Supplementary information for:

Semaphorin 4D inhibits neutrophil activation and is involved in the pathogenesis of neutrophil-mediated autoimmune vasculitis

Masayuki Nishide[1,2,3], Satoshi Nojima[2,3,4 *], Daisuke Ito[2,3,5], Hyota Takamatsu[1,2,3], Shohei Koyama[1,2,3], Sujin Kang[2,3,6], Tetsuya Kimura[1,2,3], Keiko Morimoto[1,2,3], Takashi Hosokawa[1,2,3], Yoshitomo Hayama[1,2,3], Yuhei Kinehara[1,2,3], Yasuhiro Kato[1,2,3], Takeshi Nakatani[1,2,3], Yoshimitsu Nakanishi[1,2,3], Takeshi Tsuda[2,3,7], Jeong Hoon Park[1], Toru Hirano[1], Yoshihito Shima[1], Masashi Narazaki[1,2], Eiichi Morii[4], and Atsushi Kumanogoh[1,2,3 *]

This file includes supplementary methods, supplementary table S1, and supplementary figures S1-11

Supplementary methods

Mice. C57BL/6J mice were purchased from CLEA Japan. C57BL/6J *SEMA4D*$^{-/-}$ mice (previously established) were bred at the Animal Resource Center for Infectious Diseases, Research Institute for Microbial Diseases and Immunology Frontier Research Center, Osaka University. All animal procedures were conducted according to institutional guidelines.

Reagents and antibodies. FMLP (N-formylmethionyl-leucyl-phenylalanine) was purchased from Sigma-Aldrich. Recombinant human matrix metalloproteinase (MMP)-8, MMP-9, and ADAM-17 were purchased from R&D Systems. Tapi-1 was purchased from Santa Cruz Biotechnology. Recombinant human SEMA4D (Fc chimera protein) and plexin B2 (His-tagged protein) were purchased from R&D Systems. PMA, LPS, PAM3CSK4, Zymosan, and CpG-ODN were purchased from Sigma-Aldrich. The following antibodies were used for flow cytometry: fluorescein isothiocyanate (FITC)-conjugated anti–human SEMA4D (A8; BioLegend), FITC-conjugated anti-mouse SEMA4D (BMA12; eBioscience), phycoerythrin (PE)-conjugated anti–human plexin B2 (537223; R&D Systems), PE-conjugated anti–mouse plexin B2 (3E7; BioLegend), PE-conjugated anti–mouse Gr-1 (RB6-8C5; eBioscience), allophycocyanin (APC)-conjugated anti–mouse CD11b (M1/70; eBioscience), The following antibodies were used for western blotting: anti–phospho-p44/42-MAPK (pERK; Thr202/Tyr204), anti–p44/42-MAPK (ERK), anti–phospho-p38-MAPK (Thr180/Tyr182), anti–p38-MAPK, and anti–β-actin (13E5); all of these antibodies were purchased from Cell Signaling Technology.

Induction of mouse peritoneal exudate neutrophils.

Mouse peritoneal exudate cells (PEC) were collected the indicated number of hours after i.p. injection of 4% thioglycollate broth (Sigma-Aldrich), followed by isolation of subpopulations of Gr-1^{+} CD11b^{+} neutrophils by FACS Aria (BD Biosciences).

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Total RNA was extracted using an RNeasy Mini kit (Qiagen), and complementary DNA (cDNA) was synthesized using a Super-Script III cDNA synthesis kit (Invitrogen). Quantitative RT-PCR analysis was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the TaqMan PCR protocol. Primer sets were purchased from Applied Biosystems.

Flow cytometry. Cells were prepared and stained for cell surface markers with fluorochrome-conjugated antibodies listed above, diluted in PBS (Nacalai Tesque) containing 2% FBS. To prevent nonspecific binding, cells were incubated for 20 minutes at 4˚ C with FcR Blocking Reagent (Miltenyi Biotec; for human cells) or anti-CD16/CD32 (eBioscience; for mouse cells), and then samples were labeled with antibodies against the indicated cell-surface antigens. Stained cells were analyzed on a FACS Canto II (BD Biosciences), and data were processed using the FlowJo software (Tree Star).

Vascular permeability assay. Vascular permeability assays were performed using the CultureCoat® *in vitro* Vascular Permeability Assay Kit (Trevigen; Transwell assay kit). Human umbilical vein endothelial cells (HUVEC) monolayers were grown on collagen-coated 96-well plates for 3 days, and then treated for 4 hours with control human IgG-Fc (5 μg/ml; Ctrl) or recombinant SEMA4D-Fc (10 μg/ml; SEMA4D). After incubation, permeability was measured using transmembrane FITC-Dextran passage assays. Relative permeability was determined as the ratio between the fluorescent peak and the baseline.

Adhesion Assay. Neutrophils (2×10^6 cells/ml), were suspended in RPMI 1640 (Nacalai Tesque) medium containing 10% FBS and seeded onto collagen-coated 96-well plates. Recombinant mouse TNF-α (500 ng/ml, R&D Systems) was then added. After 2 hours, wells were carefully washed with PBS, and live cells that remained attached to the well were counted on a MUSE cell analyzer (Miltenyi Biotec).

***In vivo* mice model**. *In vivo* experimental AAV model was induced by administering bovine serum albumin, according to the established experimental protocol (*28*). 7-week-old female C57BL/6J mice were pre-immunized by subcutaneous injections of BSA in complete Freund's adjuvant at weeks 0, 2, 4, and 6. Subsequently, these mice were treated with daily intraperitoneal injection of 50 mg/kg BSA at weeks 8 to 14. Histological activity index (AI) of kidney glomerulonephritis at week 14 was determined according to the modified NIH criteria as follows; the presence of cell proliferation, glomerular leucocyte infiltration, and interstitial mononuclear cell infiltration were scored in a range of 0–3 (0 = absent, 1 = mild, 2 = moderate, and 3 = severe). The presence of cellular or fibrocellular crescents was scored in a range of 0–3 (0 = absent, 1 = <20% of the glomeruli involved, 2 = 20–50% glomeruli involved, 3 = >50% glomeruli involved). The maximal activity score amounted to 12. The degree of proteinuria was determined in a range of 0–4 by dipstick test. *In vivo* zymosan-induced peritonitis model was induced by the i.p. injection of zymosan (50 μg/body), using 8-10-week-old female C57BL/6 mice. PECs were collected 3 hours after injection of zymosan.

***In vitro* gene knockdown using lentivirus**. Lentiviral particles expressing short hairpin RNA (shRNA) targeting plexin B2 or control shRNA were purchased from Santa Cruz Biotechnology. Infection of MS1 cells with lentivirus was carried out by adding lentiviral particles to cell cultures in the presence of puromycin. Cells were harvested 15 days post-infection, and then plexin B2–knockdown MS1 cells were sorted on a FACS Aria. Sorted cells were again cultured in DMEM (Nacalai Tesque) to form monolayers, and then co-culture assays were performed.

HEK-Blue™ TLR4 cell transfection assay. HEK-Blue™ TLR4 cell transfectants were established by introducing a construct expressing full-length SEMA4D (FL) or SEMA4D lacking the intracellular C-terminal domain (ΔIC) in a pMC1neo vector. Transfections were performed using Lipofectamine (Invitrogen). Cells were stimulated with LPS, lysed, and then subjected to Rac1 pull-down assay.

Western blot analysis. Neutrophils were cultured in RPMI 1640 medium containing 10% FCS. Cells were collected and lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, and 1 mM NaF). Lysates were subjected to immunoblot analyses. For SDS-PAGE, samples were boiled for 5 minutes in SDS-PAGE sample buffer containing 0.125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, and 10% 2-ME, and then loaded onto NuPAGE 4–12% Bis-Tris gels (Invitrogen). For immunoblot analysis, the gel was electroblotted onto polyvinylidene difluoride membrane, which was blocked in Blocking-1 buffer (Nacalai Tesque), incubated with specific primary Abs listed above, and then incubated with the appropriate secondary Abs.

