



Sema4D is required in both the adaptive and innate immune responses of contact hypersensitivity



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ABSTRACT

Originally recognized as a regulator of axon guidance in the nervous system, Semaphorin 4D (Sema4D, CD100) also participates in various immune responses and many immune-related diseases. However, whether Sema4D is involved in the pathogenesis of contact hypersensitivity (CHS) remains unclear. In this study, we explored the role of Sema4D in oxazolone-induced CHS using Sema4D knockout (KO) mice. We found that Sema4D KO mice developed attenuated CHS responses, as indicated by milder ear-swelling, lower expression of IL-1 β , IL-6, CXCL2 and CXCL5, and decreased recruitment of neutrophils, CD8⁺ T cells and CD4⁺ T cells. CHS was impaired in the wide type (WT) mice reconstituted with bone marrow from Sema4D KO mice, indicating that deletion of Sema4D gene in hematopoietic cells played a key role in the alleviated CHS in Sema4D KO mice. CHS was also attenuated in the WT mice transferred with draining lymph nodes (dLNs) cells from oxazolone-sensitized Sema4D KO mice, and the activation and differentiation of hapten-specific CD8⁺ T cells were impaired in Sema4D KO mice. Furthermore, Sema4D KO mice expressed less IL-1 β and CXCL2 than WT mice after oxazolone sensitization, and after transferred with dLNs cells from oxazolone-sensitized WT mice, naïve Sema4D KO mice showed attenuated CHS responses upon oxazolone challenge, indicating that the innate immune response of CHS in Sema4D KO mice was also abrogated. Taken together, our findings revealed for the first time that Sema4D positively regulated both the adaptive and innate immune responses in CHS.

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1. Introduction

Allergic contact dermatitis (ACD), a common skin disease characterized by itching, erythema, vesicles and blisters, is a T-cell dependent delayed hypersensitivity reaction (type IV hypersensitivity) caused by exposure of the skin to hapten. Advances in hapten-induced contact hypersensitivity (CHS), a murine model of ACD, have expanded our understanding of the mechanism of ACD, especially the specific roles of a variety of immune cells (Honda et al., 2013). CHS is considered as a T helper 1 (Th1)/T cytotoxic 1 (Tc1)-dominated skin inflammation, which can be induced by topical application with hapten, small molecule including oxazolone (OXA), dinitrofluorobenzene and fluorescein isothiocyanate. CHS is

composed of the sensitization and the elicitation phases, in which both the adaptive and innate immune responses are involved. During the sensitization phase, the reactive hapten covalently couples to proteins, and the haptenated proteins are captured and processed by cutaneous dendritic cells (DCs), and the DCs then migrate to the draining lymph nodes (dLNs) and prime antigen-specific skin-homing CD8⁺ T cells or CD4⁺ T cells. In the meanwhile, keratinocytes and other skin-resident cells are activated, and these cells produce various chemical mediators, which are required for the migration and maturation of DCs (Kabashima et al., 2003). The elicitation phase is initiated by the innate immune response, and exposure to the same hapten triggers a cascade of events resulting in the infiltration of neutrophils, monocytes and effector T cells into the inflamed tissues, and these cells can induce inflammation by producing large amounts of pro-inflammatory cytokines and chemokines (Martin et al., 2011). However, answers are elusive regarding how the immunoregulatory molecules are involved in the adaptive and innate immunity in CHS, and further investi-

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gations on the roles of these molecules in the pathogenesis of CHS are in demand.

Semaphorins are a family of membrane proteins that originally characterized as guidance molecules in the nervous system, and increasing evidences indicated that many members of semaphorins may also play important roles in the immune system (Kumanogoh and Kikutani, 2013). Semaphorin4D (Sema4D, also known as CD100) is the first semaphorin (Hall et al., 1996) that has been proved as an immunoregulatory molecular with diverse functions in immune system (Zhang et al., 2013). Sema4D is expressed on a wide range of cells, including multiple type of cells in nervous and epithelial tissue (Negishi-Koga et al., 2011; Soong et al., 2012; Kruger et al., 2005). In the immune system, Sema4D is expressed with high levels on T cells (Liu et al., 2015), activated B cells, macrophages, and dendritic cells (DCs) (Liu et al., 2015). Sema4D plays multiple roles in immune responses including antigen-specific T cell priming (Kumanogoh et al., 2002), antibody production (Shi et al., 2000) and cell-to-cell adhesion (Luque et al., 2015; Zhu et al., 2007). Studies using Sema4D-deficient (Sema4D KO) mice reveals that Sema4D is required in experimental autoimmune encephalomyelitis (EAE) (Okuno et al., 2010), experimental glomerulonephritis (Li et al., 2006) and lung allergic inflammation (Shanks et al., 2013). It's also demonstrated that Sema4D can promote the wound healing (Witherden et al., 2012) and the recovery of dextran sulfate sodium-induced colitis (Meehan et al., 2013) through modulating the function of $\gamma\delta$ T cells. However, to our knowledge, the relationship between Sema4D and CHS has not yet been discussed, and it still remains to be determined whether Sema4D is involved in the pathogenesis of CHS.

In the present study, we explored the role of Sema4D in CHS using Sema4D KO mice, and our data showed that CHS was significantly attenuated in Sema4D-deficient mice compared to wild type (WT) mice. Activation and differentiation of CD8⁺ T cells in CHS were impaired in Sema4D-deficient mice; moreover, deletion of Sema4D induced attenuated responses of innate cells in both the sensitization and elicitation phases of CHS. Taken together, our study reveals for the first time that Sema4D participates in both the sensitization and the elicitation phase of CHS through activating the adaptive and innate immune responses.

2. Methods

2.1. Mice

Sema4D KO mice were kindly provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan, which was originally produced by Shi et al. (Shi et al., 2000). Sema4D KO mice and C57BL/6 mice (CD45.2 and CD45.1) were housed in specific pathogen-free conditions. All animal experiments were performed in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals of the University. 8–12-week-old female mice were used in experiments.

2.2. OXA-induced CHS model

Mice were sensitized by epicutaneous application of 100 μ l of 3.0% oxazalone (dissolved in anhydrous ethanol, Sigma-Aldrich, St. Louis, MO, USA) on shaved abdomen. Five days later, baseline ear thickness of each mice was measured, and mice were then challenged with 10 μ l of 1% OXA or ethanol on both sides of one ear. Ear thickness was measured 24 h after the elicitation using an engineer's micrometer caliper.

