMI1640 supplemented with 10% FBS, 0.1 mg/ml penicillin and streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 50 μM 2-mercaptoethanol. The human mast cell leukemia cell line, LAD2, which was provided by A. Kirshenbaum (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA), was maintained in StemPro-34 SFM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in the presence of recombinant human stem cell factor (PeproTech, Rocky Hill, NJ, USA), as described previously.[3]

Antibodies and Fc fusion proteins

Expression vectors for mouse TIM-1 C57BL/6 (B6)-Fc, TIM-1 BALB/c (BALB)-Fc, TIM-2-Fc, TIM-3 B6-Fc, TIM-3 BALB-Fc, and TIM-4-Fc were generated by linking the sequence encoding the extracellular domain of each TIM molecule with that encoding the Fc portion of mouse IgG2a by inserting them into the pcDNA3.1(−) vector (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), as described previously.[4] The expression vector for mouse PD-L2-Fc was prepared as described previously.[5] All Fc fusion proteins were produced by transfection of CHO cells using FuGENE HD Transfection Reagent (Promega Corporation, Madison, WI, USA) and purified using Protein G-Sepharose (GE Healthcare, Chicago, IL, USA). A sandwich ELISA that detects the Fc domain of mouse IgG2a using an anti-mouse IgG2a mAb (R11-89) and biotinylated anti-mouse IgG2a (R19-15) was used to determine the concentration of Fc fusion protein. Monoclonal antibodies against mouse TIM-1 (RMT1-17), TIM-2 (RMT2-26), and TIM-3 (RMT3-23) were prepared as described previously.[6] Anti-mouse TIM-4 mAbs (RMT4-26, rat IgG2a/λ; RMT4-30, rat IgG2a/κ; RMT4-53, rat IgG2b/κ) were generated by immunizing Sprague Dawley rats with TIM-4-Fc protein. Control rat anti-TNP IgG2a mAb (RTG2a) and rat anti-TNP IgG2b mAb (RTG2b) were generated in our laboratory. The expression vector for CD300b-Fc comprising the extracellular domain of mouse CD300b and the Fc portion of human IgG1 was prepared as described previously.[7] The concentrations of CD300b-Fc were measured using a Human IgG ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA).

Recombinant human (rh) TIM-1, hTIM-1-Fc, hTIM-3-Fc, rhTIM-4, and hTIM-4-Fc were purchased from R&D Systems. The anti-human TIM-4 mAbs were generated by immunizing Sprague Dawley rats with rhTIM-4 emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). Three days after the final immunization, lymph node cells were fused with P3U1 myeloma cells. After hypoxanthine-aminopterin-thymidine selection, hybridomas producing anti-TIM-4 mAbs (RHT4-89, rat IgG2b/κ; RHT4-62, rat IgG2a/κ) were selected by their reactivity to human TIM-4-transfected cells, but not to parental cells, by flow cytometry and then cloned by limiting dilution.

Analysis of airway hyperreactivity (AHR)

AHR was measured using methacholine (Wako Pure Chemical Industries, Osaka, Japan)-induced airflow obstruction. Mice were placed into whole-body plethysmographs (Buxco Research Systems, Wilmington, NC, USA) interfaced with computers through differential pressure transducers. Airway resistance is expressed as follows: Penh = [(Te/0.3Tr)-1] × [2Pef/3Pif], where Penh = enhanced pause, Te = expiratory time (s), Tr = relaxation time (s), Pef = peak expiratory flow (ml), and Pif = peak inspiratory flow (ml/s). Increasing doses of methacholine were administered for 3 min using a nebulizer, and Penh values were calculated over the subsequent 5 min.

Stimulation of bronchial lymph node cells in vitro

Bronchial lymph node cells (6×10^5 cells/well) were cultured in RPMI1640 medium containing 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-mercaptoethanol in the presence or absence of the indicated doses of OVA. To determine the production of cytokines, cell-free supernatants were collected after 72 h and assayed for IL-4, IL5, IFN-γ, and IL-13 using ELISA kits.