Supplementary Table S1

Clinical characteristics of the 33 patients with ANCA-associated vasculitis, 29 patients with bacterial infection, and 16 healthy individuals

ANCA-associated vasculitis

Sample No.	Diagnosis	Age	Sex	Medication	BVAS	WBC (/μl)	CRP (mg/dl)	P-ANCA (IU/ml)	C-ANCA (IU/ml)
1	MPA	68	F	no	6	5420	0.61	266	ND
2	MPA	63	M	no	24	17790	17.71	83	ND
3	GPA	72	F	PSL/CY	9	9830	0.38	ND	ND
4	MPA	56	F	no	4	6570	4.91	19.2	10
5	GPA	56	M	PSL	10	9400	10.98	1.2	44
6	MPA	61	F	no	10	9180	0.09	ND	NA
7	GPA	74	M	no	13	7320	1.34	29.6	ND
8	MPA	81	M	no	9	13690	7.57	33.8	ND
9	GPA	56	M	PSL	4	9510	0.5	NA	31.5
10	GPA	71	M	no	6	11710	10.41	2.9	ND
11	GPA	30	F	no	11	11240	22.03	ND	ND
12	MPA	57	M	no	15	11200	6.49	59.4	ND
13	MPA	57	M	PSL	2	9410	0.04	ND	NA
14	MPA	77	F	no	12	11230	0.22	55.6	ND
15	MPA	81	F	no	13	14570	6.93	36.3	5.9
16	MPA	67	M	PSL	1	16790	10.47	117	NA
17	GPA	67	M	PSL	13	5130	1.75	ND	170
18	MPA	75	M	no	14	9990	0.44	ND	NA
19	MPA	69	F	PSL	5	7490	1.02	5.2	NA
20	MPA	64	M	PSL	0	8210	0.09	6.4	NA
21	GPA	31	F	PSL/CY	6	8270	5.53	ND	ND
22	MPA	70	F	no	14	12620	12.84	27.7	ND
23	GPA	31	F	PSL/CY	11	14130	4.46	ND	ND
24	GPA	70	F	no	12	12800	9.33	1.2	ND
25	MPA	53	F	PSL	3	6450	ND	19.3	NA
26	GPA	56	M	PSL/CY	8	10350	6.34	NA	NA
27	GPA	56	M	PSL/CY	12	8760	4.9	NA	20.2
28	MPA	67	M	PSL	9	14800	13.93	3.5	ND
29	GPA	67	M	PSL	8	6130	0.11	NA	25.3
30	MPA	69	F	no	6	8830	1.5	44.7	NA
31	MPA	81	F	no	8	9990	4.67	82.5	ND
32	GPA	48	M	no	12	7350	0.7	84.3	ND
33	MPA	83	M	no	4	5320	2.52	19.9	NA

Male/Female = 18/15
Median age: 67 years
MPA; Microscopic polyangiitis, GPA; Granulomatosis with polyangiitis, PSL; Prednisolone, CY; Cyclophosphamide, BVAS; Birmingham Vasculitis Activity Score 2008 ver.3, ND; Not detected, NA; Not assessed

Bacterial infection

Sample No.	Diagnosis	Age	Sex	WBC (/μl)	CRP (mg/dl)
1	Bacterial pneumonia	83	F	4830	3.75
2	Bacterial pneumonia	62	M	10870	12.82
3	Bacterial pneumonia	63	M	10790	19.28
4	Bacterial pneumonia	76	M	10080	5.46
5	Bacterial pneumonia	70	F	11850	28.62
6	Bacterial pneumonia	76	M	6640	15.69
7	Bacterial pneumonia	70	M	8070	9.73
8	Bacterial pneumonia	73	M	8350	6.91
9	Bacterial pneumonia	52	M	5810	6.1
10	Bacterial pneumonia	35	F	3530	7.93
11	Bacterial pneumonia	64	F	13520	13.81
12	Bacterial pneumonia	49	F	8550	16.45
13	Bacterial pneumonia	69	M	16510	39.21
14	Bacterial pneumonia	71	F	6710	11.55
15	Bacterial pneumonia	29	M	4370	2.69
16	Bacterial pneumonia	71	M	6520	5.95
17	Bacterial pneumonia	81	M	7830	16.11
18	Bacterial pneumonia	70	F	6980	2.57
19	Bacterial pneumonia	77	M	5560	0.7
20	Bacterial pneumonia	46	F	6950	9.67
21	Bacterial pneumonia	73	M	7490	2.54
22	Bacterial pneumonia	86	M	6980	0.58
23	Bacterial pneumonia	29	F	13490	14.39
24	Bacterial pneumonia	64	F	5330	5.51
25	Bacterial pneumonia	73	F	5740	2.01
26	Bacterial pneumonia	68	M	11190	18.84
27	Bacterial pneumonia	26	F	6250	4.64
28	Bacterial pneumonia	69	M	7350	6.5
29	Bacterial pneumonia	32	F	12130	5.13

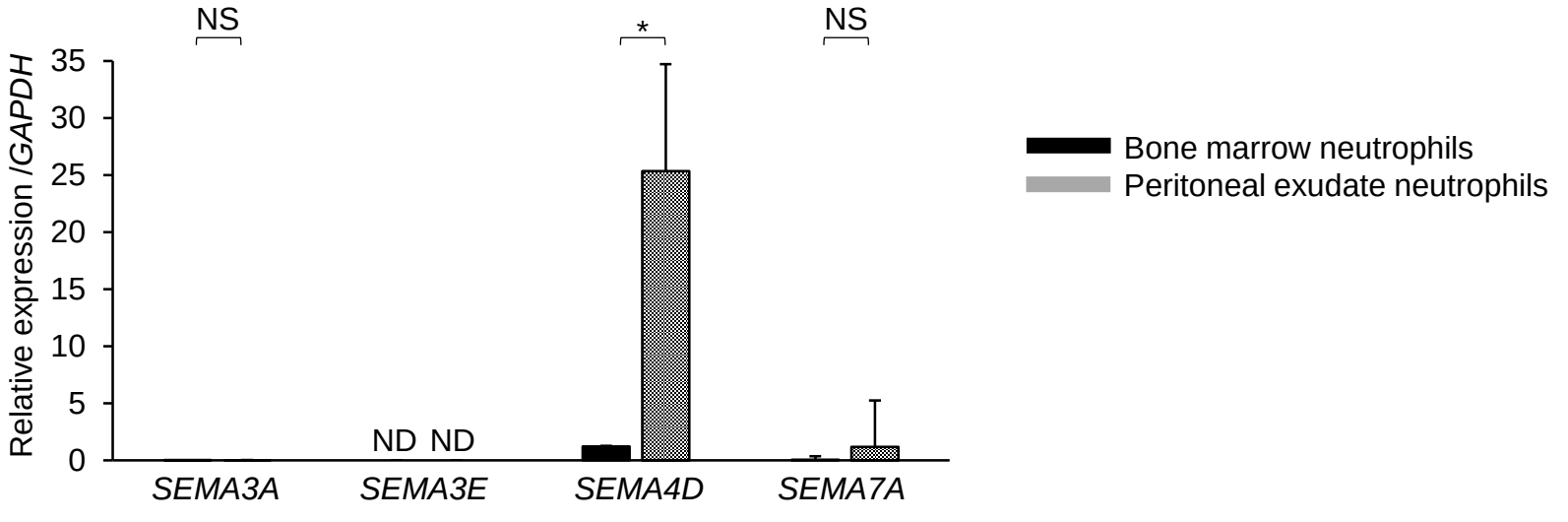
Male/Female = 16/13
Median age: 69 years

Healthy individuals

Sample No.	Age	Sex
1	33	M
2	35	M
3	50	M
4	31	M
5	28	M
6	33	M
7	38	M
8	26	F
9	25	M
10	25	F
11	27	F
12	43	M
13	30	F
14	38	M
15	35	M
16	31	M

