

An Inhibitory Role for Sema4A in Antigen-Specific Allergic Asthma

Tetsuo Morihana · Sho Goya · Masayuki Mizui ·
Teruhito Yasui · Durubaka V. R. Prasad · Atsushi Kumanogoh ·
Manabu Tamura · Takashi Shikina · Yohei Maeda ·
Yoriko Iwamoto · Hidenori Inohara · Hitoshi Kikutani

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Abstract

Purpose The class IV semaphorin Sema4A is critical for efficient T_H1 differentiation and *Sema4a*^{-/-} mice exhibit impaired T_H1 immune responses. However, the role of Sema4A in T_H2 cell-mediated allergic diseases has not been fully studied. The aim of this study was to clarify the regulatory role possessed by Sema4A in mouse models of allergic diseases, particularly allergic asthma.

Methods *Sema4a*^{-/-} mice on a BALB/c background were examined for the development of allergic diseases. To induce experimental asthma, mice were sensitized with ovalbumin (OVA) followed by intranasal challenges with OVA. After challenge, airway hyperreactivity (AHR) and airway inflammation were evaluated. The role of Sema4A in asthma was examined using *Sema4a*^{-/-} mice and Sema4A-Fc fusion

proteins. The direct effects of Sema4A-Fc on antigen-specific effector $CD4^+$ T cells were also examined.

Results A fraction of *Sema4a*^{-/-} BALB/c mice spontaneously developed skin lesions that resembled atopic dermatitis (AD) in humans. Furthermore, AHR, airway inflammation, and T_H2 -type immune responses were enhanced in *Sema4a*^{-/-} mice compared to wild type (WT) mice when immunized and challenged with OVA. In vivo systemic administration of Sema4A-Fc during the challenge period ameliorated AHR and lung inflammation and reduced the production of T_H2 -type cytokines in WT mice. The inhibitory effects of Sema4A on airway inflammation were also observed in mice deficient in Tim-2, a Sema4A receptor. Finally, we showed that Sema4A-Fc directly inhibited IL-4-producing OVA-specific $CD4^+$ T cells.

T. Morihana · M. Mizui · T. Yasui · D. V. R. Prasad ·
H. Kikutani (✉)
Department of Molecular Immunology,
Research Institute for Microbial Diseases, WPI Immunology
Frontier Research Center (IFReC), Osaka University,
3-1 Yamadaoka, Suita,
Osaka 565-0871, Japan
e-mail: kikutani@ragtime.biken.osaka-u.ac.jp

S. Goya · A. Kumanogoh
Department of Respiratory Medicine, Allergy and Rheumatic
Diseases, Graduate School of Medicine, Osaka University,
Osaka, Japan

M. Tamura
Osaka Home Care Clinic,
Osaka, Japan

T. Morihana · T. Shikina · Y. Maeda · Y. Iwamoto · H. Inohara
Department of Otorhinolaryngology-Head and Neck Surgery,
Graduate School of Medicine, Osaka University,
Osaka, Japan

Present Address:
S. Goya
Osaka Prefectural Medical Center for Respiratory and Allergic
Diseases,
Osaka, Japan

Present Address:
M. Mizui
Division of Rheumatology, Department of medicine,
Beth Israel Deaconess Medical Center,
Boston, MA, USA

Present Address:
D. V. R. Prasad
Department of Microbiology, Yogi Vemana University,
Kadapa, India

Conclusion These results demonstrate that *Sema4A* plays an inhibitory role in T_H2 -type allergic diseases, such as allergic asthma.

Keywords *Sema4A* · T_H1/T_H2 regulation · allergic asthma

Introduction

Allergic asthma, rhinitis and AD are major allergic diseases, especially in developed countries, and identifying effective therapies for these diseases is highly important [1]. These acute or chronic diseases are thought to be influenced by both genetic and environmental factors [2, 3], and share several common features, such as mast cell or eosinophil infiltration within inflammatory sites, increased T_H2 -type cytokines, and elevated serum IgE levels [4–7].

Among these allergic disorders, asthma is characterized by inflammation in the lower airways in response to a wide variety of agents, such as airborne or dietary allergens, infectious agents and chemical irritants [2, 3, 8]. The hallmarks of allergic asthma are not only typical allergic symptoms mentioned above but also mucus hypersecretion and AHR [4, 5, 9, 10]. Although other cell types, such as T_H17 cells, antigen presenting cells (APCs), and epithelial cells are involved in the pathogenesis of asthma, T_H2 cells are thought to be the essential inducer of allergic asthma [4, 5, 9, 11, 12]. IL-4 produced by T_H2 cells is required for immunoglobulin class-switching to IgE and for the induction and maintenance of strong T_H2 responses. IL-5 is also involved in the activation and recruitment of eosinophils, and IL-13 is critical for goblet cell hyperplasia and AHR [4, 13–15]. Indeed, several reports have shown that immunological T_H1/T_H2 manipulation may be used to treat allergic diseases [9, 16]. Many of these therapeutic effects were due to enhanced T_H1 and suppressed T_H2 immunity.

The semaphorin family is comprised of phylogenetically conserved secreted and membrane-bound proteins that were originally identified as axonal guidance factors. Several members of this family are expressed in the immune system and play roles in various phases of immune responses [17–19]. Among them, the class IV semaphorin *Sema4A* is highly expressed on dendritic cells (DCs) and differentiating T_H1 cells. *Sema4A* is required not only for early T cell activation but also for T_H1 differentiation. T cells from *Sema4a*^{−/−} mice differentiate poorly into IFN- γ -secreting T_H1 cells, and T_H1 immune responses are severely impaired in *Sema4a*^{−/−} mice. *Sema4a*^{−/−} mice also show higher levels of serum IgE than WT mice upon infection with *N. brasiliensis*. However, it remains unclear if moderately elevated T_H2 response in *Sema4a*^{−/−} mice is an indirect consequence of impaired T_H1 responses [20, 21].

