



Boost the immune response with
High-quality PRR ligands



Semaphorin 4C Protects against Allergic Inflammation: Requirement of Regulatory CD138⁺ Plasma Cells

This information is current as of December 12, 2016.

Di Xue, Gabriel N. Kaufman, Marieme Dembele, Marianne Beland, Amir H. Massoud, Barbara C. Mindt, Ryan Fiter, Elizabeth D. Fixman, James G. Martin, Roland H. Friedel, Maziar Divangahi, Jörg H. Fritz and Bruce D. Mazer

J Immunol published online 23 November 2016
<http://www.jimmunol.org/content/early/2016/11/23/jimmunol.1600831>

-
- | | |
|-------------------------------|---|
| Supplementary Material | http://www.jimmunol.org/content/suppl/2016/11/23/jimmunol.1600831.DCSupplemental.html |
| Subscriptions | Information about subscribing to <i>The Journal of Immunology</i> is online at: http://jimmunol.org/subscriptions |
| Permissions | Submit copyright permission requests at: http://www.aai.org/ji/copyright.html |
| Email Alerts | Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/cgi/alerts/etoc |



Semaphorin 4C Protects against Allergic Inflammation: Requirement of Regulatory CD138⁺ Plasma Cells

Di Xue,^{*,†} Gabriel N. Kaufman,^{*,†} Marieme Dembele,^{*,†} Marianne Beland,^{*} Amir H. Massoud,^{*} Barbara C. Mindt,^{‡,§} Ryan Fiter,^{*} Elizabeth D. Fixman,^{*} James G. Martin,^{*} Roland H. Friedel,[¶] Maziar Divangahi,^{*} Jörg H. Fritz,^{‡,§} and Bruce D. Mazer^{*,||}

The regulatory properties of B cells have been studied in autoimmune diseases; however, their role in allergic diseases is poorly understood. We demonstrate that Semaphorin 4C (Sema4C), an axonal guidance molecule, plays a crucial role in B cell regulatory function. Mice deficient in Sema4C exhibited increased airway inflammation after allergen exposure, with massive eosinophilic lung infiltrates and increased Th2 cytokines. This phenotype was reproduced by mixed bone marrow chimeric mice with Sema4C deficient only in B cells, indicating that B lymphocytes were the key cells affected by the absence of Sema4C expression in allergic inflammation. We determined that Sema4C-deficient CD19⁺CD138⁺ cells exhibited decreased IL-10 and increased IL-4 expression in vivo and in vitro. Adoptive transfer of *Sema4c*^{-/-} CD19⁺CD138⁺ cells induced marked pulmonary inflammation, eosinophilia, and increased bronchoalveolar lavage fluid IL-4 and IL-5, whereas adoptive transfer of wild-type CD19⁺CD138⁺IL-10⁺ cells dramatically decreased allergic airway inflammation in wild-type and *Sema4c*^{-/-} mice. This study identifies a novel pathway by which Th2-mediated immune responses are regulated. It highlights the importance of plasma cells as regulatory cells in allergic inflammation and suggests that CD138⁺ B cells contribute to cytokine balance and are important for maintenance of immune homeostasis in allergic airways disease. Furthermore, we demonstrate that Sema4C is critical for optimal regulatory cytokine production in CD138⁺ B cells. *The Journal of Immunology*, 2017, 198: 000–000.

Semaphorins are a family of proteins with a common conserved structure that are important in embryonic development, axonal guidance, angiogenesis, and organ morphogenesis (1–5). They interact with ubiquitous receptor proteins that share similar structures, known as plexins and neuropilins (6). In particular, class 4 semaphorins are implicated in multiple immunoregulatory processes. Semaphorin 4A plays a role in Th1 cell differentiation (7) and in maintaining regulatory T cells (Tregs) at sites of inflammation (8). Semaphorin 4B was shown to be a negative regulator of basophil-induced Th2 activation (9). Semaphorin 4D acts as a costimulation molecule during

T cell activation via interaction with CD72 (10). We recently found that activation of human tonsillar B cells by anti-CD40 Abs and IL-4 significantly increased the expression of semaphorin 4C (Sema4C) (11). Plexin B2, a known ligand for Sema4C, is expressed on germinal center B cells (12, 13), and Sema4C–Plexin B2 interaction plays a critical role in embryonic cerebellar (14, 15) and renal (16) development. However, there are no data describing the role of Sema4C in immune responses.

B cells are essential players in adaptive immune responses as a result of their ability to secrete specific Abs in response to infection or allergen. In allergy, plasma cells secrete IgE that binds to high-affinity FcεRI on mast cells, and cross-linking of this complex by allergens initiates allergic inflammation, including recruitment of eosinophils (17–19). B cells also function as regulatory cells by downregulating allergic and autoimmune responses in an IL-10-dependent manner (20–26). However, our understanding of regulatory B cells (Bregs) in allergic inflammation is extremely limited.

We evaluated the role of Sema4C in allergic responses by comparing wild-type (WT) and *Sema4c*^{-/-} mice using a model of OVA-driven allergic airways disease (AAD). Mice deficient in Sema4C responded to allergen by exhibiting markedly increased pulmonary inflammation and eosinophilia and enhanced Th2 cytokine production. This phenotype was rescued by provision of CD19⁺CD138⁺IL-10⁺ B cells, which are significantly diminished in *Sema4c*^{-/-} mice, and provision of exogenous Sema4C induced in vitro IL-10 production in *Sema4c*^{-/-} B cells. Taken together, our data indicate that Sema4C plays a role in B cell regulatory function, impacting Th2 responses in AAD.

Materials and Methods

Animals

WT C57BL/6 mice and IL-10^{EGFP} reporter mice C57BL/6-IL-10^{tm1.1Karp/J} (Vert-X) were purchased from the Jackson Laboratory. Sema4C heterozygote mice (*Sema4c*^{+/-}) bred on a C57BL/6 background were provided

*The Research Institute of the McGill University Health Centre, Translational Research in Respiratory Diseases Program, Meakins Christie Laboratories, Montreal, Quebec H4A 3J1, Canada; [†]Division of Experimental Medicine, Department of Medicine, McGill University, Montreal, Quebec H3A 1A3, Canada; [‡]Department of Microbiology and Immunology, McGill University, Montreal, Quebec H3A 2B4, Canada; [§]Department of Physiology, McGill University, Montreal, Quebec H3G 1Y6, Canada; [¶]Department of Neurosurgery, Icahn School of Medicine at Mount Sinai, NY 10029; and ^{||}Department of Pediatrics, Montreal Children's Hospital, Montreal, Quebec H4A 3J1, Canada

ORCID: 0000-0003-4665-968X (G.N.K.); 0000-0002-4865-7998 (M. Dembele); 0000-0003-0264-8540 (M.B.); 0000-0003-0159-5352 (R.F.); 0000-0002-7513-3604 (R.H.F.); 0000-0002-1254-6110 (M. Divangahi); 0000-0003-4018-1419 (B.D.M.).

Received for publication May 11, 2016. Accepted for publication October 27, 2016.

Address correspondence and reprint requests to Dr. Bruce D. Mazer, The Research Institute of the McGill University Health Centre, Translational Research in Respiratory Diseases Program, Meakins Christie Laboratories, 1001 Decarie Boulevard, Montreal, QC H4A 3J1, Canada. E-mail address: bruce.mazer@mcgill.ca

The online version of this article contains supplemental material.

Abbreviations used in this article: A₀, actual area; AAD, allergic airways disease; AHR, airway hyperresponsiveness; A_i, ideal area; BALF, bronchoalveolar lavage fluid; Breg, regulatory B cell; EMT, epithelial–mesenchymal transition; FVD, Fixable Viability Dye eFluor 780; i.t., intratracheally; MCh, methacholine; Sema4C, semaphorin 4C; Treg, regulatory T cell; WT, wild-type.

Copyright © 2016 by The American Association of Immunologists, Inc. 0022-1767/16/\$30.00

by Dr. Roland H. Friedel (Mount Sinai Hospital, New York, NY) (15). *Sema4c*^{+/-} mice have been back-crossed to C57BL/6 mice for >10 generations. *Sema4c*^{+/-} mice were bred, with resulting litters consisting of WT, *Sema4c*^{+/-}, and *Sema4c*^{-/-} mice. *Sema4c*^{-/-} mice were identified by genotyping, using a three-primer multiplex PCR with the following primers: 5'-TGGTGTGGCTTACCTGTGCTTGG-3' (genomic forward), 5'-AGAAAGGAGCCAGGTTGTCTGCA-3' (genomic reverse), and 5'-ACTTCCGGAGCGGATCTCAAACCTC-3' (vector reverse), which amplifies a 620-bp WT fragment and a 430-bp mutant fragment (15). WT littermate mice were used as controls for *Sema4c*^{-/-} mice. WT recipient mice used in Figs. 5 and 6 were purchased from the Jackson Laboratory.

To generate bone marrow chimeric mice, *JH*^{-/-} mice (C57BL/6-CD45.2 background) (27) were lethally irradiated (two equal doses of 450 rad) and injected i.v. with 2×10^6 bone marrow cells (20% C57BL/6-CD45.1 and 80% *JH*^{-/-} [WT-B] or 20% *Sema4c*^{-/-} and 80% *JH*^{-/-} [S4C-B]). They were fed water with neomycin (2 g/l) for 14 d after irradiation and used for experiments after 8 wk of reconstitution.

All animals were housed in a pathogen-free environment, and all experiments were in accordance with the regulations and standard guidelines of the McGill University Animal Care Committee.

AAD model

Mice were sensitized by i.p. injection with 100 μ l OVA/alum (0.5 mg/ml OVA and 4 mg/ml aluminum hydroxide in PBS) on days 0 and 14, followed by challenges with intranasal OVA from days 28 to 30 (20 μ l of 10 mg/ml OVA in PBS). For intranasal challenge, mice were anesthetized with 2% isoflurane in oxygen before OVA was delivered. Analysis of airway hyperresponsiveness (AHR; followed by sacrifice) was performed 24 h after the final challenge (28). To measure AHR, mice were exposed to saline, followed by doubling concentrations of aerosolized methacholine (MCh; 16–256 mg/ml; Sigma-Aldrich) using a small-animal ventilator (flexiVent; SCIREQ) (29). Bronchoalveolar lavage fluid (BALF) was collected following instillation of 500 μ l of PBS/0.5% BSA. Cells in BALF were centrifuged and stained with the Hema 3 kit (Fisher Scientific). Cytokine levels were measured by Milliplex (Millipore). To measure serum IgE concentrations, blood was collected by cardiac puncture and centrifuged for 15 min at 12,000 rpm/min, and serum was collected. Serum IgE levels were measured using modified commercial ELISA kits (BioLegend for total IgE and Cayman Medical for OVA-specific IgE). For cellular analysis, lung tissue was digested with 0.1 mg/ml DNase and 0.15 U/ml collagenase D (both from Roche) in 10 ml of digestion buffer (15 mM HEPES, 0.1 g/l CaCl₂ and 0.1 g/l MgCl₂ • 6 H₂O in PBS) at 37°C in a 10-cm petri dish. After 45 min, lung digests were homogenized by passing through a 3-ml syringe with an 18G needle then filtered through a 40- μ m filter.

Flow cytometry and imaging flow cytometry

The following Abs were used for phenotyping: CD19-allophycocyanin (clone 6D5), CD3-FITC (17A2), CD4-BV421 (RM4-5), CD8-PE (53-6.7), IL-10-PE (JES5-16E3), CD138-BV421 (53-7.3 and 281-2), CD11c-BV421 (N418), CD11b-PerCP or allophycocyanin (M1/70), CD25-PE (7D4), IL-7Ra-PE (A7R34), Fc ϵ RI-PE (MAR-1), TruStain FcX (93), MHCII-PerCP-eFluor 710 (M5/114.15.2), Ly6C-allophycocyanin (HK1.4), Ly6G-FITC (1A8), and Foxp3-allophycocyanin (FJK-16s) (all from BioLegend, San Diego, CA). Gr-1-PE/Cy7 (RB6-8C5), IL-4-PE (11B11), and Fixable Viability Dye eFluor 780 (FVD) were from eBioscience. Siglec-F-PE/Texas Red (E50-2440) was from BD Pharmingen. All fluorophore-labeled Abs were used at 1 μ g/ml. Fc block (TruStain FcX) was used at 2.5 μ g/ml. FVD was used at a 1:1000 dilution. For staining, 10^6 cells were stained with FVD in 100 μ l of PBS for 10 min at room temperature, followed by one wash with 1 ml of cold PBS/0.2% BSA. To avoid nonspecific binding, cells were blocked with TruStain FcX in 100 μ l of PBS/0.2% BSA at 4°C. Abs against surface Ags were added 15 min after Fc block without washing, for 30 min at 4°C, followed by three washes with 1 ml of cold PBS. For IL-10 or IL-4 intracellular staining, 10^6 cultured B cells or isolated spleen cells were permeabilized and fixed with 250 μ l of BD Cytofix/Cytoperm for 20 min at 4°C, followed by washing with 1 ml of BD Perm/Wash buffer (both from BD Pharmingen). Cells were then stained with IL-4-PE/Cy7 or IL-10-PE in 100 μ l of Perm/Wash buffer for 30 min, followed by two washes with 1 ml of Perm/Wash buffer. Data were acquired on a Becton Dickinson LSR II flow cytometer and analyzed using FlowJo software (TreeStar). Cell sorting was performed using a BD FACSARIA III, following standard procedures. Gating strategies and cell purity are shown in Supplemental Fig. 4. For intracellular cytokine staining of single-cell suspensions of spleen cells from mice following allergen challenge, CD19⁺CD138⁺ cells were stained as described above without PMA/ionomycin restimulation. We performed

imaging cytometry on an Amnis ImageStream X Mark II (EMD Millipore). Cells were stained following the same protocols as for standard cytometry, and data were acquired and analyzed with IDEAS (EMD Millipore). Cells were all pregated on focused cells, live cells, and single cells as per standard procedures.