Table 1

Primers for the analysis of mRNAs.

IL-1 β	Forward	5'-GCCACCTTTTGACAGTGATG-3'
	Reverse	5'-AAGGTCCACGGGAAAGACAC-3'
IL-6	Forward	5'-TGCAAGAGACTTCCATCCAGT-3'
	Reverse	5'-CTGCAAGTGACATCATCGTTGT-3'
IL-17A	Forward	5'-TCTGTGTCTCTGATGCTGTTGC-3'
	Reverse	5'-ATCAGGGTCTTCATTGCGGT-3'
IFN- γ	Forward	5'-ACTGGCAAAGGATGGTGAC-3'
	Reverse	5'-ACCTGTGGTGTGTTGACCTC-3'
TSLP	Forward	5'-AGGGGCTAAGTTCGAGCAAA-3'
	Reverse	5'-CGTCATTTCTCTCAGTTTCAGGG-3'
CXCL2	Forward	5'-CAACCACCAAGGCTACAGGG-3'
	Reverse	5'-GTTAGCCTTGCCTTTGTTCACT-3'
CXCL5	Forward	5'-CATCTAGCTGAAGCTGCCCC-3'
	Reverse	5'-AGCTTTCTTTTGTCACTGCC-3'
T-bet	Forward	5'-ACAAGTACCAGCCGCGATTTC-3'
	Reverse	5'-TGCTTCTTTTCTCCGACG-3'
GATA-3	Forward	5'-GAGATGGTACCGGGCAGTAC-3'
	Reverse	5'-GGTCCCCATTAGCGTTCCTC-3'
Hprt	Forward	5'-TGGATACAGGCCAGACTTTG-3'
	Reverse	5'-GATTCAACTTCGCTCATCTTA-3'

2.3. Histology

Briefly, mice were sacrificed and ear tissues were collected 24 h after the elicitation and fixed in 10% formalin for 12 h. After fixation, tissue samples were embedded in paraffin blocks, sectioned at 4 μ m thickness and stained with haematoxylin and eosin (H&E).

2.4. Quantitative real-time PCR analysis

Total RNAs were isolated using TRIzol Reagent (Invitrogen, Life Technologies, CA, USA) according to the manufacturer's protocol. cDNAs were synthesized from 2 μ g of total RNA using Prime ScriptTM RT Master Mix (Takara, Dalian, China), and quantitative real-time PCR (qRT-PCR) was performed on CFX384 Real time PCR detection system (Bio-Rad, Hercules, CA, USA) using SYBR Premix Ex Taq II (Takara, Dalian, China). The thermocycling profiles were as follows: 95 °C for 1 min, followed by 40 cycles of 95 °C for 15s, 60 °C for 15s, and 72 °C for 30s, followed by dissociation curve analysis to verify the amplification of a single product. The primers (Table 1) were synthesized by Sangon Inc (Shanghai, China). The relative expression of the genes was normalized to the levels of Hprt.

2.5. Preparation of single-cell suspension

Briefly, the skin of each ear was mechanically separated into two halves, and then minced with scissors into small pieces and digested for 40 min at 37 °C using 2 mg/ml collagenase type 11, 500 μ g/ml hyaluronidase and 100 μ g/ml DNase (all from Sigma-Aldrich, St. Louis, MO, USA). The digested tissue was then mashed over a 70 μ m filter and washed with culture media to obtain the single-cell suspension. Single-cell suspension of dLNs was obtained by mechanically dissociated and incubated in 400 U/ml Collagenase D (Roche Applied Science) for 30 min.

2.6. Flow cytometry

Single-cell suspension was stained using antibodies (All purchased from Biolegend, San Diego, CA, USA) as follows: CD8 (clone 53-6.7), Gr-1 (clone RB6-8C5), CD4 (clone GK1.5), CD45.1 (clone A20) and CD45.2 (clone 104). Dead cells were excluded with 7-AAD (Biolegend, San Diego, CA, USA). Fc receptors were blocked using Fc Blocker (clone 2.4G2, Biolegend, San Diego, CA, USA). Absolute cell counts for quantifying cell populations in the suspension were performed using CountBrightTM Absolute Counting Beads (Invitrogen,

Carlsbad, CA, USA). All FACS analyses were completed on a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) and were analyzed using FlowJo software (Tree star, Ashland, TN, USA). In addition, CD8⁺ T cells were isolated from single-cell suspension of dLNs using a MoFlo XDP sorter (Beckman Coulter, Brea, CA, USA).

2.7. Establishment of bone marrow chimera mice

Recipient mice were irradiated with 900 rads of gamma irradiation; on the same day donor bone marrow (BM) cells were isolated from the femur and tibia of donor mice and red blood cells were lysed with red blood cells lysis buffer. Each recipient was transferred with 5×10^6 WT or Sema4D-deficient BM cells through intravenous injection. Eight weeks after bone marrow transplantation, BM chimera mice were used in experiments.

2.8. Adoptive transfer

Adoptive transfer of dLNs cells was performed as previously described with some modifications (Kaplan et al., 2005). In brief, Sema4D-deficient mice, WT mice (CD45.2 or CD45.1) were sensitized with OXA. Five days after sensitization, single-cell suspension of dLNs cells was obtained, and naïve mice were injected intravenously with 5×10^7 dLNs cells. Two hours later, baseline ear thickness was measured and the mice were challenged with 10 μ l of 1.0% OXA on both sides of one ear. Ear thickness was measured 24 h later and the ear thickness above baseline was calculated.

2.9. Statistical analysis

Statistical analyses were performed using SPSS 17.0 (SPSS Corporation, Chicago, IL, USA). Data are shown as mean $\pm$ standard deviation. A Student's *t*-test or one-way ANOVA with a Tukey post test were used to calculate the statistical significance of the experimental data. The level of significance was set at $P < 0.05$. Single asterisks (*) indicate $p < 0.05$ and double asterisks (**) indicate $p < 0.01$.