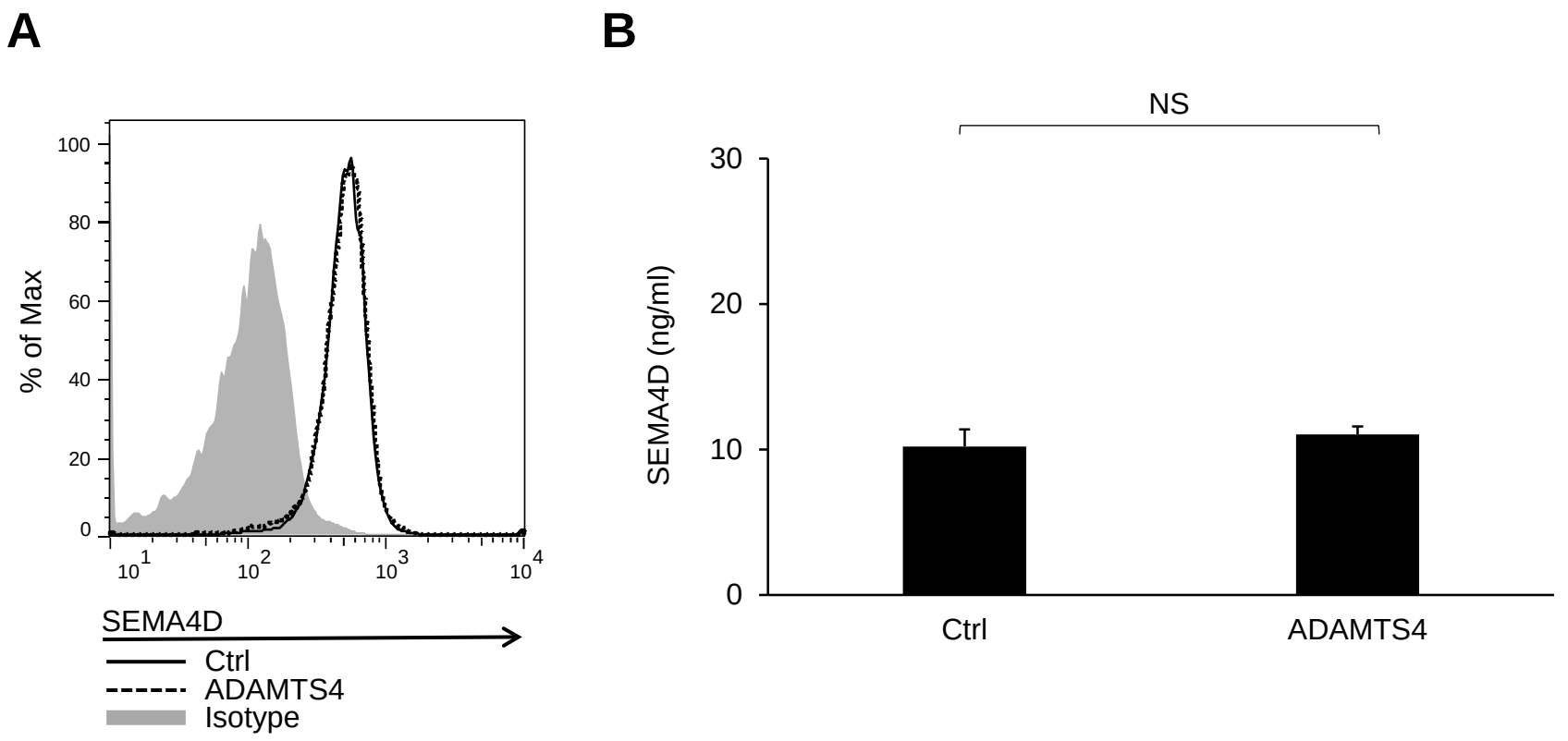
Male/Female = 12/4
Median age: 32 years

Supplementary Figure S1



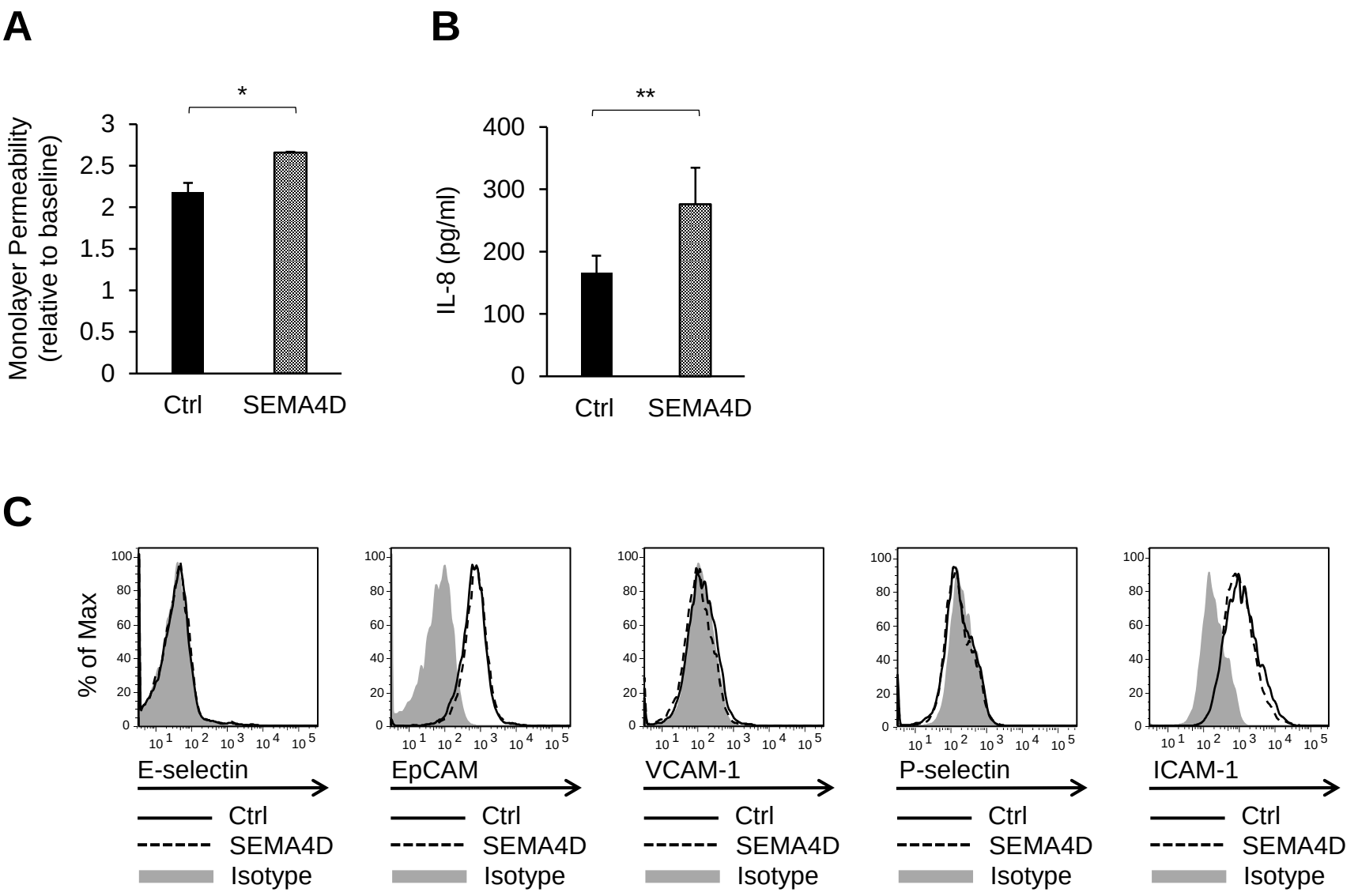
Supplementary Figure S1. Quantitative PCR analysis of immune-related Semaphorin mRNA expression in mouse bone marrow and peritoneal exudate neutrophils. Quantitative PCR analysis of the mRNA expression of Semaphorins were performed in isolated mouse bone marrow–derived neutrophils (from naïve C57BL/6 mice) and peritoneal exudate (PEC) neutrophils. Data are shown as fold change of expression from *GAPDH*. NS, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as determined by a two-tailed unpaired Student's *t* test. Values are means ± SEM. Data are representative of three independent experiments.

