The Journal of Immunology

The Inhibitory Receptor IRp60 (CD300a) Is Expressed and Functional on Human Mast Cells

This information is current as of March 18, 2019.

Ido Bachelet, Ariel Munitz, Alessandro Moretta, Lorenzo Moretta and Francesca Levi-Schaffer

References   This article **cites 42 articles**, 18 of which you can access for free at:
http://www.jimmunol.org/content/175/12/7989.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription   Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions   Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts   Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Inhibitory Receptor IRp60 (CD300a) Is Expressed and Functional on Human Mast Cells[1]

Ido Bachelet,[2]* Ariel Munitz,[2]* Alessandro Moretta,[†] Lorenzo Moretta,[‡] and Francesca Levi-Schaffer[3]*

Mast cell-mediated responses are likely to be regulated by the cross talk between activatory and inhibitory signals. We have screened human cord blood mast cells for recently characterized inhibitory receptors expressed on NK cells. We found that IRp60, an Ig superfamily member, is expressed on human mast cells. On NK cells, IRp60 cross-linking leads to the inhibition of cytotoxic activity vs target cells in vitro. IRp60 is constitutively expressed on mast cells but is down-regulated in vitro by the eosinophil proteins major basic protein and eosinophil-derived neurotoxin. An immune complex-mediated cross-linking of IRp60 led to inhibition of IgE-induced degranulation and stem cell factor-mediated survival via a mechanism involving tyrosine phosphorylation, phosphatase recruitment, and termination of cellular calcium influx. To evaluate the role of IRp60 in regulation of allergic responses in vivo, a murine model of allergic peritonitis was used in which the murine homolog of IRp60, LMIR1, was neutralized in BALB/c mice by mAbs. This neutralization led to a significantly augmented release of inflammatory mediators and eosinophilic infiltration. These data demonstrate a novel pathway for the regulation of human mast cell function and allergic responses, indicating IRp60 as a candidate target for future treatment of allergic and mast cell-associated diseases. *The Journal of Immunology,* 2005, 175: 7989–7995.

The tissue-dwelling mast cells have key roles in allergic reactions, innate immunity, inflammatory diseases, fibrosis, and autoimmunity. They arise from circulating precursors that enter the tissues, where they undergo final maturation and begin expressing the high-affinity receptor for IgE, FcεRI, the central trigger for mast cell activation in allergy. Following allergen-induced aggregation of FcεRI, the mast cells degranulate and secrete an array of preformed and newly synthesized mediators and, later on, produce and secrete additional cytokines. Mast cells can also be activated by IgE-independent stimuli, such as anaphylatoxins, neuropeptides, and eosinophil basic proteins. A main factor regulating mast cell homeostasis in humans is stem cell factor (SCF).[4] Through its tyrosine kinase receptor c-*kit* (CD117), SCF plays central roles in mast cell differentiation (1), survival (2, 3), activation (4, 5), and chemotaxis (6).

The responses coordinated by mast cells are tuned through a balance between activating and inhibitory signals. Over the last few years, it has become clear that mast cell activation can be regulated by various inhibitory receptors that are expressed and functional on the surface of murine and human mast cells (7). The functional cores of immune inhibitory receptors are specialized modules in their intracellular domain, termed ITIMs (8). Upon ligand-binding or immune complex-mediated cross-linking of the receptor, these modules undergo tyrosine phosphorylation and recruit Src homology 2-bearing phosphatases, such as Src homology 2 domain-containing phosphatase (SHP)-1/2 and the inositol phosphatase SHIP (9). These phosphatases act by dephosphorylating the docking sites for activating kinases such as Syk, or alternatively degrading phosphatidylinositides produced by phosphoinositide 3′-kinase (10), actively shutting these signals off and halting the activating cascade.

Among the known mast cell inhibitory receptors are FcγRIIB (11), gp49B1 (12), myeloid-associated Ig-like receptors 1/2 (13), the mast cell function-associated Ag (14), paired Ig-like receptor A/B (15), and CD200R (16). Of these, FcγRIIB has been extensively studied on human mast cells (17), and attempts to target it have already been made for therapeutic mast cell inhibition (18, 19). Thus, characterizing additional inhibitory receptors on human mast cells is required for a better understanding of the signals involved in mast cell regulation, as well as for defining new immunopharmacological targets for treatment of allergic diseases. Therefore, we aimed to investigate the presence and function of a series of inhibitory receptors belonging to several receptor families (Ig superfamily, lectin type). In the present study, we focus on IRp60 (CD300a), an Ig superfamily receptor that is expressed on various cells like T cells, NK cells, and neutrophils (20). Ab-mediated cross-linking of IRp60 on NK cells results in down-regulation of NK cytolytic activity in vitro.

In this study, we report that IRp60 is expressed on human mast cells and that its cross-linking inhibits IgE-induced degranulation and SCF-mediated survival through a mechanism involving tyrosine phosphorylation and phosphatase recruitment. Neutralization of the murine homolog of IRp60, LMIR1, leads to an augmented response

to allergen challenge. Altogether, this study demonstrates a novel pathway for the regulation of human mast cell function and of the allergic response.

Materials and Methods

Abs and reagents

All of the cell culture media, reagents, and buffers were purchased from Biological Industries. SCF is a kind gift from Amgen (Thousand Oaks, CA). The following mAbs recognizing various inhibitory receptors were produced as described (20): P192 and E59 (mouse monoclonal anti-IRp60; isotypes are mouse IgG2b and IgG1, respectively), Z270 (mouse anti-NKG2A), XA185 (mouse anti-CD94), 11PB6 (mouse anti-p58.1), GL183 (mouse anti-p58.2), Z27 (mouse anti-p70), Q66 (mouse anti-p140), AZ158 (mouse anti-p70/p140), F278 (mouse anti-LIR1(ILT2)). The Ab recognizing LIR3(ILT5) (15F3) was kindly provided by Dr. M. Colonna (Washington University, St. Louis, MO). The following Abs and reagents were purchased as indicated: rat anti-mouse LMIR1 (clone 224) and FITC-conjugated rat anti-mouse CCR3 from R&D Systems; anti-human CD117 Ab for human lung mast cell purification from BD Pharmingen; PE-conjugated anti-human FcεRI (clone AER-37) from eBioscience; anti-human tryptase (clone AA1) and isotype control (IgG1 and IgG2A) Abs from DakoCytomation; sheep anti-mouse F(ab′)₂ from ICN Biomedicals. Chimeric murine/human IgE anti-NP Ab from Serotec; goat anti-mouse λ-chain-specific Ab from Southern Biotech; polyclonal anti-human phosphotyrosine (pY99), SHP-1/2, and SHIP Abs from Santa Cruz; anti-human phospho-syk from Cell Signaling; HRP-conjugated anti-rabbit and anti-mouse, FITC-, Cy⁵-, and PE-conjugated secondary Abs from Jackson ImmunoResearch; chromogenic substrates from Sigma-Aldrich; Calcium Green-1AM was purchased from Molecular Probes; Ficoll-Paque from Amersham Biosciences. All other reagents were from Sigma-Aldrich, unless otherwise stated, and were of best chemical grade available.