Adoptive transfer of B lymphocytes and CD19⁺CD138⁺IL-10⁺ cells

Sema4c^{-/-} and WT CD19⁺CD138⁺ B cells. *Sema4c*^{-/-} or WT mice were sensitized with OVA, as described. Spleen cells were collected on day 27. CD138⁺CD19⁺ spleen cells were sorted by FACSARIA III. Gating strategy and purity are shown in Supplemental Fig. 4A. A total of 1×10^6 CD138⁺CD19⁺ cells from *Sema4c*^{-/-} or WT mice was transferred intratracheally (i.t.) to OVA-exposed WT recipients, which were then challenged intranasally with OVA for 3 d and sacrificed within 24 h of the last challenge. CD19⁺CD138⁺IL-10⁺ cells. Vert-X-IL-10^{EGFP} and WT mice were sensitized with OVA, as described above. Vert-X-IL-10^{EGFP} mice were sacrificed on day 27. Spleen cells (pooled from three Vert-X-IL-10^{EGFP} mice) were stained with CD19 and CD138. CD19⁺CD138⁺GFP⁺ cells were sorted using a FACSARIA III. Gating strategy and purity are shown in Supplemental Fig. 4C. For adoptive transfer, 10^5 cells in 50 μ l of saline, or vehicle alone, were transferred i.t. to OVA-sensitized WT animals 24 h before their first OVA challenge. Before receiving the i.t. cell transfer, recipients were anesthetized with 2% isoflurane in oxygen. Recipients were sacrificed 24 h following the last OVA challenge.

To ensure that the donor cells were successfully delivered into the lungs of recipients, donor cells were labeled with CellTrace Far Red (Invitrogen) before transferring, following the manufacturer's instructions. A total of 10^6 cells was incubated in 1 μ M CellTrace Far Red in 1 ml of PBS for 20 min at 37°C. The reaction was stopped with 5 ml of PBS/10% FBS. Cells were washed and resuspended for infusion, as above.

Histological analysis

Murine lungs were inflated by injecting 10 ml of ice-cold PBS/0.5% BSA into the lungs through the right ventricle to remove blood and then injecting 2 ml of 10% formalin through the trachea to inflate the lungs. The intact lungs were carefully removed, fixed in 10% formalin, and embedded in paraffin. Five-micron sections were prepared, stained with H&E, and examined using light microscopy. For airway caliber measurements, the actual area (A_a) and the perimeter of 10 randomly selected airways (P_a) were measured by ImageJ. The ideal area (A_i) of the airway was calculated using the formula $A_i = (P_a)^2/4\pi$. The degree of airway narrowing was calculated using A_a/A_i (30). A lower A_a/A_i indicates increased bronchospasm. Histopathological scores were calculated as described by Cimolai et al. (31).

Cell culture

B cell culture. Splenic B cells were purified with an EasySep Mouse B Cell Enrichment Kit (STEMCELL Technologies). A total of 0.25×10^6 B cells per milliliter was stimulated with anti-CD40 Abs (1 μ g/ml; clone HM40-3; eBioscience), with or without IL-4 (40 ng/ml) and/or IL-21 (40 ng/ml) (Prospec), in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-ME, and 25 mM HEPES (Fisher Scientific). For intracellular staining, PMA (50 ng/ml) and ionomycin (1 μ g/ml; both from Sigma-Aldrich), in the presence of 2 μ M GolgiStop (BD), were added to culture 5 h prior to harvest.

HEK-S4C and B cell coculture. HEK293 cells were stably transfected using Lipofectamine 3000 (Life Technologies) with a full-length murine Sema4C construct using expression vector pReceiver-M77 (GeneCopoeia) (32). HEK293 cells stably transfected with Sema4C or untransfected HEK293 cells were treated with mitomycin C (10 μ g/ml) for 2 h and then cultured with B cells (1:10) in the presence of anti-CD40 Abs and IL-4 and IL-21, as above. After 96 h, supernatants were harvested, and the percentage of CD138⁺IL-10⁺ cells among CD138⁺ cells was determined by flow cytometry.

Western blotting

Cells were washed in ice-cold PBS and incubated in 1 $\times$ RIPA buffer (Abcam) supplemented with Roche cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich). A total of 20 μ g of protein was resolved by 10% SDS-PAGE and electro-transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with 3% BSA in PBS (pH 7.2) for 1 h at room temperature and incubated overnight at 4°C with sheep anti-mouse Sema4C (1/1000; R&D Systems). After washing, membranes were treated with anti-sheep HRP at room temperature for 2 h. Blots were developed using Clarity ECL Blotting Substrate (Bio-Rad), with actin as the control protein.

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical analysis for AHR was performed using repeated-measures ANOVA. Comparisons among several means were analyzed using one-way ANOVA with the Bonferroni post hoc test. Two-tailed *t* tests were performed for other assays. The *p* values $\leq$ 0.05 were regarded as statistically significant. Statistical analyses were performed with GraphPad Prism 6.0 software.

Results

Sema4C-deficient mice develop augmented Th2 responses following allergen exposure

We examined murine splenic B lymphocytes for their expression of Sema4C. Resting splenic B cells exhibited little Sema4C mRNA or protein (Fig. 1A, 1B); however, Sema4C expression was induced significantly when B cells were stimulated with anti-CD40 Abs in the presence of IL-4 and IL-21.

To examine the role of Sema4C in Th2-mediated allergic responses, we exposed WT and *Sema4C*^{-/-} mice to Ag using an OVA-driven model of AAD (28). Of note, no significant difference in immune cell counts in spleens or lymph nodes was detected between naive WT and *Sema4C*^{-/-} mice (Supplemental Fig. 1A, 1B). OVA-sensitized and -challenged *Sema4C*^{-/-} mice exhibited significantly higher airway resistance and elastance in response to MCh inhalation compared with WT mice (Fig. 1C, 1D), as well as significant decreases in airway caliber (Fig. 1E, 1F). The increased AHR in *Sema4C*^{-/-} mice was also associated with enhanced infiltration of mononuclear and granulocytic cells into the lungs (Fig. 1E). The percentage of T cells and Tregs in the lungs was similar between WT and *Sema4C*^{-/-} mice (Supplemental Fig. 1C, 1D); however, the numbers of B cells and eosinophils (CD11b⁺CD11c^{-dim}Siglec-F⁺SSC-A^{high}) were significantly increased in inflamed lungs of *Sema4C*^{-/-} mice (Fig. 1G–J). No difference in CD11b⁺Siglec-F⁻ cells was detected between WT and *Sema4C*^{-/-} mice (Supplemental Fig. 1E, 1F). The majority of B cells detected in the lungs from mice with AAD exhibited the phenotype of follicular B cells; however, we were able to detect CD19⁺CD138⁺ plasma cells in lung cells isolated using a collagenase-free method (Supplemental Fig. 1G). Levels of Th2 cytokines, including IL-4 and IL-5, in BALF, as well as total and OVA-specific serum IgE, were also significantly increased in *Sema4C*^{-/-} mice following OVA challenge (Fig. 1K–M, Supplemental Fig. 1H, 1I). Serum IgE was not detectable in the absence of allergen exposure in PBS-treated *Sema4C*^{-/-} mice (Supplemental Fig. 1H). No significant difference in IL-17 or IFN- γ in BALF was detected between WT and *Sema4C*^{-/-} mice following allergen challenge (Supplemental Fig. 1J).

Sema4C-deficient B cells enhance pulmonary inflammation in response to OVA

To determine whether the augmented response to Ag inhalation in *Sema4C*^{-/-} mice was due to the lack of Sema4C expression on B cells, we generated B cell-specific Sema4C-deficient mixed bone marrow chimeric mice (S4C-B). WT-B mice were used as controls (Fig. 2A). Six weeks after reconstitution, no overt phenotypic defects were observed in chimeric mice. All peripheral B cells from WT-B mice expressed the congenic marker CD45.1 (Fig. 2B), indicating that the B cells in recipients were differentiated from donor bone marrow cells. Twenty percent of T cells from WT-B mice expressed CD45.1, and 80% expressed CD45.2 (Fig. 2B), indicating proportional repopulation of the transplanted bone marrow cells. No differences were detected in baseline splenic or lung B cells, including follicular B cells and marginal zone B cells, CD4⁺ T cells, CD8⁺ T cells, dendritic cells, and macrophages, between naive S4C-B or WT-B mice (Supplemental

Fig. 2B–F). Collectively, these data demonstrate that the chimeric mice were generated successfully.

S4C-B mice, sensitized and challenged with OVA, reproduced the enhanced pulmonary inflammation seen in *Sema4C*^{-/-} mice, exhibiting markedly increased eosinophils (Fig. 2C–E) and B cells in the lungs, with no difference in T cell populations (Fig. 2F, Supplemental Fig. 2F). Compared with WT-B mice, S4C-B mice with AAD exhibited elevated levels of BALF IL-4 and IL-5, as well as total and OVA-specific serum IgE (Fig. 2G–I, Supplemental Fig. 2G). These results indicate that the increased Th2 inflammation observed in *Sema4C*^{-/-} mice was primarily due to the defect in Sema4C specifically in B cells.

Sema4C^{-/-} B cells exhibit a deviation from regulatory to proinflammatory cytokine profiles

Elevated Th2 responses are associated with decreased regulatory cells and diminished IL-10 production (33). However, upon analysis of Treg numbers, we observed no difference in Treg in the lungs of sensitized and challenged WT mice compared with *Sema4C*^{-/-} mice or in chimeric WT-B mice versus S4C-B mice (Supplemental Fig. 1B). In contrast, >50% of the IL-10⁺ cells detected in spleens and draining lymph nodes in WT-B mice with AAD postchallenge were CD19⁺CD138⁺IL-10⁺ B cells (Fig. 3A–C). The presence of significant numbers of IL-10⁺ B cells was confirmed by performing our experiments using IL-10–GFP Vert-X reporter mice, which revealed that 40% of the GFP-expressing cells were CD19⁺CD138⁺ (Supplemental Fig. 3A). CD138⁺ B cells exhibited increased median fluorescent intensity for IL-10 compared with CD138⁻ B cells (Fig. 3D), suggesting greater cytokine production. Using imaging flow cytometry, IL-10 was demonstrable in CD19⁺CD138⁺ cells (Fig. 3E). IL-10 production was confirmed using sorted cultured CD19⁺CD138⁺ cells (Supplemental Fig. 3B). In S4C-B mice with a selective absence of Sema4C expression on B cells, the percentage and absolute number of CD19⁺CD138⁺IL-10⁺ cells were significantly decreased (Fig. 3F–H) compared with WT-B mice. No difference was noted in the percentage or frequency of nonplasma cells (CD19⁺CD138⁻) between S4C-B and WT-B mice (Supplemental Fig. 3C, 3D). In contrast to the ~40% of CD19⁺CD138⁺ cells that produced IL-10, only 0.3% of CD19⁺CD138⁻ cells are IL-10⁺. The frequency of IL-10–producing cells among CD19⁺CD138⁻ cells was comparable between S4C-B and WT-B mice with AAD (Supplemental Fig. 3E).

In contrast to the significant numbers of IL-10–producing cells in WT-B mice, S4C-B mice exhibited increased CD19⁺CD138⁺IL-4⁺ cells in spleens following OVA exposure (Fig. 3I–K). This led to an increase in the ratio of CD19⁺CD138⁺IL-4⁺/CD19⁺CD138⁺IL-10⁺ cells in S4C-B mice (Fig. 3L). Compared with CD138⁺CD19⁺ cells, there were lower significant numbers of IL-4⁺CD19⁺CD138⁻ or IL-4⁺CD19⁺ cells (Supplemental Fig. 3F). IL-4⁺ and IL-10⁺–producing CD19⁺CD138⁺ cells appeared to be two distinct populations (Supplemental Fig. 3G). These results demonstrate that Sema4C-deficient CD19⁺CD138⁺ cells exhibit a marked deviation from regulatory cytokine-producing cells to cells that primarily produce proinflammatory cytokines, such as IL-4 (34, 35).

