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J Immunol 2011; 187:2268-2277; Prepublished online 20 July 2011; doi: 10.4049/jimmunol.1101008
http://www.jimmunol.org/content/187/5/2268

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/07/20/jimmunol.1101008.DC1

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SIRPα/CD172a Regulates Eosinophil Homeostasis

Noel Verjan Garcia,* Eiji Umemoto,*† Yasuyuki Saito,‡,† Mikako Yamasaki,† Erina Hata,*† Takashi Matozaki,‡,† Masaaki Murakami,§ Yun-Jae Jung,‡ So-Youn Woo,‖ Ju-Young Seoh,‖ Myoung Ho Jang,‖‖* Katsuyuki Aozasa,‖‖ and Masayuki Miyasaka*†

Eosinophils are abundant in the lamina propria of the small intestine, but they rarely show degranulation in situ under steady-state conditions. In this study, using two novel mAbs, we found that intestinal eosinophils constitutively expressed a high level of an inhibitory receptor signal regulatory protein α (SIRPα)/CD172a and a low, but significant, level of a tetraspanin CD63, whose upregulation is closely associated with degranulation. Cross-linking SIRPα/CD172a on the surface of wild-type eosinophils significantly inhibited the release of eosinophil peroxidase induced by the calcium ionophore A23187, whereas this cross-linking effect was not observed in eosinophils isolated from mice expressing a mutated SIRPα/CD172a that lacks most of its cytoplasmic domain (SIRPα Cyto−/−). The SIRPα Cyto−/− eosinophils showed reduced viability, increased CD63 expression, and increased eosinophil peroxidase release with or without A23187 stimulation in vitro. In addition, SIRPα Cyto−/− mice showed increased frequencies of Annexin V-binding eosinophils and free MBP+CD63+ extracellular granules, as well as increased tissue remodeling in the small intestine under steady-state conditions. Mice deficient in CD47, which is a ligand for SIRPα/CD172a, recapitulated these phenomena. Moreover, during Th2-biased inflammation, increased eosinophil cell death and degranulation were obvious in a number of tissues, including the small intestine, in the SIRPα Cyto−/− mice compared with wild-type mice. Collectively, our results indicated that SIRPα/CD172a regulates eosinophil homeostasis, probably by interacting with CD47, with substantial effects on eosinophil survival. Thus, SIRPα/CD172a is a potential therapeutic target for eosinophil-associated diseases. The Journal of Immunology, 2011, 187: 2268–2277.

The eosinophil is a multifunctional granulocyte associated with innate and Th2 immune responses against allergens and gastrointestinal helminth infections (1). Under steady-state conditions, eosinophils are particularly abundant in the small intestinal lamina propria, which they colonize prenatally in a manner dependent on CC-chemokine eotaxin-1 (2, 3) and its receptor, CCR3 (4, 5). The colonization is independent of viable commensal bacteria in the intestinal lumen (3). Eosinophils play a role in host defense against migratory helminths, particularly during secondary immune responses (6, 7). They are also involved in the development and pathogenesis of allergic diseases and gastrointestinal dysfunction through their secretion of cytotoxic proteins (8, 9).

The release of eosinophil granules, “degranulation,” is an immune response common to many human diseases, including asthma (10), allergy and atopic dermatitis (11), and inflammatory bowel diseases (12, 13); little degranulation is observed under basal physiological conditions. During degranulation, activated human eosinophils release preformed cytotoxic proteins, such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin, and eosinophil cationic protein, as well as other cytokines, chemokines, and growth factors, from cytoplasmic granules (14). Agonists, including Lgs (15, 16); cytokines, such as IFN-γ (17) and IL-6 (18); lipid mediators, such as platelet activating factor (18, 19), and the calcium ionophore A23187 (19) were shown to induce the release of eosinophil granule proteins. The stimulus-induced release of eosinophil proteins is a tightly regulated and highly selective process that occurs within minutes of agonist stimulation (14), although its regulatory mechanisms remain to be fully explored.

The survival of tissue-resident eosinophils seems to be self-regulated by tissue-specific or autocrine survival signals that the eosinophils secrete following contact with extracellular matrix proteins (20) or upon activation (16). In β common chain gene-targeted mice, which are deficient in the functional receptor for cytokines that include IL-5 and GM-CSF, eosinophils are markedly reduced in the gastrointestinal tract and peripheral blood (3). In addition, Rag-2/common γ-chain double-deficient mice were recently reported to have reduced eosinophil numbers in the

Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; BM, bone marrow; EPO, eosinophil peroxidase; FSC, forward light scatter; LC/MS, liquid chromatography-mass spectrometry; MBP, major basic protein; PIR-B, paired Ig-like receptor B; RT, room temperature; Siglec F, sialic acid-binding Ig-like lectin F; SIRPα, signal regulatory protein α; SSC, side scatter; WT, wild-type.

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intestinal, but not in the lung, bone marrow (BM), or PBLs, suggesting that tissue-specific cytokine signals dependent on the common γ-chain contribute to eosinophil survival in the intestine (21).

Signaling via inhibitory receptors also seems to play a role in eosinophil homeostasis. The cross-linking of an inhibitory receptor IRP60 (CD300a) inhibits both the survival signals provided by IL-5/GM-CSF and eotaxin-dependent eosinophil migration (22). Genetic inactivation of the inhibitory paired Ig-like receptor B (PIR-B) is associated with an increased recruitment of eosinophils to the gastrointestinal tract, and PIR-B negatively regulates eotaxin-dependent eosinophil chemotaxis in vitro and in vivo (23). Given the importance of pair activation and inhibition in the development of immune responses (24), inhibitory receptors are likely to constitute a major regulatory mechanism for counter-balancing the activating signals encountered by eosinophils to maintain tissue homeostasis.

