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Identification of Semaphorin 4B as a Negative Regulator of Basophil-Mediated Immune Responses

Yukinobu Nakagawa,^{*,†,1} Hyota Takamatsu,^{*,1} Tatsusada Okuno,^{*,‡} Sujin Kang,^{*} Satoshi Nojima,^{*,§} Tetsuya Kimura,^{*} Tatsuki R. Kataoka,[¶] Masahito Ikawa,^{||} Toshihiko Toyofuku,^{*} Ichiro Katayama,[†] and Atsushi Kumanogoh^{*,#}

Basophils are strong mediators of Th2 responses during helminthic infections. Recently, basophils were shown to function as APCs and promote both Th2 skewing and humoral memory responses. However, the mechanisms that regulate basophils are still unclear. In this article, we show that a class IV semaphorin, *Sema4B*, negatively regulates basophil functions through T cell–basophil contacts. In a screen to identify semaphorins that function in the immune system, we determined that *Sema4B* is expressed in T and B cells. Interestingly, *Sema4B*^{−/−} mice had considerably increased serum IgE levels despite normal lymphocyte and dendritic cell functions. Recombinant *Sema4B* significantly inhibited IL-4 and IL-6 production from basophils in response to various stimuli, including IL-3, papain, and FcεRI cross-linking. In addition, T cell-derived *Sema4B*, which accumulated at contact sites between basophils and CD4⁺ T cells, suppressed basophil-mediated Th2 skewing, suggesting that *Sema4B* regulates basophil responses through cognate cell–cell contacts. Furthermore, *Sema4B*^{−/−} mice had enhanced basophil-mediated memory IgE production, which was abolished by treating with an anti-FcεRIα Ab. Collectively, these results indicate that *Sema4B* negatively regulates basophil-mediated Th2 and humoral memory responses. *The Journal of Immunology*, 2011, 186: 000–000.

Basophils are rare granulocytes that are found in the circulation and are effector cells of the innate immune system that are associated with allergic inflammation and infections with helminth parasites (1–4). Recent studies indicated that basophils regulate Th1/Th2 homeostasis (5, 6) and humoral immunity (7). Basophils produce large amounts of IL-4, a key cytokine in Th2 skewing, in response to various stimuli, including IL-3, the protease allergen papain, and cross-linking of surface-

bound IgE molecules (8, 9). In addition, basophils have been shown to express MHC class II and costimulatory molecules and to function as APCs (10–12), although the role of basophils as APCs in vivo is still controversial (13–16). Previous studies have shown that basophil-mediated Th2 skewing can be promoted when these cells are activated by papain (11) or internalize Ags through Ag-specific IgE on their cell surface (12). In addition, basophils have been implicated in humoral memory responses; on re-exposure to Ags, Ag and Ag-specific IgE complexes activate basophils to release IL-4 and IL-6, resulting in enhanced humoral immune responses (7). However, the mechanisms that regulate basophil-mediated Th2 responses are still unknown.

Semaphorins were originally identified as axon guidance molecules during neuronal development (17). However, cumulative findings indicate that semaphorins have diverse functions in many physiological processes (18–22). Semaphorins have been shown to be involved in various phases of immune responses, including the activation of B cells (23), T cells (24), and dendritic cells (DCs) (25); the regulation of Th differentiation (26); and the navigation of immune cell trafficking (27). In particular, membrane-bound class IV semaphorins, *Sema4A* and *Sema4D*/CD100, have been extensively investigated. *Sema4D* has been shown to be important for B cell and DC activation (23). In addition, *Sema4A* has been demonstrated to be critical for Th differentiation (26). However, the impact of other class IV semaphorins on other immune functions has not been determined.

In this study, we searched for semaphorins that function in the immune system and identified a class IV semaphorin, *Sema4B*, as a novel immune semaphorin. To determine the physiological roles of *Sema4B*, we generated *Sema4B*^{−/−} mice and determined that *Sema4B* negatively regulates IL-4 and IL-6 production by basophils. We also determined that T cell-derived *Sema4B* suppresses basophil-mediated Th2 skewing. In addition, *Sema4B*^{−/−} mice not only had increased serum IgE levels under steady-state conditions but also enhanced memory IgE responses caused by defects in *Sema4B*-mediated negative regulation of basophils.

^{*}Department of Immunopathology, World Premier International Immunology Frontier Research Center, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan; [†]Department of Dermatology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan; [‡]Department of Neurology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan; [§]Department of Pathology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan; [¶]Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto 606-8507, Japan; ^{||}Animal Resource Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan; and [#]Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan

¹Y.N. and H.T. contributed equally to this study.

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Address correspondence and reprint requests to Prof. Atsushi Kumanogoh, Department of Immunopathology, World Premier International Immunology Frontier Research Center, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail address: kumanogo@ragtime.biken.osaka-u.ac.jp

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Abbreviations used in this article: BM, bone marrow; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; hIgG, human IgG; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; NP, 4-hydroxy-3-nitrophenyl acetyl; NP-CGG, 4-hydroxy-3-nitrophenylacetyl-chicken-γ-globulin conjugate; PSD, postsynaptic density; rSema4B, recombinant *Sema4B*; Tg, transgenic; WT, wild-type.

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Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from CLEA Japan. Sema4B^{-/-} mice on C57BL/6 and BALB/c backgrounds were generated as described later. Sema4B^{-/-} mice were backcrossed >6× onto the C57BL/6 or BALB/c background. Transgenic (Tg) mice expressing the TCR specific for OVA (323–339; BALB/c background) were kindly provided by S. Habu (28). OVA-TCR Tg mice on a Sema4B^{-/-} background were established by crossing the OVA-TCR Tg mice with Sema4B^{-/-} BALB/c mice. All mice were bred in specific pathogen-free conditions. All animal experimental protocols were reviewed and approved by our institutional animal care committees.

Generation of Sema4B^{-/-} mice

A targeting vector was designed to replace the fifth to eighth exons of the Sema4B gene with a Neo resistance cassette. In addition, the HSV thymidine kinase gene was inserted to select against random integration of this targeting vector. The linearized targeting plasmid DNA was transfected into embryonic stem cells by electroporation. After double selection with G418 and ganciclovir, we screened for homologous recombination of the Sema4B allele by PCR and FACS analysis. PCR was performed using 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s. The following oligonucleotide primers were used to identify the rearranged Sema4B locus: forward primer (P1): 5'-TAGTGGCATATGTGGACCTG-3'; reverse primer (P2): 5'-TCCCTGGAAGCTACTGACTGTT-3'; and reverse primer (P3) that includes the Neo cassette: 5'-TGCTCGACGTTGTCACTGAA-3'.

RT-PCR

Sema4B mRNA expression was examined by RT-PCR using a panel of multiple mouse tissue cDNAs (Clontech). RT-PCR was performed using 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with the forward primer (5'-AACAGCAACCTCAGCTTCTTGC-3') and reverse primer (5'-GGCCTCATCTTGGGCTAAAGTA-3').

Generation of the anti-Sema4B Ab

Rats were repeatedly immunized with purified mouse recombinant Sema4B (rSema4B) and alum, and their spleens were harvested to generate hybridomas using standard methodologies. The hybridomas were screened by ELISA for the production of Sema4B-specific Abs. To confirm the specificity of the Ab for Sema4B, we transfected COS7 cells with Sema4B, Sema4A, Sema4D, or the control vector using Lipofectamine (Invitrogen). After 48 h, the transfectants were stained with the biotinylated anti-Sema4B Ab (TK-2) followed by allophycocyanin-conjugated streptavidin.