3. Results

3.1. Attenuated CHS responses in Sema4D-deficient mice

To investigate the role of Sema4D in CHS, Sema4D-deficient (CD100^{-/-}, KO) mice and WT mice were sensitized and elicited with the hapten oxazolone (OXA). After challenge, the ear thickness of Sema4D KO mice was approximately half of that of WT mice (Fig. 1A). H&E staining of ear specimens obtained 24 h after elicitation revealed that the ears of Sema4D KO mice had less inflammatory cell infiltration and appeared thinner than that of WT mice (Fig. 1B). Cytokines and chemokines in the ears after elicitation were investigated by qRT-PCR and the results revealed that the expression levels of IL-1 β , IL-6, CXCL2 and CXCL5 were significantly lower in Sema4D KO mice compared to WT mice (Fig. 1C). There was no significant difference in the relative expression of IFN- γ or TSLP between the two groups. Moreover, FACS analysis of single-cell suspension indicated that 24 h after OXA challenge, the numbers of infiltrating Gr1⁺, CD8⁺ or CD4⁺ cells (Fig. 2A–B) in the ears of Sema4D KO mice were significantly lower compared to WT mice. Collectively, our data proved that KO of Sema4D attenuated the hapten-induced CHS responses in mice.

3.2. Attenuated CHS response in WT mice reconstituted with BM from Sema4D KO mice

Next, we performed BM chimera experiments to explore whether KO of Sema4D in radio-sensitive hematopoietic cells could

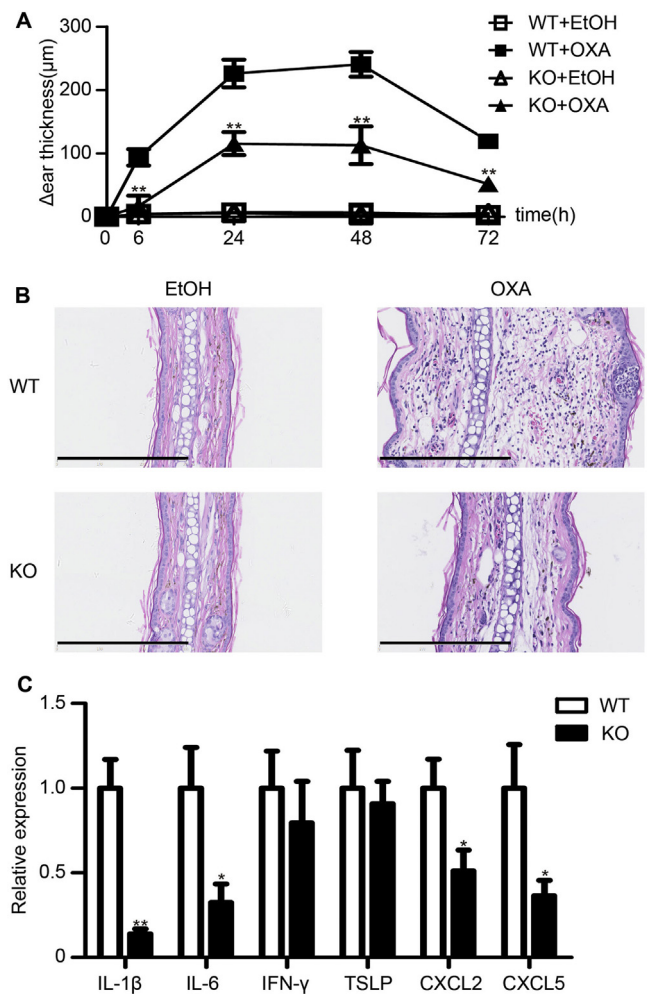


Fig. 1. Attenuated CHS responses in Sema4D-deficient mice. WT and Sema4D KO mice were sensitized with 3% oxazolone. Five days later, the mice were challenged with 1% oxazolone or vehicle (ethanol, EtOH) on the ears. (A) Ear thickness was measured at 6, 24, 48 and 72 h after challenge, and the increase in ear thickness from baseline was shown. (B) H&E staining of ear samples collected 24 h after challenge. (Original magnification 200 $\times$, scale bar = 300 μ m) (C) The relative expression of IL-1 β , IL-6, INF- γ , TSLP, CXCL2 and CXCL5 in the ear samples 24 h after challenge were examined using quantitative real-time PCR. Data are shown as mean $\pm$ SD ($n = 5$ –6 mice/group) and are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

attenuate CHS responses. Eight weeks after BM reconstitution, the mice were subjected to CHS, and the chimeric mice reconstituted with Sema4D-deficient BM showed milder ear swelling after OXA challenge compared to their irradiated control mice reconstituted with WT BM (Fig. 3A). Moreover, there were less infiltrating Gr1⁺, CD8⁺, or CD4⁺ cells in the chimeric mice reconstituted with Sema4D-deficient BM (Fig. 3B–D). And the expression of pro-inflammatory cytokines and chemokines, including IL-1 β , IL-6, CXCL2 and CXCL5, in ears samples after elicitation was also significantly lower in Sema4D KO mice compared with WT mice, as shown by qRT-PCR (Fig. 3E). There was no significant difference in the expression of IFN- γ between the two groups. Thus, our data indicated that deletion of Sema4D in hematopoietic cells contributed to the attenuated CHS responses in Sema4D KO mice.

3.3. Impaired sensitization of CHS in the absence of Sema4D

To further explore the involvement of Sema4D in the sensitization phase of CHS *in vivo*, we carried out adoptive transfer experiments. Cell suspension from dLNs was prepared from