In this study, we found that intestinal eosinophils express an inhibitory receptor signal regulatory protein α (SIRPα/CD172a) at high levels and a degradation marker CD63 at low, but significant, levels and that SIRPα/CD172a contributes to eosinophil homeostasis by regulating the degradation in these cells, with substantial effects on their survival.

Materials and Methods

Mice
Female 6–12 wk-old BALB/c and C57BL/6 mice (SLC, Hamamatu, Japan) were used. A C57BL/6 mouse strain expressing a mutant SIRPα/CD172a lacking most of its cytoplasmic domain (25) was backcrossed onto the BALB/c background for >10 generations (hereafter referred to as SIRPα Cyto-). The CD47+ mice on the C57BL/6 background were kindly provided by Dr. Per-Arne Oldenborg (Umea University, Umea, Sweden). All animal experiments were approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine.

Reagents

O-phenylenediamine, cetyltrimethylammonium bromide, and calcium ionophore A23187 were purchased from Sigma-Aldrich (St. Louis, MO). The stock solution of A23187 (10 mM) was prepared in DMSO, and subsequent dilutions were prepared in RPMI 1640 without phenol red (Invitrogen Life Technologies, Carlsbad, CA). Recombinant murine stem cell factor, recombinant murine Flt3 ligand, and recombinant murine IL-5 were all purchased from PeproTech (Rocky Hill, NJ) and used at the concentrations described previously (26). Recombinant murine IL-25 was from R&D Systems.

Cell isolation

The small intestine was isolated, and after removal of the fat tissue and mesentery, the intestine was opened longitudinally, rinsed in cold PBS, and cut into 1–2-cm lengths. The epithelial cell layer was isolated and digested with trypsin. The eluted peptides were analyzed by mass spectrometry. The CD47+ mice were used. A C57BL/6 mouse strain expressing a mutant SIRPα/CD172a lacking most of its cytoplasmic domain (25) was backcrossed onto the BALB/c background for >10 generations (hereafter referred to as SIRPα Cyto-). The CD47+ mice on the C57BL/6 background were kindly provided by Dr. Per-Arne Oldenborg (Umea University, Umea, Sweden). All animal experiments were approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine.

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Cell isolation

The small intestine was isolated, and after removal of the fat tissue and mesentery, the intestine was opened longitudinally, rinsed in cold 5 mM EDTA in PBS, and cut into 1–2-cm lengths. The epithelial cell layer was removed by vigorous stirring in FACS buffer (PBS containing 10% FCS, 2 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 10 mM EDTA) for 20 min at 37°C. After shaking, in PBS, the intestinal fragments were incubated in complete RPMI 1640 medium for 10 min at room temperature (RT), rinsed, and digested in 400 μl collagenase D and 10 μg/ml DNase I (Roche, Mannheim, Germany) at 37°C for 45 min with continuous stirring. The cell suspension was filtered through a 40-μm cell strainer and subjected to 40% to 75% Percoll (Amersham Biosciences) density-gradient centrifugation. Cells at the interface (hereafter referred to as “light-density cells”) were collected, washed, and used for phenotypic analysis, immunoprecipitation, coinoculation, eosinophil degranulation assay, or culture. BC cells were collected by flushing the fumus and tissue with complete RPMI 1640 medium. The red cells in the BM and blood were lysed with ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 M Na₂EDTA [pH 7.3]), and the remaining leukocytes were subjected to enzymatic digestion, as described above for the lamina propria leukocytes, or used directly for phenotypic analysis. BM-derivative eosinophils were obtained by culturing BM cells in the presence of the stem cell factor, Flt3 ligand, and IL-5, following a previously described method (26).

Generation of MY-1 and NVG-2 mAbs

Sprague-Dawley rats were immunized with an eosinophil-enriched cell fraction, obtained by sorting granulocytes (FSChigh and SSCsmall) from the mouse small intestinal lamina propria by FACS. Two weeks after the last booster immunization, the popliteal lymph nodes were used to generate hybridomas, following standard methods. Hybridomas were screened in hypoxanthine–aminopterin–thymidine medium supplemented with IL-6, and the culture supernatants were screened for Abs reactive with small intestinal lamina propria eosinophils by flow cytometry. The MY-1 and NVG-2 mAbs were produced in ascitic fluid from ICR nu/nu mice. They were both determined to be of the IgG2a subclass and purified using protein G-affinity chromatography.

Cell lines

CHO cells stably expressing an active form of H-Ras (CHO-Ras cells) and those stably expressing mouse SIRPα/CD172a (CHO-Ras-SIRPα-ICR) were cultured in α-MEM medium (Sigma-Aldrich) supplemented with 10% FCS, 2 mM l-glutamine, 10 mM HEPES, 500 μg/ml Geneticin, and 500 μg/ml Zeocin, as previously described (27). COS-7 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, l-glutamine, and 0.1 mM nonessential amino acids at 37°C with 5% CO₂ and transfected with a pCMV-SPORT6 expression vector encoding mouse CD63 Ag (Invitrogen Life Technologies) to generate a stable cell line expressing CD63 on the cell surface.