Reagents and Abs

Anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-CD49b (DX5), anti-FcεRIα (MAR-1), anti-CD62L (MEL-14), anti-c-Kit (2B8), MHC class II (I-A/I-E; M5/114.15.2), and allophycocyanin-, FITC-, and Cy5-conjugated streptavidin were obtained from eBioscience. Anti-CD16/CD32 (2.4G2), anti-CD11c (HL3), anti-CD40 (HM40-3), and anti-CD3e (145-2c11) were from BD Pharmingen. Anti-T1/ST2 was from MD Biosciences. Anti-p44/42 MAPK (SC-154) and anti-STAT5 (SC-835) were from Santa Cruz Biotechnology. Anti-phospho-p44/42 MAPK (9101) was from Cell Signaling Technology. Anti-phospho-STAT5 (47) was from BD Biosciences. Papain was purchased from Calbiochem. The monoclonal IgE anti-DNP Ab (SPE-7), OVA, DNP-human serum albumin (HSA), and LPS were from Sigma. 4-Hydroxy-3-nitrophenylacetyl-chicken-γ-globulin conjugate (NP-CGG), NP-BSA, and DNP-OVA were from Biosearch Technologies.

Cell purification

To prepare bone marrow (BM)-derived basophils, BM cells were cultured for 14 d with IL-3 (30 ng/ml, conditioned medium from a mouse IL-3-producing cell line) in RPMI 1640 medium supplemented with 10% (v/v) FBS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (29). Samples were enriched for basophils by single-cell sorting on CD11c⁻c-Kit⁺FcεRIα⁺ cells or by positively selecting CD11c⁻c-Kit⁺DX5⁺ cells with MACS (Miltenyi Biotech). Otherwise, BM-derived basophils were enriched by depleting mast cells using anti-c-Kit-biotin and streptavidin-coupled Dynabeads (Invitrogen) (30). T cells, B cells, and DCs were isolated from the spleen by MACS. The resulting purity was >95% for each experiment. BM-derived DCs (BMDCs) were generated by stimulating with GM-CSF as previously described (31).

Flow cytometry

For the rSema4B binding assay, BM-derived basophils were incubated with either biotinylated rSema4B or biotinylated human IgG (hIgG), followed by streptavidin-allophycocyanin. For *in vivo* Th2 skewing, single-cell suspensions from the spleens of adoptively transferred mice were stained with allophycocyanin-anti-CD4 and FITC-anti-T1/ST2. To evaluate Sema4B expression in specific immune cell populations, we identified CD4⁺T cells (CD4⁺CD8⁻), CD8⁺T cells (CD4⁻CD8⁺), B cells (B220⁺CD11c⁻), DCs (CD11c⁺B220⁻), and basophils (FcεRIα⁺DX5⁺) using the indicated markers.

In vitro stimulation assays

c-Kit-depleted BM-derived basophils were stimulated with IL-3 (30 ng/ml) after being starved for 8 h, or stimulated with papain (20 μg/ml) or IgE cross-linking without starvation. Basophils were activated with IgE cross-linking by incubating with IgE anti-DNP (1 μg/ml), followed by various concentrations of DNP-HSA for 16 h. Basophils were stimulated on plates coated with rSema4B (10 μg/ml) or hIgG (10 μg/ml). The concentrations of IL-4 and IL-6 in the culture supernatants were measured by ELISA (R&D Systems). B cells were cultured with anti-CD40 (0.2 μg/ml) and various concentrations of IL-4 for 7 d (32). The IgM, IgG1, and IgE levels in the culture supernatants were measured by ELISA (Bethyl Laboratories).

In vitro and in vivo basophil-mediated Th2 skewing assays

For the *in vitro* basophil-mediated Th2 skewing assay, BM-derived basophils (CD11c⁻c-Kit⁺FcεRIα⁺) were enriched by flow cytometry sorting. Naive CD4⁺CD62L⁺T cells from OVA-TCR Tg mice (4 × 10⁵ cells/ml) and irradiated basophils (5 × 10⁵ cells/ml) were cultured with the OVA peptide (1 μM), OVA (100 μg/ml) and papain (20 μg/ml), or IgE anti-DNP (1 μg/ml) and DNP-OVA (100 μg/ml) (11, 12) in plates coated with Sema4B (10 μg/ml) or hIgG (10 μg/ml). In some experiments, naive CD4⁺CD62L⁺T cells from wild-type (WT) or Sema4B^{-/-} OVA-TCR Tg mice were used instead of rSema4B. After 5 d of coculture, CD4⁺T cells isolated by anti-CD4-conjugated magnetic beads (autoMACS) were restimulated with immobilized anti-CD3 for 24 h. For the *in vivo* basophil-mediated Th2 skewing assay, naive CD4⁺CD62L⁺T cells (5 × 10⁶ cells/mouse) from WT or Sema4B^{-/-} OVA-TCR Tg mice were *i.v.* transferred into nude mice. The next day, basophils (CD11c⁻c-Kit⁺FcεRIα⁺, 5 × 10⁶ cells/mouse) pulsed with IgE anti-DNP (2 μg/ml) and DNP-OVA (100 μg/ml) were transferred through the tail vein into these recipient nude mice. After 4 d, CD4⁺T cells (1 × 10⁵ cells/well) isolated from the spleen by flow cytometry sorting were cultured with BMDC (1 × 10⁵ cells/well) and DNP-OVA (100 μg/ml) in 96-well plates for 36 h. Cytokine concentrations in the supernatants were measured by ELISA (R&D Systems).

In vivo T cell priming and Ab production assays

Mice were immunized in the hind footpads with keyhole limpet hemocyanin (KLH; 100 μg) in CFA or alum. Five days after priming, cells were purified from the draining lymph nodes. Lymph node cells (1 × 10⁵ cells/well) were stimulated for 48 h with various concentrations of KLH. For proliferation assays, the cells were pulsed with 2 μCi [³H]thymidine for the last 14 h (26). To induce Ab responses to T cell-dependent Ags, we immunized mice *i.p.* with 50 μg NP-CGG as an alum-precipitated complex on day 0 and then boosted on day 14. Serum was collected on days 0, 14, and 21. NP-specific Abs were detected with NP-BSA-coated ELISA plates and quantified using isotype-specific Abs as previously described (33).

Measurement of serum Igs

The concentrations of serum Igs were measured using a mouse IgE ELISA quantitation kit (Bethyl Laboratories).

In vivo memory response assays

WT and Sema4B^{-/-} mice were immunized *i.p.* with 100 μg OVA without adjuvant. Four weeks after the primary immunization, the mice were administered 10 μg OVA *i.v.* (7). Serum was collected on days 0, 5, 7, and 9 after rechallenging with the Ag. To deplete basophils, we injected primary immunized mice twice daily for 3 d with 5 μg anti-FcεRIα. The mice were allowed to rest for 2 d and then were *i.v.* injected with 10 μg OVA. OVA-specific serum IgE was measured with a mouse OVA-IgE ELISA kit (Dainippon Sumitomo Pharma). OVA-specific serum IgG1 was measured with a mouse OVA-IgG1 ELISA kit (Shibayagi).

Immunohistochemistry

Sema4B^{-/-} BM-derived basophils (CD11c⁻c-Kit⁺DX5⁺) and OVA-TCR Tg-derived CD4⁺T cells were cultured with OVA peptide for 1 h. Then the

cells were fixed with 4% paraformaldehyde in phosphate buffer for 15 min, attached to coverslips by cytospin, stained with FITC-anti-CD3 ϵ , PE-anti-I-A/I-E, and anti-Sema4B-biotin plus streptavidin-Cy5, and then examined by confocal microscopy (Zeiss Exciter).

Western blot analysis

BM-derived basophils were starved for 16 h and then stimulated with IL-3 (30 ng/ml) in the presence of rSema4B- or hIgG-coated Dynabeads (Invitrogen). The cells were lysed at the indicated times with lysis buffer containing 1% Nonidet P40, 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM Na₃VO₄, 0.5 mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM sodium orthovanadate, and a protease inhibitor mixture (Roche). Whole-cell lysates were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes. The membranes were immunoblotted with various Abs.

Statistical analysis

Data are presented as mean $\pm$ SD. The *p* values were calculated with the two-tailed Student *t* test after the data were confirmed to fulfill the criteria. Otherwise, Mann-Whitney *U* test was performed.