Development of CD138⁺ B cells is highly dependent on optimal signals from T follicular helper cells (36). To investigate whether the cytokine profile from *Sema4C*^{-/-} CD19⁺CD138⁺ cells is due to an intrinsic defect or to incomplete T follicular helper cell signals in the germinal center, we provided optimal cytokine conditions by stimulating splenic B cells with anti-CD40 Abs with the addition of IL-4 and IL-21. After 96 h, similar to in vivo conditions, ~50% of WT IL-10–producing CD19⁺ cells were CD138⁺ (Fig. 4A). In contrast, few CD19⁺CD138⁻ cells produced

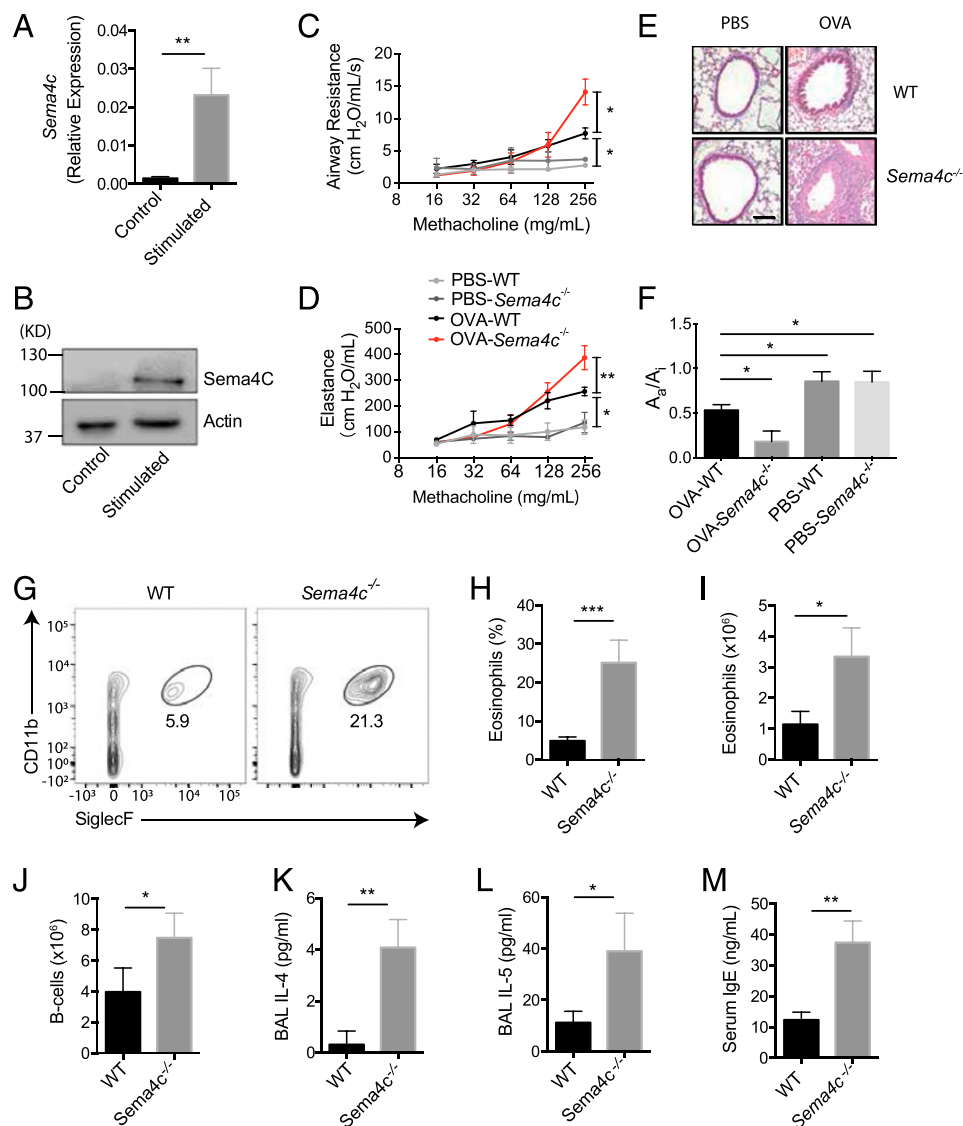


FIGURE 1. *Sema4c*^{-/-} mice develop severe AAD. (A and B) Murine B cells express Sema4C. (A) Sema4C expression in unstimulated B cells (Control) and B cells stimulated with anti-CD40+IL-4+IL-21 for 24 h, measured by RT-PCR, normalized to GAPDH. Data are mean $\pm$ SEM, $n = 3$. (B) Representative Western blot showing Sema4C protein in unstimulated B cells (Control) and B cells stimulated as above for 72 h ($n = 3$). (C–M) *Sema4c*^{-/-} mice exhibit an enhanced response to allergen challenge. WT and *Sema4c*^{-/-} mice were sensitized and challenged with OVA, as described. Lung function and pulmonary inflammation were examined 24 h after the last challenge. Airway resistance (C) and elastance (D) showing increased AHR in *Sema4c*^{-/-} mice compared with WT mice in response to MCh. (E) Representative H&E staining of lung sections from PBS- and OVA-exposed WT mice (upper panel) and *Sema4c*^{-/-} mice (lower panel) showing marked pulmonary inflammation in *Sema4c*^{-/-} mice in response to OVA. Scale bar, 50 μ m. (F) Calculation of severity of airway narrowing in WT and *Sema4c*^{-/-} naive mice and those with AAD ($n = 10$ airways per mouse), indicating greater airway narrowing in *Sema4c*^{-/-} mice. Percentage (G and H) and frequency (I) of eosinophils in lung digests from WT and *Sema4c*^{-/-} mice with AAD. All cells were pregated using viability dyes for live cells and singlets, and populations were compared using fluorescence minus one. Eosinophils were defined as CD11b⁺Siglec-F⁺CD11c⁻/midSSC-A^{high}. Pulmonary B cells (CD19⁺SSC-A^{low}) (J) and BALF IL-4 (K), BALF IL-5 (L), and serum IgE (M) levels in WT and *Sema4c*^{-/-} mice with AAD; all indicate greater Th2 inflammation in *Sema4c*^{-/-} mice. Data are mean $\pm$ SEM, $n = 6$, two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IL-10 (Supplemental Fig. 3H). The number and percentage of total CD138⁺ cells were similar between WT and *Sema4c*^{-/-} mice (Fig. 4B, 4C). However, among CD138⁺ cells, $13.47 \pm 1.27\%$ of cells in WT culture expressed IL-10 compared with $7.09 \pm 0.63\%$ in *Sema4c*^{-/-} culture (Fig. 4D, 4E). IL-10 production from *Sema4c*^{-/-} mice was also diminished in cell culture supernatants compared with those from WT mice (Fig. 4F).

Exogenous Sema4C rescues IL-10 production in *Sema4c*^{-/-} B lymphocytes

Semaphorin 4 family members have short cytoplasmic tails and primarily induce signals via interaction with receptors, such as Plexin B2 (16). In our study, nearly 100% of CD19⁺CD138⁺ cells

express Plexin B2 (Supplemental Fig. 3I). Therefore, we hypothesized that the loss of Sema4C–Plexin B2 contact within spleen or lymph nodes could influence the observed dysregulated cytokine production. To determine whether we could restore normal cytokine production by provision of a source of cell-bound Sema4C to Plexin B2–expressing B cells, we created stably transfected HEK293 cells with a full-length construct of Sema4C. Fig. 4G demonstrates that coculturing *Sema4c*^{-/-} B cells with Sema4C stably transfected HEK293 cells successfully rescued IL-10 production in *Sema4c*^{-/-} CD138⁺ B cells to WT levels, whereas control untransfected HEK293 cells had no effect on IL-10 production. This suggests that Sema4C induces CD138⁺ Bregs through contact with a cognate receptor, such as Plexin B2.

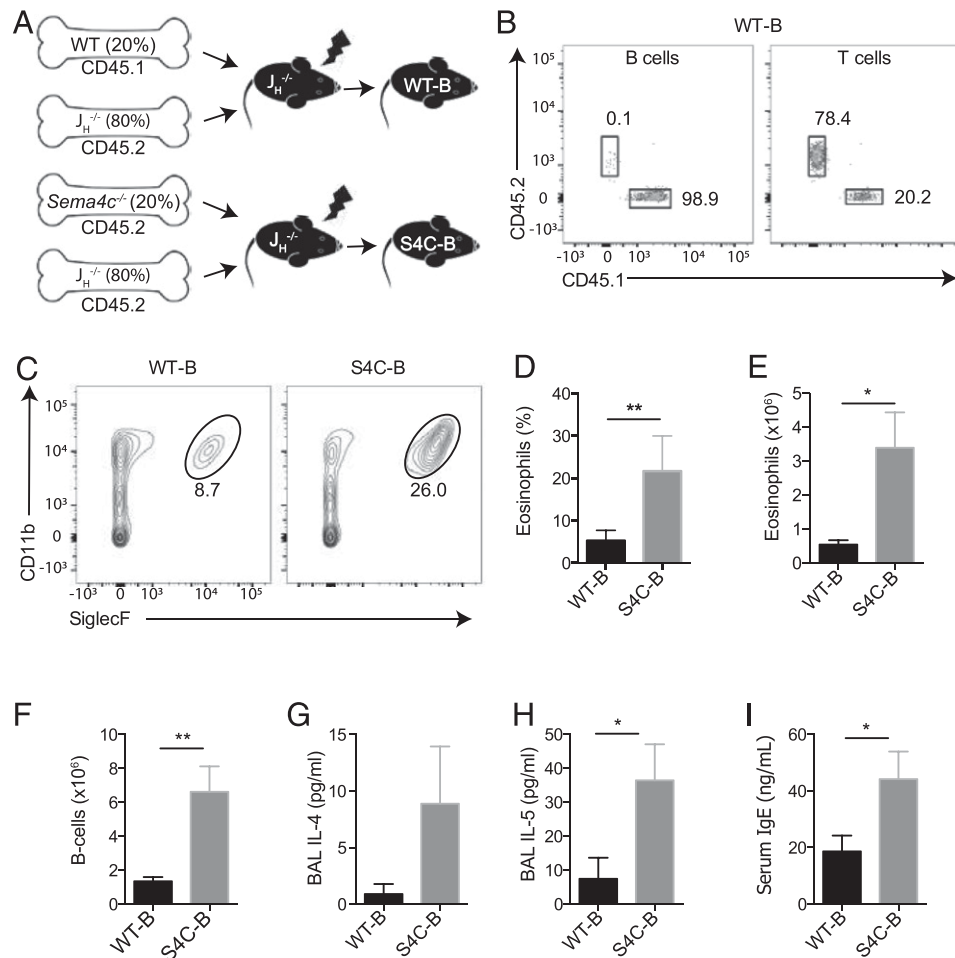


FIGURE 2. Sema4C-deficient B cells (S4C-B) induce severe Th2 inflammation in AAD. (A and B) Generation of B cell-specific Sema4C-deficient mice. B cell-deficient mice ($J_H^{-/-}$ -CD45.2) were lethally irradiated and reconstituted with bone marrow from $J_H^{-/-}$ (80%) and $Sema4C^{-/-}$ (CD45.2) (20%) mice. $J_H^{-/-}$ mice reconstituted with $J_H^{-/-}$ (80%) and C57BL/6-CD45.1 WT (20%) bone marrow (WT-B) were used as controls. After 6 wk of reconstitution, blood was collected from the saphenous vein and examined. (B) Dot plot showing congenic marker expression on B cells and T cells from WT-B mice, which demonstrates that reconstitution was successful. (C–I) S4C-B mice had elevated pulmonary inflammation compared with WT-B mice with AAD. S4C-B and WT-B mice were sensitized and challenged with OVA, and pulmonary inflammation was examined 24 h after the final challenge. Percentage (C and D) and frequency (E) of CD11b⁺SiglecF⁺CD11c^{mid}SSC-A^{high} eosinophils in lung digests of WT-B and S4C-B mice with AAD. Cells were pregated on live cells and singlets. Fluorescence minus one and gating strategy were as in Fig. 1. (F) B cell counts in pulmonary digests of WT-B and S4C-B mice with AAD. BALF IL-4 (G), BALF IL-5 (H), and serum IgE (I) levels in WT-B and S4C-B mice with AAD, all indicating greater Th2 inflammation in S4C-B mice. Data are mean $\pm$ SEM, $n = 6$ per group, two independent experiments. * $p < 0.05$, ** $p < 0.01$.

Sema4C-deficient CD19⁺CD138⁺ cells augment AHR following allergen challenge

Given the decreased IL-10 and increased IL-4 in $Sema4C^{-/-}$ CD19⁺CD138⁺ B cells, we hypothesized that $Sema4C^{-/-}$ CD19⁺CD138⁺ cells played an important role in the augmented Th2 response to OVA. To test this, we adoptively transferred CD19⁺CD138⁺ cells from spleens of OVA-sensitized WT or $Sema4C^{-/-}$ mice to recipient WT mice i.t. 24 h prior to the first OVA challenge. Mice receiving $Sema4C^{-/-}$ CD19⁺CD138⁺ cells exhibited markedly increased airway inflammation (Fig. 5A, 5B) and BALF eosinophilia (Fig. 5C). The levels of IL-4 and IL-5 in BALF were also significantly elevated in mice receiving $Sema4C^{-/-}$ cells compared with those receiving WT cells or PBS (Fig. 5D, 5E). Consistent with $Sema4C^{-/-}$ and S4C-B mice, mice receiving $Sema4C^{-/-}$ CD19⁺CD138⁺ cells had a markedly increased pulmonary eosinophilia (Fig. 5F–H), whereas mice receiving WT cells or vehicle control had statistically similar measures of airway inflammation. Collectively, these data directly link Sema4C to the production of optimal regulatory cytokines in CD19⁺CD138⁺ cells, as well as to the downstream consequences on Th2 responses in AAD.

To confirm that WT CD19⁺CD138⁺IL-10⁺ plasma cells could act as regulatory cells and inhibit Th2 inflammation, we sorted CD19⁺CD138⁺IL-10⁺ or CD19⁺CD138⁺IL-10⁻ cells from OVA-exposed IL-10-GFP reporter mice. Sorted cells or PBS control were transferred i.t. to OVA-sensitized mice 24 h before the first OVA challenge, as above. OVA-sensitized and -challenged mice receiving CD19⁺CD138⁺IL-10⁺ cells had significantly lower pulmonary histological inflammation scores compared with mice that received the vehicle control (Fig. 6A, 6B). CD19⁺CD138⁺IL-10⁺ recipients also exhibited lower serum IgE and decreased eosinophilic BALF infiltrates (Fig. 6C–E). OVA-sensitized and -challenged WT mice that received CD19⁺CD138⁺IL-10⁻ cells exhibited significantly increased lung inflammation, increased BALF eosinophils, and elevated serum IgE compared with those receiving CD19⁺CD138⁺IL-10⁺ cells (Fig. 6).