Supplementary Figure S2



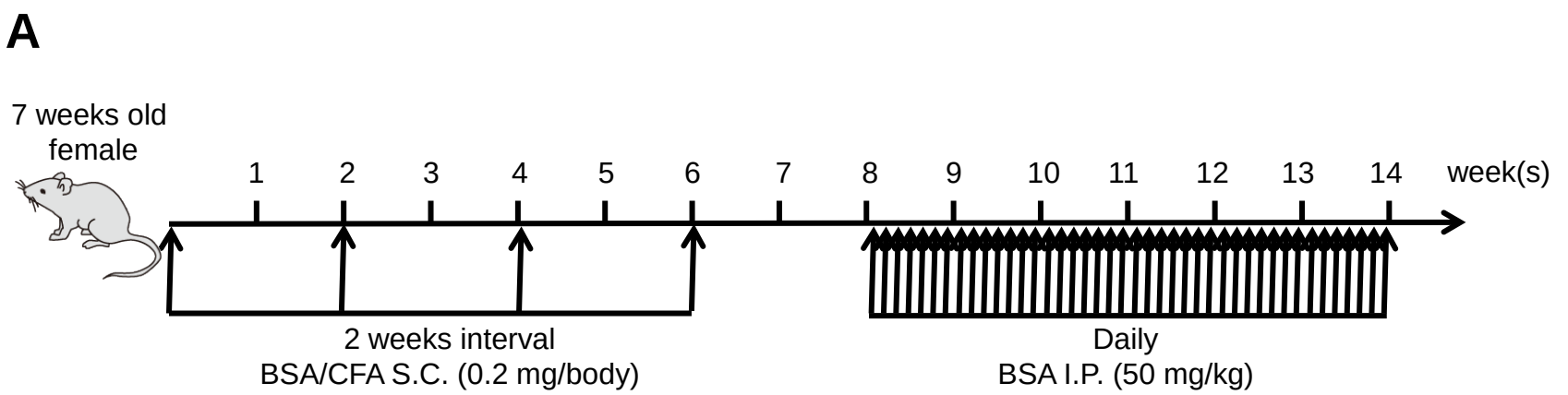
Supplementary Figure S2. Effects of ADAMTS4 on cell surface cleavage of neutrophil SEMA4D. (A) SEMA4D cell surface expression on isolated human neutrophils stimulated with ADAMTS-4. Isolated neutrophils were primed with TNF-α (2 ng/ml) for 30 minutes, and then stimulated with recombinant matrix metalloprotease ADAMTS-4 (5 µg/ml) for 60 minutes. Flow cytometry was performed similarly to **(figure 2A)**. Soluble SEMA4D levels in culture supernatant was determined by ELISA **(B)**. NS, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as determined by a two-tailed unpaired Student's *t* test. Values are means ± SEM. Data are representative of two independent experiments.

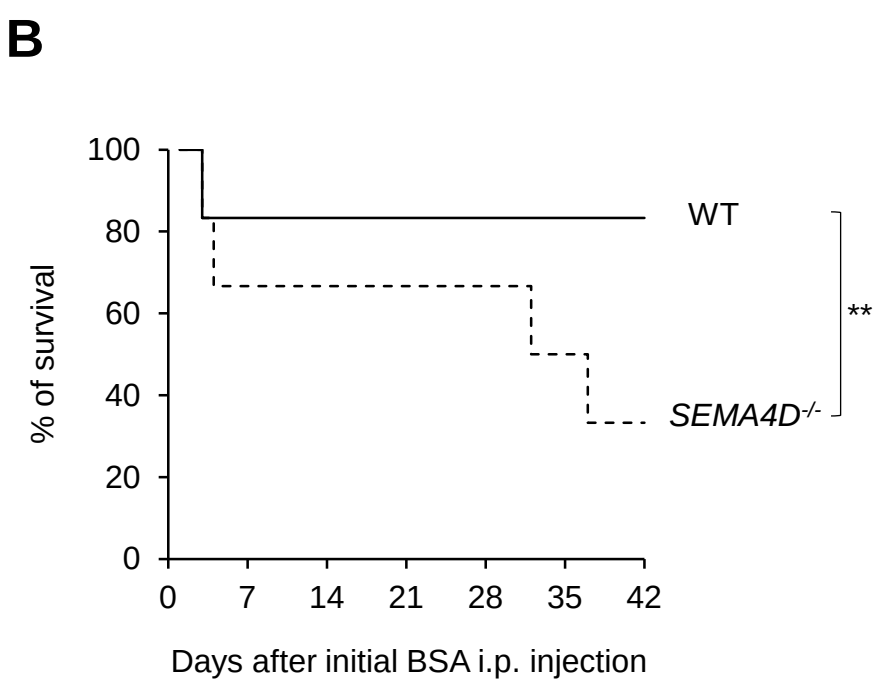
Supplementary Figure S3

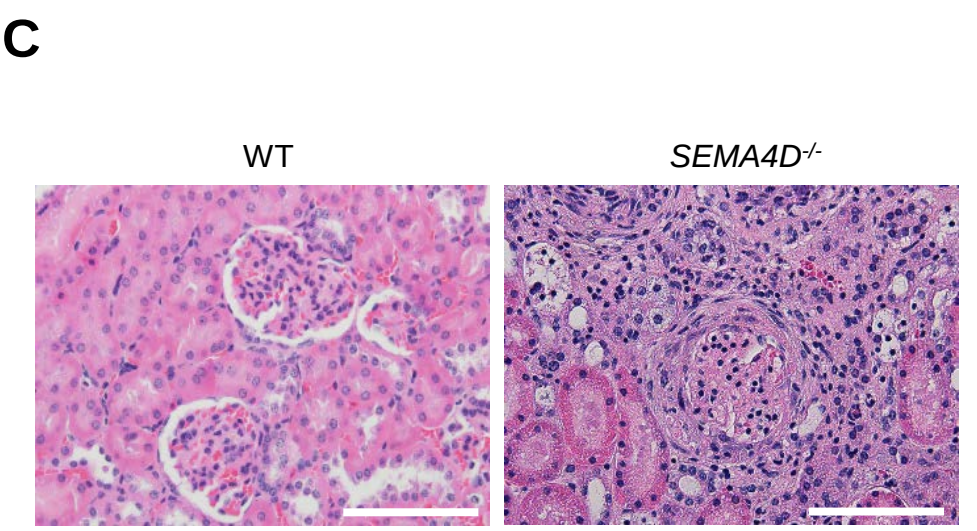


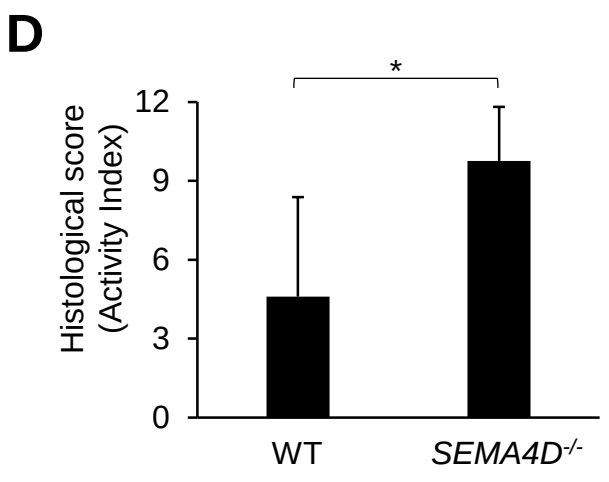
Supplementary Figure S3. Effects of soluble SEMA4D on endothelial cells. (A) Effects of recombinant SEMA4D proteins on vascular permeability (Transwell assay). Human umbilical vein endothelial cell (HUVEC) monolayers were grown on a collagen-coated 96-well plate for 3 days and treated with control human IgG-Fc (5 μg/ml; Ctrl) or recombinant SEMA4D-Fc (10 μg/ml; SEMA4D) for 4 hours. After incubation, permeability was measured using transmembrane FITC-dextran passage assays. Relative permeability was determined as the ratio of fluorescent peak to baseline. **(B)** Effects of recombinant SEMA4D proteins on IL-8 secretion from vascular endothelium. Human umbilical vein endothelial cell (HUVEC) monolayers were grown and treated with control human IgG-Fc or recombinant SEMA4D-Fc similar to **(A)**. After incubation for 24 hours, IL-8 levels in the culture supernatants were determined using ELISA. **(C)** Cell-surface expression of adhesion molecules on HUVEC after cells were either treated with control human IgG-Fc or recombinant SEMA4D-Fc (10 μg/ml) for 24 hours. The expression level of each indicated adhesion molecule was analyzed using flow cytometry (Ctrl: solid line; SEMA4D treated: dotted line). Cells were also stained with an isotype-matched control antibody (Isotype; gray-filled histogram). NS, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as determined by a two-tailed unpaired Student's *t* test. Values are means ± SEM. Data are representative of three independent experiments **(A** and **B)** and two independent experiments **(C)**.