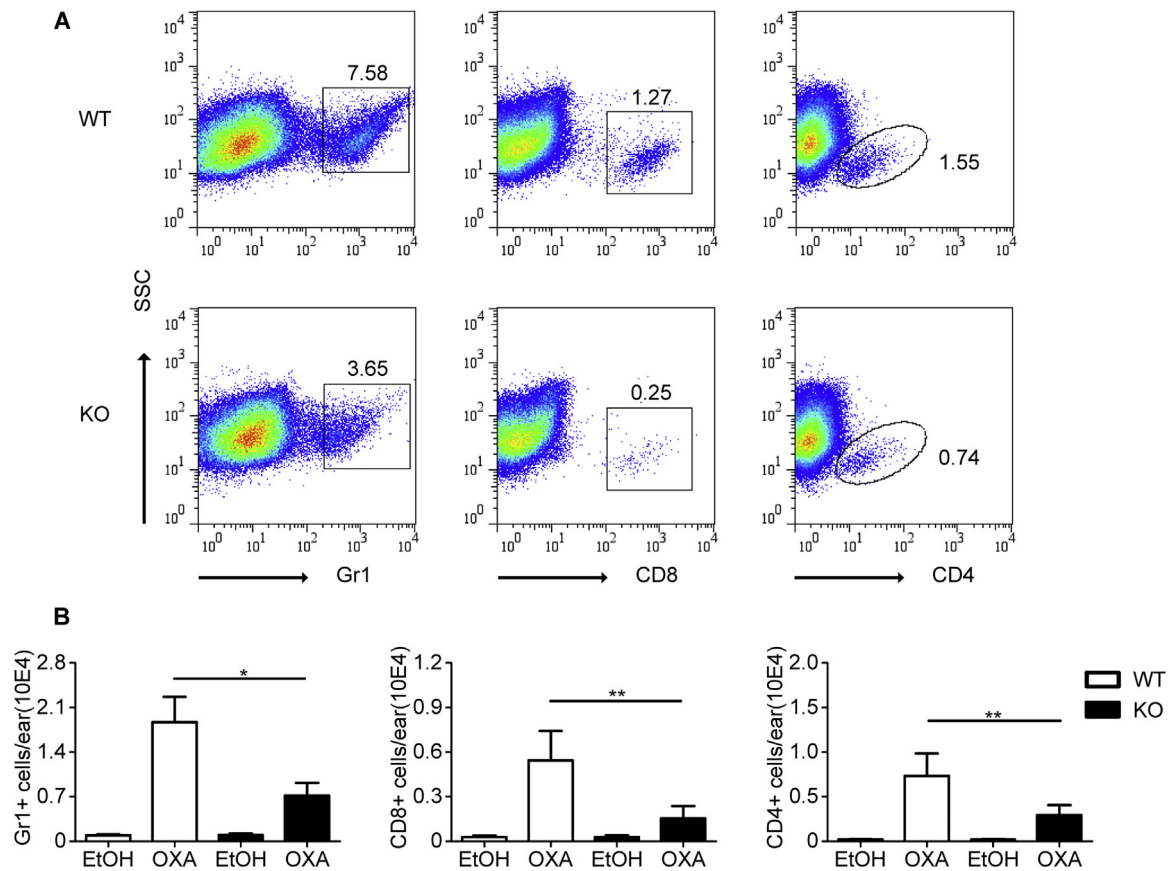


Fig. 2. Impaired inflammatory cell infiltration in Sema4D KO mice in CHS. WT and Sema4D KO mice were sensitized and challenged with OXA as depicted in Fig. 1. Twenty-four hours after challenge, single-cell suspension was prepared from the ears and stained with antibodies against Gr1 (Ly-6G/Ly-6C), CD8 or CD4. (A) FACS result showing Gr1⁺, CD8⁺ or CD4⁺ cells gated from live cells. (B) The absolute numbers of Gr1⁺, CD8⁺ or CD4⁺ cells in one ear. Data are shown as mean $\pm$ SD (n = 5–6 mice/group) and are representative of three independent experiments. *P < 0.05, **P < 0.01.

Sema4D KO mice or WT mice with CD45.2 allotype 5 days after hapten sensitization, and transferred intravenously to naïve WT mice with CD45.1 allotype. After OXA challenge, the mice receiving dLNs cells from sensitized Sema4D KO mice showed significantly milder ear swelling compared to the mice receiving dLNs cells from WT mice (Fig. 4A). Moreover, there were less infiltrating Gr1⁺, CD8⁺ or CD4⁺ cells in naïve CD45.1 mice receiving dLNs cells from sensitized Sema4D KO mice than WT mice (Fig. 4B–D), and most of the infiltrating CD4⁺ or CD8⁺ cells were CD45.2⁺ (donor) origin (data not shown). These data indicated that dLNs cells from Sema4D-deficient mice after sensitization transferred attenuated CHS responses.

3.4. Attenuated activation and differentiation of CD8⁺ T cells in Sema4D-deficient mice

A key event in CHS is the activation and differentiation of hapten-specific naïve CD8⁺ T cells into effector T cell subsets. Therefore, we assessed the IFN- γ -producing CD8⁺ T cells from the skin dLNs of Sema4D KO mice and WT mice 24 h after elicitation. Both the percentage and absolute number of IFN- γ -producing CD8⁺ T cells in dLNs of Sema4D KO mice were significantly lower than that of WT mice, and the mean fluorescence intensity (MFI) of IFN- γ staining in CD8⁺ T cells of Sema4D KO mice was also lower than that of WT mice (Fig. 5A–B), indicating that the activation and differentiation of IFN- γ -producing CD8⁺ T cells in Sema4D KO mice were impaired. T-bet and GATA-3 are critical transcription factors for the polarization of Th1 cells and Th2 cells, respectively. Our data showed that compared to WT mice, the expression of T-bet was

significantly lower in the dLNs of Sema4D KO mice 24 h after hapten challenge, indicating that Th1/Tc1 polarization was hindered (Fig. 5C). However, the expression of GATA-3 was not affected in Sema4D KO mice, in sharp contrast to the decreased expression of GATA-3 in WT mice due to Th1 polarization (Fig. 5C). In addition, the expression of IFN- γ , but not IL-17A, was also lower in Sema4D KO mice (Fig. 5C). Taken together, these results indicated that KO of Sema4D impeded Th1/Tc1 cell polarization.

To further investigate the role of Sema4D in CD8⁺ cell differentiation, we isolated CD8⁺ T cells from dLNs by FACS 5 days after OXA sensitization and examined the expression of T-bet, GATA-3 and IFN- γ by qRT-PCR. While there was no significant difference in the expression of IFN- γ in CD8⁺ T cells between Sema4D KO mice and WT mice, the relative expression of T-bet was significantly lower in CD8⁺ T cells of Sema4D KO mice than that of WT control (Fig. 5D). These results suggested that KO of Sema4D impeded the differentiation of naïve CD8⁺ T cell into cytotoxic T cells in CHS.

3.5. KO of Sema4D retarded the innate immune response in CHS

Haptens or haptenated self-proteins are recognized by the innate immune system in the skin, which leads to the elaboration of a number of pro-inflammatory cytokines and chemokines (Kaplan et al., 2012). To investigate the innate responses in Sema4D KO and WT mice after OXA application, ears of naïve Sema4D KO or WT mice were treated with 1.0% OXA solution, and after 0, 2, 4 and 6 h, mice were sacrificed and ears were harvested for qRT-PCR analysis of IL-1 β and CXCL2 expression. As shown in Fig. 6A and B, levels of IL-1 β and CXCL2 were lower in Sema4D KO mice compared to