Immunoprecipitation and western blotting

The culture supernatants of TIM-4/L5178Y or L5178Y cells were collected after incubation with or without 10 μM BB-94 (batimastat; Abcam, Cambridge, UK) or 0.1% DMSO for 24 h (5×10^6 cells/5 ml). The cells were lysed in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. The cleared lysates or culture supernatants were incubated with anti-TIM-4 mAb (RMT4-26) or control IgG (RTG2a)-protein G-Sepharose. The beads were washed with the lysis buffer, and bound proteins were eluted with 1% SDS sample buffer, subjected to 14% SDS-PAGE under reducing conditions, and blotted onto polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA). The proteins on the blots were detected using biotinylated anti-TIM-4 polyclonal Ab (R&D Systems, Minneapolis, MN, USA), Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA), and SuperSignal West Dura

Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The images were acquired using the ImageQuant LAS-4000 (Fujifilm Corporation, Tokyo, Japan).

The culture supernatants of TIM-4/K562 or mock/K562 cells were collected after incubation with or without 10 μM BB-94 or 0.1% DMSO for 24 h (5×10^6 cells/5 ml). The cells were lysed in RIPA buffer (Nacalai Tesque, Kyoto, Japan) and cleared lysates or culture supernatants were incubated with anti-TIM-4 mAb (RHT4-89) or control IgG (RTG2b)-protein G-Sepharose. The beads were washed with RIPA buffer, and bound proteins were eluted with 1% SDS sample buffer, subjected to 14% SDS-PAGE under reducing conditions, and blotted onto polyvinylidene difluoride membrane. The proteins on the blots were detected using horseradish peroxidase (HRP) labeled-anti-TIM-4 (RHT4-62) and SuperSignal West Dura Chemiluminescent Substrate. Images were acquired using the ImageQuant LAS-4000.

Analysis of sTIM-4

The culture supernatants of TIM-4/L5178Y (1×10^6 cells), L5178Y cells, TIM-4/NRK (5×10^4 cells), NRK cells, or peritoneal macrophages (3×10^5 cells) were collected after treatment with 10 μM BB-94 or 0.1% DMSO for 24 h. BAL fluids or sera were collected from naïve mice or mice sensitized and challenged with OVA as described below. The concentrations of sTIM-4 in the culture supernatants, BAL fluids, or sera were measured by ELISA. Immulon 2 HB 96-well microtiter plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 10 μg/ml anti-TIM-4 mAb (RMT4-26) and incubated at 4°C overnight. Wells were then blocked with 1% BSA in PBS for 1 h at room temperature. Serially diluted samples were added to each well followed by incubation for 2 h at room temperature. After washing with 0.05% Tween 20 in PBS, the wells were incubated with biotin-conjugated anti-TIM-4 mAb (RMT4-30) for 1 h and washed, and the antigen–antibody complexes were detected using 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, the absorbance at 490/595 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). We defined 1 ng of purified TIM-4-Fc as 1 unit (U) of sTIM-4.

The culture supernatants of TIM-4/K562 (1×10^6 cells) or mock/K562 cells were collected after treatment with 10 μM BB-94 or 0.1% DMSO for 24 h. ELISA plates were coated with 10 μg/ml anti-human TIM-4 mAb (RHT4-89) and incubated at 4°C overnight. The wells were then blocked with 1% BSA in PBS for 1 h at room temperature. Serially diluted purified recombinant hTIM-4 or 50 ng/ml human IgG1 (hIgG1), rhTIM-1, hTIM-1-Fc, hTIM-3-Fc, rhTIM-4, or hTIM-4-Fc were added to each well, followed by incubation for 2 h at room temperature. After washing with 0.05 % Tween 20 in PBS, the wells were incubated with HRP-labeled anti-human TIM-4 mAb (RHT4-62) for 1 h, washed again, and then developed using tetramethylbenzidine (Sigma–Aldrich, St. Louis, MO, USA). After terminating the reaction with 2N H_2SO_4, absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). We defined 1 ng of rhTIM-4 as 1 U of sTIM-4.