Abs and flow cytometry

Unlabeled mAbs used in EPO-release experiments were anti-mouse sialic acid-binding Ig-like lectin F (Siglec F; 238047), anti-mouse PIR-B (326414) from R&D Systems, rat IgG2a isotype control (eBioscience), and MY-1. Abs used in flow cytometry were Alexa Fluor 647- or FITC-conjugated MY-1, Alexa Fluor 647- or FITC-conjugated NVG-2, anti-CD63 (MBL, Woburn, MA), and FITC-conjugated anti-CCR3 (83101) from R&D Systems. PE-conjugated anti-Siglec F (E50-2440), PE-conjugated anti-CD121 (T21), allophycocyanin- or PE-conjugated anti-CD11c (HL3), and FITC-conjugated Annexin V were from BD Pharmingen. Allophycocyanin- or PE-conjugated anti-CD11b (M1/70) and FITC-conjugated rat IgG2a isotype control were from eBioscience. PE- or Alexa Fluor 647-conjugated rat IgG2a κ (RTK2758) isotype control Abs were from BioLegend. Biotin-conjugated P84 mAb was produced from hybridoma cells kindly provided by Dr. C.F. Lagena (University of Pittsburgh, Pittsburgh, PA). FCr blocking was performed using anti-CD16/32 (2.4G2) or mouse γ-globulins when necessary. After addition of a nucleic acid dye 7-aminoactinomycin D (7AAD), flow cytometric analysis was performed on a FACSCanto II (BD Biosciences) or Gallios (Beckman Coulter), and the data were processed with FlowJo (Tree Star) or Kaluza (Beckman Coulter) software. The geometric mean fluorescence intensity values of isotype control Abs were subtracted from those of specific Abs.

Immunoprecipitation, Western blot, and liquid chromatography mass spectrometry analysis

The MY-1 and NVG-2 mAbs were covalently linked to Sepharose beads (GE Healthcare) and used to immunoprecipitate Ags from lysates of lamina propria light-density cells. The immunoprecipitated Ag was washed in PBS containing 0.1% Triton X-100 and 500 mM NaCl, eluted with glycine-HCl (pH 2.5), neutralized immediately with 3 μl Tris-HCl (pH 9.0), and concentrated 10 times using a CC-105 centrifugal concentrator (Tomy Tech). Cell lysates and the immunoprecipitated materials were resuspended in 2× SDS PAGE sample buffer and separated on a 5–20% SDS-PAGE gel under reducing or nonreducing conditions. After blotting, the filters were incubated with the MY-1 or NVG-2 mAbs, followed by HRP-conjugated goat anti-rat IgG, and detected with ECL Western blotting detection reagents (GE Healthcare). An additional gel was stained with the Sipro Ruby protein gel stain (Invitrogen Life Technologies), and the protein band was isolated and digested with trypsin. The eluted peptides were analyzed by liquid chromatography mass spectrometry (LC/MS). The mass spectrometry data were compared with primary sequence databases using the Mascot search (http://www.matrixscience.com).

Eosinophil-degranulation assay

Small intestinal lamina propria light-density cells (5 × 10⁶ cells/well, 30 ± 5% eosinophils) and in vitro-differentiated BM-derived eosinophils (2.5 × 10⁵ cells/well, >90% eosinophils) were assayed for EPO release following a previously described method that measured extracellular EPO (28). The cells were resuspended in RPMI 1640 medium without phenol red and
incubated with 50 μg/ml mouse γ-globulins for 15 min at RT. The cells were then incubated with 2.5 μg/ml MY-1, anti-Siglec-F, anti-PIR-B, rat IgG2a isotype control, or the indicated Ab combination for 10 min at RT. After washing with RPMI 1640 medium without phenol red, the cells were incubated with 2.5 μg/ml goat anti-rat IgG for 10 min at RT. After the removal of unbound Abs, the cells were stimulated with 10 μM A23187 (29) for 30 min at 37°C in a 5% CO₂ incubator. Subsequently, a substrate solution (0.4 M Tris-HCl, 0.2 mM o-phenylenediamine, 0.005% H₂O₂) was added, and the mixture was left for 15 min at RT before stopping the enzymatic reaction with cold 4 M H₂SO₄. The total EPO activity was determined from cells lysed in 0.3 M sucrose containing 0.22% cetyltrimethylammonium bromide and 0.2% Triton X-100. The absorbance was determined at 492 nm in a microplate reader, and the data are presented as the percentage of total EPO activity in lysed cells.

**Immunofluorescence staining**

Cryosections of the small intestine and spleen were blocked with 10% FCS in PBS containing 20 μg/ml mouse γ-globulins for 12 h at 4°C. The sections were stained with 2 μg/ml rat anti-mouse MBP mAb (kindly provided by D.J. Lee, Mayo Clinic, Scottsdale, AZ) for 1 h at RT, followed by Alexa Fluor 594-conjugated goat anti-rat IgG. After a second blocking with 10 μg/ml rat IgG, the sections were stained with FITC-labeled anti-CD63 (NVG-2 mAb) or Alexa Fluor 647-conjugated MY-1, followed by the nucleic acid dye Hoechst 33342. Apoptotic eosinophils were detected by TUNEL, using the In Situ Cell Death Detection Kit (Roche). Pictures were acquired by an Olympus FV-1000 confocal laser-scanning microscope.

**Induction of Th2-type inflammation**

IL-25 was administered i.p. (400 ng/mouse) daily for 3 d (30), and the peritoneal cells and tissues were collected 24 h after each treatment.

**Statistical analysis**

Flow cytometry data were collected from individual mice or from pooled samples from three to five mice. In the EPO-release experiments, the mean ± SD was calculated from the data collected from six to eight wells per treatment, and the experiment was repeated at least three times. Significant differences were determined using the Student t test. Statistical analysis was performed with GraphPad Prism 5 software (GraphPad).