Mast cells

Human cord blood mast cells (CBMC) were obtained by culturing umbilical cord blood mononuclear cells as described previously (21). Briefly, fresh cord blood was diluted with Hank's solution, loaded on Ficoll-Paque, and centrifuged ($350 \times g$, 25 min). Mononuclear cells were washed twice with Hank's and resuspended with 100 ml of MEM-Alpha containing 10% v/v heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), ribonucleosides/deoxyribonucleosides, SCF (100 ng/ml), IL-6 (10 ng/ml) (PeproTech), and PGE_2 (0.3 μM) (Sigma-Aldrich). The culture medium was replaced weekly. CBMC were used after 8–12 wk of culture, when >97% were positive for tryptase as assessed by intracellular flow cytometry (FACS; see below). Human lung mast cells (HLMC) were purified from healthy-looking lung specimens surgically removed from lung cancer patients undergoing lobectomy, using proteolysis, gradient and positive magnetic selection as described (21). Nasal polyps underwent proteolysis using the same protocol described for lungs, and mast cells (NPMC) were detected in FACS by gating of FcεRI⁺ cells. Murine bone marrow-derived mast cells (BMMC) were kindly provided by Dr. E. Razin (The Hebrew University of Jerusalem). Human tissues and cord blood were obtained according to the Institutional Helsinki Committee guidelines, and their use was approved by the Committee.

Flow cytometry (FACS)

FACS analysis was performed using BD Biosciences FACSCalibur and CellQuest software. All stages were performed in a round-bottom 96-well culture plate (Nunc) in a volume of 100 μl. Cells (~10^5/sample) were washed with ice-cold HBA buffer (Hank's solution containing BSA (0.1% w/v) and NaN₃ (0.01% w/v)), and then incubated with either anti-IRp60 or the appropriate isotype control (4°C, 30 min) followed by two washes with cold HBA. The cells were then incubated with secondary Abs, FITC- or Cy⁵-conjugated anti-mouse at the recommended dilutions (4°C, 30 min) followed by two additional washes, and analyzed immediately by FACS. Intracellular FACS was performed essentially the same, except for the two stages before the addition of the primary Ab. In this case, the cells were first fixed in 2% formaldehyde (4°C, 10 min) and blocked with HBA containing BSA (10% w/v), goat serum (0.1% v/v), saponin (0.1% w/v), and HEPES (10 mM) (4°C, 10 min). HBA containing saponin (0.1% w/v) and HEPES (10 mM) was also used for incubation and washing.

Mast cell activation and inhibition

Five days before activation, CBMC (7.5×10^4 per 200 μl) were incubated with chimeric murine/human IgE anti-NP Ab (5 μg/ml). For activation, an Immunolon-2HB 96-well plate (ThermoLabsystems) was incubated with

or without sheep anti-mouse F(ab′)₂ (25 μg/ml in PBS) (37°C, 4 h), followed by two washes with PBS. Next, the plate was incubated with either anti-IRp60 (2–5 μg/ml), or isotype control or PBS (37°C, 4 h) and washed twice with PBS. On the day of activation, cells (10^5) were washed twice in warm Tyrode's gelatin-calcium buffer (137 mM NaCl, 12 mM NaHCO₃, 5.5 mM L-glucose, 2 mM KCl, 0.3 mM Na₂HPO₄, 0.1% w/v gelatin, 1.8 mM CaCl₂, 0.9 mM MgCl₂). The cells were then blocked in human serum 10% v/v in MEM-Alpha (10 min on ice) and washed, transferred to the coated plate (7.5×10^4 per 100 μl), and either anti-mouse λ-chain-specific Ab (5 μg/ml) or compound 48/80 (15 μg/ml) were added, and the cells were incubated for 30 min at 37°C. Then, the cells were centrifuged ($1500 \times g$, 1 min), separated from the supernatant, and lysed by three freeze/thaw cycles. Supernatant and lysate were recovered and stored at −80°C until assessed for the amounts of mediators released.

Mediator release assays

β-Hexosaminidase and tryptase were measured by chromogenic assays as described (22, 23) with slight modifications. For β-hexosaminidase, 18 μl of sample (supernatant or cell lysate) were mixed with 42 μl of substrate solution (8 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide in 48 mM citric acid and 56 mM Na₂HPO₄ (pH 4.5)) and incubated for 2 h at 37°C. The reaction was stopped by addition of 120 μl of ice-cold glycine (0.2 M) (pH 10.7), and the OD was immediately read in a standard spectrophotometer at 410 nm absorbance. For tryptase, 48 μl of sample were mixed with 2 μl of substrate solution (25 mM N-p-tosyl-gly-pro-lys-p-nitroanilide in 100% DMSO) and incubated at 37°C until chromogenesis and immediately read as above. Samples in which counts exceeded the reliable detection OD range were diluted and reread. Percent release was calculated as follows: $\%R = 100 \times \text{supernatant}/(\text{lysate} + \text{supernatant})$. IL-4 release was measured by a commercial ELISA kit (Diaclone). Eotaxin-2 content was measured by a commercial ELISA kit (Duoset; R&D Systems).

Survival assay

CBMC (10^5) were washed with MEM-Alpha without growth factors and incubated in 200 μl MEM-Alpha with or without SCF (100 ng/ml) in a culture plate coated as described above. At 24 and 48 h, all samples were stained by addition of 5 μl of propidium iodide solution in PBS (10% v/v) and immediately analyzed by FACS.

Modulation of receptor expression

CBMC (2×10^5 per 200 μl) were cultured in the presence of 10–100 ng/ml (ranging between ~1 and 15 nM) of either of the following proteins: eosinophil major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), or poly-L-arginine (15,000–70,000 kDa; Sigma-Aldrich; at 1 ng/ml, equal to 14–66 mM) for 12, 24, and 48 h at 37°C. At these time points, IRp60 expression and viability were assessed by FACS using anti-IRp60/propidium iodide double staining.

Intracellular Ca²⁺ mobilization

Before loading with calcium sensor, 3×10^5 IgE-sensitized CBMC were blocked with 10% v/v human serum in MEM-Alpha (10 min on ice). Later, IRp60 was cross-linked by incubation of the CBMC with anti-IRp60 or isotype control Ab (5 μg/ml, 30 min on ice). Subsequently, cells were washed and incubated with sheep anti-mouse F(ab′)₂ (25 μg/ml, 30 min on ice) in MEM-Alpha. The cells were loaded with Calcium Green-1AM (5 μM; 45 min, 37°C) in MEM-Alpha (FCS, 2% v/v), washed, and resuspended in 300 μl of Tyrode's gelatin-calcium buffer (prewarmed to 37°C). The cells were allowed to flow freely in the cytometer for 40 s, at which time anti-IgE (5 μg/ml) was added. Changes in FL-1 geo mean were recorded for a total of 5 min.