In this study, we examined the role of *Sema4A* in T_H2 responses using a model of OVA-specific experimental asthma. In this model, *Sema4a*^{−/−} mice showed enhanced AHR

with increased pulmonary eosinophil infiltration compared to WT mice. The levels of T_H2 -type cytokines in bronchoalveolar lavage (BAL) fluid and antigen-specific IgE in the serum were elevated in asthmatic *Sema4a*^{−/−} mice. Lung samples from asthmatic *Sema4a*^{−/−} mice showed greater infiltration of inflammatory granulocytes than WT mice. Moreover, *Sema4A*-Fc-treated mice displayed suppressed AHR and inflammatory cells infiltration with reduced T_H2 -type cytokine production in the BAL fluid and from lymphocytes in the regional lymph nodes. Furthermore, *Sema4A*-Fc appeared to directly suppress IL-4 production by effector CD4⁺ T cells.

Materials and Methods

Mice

BALB/c WT mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). *Sema4a*^{−/−} mice, which were previously described [20], were backcrossed with BALB/c mice. *Rag2*^{−/−} *Il-2r γ* ^{−/−} mice on a BALB/c background, which lack T cells, B cells, NK cells and NKT cells, were kindly provided by Dr. Ito (Central Institute for Experimental Animals, Yokohama, Japan). OVA-TCR Tg mice on a BALB/c background, which express a transgenic TCR that recognizes a peptide from the chicken egg OVA peptide (323–339) in the context of I-Ad, were kindly provided by Dr. Sonoko Habu (Tokai University, Kanagawa, Japan). *Tim-2*^{−/−} mice on a BALB/c background were kind gift from Dr. Paul Rennert (Biogen-Idec, Boston, MA). To induce experimental asthma, age-matched (8–12 weeks of age) mice were maintained under Specific Pathogen-Free (SPF) conditions. All mice were housed in the Animal Resource Center for Infectious Diseases in the Research Institute for Microbial Diseases, Osaka University, and all experimental procedures were consistent with the institutional guidelines.

Reagents

The *Sema4A*-Fc fusion protein was produced as previously reported [20]. A Detoxi-Gel Endotoxin Removal Gel kit (Thermo Scientific, Waltham, MA) was used to remove endotoxins from the purified protein.

Sensitization and Airway Challenge

All procedures for experimental allergic asthma were in accordance with previously reported methods [22, 23]. Mice were immunized intraperitoneally with 50 μ g of OVA (grade V, Sigma-Aldrich, St. Louis, MO) emulsified in 2 mg of Imject Alum (Pierce Biochemicals, Rockford, IL) on day 0. The sensitized mice were intranasally challenged with 50 μ g of OVA in 50 μ l of saline on days 7, 8 and 9. In the control

group, mice were immunized with OVA and challenged with saline. For the Sema4A-Fc experimental therapy, mice were intravenously injected with 10 µg of Sema4A-Fc (in 100 µl of saline) simultaneously with the OVA challenge. As a control, 5 µg of human IgG isotype control (MBL, Aichi, Japan) was administered instead of Sema4A-Fc.

Determination of Airway Responsiveness (Invasive Method)

Airway function was assessed as follows. Twenty-four hours after the last OVA challenge, the mice were anesthetized, tracheotomized with a 20-gauge cannula, and mechanically ventilated. Airway resistance (R_L) and lung compliance (C_{dyn}) were measured in response to increasing doses of inhaled methacholine chloride (Tokyo Kasei, Tokyo, Japan) using a BUXCO RC system with R/C analyzer and BioSystem XA software (Buxco Research Systems, Wilmington, NC). Mice in the control groups were immunized with OVA and challenged with saline. Data are expressed as the percent change from the baseline values that were obtained after saline inhalation. After measuring AHR, the BAL, BAL fluid, sera, regional lymph nodes, and lung tissues were obtained for further analysis.

BAL Analysis and BAL Fluid Preparation

Immediately after assessing airway function, the lungs were lavage via a trachea tube with 2.5 ml (5×0.5 ml) of saline at room temperature. Isolated BAL cells (5×10^4) were centrifuged using Cytospin4 (Thermo Scientific) and then placed on slides and stained with Diff-Quik (Siemens Healthcare, Malvern, PA). The numbers of macrophages, neutrophils, eosinophils and lymphocytes were manually determined by microscopy (100× magnification). For each mouse, averages of mast cell numbers were calculated in three microscopic sights. The BAL supernatant was used as the BAL fluid to measure cytokine concentrations.

Stimulation of Cells from the Regional Lymph Nodes

To measure cytokine production from the regional lymph node, a single cell suspension was obtained from the bronchial lymph nodes. The cells (1×10^5) were cultured with OVA (1 µM) for 48 h in vitro. The supernatants were used for cytokine measurements. All in vitro cultures were performed in RPMI 1640 medium with 10 % FCS and 1 mM penicillin and streptomycin.

Histological Examination

Tissue samples from skins and lungs were fixed with 10 % formalin and embedded in paraffin using the conventional

method, cut into 5-µm thick sections, and stained with hematoxylin-eosin (H-E) and/or toluidine blue (TB). To visualize the dermal infiltration of mast cells, the number of cells in a microscopic sight (200× magnification) were counted manually. For each mouse, averages of mast cell numbers were calculated in three microscopic sights.

Measurement of Cytokines and IgE Levels

Cytokine levels were measured using mouse ELISA kits (R&D Systems, Minneapolis, MN) or the BioPlex Suspension Array System (Bio-Rad Laboratories, Benicia, CA). The concentrations of total IgE or OVA-specific IgE in the sera were evaluated using a mouse IgE ELISA kit (R&D Systems) or OVA-IgE ELISA kit (Shibayagi, Gunma, Japan), respectively.