**FIGURE 1.** The small intestinal lamina propria eosinophils express SIRPα/CD172a and CD63. A, The small intestinal lamina propria light-density cells of naive BALB/c mice were stained with FITC-conjugated anti–CCR-3 and anti–CD11b, PE-conjugated anti–CD125 (IL-5Rα), anti–Siglec F, or anti–CD11c and analyzed by flow cytometry. B, The expression levels of SIRPα/CD172a and CD63 in the small intestinal lamina propria eosinophils were determined using Alexa Fluor 647-conjugated MY-1 or NVG-2 mAbs, respectively. Numbers inside the outlined areas or within each quadrant indicate the percentage of gated cells. Data are from one experiment representative of more than three with three mice each.

**FIGURE 2.** SIRPα/CD172a is more abundantly expressed in intestinal eosinophils than in BM or PBL eosinophils. A, Flow cytometry of the BM, PBL, and small intestinal lamina propria eosinophils from BALB/c and C57BL/6 mice, stained with Alexa Fluor 647-conjugated MY-1 (anti-SIRPα/CD172a). B, Geometric mean fluorescence intensity values of SIRPα/CD172a, Siglec F, CD125, and CCR3 in BM and PBL eosinophils that had been subjected to treatment with collagenase D and DNase I, similar to that used for the lamina propria eosinophils. Data are from one experiment representative of three with three mice each.
Results

Eosinophils express SIRPa/CD172a and CD63 in the small intestinal lamina propria

Analysis of the small intestinal lamina propria light-density cells by flow cytometry indicated the presence of at least three main cell populations based on the forward light scatter (FSC)/side scatter (SSC) pattern of live cells: a granulocyte population with medium to high SSC (R1; 33 ± 5%), a mononuclear cell population with low SSC (R2; 56 ± 4%), and a minor cell population with low FSC/SSC (R3; 5.5 ± 7%) (Fig. 1A). Staining with mAbs against CCR3, IL-5Rα, and Siglec F verified that >90% of the granulocytes in the R1 fraction were eosinophils expressing all three of these markers. The majority of the R2 cells (>80%) were negative for CCR3, IL-5Rα, and Siglec F, although there were minor cell populations with the phenotype of CCR3−IL-5Rα+ (11%), CCR3+IL-5Rα− (6.4%), CCR3+Siglec F− (4.3%), CCR3−Siglec F+ (8%), and CCR3−Siglec F+ (4%), some of which might represent immature-type eosinophils. Additional staining indicated that almost all of the cells in the R1 gate (eosinophil-dominant subpopulation) expressed the integrin CD11c and reacted with two novel mAbs, MY-1 and NVG-2, which were generated against mouse eosinophils from the small intestinal lamina propria (see Materials and Methods). The lamina propria eosinophils had a high reactivity with MY-1 (95%) and a low, but significant, reactivity with NVG-2 (90%) (Fig. 1B), whereas the mononuclear cells in the R2 gate showed only minor or no reactivity with these mAbs.

When the Ags recognized by the mAbs MY-1 and NVG-2 were analyzed by Western blotting, MY-1 was found to recognize a major band with a molecular mass of ~100–120 kDa and a minor band of ~40 kDa, whereas NVG-2 reacted with a band of ~60–65 kDa in lamina propria cell lysates (Supplemental Fig. 1A). LC/MS analysis indicated that the major band recognized by MY-1 was an immune-inhibitory protein, SIRPa/CD172a (score 96.29, p = 5.00E−15), whereas NVG-2 reacted with tetraspanin CD63 (score 33, p = 4.19E−05), which appears on degranulating leukocytes. Confirming these data, the MY-1 mAb also reacted with CHO-Ras cells stably expressing mouse SIRPa/CD172a, as did a commercially available anti-SIRPa/CD172a mAb P84, in immunofluorescence and flow cytometric analyses (Supplemental Fig. 1B, 1C). The NVG-2 mAb reacted with COS-7 cells expressing the mouse CD63 Ag, as did a commercially available anti-CD63 (MBL) Ab (Supplemental Fig. 1D). Collectively, these results

FIGURE 3. Cross-linking of SIRPa/CD172a on eosinophils leads to an inhibition of peroxidase release. A, A23187 (10 μM)-induced EPO release from lamina propria eosinophils that had been pretreated with 2.5 μg/ml MY-1, anti-Siglec F, anti–PIR-B, rat IgG2a isotype control, or the indicated Ab combinations, with or without subsequent cross-linking with 2.5 μg/ml goat anti-rat IgG. Data are presented as the percentage of total EPO activity from lysed cells. B, EPO release from the lamina propria eosinophils of WT and SIRPa Cyto−/− mice treated as in A and stimulated with 10 μM A23187. Data are the mean values ± SD of six to eight wells from one experiment representative of three. Statistical significance of the differences was determined using the Student unpaired t test. *p < 0.05, **p < 0.001, ***p < 0.0001, compared with rat IgG2a isotype control Ab or between the indicated groups.
indicated that eosinophils in the lamina propria of the small intestine express SIRPα/CD172a at a high level and CD63 at a low, but significant, level.