Immunoprecipitation and Western blot

CBMC (5×10^5) were lysed using a commercial lysis buffer (Pierce), run on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Pierce) and blotted vs IRp60 (anti-IRp60; 1 μg/ml). For IRp60 precipitation, CBMC (8×10^6) were treated with sodium orthovanadate (4 mM; 10 min, 37°C) or incubated with an immune complex (25 μg/ml sheep anti-mouse, 5 μg/ml anti-human IRp60, or isotype control Ab) for various time periods (0, 15, 30, 60, and 120 s). IRp60 was precipitated from CBMC using a commercial kit (Seize Classic Mammalian kit; Pierce) according to the manufacturer's instructions. The samples were run as described and blotted vs phosphotyrosine (pY99), SHP-1/2, and SHIP. For detection, HRP-conjugated anti-mouse or anti-rabbit Abs were used as recommended by the manufacturer.

Murine allergic peritonitis model

Allergic peritonitis was induced in 8- to 10-wk-old female BALB/c mice by OVA essentially as described (24). On days 0 and 7, mice were sensitized intradermally with 100 μg of OVA adsorbed on 1.6 mg of alum hydroxide in 300 μl of saline. On day 11, 30 min before allergen challenge (30 μg of OVA in 300 μl of saline i.p.), a group of mice were injected i.p. with anti-LMIR1 neutralizing Ab (5 μg/mouse). Mice were sacrificed either 45 min (for tryptase) or 24 h (for eotaxin-2 and cell counts) later. At these time points, the peritoneal cavity was washed with 5 ml of Tyrode's gelatin buffer. The recovered (4 ml) peritoneal lavage fluid was centrifuged (150 × g, 5 min), and cell pellets were resuspended in 2 ml of Tyrode's gelatin buffer for mediator analysis and total cell counts. Peritoneal lavage cells were counted under a light microscope in a hemocytometer following trypan blue staining. Eosinophils were analyzed by FACS gating SSC^{high}-CCR3$^+$. Eosinophil numbers in the lavage fluid were thereafter calculated by multiplying the total cell counts with the percent population gated as eosinophils. All experimental protocols were approved by the Animal Experimentation Committee of The Hebrew University of Jerusalem.

Statistical analysis

Activation, survival, and mediator release assays were performed in triplicate or quadruplicate. In vitro mast cell experiments were repeated from at least three different donors. Data are expressed as mean ± SD. Data were analyzed by ANOVA, followed by paired Student's *t* test (assuming equal variances).

Results

Human mast cells express IRp60

To investigate the expression pattern of inhibitory receptors on human mast cells, CBMC were incubated with a large panel of mAbs recognizing various inhibitory receptors characterized on NK cells. As shown in Fig. 1*a*, FACS analysis revealed that CBMC express high levels of IRp60, but not LIR1(ILT2), LIR3(ILT5), p58.1, p58.2, p70, p140, or NKG2A/CD94. To evaluate whether tissue mast cells express IRp60, HLMC and NPMC were stained for IRp60. As shown in Fig. 1*b*, HLMC and NPMC displayed significant levels of IRp60.

Eosinophil MBP and EDN down-regulate IRp60 expression on CBMC

We next investigated the capability of eosinophil-derived cytotoxic proteins, found in the allergic inflammatory milieu, to modulate IRp60 expression on CBMC. For this, CBMC were cultured with subactivating concentrations (1–10 ng/ml) of eosinophil MBP, ECP, EDN, EPO, and of poly-L-arginine at an equimolar concentration. As shown in Fig. 2, MBP and EDN selectively induced a decrease in the expression level of IRp60 in a dose-response manner (MFI of 9.12 ± 2.27 vs 2.69 ± 0.24 and 2.22 ± 0.25 for MBP at 10 and 100 ng/ml, respectively; or vs 3.03 ± 0.68 and 2.79 ± 0.71 for EDN at 10 and 100 ng/ml, respectively; *p* < 0.05 for all of the cases).

IRp60 cross-linking inhibits IgE-dependent mediator release from CBMC

Based on the observed inhibitory effect of IRp60 on NK-mediated cytotoxicity, we hypothesized that IRp60 inhibits CBMC activity as well. To induce coaggregation of IRp60 with FcεRI (as described for other inhibitory receptors) (11), we used chimeric IgE for sensitization and an anti-mouse cross-linking Ab. CBMC were sensitized with IgE and then triggered to degranulate by an anti-IgE Ab or by compound 48/80 in an anti-IRp60-coated plate. As shown in Fig. 3, *a* and *b*, cross-linked IRp60 strongly and significantly inhibited IgE-mediated release of β-hexosaminidase and tryptase (degranulation inhibited by 86.04% with anti-IRp60; *p* < 0.005) and IL-4 (release inhibited by 99.78% with anti-IRp60; *p* < 0.005). Interestingly, IRp60 did not inhibit compound 48/80-me-
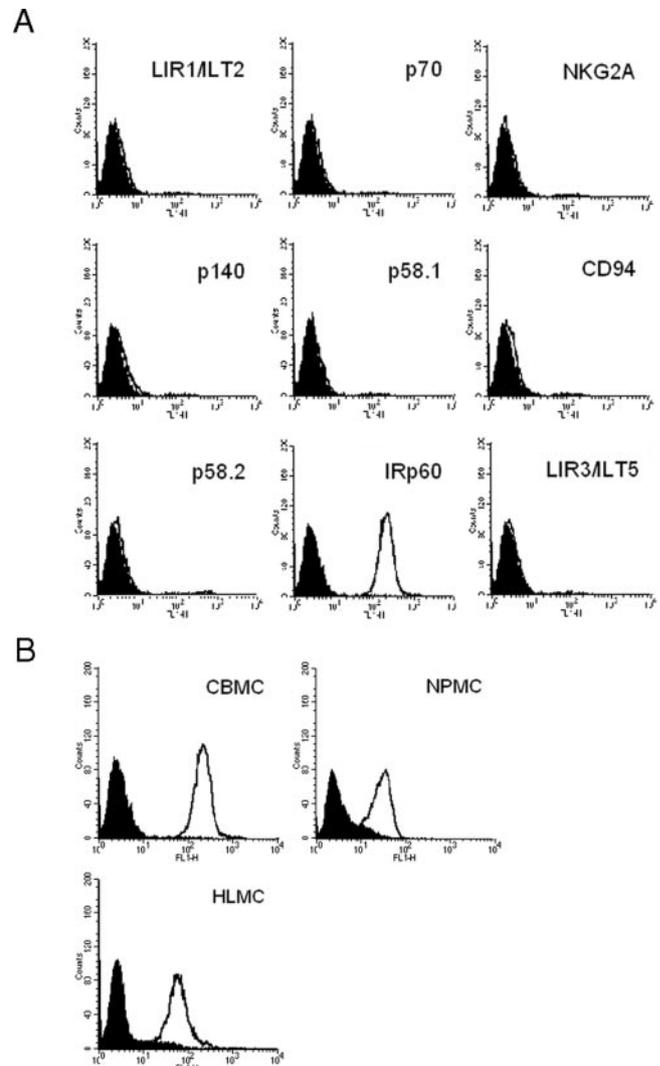

