Wegmann et al., http://www.jem.org/cgi/content/full/jem.20060565/DC1

SUPPLEMENTAL MATERIALS AND METHODS

Generation of ESAM-deficient mice

A genomic library (no. 121) derived from adult mouse spleen (strain: 129/ola) was screened with a full-length mouse ESAM cDNA probe at the Deutsches Ressourcenzentrum für Genomforschung (RZPD). The resulting 11 positive cosmid clones were PCR screened with the primer pairs 5′-ATGATTCTTCAGGCTGGAACCCCCGAG-3′ and 5′-AAGATAGTTGTCCTGGAGAGCTCACC-3′ as well as 5′-GCTTTGTACTTGTGGGAAGAATGTC-3′ and 5′-AACAAGAAGGGAAGGAACCAAACCAGG-3′ for the presence of exons 1 and 2 of the murine ESAM gene. To construct the targeting vector, a 2.7-kb BcuI/ScaI fragment, located 5′ of the start codon, was cloned into the targeting vector pPGKneo/loxP (provided by A. Gossler, Hannover Medical School, Hannover, Germany). The 3′ arm consisting of a 5.9-kb TaqI/Eco52I fragment was subcloned into pBlueskript II KS+ (Stratagene) and combined with the first construct to give the final targeting construct (Fig. 1 A). Electroporation, selection, and blastocyst injection of R1 embryonic stem (ES) cells were performed essentially as described (Fassler, R., and M. Meyer. 1995. *Genes Dev.* 9:1896–1908). To check for homologous recombination, Southern blots of EcoRV-digested ES cell clones were hybridized with a 0.9-kb 5′ probe, which was PCR generated from one of the above cosmid clones using the primers 5′-GCACTTTGATTCTCCATGTT-3′ and 5′-CACTGGTGACCATGAATTTA-3′. Homologous recombinant clones were identified by the detection of a band of 7.3 kb in addition to the 16.2-kb wild-type band. Targeted ES cells were injected into blastocysts to generate chimeras, which were subsequently mated with C57BL/6 females. Transmission of the targeted ESAM locus was confirmed by Southern blotting. Subsequent genotyping was performed by genomic PCR using the primers P1 (5′-AAGGGAAGAAAGCGAAAGGAG-3′), P2 (5′-GGGCTGTTCCAGGTTTATTTG-3′), and P3 (5′-GCGGGTGGGTGAGAAGATA-3′). ESAM$^{+/-}$ mice were backcrossed into C57BL/6 background for four generations and used to generate ESAM$^{-/-}$ and ESAM$^{+/+}$ littermates that were further bred among each other to generate sufficient numbers of mice for all experiments requiring groups of mice larger than four. Several peritonitis experiments, testing of blood vessel leakiness, and the experiments with platelet-depleted mice were performed with ESAM$^{-/-}$ mice backcrossed into the C57BL/6 background for eight generations.

Contact hypersensitivity and T cell immigration into inflamed skin

Experiments were performed essentially as described previously (Bixel, G., S. Kloep, S. Butz, B. Petri, B. Engelhardt, and D. Vestweber. 2004. *Blood.* 104:3205–3213), except for the schedule of stimulation with 2,4-dinitrofluorobenzene (DNFB). Mice were sensitized by skin painting of the shaved abdomen on days −15 and −14, and rechallenged on day −10 on both shaved flanks for isolation of T cells, or on day −1 on the front and dorsal surface of the right ear for the DTH experiment. T cells were isolated from axilar and inguinal lymph nodes of sensitized mice, radioactively labeled with [^{51}Cr] chromate, and injected i.v. at day 0 into mice whose ears had been challenged at day −1 as described previously (Bixel, G., S. Kloep, S. Butz, B. Petri, B. Engelhardt, and D. Vestweber. 2004. *Blood.* 104:3205–3213). At 15, 5, or 2 h after T cell injection, mice were killed and the radioactivity in the inflamed and the non-inflamed ear, as well as in various other organs (spleen, liver, and lung), was measured (Wallac 1470 Wizard; PerkinElmer). Each genotype group consisted of at least five mice.