Results

Sema4B is expressed in T and B cells

In a screen to identify semaphorins in the immune system, we isolated a cDNA fragment encoding a class IV semaphorin,

Sema4B, through PCR-based cloning using degenerate oligonucleotide primers derived from motifs that are conserved across the semaphorin family. RT-PCR analyses showed that Sema4B was abundantly expressed in the spleen (Fig. 1A). Sema4B has been reported to play a role in assembling the postsynaptic specialization at glutamatergic and GABAergic synapses in the nervous system (34). However, the involvement of Sema4B in immune cell regulation has not been determined, and this led us to investigate the immunological function of Sema4B. To examine the expression of Sema4B in more detail, we generated an anti-Sema4B mAb that specifically recognizes Sema4B and does not cross-react with Sema4A or Sema4D (Fig. 1B). As shown in Fig. 1C, Sema4B is constitutively expressed in B and T cells, but not in DCs or basophils. To investigate the physiological role of Sema4B, we generated Sema4B^{-/-} mice by deleting the fifth to eighth exons in the *Sema4B* gene (Fig. 1D–F). Sema4B^{-/-} mice were born at the expected Mendelian ratio and were fertile. There were no apparent abnormalities based on gross macroscopic or histological examinations of all the tissues that express *Sema4B* transcripts.

Sema4B^{-/-} mice have increased serum IgE levels

Immune cell populations, such as T cells, B cells, DCs, NK cells, and subpopulations of T cells, were normal in Sema4B^{-/-} mice

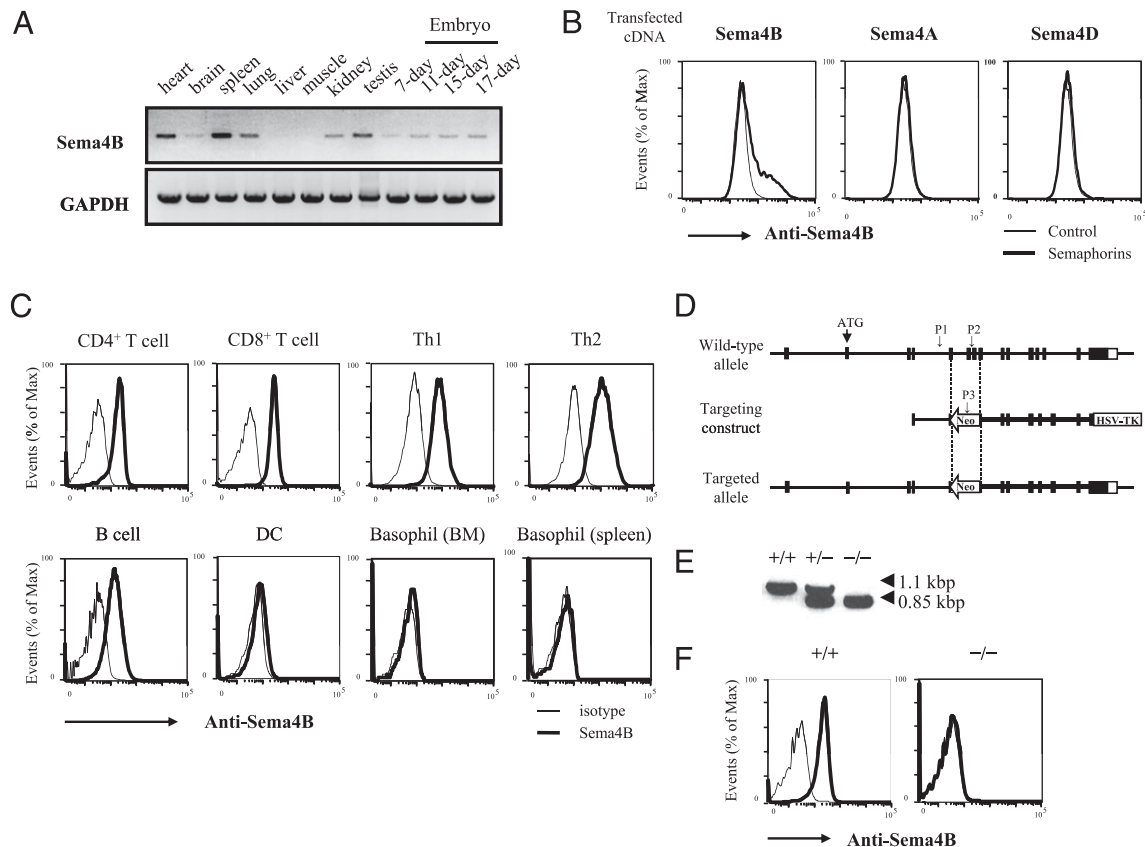


FIGURE 1. Sema4B is expressed in immune cells, especially T and B cells. *A*, Expression profiles of Sema4B. Sema4B transcripts were examined by RT-PCR using a panel of multiple mouse tissue cDNAs. GAPDH transcripts were used as controls. *B*, The anti-Sema4B Ab specifically binds to Sema4B. COS7 cells transfected with Sema4B, Sema4A, Sema4D expression constructs, or the control vector were stained with a biotinylated anti-Sema4B Ab (thick lines) or the isotype-matched control Ab (thin lines) followed by streptavidin-FITC. *C*, Sema4B is constitutively expressed in T and B cells. Mouse splenocytes, as well as BM-derived or fully differentiated Th1 or Th2 cells, were stained with anti-Sema4B (thick lines) or isotype-matched control (thin lines) Abs. *D*, Disruption of the *Sema4B* gene. The gene structures of the WT *Sema4B* allele (top), *Sema4B*-targeting construct (middle), and *Sema4B* mutant allele (bottom) are shown. Filled boxes denote the coding sequences. Exons 5 to 8 were replaced with a Neo cassette. The HSV thymidine kinase gene was added to select against random integration. *E*, Genomic typing analyses. Genomic DNA was isolated from the tails of WT (+/+), Sema4B heterozygous (+/-), and homozygous mutant mice (-/-), and the genotype was determined by PCR using the indicated primers. Arrowheads indicate the 1.1- and 0.85-kbp fragments that represent the WT allele and the targeted allele, respectively. *F*, Splenocytes from WT (+/+) and Sema4B^{-/-} (-/-) mice were stained with FITC-anti-CD3 and biotinylated anti-Sema4B mAbs (thick lines) or isotype-matched controls (thin lines) plus streptavidin-allophycocyanin. The CD3⁺ cells were gated and analyzed for Sema4B expression by flow cytometry.

(Supplemental Fig. 1). In vitro T cell-, B cell-, and DC-proliferative responses and cytokine production and T cell–DC interactions were not affected by the absence of Sema4B (Supplemental Fig. 2). However, Sema4B^{-/-} mice had considerably increased serum IgE levels at steady-state, and these concentrations gradually increased as the mutant mice aged (Fig. 2A). We next examined T cell-dependent Ab responses in vivo, in which WT and Sema4B^{-/-} mice were immunized with NP-CGG in alum and then boosted 2 wk after the first immunization. Interestingly, Sema4B^{-/-} mice had considerably greater serum levels of IgE than WT mice, although the serum titers of NP-specific IgM, IgG1, and IgG2a were comparable between WT and Sema4B^{-/-} mice (Fig. 2B). IgE production is tightly regulated by cell–cell interactions among T cells, B cells, and DCs (35). To explore the involvement of intrinsic Sema4B in IgE production by B cells, we examined Ab production from Sema4B^{-/-} B cells. However, IgM, IgG1, and IgE production was comparable between WT and Sema4B^{-/-} B cells (Fig. 2C) that were cultured with anti-CD40 and IL-4 in vitro. We then performed in vivo priming experiments by immunizing WT and Sema4B^{-/-} mice in the hind footpads with KLH with CFA or alum. As shown in Fig. 2D and 2E, there were no differences in the generation of Ag-specific T cells in terms of proliferation and cytokine production between WT and Sema4B^{-/-} mice. Furthermore, rSema4B had no effect on B cells, T cells, DCs, and T cell–DC interactions (Supplemental Fig. 3). These results strongly imply that non-B cell, non-T cell, and non-DC populations are responsible for the enhanced IgE production in Sema4B^{-/-} mice.