FIGURE 1. Human mast cells express IRp60. *a*, FACS analysis of NK cell inhibitory receptors was performed on CBMC using a panel of mAbs. All Abs are monoclonal mouse anti-human, followed by FITC-conjugated anti-mouse. Representative of *n* = 3. *b*, FACS analysis of IRp60 expression on CBMC, human lung and nasal polyp mast cells. Black peaks represent controls. White peaks represent anti-IRp60 staining. Representative of 10 (CBMC) and 3 (HLMC and NPMC) experiments.

diated release of either β-hexosaminidase (as shown in Fig. 3*c*), tryptase, or IL-4 (data not shown).

IRp60 cross-linking inhibits SCF-mediated CBMC survival

SCF is the most important survival factor for human mast cells. SCF signals upon binding to c-*kit* and initiating a signaling cascade that involves phosphatidylinositol-3′ kinase, protein kinase B, and syk. To examine the ability of IRp60 to interfere with c-*kit* signaling, CBMC were cultured in the presence or absence of SCF (100 ng/ml) in an anti-IRp60-coated plate, followed by FACS analysis of propidium iodide (PI)-positive cells. As shown in Fig. 4, IRp60 cross-linking significantly inhibited mast cell survival at 24 h (5.5-fold increase in number of PI$^+$ cells with anti-IRp60; *p* < 0.005) and at 48 h (10.3-fold increase in PI$^+$ cells with anti-IRp60; *p* < 0.005).

IRp60 cross-linking inhibits IgE-induced [Ca^{2+}] influx

One of the initial steps in FcεRI-dependent activation of CBMC is intracellular calcium influx due to phosphatidylinositide formation
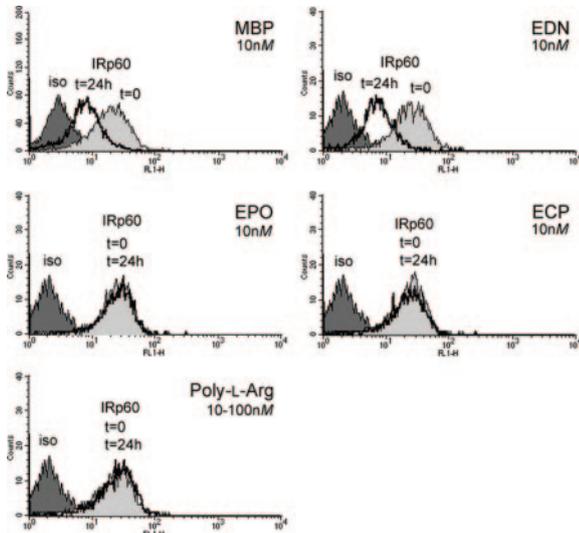
FIGURE 2. Eosinophil-derived MBP and EDN induce down-regulation of IRp60 expression on CBMC. CBMC were incubated with either eosinophil MBP, ECP, EDN, EPO (1–10 ng/ml), or poly-L-arginine for 24 and 48 h, followed by staining with anti-IRp60 and analyzed by FACS. Dark gray peaks represent controls. Light gray peaks represent IRp60 levels without eosinophil mediators. White peaks represent IRp60 levels following incubation with eosinophil mediators. Representative of three experiments.

and store-operated channel opening. Therefore, the effect of IRp60 cross-linking on calcium influx was examined using the fluorescent sensor Calcium Green-1AM. As shown in Fig. 5 within
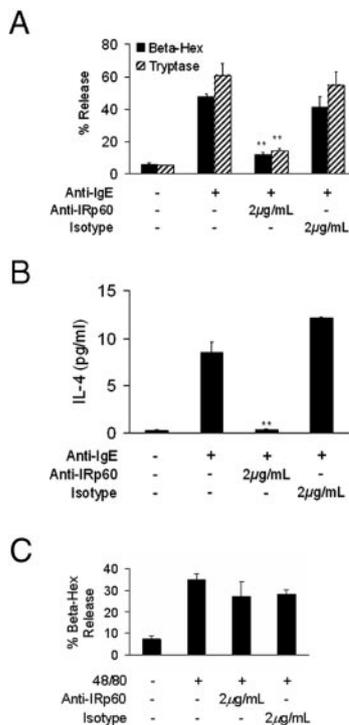

FIGURE 3. The cross-linking of IRp60 inhibits IgE-dependent, but not IgE-independent, CBMC degranulation. CBMC were sensitized with IgE for 5 days and activated with anti-IgE Ab for 30 min in the presence of anti-IRp60 or isotype control Abs. The release levels of β-hexosaminidase and tryptase (*A*) were evaluated by enzymatic-chromogenic assays and expressed as percent release. IL-4 release (*B*) was evaluated by ELISA, and expressed in picograms per milliliter ($n > 5$; **, $p < 0.005$). *C*, IgE-independent activation is not inhibited by IRp60 ($n > 3$).
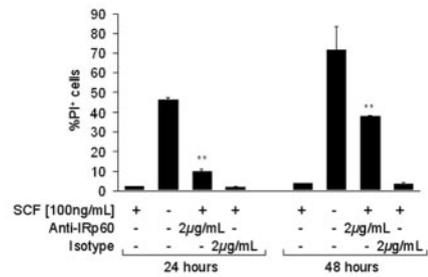

FIGURE 4. The cross-linking of IRp60 inhibits SCF-induced CBMC survival. CBMC were incubated with or without SCF (100 ng/ml) in the presence of anti-IRp60 or isotype control Abs for 24 and 48 h, stained for PI, and analyzed by FACS ($n = 3$; **, $p < 0.005$)

10–20 s of anti-IgE addition, a marked $[Ca^{2+}]$ increase was observed. This increase was completely abolished in response to IRp60 cross-linking compared with the isotype control.