Transfer Experiments and Ex Vivo Culture

Naïve $CD4^+$ T cells (3×10^6) from OVA-TCR Tg mice were injected intravenously into recipient *Rag2^{-/-}Il-2rγ^{-/-}* mice on day 0. On day 1, the mice were immunized intraperitoneally with OVA as described above. The spleens and peritoneal lymph nodes were collected on day 8 and $CD4^+$ T cells were purified using autoMACS with anti-mouse $CD4$ microbeads (L3T4) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) or a Murine T Cell $CD4$ Subset Column Kit (R&D Systems). $CD4^+$ T cells (1×10^5) were stimulated with OVA peptide^{323–339} (MBL)-loaded bone marrow DCs (1×10^6), which were obtained from bone marrow cells cultured with GM-CSF (eBioscience, San Diego, CA), with or without Sema4A-Fc (10 µg/ml) for 48 h to evaluate IL-4 production. As a control, human IgG (5 µg/ml) was used instead of Sema4a-Fc.

In Vitro Differentiation of Primary T_H1 and T_H2 Cells

$CD4^+$ and $CD62L^{high}$ naïve $CD4^+$ T cells were collected from OVA-TCR Tg mouse spleens using a FACSaria (BD Biosciences, Franklin Lakes, NJ) sorting system or a mouse $CD4$ naïve T cell column kit (R&D Systems). T cell polarization was performed under the following conditions: anti- $CD28$ (5 µg/ml), IL-12 (20 ng/ml) and anti-IL-4 (10 µg/ml) for T_H1 differentiation and anti- $CD28$ (5 µg/ml), IL-4 (20 ng/ml), anti-IL-12 (10 µg/ml) and anti-IFN-γ (10 µg/ml) for T_H2 differentiation in anti- $CD3$ -coated (1 µg/ml) 12-well plates. All mouse cytokines and antibodies (anti-mouse) were purchased from eBioscience.

Evaluation of Binding Ability (Flow Cytometry)

Various stages of in vitro polarized helper T cells and helper T cell clones (T_H1 -clone; 35-9D and T_H2 -clone;

D10.G4.1) were stained with either biotin-conjugated Sema4A or biotin-conjugated human IgG (Isotype-Ig) as a control, followed by Streptavidin-APC (BD Pharmingen). These samples were analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Statistical Analysis

Data are shown as the mean $\pm$ SEM of each group. The statistical analysis was performed using Student's *t*-tests or Welch's *t*-tests. Asterisks indicate statistically significant differences (single asterisk; $p < 0.05$, double asterisks; $p < 0.01$).

Results

Sema4A-Deficient Mice Developed Spontaneous AD-like Skin Lesions

To study the role of Sema4A in T_H2 responses, *Sema4a*^{-/-} mice on a C57BL/6 background were backcrossed onto the

T_H2 -prone BALB/c strain. We noticed that a fraction of male but not female *Sema4a*^{-/-} mice that were housed in a SPF facility developed dermatitis with age (at approximately 30 weeks of age) (Fig. 1a). We observed that 62.5 % of male *Sema4a*^{-/-} mice developed severe AD-like dermatitis, and rest 37.5 % had mild skin changes, and none was intact. Histological analyses of skin lesions revealed severe acanthosis, ulceration and hyperkeratosis (Fig. 1b). Furthermore, diseased *Sema4a*^{-/-} mice had severe mast cell infiltration in skin samples and high concentrations of serum IgE, which are hallmarks of AD (Fig. 1c). It is also noteworthy that these AD-like skin lesions were not observed when each *Sema4a*^{-/-} male mouse was reared separately in distinct cages, suggesting that physical aggression by the male mice at least partially contributed to the development of AD-like skin lesions. These observations prompted us to perform a further detailed analysis of *Sema4a*^{-/-} BALB/c mice using an experimentally-induced acute model of allergic asthma which is one of the most common airway allergic diseases.