**SIRPα/CD172a is more abundantly expressed in intestinal eosinophils than in BM or PBL eosinophils**

We next asked whether SIRPα/CD172a is expressed in eosinophils in other tissues. As shown in Fig. 2A, SIRPα/CD172a expression was comparable between the BM and blood eosinophils, whereas its expression was substantially higher in the lamina propria eosinophils in BALB/c and C57BL/6 mice (Fig. 2A). To exclude the possibility that the higher levels of SIRPα/CD172a observed in lamina propria eosinophils were due to differences in the isolation method, we subjected PBL and BM leukocytes to an isolation method similar to that used for the lamina propria cells and analyzed the expression of SIRPα/CD172a comparatively with other markers. Subtracting the geometric mean fluorescence intensity values of isotype control Abs from the values of the specific Abs, we found that the collagenase treatment did not significantly alter the expression levels of SIRPα/CD172a, Siglec F, CD125 (IL-5Rα), or CCR3 in the PBL or BM eosinophils. Furthermore, the intestinal eosinophils expressed both SIRPα/CD172a and Siglec F at higher levels and CD125 and CCR3 at lower levels than did those obtained from the PBL and BM (Fig. 2B).

**Cross-linking of SIRPα/CD172a leads to the inhibition of EPO release**

To understand the role of SIRPα/CD172a in intestinal eosinophils, we asked whether cross-linking SIRPα/CD172a on the cell surface has any effect on the A23187-induced release of EPO (28). As shown in Fig. 4, mice expressing SIRPα Cyto−/− show increased eosinophil degranulation and lamina propria remodeling. A. Flow cytometry of lamina propria leukocytes from WT and SIRPα Cyto−/− mice stained with anti–CCR-3, anti-CD125, anti-Siglec F, anti-SIRPα/CD172a (MY-1), and anti-CD63 (NVG-2) mAbs or rat IgG isotype control Abs. Numbers inside the outlined areas or within each quadrant indicate the percentage of gated cells. The total number of lamina propria light-density cells and of eosinophils in WT and SIRPα Cyto−/− mice is shown in the far right panel. Statistical significance of the differences was determined using the Student unpaired t test. *p < 0.05, ***p < 0.0001. Data are from one experiment representative of more than three with three mice per group. B. Confocal laser-scanning microscopy images of frozen sections from the small intestine of WT and SIRPα Cyto−/− mice immunostained for MBP (red), CD63 (green), and nuclei (Hoechst 33342, blue). Dotted circles demarcate MBP+CD63+ amorphous structures compatible with clusters of free extracellular granules. Arrows indicate free MBP+CD63+ granules, and white arrowhead indicates MBP+CD63+ granules apparently in the cytoplasm of mononuclear leukocytes. Scale bars, 20 μm. Data are from one experiment representative of more than three. C. Paraffin wax-embedded sections of the small intestine of WT and SIRPα Cyto−/− mice stained with Masson’s trichrome. Collagen is stained blue, and white brackets indicate the submucosa (SM) and circular muscle layer (CM). Original magnification ×200 (first and third panels) and ×1000 (second and fourth panels). Data are from one experiment representative of two.
shown in Fig. 3A, intestinal eosinophils obtained from un-  
perturbed wild-type (WT) mice showed a modest, but significant,  
reduction in EPO release upon the cross-linking of SIRPα/CD172a. In contrast, intestinal eosinophils from mice expressing  
SIRPα CytO−/−, which is a mutated SIRPα/CD172a that lacks  
most of its cytoplasmic domain and, hence, has no signal-  
transducing ability, did not show a significant reduction in EPO  
release after SIRPα/CD172a cross-linking (Fig. 3B); instead,  
the mutant cells showed a tendency to release more EPO with  
or without stimulation. This inhibitory effect on EPO release in WT  
eosinophils was found only when the cells were treated with anti-  
SIRPα/CD172a mAb, followed by a secondary Ab, but not with  
anti-SIRPα/CD172a mAb alone (Fig. 3A). A comparable  
inhibition of EPO release was observed by SIRPα/CD172a cross-  
linking in WT BM-derived eosinophils (data not shown). These  
findings are consistent with the hypothesis that SIRPα/CD172a  
negatively regulates eosinophil degranulation via its cytoplasmic  
domain.  

The cross-linking of other inhibitory receptors, Siglec F and  
PIR-B, also inhibited eosinophil degranulation at low levels, and  
the simultaneous cross-linking of SIRPα/CD172a enhanced the  
inhibition by either of these receptors slightly; cross-linking all  
three receptors did not enhance the inhibition further (Fig. 3A).  
These results suggested that there are multiple arms of regulation  
in eosinophil degranulation in the intestine, and these inhibitory  
receptors may act independently.  

Mice expressing SIRPα CytO−/− show increased eosinophil  
degranulation and lamina propria remodeling  

We next asked whether the absence of SIRPα/CD172a-mediated inhibitory signals compromises eosinophil homeostasis in vivo.  
As shown in Fig. 4A, we found a decrease in SSCbright light-density  
cells in the lamina propria of SIRPα CytO−/− mice compared with WT mice (24% versus 37%), which mainly reflected a de-  
crease in eosinophils, as revealed by staining with anti-CCR3 and  
anti-CD125 mAbs. Manual counting of live cells obtained from  
the small intestinal lamina propria confirmed that there was a  
lower recovery of total cells and eosinophils from the SIRPα  
CytO−/− mice. Eosinophils from the small intestine of the SIRPα  
CytO−/− mice showed increased expression of the tetraspanin  
CD63 and reduced levels of the mutated SIRPα/CD172a. The  
cells in the R2 fraction, which were mainly macrophages and dendritic cells expressing SIRPα/CD172a, did not show obvious  
changes in cell number, but they showed reduced expressions of  
MHC class II and F4/80 in SIRPα CytO−/− mice compared with WT mice (data not shown). These results indicated that the  
number of intestinal eosinophils and the expression of the de-  
granulation marker CD63 were altered in mice expressing a mu-  
tant SIRPα/CD172a protein.  