In addition, adoptive transfer of CD19⁺CD138⁺IL-10⁺ cells from OVA-exposed IL-10-GFP reporter mice to OVA-sensitized $Sema4C^{-/-}$ mice with AAD 24 h before the first OVA challenge virtually reversed the pulmonary inflammation that characterized $Sema4C^{-/-}$ mice. As shown in Fig. 7, $Sema4C^{-/-}$ mice receiving

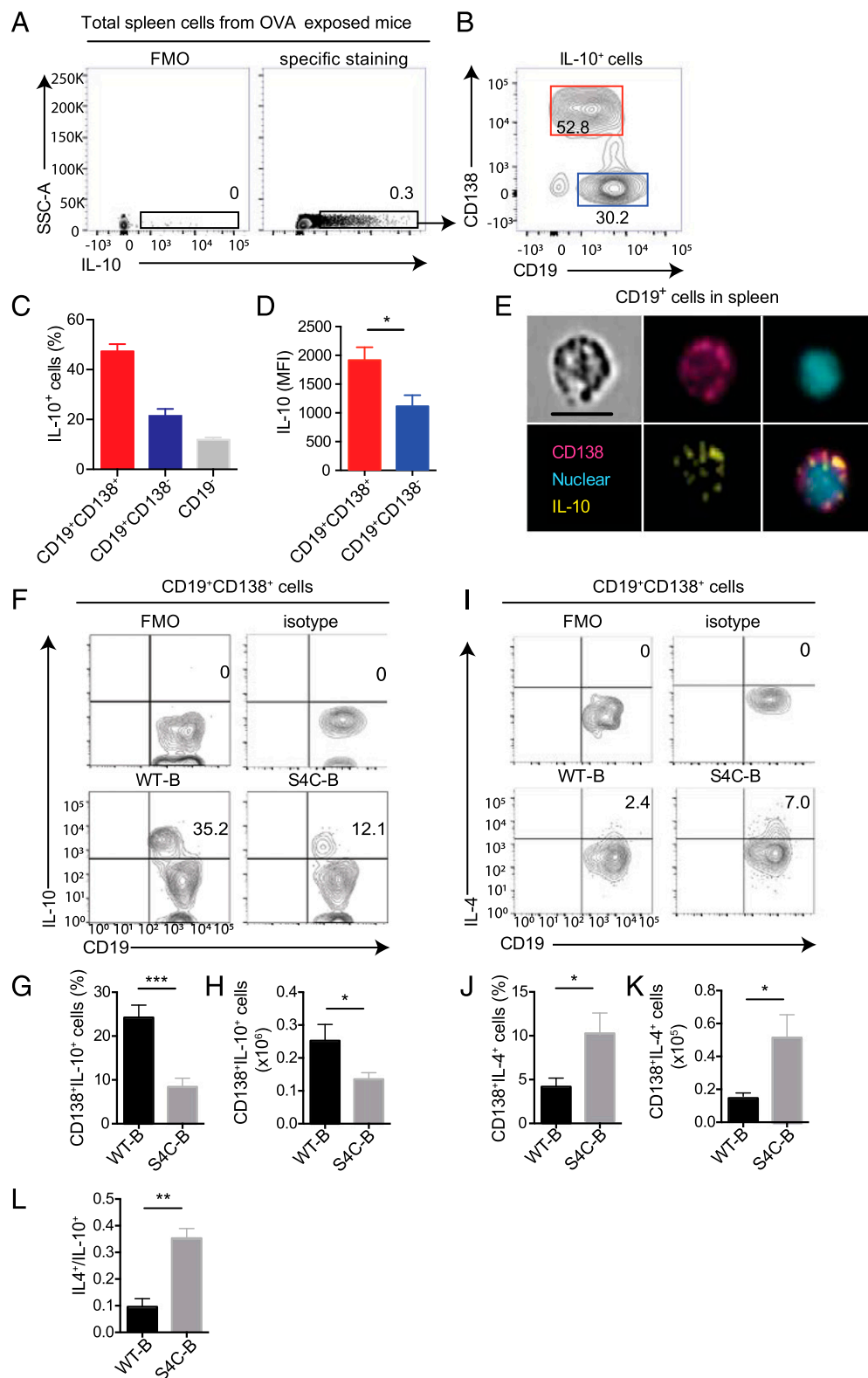
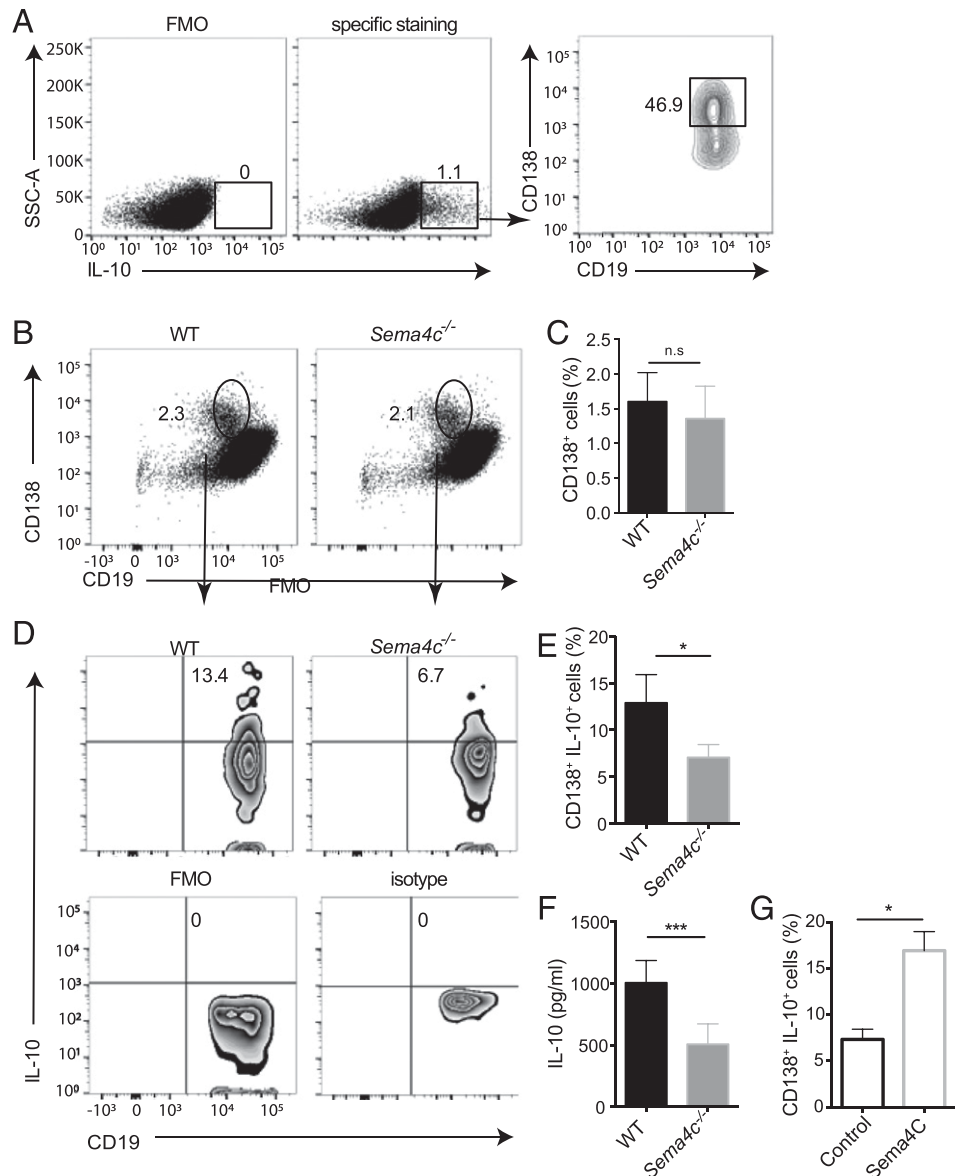


FIGURE 3. *Sema4c*^{-/-} B cells exhibit deviations in cytokine profile from regulatory to proinflammatory cytokines. Splenocytes were isolated 24 h following OVA challenge from WT-B and S4C-B mice. (A–D) Demonstration of CD19⁺CD138⁺ IL-10⁺ splenocytes in AAD. (A) Fluorescence minus one (FMO) and identification of IL-10⁺ population in spleens following OVA sensitization and challenge in WT mice. (B) Dot plot indicating that >50% of IL-10–producing cells in the spleens were CD19⁺CD138⁺ cells. IL-10⁺ gating was based on FMO in (A). (C) Percentage of specific cells among total IL-10⁺ cells, indicating that the CD19⁺CD138⁺ cell population accounted for the majority of IL-10⁺ cells. (D) IL-10 median fluorescent intensity (MFI) from IL-10⁺CD138⁺ B cells and IL-10⁺CD138⁻ B cells. (E) Representative image taken by imaging flow cytometry showing IL-10 colocalized in CD19⁺CD138⁺ cells. Imaging cytometry was performed by pregating only on cells in focus, live cells, singlets, and CD19⁺ cells. Scale bar, 10 μ m. (F–K) *Sema4c*-deficient B cells express lower IL-10 and increased IL-4. (F and G) Percentage of IL-10⁺ cells among CD19⁺CD138⁺ cells. IL-10⁺ gating was based on FMO and isotype controls. (H) Total CD19⁺CD138⁺IL-10⁺ cells in spleens of WT-B and S4C-B mice with AAD. (I and J) Percentage of IL-4⁺ cells among CD19⁺CD138⁺ cells. IL-4⁺ gating was based on FMO and isotype controls. (K) Total CD19⁺CD138⁺IL-4⁺ cells in spleens of WT-B and S4C-B mice with AAD. (L) Ratio of CD19⁺CD138⁺IL-4⁺/CD19⁺CD138⁺IL-10⁺ cells in spleens of WT-B and S4C-B mice with AAD. Data are mean $\pm$ SEM, $n = 8$, two independent experiments. *** $p < 0.001$.

B cells from naive mice were stimulated with α CD40+IL-4+IL-21 for 96 hours

FIGURE 4. *Sema4c*^{-/-} B cells demonstrate impaired differentiation into CD19⁺CD138⁺IL-10⁺ cells. Splenic B cells from naive WT or *Sema4c*^{-/-} mice were stimulated with anti-CD40+IL-4+IL-21. After 96 h, cells were analyzed by flow cytometry, and culture supernatants were analyzed by ELISA. For cell analysis, GolgiStop and PMA/ionomycin were added 5 h prior to harvesting. (A–F) *Sema4c*^{-/-} B cells produced decreased IL-10 in vitro. (A) Dot plot showing that 46.9% of IL-10–producing B cells were CD138⁺ in WT culture. IL-10 gating was based on fluorescence minus one (FMO). (B and C) Percentage of total CD19⁺CD138⁺ cells in WT and *Sema4c*^{-/-} culture, demonstrating no apparent defect in overall production of CD138⁺ cells in *Sema4c*^{-/-}. (D and E) Percentage of IL-10⁺ cells among CD19⁺CD138⁺ cells. (F) IL-10 concentration in culture supernatants of B cells from WT and *Sema4c*^{-/-} mice, showing diminished IL-10 in *Sema4c*^{-/-} mice. (G) Exogenous Sema4C rescued CD138⁺ IL-10⁺ cell induction in cultured B cells from *Sema4c*^{-/-} mice. *Sema4c*^{-/-} B cells were cocultured with nontransfected HEK293 cells (Control) or Sema4C stably transfected HEK293 cells (Sema4C) in the presence of anti-CD40+IL-4+IL-21. After 96 h, percentages of CD138⁺IL-10⁺ cells among CD138⁺ B cells were measured by flow cytometry. Data are mean $\pm$ SEM, $n = 5$, three independent experiments. * $p < 0.05$, *** $p < 0.001$. n.s., not significant.



CD19⁺CD138⁺IL-10⁺ cells had significantly lower pulmonary histological inflammation scores (Fig. 7A, 7B) and markedly fewer eosinophils in BALF (Fig. 7C, 7D). These results demonstrate that a defect in IL-10–producing plasma cells contributes to the pathology observed in mice deficient in Sema4C.

Discussion

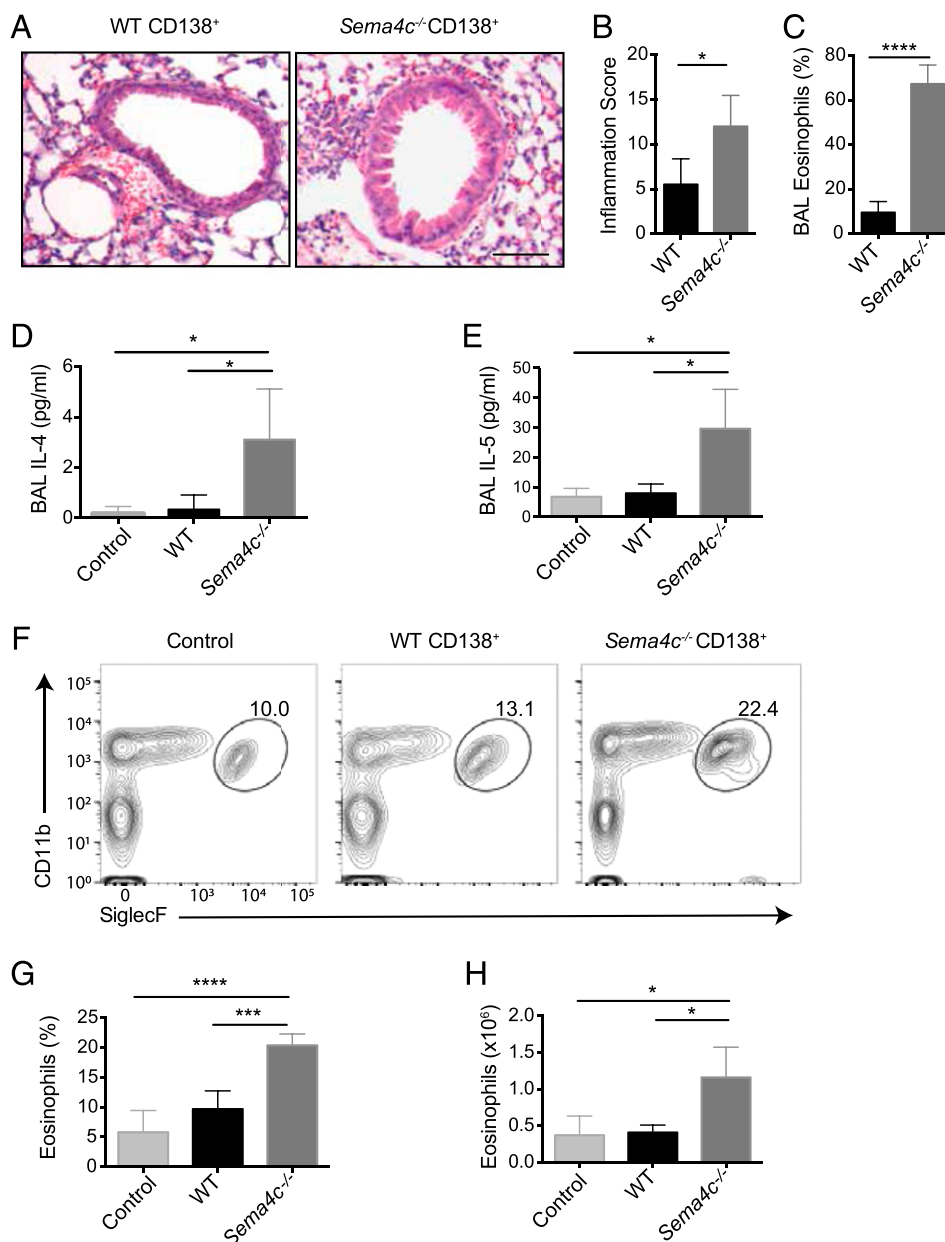
In this study, using knockout mice, mixed bone marrow chimeric mice, and adoptive-transfer models, we demonstrated that expression of Sema4C on B cells played a critical and protective role in AAD. The absence of Sema4C specifically on B cells led to significantly elevated IL-4, IL-5, and IgE and a robust eosinophilia in the lung. Adoptive transfer of CD19⁺CD138⁺ cells from *Sema4c*^{-/-} mice induced a marked influx of eosinophils to the airways, with strikingly increased BALF IL-4 and IL-5. The phenotype of the *Sema4c*^{-/-} was normalized with the transfer of WT CD19⁺CD138⁺IL-10⁺ cells.