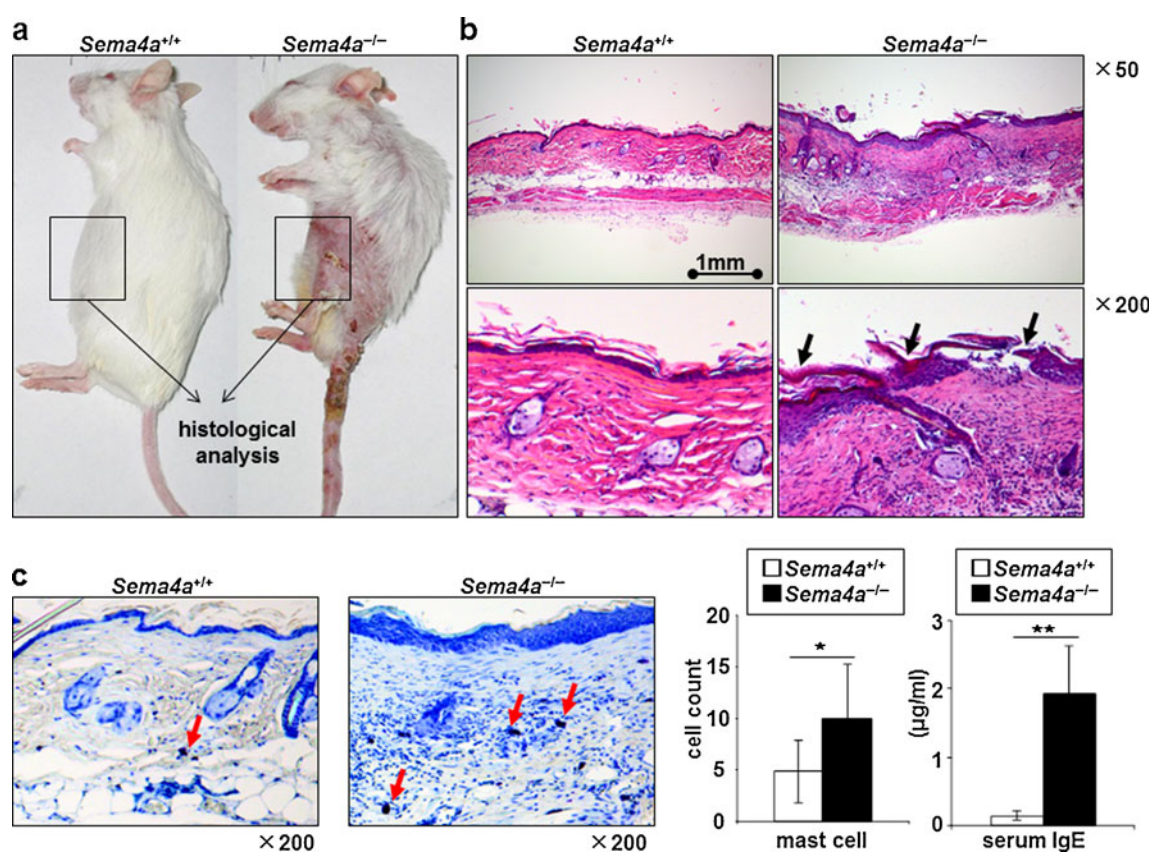


Fig. 1 *Sema4a*^{-/-} mice develop spontaneous AD-like skin lesions. **a** Representative states of skin lesions in *Sema4a*^{+/+} and *Sema4a*^{-/-} male mice maintained in the same cage. **b** H-E staining (50 $\times$ and 200 $\times$ magnification) of a skin sample (acanthosis, ulceration and hyperkeratosis are indicated by black arrows). **c** TB staining of skin samples (left

panels, mast cells are indicated by red arrows). Mast cell numbers were counted by microscopy (center panel, 200 $\times$ magnification). Serum IgE levels are shown in right panel. Data are shown as the mean $\pm$ SEM of 8 mice (mast cells) and 5 mice (serum IgE) per group. Asterisks indicate statistically significant differences (* $p < 0.05$, ** $p < 0.01$)

Sema4a^{-/-} Mice are more Susceptible to OVA-Specific Airway Allergy than WT Mice

To induce antigen-specific AHR and lung inflammation, *Sema4a*^{-/-} and WT mice were immunized intraperitoneally with OVA in alum and then challenged intranasally with OVA for three consecutive days starting on day 7. One day after the last OVA challenge, the mice were analyzed for AHR and airway inflammation (see **Materials and Methods**). Although intranasal challenges with OVA could induce AHR, characterized by increased airway resistance (R_L) and reduced airway compliance (C_{dyn}), in both OVA-sensitized and challenged *Sema4a*^{-/-} and WT mice, the severity of AHR was enhanced in *Sema4a*^{-/-} mice (Fig. 2a). A histological analysis of lung samples also revealed much more severe inflammatory cell infiltration around the small airways in OVA-sensitized and challenged *Sema4a*^{-/-}

mice (Fig. 2b). To quantitatively characterize the inflammatory cells accumulating in the lung, cells in the BAL from these mice were examined. The total number of cells was higher in the BAL from *Sema4a*^{-/-} mice than WT mice. In particular, BAL from *Sema4a*^{-/-} mice contained approximately two-fold more eosinophils, a signature of allergic inflammation, than WT mice (Fig. 2c), but there were no significant differences in other subpopulations. These findings indicate that a *Sema4A* deficiency results in exacerbated AHR and allergic airway inflammation.

T_H2 -Type Immune Responses are Enhanced in *Sema4a*^{-/-} Mice

To determine if exacerbated AHR and inflammation in *Sema4a*^{-/-} mice reflects enhanced T_H2 responses, the cytokine levels in the BAL fluids were analyzed. The BAL fluid of