A close examination of the small intestine of the SIRPα CytO−/−  
mice confirmed the increased eosinophil degranulation, with markedly more MBP+ CD63+ granules and amorphous structures  
in the lamina propria than in the WT intestine (Fig. 4B). Some of  
the MBP+ CD63+ granules in the SIRPα CytO−/− lamina propria  
were found within the cytoplasm of other leukocytes, indicating  
that the eosinophils’ granular contents were released extracellularly  
and phagocytosed by other cells. Masson’s trichrome staining  
showed increased collagen deposition in the submucosa and  
smooth muscle hypertrophy (Fig. 4C). Taken together, these data  
indicated that, in the absence of SIRPα/CD172a signaling, in-  
testinal eosinophils had an increased tendency to degranulate,  
leading to reduced eosinophil viability and increased expression  
of the degranulation marker CD63. The lamina propria showed  
increased tissue remodeling under steady-state conditions.

CD47−/− mice show increased eosinophil degranulation and reduced numbers of lamina propria eosinophils  

Given that SIRPα/CD172a transmits negative signals upon ligation  
by the transmembrane glycoprotein CD47 (31), which is  
expressed in a number of cell types (32), including intestinal epithelial cells (33), we examined whether CD47−/− mice re-  
capitulate the changes observed in the SIRPα CytO−/− mice. In  
this experiment, because the only CD47−/− mice available were  
on the C57BL/6 background, we made the comparisons using  
SIRPα CytO−/− mice on the C57BL/6 background, instead of the  
BALB/c background. As shown in Fig. 5A, both CD47−/− and SIRPα CytO−/− mice showed a substantial reduction in the  
number of eosinophils and increased degranulation in the lamina  
propria compared with age- and sex-matched WT mice. In the  
spleen, the number of degranulating eosinophils was also in-  
creased in both the SIRPα CytO−/− and CD47−/− mice (Fig. 5B).  
These results are consistent with the hypothesis that a lack of  
signals provided by the SIRPα/CD172a–CD47 interaction leads to  
increased eosinophil degranulation and reduced eosinophil num-  
bers in the intestine.  

SIRPα/CD172a mutation negatively affects eosinophil survival  

We next asked whether eosinophils show decreased survival in the  
absence of SIRPα/CD172a signaling. The small intestinal lamina  
propria light-density cells from SIRPα CytO−/− mice showed a  
higher incidence of cell death, irrespective of the presence of IL-5,  
after 24 h of culture (Fig. 6A). In the absence of IL-5, SIRPα  
CytO−/− lamina propria eosinophils showed increased early  
(7AAD−Annexin V+ cells; 13% versus 9.8%) and late (7AAD+  
Annexin V+ cells; 50% versus 34%) apoptotic cell death com-  
pared with their WT counterparts. IL-5 showed little survival.
ROLE OF SIRPα/CD172a IN EOSINOPHIL HOMEOSTASIS

Effect on cultured lamina propria eosinophils and yielded only a small reduction in early (9.8% versus 6.7%) and late (50% versus 41%) apoptosis in WT and SIRPα Cyto−/− eosinophils, respectively (Fig. 6A, upper panels). These observations were also apparent when the absolute cell numbers were compared (Fig. 6A, lower panels). A similar tendency was observed after 48 h of culture (data not shown). Furthermore, the small intestinal eosinophils from SIRPα Cyto−/− mice showed increased Annexin V binding at the time of cell harvest compared with those from WT mice (total Annexin V+ eosinophils: 27.7% versus 13.1%) (Fig. 6B). Finally, MBP+ TUNEL+ apoptotic eosinophils were frequently observed in the small intestine of SIRPα Cyto−/− mice but not WT mice (Fig. 6C). These results demonstrated that eosinophil survival was compromised in the absence of SIRPα/CD172a signaling, both in vitro and in vivo.

Reduced survival of eosinophils in the absence of SIRPα/CD172a signaling in inflammation

We next asked whether the absence of appropriate SIRPα/CD172a signaling would affect eosinophil homeostasis in inflammation. To this end, we injected IL-25 i.p. into WT and SIRPα Cyto−/− mice to induce a Th2-type inflammatory response (30, 34). The WT mice showed a significant increase in eosinophils in the small intestinal lamina propria after 3 d of daily IL-25 administration, and a substantial proportion of the eosinophils was degranulating in the villus (Fig. 7A). In contrast, the SIRPα Cyto−/− mice showed a much lower frequency of lamina propria eosinophils but a marked increase in MBP+ CD63+ amorphous material outside the intestinal villi (Fig. 7A). These observations indicated that, in the absence of SIRPα/CD172a signaling, the eosinophils rapidly degranulated, and their cytoplasmic contents were released into the lumen in the inflamed intestine.

Corroborating these observations, marked eosinophil infiltration was seen in the peritoneal cavity in both the WT and SIRPα Cyto−/− mice upon IL-25 administration (Fig. 7B). The SIRPα Cyto−/− mice showed a prominent reduction in Siglec F expression and a concomitant increase in Annexin V-binding cells in the R1, but not the R2, fraction (Fig. 7B), indicating an increase in dying eosinophils. Collectively, these results indicated that SIRPα/CD172a signaling significantly contributes to eosinophil survival in inflammation.