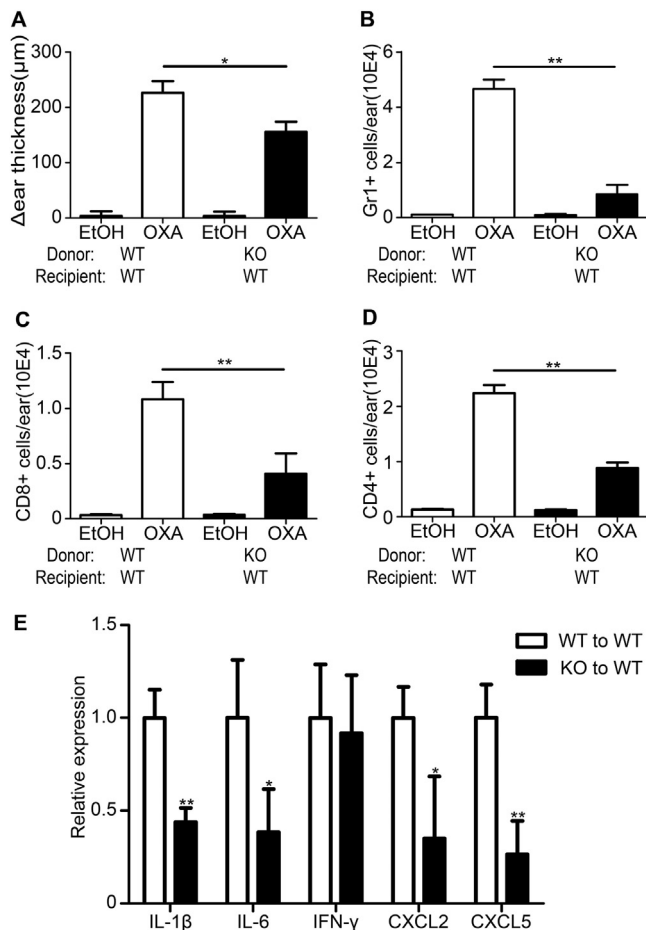


Fig. 3. Attenuated CHS in chimeric mice reconstituted with BM from Sem4D KO mice. WT mice were lethally irradiated and reconstituted with BM from WT or Sem4D KO mice, and OXA-induced CHS was evaluated. (A) Ear thickness was measured 24 h after the elicitation. The increase in ear thickness from baseline was shown. (B–D) The numbers of Gr1⁺, CD8⁺ or CD4⁺ cells in one ear was shown. (E) The levels of IL-1β, IL-6, INF-γ, CXCL2 and CXCL5 in the ears were examined using quantitative real-time PCR. Data are shown as mean ± SD (n = 3–4 mice/group) and are representative of three independent experiments. *P < 0.05, **P < 0.01.

WT mice, indicating that Sem4D deficiency resulted in a defect in the production of pro-inflammatory cytokines and chemokines by innate cells in the skin, which might attenuate the CHS responses.

The elicitation phase of CHS can be divided into the early phase (6–12 h) which is dominated primarily by innate response, and the late phase (24–48 h) which is dominated largely by hapten-specific T-cell response (Christensen and Haase, 2012). To further investigate the role of Sem4D in the innate response during the elicitation phase of CHS, naïve Sem4D KO or WT mice with CD45.2 allotype were transferred with dLNs cells from OXA-sensitized mice with CD45.1 allotype. The recipient Sem4D KO mice showed significantly milder ear swelling 24 h after elicitation compared to WT control (Fig. 6C). There were less infiltrating CD8⁺ or CD4⁺ cells, but not Gr1⁺ cells in Sem4D KO mice than WT control (Fig. 6D–F), and approximately 90% of CD8⁺ and 50% of CD4⁺ T cells were CD45.1⁺ (donor origin, data not shown). This result indicated that KO of Sem4D in the recipient mice retarded the recruitment of hapten-specific T cells, which may mainly due to the aberrant activation of innate immune response.

4. Discussion

In the present study, we proved that Sem4D played a critical role in OXA-induced CHS. The CHS was significantly attenuated

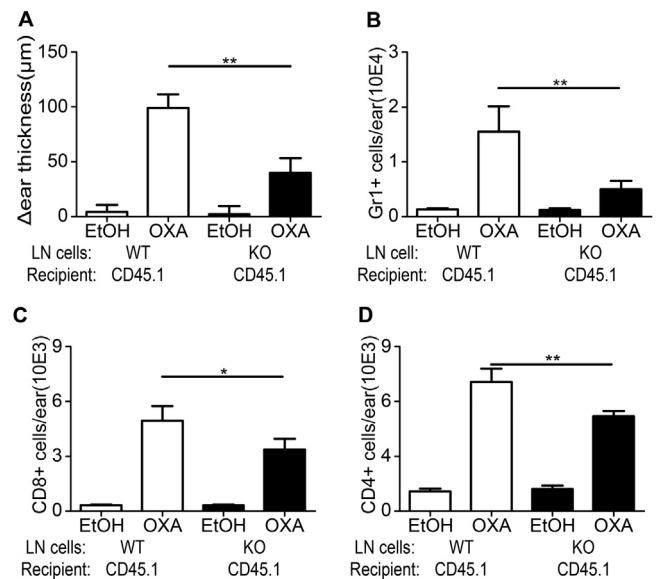


Fig. 4. Attenuated CHS in WT mice transferred with sensitized dLNs cells from Sem4D KO mice. Naïve WT mice with 45.1 allotype were adoptively transferred with dLNs cells isolated from OXA-sensitized WT or Sem4D KO mice, followed by treatment of the ears of recipient mice with OXA two hours after the transfer. (A) Twenty-four hours after OXA treatment, ear swelling was determined. The increase in ear thickness from baseline was shown. (B–D) The numbers of infiltrating Gr1⁺, CD8⁺ or CD4⁺ cells in one ear were shown. Data are shown as mean ± SD (n = 3–4 mice/group), and are representative of three independent experiments. *P < 0.05, **P < 0.01.

in Sem4D KO mice, which may mainly due to the absence of Sem4D in hematopoietic cells; moreover, Sem4D-deficiency not only impeded the activation and differentiation of CD8⁺ T cells in the sensitization phase of CHS, but also impaired the activation of skin-resident innate cells in both the sensitization and elicitation phases. Taken together, our study revealed for the first time that Sem4D was required in both the adaptive and innate immune responses in CHS.