Sema4B inhibits cytokine production from basophils

We then analyzed the basophil populations in the spleen and BM because basophil numbers have been shown to be critical for Th2

polarization and IgE production (5, 6). The proportion of basophils in WT and Sema4B^{-/-} mice was comparable (Fig. 3A). We next examined whether rSema4B affects basophil responses. As shown in Fig. 3B, rSema4B bound to BM-derived basophils and significantly inhibited the ability of these cells to produce IL-4 and IL-6 in response to IL-3 stimulation (Fig. 3C), and these inhibitory effects were proportional to the rSema4B concentrations (Supplemental Fig. 4). It was previously reported that the FcR γ -mediated ITAM-spleen tyrosine kinase and its downstream mediator, ERK, are involved in IL-3-induced IL-4 production (30). In addition, IL-3R β -mediated JAK–STAT5 pathways are crucial for IL-3-induced proliferation (36, 37). As shown in Fig. 3D, ERK phosphorylation was inhibited by rSema4B, and similarly, STAT5 phosphorylation was inhibited by rSema4B (Fig. 3E). Basophils produce large amounts of IL-4 and IL-6 after being stimulated with cysteine proteases such as papain (9) or cross-linking of their surface IgE (12). rSema4B also inhibited IL-4 and IL-6 production from BM-derived basophils that were stimulated with papain or IgE anti-DNP and DNP-HSA (Fig. 3F, 3G). These results indicate that Sema4B negatively regulates cytokine production from basophils.

Sema4B suppresses basophil-mediated Th2 skewing

We examined whether rSema4B suppressed basophil-mediated Ag-specific Th2 skewing because basophils have been reported to promote Th2 polarization by functioning as APCs (38). To clarify this point, we cocultured OVA-TCR Tg-derived naive CD4⁺ T cells with OVA peptide-pulsed BM-derived basophils for 5 d in the presence of rSema4B or hIgG. Interestingly, rSema4B significantly suppressed IL-4 but not IFN- γ production from T cells (Fig. 4A). Furthermore, when the basophil–T cell cocultures were

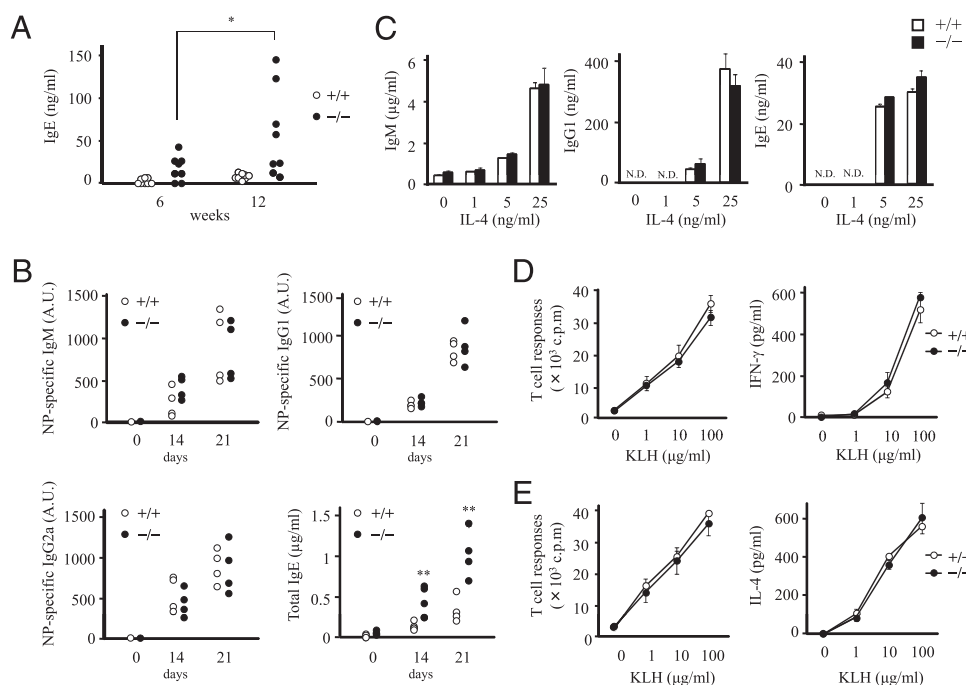


FIGURE 2. Sema4B^{-/-} mice have increased serum IgE levels. **A**, Serum IgE levels in WT (open circles) and Sema4B^{-/-} (closed circles) mice were measured at 6 and 12 wk by ELISA. **p* < 0.05 (Mann–Whitney *U* test). **B**, WT (open circles) and Sema4B^{-/-} (closed circles) mice were immunized i.p. with NP-CGG as an alum-precipitated complex on days 0 and 14, and bled at the indicated times. The anti-NP Ab titers were determined using NP-BSA-coated ELISA plates. The total IgE levels were determined by ELISA. ***p* < 0.01, ****p* < 0.005 (Mann–Whitney *U* test). **C**, Purified splenic B cells from WT (open bars) or Sema4B^{-/-} (closed bars) mice were stimulated with anti-CD40 and the indicated concentrations of IL-4. After 7 d, the IgM, IgG1, and IgE titers in the culture supernatants were measured by ELISA. **D** and **E**, WT (open circles) and Sema4B^{-/-} (closed circles) mice were immunized in the hind footpads with KLH in CFA (**D**) or alum (**E**). Five days after priming, CD4⁺ T cells prepared from the draining lymph nodes were restimulated with various concentrations of KLH and then examined for proliferation and cytokine production. Data are representative of two (**A**) or three (**B**–**E**) independent experiments.

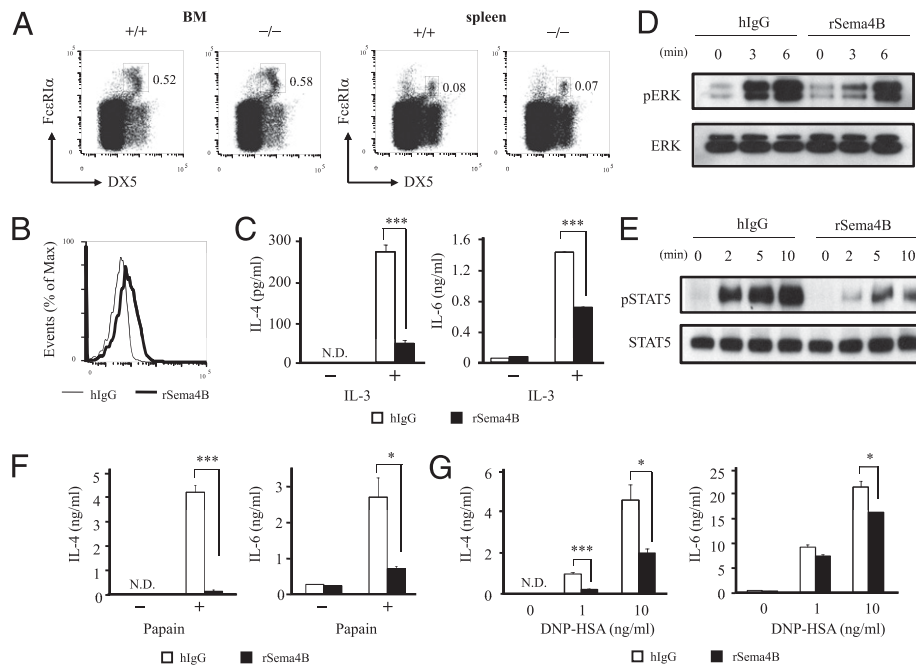


FIGURE 3. rSema4B inhibits cytokine production from basophils. **A**, Frequency of basophils in WT (+/+) and Sema4B^{-/-} (-/-) mice. The numbers above the outlined areas indicate the percentages of FcεRIα⁺DX5⁺ cells in the spleen or BM. **B**, BM-derived basophils were stained with biotinylated rSema4B (thick lines) or hlgG (thin lines) plus streptavidin-allophycocyanin. **C**, c-Kit-depleted and starved BM-derived basophils were stimulated with IL-3 in the presence of rSema4B (closed bars) or hlgG (open bars) for 16 h. The cytokine concentrations in the culture supernatants were measured by ELISA. **D** and **E**, c-Kit-depleted and starved BM-derived basophils were stimulated with IL-3 in the presence of rSema4B- or hlgG-coated beads. Cell lysates were prepared at the indicated times. The same amounts of protein extracts were immunoblotted using Abs against phospho-ERK and total ERK (**D**), or phospho-STAT5 and total STAT5 (**E**). **F** and **G**, c-Kit-depleted, BM-derived basophils were stimulated with papain (**F**) or IgE anti-DNP and various concentrations of DNP-HSA (**G**) for 16 h in the presence of rSema4B (closed bars) or hlgG (open bars). The cytokine concentrations in the culture supernatants were measured by ELISA. **p* < 0.05, ****p* < 0.005 (Student *t* test). Data are representative of at least three independent experiments.