Discussion

In this study, through the generation and use of two novel mAbs, we identified an inhibitory receptor, SIRPα/CD172a (35), and the tetraspanin CD63 (36) as proteins expressed on the surface of small intestinal lamina propria eosinophils. We also showed that SIRPα/CD172a regulates eosinophil homeostasis by inhibiting degranulation, a mechanism that seems to increase eosinophil survival under both physiological and inflammatory conditions.

SIRPα/CD172a (also known as SHPS-1, P84, or BIT), possesses two ITIM motifs in its cytoplasmic domain that can transmit negative signals in various cellular events, including proliferation (35, 37), cytoskeletal reorganization and cell motility (38), and phagocytosis and oxidative burst (25, 39, 40). In the current study, we found that cross-linking SIRPα/CD172a on lamina propria eosinophils significantly inhibited calcium granules and amorphous structures shown in Figs. 4 and 5 were present but not readily visible without CD63 staining. Scale bars, 100 μm (upper panels) and 40 μm (lower panels). Data are from one experiment representative of two.
ionophore-induced EPO release (Fig. 3). Although this inhibition was modest, it was consistently observed in both intestinal eosinophils and BM-derived eosinophils from normal mice but not in those from SIRPα/CD172α−/− mice expressing a mutant SIRPα/CD172α that lacks most of its cytoplasmic domain, consistent with the notion that SIRPα/CD172α inhibits eosinophil degranulation via its cytoplasmic domain. The lower expression of the SIRPα/CD172α protein in SIRPα/CD172α−/− eosinophils might have contributed to the lack of inhibition of EPO release upon cross-linking. However, the finding that SIRPα/CD172α−/− eosinophils consistently showed a higher tendency to degranulate, even in the absence of cross-linking, argued against this possibility and instead indicated that they are more prone to degranulate than are their WT counterparts.

There are at least three possible explanations for the modest effect of the SIRPα/CD172α cross-linking. First, the SIRPα/CD172α-mediated degranulation inhibition seems to represent just one arm of multiple inhibitory pathways. As shown in Fig. 3A, the cross-linking of two other inhibitory receptors, Siglec F (41) and PIR-B, yielded a comparable or smaller degranulation inhibition, and the additional cross-linking of SIRPα/CD172α tended to increase the inhibition. Furthermore, other inhibitory receptors have been reported on the surface of eosinophils (42). Second, mouse eosinophils are much less susceptible to degranulation induction than are their human counterparts in vitro and in vivo (43). Third, eosinophils represented no more than one third of the light-density cells present in the membrane of intracellular crystalloid granules and appears on the cell surface during the piecemeal degranulation of human eosinophils (17, 44). In addition, the SIRPα/CD172α−/− mice showed an increased frequency of extracellular MBP+CD63+ amorphous materials in the lamina propria, increased collagen deposition in the submucosa, and smooth muscle hypertrophy in the jejunum, which are characteristic features of the increased tissue remodeling induced by eosinophil degranulation (45). Furthermore, the CD47−/− mice showed reduced numbers of lamina propria eosinophils and frequent MBP+CD63+ extracellular granules. Although MBP+CD63+ amorphous structures were not found in the lamina propria of the CD47−/− mice, they were observed in the spleen of these mice, where eosinophil degranulation was more prominent. These results provide strong support for the inhibitory role of SIRPα/CD172α in eosinophil degranulation.

Despite the increased eosinophil degranulation and tissue remodeling in the small intestine of the SIRPα/CD172α−/− mice, we did not find significant inflammation or intestinal tissue damage resulting from the release of eosinophil granule proteins. This observation might be explained, in part, by a species difference, because mouse models of human asthma show only mild eosinophil degranulation (46). However, extensive eosinophil degranulation and pulmonary pathologies could be induced in double-transgenic mice expressing IL-5 and eotaxin-2 coordinately by mature T cells and lung epithelial cells, respectively (47), implying that in vivo eosinophil effector functions mediated by degranulation might involve multiple costimulatory receptor–ligand interactions. In addition, previous studies in man indicated that eosinophil granules are secreted extracellularly as intact membrane-bound structures and that external stimulation is required to elicit...
secretion from the granules (14, 48). The small intestine may lack the endogenous activating signal necessary for the ejection of toxic granular contents from the released MBP\(^+\)CD63\(^+\) granules, preventing any obvious tissue damage. In addition, given that some MBP\(^+\)CD63\(^+\) granules were found within the cytoplasm of other types of cells, a substantial proportion of the released MBP\(^+\)CD63\(^+\) granules might have been rapidly taken up by phagocytes before they ejected toxic materials. Although it has been widely believed that eosinophil degranulation leads directly to tissue damage, these data collectively support the idea that, under certain conditions, eosinophil degranulation leads to tissue remodeling in the absence of noticeable inflammatory cell infiltration and the resulting tissue damage (43).

Eosinophils are terminally differentiated leukocytes that are believed to possess a limited ability to survive in tissues in the absence of survival-promoting cytokine signals (49), and cytokine signaling through the common \(\gamma\)-chain is reported to increase the survival of intestinal eosinophils (21). Although it is possible that inhibitory receptors inhibit these survival signals in eosinophils under certain situations (22), our results indicated that SIRP\(^\alpha\)/CD172a promotes eosinophil survival instead, because in vitro-cultured SIRP\(^\alpha\)/CD172a effector cells showed reduced viability compared with WT eosinophils, and SIRP\(^\alpha\)/CD172a effector cells showed reduced intestinal eosinophil recruitment and eosinophil survival signals with increased Annexin V binding or TUNEL\(^+\) staining (Fig. 6). Although IL-5 treatment seemed to have little effect on the survival of both WT and SIRP\(^\alpha\)/CD172a lamina propria eosinophils (Fig. 6a), this was probably due to the short time (24 h) of cell culture. Significant survival effects of IL-5 on WT lamina propria eosinophils were apparent when the cells were cultured for an extended period of time (48–72 h; data not shown). Although it remains to be analyzed whether SIRP\(^\alpha\)/CD172a lamina propria eosinophils respond favorably to the pro-survival signals of IL-5 in culture, technical difficulties with the isolation of sufficient numbers of viable SIRP\(^\alpha\)/CD172a eosinophils have impeded these experiments.