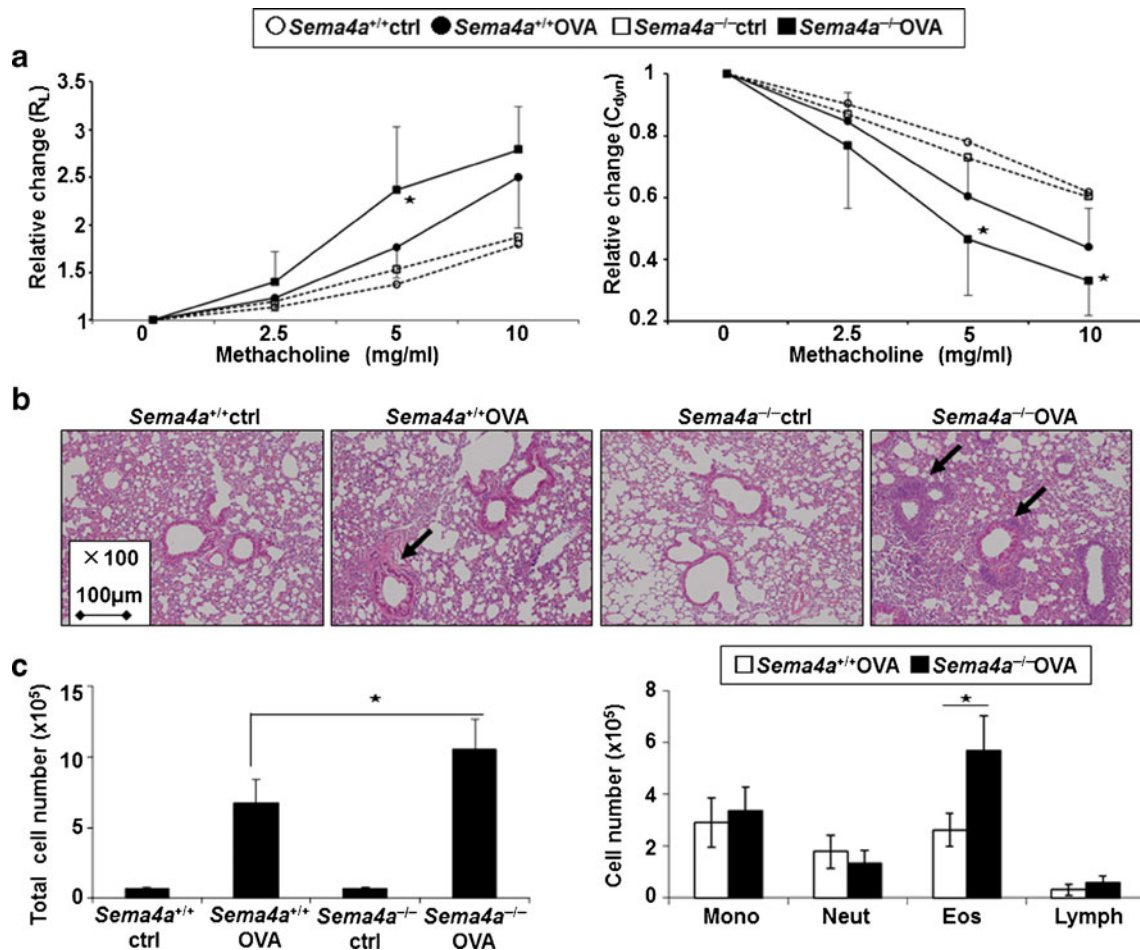


Fig. 2 OVA-sensitized and challenged *Sema4a*^{-/-} mice exhibit severe asthma symptoms. **a** AHR in *Sema4a*^{+/+} and *Sema4a*^{-/-} mice. Airway resistance (left panel, lung resistance, R_L) and lung compliance (right panel, dynamic compliance, C_{dyn}) were measured using an invasive method (see **Materials and Methods**). Data are shown as the mean $\pm$ SEM of 10–12 mice per group. Asterisks indicate statistically significant differences (* p <0.05). **b** H-E staining of asthma-induced lung tissue. The

infiltration of inflammatory cells around small airways and epithelium thickness are shown by arrows. (100 $\times$ magnification). **c** Total BAL cell numbers (left panel) and cellular fractions (right panel). Mono, Monocyte or macrophage; neu, neutrophil; eos, eosinophil; lymph, lymphocyte. Data are the mean $\pm$ SEM of 8 mice (BAL cell numbers) and 3 mice (BAL subpopulations) per group. Data are representative of two independent experiments. Asterisks indicate statistically significant differences (* p <0.05)

Sema4a^{-/-} mice had higher levels of T_H2-type cytokines, such as IL-4, IL-5 and IL-13, than WT mice, while the concentrations of IFN- γ and IL-17 in the BAL fluid were low and below the detection limit in both groups, respectively (Fig. 3a). Then, total cells from the bronchial lymph nodes were stimulated in vitro with OVA to evaluate antigen-specific cytokine production. Lymph node cells from *Sema4a*^{-/-} mice produced greater IL-4 and IL-5 levels and reduced IL-13 levels than lymph node cells from WT mice (Fig. 3b). The serum levels of OVA-specific IgE were also higher in asthmatic *Sema4a*^{-/-} mice than in WT mice (Fig. 3c). These results indicate that enhanced production of T_H2-type cytokines can contribute to enhanced allergic asthma of *Sema4a*^{-/-} mice in this experimental model.

Sema4A-Fc Administration Ameliorates Experimental Asthma with Suppressed T_H2 Immune Responses

To test the effects of exogenous Sema4A on OVA-induced AHR and airway inflammation, the Sema4A-Fc protein was

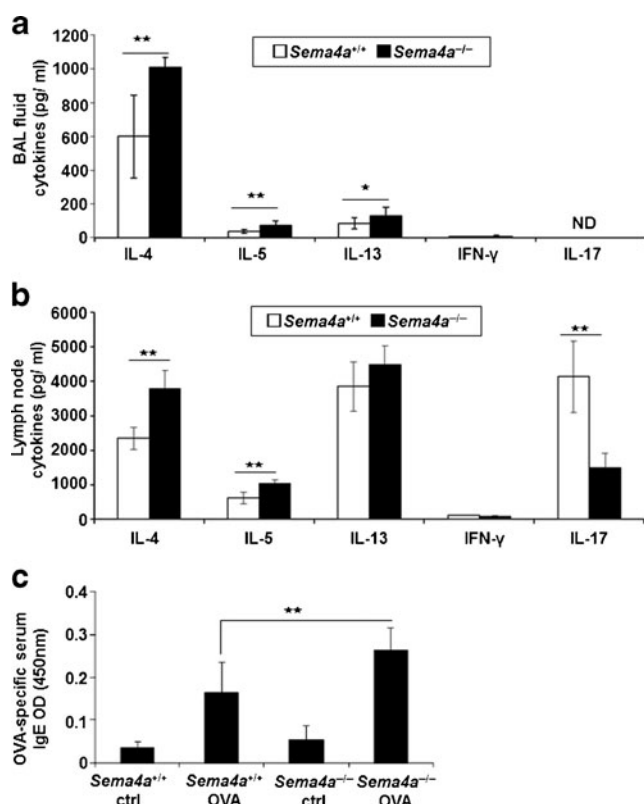


Fig. 3 T_H2-type immune responses are dominant in OVA-challenged *Sema4a*^{-/-} mice. **a** Levels of cytokines in the BAL fluid. ND, not detected. Data are shown as the mean $\pm$ SEM of at least 7 mice per group. Asterisks indicate statistically significant differences (* p <0.05, ** p <0.01). **b** Cytokine production from regional lymph node cells. Data are shown as the mean $\pm$ SEM of 5 mice per group. Asterisks indicate statistically significant differences (** p <0.01). **c** Levels of OVA-specific serum IgE. Data are presented as the mean $\pm$ SEM of 4 mice in *Sema4a*^{+/+}-ctrl, 4 mice in *Sema4a*^{-/-}-ctrl, 9 mice in *Sema4a*^{+/+}-OVA, and 12 mice in *Sema4a*^{-/-}-OVA. Asterisks indicate statistically significant differences (** p <0.01)

intravenously administered to OVA-sensitized WT BALB/c mice during the challenge period (see **Materials and Methods**). Mice that received Sema4A-Fc exhibited attenuated AHR (Fig. 4a). Sema4A-Fc-treated mice also had significantly reduced numbers of infiltrating cells, particularly eosinophils, in the BAL fluid. There were no remarkable differences in other subpopulations (Fig. 4b). Furthermore, Sema4A-Fc administration resulted in decreased levels of T_H2-type cytokines produced not only in the BAL fluid but also from in vitro stimulated lymph node cells (Fig. 4c). Together, these results suggest that exogenous Sema4A has a therapeutic effect on allergic asthma by directly or indirectly suppressing T_H2 immune responses.