RNA interference (RNAi)

RNAi knockdown of ESAM expression in bEnd.3 cells was achieved most efficiently by a siRNA duplex targeting the sequence 5′-AAGGAGUAAACCUACUGCUCA-3′ (nucleotide positions 539–559, relative to the start codon) of the ESAM mRNA. Negative control siRNA was designed as a random sequence that does not show significant homology to any murine gene sequence. The siRNA oligonucleotides were obtained from Ambion. Transfection was performed by electroporation using the Nucleofector device II and the Cell Line

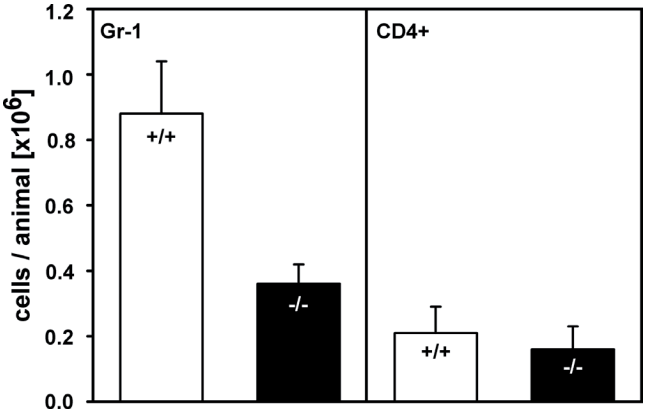

Figure S1. Lack of ESAM impairs neutrophil, but not lymphocyte, recruitment into IL-1β/CCL19-stimulated peritoneum. To attract neutrophils and lymphocytes at the same time into inflamed peritoneum, we stimulated mice intraperitoneally with IL-1β and the chemokine CCL19. Peritoneal leukocytes were removed 2 h later, and Gr-1$^+$ neutrophils and CD4$^+$ T cells were determined and counted by FACS analysis. The data represent the mean ± SD of four or more mice in each group, and the depicted experiment represents one of three independent experiments with similar results.

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Nucleofector kit V (Amaxa) according to the manufacturer's instructions.

GTPase activation assay

To test for the activation levels of the small GTPases Rac1, Rho, Cdc42, and Rap1 in bEnd.3 cells upon siRNA-mediated down-regulation of ESAM, binding assays with immobilized recombinant effector proteins were performed. The GST-Pak1-PBD pull-down of Cdc42-GTP and Rac1-GTP was done with the Cdc42 Activation kit, the GST-RalGDS-RBD pull-down of Rap1-GTP with the Rap1 Activation kit, and the GST-Rhotekin-RBD pull-down of Rho-GTP with the Rho Activation kit from StressGen Biotechnologies. Alternatively, activated Rho was detected using the Rho Assay Reagent from Upstate Biotechnology. Cell lysis, performed 36 h after siRNA transfection, pull-downs with GST effector fusion proteins, SDS-PAGE, and immunoblotting, was performed essentially as described by the manufacturers. Rho, Cdc42, and Rap1 were detected with antibodies supplied with the kits. Anti-Rac1 mAb (clone 102) was obtained from BD Biosciences.

Immunohistology

Immunohistology was performed essentially as described previously (Bixel, G., S. Kloep, S. Butz, B. Petri, B. Engelhardt, and D. Vestweber. 2004. *Blood.* 104:3205–3213).

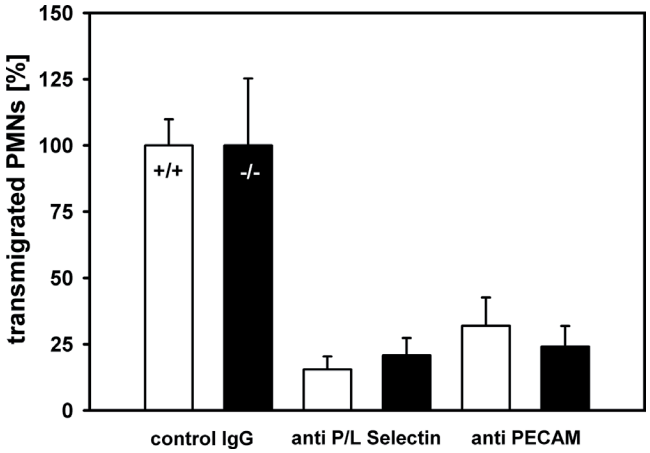

Figure S2. Antibodies against PECAM-1 block neutrophil recruitment into IL-1β–stimulated peritoneum in ESAM$^{-/-}$ as well as in ESAM$^{+/+}$ mice. ESAM$^{+/+}$ (white bars) and ESAM$^{-/-}$ (black bars) mice were i.v. injected with isotype-matched negative control antibodies for positive controls with a mixture of a mAb against L-selectin and against P-selectin, and with mAbs against PECAM-1. Immediately after antibody injection, the mice were intraperitoneally stimulated with IL-1β and peritoneal leukocytes were removed 4 h later, stained for Gr-1, and counted by FACS analysis. The number of neutrophils recruited in the presence of the negative control antibodies was set as 100%. Note that the lack of ESAM did not affect the inhibitory effect of the anti–PECAM-1 mAb. The data represent the mean ± SD of four or more mice in each group, and the depicted experiment represents one of three independent experiments with similar results.