The reduced eosinophil survival was also obvious when inflammation was induced in SIRP\(^\alpha\)/CD172a mice by IL-25 administration. Taken together, these findings suggested that the absence of appropriate negative signaling via the cytoplasmic region of SIRP\(^\alpha\)/CD172a accelerated the rate of eosinophil cell death in vivo under steady-state or pathological conditions, probably because of their accelerated degranulation, and we excluded the possibility that this phenomenon was induced during the isolation process. Moreover, given that CD47, which binds SIRP\(^\alpha\)/CD172a to induce negative signals, is expressed in the basal aspect of intestinal epithelial cells, and that CD47\(^-/-\) mice showed changes in the intestinal eosinophils that were very similar to those observed in the SIRP\(^\alpha\)/CD172a mice, the interaction of eosinophil SIRP\(^\alpha\)/CD172a and epithelial cell CD47 is likely to be involved in the regulation of eosinophil survival. A recent study indicated that both SIRP\(^\alpha\)/CD172a and CD47 are also important for dendritic cell homeostasis (50). In addition, not excluding the above-mentioned possibility, the absence of inhibitory signals provided by SIRP\(^\alpha\)/CD172a may also render eosinophils susceptible to death signals following the ligation of Fas (51) or of other receptors, such as Siglec F (52). The regulatory mechanism of eosinophil survival warrants further investigation.

A previous study indicated that human polymorphonuclear cell migration is regulated by CD47 through SIRP\(^\alpha\)/CD172a-dependent and -independent mechanisms (53). Abs against SIRP\(^\alpha\)/CD172a partially inhibited neutrophil migration across both human epithelial monolayers and collagen-coated filters, whereas Abs against CD47 inhibited neutrophil migration almost completely (53). In addition, mouse neutrophils deficient in CD47 showed reduced migration into the inflamed peritoneal cavity (40). However, in the current study, we found no evidence that SIRP\(^\alpha\)/CD172a regulates eosinophil migration. First, SIRP\(^\alpha\)/CD172a eosinophils efficiently migrated toward CCL24/eotaxin-2, as did WT eosinophils in vitro (data not shown). Second, although there was a slight delay in eosinophil recruitment into the peritoneal cavity in SIRP\(^\alpha\)/CD172a mice at 24 and 48 h after IL-25 injection, there was no such a delay at 72 h after inflammation induction. Instead, the only prominent finding at 72 h was the reduced viability of the infiltrated eosinophils (Fig. 7). Hence, our data do not support the role of SIRP\(^\alpha\)/CD172a in eosinophil migration regulation but rather support its role in eosinophil degranulation and cell survival.

In summary, we identified an inhibitory receptor, SIRP\(^\alpha\)/CD172a, and tetraspanin CD63 as proteins that are expressed by small intestinal lamina propria eosinophils. SIRP\(^\alpha\)/CD172a seems to play a role in inhibiting eosinophil degranulation, which leads to the prolongation of tissue-dwelling eosinophil survival. Given its prominent expression in human myeloid cells, including granulocytes (54), SIRP\(^\alpha\)/CD172a is also likely to be expressed in human eosinophils. If so, SIRP\(^\alpha\)/CD172a should be an interesting therapeutic target for eosinophil-associated diseases through the inhibition of eosinophil degranulation.

Acknowledgments
We thank Dr. Per-Arne Oldenborg for providing the CD47\(^-/-\) mice and the members of Dr. Masaaki Murakami’s laboratory for invaluable assistance with the LC/MS.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Fig. 1. The MY-1 mAb recognizes SIRPα/CD172a, whereas the NVG-2 mAb recognizes a tetraspanin CD63. A. Left panels: Western blot analysis of total cell lysates derived from small intestinal lamina propria with the mAbs MY-1 and NVG-2. Right panels: Silver staining of the materials immunoprecipitated with MY-1 or NVG-2 (major immunoreactive products are indicated by red circles). B. The MY-1 mAb binds to SIRPα/CD172a. CHO-Ras cells stably expressing mouse SIRPα/CD172a and the parental cells were fixed and stained with unlabeled MY-1 or P84 mAb (a commercial anti-SIRPα/CD172a). Subsequently, Alexa 488-conjugated anti-rat IgG secondary antibody or Rhodamine-phalloidin was added, and the cells were analyzed by fluorescence microscopy. C. Flow cytometry of the CHO-Ras cells stably expressing mouse SIRPα/CD172a. Cells were stained with unlabeled MY-1, P84, or rat IgG2a isotype control antibody (shaded histograms) followed by PE-conjugated anti-rat IgG. The mean fluorescent intensity (MFI) for MY-1 and P84 was 644 and 536, respectively. D. The NVG-2 mAb binds to CD63. Flow cytometry of the COS-7 cells expressing mouse CD63 antigen on the cell surface. Transfected and control (untransfected) COS-7 cells were stained with unlabeled anti-CD63 (MBL) or NVG-2 mAb followed by FITC-conjugated goat anti-rat IgG or rat IgG isotype control antibody (shaded histograms).