Semaphorins were first identified as axonal guidance molecules in embryonic neurodevelopment. There has been an increased interest in semaphorin molecules in allergic diseases and, in par-

ticular, in airway inflammation, especially asthma (37–39). In a murine allergic rhinitis model, semaphorin 3A expression was decreased in the inflamed nasal mucosa, and treatment with exogenous semaphorin 3A improved mucosal histological changes, nasal rubbing, and sneezing (40). Semaphorin 3E augmented airway smooth muscle hypertrophy by inhibiting cell migration and signaling (2), with similar inhibitory effects on macrophages (41). Nkyimbeng-Takwi et al. (42) found that *Sema4a*^{-/-} mice had higher baseline AHR and were hyperresponsive to OVA challenge. *Sema4d*^{-/-} mice had low airway eosinophilia, normal basal AHR, and modest decreases in airway inflammation (43).

Compared with data for other members of the semaphorin 4 family, the phenotype of *Sema4c*^{-/-} mice following allergen challenge is the most striking, characterized by markedly increased airway narrowing and a profound eosinophilic infiltrate. Our interest in Sema4C was triggered by our findings of strong upregulation of this molecule on human B cells following activation with anti-CD40 and IL-4 (11). This led us to further characterize Sema4C-sufficient and -knockout B cells for their pro- and anti-inflammatory potential. In our models, the response

FIGURE 5. Airway inflammation is exacerbated by adoptive transfer of *Sema4c*^{-/-} CD138⁺ cells. CD19⁺ CD138⁺ cells from OVA-sensitized WT and *Sema4c*^{-/-} mice or Control (PBS) mice were transferred i.t. to OVA-sensitized WT mice 24 h before the first of three OVA challenges. Analysis was performed 24 h after the last OVA challenge. (A) Representative H&E staining of lung sections from mice receiving CD19⁺ CD138⁺ WT cells or CD19⁺ CD138⁺ *Sema4c*^{-/-} cells, showing exacerbated inflammation in mice receiving CD19⁺ CD138⁺ *Sema4c*^{-/-} cells. Scale bar, 200 μ m. Histopathology scores (B) and BALF eosinophil counts (C) following sacrifice. Mice receiving PBS were not significantly different from mice receiving WT CD19⁺ CD138⁺ cells. IL-4 (D) and IL-5 (E) concentrations in BALF from mice receiving PBS (Control) or WT or *Sema4c*^{-/-} CD19⁺ CD138⁺ cells. Percentage (F and G) and frequency (H) of CD11b⁺ Siglec-F⁺ CD11C^{-mid} SSC-A^{high} eosinophils in lung digests from mice receiving PBS (Control) or WT or *Sema4c*^{-/-} CD19⁺ CD138⁺ cells. Cells were pre-gated on live cells and singlets. Data are mean $\pm$ SEM, $n = 4$, two independent experiments. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.



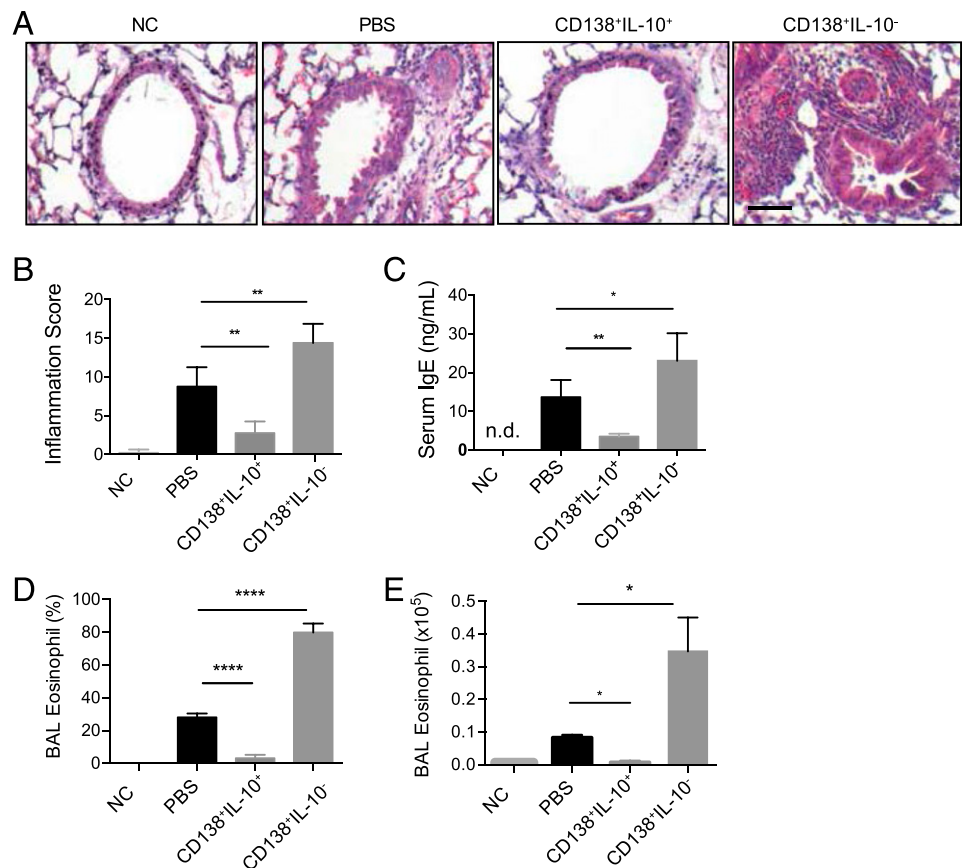
to inhaled allergen is characterized by differentiation of specific cytokine-secreting CD19⁺CD138⁺ populations. WT B cells develop significant numbers of CD19⁺CD138⁺IL-10⁺ cells that counter-balance the induction of CD19⁺CD138⁺IL-4⁺ cells, whereas *Sema4c*^{-/-} cells exhibit a higher number and percentage of CD19⁺CD138⁺ cells that express IL-4, with decreased numbers of IL-10⁺ cells. These CD19⁺CD138⁺ subsets are analogous to the CD138⁺ regulatory plasmablasts or plasma cells described by Shen et al. (44) and Matsumoto et al. (45). The significant shift in the CD19⁺CD138⁺IL-4⁺/CD19⁺CD138⁺IL-10⁺ ratio is clearly associated with worsening airway disease and suggests a key role for Sema4C in cytokine production of CD138⁺ B cells, particularly with regard to the induction of CD19⁺CD138⁺IL-10⁺ cells following allergen exposure.

Although little is known about Sema4C in the immune system, it was shown to influence cell migration and be a positive regulator of epithelial-mesenchymal transition (EMT). Sema4C mediates TGF- β -driven EMT in renal tubular cells and induces EMT in malignant cell lines, including breast cancer and small cell lung

carcinoma (46, 47). Among Sema4 family members, it is the only one with a PDZ-binding domain, which allows it to bind certain leucine-rich proteins, such as Erbin (48). Plexin B2, the main binding partner for Sema4C, is highly expressed on B cells in germinal centers following T cell-dependent Ag stimulation (13), and we show that expression of Plexin B2 on CD19⁺CD138⁺ splenocytes is nearly 100%. Plexin B2 signaling is via the GAP domain, and plexin-mediated signaling appears to account for most properties of plexin-semaphorin interaction (49). Within the lymphoid tissue, T follicular cells produce IL-4 and IL-21, cytokine signals that facilitate the development of memory B cells and plasma cells (36). Indeed, these cytokines result in strong upregulation of Sema4C, which then can interact with Plexin B2-expressing B cells in a paracrine or autocrine manner. This model was tested by the use of exogenous Sema4C expressed on HEK293 cells, which augmented IL-10 production by Sema4C-deficient B cells. In these experiments, the source of Plexin B2 was the B cells in culture, and the Sema4C binding partner was provided solely by the transfected HEK293 cells.

Recipient: WT mice

FIGURE 6. Adoptive transfer of CD138⁺IL-10⁺ cells attenuates airway inflammation in WT mice. CD138⁺IL-10⁺ cells and CD138⁺IL-10⁻ cells were harvested from IL-10-GFP mice exposed to OVA or saline and transferred i.t. to WT mice with AAD 1 d prior to OVA challenge. Controls included OVA-exposed and -challenged mice given PBS i.t. and PBS-exposed and -challenged mice (NC). Representative H&E staining of lung sections (**A**) and histopathology scores (**B**) following challenge. CD138⁺IL-10⁺ cells abrogated airway inflammation, whereas CD138⁺IL-10⁻ cells markedly increased pulmonary inflammation. Scale bar, 200 μ m. Serum IgE concentrations (**C**), BALF eosinophil percentages (**D**), and eosinophil absolute counts (**E**) demonstrating increased IgE and eosinophilia in mice receiving CD138⁺IL-10⁻ cells. Data are mean $\pm$ SEM, $n = 4$, two independent experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. n.d., not detectable.



Indeed, in vivo there may be multiple sources of Plexin B2 in B cell follicles that can interact with Sema4C on B cells; thus, Sema4C–Plexin B2 binding may occur via paracrine and autocrine interaction. The role of Sema4C–Plexin B2 signaling in the development of Bregs is being explored in our laboratory.

Beyond Ig synthesis and Ag presentation, B cells are often ignored as effectors of other facets of immune responses, particularly their role as cytokine-producing cells. The initial descriptions of murine B effector cells (50) led to extensive study of the contribution of B cells to the cytokine network. Not surprisingly, pro- and anti-inflammatory cytokines can be produced by human and murine B cells, including regulatory cytokines, such as TGF- β and IL-10 (51). In particular, Bregs or B10 cells were investigated in autoimmune conditions (51–53).

There is no single definition for Bregs; similar to Tregs, the term Breg likely reflects different populations. Bregs in mice and humans produce IL-10 and express surface Ags, such as CD19⁺CD1d^{high}CD21^{high}CD23⁺sIgM^{high} cells in mice or CD19⁺CD38⁺CD24⁺ cells in humans. Alternative populations, including CD19⁺CD5⁺ (18), CD19⁺CD25⁺ (54), CD19⁺PDL-1⁺ (55), and CD25⁺CD71⁺CD73⁻ subsets (56), were suggested as potential Bregs. CD38⁺CD27⁺ B cells were demonstrated to produce significant amounts of IL-10 in humans (45, 57). Shen and Fillatreau (58) proposed that Bregs defined via in vitro–stimulation assays and adoptive-transfer experiments are distinct from those identified as IL-10- or IL-35-expressing cells in vivo. In our studies, CD19⁺CD138⁺ cells appear to be a crucial IL-10-producing B cell subset in vivo. CD19⁺CD138⁺ cells account for 2% of splenic B cells, yet they account for >50% of IL-10-producing B cells in our AAD

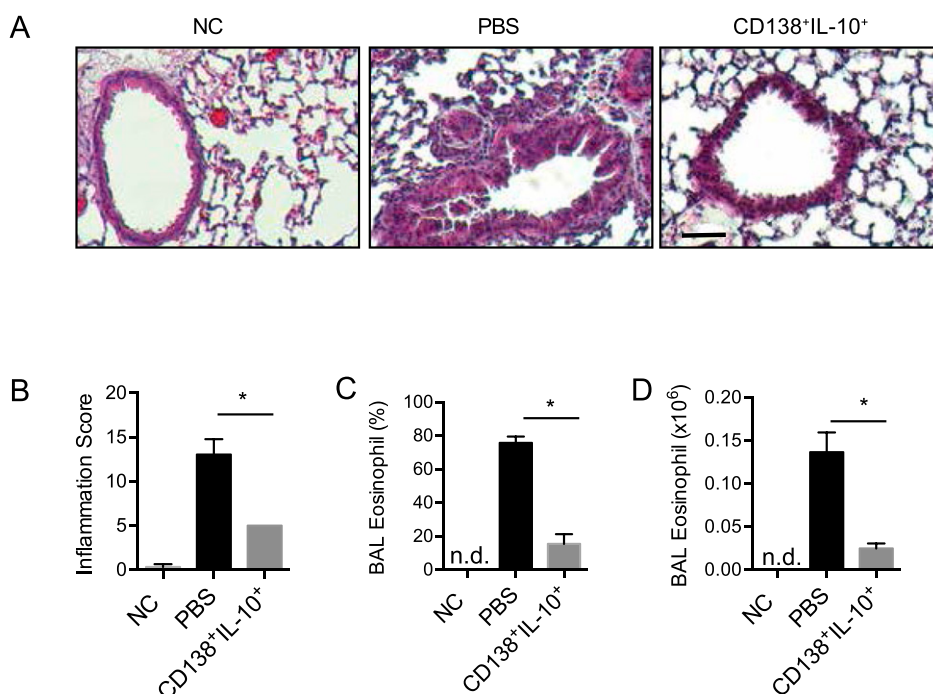
model. This is consistent with work in multiple sclerosis models, in which IL-10-producing CD138⁺ cells have a remissive effect on experimental autoimmune encephalomyelitis (44, 45). The regulatory cytokine IL-35 also may play a role in these regulatory processes (44). We are in the process of determining whether this cytokine is present in CD19⁺CD138⁺IL-10⁺ B cells from *Sema4c*^{-/-} mice.