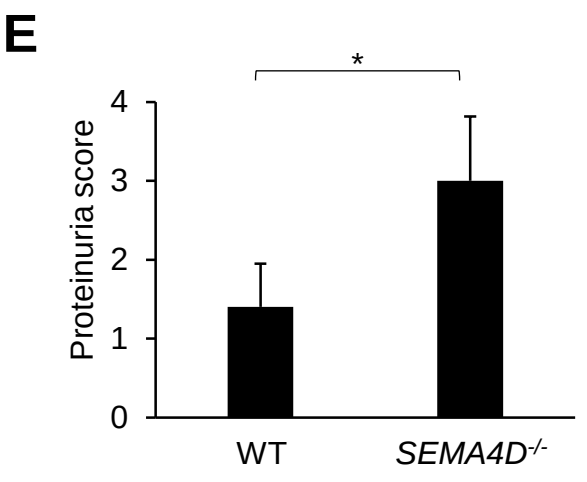
Supplementary Figure S4





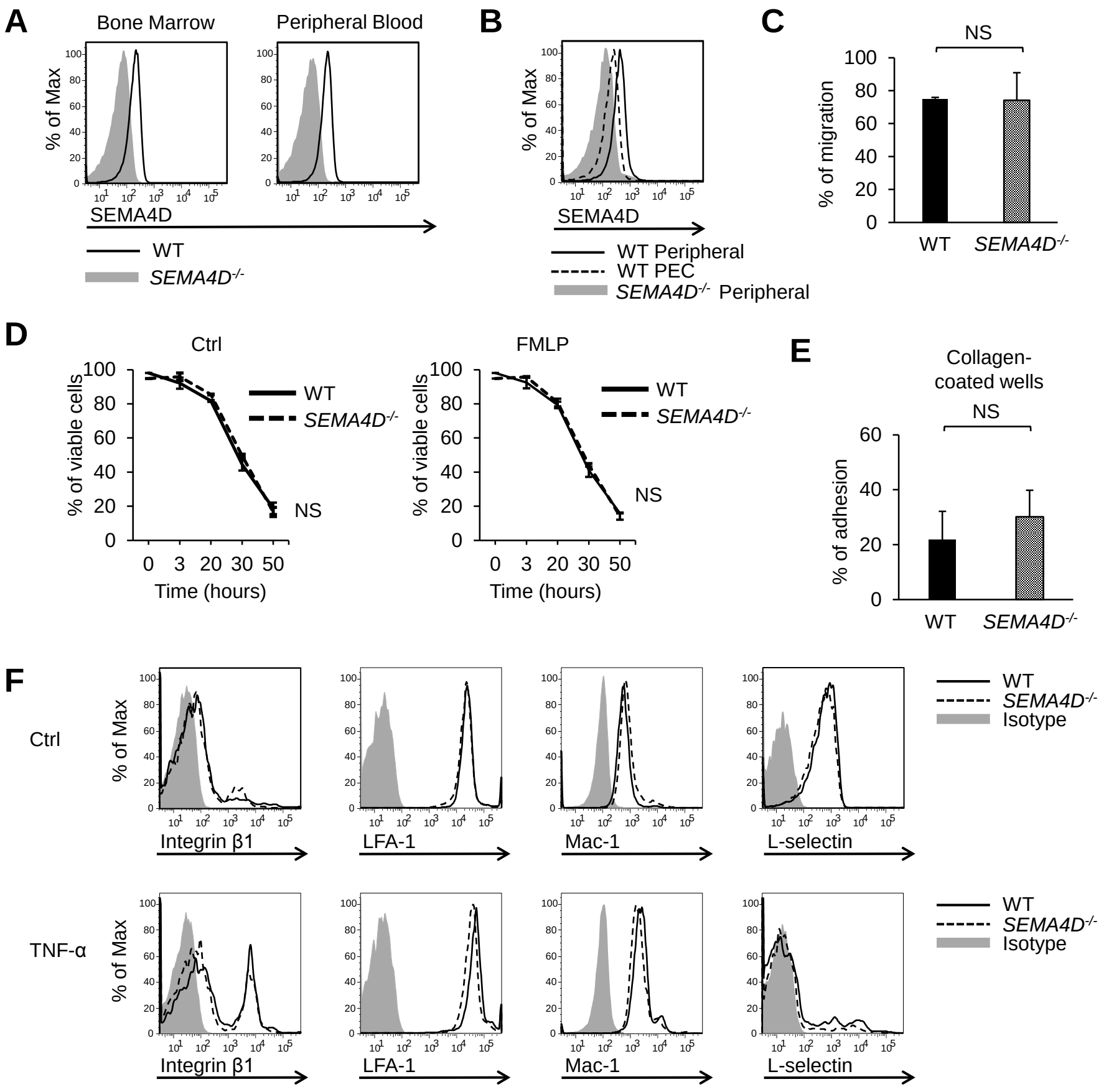






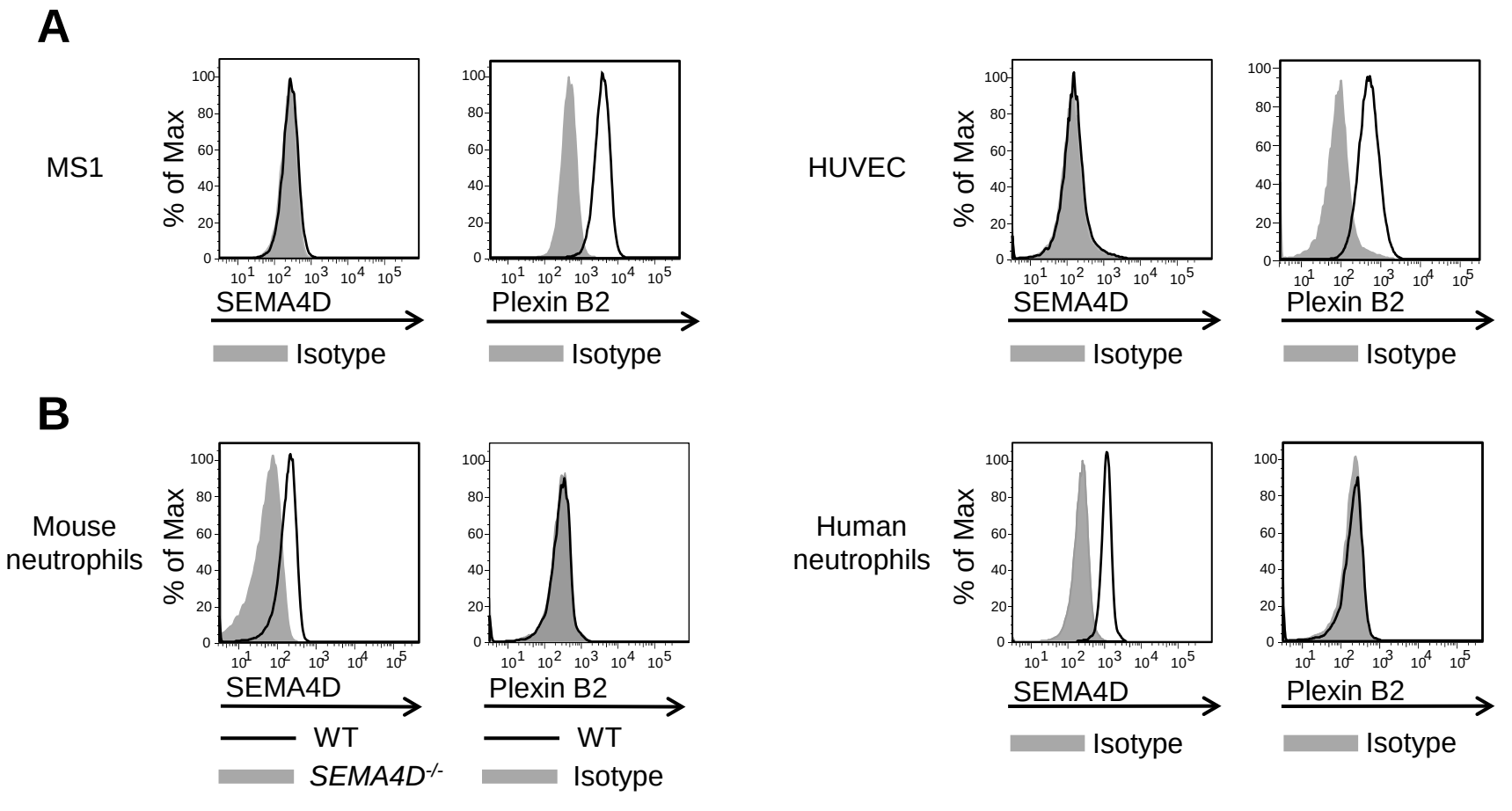
Supplementary Figure S4. ***In vivo*** **experimental AAV model using WT and** ***SEMA4D*****$^{-/-}$** **mice. (A)** Experimental protocol for BSA-induced AAV model mice. 7-week-old female C57BL/6J mice were pre-immunized by subcutaneous injections of BSA in complete Freund's adjuvant at weeks 0, 2, 4, and 6. Subsequently, these mice were treated with daily intraperitoneal injection of 50 mg/kg BSA at weeks 8 to 14. **(B)** Survival rate in WT and *SEMA4D*$^{-/-}$ mice after initial i.p. injection of BSA. N = 6 in each group. **(C)** Histological images of kidney glomeruli (Hematoxylin and eosin staining) at week 14. Scale bar; 100 μm. **(D)** Histological scores of kidney glomerulonephritis at week 14. Activity index (AI) was determined according to the modified NIH criteria as follows; the presence of cell proliferation, glomerular leucocyte infiltration, and interstitial mononuclear cell infiltration were scored in a range of 0–3 (0 = absent, 1 = mild, 2 = moderate, and 3 = severe). The presence of cellular or fibrocellular crescents was scored in a range of 0–3 (0 = absent, 1 = <20% of the glomeruli involved, 2 = 20–50% glomeruli involved, 3 = >50% glomeruli involved). The maximal activity score amounted to 12. **(E)** The degree of proteinuria. Proteinuria score was determined in a range of 0–4 by dipstick test. NS, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as determined by a two-tailed unpaired Student's *t* test. Values are means ± SEM.