treated with papain and OVA or IgE cross-linking with IgE anti-DNP and DNP-OVA, Sema4B also significantly inhibited basophil-mediated Th2 skewing (Fig. 4*B*, 4*C*). Collectively, these findings suggest that Sema4B inhibits IL-4 production by basophils, resulting in suppression of basophil-mediated Th2 skewing.

Sema4B is highly expressed in CD4⁺ T cells (Fig. 1*C*). However, intrinsic Sema4B did not affect Th1 and Th2 differentiation in vitro (Supplemental Fig. 2*C*). To analyze whether T cell-derived Sema4B is critical for regulating basophil-mediated Th2 skewing, we cultured WT or Sema4B^{-/-} OVA-TCR Tg-derived naive CD4⁺ T cells with BM-derived basophils and OVA peptide or DNP-OVA and IgE anti-DNP for 5 d. Sema4B^{-/-} T cells showed considerably enhanced Th2 skewing (Fig. 4*D*, 4*E*). Next, to examine basophil-mediated Th2 skewing in vivo, we adoptively transferred WT or Sema4B^{-/-} OVA-TCR Tg-derived naive CD4⁺ T cells into nude mice; then BM-derived basophils pulsed with DNP-OVA and IgE anti-DNP were intravenously transferred into these recipients. Four days later, we isolated CD4⁺ T cells from the spleen and examined the in vivo Th2 skewing of these CD4⁺ T cells. As shown in Fig. 4*F*, Sema4B^{-/-} T cells had more T1/ST2⁺ Th2 cells and enhanced IL-4 production after being stimulated with DNP-OVA and BMDC (Fig. 4*G*). These results suggest that T cell-derived Sema4B is important for basophil-mediated Th2 skewing.

Basophils have been shown to form an immunological synapse with cognate T cells (11). The cytoplasmic tail of Sema4B contains a PDZ-binding motif and binds to postsynaptic density (PSD)-95 (39) that accumulates at contact sites between thymocytes and DCs (40). Thus, we next examined the localization of Sema4B during basophil and T cell interactions. Consistent with previous reports, papain-activated BM-derived basophils formed an immunological synapse with OVA-TCR Tg-derived CD4⁺

T cells in the presence of OVA peptide. Of note, Sema4B in T cells colocalized with CD3 and clustered at T cell–basophil contact sites (Fig. 4*H*). These results suggest that Sema4B in T cells accumulates at the immunological synapse and suppresses basophil functions in a cell–cell contact-dependent manner.

Sema4B^{-/-} mice have enhanced IgE memory responses

During secondary Ag exposure, Ag-specific, IgE-bearing basophils capture Ag, become activated, and secrete cytokines, which subsequently facilitates memory B cell responses (7, 41). Although WT and Sema4B^{-/-} basophils produced comparable levels of IL-4 and IL-6 on FcεRI cross-linking (Supplemental Fig. 5), the serum IgE concentrations increased with age in Sema4B^{-/-} mice (Fig. 2*A*). We hypothesized that this phenomenon can be caused not only by basophil-mediated priming responses but by basophil-mediated memory responses. To determine whether Sema4B is involved in immunological memory responses, we immunized WT and Sema4B^{-/-} mice with OVA proteins and then boosted them with OVA proteins. Sema4B^{-/-} mice had significantly greater levels of serum OVA-specific IgG1 and IgE than WT mice (Fig. 5*A*). Furthermore, the increased IgG1 and IgE responses in Sema4B^{-/-} mice were suppressed when basophils were depleted before the secondary immunization (Fig. 5*B*), which suggests that basophils are responsible for the enhanced memory responses in Sema4B^{-/-} mice. Taken together, these results indicate that Sema4B also negatively regulates basophil-mediated humoral memory responses.

Discussion

Semaphorins have been shown to play crucial roles in the immune system (21). In this study, we performed a screen to identify novel