In allergy, a relatively limited number of studies examined the role of CD138⁺IL-10⁺ cells in airway disease. van der Vlugt et al. (57) suggested that a key feature of atopic asthma is a lack of Bregs. Natural history studies that follow beekeepers over extended periods of time, as well as in vitro gene-overexpression studies, indicate regulatory and therapeutic potential for IL-10⁺ B cells (56, 59, 60). We and other investigators showed that IgE-producing B cells and plasma cells are found in asthmatic airways, and large B cell follicles were found in the airways of severe asthmatics (61). Our current data underline the need to understand the potential role that B cell subsets play in airway inflammation (59).

This study identifies a novel pathway by which Th2-mediated immune responses are regulated. We demonstrate that Sema4C is critical for optimal regulatory cytokine production from CD138⁺ B cells. Our study suggests that CD138⁺ B cells contribute to cytokine balance and that they are important for maintenance of immune homeostasis in AAD. Furthermore, interaction between Sema4C and Plexin B2 may be required for regulation of cytokine production by activated B cells. Our study also highlights the importance of plasma cells as regulatory cells in allergic inflammation and provides a clue for exploring a link toward defining key molecules that can induce Breg development.

Recipient: *Sema4C*^{-/-} mice

FIGURE 7. Adoptive transfer of CD138⁺IL-10⁺ cells attenuates airway inflammation in *Sema4C*^{-/-} mice with AAD. CD138⁺IL-10⁺ cells were harvested from OVA-sensitized IL-10-GFP mice. CD138⁺IL-10⁺GFP cells or PBS were transferred i.t. to OVA-sensitized *Sema4C*^{-/-} mice 1 d prior to OVA challenge. Sham-sensitized and -challenged mice were used as negative controls (NC). Representative H&E staining of lung sections (**A**) and histopathology scores (**B**) following challenge in mice receiving saline or CD138⁺IL-10⁺ cells i.t., indicating that CD138⁺IL-10⁺ cells alleviated airway inflammation. Scale bar, 200 μ m. Percentage (**C**) and absolute cell counts (**D**) of eosinophils in BALF, confirming the effect of CD138⁺IL-10⁺ cells on pulmonary inflammation in *Sema4C*^{-/-} mice. Data are mean $\pm$ SEM, $n = 3$, two independent experiments. * $p < 0.05$. n.d., not detectable.



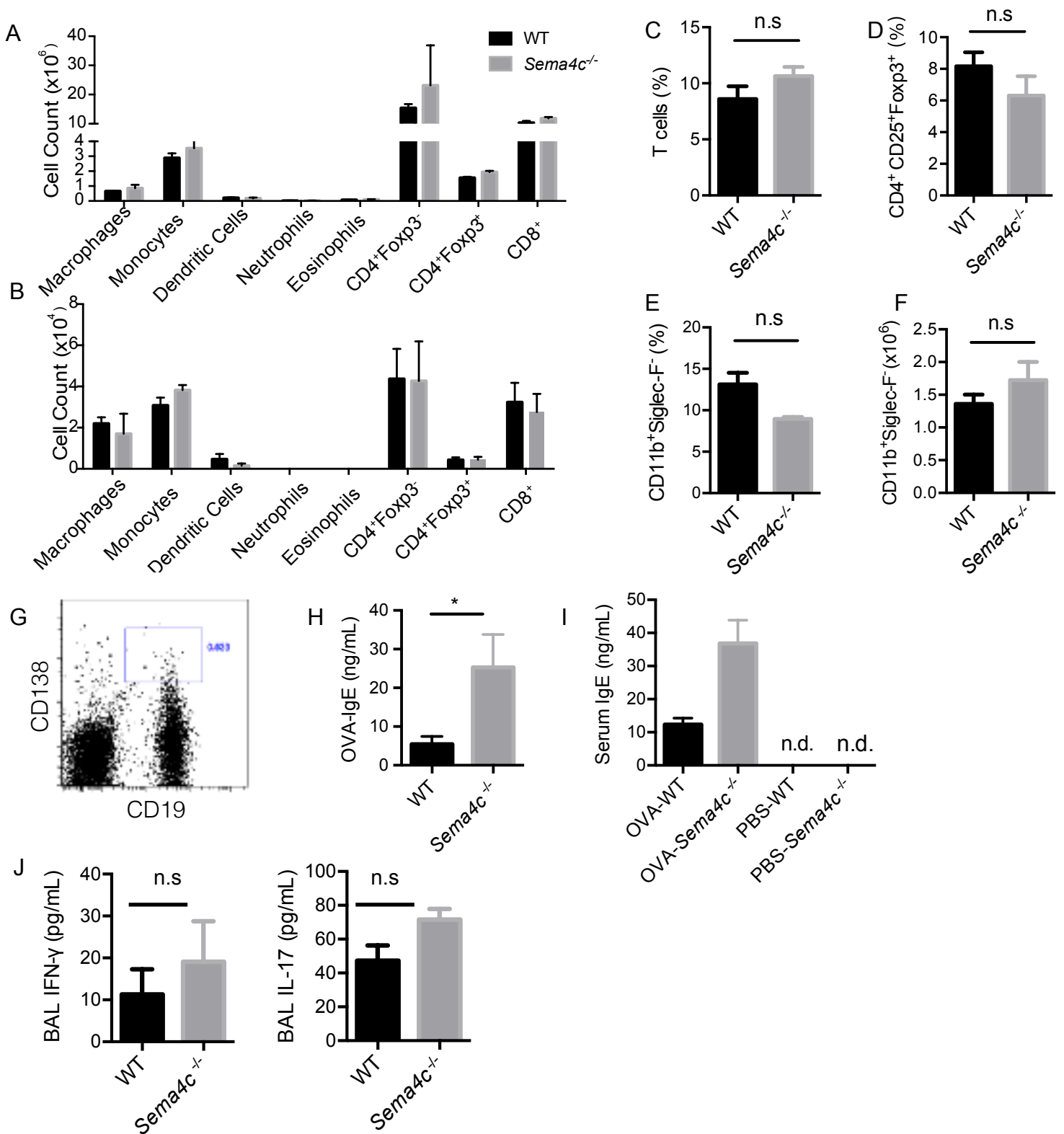
Disclosures

The authors have no financial conflicts of interest.

References

- Vadivel, A., R. S. Alphonse, J. J. Collins, T. van Haaften, M. O'Reilly, F. Eaton, and B. Thébaud. 2013. The axonal guidance cue semaphorin 3C contributes to alveolar growth and repair. *PLoS One* 8: e67225.
- Movassagh, H., L. Shan, A. J. Halayko, M. Roth, M. Tamm, J. Chakir, and A. S. Gouni. 2014. Neuronal chemorepellent Semaphorin 3E inhibits human airway smooth muscle cell proliferation and migration. *J. Allergy Clin. Immunol.* 132: 560–567.
- Masuda, T., M. Taniguchi, C. Sakuma, T. Yamagishi, S. Ueda, M. Kawaguchi, and H. Yaginuma. 2013. Development of the dorsal ramus of the spinal nerve in the mouse embryo: involvement of semaphorin 3A in dorsal muscle innervation. *Congenit. Anom. (Kyoto)* 53: 122–126.
- Fukuda, T., S. Takeda, R. Xu, H. Ochi, S. Sunamura, T. Sato, S. Shibata, Y. Yoshida, Z. Gu, A. Kimura, et al. 2013. Sema3A regulates bone-mass accrual through sensory innervations. *Nature* 497: 490–493.
- Bender, R. J., and F. Mac Gabhann. 2013. Expression of VEGF and semaphorin genes define subgroups of triple negative breast cancer. *PLoS One* 8: e61788.
- Negishi, M., I. Oinuma, and H. Katoh. 2005. Plexins: axon guidance and signal transduction. *Cell. Mol. Life Sci.* 62: 1363–1371.
- Kumanogoh, A., T. Shikina, K. Suzuki, S. Uematsu, K. Yukawa, S. Kashiwamura, H. Tsutsui, M. Yamamoto, H. Takamatsu, E. P. Ko-Mitamura, et al. 2005. Nonredundant roles of Sema4A in the immune system: defective T cell priming and Th1/Th2 regulation in Sema4A-deficient mice. *Immunity* 22: 305–316.
- Delgoffe, G. M., S. R. Woo, M. E. Turnis, D. M. Gravano, C. Guy, A. E. Overacre, M. L. Bettini, P. Vogel, D. Finkelstein, J. Bonnevier, et al. 2013. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature* 501: 252–256.
- Nakagawa, Y., H. Takamatsu, T. Okuno, S. Kang, S. Nojima, T. Kimura, T. R. Kataoka, M. Ikawa, T. Toyofuku, I. Katayama, and A. Kumanogoh. 2011. Identification of semaphorin 4B as a negative regulator of basophil-mediated immune responses. *J. Immunol.* 186: 2881–2888.
- Kumanogoh, A., C. Watanabe, I. Lee, X. Wang, W. Shi, H. Araki, H. Hirata, K. Iwahori, J. Uchida, T. Yasui, et al. 2000. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* 13: 621–631.
- Xue, D., M. Desjardins, G. N. Kaufman, M. Beland, S. Al-Tamemi, E. Ahmed, S. Tao, R. Friedel, W. Mourad, and B. D. Mazer. Semaphorin 4C: a novel component of B-cell polarization in Th2 driven immune responses. *Front. Immunol.* In press.
- Weisel, F. J., G. V. Zuccarino-Catania, M. Chikina, and M. J. Shlomchik. 2016. A temporal switch in the germinal center determines differential output of memory B and plasma cells. *Immunity* 44: 116–130.
- Yu, D., M. C. Cook, D. M. Shin, D. G. Silva, J. Marshall, K. M. Toellner, W. L. Havran, P. Caroni, M. P. Cooke, H. C. Morse, et al. 2008. Axon growth and guidance genes identify T-dependent germinal centre B cells. *Immunol. Cell Biol.* 86: 3–14.
- Deng, S., A. Hirschberg, T. Worzfeld, J. Y. Penachioni, A. Korostylev, J. M. Swiercz, P. Vodrazka, O. Mauti, E. T. Stoeckli, L. Tamagnone, et al. 2007. Plexin-B2, but not Plexin-B1, critically modulates neuronal migration and patterning of the developing nervous system in vivo. *J. Neurosci.* 27: 6333–6347.
- Maier, V., C. Jolicœur, H. Rayburn, N. Takegahara, A. Kumanogoh, H. Kikutani, M. Tessier-Lavigne, W. Wurst, and R. H. Friedel. 2011. Semaphorin 4C and 4G are ligands of Plexin-B2 required in cerebellar development. *Mol. Cell. Neurosci.* 46: 419–431.
- Perala, N., M. Jakobson, R. Ola, P. Fazzari, J. Y. Penachioni, M. Nymark, T. Tanninen, T. Immonen, L. Tamagnone, and H. Sariola. 2011. Sema4C-Plexin B2 signalling modulates ureteric branching in developing kidney. *Differentiation* 81: 81–91.
- Prussin, C., and D. D. Metcalfe. 2003. 4. IgE, mast cells, basophils, and eosinophils. *J. Allergy Clin. Immunol.* 111(2 Suppl.): S486–S494.
- Yanaba, K., J. D. Bouaziz, K. M. Haas, J. C. Poe, M. Fujimoto, and T. F. Tedder. 2008. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity* 28: 639–650.
- Yoshizaki, A., T. Miyagaki, D. J. DiLillo, T. Matsushita, M. Horikawa, E. I. Kountikov, R. Spolski, J. C. Poe, W. J. Leonard, and T. F. Tedder. 2012. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* 491: 264–268.
- Amu, S., S. P. Saunders, M. Kronenberg, N. E. Mangan, A. Atzberger, and P. G. Fallon. 2010. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J. Allergy Clin. Immunol.* 125: 1114–1124 e1118.
- Fillatreau, S., C. H. Sweeney, M. J. McGeachy, D. Gray, and S. M. Anderson. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat. Immunol.* 3: 944–950.
- Haas, K. M., R. Watanabe, T. Matsushita, H. Nakashima, N. Ishiura, H. Okochi, M. Fujimoto, and T. F. Tedder. 2010. Protective and pathogenic roles for B cells during systemic autoimmunity in NZB/W F1 mice. *J. Immunol.* 184: 4789–4800.
- Matsushita, T., K. Yanaba, J. D. Bouaziz, M. Fujimoto, and T. F. Tedder. 2008. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J. Clin. Invest.* 118: 3420–3430.
- Saeki, K., Y. Miura, D. Aki, T. Kurosaki, and A. Yoshimura. 2003. The B cell-specific major raft protein, Raftlin, is necessary for the integrity of lipid raft and BCR signal transduction. *EMBO J.* 22: 3015–3026.
- Mizoguchi, A., E. Mizoguchi, H. Takedatsu, R. S. Blumberg, and A. K. Bhan. 2002. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 16: 219–230.
- Watanabe, R., N. Ishiura, H. Nakashima, Y. Kuwano, H. Okochi, K. Tamaki, S. Sato, T. F. Tedder, and M. Fujimoto. 2010. Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. *J. Immunol.* 184: 4801–4809.