Sema4A-Fc Directly Suppresses IL-4 Production from Effector T_H2 Cells

Our previous study showed that Sema4A is necessary for the efficient development of T_H1 responses [21]. However, the suppressive effects of Sema4A-Fc on AHR and airway inflammation also suggest that Sema4A-Fc could directly inhibit T_H2 functions. Thus, we analyzed the effects of Sema4A on allergen-specific T cells that were differentiated in vivo. Naïve CD4⁺ T cells from OVA-TCR Tg BALB/c mice were adoptively transferred to *Rag2*^{-/-}*Il-2 γ* ^{-/-} BALB/c mice and then the mice were immunized with OVA. After 1 week, CD4⁺ T cells collected from the spleens and regional lymph nodes of the recipients were stimulated with bone marrow-derived DCs and OVA peptide in the presence or absence of Sema4A-Fc for 48 h (see **Materials and Methods**). Sema4A-Fc selectively and significantly suppressed IL-4 production from CD4⁺ T cells, and no significant change was seen in IL-5, IL-13, IL-17 and IFN- γ (Fig. 5a). Furthermore, to determine whether Sema4A-Fc directly bound to helper T lymphocytes, we performed flow cytometry using primary T_H1 and T_H2 polarized cells and T_H1 and T_H2 cell clones. Sema4A-Fc preferentially bound to T_H2 cells as T_H2 polarization occurred (Fig. 5b). This result supports the hypothesis that Sema4A-Fc directly suppresses the functions of T_H2 cells.

Sema4A-Fc Ameliorates Airway Inflammation even in *Tim-2*^{-/-} Mice

Sema4A interacts with multiple receptor molecules including Plexins as well as Tim-2 [19, 20]. Notably, Tim-2 can be induced on activated T cells [20]. Thus, to determine whether Sema4A exerts its inhibitory activity on allergic airway inflammation through Tim-2, we tested the effects of Sema4A-Fc in the asthma model as in Fig. 4 using *Tim-2*^{-/-} mice on a BALB/c background. Sema4A-Fc attenuated airway inflammation and T_H2 immune responses even in *Tim-2*^{-/-} mice (Fig. 6a, b). This result implies that Sema4A may have a

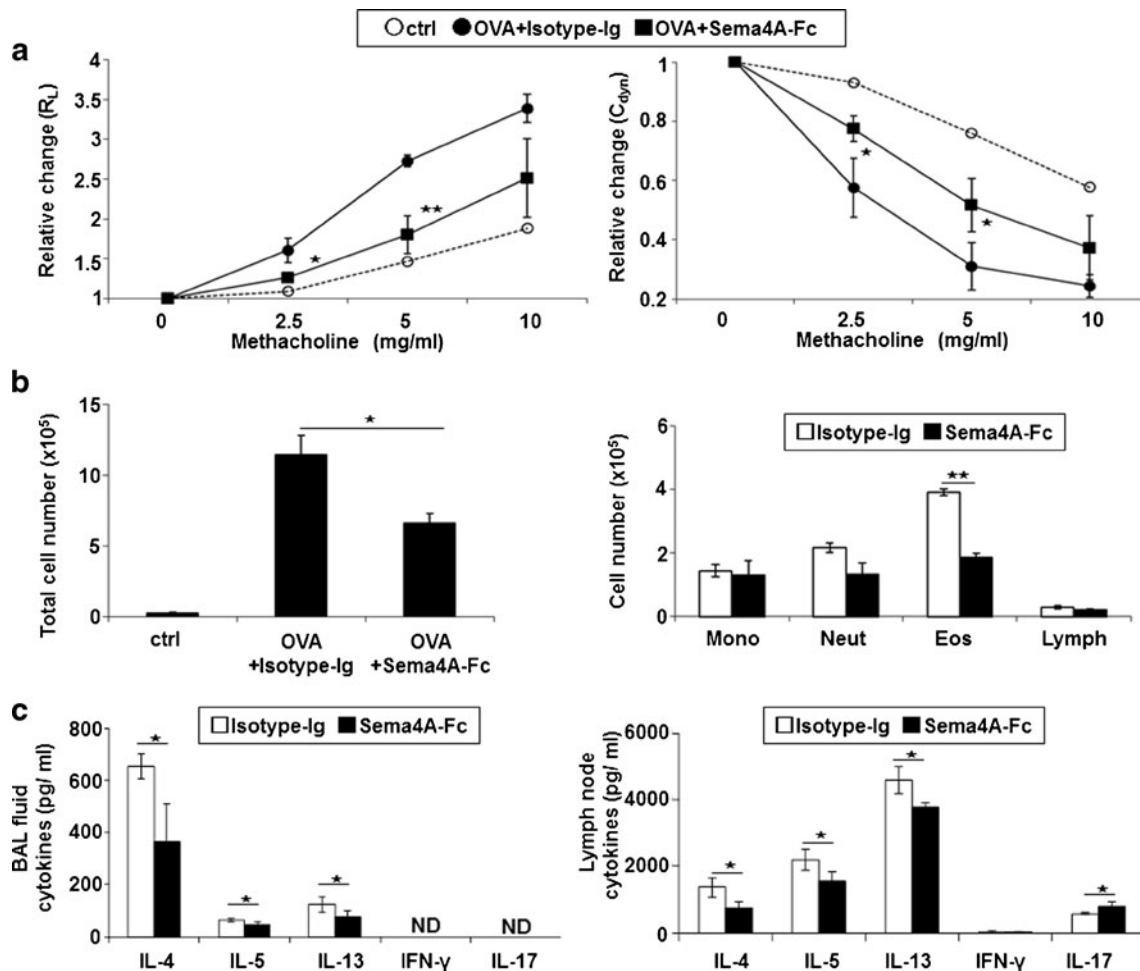


Fig. 4 Systemic administration of Sema4A-Fc ameliorates experimental asthma by suppressing T_H2 immune responses. **a** WT mice were challenged intranasally with either OVA (shown as OVA) or PBS (shown as ctrl) 7 days after OVA immunization. In the challenge phase, OVA-challenged mice were injected intravenously with either Sema4A-Fc (Sema4A-Fc) or human IgG (Isotype-Ig). Airway resistance (left panel) and lung compliance (right panel) were measured using an invasive method. Data are shown as the relative changes from baseline. Data are shown as the mean $\pm$ SEM of 4 mice per group. Data are representative of two independent experiments. Asterisks indicate statistically significant differences (* p <0.05, ** p <0.01). **b** The cell