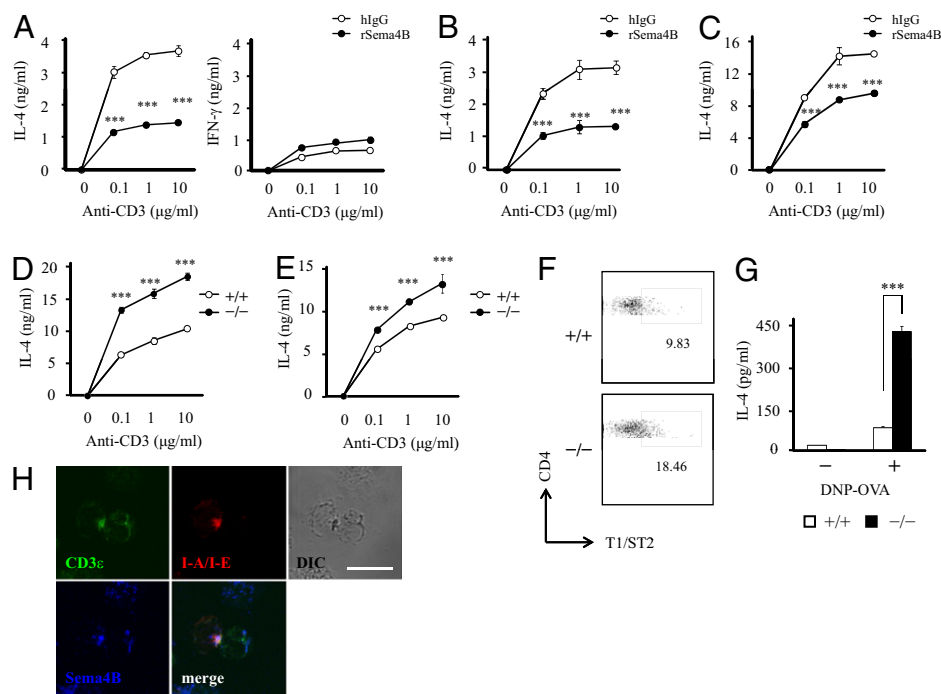


FIGURE 4. Sema4B negatively regulates basophil-mediated Th2 skewing. *A–C*, OVA-TCR Tg-derived naive CD4⁺CD62L⁺ T cells and irradiated BM-derived basophils (CD11c⁺c-Kit⁺FcεRIα⁺) were cultured with OVA peptide (*A*), papain and OVA (*B*), or IgE anti-DNP and DNP-OVA (*C*) in the presence of rSema4B (closed circles) or hlgG (open circles) for 5 d. Then the CD4⁺ T cells isolated by anti-CD4-conjugated magnetic beads were restimulated with various concentrations of immobilized anti-CD3 for 24 h. The cytokine concentrations in the culture supernatants were measured by ELISA. *D* and *E*, WT (open circles) or Sema4B^{-/-} (closed circles) OVA-TCR Tg-derived naive CD4⁺CD62L⁺ T cells and irradiated BM-derived basophils (CD11c⁺c-Kit⁺FcεRIα⁺) were cultured with OVA peptide (*D*) or IgE anti-DNP and DNP-OVA (*E*) for 5 d. Then the CD4⁺ T cells isolated by anti-CD4-conjugated magnetic beads were restimulated with various concentrations of immobilized anti-CD3 for 24 h. The cytokine concentrations in the culture supernatants were measured by ELISA. *F* and *G*, WT (+/+ or open bars) or Sema4B^{-/-} (-/- or closed bars) OVA-TCR Tg-derived naive CD4⁺CD62L⁺ T cells were i.v. transferred into nude mice. The next day, BM-derived basophils (CD11c⁺c-Kit⁺FcεRIα⁺) pulsed with IgE anti-DNP and DNP-OVA were i.v. transferred into these recipients. Four days later, CD4⁺ T cells isolated from these mice were stained with anti-CD4 and anti-T1/ST2 (*F*), and cultured with BMDC and DNP-OVA for 36 h (*G*). The cytokine concentrations in the culture supernatants were measured by ELISA. ****p* < 0.005 (Student *t* test). *H*, Papain-activated, BM-derived basophils (CD11c⁺c-Kit⁺DX5⁺) and OVA TCR Tg-derived CD4⁺ T cells were cocultured with OVA peptide for 1 h and then fixed in 4% paraformaldehyde phosphate buffer. Then the cells were attached to coverslips by cytopsin and stained with FITC-anti-CD3e (green), PE-anti-I-A/I-E (red) and anti-Sema4B-biotin plus streptavidin-Cy5 (blue). The localization of Sema4B was evaluated by confocal microscopy. Scale bar, 10 μ m.

immune semaphorins that function in the immune system and determined that Sema4B, which is abundantly expressed in lymphocytes, suppresses the functions of basophils and regulates both

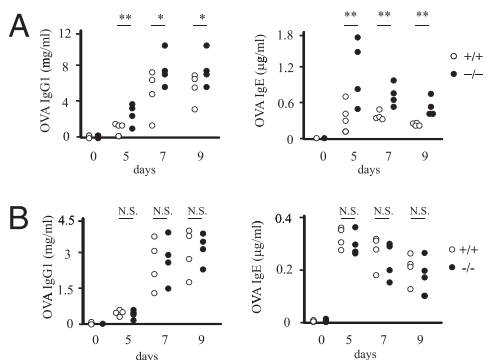


FIGURE 5. Sema4B^{-/-} mice exhibit enhanced humoral memory responses. *A*, WT (open circles) and Sema4B^{-/-} (closed circles) mice were immunized i.p. with OVA without adjuvant and then rechallenged i.v. with OVA after 4 wk. OVA-specific IgG1 and IgE in the serum were measured at the indicated times by ELISA. *B*, WT (open circles) and Sema4B^{-/-} (closed circles) mice were primarily immunized as described earlier and then depleted of basophils. Two days after the final injection of anti-FcεRIα, the mice were rechallenged with OVA. The OVA-specific IgG1 and IgE levels were measured by ELISA. **p* < 0.05, ***p* < 0.01 (Mann-Whitney *U* test).

the homeostatic Th1/Th2 balance and immunological memory responses. Our findings identified not only a novel immune semaphorin but also an important regulatory mechanism for basophils.

In this article, we show that Sema4B significantly inhibited IL-4 and IL-6 production by basophils in response to various stimuli, including IL-3, papain, and FcεRI cross-linking. Because of the fact that Sema4B regulates cytokine production, basophil-mediated Th2 skewing was also inhibited. In addition, we showed that a Sema4B deficiency in T cells results in increased Th2 responses, suggesting that Sema4B negatively regulates basophils. Basophils are shown to facilitate Th2 skewing and IgE production, and serve as a cellular source for IL-4 and IL-6 (4). In addition, recent reports have shown that Ag-capturing basophils can function as APCs and induce Th2 responses in an IL-4-dependent manner (12). In contrast, several reports have claimed that basophils only minimally function as APCs during in vivo inflammatory conditions such as helminthic infections (15) and allergic responses induced by the house dust mite allergen (13), in which DCs played vital roles in Th2 development. In addition, CD11c⁺FcεRIα⁺ DCs have been detected in IL-3-conditioned BM cultures (13), which raise a possibility that a subpopulation of DCs remained in the basophil fraction. To exclude this possibility, we prepared BM-derived basophils by sorting for the CD11c⁺c-Kit⁺FcεRIα⁺ fraction. However, these cells still induced naive CD4⁺ T cells to become Th2 cells. In addition, rSema4B did not affect DCs in terms of cytokine production and cognate T cell

activation. Although we cannot completely exclude a possible involvement of endogenous DCs, our findings indicate that the effects of T cell-derived Sema4B on basophils would be primarily responsible for in vivo Th2 skewing in our adoptive transfer experiments. In addition, we found that Sema4B expression on T cells was upregulated by anti-CD3 stimulation (data not shown), and that Sema4B localized at the contact sites between basophils and CD4⁺ T cells. It was reported that the PDZ-binding motif in the C terminus of Sema4B binds to PSD-95 (39), and that PSD-95 in T cells preferentially localizes at contact sites during cell–cell interactions (40). In this context, our data strongly imply that basophils are regulated through cell–cell contact, in which Sema4B negatively regulates basophil functions.

It has been thought that basophils are predominant Ag-capturing cells (41), and that Ag and Ag-specific IgE complexes activate basophils to produce IL-4 and IL-6, leading to enhanced humoral memory immune responses (7). However, a recent report showed that basophil-deficient mice still have efficient humoral immune responses (14). It has been reported that DCs have a considerably greater potential to function as APCs than basophils (13, 15, 16), suggesting that DCs overcome basophil functions depending on the experimental conditions, including the Ag dose and the use of adjuvants. However, Sema4B^{−/−} mice that were immunized with Ag without adjuvant and administered small amounts of Ag i.v. displayed enhanced Ag-specific IgE production. In addition, depleting basophils with an anti-FcεRIα Ab abolished the enhanced IgE production in Sema4B^{−/−} mice. It thus appears that the impaired negative regulation on basophils is responsible for enhanced humoral memory responses in Sema4B^{−/−} mice.

Sema4B not only suppressed FcεRI cross-linking-induced cytokine production by basophils (Fig. 3G) but negatively regulated Th2 skewing in a cell–cell contact-dependent manner (Fig. 4D, 4E). Therefore, it is plausible that enhanced humoral memory responses in Sema4B-deficient mice are due to soluble cytokines, as well as cell–cell contact-dependent mechanisms, although further studies would be required to determine the definitive Sema4B mode. Of note, recent reports have shown that more basophils enhanced steady-state Th2 polarization and increased serum IgE levels (5, 6), suggesting that basophils control the homeostatic Th1/Th2 balance even in the steady-state conditions. In this context, it appears that the defects in the regulatory effects of Sema4B on basophils during both priming and memory phases can contribute to the increased serum IgE levels in Sema4B^{−/−} mice as they aged.

Regarding the signaling mechanism for Sema4B-mediated basophil functions, we showed that Sema4B suppressed the IL-3-induced phosphorylation of ERK and STAT5, suggesting that Sema4B regulates IL-3 signaling. IL-3-mediated signals in basophils are reportedly involved in the ITAM in the FcRγ-spleen tyrosine kinase-ERK pathway to produce IL-4 and the IL-3Rβc-JAK-STAT5 pathway to promote proliferation and differentiation (30, 36, 37). Recently, it was shown that SHIP inhibited IL-4 production from basophils (42). In addition, Src homology 2 domain-containing protein tyrosine-phosphatase 1 (SHP-1) can interact with and inhibit the phosphorylation of STAT5 (43, 44). These inhibitory molecules are phosphatases that are mainly recruited to ITIM-containing receptors (45). Although it remains unclear whether ITIM-containing receptor is directly involved in IL-3-mediated signals in basophils, it is possible that Sema4B may regulate basophil functions through ITIM-containing molecules. Regarding the receptors for class IV semaphorins, several molecules such as plexin-Bs (B1, B2, B3) (46), plexin-D1 (47), T cell Ig and mucin domain-containing molecule-2 (Tim2) (24), and CD72 (23) have been shown to bind to class IV semaphorins. In fact, many of these molecules are expressed by basophils as

determined by RT-PCR (data not shown). Additional investigations, including the identification of the Sema4B receptor, are required to further examine these mechanisms.