IRp60 undergoes tyrosine phosphorylation, recruits SHP-1 and SHIP, and induces syk dephosphorylation

In NK cells, IRp60 has been shown to mediate its inhibitory effect via recruitment of SHP-1 and SHP-2 (20). To establish the mechanism of the inhibitory activity of IRp60 on human mast cells, CBMC were treated with sodium orthovanadate or alternatively cross-linked, precipitated, and blotted for phosphotyrosine, SHP-1 and -2, and SHIP. Upon orthovanadate pretreatment, IRp60 underwent tyrosine phosphorylation (Fig. 6*a*). In addition, IRp60 coprecipitated with SHP-1 and SHIP, but not with SHP-2. Upon Ab-driven cross-linking (Fig. 6*b*), IRp60 underwent tyrosine phosphorylation and precipitated with SHIP, but not SHP-1. Moreover, by intracellular FACS analysis of phosphorylated syk, it was detected that IRp60 cross-linking with immune complex, but not the isotype control, caused a rapid dephosphorylation of syk (Fig. 6*c*).

The murine homolog of IRp60 regulates mast cells activation and consequent inflammatory response in an allergic-peritonitis mouse model

Bioinformatic analysis that included sequence homology comparison revealed a murine homolog for IRp60. This receptor has been cloned, characterized, and termed LMIR1 (25). We reconfirmed that murine mast cells from both bone marrow and peritoneum express LMIR1. Furthermore, LMIR1 cross-linking specifically inhibited β-hexosaminidase release from IgE-activated BMMC in
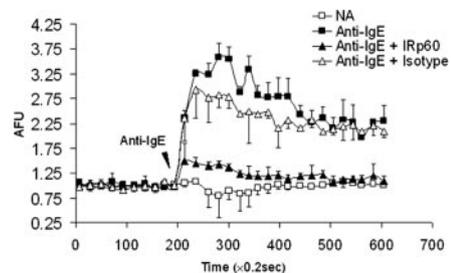
FIGURE 5. The cross-linking of IRp60 inhibits IgE-induced (Ca^{2+}) influx. CBMC were sensitized with IgE for 5 days, IRp60 on CBMC was cross-linked by incubation with immune complex (5 μg/ml anti-IRp60, followed by 25 μg/ml sheep anti-mouse), and the cells were loaded with Calcium Green-1AM. Forty seconds after initiation of flow cytometry, anti-IgE was added. $[Ca^{2+}]$ is expressed as arbitrary fluorescence units (AFU), representing fold change in mean fluorescence intensity over basal level. NA, Not activated ($n = 4$).

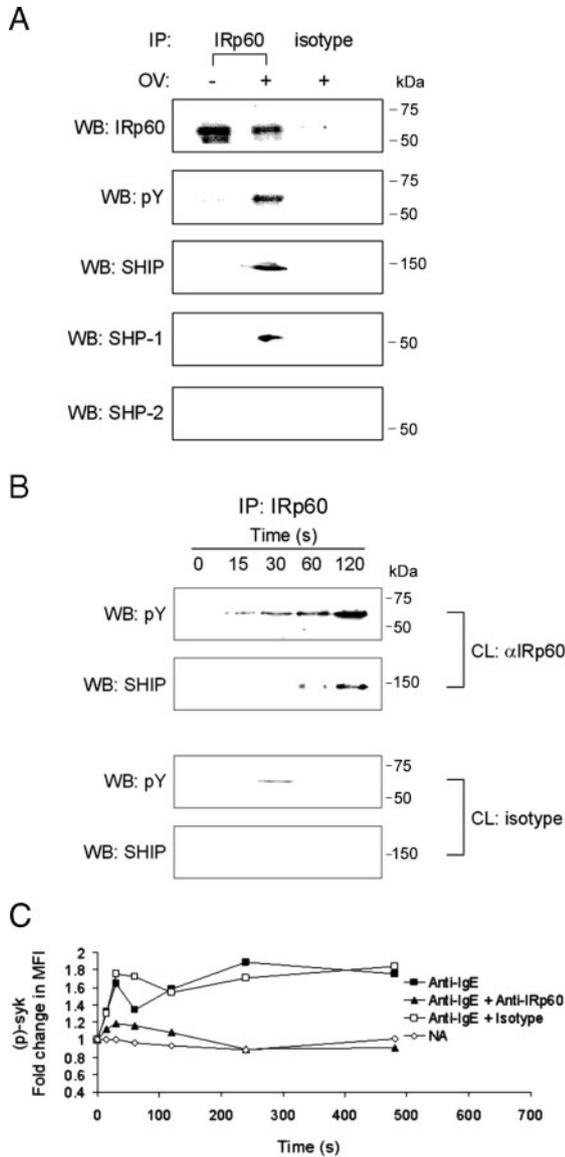**FIGURE 6.** IRp60 undergoes tyrosine phosphorylation and recruits phosphatases. After orthovanadate (*a*) or immune complex (5 μg/ml anti-IRp60, followed by 25 μg/ml sheep anti-mouse) (*b*) treatment, IRp60 was precipitated and blotted vs phosphotyrosine, SHP-1/2, and SHIP. CBMC were activated by anti-IgE Ab with IRp60 cross-linking, and cells were fixed and stained with Abs against phospho-syk (*c*). NA, Not activated. CL, Cross-linking agent. Representative of three experiments.

FIGURE 7. LMIR1 neutralization leads to an augmented mast cell activation and subsequent eosinophil infiltration in a mouse model of allergic peritonitis. OVA-induced allergic peritonitis was induced, anti-LMIR1 was injected 30 min before challenge, and mice were sacrificed at 45 min or 24 h after challenge. The peritoneal cavity was lavaged for mediator analysis and cell counts. *a*, Tryptase activity was evaluated using enzymatic-chromogenic assay. *b*, Eotaxin-2 was measured by ELISA. *c*, Cells from lavage collected 24 h following the challenge were stained for CCR3 and analyzed by FACS. Representative of three experiments; four to six mice per group. *, $p < 0.05$; **, $p < 0.005$.

vitro (data not shown). To assess whether IRp60 has a role in regulating mast cell activation in vivo, a murine model of experimental allergic peritonitis was used (24). Mice injected with neutralizing anti-LMIR1 mAb before allergen challenge, displayed enhanced mast cell activation in response to allergen challenge as demonstrated by the early (45-min) increase in tryptase activity in the peritoneal lavage (Fig. 7*a*). Eotaxin-2 levels 24 h after allergen challenge were significantly increased (Fig. 7*b*). Also, the peritoneal eosinophilic infiltration was doubled (eosinophil counts $4.199 \pm 0.825 \times 10^6$ with anti-LMIR1 vs $2.056 \pm 0.829 \times 10^6$ without; $p < 0.05$) (Fig. 7*c*).