numbers in the BAL (left panel) and the subpopulations in the BAL (right panel). Mono, Monocyte or macrophage; neu, neutrophil; eos, eosinophil; lymph, lymphocyte. Data are shown as the mean $\pm$ SEM of 3 mice (left panel) and 5 mice (right panel) per group. Data are representative of two independent experiments. Asterisks indicate statistically significant differences (* p <0.05, ** p <0.01). **c** Cytokine levels in the BAL fluid and from regional lymph node cells. ND, not detected. Data are shown as the mean $\pm$ SEM of 4 mice per group. Data are representative of two independent experiments. Asterisks indicate statistically significant differences (* p <0.05)

distinct functional receptor from Tim-2 in this mouse model of experimental acute asthma.

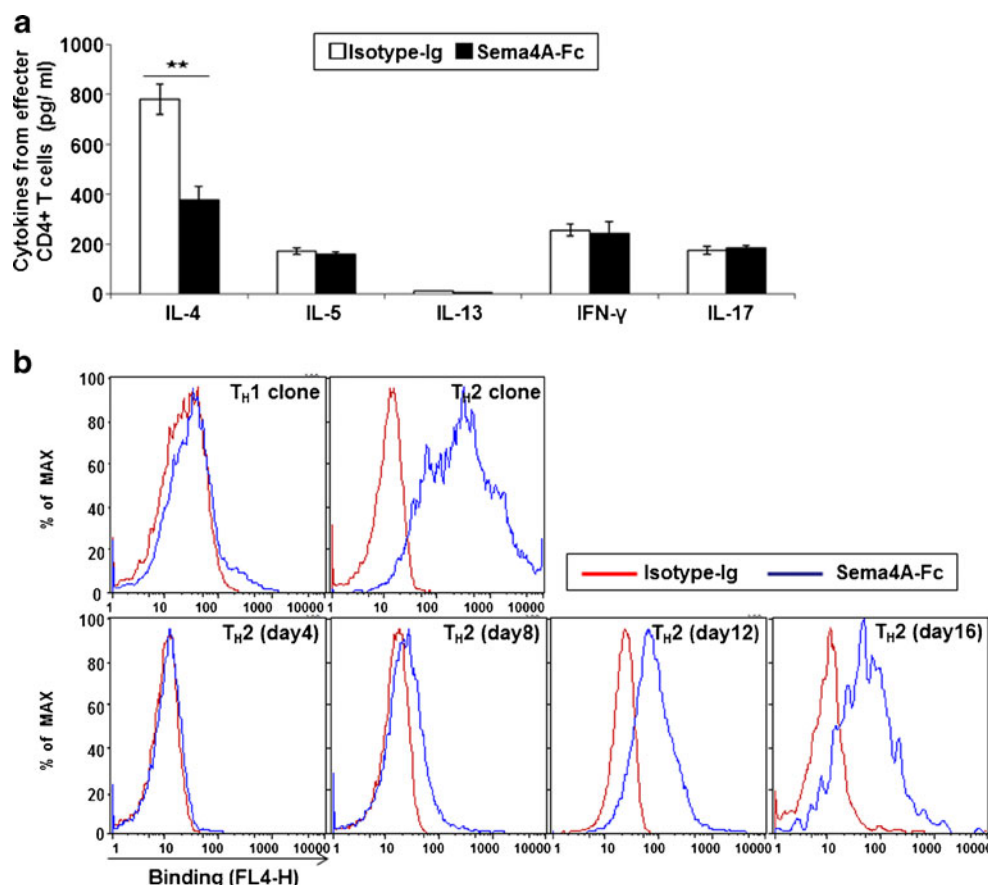
Discussion

In the present study, we explored the role of Sema4A in T_H2 immune responses using *Sema4a*^{-/-} mice on a BALB/c background. *Sema4a*^{-/-} BALB/c mice were not only prone to AD-like skin lesions but also showed enhanced AHR and airway inflammation when immunized and challenged with OVA. Furthermore, systemic administration of Sema4A-Fc suppressed AHR and airway inflammation in WT BALB/c mice.

These findings demonstrate that Sema4A plays an inhibitory role in allergic responses, particularly in allergen-induced AHR and airway inflammation.

T_H2 cell-mediated immune responses as well as T_H17 cell-mediated neutrophil recruitment have been shown to be involved in the pathogenesis of mouse models of asthma [24–26]. In this study, we employed a short-term protocol suitable to analyze acute airway inflammation that is predominantly mediated by T_H2 cells. Indeed, higher levels of T_H2 -type cytokines were observed in *Sema4a*^{-/-} mice, while IL-17 was not detectable in the BAL fluid of either *Sema4a*^{-/-} or WT mice. Furthermore, IL-17 production within regional lymphocytes was lower in *Sema4a*^{-/-} than in WT mice.