Analyses of cytokine production

BMMCs (5×10^6/ml) were sensitized with 100 ng/ml mouse IgE (IgE-3; BD Biosciences, San Jose, CA, USA) for 1 h at 4°C and then washed. IgE-sensitized cells (2×10^6/ml) were incubated with an anti-mouse IgE mAb (R35–72; BD Biosciences) in the presence of TIM-4-Fc, PD-L2-Fc, mouse IgG2a, anti-TIM-4 mAb (RMT4-53), or control IgG (RTG2b) for 6 h. In some experiments, BMMCs were incubated for 6 h in culture supernatants obtained from L5178Y or TIM-4/L5178Y cells. Culture supernatants were assayed for IL-6 using an OptEIA Kit (BD Biosciences) and for IL-13 using a Ready-SET-Go! ELISA Kit (eBioscience, San Diego, CA, USA), respectively, according to the manufacturers' instructions.

The culture supernatants of LAD2 cells (1×10^5 cells) were collected after incubation with culture supernatants obtained from mock/K562 or TIM-4/K562 cells and anti-TIM-4 mAb (5 μg/ml) for 24 h. Culture supernatants were assayed for IL-6 using a Human IL-6 DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions.

Degranulation by BMMCs

Degranulation of the BMMCs was measured using the β-hexosaminidase release assay.[8] BMMCs (5×10^6 cells/ml) were sensitized with 1 μg/ml mouse IgE for 1 h at 4°C, washed with Tyrode's buffer (10 mM HEPES buffer, pH 7.4, 130 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.1% BSA), resuspended in Tyrode's buffer containing 1 mM $CaCl_2$ and 0.6 mM $MgCl_2$, and incubated for 40 min at 37°C with anti-mouse IgE in the presence of TIM-4-Fc (10 μg/ml) or mouse IgG2a. The supernatants were collected by centrifugation at 4°C and incubated with 1.3 mg/ml *4*-nitrophenyl-*N*-acetyl-b-D-gulcopyranoside (Sigma–Aldrich, St. Louis, MO, USA) in 0.1 M sodium citrate buffer (pH 4.5) for 60 min at 37°C. The reaction was terminated by adding 0.2 M glycine buffer (pH 10.7). The release of the product 4-*p*-nitrophenol was monitored by optical absorbance at 405 nm. The percentage of β-hexosaminidase release was calculated as follows: % release = supernatant OD value of stimulated cells/total cell lysate OD value × 100. Total cell lysates were prepared using 1% Triton-X 100.

Induction of PCA

Mice were intradermally sensitized with 50 ng anti-DNP IgE (H1-e-26; gifted from T. Kitamura, University of Tokyo) or PBS, and each ear was treated with 10 μg anti-TIM-4 mAb (RMT4-53) or control IgG (RTG2b). The mice were intravenously injected with 1% Evans blue dye (Sigma–Aldrich, St. Louis, MO, USA) containing 250 μg DNP-HSA (Sigma–Aldrich) 24 h after sensitization. The amounts of extravasated dye 30 min after antigen challenge were measured from absorbance at 620 nm.

Knockdown of mRNA expression using siRNAs

Cd300lb siRNA (Stealth RNAi, MSS238415 and MSS238417), *Tim3* siRNA (MSS206431 and MSS275596), and control siRNA (siRNA negative control, low and medium GC–content duplexes) were purchased from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). A 10 μl aliquot of 20 μM siRNA was introduced into 2×10^6 cells using a Neon 100 μl kit with a Neontransfection System (Invitrogen, Thermo Fisher Scientific) set at 1700 V, 20 msec, and 1 pulse. The electroporated BMMCs were harvested 72 h after transfection.

Real-time PCR analysis of mRNAs

Total RNA was extracted from cells using TRIzol reagents (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. The levels of *Cd300b* and *Tim3* mRNA were quantified using a 7500 FAST Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with TaqMan gene expression assays (Mm01701740_m1 for *Cd300lb* and Mm00454540_m1 for *Tim3*; Applied Biosystems, Thermo Fisher Scientific) and TaqMan Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific). The mRNA levels were evaluated as their ratios to those of the housekeeping gene *Gapdh* (4352339E; Applied Biosystems, Thermo Fisher Scientific) by calculating cycle threshold values in amplification plots using 7500 SDS software (Applied Biosystems, Thermo Fisher Scientific).

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