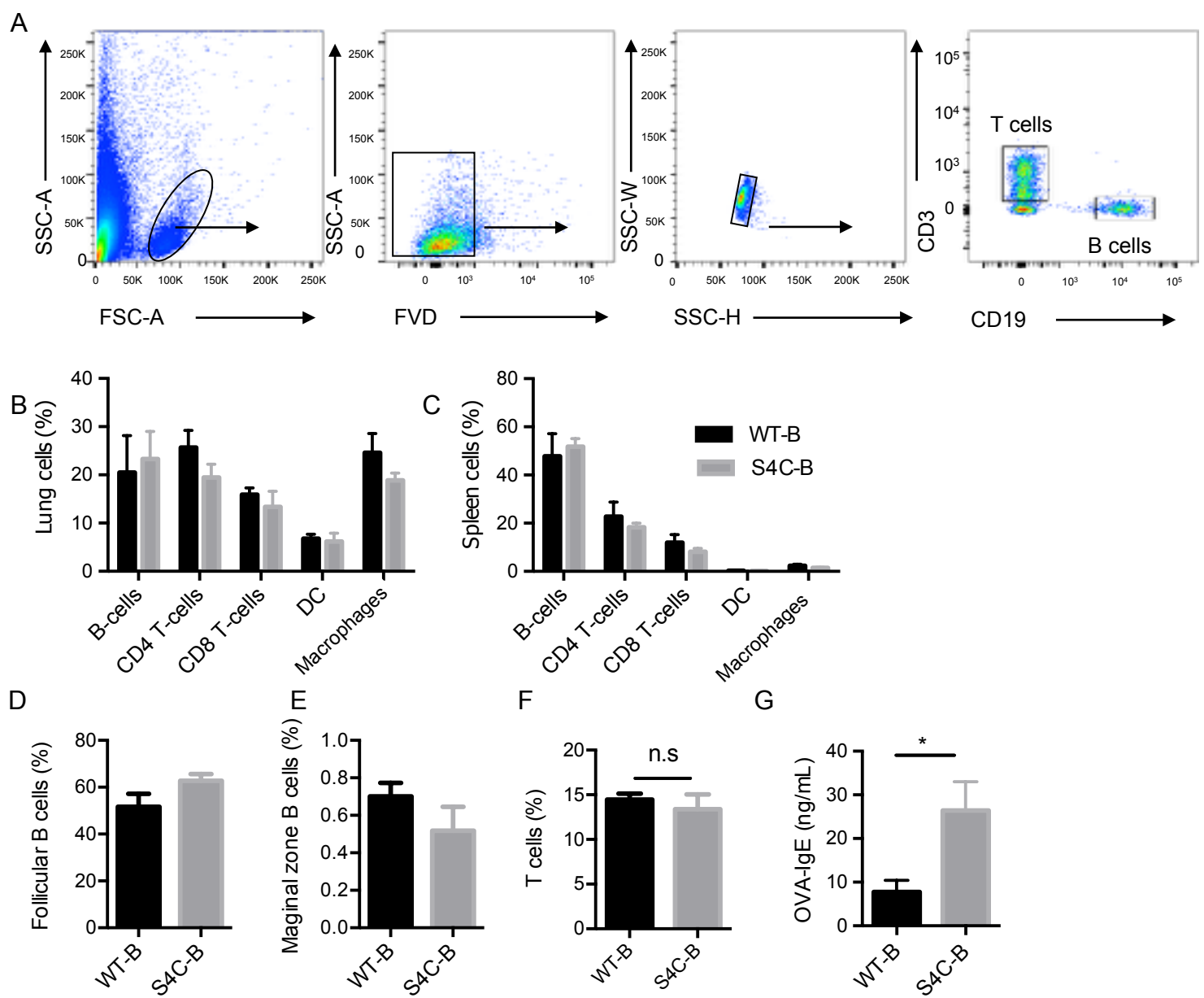
27. Fritz, J. H., O. L. Rojas, N. Simard, D. D. McCarthy, S. Hapfelmeier, S. Rubino, S. J. Robertson, M. Larjani, J. Gosselin, I. I. Ivanov, et al. 2011. Acquisition of a multifunctional IgA+ plasma cell phenotype in the gut. *Nature* 481: 199–203.
28. Massoud, A. H., J. Guay, K. H. Shalaby, E. Bjur, A. Ablona, D. Chan, Y. Nouhi, C. T. McCusker, M. W. Mourad, C. A. Piccirillo, and B. D. Mazer. 2012. Intravenous immunoglobulin attenuates airway inflammation through induction of forkhead box protein 3-positive regulatory T cells. *J Allergy Clin Immunol* 129: 1656–1665.e3.
29. Kaufman, G. N., A. H. Massoud, S. Audusseau, A. A. Banville-Langelier, Y. Wang, J. Guay, J. A. Garellek, W. Mourad, C. A. Piccirillo, C. McCusker, and B. D. Mazer. 2011. Intravenous immunoglobulin attenuates airway hyperresponsiveness in a murine model of allergic asthma. *Clin. Exp. Allergy* 41: 718–728.
30. Siddiqui, S., T. Jo, M. Tamaoka, K. H. Shalaby, H. Ghezzi, M. Bernabeu, and J. G. Martin. 2010. Sites of allergic airway smooth muscle remodeling and hyperresponsiveness are not associated in the rat. *J. Appl. Physiol.* 109: 1170–1178.
31. Cimolai, N., G. P. Taylor, D. Mah, and B. J. Morrison. 1992. Definition and application of a histopathological scoring scheme for an animal model of acute *Mycoplasma pneumoniae* pulmonary infection. *Microbiol. Immunol.* 36: 465–478.
32. Al Heialy, S., M. Zeroual, S. Farahnak, T. McGovern, P. A. Risse, M. Novali, A. M. Lauzon, H. N. Roman, and J. G. Martin. 2015. Nanotubes connect CD4+ T cells to airway smooth muscle cells: novel mechanism of T cell survival. *J. Immunol.* 194: 5626–5634.
33. Laouini, D., H. Alenius, P. Bryce, H. Oettgen, E. Tsitsikov, and R. S. Geha. 2003. IL-10 is critical for Th2 responses in a murine model of allergic dermatitis. *J. Clin. Invest.* 112: 1058–1066.
34. Harris, D. P., S. Goodrich, K. Mohrs, M. Mohrs, and F. E. Lund. 2005. Cutting edge: the development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor alpha, and Th2 cells. *J. Immunol.* 175: 7103–7107.
35. Bastien, Y., B. J. Toledano, N. Mehio, L. Cameron, B. Lamoukhaid, P. Renzi, Q. Hamid, and B. D. Mazer. 1999. Detection of functional platelet-activating factor receptors on human tonsillar B lymphocytes. *J. Immunol.* 162: 5498–5505.
36. Desjardins, M., A. Clarke, R. Alizadehfar, D. Grenier, H. Eisman, S. Carr, T. K. Vander Leek, L. Teperman, N. Higgins, L. Joseph, et al. 2013. Canadian allergists' and nonallergists' perception of epinephrine use and vaccination of persons with egg allergy. *J. Allergy Clin. Immunol. Pract.* 1: 289–294.
37. Esnault, S., E. A. Kelly, M. W. Johansson, L. Y. Liu, S. T. Han, M. Akhtar, N. Sandbo, D. F. Mosher, L. C. Denlinger, S. K. Mathur, et al. 2014. Semaphorin 7A is expressed on airway eosinophils and upregulated by IL-5 family cytokines. *Clin. Immunol.* 150: 90–100.
38. Gan, Y., R. Reikoff, X. Peng, T. Russell, Q. Chen, S. K. Mathai, R. Homer, M. Gulati, J. Siner, J. Elias, et al. 2011. Role of semaphorin 7a signaling in transforming growth factor β 1-induced lung fibrosis and scleroderma-related interstitial lung disease. *Arthritis Rheum.* 63: 2484–2494.
39. Becker, P. M., T. S. Tran, M. J. Delannoy, C. He, J. M. Shannon, and S. McGrath-Morrow. 2011. Semaphorin 3A contributes to distal pulmonary epithelial cell differentiation and lung morphogenesis. *PLoS One* 6: e27449.
40. Sawaki, H., F. Nakamura, M. Aihara, Y. Nagashima, J. Komori-Yamaguchi, N. Yamashita, M. Nakazawa, Y. Goshima, and Z. Ikezawa. 2011. Intranasal administration of semaphorin-3A alleviates sneezing and nasal rubbing in a murine model of allergic rhinitis. *J. Pharmacol. Sci.* 117: 34–44.
41. Wanschel, A., T. Seibert, B. Hewing, B. Ramkhalawon, T. D. Ray, J. M. van Gils, K. J. Rayner, J. E. Feig, E. R. O'Brien, E. A. Fisher, and K. J. Moore. 2013. Neuroimmune guidance cue Semaphorin 3E is expressed in atherosclerotic plaques and regulates macrophage retention. *Arterioscler. Thromb. Vasc. Biol.* 33: 886–893.
42. Nkyimbeng-Takwi, E. H., K. Shanks, E. Smith, A. Iyer, M. M. Lipsky, L. J. Detolla, H. Kikutani, A. D. Keegan, and S. P. Chapoval. 2012. Neuroimmune semaphorin 4A downregulates the severity of allergic response. *Mucosal Immunol.* 5: 409–419.
43. Shanks, K., E. H. Nkyimbeng-Takwi, E. Smith, M. M. Lipsky, L. J. DeTolla, D. W. Scott, A. D. Keegan, and S. P. Chapoval. 2013. Neuroimmune semaphorin 4D is necessary for optimal lung allergic inflammation. *Mol. Immunol.* 56: 480–487.
44. Shen, P., T. Roch, V. Lampropoulou, R. A. O'Connor, U. Stervbo, E. Hilgenberg, S. Ries, V. D. Dang, Y. Jaimes, C. Daridon, et al. 2014. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* 507: 366–370.
45. Matsumoto, M., A. Baba, T. Yokota, H. Nishikawa, Y. Ohkawa, H. Kayama, A. Kallies, S. L. Nutt, S. Sakaguchi, K. Takeda, et al. 2014. Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. *Immunity* 41: 1040–1051.
46. Zeng, R., M. Han, Y. Luo, C. Li, G. Pei, W. Liao, S. Bai, S. Ge, X. Liu, and G. Xu. 2011. Role of Semaphorin 4C in TGF- β 1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *Nephrol. Dial. Transplant.* 26: 1149–1156.
47. Li, J., Q. Wang, R. Wen, J. Liang, X. Zhong, W. Yang, D. Su, and J. Tang. 2015. MiR-138 inhibits cell proliferation and reverses epithelial-mesenchymal transition in non-small cell lung cancer cells by targeting GIT1 and SEMA4C. *J. Cell. Mol. Med.* 19: 2793–2805.
48. Zhou, Q. D., Y. Ning, R. Zeng, L. Chen, P. Kou, C. O. Xu, G. C. Pei, M. Han, and G. Xu. 2013. Erbin interacts with Semaphorin 4C and inhibits Semaphorin 4C-induced epithelial-mesenchymal transition in HK2 cells. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 33: 672–679.
49. Worzfeld, T., J. M. Swiercz, A. Sentürk, B. Genz, A. Korostylev, S. Deng, J. Xia, M. Hoshino, J. A. Epstein, A. M. Chan, et al. 2014. Genetic dissection of plexin signaling in vivo. *Proc. Natl. Acad. Sci. USA* 111: 2194–2199.
50. Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain, and F. E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat. Immunol.* 1: 475–482.
51. Kalampokis, I., A. Yoshizaki, and T. F. Tedder. 2013. IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. *Arthritis Res. Ther.* 15(Suppl. 1): S1.
52. Ries, S., E. Hilgenberg, V. Lampropoulou, P. Shen, V. D. Dang, S. Wilantri, I. Sakwa, and S. Fillatreau. 2014. B-type suppression: a role played by “regulatory B cells” or “regulatory plasma cells”? *Eur. J. Immunol.* 44: 1251–1257.
53. Vadasz, Z., T. Haj, A. Kessel, and E. Toubi. 2013. B-regulatory cells in autoimmunity and immune mediated inflammation. *FEBS Lett.* 587: 2074–2078.
54. de Andrés, C., M. Tejera-Alhambra, B. Alonso, L. Valor, R. Teijeiro, R. Ramos-Medina, D. Mateos, F. Faure, and S. Sánchez-Ramón. 2014. New regulatory CD19(+)CD25(+) B-cell subset in clinically isolated syndrome and multiple sclerosis relapse. Changes after glucocorticoids. *J. Neuroimmunol.* 270: 37–44.
55. Khan, A. R., E. Hams, A. Floudas, T. Sparwasser, C. T. Weaver, and P. G. Fallon. 2015. PD-L1hi B cells are critical regulators of humoral immunity. *Nat. Commun.* 6: 5997.
56. van de Veen, W., B. Stanic, G. Yaman, M. Wawrzyniak, S. Söllner, D. G. Akdis, B. Rückert, C. A. Akdis, and M. Akdis. 2013. IgG4 production is confined to human IL-10–producing regulatory B cells that suppress antigen-specific immune responses. *J. Allergy Clin. Immunol.* 131: 1204–1212.
57. van der Vlugt, L. E., E. Mlejnek, A. Ozir-Fazalalikhan, M. Janssen Bonas, T. R. Dijkman, L. A. Labuda, R. Schot, B. Guigas, G. M. Möller, P. S. Hiemstra, et al. 2014. CD24(hi)CD27(+) B cells from patients with allergic asthma have impaired regulatory activity in response to lipopolysaccharide. *Clin. Exp. Allergy* 44: 517–528.
58. Shen, P., and S. Fillatreau. 2015. Antibody-independent functions of B cells: a focus on cytokines. *Nat. Rev. Immunol.* 15: 441–451.
59. Mazer, B. 2014. Is there a place for B cells as regulators of immune tolerance in allergic diseases? *Clin. Exp. Allergy* 44: 469–471.
60. Stanic, B., W. van de Veen, O. F. Wirz, B. Rückert, H. Morita, S. Söllner, C. A. Akdis, and M. Akdis. 2015. IL-10-overexpressing B cells regulate innate and adaptive immune responses. *J. Allergy Clin. Immunol.* 135: 771–780.e8.
61. Drolet, J. P., H. Frangie, J. Guay, O. Hajoui, Q. Hamid, and B. D. Mazer. 2010. B lymphocytes in inflammatory airway diseases. *Clin. Exp. Allergy* 40: 841–849.