Previous studies have indicated that Sem4D is necessary for the optimal T cell activation (Shi et al., 2000). In the mouse model of EAE, a Th1-dominant immune response, deficiency of Sem4D attenuated the antigen-specific activation of T cells (Kumanogoh et al., 2002); and in the mice model of lung allergic inflammation, Th2-type of immune response induced by OVA was also attenuated in Sem4D KO mice (Shanks et al., 2013). However, these studies mainly focused on the function of Sem4D in the activation of CD4⁺ cells, and the role of Sem4D in CD8⁺ cell activation remains largely unknown. CHS is characterized as a Th1/Tc1-dominant inflammation, in which CD8⁺ T cells are mainly involved and have proinflammatory effector functions. Our results showed that Sem4D was required for the differentiation of hapten-specific naïve CD8⁺ T cells into effector T cells, proposing that Sem4D may play an important role in the regulation of the function of CD8⁺ T cells. Several lines of evidence from human infectious diseases also suggested that Sem4D might be involved in CD8⁺ T cell function. In patients suffering from Hantaan virus induced hemorrhagic fever with renal syndrome (HFRS), elevated level of soluble Sem4D was correlated with the reduced expression of Sem4D on CD4⁺ and CD8⁺ T cells in the acute phase of infection (Liu et al., 2013). In HIV patients, the percentage of Sem4D-expressing CD4⁺ and CD8⁺ T cells was reduced, and Sem4D negative CD8⁺ T cells were functionally impaired and present in increased numbers (Eriksson et al., 2012). CD8⁺ cytotoxic T cells are key players in the immune responses of both the CHS and the virus infection (Andersen et al., 2006). Thus, our results obtained from Sem4D KO mice confirm the observations in human studies, in that Sem4D plays an impor-

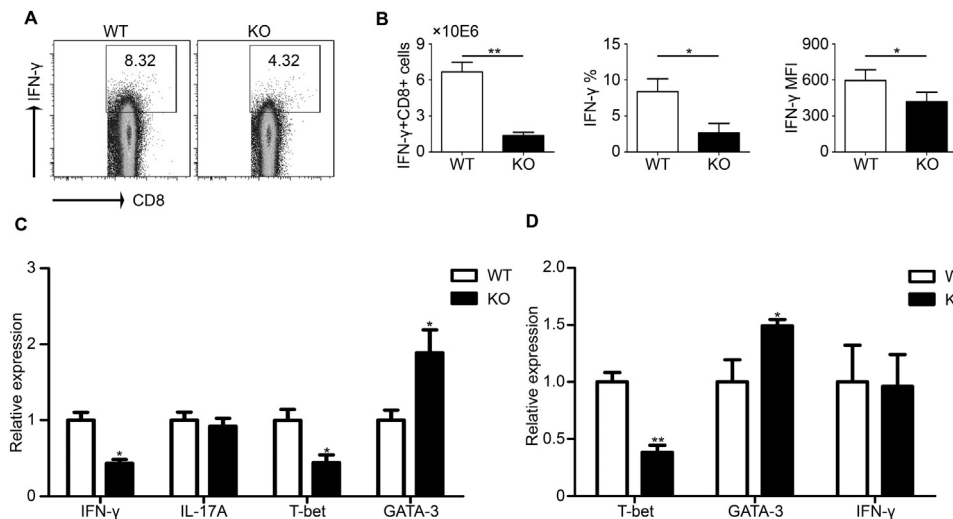


Fig. 5. Impaired activation and differentiation of hapten-specific CD8⁺ T cell in Sema4D KO mice. WT and Sema4D KO mice were sensitized and challenged with OXA as depicted in Fig. 1. Twenty-four hours after challenge, single-cell suspension was prepared from the dLNs and analyzed for intracellular IFN-γ production. (A) FACS result of IFN-γ-producing cells gated from CD8⁺ cells in dLNs. (B) Percentage and total number of IFN-γ-producing CD8⁺ T cells and MFI of IFN-γ expression in CD8⁺ cells from (A). (C) Cells from dLNs were harvested and the expression of IFN-γ, IL-17A, T-bet and GATA-3 were examined. (D) Five days after sensitization with OXA, CD8⁺ T cells in dLNs were sorted by FACS and the expression of T-bet, GATA-3 and IFN-γ were evaluated by quantitative real-time PCR. Data are shown as mean ± SD (n = 5–6 mice/group) and are representative of three independent experiments. *P < 0.05, **P < 0.01.

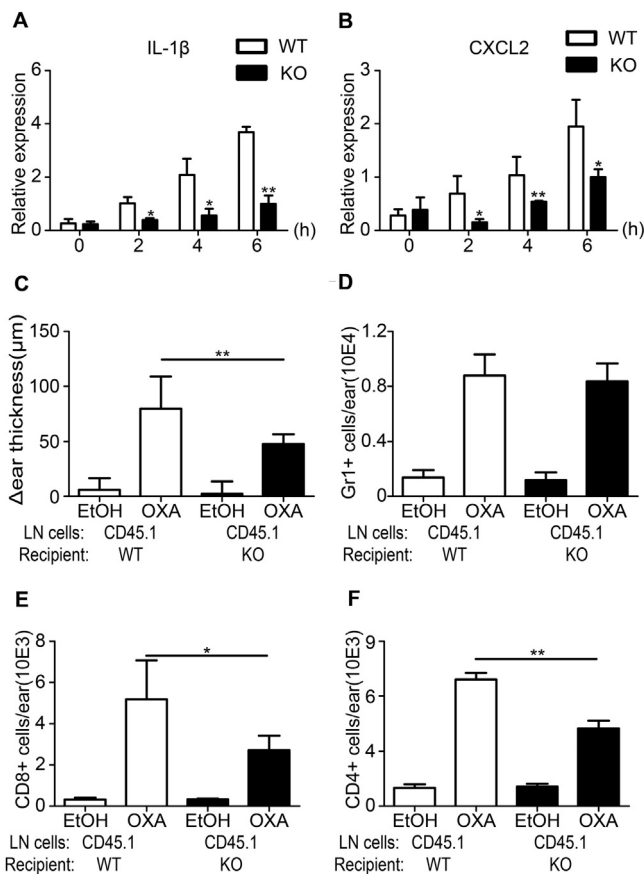


Fig. 6. Attenuated CHS in the elicitation phase in Sema4D KO mice. (A–B) Naïve Sema4D KO or WT mice were treated with 1.0% OXA solution, and after 0, 2, 4 and 6 h, mice were sacrificed and ears were harvested for qRT-PCR analysis of IL-1β (A) and CXCL2 (B) expression. (C–F) Adoptive transfer of dLNs cells from sensitized WT mice with CD45.1 allotype to WT or Sema4D KO mice with CD45.2 allotype was performed as described in Fig. 4. Ear thickness was measured 24 h after OXA application. The increase in ear thickness from baseline was shown (C). Numbers of Gr1⁺, CD8⁺ or CD4⁺ cells in one ear 24 h after the elicitation were shown (D–F). Data are shown as mean ± SD (n = 3 mice/group) and are representative of three independent experiments. *P < 0.05, **P < 0.01.

tant role in regulating the function of CD8⁺ T cells. However, in our *in vivo* system, we couldn't discriminate the defect in CD8⁺ T cell activation was CD8⁺ T cell intrinsic or due to the aberrant signals from innate cells or Th cells. Further studies are needed to explore the exact role and mechanism of Sema4D in the activation and differentiation of CD8⁺ T cells.