Discussion

A major issue in modulating mast cell responses is the identification of new inhibitory pathways that counteract their activity. In the present study, we have found that the inhibitory receptor IRp60
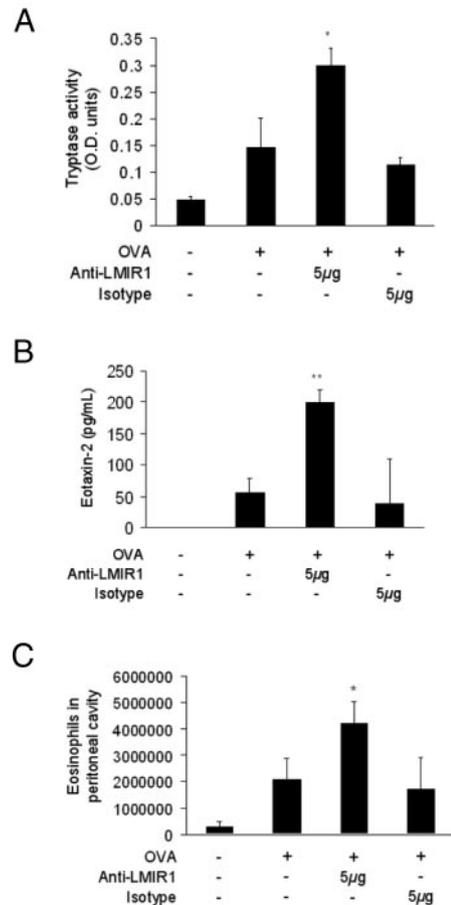
is expressed and functional on human mast cells. We found that CBMC selectively express IRp60. To date, the functionally characterized inhibitory receptors on human mast cells are the tetraspanins CD63 (26) and CD81 (27), CD200R (16), immune receptor expressed on myeloid cells 1 (28), FcγRIIB (11), and signal regulatory protein-α (29).

CBMC are in vitro-derived, mostly tryptase-containing mast cells, with a phenotype similar to HLMC (30). We therefore aimed to verify whether tissue in vivo-derived mast cells express IRp60 as well. Indeed, IRp60 was found to be expressed both on lung (tryptase-positive) and nasal polyp (tryptase- and chymase-positive) (31) mast cells, indicating that IRp60 may have a functional significance in mast cell regulation in vivo, of the two mast cell subtypes. Still, because HLMC in this study were obtained from lung specimens taken from cancer patients, it is possible that these mast cells display an abnormal pattern of IRp60 expression and function.

Because we believe that mast cell-eosinophil interactions form a crucial axis in allergic inflammatory and fibrotic diseases, we have examined the influence of eosinophil-derived granule proteins on

the expression of IRp60 on mast cells. Although IRp60 is expressed constitutively, it was selectively down-regulated by eosinophil-derived MBP and EDN, but not by ECP or EPO. This effect is not likely due to the positive charge shared by the four molecules, because poly-L-arginine in an equivalent concentration did not induce a significant effect. This effect is also receptor specific, because CD117 was not affected by eosinophil granule proteins (data not shown). Furthermore, although MBP and ECP share a similar isoelectric point (pI $\approx$ 10.9) and a similar net charge at pH 7.0 ($e \approx$ 15), the traits of EDN differ significantly from those values (pI = 8.9; e = 7.5) (32). In fact, selective activities of the four eosinophil basic proteins have been described. For example, MBP and EPO induce eosinophil degranulation, EDN release, and synthesis of IL-8 (33). MBP also activates neutrophils to release superoxides by a mechanism involving phosphoinositide 3′-kinase and protein kinase Cζ (34). We believe that the decrease in IRp60 expression induced by eosinophil granule proteins is extremely important in the context of chronic allergic-inflammation where mast cells and eosinophils interact (35).

Mast cells are principally activated in allergy through IgE, but they are also able to degranulate upon IgE-independent activation (21). Therefore, the effect of IRp60 was examined on both modalities. The cross-linking of IRp60 inhibited β-hexosaminidase, tryptase, and IL-4 release from IgE-activated CBMC. However, it did not influence the IgE-independent compound 48/80-mediated CBMC activation. This differential effect indicates that IRp60 interferes with pathways involving tyrosine phosphorylation, but not with GTP/GDP-dependent pathways.

An important question arising from the observed inhibitory effect of IRp60 is whether it should or should not be coaggregated with the activatory receptor, such as FcϵRI and c-*kit*, to achieve an inhibitory effect. Although such coaggregation is considered as mandatory for inhibitory receptors, several observations have clearly demonstrated that this is not the case (36, 37). Our results demonstrate that, when IRp60 is coaggregated with FcϵRI, it elicits a much stronger effect than that achieved when IRp60 is self-ligated (data not shown). Interestingly, the effect on mast cell survival did not vary significantly when IRp60 was coaggregated with c-*kit*.

Another important tyrosine kinase-dependent pathway in mast cell regulation is the SCF-CD117 axis, mediated primarily by phosphatidylinositol 3-OH kinase, Akt, and syk. Consistent with this, we have examined the effect of IRp60 on the SCF-mediated survival of CBMC, and found that IRp60 cross-linking led to increased cell death. These data are in accordance with reports that demonstrated the ability of FcγRIIB to block SCF-mediated survival of mast cells (11). Moreover, gp49B1 was reported to decrease the activation of mast cells in vivo via SCF (4). It is noteworthy that IRp60 did not actively induce apoptosis but rather blocked the survival-inducing signals generated by the SCF-CD117 axis.

By orthovanadate treatment, IRp60 undergoes tyrosine phosphorylation and recruits the protein phosphatases SHP-1 and the inositol phosphatases SHIP, two well-established negative regulators of cell activation and survival. In addition, by immune complex-derived cross-linking, IRp60 recruited SHIP but not SHP-1. This could be due to the lower level of phosphorylation induced by immune complex compared with orthovanadate. Yet, syk was dephosphorylated by immune complex-driven cross-linking, and therefore it is possible to assume that SHP-1 is recruited by this stimulus as well. The observation that IRp60 does not recruit SHP-2, unlike in NK cells, supports recently reported data in which IgSF13, a close family member of IRp60 expressed on dendritic cells, follows exactly the same phosphatases deployment pat-

tern (38). By phosphatase recruitment and activation, IRp60 prevents IgE-mediated syk phosphorylation, thus blocking mast cell activation in an early stage. Interestingly, Ab-cross-linked IRp60 did not precipitate with SHP-1. Although this may be related to the robust phosphorylation induced by orthovanadate rather than by immune complex, other factors might contribute to this observation. However, it is likely that SHP-1 is recruited by IRp60, because syk phosphorylation was observed. We are currently using biotinylated ITIMs as described (16) to clarify this issue.

IRp60 represents an allelic isoform of CMRF-35H (39). Although no in vivo function has been attributed to IRp60 in humans yet, a murine CMRF-35H family member (termed CMRF-like molecule 1 (CLM-1)) has been shown to inhibit osteoclast formation in vivo through SHP-1 recruitment (40). It has been recently reported that murine mast cells express an Ig-superfamily receptor termed LMIR1 (25), which is ~70% identical with human IRp60, with the important functional residues in the V-type Ig-fold and ITIM sequences well conserved, and is able to recruit SHP-1 and SHP-2 via tyrosine phosphorylation. We have conducted a functional bioinformatics comparison between LMIR1 and IRp60 (data not shown) and concluded that there is a very high probability that LMIR1 actually represents a murine homolog of the human IRp60. Neutralization of LMIR1 enhanced mast cell activation and consequent eosinophilic infiltration in the peritoneal lavage of these mice. This indicates that LMIR1 and its yet-undefined ligand have a key role in regulation of allergy.