Supplementary Figure S5



Supplementary Figure S5. Comparing the functions of wild-type (WT) and SEMA4D-deficient (*SEMA4D*$^{-/-}$) mouse neutrophils. **(A)** SEMA4D expression in mouse Gr-1$^+$ CD11b$^+$ neutrophils. Bone marrow–derived neutrophils were isolated from wild-type (WT) and SEMA4D-deficient (*SEMA4D*$^{-/-}$) mice. The expression level of mouse SEMA4D on each neutrophil was analyzed using flow cytometry (WT: solid line; *SEMA4D*$^{-/-}$: gray-filled histogram). **(B)** Cell surface expression of SEMA4D on peritoneal exudate neutrophils. Mouse peripheral blood neutrophils and peritoneal exudate cells (PEC) were harvested 3 hours after i.p. injection of 4% thioglycollate broth. Subsequently, flow cytometry was performed to analyze SEMA4D expression levels on Gr-1$^+$ CD11b$^+$ neutrophils (WT peripheral blood neutrophils: solid line; WT PEC neutrophils: dotted line; *SEMA4D*$^{-/-}$ PEC neutrophils: gray-filled histogram). **(C)** The populations of migratory PEC neutrophils. PECs were collected 3 hours after i.p. injection of 4% thioglycollate broth. Total PECs were counted by MUSE cell analyzer and flow cytometry was subsequently performed to identify the rate of Gr-1$^+$ CD11b$^+$ neutrophils. **(D)** Viability profiles of WT and *SEMA4D*$^{-/-}$ neutrophils as measured by a MUSE cell analyzer. Bone marrow–derived neutrophils prepared from WT (solid line) or *SEMA4D*$^{-/-}$ (dotted line) mice were incubated with and without 2 μM FMLP for 50 hours. Cell viability was monitored at the indicated time points. **(E)** Adhesion capacity of WT and *SEMA4D*$^{-/-}$ neutrophils. Neutrophils were suspended in RPMI 1640 medium containing 10% FBS and seeded onto collagen-coated 96-well plates. Recombinant mouse TNF-α (500 ng/ml) was then added. After 2 hours, wells were washed with PBS, and cells that remained attached to the well were counted on a MUSE cell analyzer. **(F)** Cell-surface expression of adhesion molecules on WT and *SEMA4D*$^{-/-}$ neutrophils after cells were either stimulated with recombinant mouse TNF-α (1 μg/ml) for 4 hours or left unstimulated. Flow cytometry was performed as in (**A**). Cells were also stained with an isotype-matched control antibody (Isotype; gray-filled histogram). NS, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as determined by a two-tailed unpaired Student's *t* test. Values are means ± SEM. Data are representative of three independent experiments.

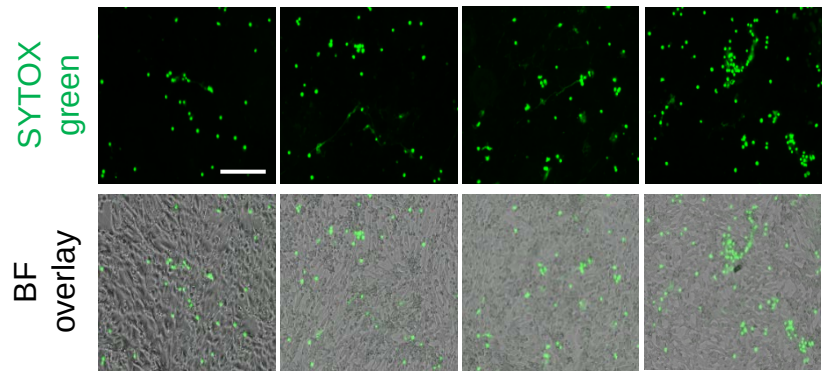
Supplementary Figure S6



Supplementary Figure S6. Expression profiles of SEMA4D and plexin B2 in endothelial cells and neutrophils. (A) Expression of SEMA4D and plexin B2 in MS-1 and HUVEC. Cells were also stained with an isotype-matched control antibody (Isotype; gray-filled histogram). **(B)** Expression of SEMA4D and plexin B2 in mouse and human neutrophils. Data are representative of three independent experiments.

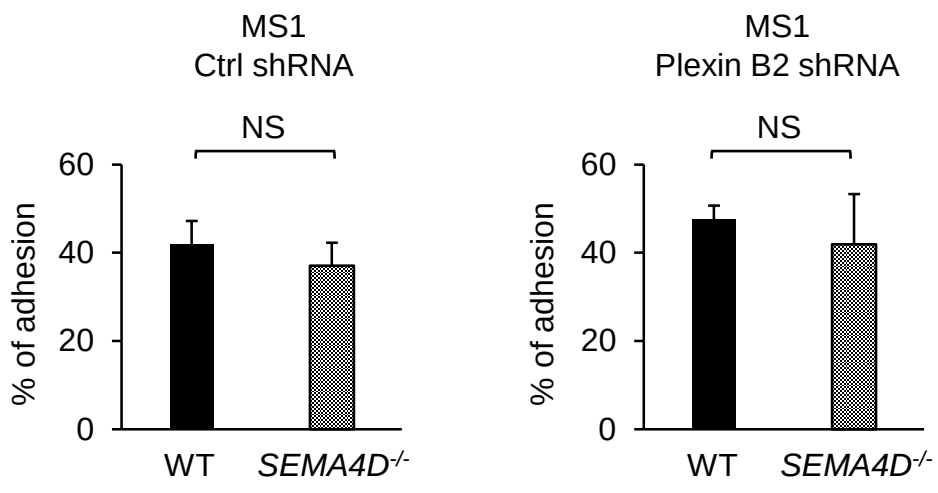
Supplementary Figure S7

A



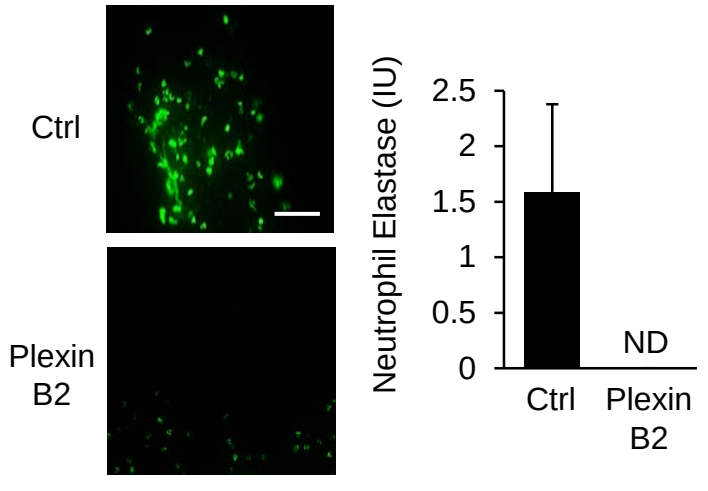
Neu	WT		*SEMA4D*$^{-/-}$	
MS1	+	+	+	+
shRNA	Ctrl	Plexin B2	Ctrl	Plexin B2