CHS was once considered primarily an adaptive immune response; however, innate cells also play important roles in CHS (Kaplan et al., 2012). With the application of hapten, DCs and other skin-resident cells are activated and produce various pro-inflammatory cytokines and chemokines, which are critical for both the sensitization phase and the elicitation phase of CHS (Christensen and Haase, 2012). Recent study demonstrates that Sema4D is also involved in innate immunity (Witherden et al., 2012). DCs from Sema4D KO mice express lower levels of costimulatory molecules and produce reduced IL-12 in EAE (Kumanogoh et al., 2002). In the present study, naïve Sema4D KO mice produced much less of IL-1β and CXCL2 upon OXA application, and developed significant milder inflammation after transferred with dLNs cells from sensitized WT mice. These results indicated that the decreased release of pro-inflammatory cytokines and chemokines in Sema4D KO mice may in part participate in the alleviated induction of CHS in the elicitation phase. Therefore, the hypo-responsiveness of innate immune response in Sema4D KO mice not only play a role in the sensitization phase, but also serve as a potential trigger or amplifier in the elicitation phase.

One important phenotype of Sema4D KO mice is that the number of B-1 cells in the peritoneal cavity and spleen is significantly reduced (Shi et al., 2000). However, previous studies report that peritoneal B-1 cells are also involved in CHS (Campos et al., 2003). B-1 cells are radiation resistant, and would survive in the chimeric mice reconstituted with Sema4D KO BM in our experiment, and the effect of the reduced number of B-1 cells in Sema4D KO mice could be excluded. Dendritic epidermal T cells (DETCs), a subset of skin-resident γδ T cells (MacLeod and Havran, 2011), can be activated in an IL-1β-dependent manner and produce IL-17 in CHS (Nielsen et al., 2014). Previous study demonstrates that there is delayed wound healing in Sema4D KO mice, which is due to the failed activation and migration of DETCs (Witherden et al., 2012). In OXA-induced CHS model, activation of DETCs might be attenuated

due to the aberrant production of IL-1 β in Sema4D KO mice, which may contribute to the reduction of CHS response. It's also reported that Sema4D and its receptors mediate the monocyte-endothelial cell adhesion (Luque et al., 2015), and this cell-to-cell adhesion may play a role in the recruitment of leukocytes from blood vessels to tissues and might contribute to the alleviated response of CHS in Sema4D KO mice.

In summary, our study proved that Sema4D positively regulated both the adaptive and innate immune response in CHS, and Sema4D targeting might be alternative treatment for ACD in the future. As deletion of Sema4D is not in a cell-specific manner in Sema4D KO mice, we were unable to investigate the function of Sema4D in a particular type of cells *in vivo*, and future studies using conditional Sema4D KO mice would help to further our understanding on the cell-specific function of Sema4D.

Conflict of interest

The authors do not declare a conflict of interest.