The gene encoding for IRp60, *IRC1*, is located on chromosome 17q24.25 (41) in close linkage with a recently identified locus dictating susceptibility to psoriasis (41), arthritis, and atopic dermatitis (42). The possibility that IRp60 is the actual gene responsible for these diseases is strengthened by the observation that neutralizing its activity in vivo augments the allergen-dependent response in murine allergic peritonitis. It is therefore conceivable that IRp60 represents an important family of immune negative regulators that has long been overlooked.

In conclusion, this work demonstrates a novel pathway for the negative regulation of human mast cell function. This issue is important not just academically, because the ability to target IRp60 and consequently inhibit mast cell inflammatory functions opens the potential for novel therapeutic strategies for the management of mast cell-dependent pathologies.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

References

1. Kirshenbaum, A. S., J. P. Goff, S. W. Kessler, J. M. Mican, K. M. Zsebo, and D. D. Metcalfe. 1992. Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from CD34$^+$ pluripotent progenitor cells. *J. Immunol.* 148: 772–777.
2. Iemura, A., M. Tsai, A. Ando, B. K. Wershil, and S. J. Galli. 1994. The c-*kit* ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *Am. J. Pathol.* 144: 321–328.
3. Asai, K., J. Kitaura, Y. Kawakami, N. Yamagata, M. Tsai, D. P. Carbone, F. T. Liu, S. J. Galli, and T. Kawakami. 2001. Regulation of mast cell survival by IgE. *Immunity* 14: 791–800.
4. Feldweg, A. M., D. S. Friend, J. S. Zhou, Y. Kanaoka, M. Daheshia, L. Li, K. F. Austen, and H. R. Katz. 2003. gp49B1 suppresses stem cell factor-induced

mast cell activation-secretion and attendant inflammation in vivo. *Eur. J. Immunol.* 33: 2262–2268.

5. Pandey, V., S. Mihara, A. Fensome-Green, S. Bolsover, and S. Cockcroft. 2004. Monomeric IgE stimulates NFAT translocation into the nucleus, a rise in cytosol Ca^{2+}, degranulation, and membrane ruffling in the cultured rat basophilic leukemia-2H3 mast cell line. *J. Immunol.* 172: 4048–4058.

6. Sundstrom, M., J. Alfredsson, N. Olsson, and G. Nilsson. 2001. Stem cell factor-induced migration of mast cells requires p38 mitogen-activated protein kinase activity. *Exp. Cell. Res.* 267: 144–151.

7. Katz, H. R. 2002. Inhibitory receptors and allergy. *Curr. Opin. Immunol.* 14: 698–704.

8. Ravetch, J. V., and L. L. Lanier. 2000. Immune inhibitory receptors. *Science* 290: 84–89.

9. Nakamura, K., A. Brauweiler, and J. C. Cambier. 2000. Effects of Src homology domain 2 (SH2)-containing inositol phosphatase (SHIP), SH2-containing phosphotyrosine phosphatase (SHP)-1, and SHP-2 SH2 decoy proteins on $Fc\gamma RIIB1$-effector interactions and inhibitory functions. *J. Immunol.* 164: 631–638.

10. Maeda, A., A. M. Scharenberg, S. Tsukada, J. B. Bolen, J. P. Kinet, and T. Kurosaki. 1999. Paired immunoglobulin-like receptor B (PIR-B) inhibits BCR-induced activation of Syk and Btk by SHP-1. *Oncogene* 18: 2291–2297.

11. Malbec, O., J. P. Attal, W. H. Fridman, and M. Daeron. 2002. Negative regulation of mast cell proliferation by $Fc\gamma RIIB$. *Mol. Immunol.* 38: 1295–1299.

12. Lu-Kuo, J. M., D. M. Joyal, K. F. Austen, and H. R. Katz. 1999. gp49B1 inhibits IgE-initiated mast cell activation through both immunoreceptor tyrosine-based inhibitory motifs, recruitment of src homology 2 domain-containing phosphatase-1, and suppression of early and late calcium mobilization. *J. Biol. Chem.* 274: 5791–5796.

13. Yotsumoto, K., Y. Okoshi, K. Shibuya, S. Yamazaki, S. Tahara-Hanaoka, S. Honda, M. Osawa, A. Kuroiwa, Y. Matsuda, S. G. Tenen, et al. 2003. Paired activating and inhibitory immunoglobulin-like receptors, MAIR-I and MAIR-II, regulate mast cell and macrophage activation. *J. Exp. Med.* 198: 223–233.

14. Abramson, J., R. Xu, and I. Pecht. 2002. An unusual inhibitory receptor—the mast cell function-associated antigen (MAFA). *Mol. Immunol.* 38: 1307–1313.

15. Uehara, T., M. Blery, D. W. Kang, C. C. Chen, L. H. Ho, G. L. Gartland, F. T. Liu, E. Vivier, M. D. Cooper, and H. Kubagawa. 2001. Inhibition of IgE-mediated mast cell activation by the paired Ig-like receptor PIR-B. *J. Clin. Invest.* 108: 1041–1050.

16. Cherwinski, H. M., C. A. Murphy, B. L. Joyce, M. E. Bigler, Y. S. Song, S. M. Zurawski, M. M. Moshrefi, D. M. Gorman, K. L. Miller, S. Zhang, et al. 2004. The CD200 receptor is a novel and potent regulator of murine and human mast cell function. *J. Immunol.* 173: 6786–6793.

17. Kepley, C. L., S. Taghavi, G. Mackay, D. Zhu, P. A. Morel, K. Zhang, J. J. Ryan, L. S. Satin, M. Zhang, P. P. Pandolfi, and A. Saxon. 2004. Co-aggregation of $Fc\gamma RII$ with $Fc\epsilon RI$ on human mast cells inhibits antigen-induced secretion and involves SHIP-Grb2-Dok complexes. *J. Biol. Chem.* 279: 35139–35149.

18. Tam, S. W., S. Demissie, D. Thomas, and M. Daeron. 2004. A bispecific antibody against human IgE and human $Fc\gamma RII$ that inhibits antigen-induced histamine release by human mast cells and basophils. *Allergy* 59: 772–780.

19. Zhu, D., C. L. Kepley, M. Zhang, K. Zhang, and A. Saxon. 2002. A novel human immunoglobulin $Fc\gamma$ $Fc\epsilon$ bifunctional fusion protein inhibits $Fc\epsilon RI$-mediated degranulation. *Nat. Med.* 8: 518–521.