In conclusion, we demonstrated that Sema4B suppressed IL-4 production from basophils, in which T cell-derived Sema4B inhibited basophil-mediated Th2 skewing. In addition, a Sema4B deficiency significantly affected Ag-specific IgE memory responses, possibly in a cell–cell contact-dependent manner. Thus, Sema4B negatively regulates basophil functions during both primary and memory responses. These findings not only provide new insight into mechanisms that regulate basophils but identify a novel therapeutic target for allergic diseases.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure Legends

Supplemental Figure 1. $\text{Sema4B}^{-/-}$ mice have normal immune cell populations.

Cells were prepared from the spleens of WT and $\text{Sema4B}^{-/-}$ mice, stained with various antibodies and then analyzed by flow cytometry.

Supplemental Figure 2. $\text{Sema4B}^{-/-}$ T cells, B cells and DCs have normal proliferation, cytokine production and cellular interactions. *A, B*, Normal proliferative responses in $\text{Sema4B}^{-/-}$ B cells and T cells. Splenic B cells from WT (open circles) and $\text{Sema4B}^{-/-}$ (closed circles) mice were cultured with various concentrations of anti- μ or anti-CD40 for 36 h (*A*). Splenic CD4^+ T cells from WT (open circles) and $\text{Sema4B}^{-/-}$ (closed circles) mice were cultured with immobilized anti-CD3 with or without anti-CD28 for 48 h (*B*). [^3H] thymidine was added for the last 14 h. *C*, Th1/Th2 skewing was not impaired in $\text{Sema4B}^{-/-}$ T cells. WT (open circles) or $\text{Sema4B}^{-/-}$ (closed circles) naïve $\text{CD62L}^+\text{CD4}^+$ T cells were cultured under Th1- (left panel) or Th2- (right panel) skewing conditions for 5 days. Then, CD4^+ T cells were restimulated with immobilized anti-CD3 for 24 h. The cytokine concentrations in the culture supernatants were measured by ELISA. *D*, Normal cytokine production of $\text{Sema4B}^{-/-}$ DCs. Splenic DCs (left panel) or BMDCs (right panel) from WT (open circles) or

Sema4B^{-/-} (closed circles) mice were stimulated with anti-CD40 for 48 h. The concentration of IL-12p40 in the culture supernatants was measured by ELISA. *E*, Sema4B was not involved in normal MLRs between T cells and DCs. CD4⁺ T cells from BALB/c mice were cultured with irradiated allogeneic C57BL/6 WT (open circles) or Sema4B^{-/-} (closed circles) DCs for 60 h (upper panel). CD4⁺ T cells from C57BL/6 WT (open circles) or Sema4B^{-/-} (closed circles) mice were cultured with irradiated allogeneic BALB/c DCs for 60 h (lower panel). [³H] thymidine was added for the last 14 h.

Supplemental Figure 3. rSema4B does not affect B cells, T cells and DCs in terms of proliferation, cytokine production and antibody production. B cell proliferation (*A*), B cell antibody production (*B*), T cell proliferation (*C*), splenic DC (left panel) and BMDC (right panel) cytokine production (*D*) and MLRs between CD4⁺ T cells and allogeneic DCs (*E*) were analyzed in the presence of rSema4B (closed bars or circles) or hIgG (open bars or circles). *F*, rSema4B did not affect DC-mediated Th1/Th2 differentiation. Naïve CD62L⁺CD4⁺ T cells derived from OVA-TCR Tg mice and BMDCs were cultured with OVA peptide for 4 days in the presence of rSema4B (closed circles) or hIgG (open circles). Then, CD4⁺ T cells were isolated and stimulated with

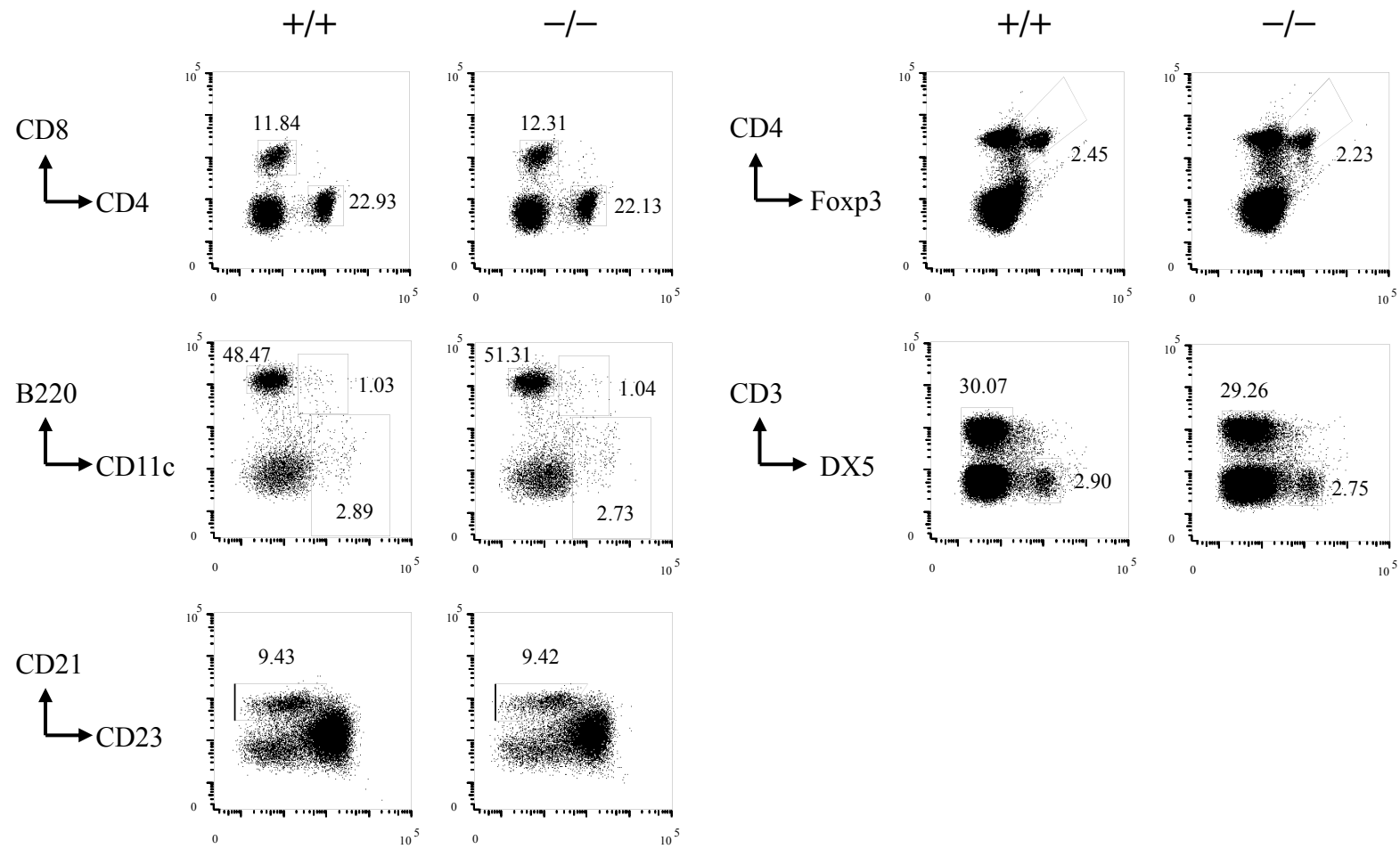
immobilized anti-CD3. The concentrations of IFN- γ (left panel) and IL-4 (right panel) in the culture supernatants were measured by ELISA.

Supplemental Figure 4. rSema4B affects basophils in a dose-dependent manner.