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References

- Andersen, M.H., Schrama, D., Thor, S.P., Becker, J.C., 2006. Cytotoxic T cells. *J. Invest. Dermatol.* 126, 32–41, <http://dx.doi.org/10.1038/sj.jid.5700001>.
- Campos, R.A., Szczepanik, M., Itakura, A., Akahira-Azuma, M., Sidobre, S., Kronenberg, M., Askenase, P.W., 2003. Cutaneous immunization rapidly activates liver invariant Valpha14 NKT cells stimulating B-1 B cells to initiate T cell recruitment for elicitation of contact sensitivity. *J. Exp. Med.* 198, 1785–1796, <http://dx.doi.org/10.1084/jem.20021562>.
- Christensen, A.D., Haase, C., 2012. Immunological mechanisms of contact hypersensitivity in mice. *APMIS* 120, 1–27, <http://dx.doi.org/10.1111/j.1600-0463.2011.02832.x>.
- Eriksson, E.M., Milush, J.M., Ho, E.L., Batista, M.D., Holditch, S.J., Keh, C.E., Norris, P.J., Keating, S.M., Deeks, S.G., Hunt, P.W., Martin, J.N., Rosenberg, M.G., Hecht, F.M., Nixon, D.F., 2012. Expansion of CD8+ T cells lacking Sema4D/CD100 during HIV-1 infection identifies a subset of T cells with decreased functional capacity. *Blood* 119, 745–755, <http://dx.doi.org/10.1182/blood-2010-12-324848>.
- Hall, K.T., Boumsell, L., Schultze, J.L., Boussiotis, V.A., Dorfman, D.M., Cardoso, A.A., Bensussan, A., Nadler, L.M., Freeman, G.J., 1996. Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. *Proc. Natl. Acad. Sci. U. S. A.* 93, 11780–11785.
- Honda, T., Egawa, G., Grabbe, S., Kabashima, K., 2013. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J. Invest. Dermatol.* 133, 303–315, <http://dx.doi.org/10.1038/jid.2012.284>.
- Kabashima, K., Sakata, D., Nagamachi, M., Miyachi, Y., Inaba, K., Narumiya, S., 2003. Prostaglandin E2-EP4 signaling initiates skin immune responses by promoting migration and maturation of Langerhans cells. *Nat. Med.* 9, 744–749, <http://dx.doi.org/10.1038/nm872>.
- Kaplan, D.H., Jenison, M.C., Saeland, S., Shlomchik, W.D., Shlomchik, M.J., 2005. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* 23, 611–620, <http://dx.doi.org/10.1016/j.immuni.2005.10.008>.
- Kaplan, D.H., Igyártó, B.Z., Gaspari, A.A., 2012. Early immune events in the induction of allergic contact dermatitis. *Nat. Rev. Immunol.*, <http://dx.doi.org/10.1038/nri3150>.
- Kruger, R.P., Aurandt, J., Guan, K.L., 2005. Semaphorins command cells to move. *Nat. Rev. Mol. Cell Biol.* 6, 789–800, <http://dx.doi.org/10.1038/nrm1740>.
- Kumanogoh, A., Kikutani, H., 2013. Immunological functions of the neuropilins and plexins as receptors for semaphorins. *Nat. Rev. Immunol.* 13, 802–814.
- Kumanogoh, A., Suzuki, K., Ch'Ng, E., Watanabe, C., Marukawa, S., Takegahara, N., Ishida, I., Sato, T., Habu, S., Yoshida, K., Shi, W., Kikutani, H., 2002. Requirement for the lymphocyte semaphorin, CD100, in the induction of antigen-specific T cells and the maturation of dendritic cells. *J. Immunol.* 169, 1175–1181.
- Li, M., O'Sullivan, K.M., Jones, L.K., Sempole, T., Kumanogoh, A., Kikutani, H., Holdsworth, S.R., Kitching, A.R., 2006. CD100 enhances dendritic cell and CD4+ cell activation leading to pathogenetic humoral responses and immune complex glomerulonephritis. *J. Immunol.* 177, 3406–3412.
- Liu, B., Ma, Y., Yi, J., Xu, Z., Zhang, Y.S., Zhang, C., Zhuang, R., Yu, H., Wang, J., Yang, A., Zhang, Y., Jin, B., 2013. Elevated plasma soluble Sema4D/CD100 levels are associated with disease severity in patients of hemorrhagic fever with renal syndrome. *PLoS One* 8, e73958, <http://dx.doi.org/10.1371/journal.pone.0073958>.
- Liu, B., Ma, Y., Zhang, Y., Zhang, C., Yi, J., Zhuang, R., Yu, H., Yang, A., Zhang, Y., Jin, B., 2015. CD8low CD100- T cells identify a novel CD8 T cell subset associated with viral control during human hantaan virus infection. *J. Virol.* 89, 11834–11844, <http://dx.doi.org/10.1128/JVI.01610-15>.
- Luque, M.C.A., Gutierrez, P.S., Debbas, V., Kalil, J., Stolf, B.S., 2015. CD100 and plexins B2 and B1 mediate monocyte-endothelial cell adhesion and might take part in atherogenesis. *Mol. Immunol.* 67, 559–567, <http://dx.doi.org/10.1016/j.molimm.2015.07.028>.
- MacLeod, A.S., Havran, W.L., 2011. Functions of skin-resident gammadelta T cells. *Cell. Mol. Life Sci.* 68, 2399–2408, <http://dx.doi.org/10.1007/s00018-011-0702-x>.
- Martin, S.F., Esser, P.R., Weber, F.C., Jakob, T., Freudenberger, M.A., 2011. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy*.
- Meehan, T.F., Witherden, D.A., Kim, C., Sendaydiego, K., Ye, I., Garijo, O., Komori, H.K., Kumanogoh, A., Kikutani, H., Eckmann, L., Havran, W.L., 2013. Protection against colitis by CD100-dependent modulation of intraepithelial $\gamma\delta$ T lymphocyte function. *Mucosal Immunol.* 7, 134–142, <http://dx.doi.org/10.1038/mi.2013.32>.
- Negishi-Koga, T., Shinohara, M., Komatsu, N., Bito, H., Kodama, T., Friedel, R.H., Takayanagi, H., 2011. Suppression of bone formation by osteoclastic expression of semaphorin 4D. *Nat. Med.* 17, 1473–1480, <http://dx.doi.org/10.1038/nm.2489>.
- Nielsen, M.M., Lovato, P., MacLeod, A.S., Witherden, D.A., Skov, L., Dyring-Andersen, B., Dabelsteen, S., Woetmann, A., Odum, N., Havran, W.L., Geisler, C., Bonefeld, C.M., 2014. IL-1beta-dependent activation of dendritic epidermal T cells in contact hypersensitivity. *J. Immunol.* 192, 2975–2983, <http://dx.doi.org/10.4049/jimmunol.1301689>.
- Okuno, T., Nakatsuji, Y., Moriya, M., Takamatsu, H., Nojima, S., Takegahara, N., Toyofuku, T., Nakagawa, Y., Kang, S., Friedel, R.H., Sakoda, S., Kikutani, H., Kumanogoh, A., 2010. Roles of Sema4D-plexin-B1 interactions in the central nervous system for pathogenesis of experimental autoimmune encephalomyelitis. *J. Immunol.* 184, 1499–1506, <http://dx.doi.org/10.4049/jimmunol.0903302>.
- Shanks, K., Nkyimbeng-Takwi, E.H., Smith, E., Lipsky, M.M., DeTolla, L.J., Scott, D.W., Keegan, A.D., Chapoval, S.P., 2013. Neuroimmune semaphorin 4D is necessary for optimal lung allergic inflammation. *Mol. Immunol.* 56, 480–487, <http://dx.doi.org/10.1016/j.molimm.2013.05.228>.
- Shi, W., Kumanogoh, A., Watanabe, C., Uchida, J., Wang, X., Yasui, T., Yukawa, K., Ikawa, M., Okabe, M., Parnes, J.R., Yoshida, K., Kikutani, H., 2000. The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. *Immunity* 13, 633–642.
- Soong, J., Chen, Y., Shustef, E.M., Scott, G.A., 2012. Sema4D, the ligand for Plexin B1, suppresses c-Met activation and migration and promotes melanocyte survival and growth. *J. Invest. Dermatol.* 132, 1230–1238, <http://dx.doi.org/10.1038/jid.2011.414>.
- Witherden, D.A., Watanabe, M., Garijo, O., Rieder, S.E., Sarkisyan, G., Cronin, S.J., Verdino, P., Wilson, I.A., Kumanogoh, A., Kikutani, H., Teyton, L., Fischer, W.H., Havran, W.L., 2012. The CD100 receptor interacts with its plexin B2 ligand to regulate epidermal gammadelta T cell function. *Immunity* 37, 314–325, <http://dx.doi.org/10.1016/j.immuni.2012.05.026>.
- Zhang, Y., Liu, B., Ma, Y., Jin, B., 2013. Sema 4D/CD100-plexin B is a multifunctional counter-receptor. *Cell Mol. Immunol.* 10, 97–98, <http://dx.doi.org/10.1038/cmi.2012.65>.
- Zhu, L., Bergmeier, W., Wu, J., Jiang, H., Stalker, T.J., 2007. Regulated surface expression and shedding support a dual role for semaphorin 4D in platelet responses to vascular injury. *Proc. Natl. Acad. Sci.*