Supplemental Figure 1.

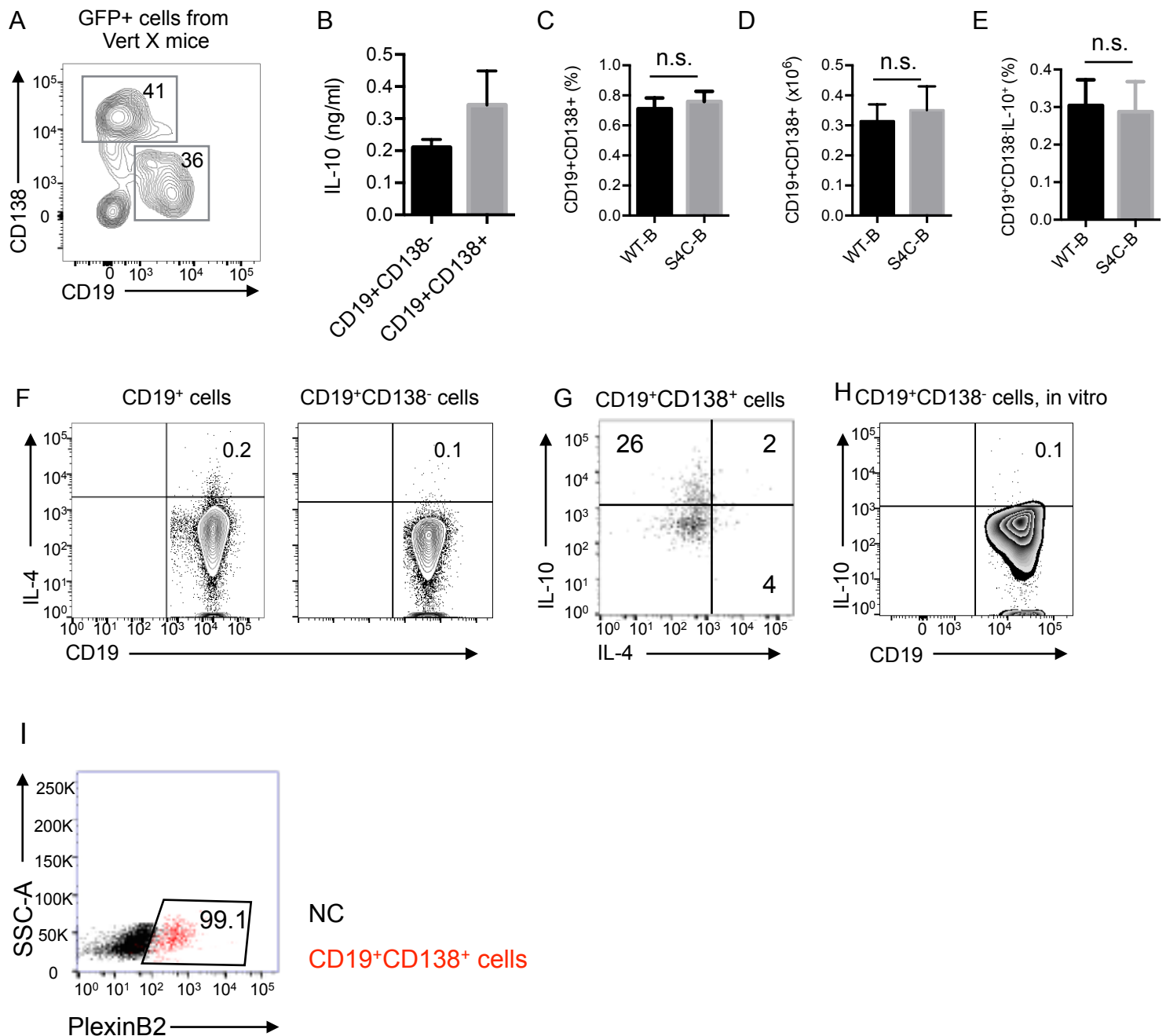
(A and B) Cells isolated from spleen (A) or cervical lymph nodes (B) of 8-week old naive WT or *Sema4c*^{-/-} mice. Macrophages (F4/80⁺MHCII⁺CD11b⁺CD19⁻), Monocytes (Ly6C^{high/low}Ly6G⁺CD11b⁺MHCII⁺CD11c⁻F4/80^{low/int}), Dendritic cells (CD11b⁺CD11c⁺F4/80⁻MHCII⁺), Neutrophils (Ly6C^{high}Ly6G⁺CD11b⁺MHCII⁺CD11c⁻F4/80⁻), Eosinophils (CD11b⁺SiglecF⁺SSC-A^{high}), CD4⁺Foxp3⁺ (CD3⁺CD4⁺CD25⁺Foxp3⁺), CD4⁺Foxp3⁻ (CD3⁺CD4⁺CD25⁺Foxp3⁻), CD8⁺ (CD3⁺CD8⁺). n=3 each group. No significant difference was detected among any population in the graph between WT and *Sema4c*^{-/-} mice.

(C-I) WT and *Sema4c*^{-/-} mice were sensitized and challenged to OVA as described. Lung function and pulmonary inflammation were examined 24 hours after the last challenge. (C) Percentage of T cells among total lung cells and (D) percentage of CD25⁺Foxp3⁺ T cells among lung CD4⁺ T cells in WT and *Sema4c*^{-/-} AAD mice. (E) Percentage of CD11b⁺SiglecF⁻ cells among lung cells and (F) count of CD11b⁺SiglecF⁻ cells in the lungs of WT and *Sema4c*^{-/-} AAD mice. (G) Representative plot of plasma cells in lungs of OVA challenged WT mice. Lung cells were isolated using a collagenase-free method. (H) OVA-specific IgE level in the serum of WT and *Sema4c*^{-/-} AAD mice. (I) Total serum IgE from OVA exposed mice and sham control. (J) BAL IFN γ and BAL IL-17 concentration from WT and *Sema4c*^{-/-} AAD mice. Means $\pm$ SEM, 2 independent experiments. n=5. n.s. not significant. n.d. not detectable.



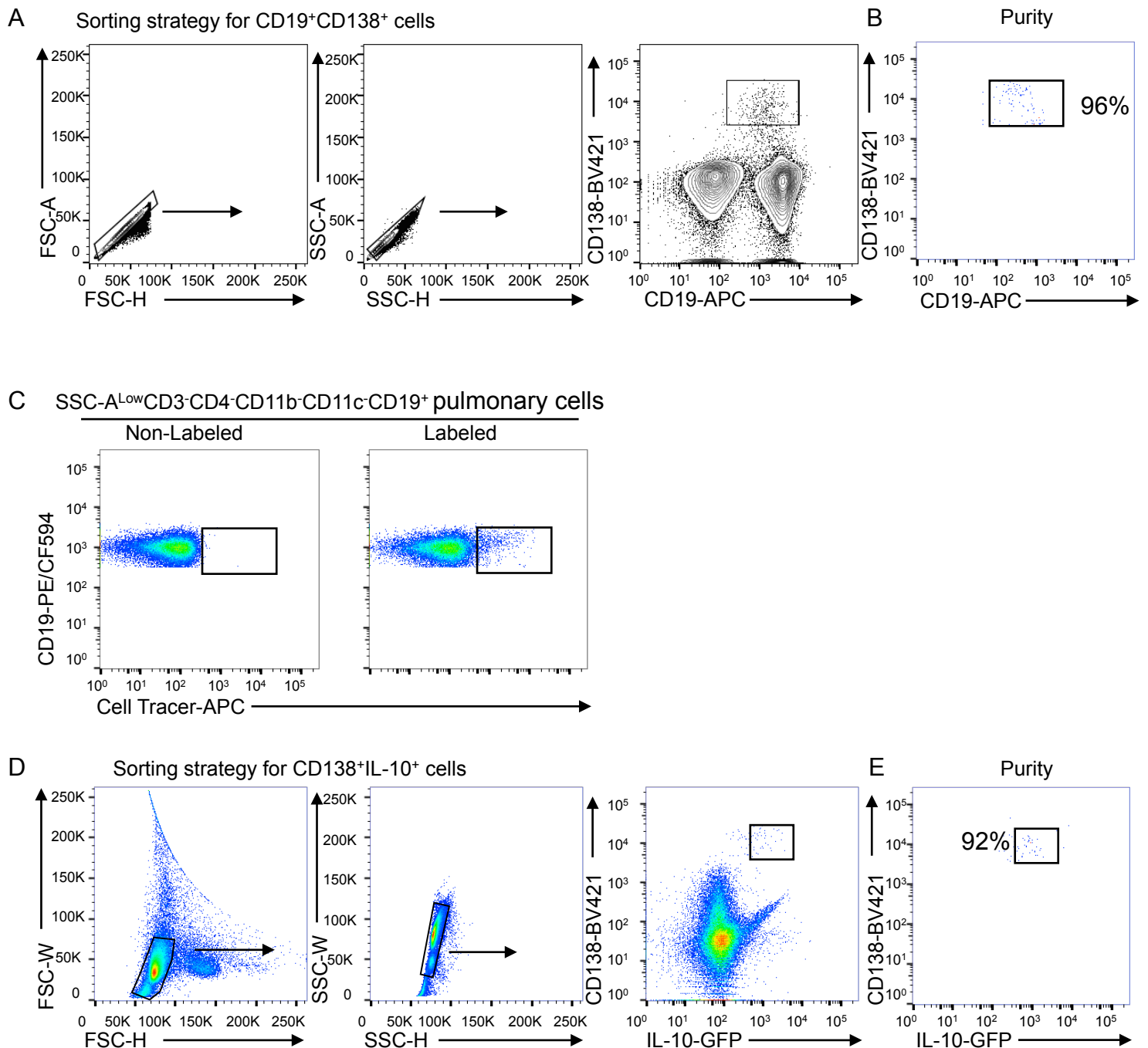
Supplemental Figure 2. S4C-B chimeric mice have normal immune cell parameters

S4C-B and WT-B chimeric mice were generated as described in material and methods. (A) Gating strategy of T cells and B cells shown in Figure 2B. Cells were from blood 6 weeks after reconstitution. (B-F) Splenocytes and lung cells from naïve chimeric mice were examined by flow cytometry 8-weeks after reconstitution. (B and C) Percentage of total B-cells (B220⁺CD19⁺), CD4 T-cells (CD3⁺CD4⁺), CD8 T-cells (CD3⁺CD8⁺), dendritic cells (CD11b⁺CD11c⁺F4/80⁺MHCII⁺), or macrophages (F4/80⁺MHCII⁺CD11b⁺CD19⁻) among total lung cells (B) or spleen cells (C) from naïve WT-B and S4C-B mice. (D and E) Percentage of follicular B cells (CD19⁺CD21⁻IgM^{Low}IgD^{High}CD23⁺CD93⁻), marginal zone B cell (CD19⁺CD21⁺IgM^{High}IgD^{Low}CD23⁻CD93⁻) among total spleen cells from naïve WT-B and S4C-B mice. (F) percentage of CD4⁺ T cells among total lung cells in WT-B and S4C-B AAD mice. (G) OVA specific IgE level in the serum of WT-B and S4C-B AAD mice. Means ± SEM. 2 independent experiments, n=6.



Supplemental Figure 3. S4C-B mice had pro-inflammatory cytokine profile post allergen exposure.

Splenocytes were isolated 24 hours following the last OVA challenge. (A) IL-10⁺GFP VertX mice were sensitized and challenged to OVA as described. A representative plot showing the percentage of CD19⁺CD138⁺ and CD19⁺CD138⁻ cells among IL-10⁺ spleen cells. (B) Sorted CD19⁺CD138⁺ and CD19⁺CD138⁻ cells were cultured in vitro for 4 hours, IL-10 concentration in supernatant was measured using ELISA. (C) Percentage of CD19⁺CD138⁺ cells among spleen B cells from WT-B and S4C-B AAD mice. (D) Count of CD19⁺CD138⁺ cells among spleen B cells from WT-B and S4C-B AAD mice. (E) Percentage of IL-10 producing cells among CD19⁺CD138⁻ spleen cells from WT-B and S4C-B AAD mice. (F) Percentage of IL-10 producing cells among CD19⁺ spleen cells from WT-B and S4C-B AAD mice. (F) Representative plots showing the percentage of IL-4 producing cells among CD19⁺ and CD19⁺CD138⁻ spleen cells from WT-B AAD mice. (G) A representative dot plot showing that 2% of CD19⁺CD138⁺ cells in spleen of a WT mice. produce both IL-4 and IL-10 following OVA sensitization and challenge. (H) WT B cells were isolated and stimulated with anti-CD40 antibody, in the presence of IL-4 and IL-21. A representative plot showing the percentage of IL-10 producing cells among CD19⁺CD138⁻ cells after 72 hours stimulation. (I) PlexinB2 expression level in spleen CD19⁺CD138⁺ cells (red) from WT-B AAD mice, black cells indicate FMO control, demonstrating the majority of CD19⁺CD138⁺ cells express cell surface PlexinB2. Means $\pm$ SEM. 2 independent experiments, n=6.



Supplemental Figure 4. CD138⁺IL-10⁺ cells abrogate AAD in WT and *Sema4c*^{-/-} AAD mice.

(A) Gating strategy for CD19⁺CD138⁺ spleen cells sorted from WT or *Sema4c*^{-/-} AAD mice. (B) Representative plot showing the purity of sorted cells in (A). (C) Cells sorted in (B) were labeled with CellTrace-APC before transferred to OVA exposed recipients. Lung cells from OVA i.n. challenged recipients were isolated and traced for APC⁺ cells. Cells were gated on SSC-A^{Low}CD3-CD4-CD11b-CD11c-CD19⁺ populations. Lung cells from recipients who were transferred with Non-labeled donor cells were used as control. (D) Gating strategy for CD138⁺IL-10⁺ spleen cells sorted from VertX mice. (E) Representative plot showing the purity of sorted cells in (D). 2 independent experiments.