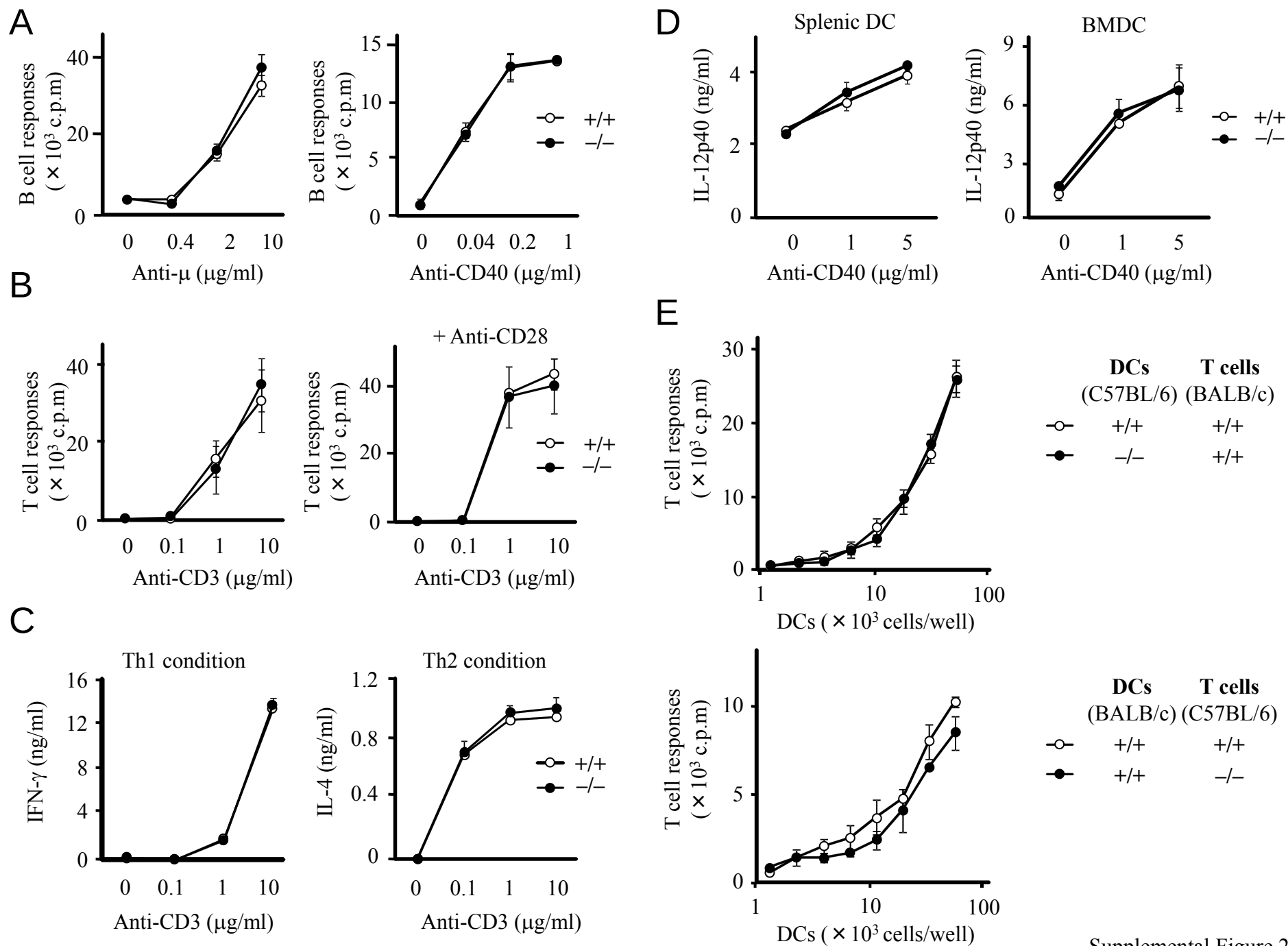
BM-derived basophils were stimulated with IL-3 in the presence of various concentrations of rSema4B (closed and semi-closed bars) or hIgG (open and semi-open bars) and then examined for IL-4 production.

Supplemental Figure 5. Sema4B^{-/-} basophils have normal IL-4 production. WT

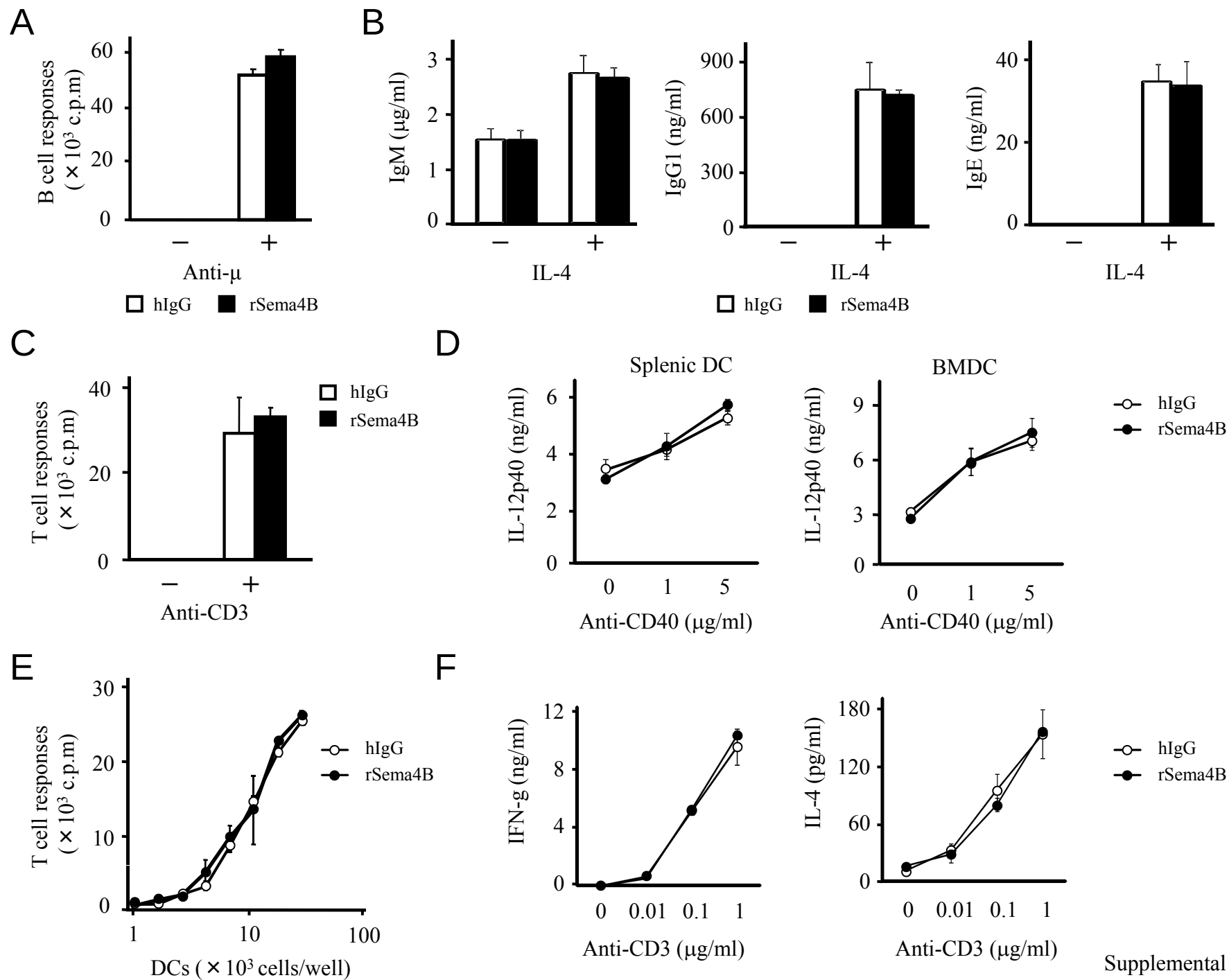
(open bars) or Sema4B^{-/-} (closed bars) BM-derived basophils were cultured with IgE anti-DNP plus various concentrations of DNP-HSA for 16 h. The cytokine concentrations in the culture supernatants were measured by ELISA.



Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