B



Supplementary Figure S7. NET formation assay and adhesion assay on neutrophils co-cultured with plexin B2–knocked down MS1 cells. (A) SYTOX green immunofluorescence analysis was performed similarly to **(figure 3A)**. Neutrophils were co-cultured with control or plexin B2–knocked down MS1 cells and treated with LPS (10 μg/ml) for 4 hours. Scale bar: 200 μm. **(B)** Adhesion capacity of WT and *SEMA4D*$^{-/-}$ neutrophils seeded onto MS1. Mouse neutrophils were suspended in RPMI 1640 medium containing 10% FBS and co-cultured with control or plexin B2–knocked down MS1 cells. Recombinant mouse TNF-α (500 ng/ml) was then added. After 2 hours, wells were washed with PBS, and neutrophils that remained attached to the well were counted by flow cytometry. NS, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as determined by a two-tailed unpaired Student's *t* test. Values are means ± SEM. Data are representative of three independent experiments **(A)** and two independent experiments **(B)**.

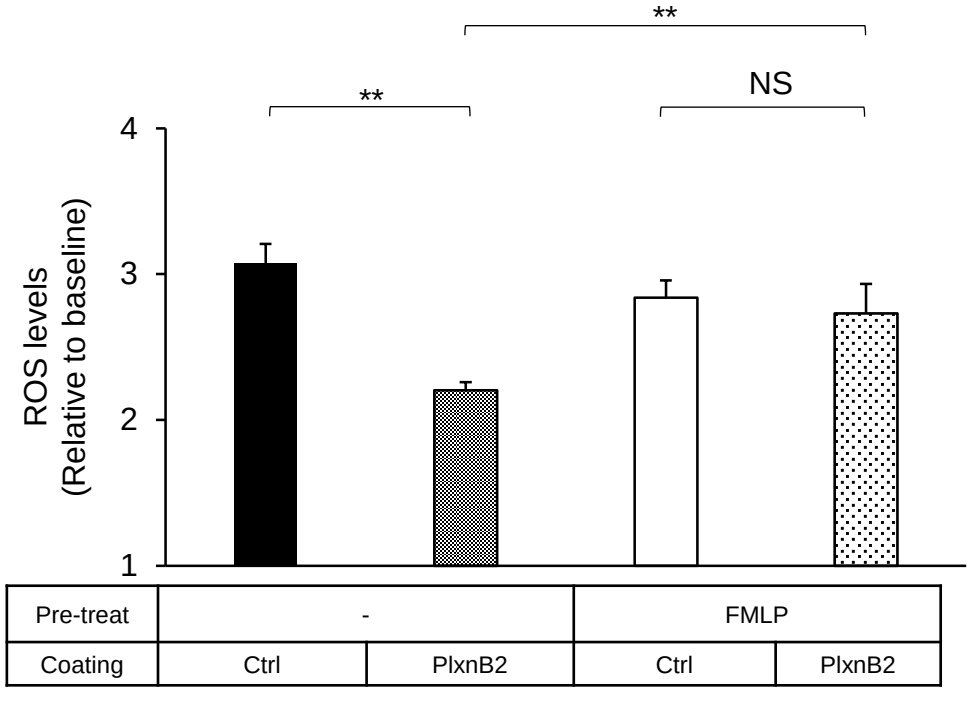
Supplementary Figure S8



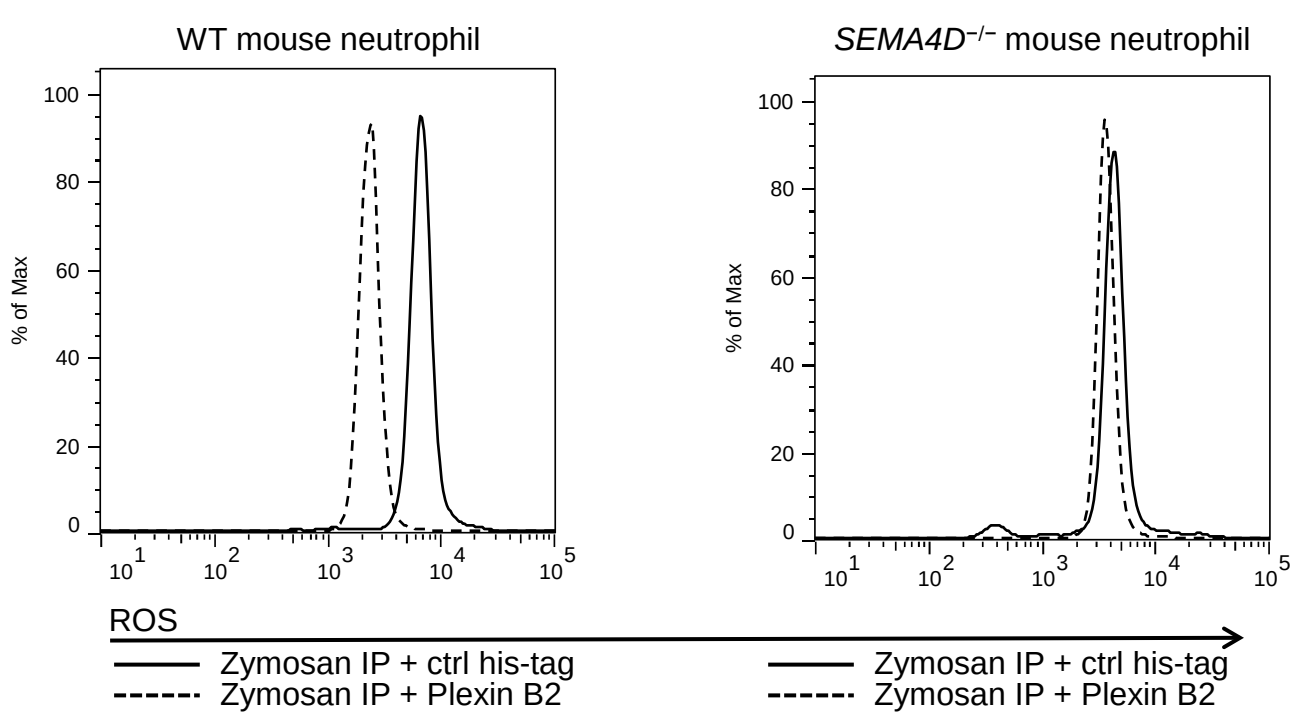
Supplementary Figure S8. **Effects of plexin B2 on human NET formation.** Isolated neutrophils derived from healthy donors were seeded onto culture plates pre-coated with recombinant human plexin B2 protein or control his-tag protein and stimulated with LPS (10 µg/ml) for 24 hours. SYTOX green immunofluorescence analysis was performed similarly to **(figure 3A)**. Scale bar: 100 µm. NET formation by these cells was quantified using the level of NET-associated neutrophil elastase in the culture supernatants. ND, not detected. Values are means ± SEM. Data are representative of three independent experiments.