Fig. 5 Exogenous Sema4A exerts therapeutic effects via effector CD4⁺ T cells. **a** OVA-specific T_H2 cells were re-stimulated with OVA peptide-loaded bone marrow DCs with or without Sema4A-Fc. Cytokine production from effector T_H2 cells was examined in vitro. Data are the mean $\pm$ SEM of 3 mice per group. Data are representative of three independent experiments. Asterisks indicate statistically significant differences (** p <0.01). **b** The ability of Sema4A to bind to helper T cells at various polarizing stages was evaluated by flow cytometry. Sema4A binding to helper T cell clones (*upper panel*) and primary polarized helper T cells (*lower panel*) are shown. Red line, binding of Isotype-Ig; blue line, binding of Sema4A-Fc



Therefore, exacerbated AHR and airway inflammation in *Sema4a*^{-/-} mice in this model appeared to be due to enhanced T_H2 responses.

Our previous study showed that Sema4A has a role in T_H1 differentiation [21], suggesting that enhanced AHR and T_H2-type cytokine production may be due to a lack of counteraction by T_H1 cells in *Sema4a*^{-/-} mice. However, IFN-γ was not detectable in either *Sema4a*^{-/-} or WT mice treated with OVA. Administering Sema4A-Fc during the challenge phase suppressed both AHR and T_H2-type cytokine production but did not induce IFN-γ production. Sema4A-Fc also suppressed IL-4 secretion from OVA-specific IL-4-producing cells in vitro.

These findings strongly suggest that Sema4A has a direct inhibitory effect on T_H2 cells. Indeed, Sema4A bound preferentially to T_H2-polarized cells. On the other hand, it was previously shown that Sema4A is expressed on T_H1-polarized cells and DCs [21], and in inflammatory lung tissues [27]. Therefore, Sema4A expressed on APCs, infiltrating T_H1 cells and lung tissue cells may play an inhibitory role in allergic lung inflammation by suppressing T_H2 function.

Like other semaphorins, Sema4A interacts with several Plexin molecules such as members of the PlexinB subfamily and PlexinD1 [18, 19]. Sema4A has also been shown to bind to Tim-2 expressed on activated T cells in the immune

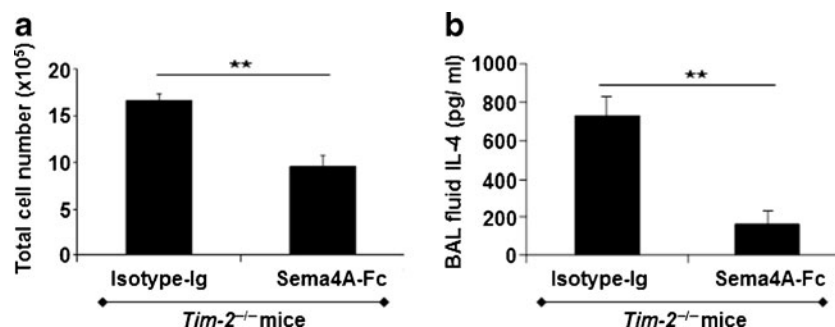


Fig. 6 Sema4A-Fc exerts therapeutic effects on airway inflammation independent of Tim-2. **a** Total cell numbers in the BAL and **b** IL-4 concentrations in the BAL fluid from OVA-challenged *Tim-2*^{-/-} mice.

Data are the mean $\pm$ SEM of 3 mice per group. Data are representative of two independent experiments. Asterisks indicate statistically significant differences (** p <0.01)

system [20]. Furthermore, Freedman and colleagues reported that polymorphisms within the *Tim* locus were highly linked to AHR in laboratory mouse strains [28]. Moreover, it was recently shown that *Tim-2*^{-/-} mice develop enhanced airway allergic inflammation compared to WT mice [29]. Thus, one attractive possibility is that Sema4A suppresses allergic lung inflammation by binding to Tim-2 on T_H2 cells. However, the suppressive effects of Sema4A-Fc were also observed in *Tim-2*^{-/-} mice, indicating that Sema4A exerts its inhibitory activity on allergic responses through receptor molecules other than Tim-2. Further studies will be necessary to determine what molecules, including Plexins, are responsible for this activity of Sema4A.

T_H2 cells as well as epithelial cells and APCs are involved in the pathogenesis of allergic asthma. In fact, cytokines such as TSLP, IL-25 or IL-33 derived from these cells have been reported to promote airway allergy in mice [12, 30, 31]. Moreover, nuocytes, newly identified type 2 innate lymphoid cells (ILCs) have been shown to produce much IL-5 and IL-13 and little IL-4. They are reported to reside in inflammatory site and are hardly found in LNs, and are supposed to assist T_H2-type immune responses such as AHR [32, 33]. Although Sema4A-Fc could suppress IL-4 production of induced effector T cells in vitro, we cannot exclude a possibility that Sema4A may also be involved in the regulation of IL-5 and IL-13 secretion from type 2 ILCs. At present, it remains unknown if Sema4A might influence the functions of these cells, which is necessary to be elucidated in future.

Despite the complexity of asthma pathogenesis, several experimental therapies have been reported for airway allergic diseases. For example, administering IL-27, bisperoxovanadium and a Janus kinase inhibitor attenuated airway inflammation or AHR in mouse models of asthma [34–36]. Furthermore, GATA-3 antisense oligonucleotides, adenovirus-mediated *IL-18* gene transfer and T-bet overexpression in transgenic mice were shown to have therapeutic efficacy in mouse asthma models [37–39]. Most successful therapies, like those described above, are designed to modify the T_H1/T_H2 immune balance to favor T_H1 responses as demonstrated in this article.

Recently, involvement of Sema4A in helper-T regulation has been shown in not only mouse but also human pathology. Actually, a report has exhibited that higher concentrations of soluble Sema4A were detected in sera from patients of multiple sclerosis than normal controls [40]. Similarly, to see the involvement of Sema4A in human allergic diseases, it will be necessary to evaluate the Sema4A expression in human specimen such as lymphocytes or lung tissues.

In conclusion, Sema4A regulates allergic responses by inhibiting T_H2 cells directly in antigen-specific asthma model. This study provides new insights into mechanisms of allergic immune responses and identifies Sema4A as a potential

therapeutic target. Further study would lead to development of Sema4A-targeted novel immunotherapy in human allergic diseases.

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