20. Cantoni, C., C. Bottino, L. Augugliaro, L. Morelli, E. Marcenaro, R. Castriconi, M. Vitale, D. Pende, S. Sivori, R. Millo, et al. 1999. Molecular and functional characterization of IRp60, a member of the immunoglobulin superfamily that functions as an inhibitory receptor in human NK cells. *Eur. J. Immunol.* 29: 3148–3159.

21. Piliponsky, A. M., G. J. Gleich, A. Nagler, I. Bar, and F. Levi-Schaffer. 2003. Non-IgE-dependent activation of human lung- and cord blood-derived mast cells is induced by eosinophil major basic protein and modulated by the membrane form of stem cell factor. *Blood* 101: 1898–1904.

22. Woolhiser, M. R., K. Brockow, and D. D. Metcalfe. 2004. Activation of human mast cells by aggregated IgG through $Fc\gamma RI$: additive effects of C3a. *Clin. Immunol.* 110: 172–180.

23. Greenfeder, S., H. Gilchrest, B. Cheewatrakoolpong, S. Eckel, M. Billah, R. W. Egan, and J. C. Anthes. 2003. Real-time assay of tryptase release from human umbilical cord blood-derived mast cells. *BioTechniques* 34: 910–914.

24. Zuany-Amorim, C., D. Leduc, B. B. Vargaftig, and M. Pretolani. 1993. Characterization and pharmacological modulation of antigen-induced peritonitis in actively sensitized mice. *Br. J. Pharmacol.* 110: 917–924.

25. Kumagai, H., T. Oki, K. Tamitsu, S. Z. Feng, M. Ono, H. Nakajima, Y. C. Bao, Y. Kawakami, K. Nagayoshi, N. G. Copeland, et al. 2003. Identification and characterization of a new pair of immunoglobulin-like receptors LMIR1 and 2 derived from murine bone marrow-derived mast cells. *Biochem. Biophys. Res. Commun.* 307: 719–729.

26. Kraft, S., T. J. Fleming, J. M. Billingsley, S. Y. Lin, M. H. Jouvin, P. Storz, and J. P. Kinet. 2005. Anti-CD63 antibodies suppress IgE-dependent allergic reactions in vitro and in vivo. *J. Exp. Med.* 201: 385–396.

27. Fleming, T. J., E. Donnadieu, C. H. Song, F. V. Laethem, S. J. Galli, and J. P. Kinet. 1997. Negative regulation of $Fc\epsilon RI$-mediated degranulation by CD81. *J. Exp. Med.* 186: 1307–1314.

28. Alvarez-Errico, D., H. Aguilar, F. Kitzig, T. Brckalo, J. Sayos, and M. Lopez-Botet. 2004. IREM-1 is a novel inhibitory receptor expressed by myeloid cells. *Eur. J. Immunol.* 34: 3690–3701.

29. Lienard, H., P. Bruhns, O. Malbec, W. H. Fridman, and M. Daeron. 1999. Signal regulatory proteins negatively regulate immunoreceptor-dependent cell activation. *J. Biol. Chem.* 274: 32493–32499.

30. Saito, H., M. Ebisawa, N. Sakaguchi, T. Onda, Y. Iikura, M. Yanagida, H. Uzumaki, and T. Nakahata. 1995. Characterization of cord-blood-derived human mast cells cultured in the presence of Steel factor and interleukin-6. *Int. Arch. Allergy Immunol.* 107: 63–65.

31. Kawabori, S., J. A. Denburg, L. B. Schwartz, A. A. Irani, D. Wong, G. Jordana, S. Evans, and J. Dolovich. 1992. Histochemical and immunohistochemical characteristics of mast cells in nasal polyps. *Am. J. Respir. Cell Mol. Biol.* 6: 37–43.

32. Barker, R. L., D. A. Loegering, R. M. Ten, K. J. Hamann, L. R. Pease, and G. J. Gleich. 1989. Eosinophil cationic protein cDNA: comparison with other toxic cationic proteins and ribonucleases. *J. Immunol.* 143: 952–955.

33. Kita, H., R. I. Abu-Ghazaleh, S. Sur, and G. J. Gleich. 1995. Eosinophil major basic protein induces degranulation and IL-8 production by human eosinophils. *J. Immunol.* 154: 4749–4758.

34. Shenoy, N. G., G. J. Gleich, and L. L. Thomas. 2003. Eosinophil major basic protein stimulates neutrophil superoxide production by a class IA phosphoinositide 3-kinase and protein kinase C-ζ-dependent pathway. *J. Immunol.* 171: 3734–3741.

35. Munitz, A., A. M. Piliponsky, and F. Levi-Schaffer. 2003. IgE-independent activation of human mast cells indicates their role in the late phase reaction of allergic inflammation. *Cell Tissue Bank* 4: 25–28.

36. Vitale, C., C. Romagnani, A. Puccetti, D. Olive, R. Costello, L. Chiossone, A. Pitto, A. Bacigalupo, L. Moretta, and M. C. Mingari. 2001. Surface expression and function of p75/AIRM-1 or CD33 in acute myeloid leukemias: engagement of CD33 induces apoptosis of leukemic cells. *Proc. Natl. Acad. Sci. USA* 98: 5764–5769.

37. Nutku, E., H. Aizawa, S. A. Hudson, and B. S. Bochner. 2003. Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. *Blood* 101: 5014–5020.

38. Sui, L., N. Li, Q. Liu, W. Zhang, T. Wan, B. Wang, K. Luo, H. Sun, and X. Cao. 2004. IgSF13, a novel human inhibitory receptor of the immunoglobulin superfamily, is preferentially expressed in dendritic cells and monocytes. *Biochem. Biophys. Res. Commun.* 319: 920–928.

39. Biassoni, R., C. Cantoni, D. Marras, J. Giron-Michel, M. Falco, L. Moretta, and N. Dimasi. 2003. Human natural killer cell receptors: insights into their molecular function and structure. *J. Cell. Mol. Med.* 7: 376–387.

40. Chung, D. H., M. B. Humphrey, M. C. Nakamura, D. G. Ginzinger, W. E. Seaman, and M. R. Daws. 2003. CMRF-35-like molecule-1, a novel mouse myeloid receptor, can inhibit osteoclast formation. *J. Immunol.* 171: 6541–6548.

41. Speckman, R. A., J. A. Wright Daw, C. Helms, S. Duan, L. Cao, P. Taillon-Miller, P. Y. Kwok, A. Menter, and A. M. Bowcock. 2003. Novel immunoglobulin superfamily gene cluster, mapping to a region of human chromosome 17q25, linked to psoriasis susceptibility. *Hum. Genet.* 112: 34–41.

42. Bowcock, A. M., and W. O. Cookson. 2004. The genetics of psoriasis, psoriatic arthritis and atopic dermatitis. *Hum. Mol. Genet.* 13(Spec. 1): R43–R55.