Supplementary Figure S9

A

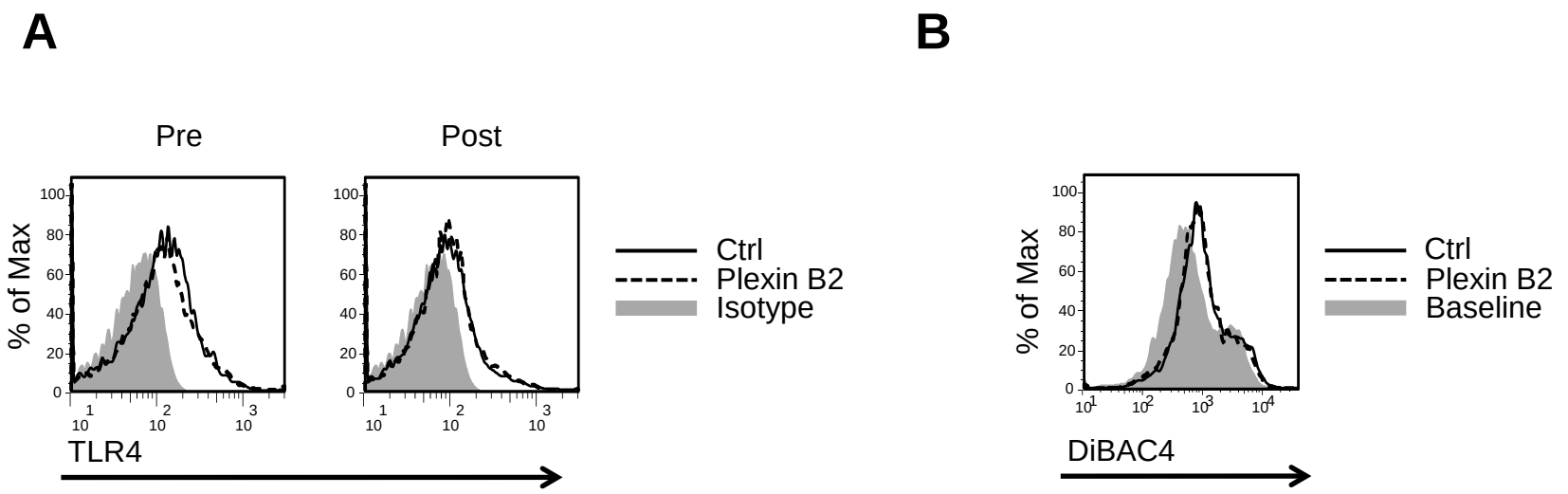


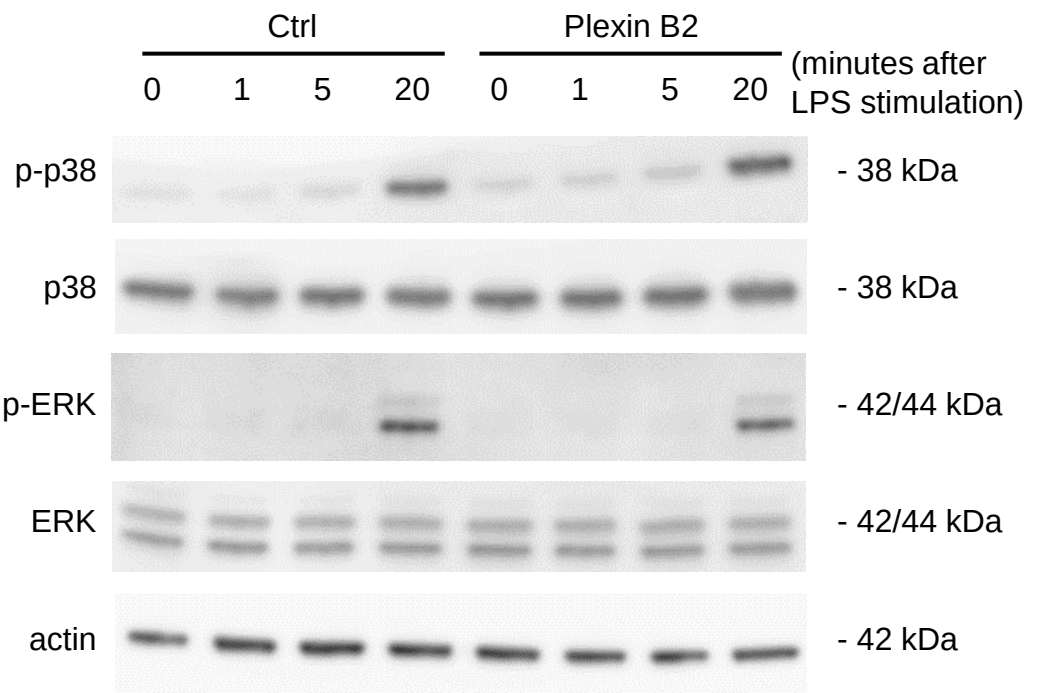
B



Supplementary Figure S9. Effects of recombinant plexin-B2 on the generation of reactive oxygen species (ROS) in human and mouse neutrophils. (A) Effects of plexin B2 on the generation of ROS in human neutrophils. Neutrophils isolated from healthy donors were pre-treated with or without 2 μM FMLP for 15 minutes, washed and then seeded onto culture plates pre-coated with recombinant human plexin B2 or control his-tag protein. These cells were incubated for 60 minutes, then stimulated with LPS (1 μg/ml) for 2 hours. ROS levels were monitored every 1 minute for a total of 120 cycles, and relative ROS levels were determined as the ratio of fluorescent peak to baseline. **(B)** Effects of plexin B2 on the generation of ROS in PEC neutrophils. 8-10-week-old female C57BL/6 mice were pre-treated by i.p. injections of recombinant plexin B2 protein (5 μg/body) 1 hour before the i.p. injection of zymosan (50 μg/body). PECs were collected 3 hours after injection of zymosan, and ROS production in peritoneal Gr-1$^+$ CD11b$^+$ neutrophils were detected by flow cytometry of CM-H2DCFDA. (Control PECs: solid line; plexin-B2 treated PECs: dotted line). NS, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as determined by a two-tailed unpaired Student's *t* test. Values are means ± SEM. Data are representative of two independent experiments.

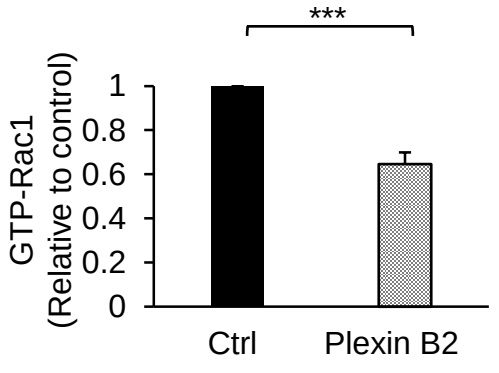
Supplementary Figure S10





Supplementary Figure S10. Evaluation of LPS-mediated signaling in human neutrophils. (A) Expression of Toll-like receptor 4 (TLR4) on neutrophils. Neutrophils isolated from healthy donors were seeded onto culture plates pre-coated with recombinant human plexin B2 or control his-tag protein. These cells were incubated for 60 minutes (Pre), then stimulated with LPS (1 μg/ml) for 2 hours (Post). Flow cytometry was performed at each time point on neutrophils cultured in the wells with plexin B2 (Plexin B2; dotted line) or with control his-tag protein (Ctrl; solid line). Cells from control wells were also stained with an isotype-matched control antibody (Isotype; gray-filled histogram). **(B)** Evaluation of neutrophil membrane depolarization using DiBAC4 staining. Neutrophils were seeded similarly to **(A)**, and these plates were incubated for 60 minutes (Baseline; gray-filled histogram). Subsequently, cells were stimulated with LPS for 20 minutes (1 μg/ml). Neutrophils were stained with DiBAC4, and the intensity of the fluorescence was determined using flow cytometry at each time point on neutrophils cultured with plexin B2 (dotted line) or with control his-tag protein (Ctrl; solid line). **(C)** Western blot analysis of LPS-mediated MAPK signaling pathways. Neutrophils were seeded similarly to **(A)**, and these plates were incubated for 60 minutes (Pre; 0 minute). Subsequently, cells were stimulated with LPS for 20 minutes (1 μg/ml). Cells were lysed at the indicated time points (0, 1, 5, or 20 minutes after LPS) as described. Proteins were separated by SDS-PAGE (1×10^6 cells/lane) and blotted using the indicated antibodies. Data are representative of three independent experiments.

Supplementary Figure S11



Supplementary Figure S11. Densitometry analysis of Rac-1 pull down assay. The band intensity of the Rac-1 pull down assay shown in **(figure 4D)**. Densitometry analysis of blotted bands was performed using Image J software. NS, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as determined by a two-tailed unpaired Student's *t* test. Values are means ± SEM. The density ratio of plexin B2–treated cells to control his-tag-treated cells was evaluated from three independent experiments.