

Peritoneal Cell-Derived Mast Cells: An In Vitro Model of Mature Serosal-Type Mouse Mast Cells¹

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Bone marrow-derived mast cells (BMMC) have been used extensively as a mast cell model. BMMC, however, are immature cells that have no known physiological equivalent in tissues. They do not respond to IgG immune complexes. They may therefore not be appropriate for studying the physiopathology of IgE-induced allergies or IgG-induced tissue-specific inflammatory diseases which both depend on mature mast cells. Resident peritoneal mast cells are a minor population of differentiated cells that are not readily purified. They, however, can be expanded in culture to generate large numbers of homogeneous cells. We show here that these peritoneal cell-derived mast cells (PCMC) are mature serosal-type mouse mast cells which retain most morphological, phenotypic, and functional features of peritoneal mast cells. Like peritoneal mast cells, PCMC respond to IgG Abs. IgG immune complex-induced responses depended on Fc γ RIIIA and were negatively regulated by Fc γ RIIB. We found that a moderate Fc γ RIIB-dependent negative regulation, due not to a higher Fc γ RIIIA/Fc γ RIIB ratio, but to a relatively inefficient use of the lipid phosphatase SHIP1, determines this property of PCMC. PCMC also respond to IgE Abs. IgE-induced PCMC responses, however, differed quantitatively and qualitatively from BMMC responses. PCMC secreted no or much lower amounts of lipid mediators, chemokines, and cytokines, but they contained and released much higher amounts of preformed granular mediators. PCMC, but not BMMC, also contained and, upon degranulation, released molecules with a potent proteolytic activity. These properties make PCMC a useful new model for understanding the physiopathology of mast cells in IgE- and IgG-dependent tissue inflammation. *The Journal of Immunology*, 2007, 178: 6465–6475.

The incidence of both allergies and autoimmune diseases has dramatically increased over the last three to four decades (1), calling for better understanding of the cellular and molecular immunopathological processes underlying these diseases. A recent advance has been the discovery that Ab-dependent mast cell activation plays a pivotal role not only in IgE-dependent allergies (2), but also, as observed in murine models of arthritis (3) and encephalitis (4), in IgG-dependent tissue-specific autoimmune diseases. These unexpected findings call for better understanding of mast cell biology. To this aim, genetically engineered mice provide powerful analytical tools. Mast cells from such mice indeed make it possible to dissect the molecular mechanisms involved in the generation of pathological responses. Mast cells, however, represent a minor population in tissues, from which

they are not readily purified. Moreover, the biological properties of distinct mast cell populations that reside in different tissues are poorly known. Reliable models of defined types of murine mast cells, that can be obtained in high numbers and that can account for immunopathological processes, are therefore a requirement. Such models are not currently available.

Like tissue-specific autoimmune diseases, most allergies have a local expression. Clinical symptoms, indeed, primarily depend on the anatomic site where mast cells are initially activated. The reasons are 2-fold. One reason is that different target organs do not respond identically to the same mediators. Activated mast cells release and secrete a variety of inflammatory molecules. These include preformed mediators stored in mast cell granules, among which are vasoactive amines and enzymes (5), newly formed lipid-derived mediators, such as PGs, thromboxanes, and leukotrienes (LT) (6), newly transcribed cytokines, mostly but not exclusively of the TH2 type (7), growth factors, and chemokines (8). These mediators generate a wide array of biological effects. They increase vascular permeability, trigger the contraction of smooth muscles, and attract and activate numerous inflammatory cells. Altogether, they concur to generate an acute reaction within minutes, followed by a late reaction within hours, a chronic reaction within days, and tissue remodeling within months. Another reason is that mast cells are not identical in different tissues and different mast cells may not secrete the same mediators. Mast cells indeed differentiate and mature in peripheral tissues, into which mast cell-committed bone marrow progenitors migrate and where they receive tissue-specific signals (9). Thus, mucosal-type mast cells develop in the mucosa of the gastrointestinal tract and in the lamina propria of the respiratory tract where their differentiation depends on T cell-derived cytokines, among which IL-3 is critical (10), while serosal-type mast cells develop in the skin, in the submucosa of the respiratory tract, in joint synovia, and in the

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Table I. Oligonucleotides used for RT-PCR analysis of mMCP, SHIP1, and β_2 -microglobulin transcripts

	Sense	Antisense
mMCP-1	ATGCAGGCCCTACTATTC	TTCTTTCTCTCCAGTTT
mMCP-2	GCCCTACTATTCCTGATG	TCTTTCTGTTCCTCC
mMCP-4	GGTGGTGTGAGTCTAGA	GCACATATGAGGAGATTCGG
mMCP-5	CCTGGGTTCAGCACCAAG	TGGACAACCAATTCATC
mMCP-6	GCACTGTCCCTCCTGGCT	TGGGAACCTTCACCTGCT
mMCP-7	TCACTGTGTCCAAATGCTAA	AAGGTGGTTTCTATAATGG
mMCP-8	CAACGCTGAAGGAGGGGA	TGGGACATGCTGCGACAC
mMCP-9	CCCCTGGAATGAAAAGA	TGGCTGTGAGAGAAAAA
mMCP-10	GCCCTACTATTCCTGATG	GCCTATCCTTGTAAATGCT
SHIP1	CGAGTCCTCTGGAAGTCTTCAC	TGAGAGGCGTGTAGATAGGATGC
β_2 -Microglobulin	TGACCGGCTTGTATGCTATC	CAGTGTGAGCCAGGATATAG

peritoneum where their differentiation primarily depends on fibroblast-derived stem cell factor (SCF)³ (11). Besides being dependent on different growth factors, mucosal- and serosal-type mast cells can be distinguished by their morphology, by their histamine content, and by a differential expression of mast cell-specific chymases and tryptases (12). The respective contributions of these two mast cell types to inflammation are not known.

Ab-dependent mast cell activation results from the engagement of receptors for the Fc portion of Abs (FcRs). Mouse mast cells express activating FcRs not only for IgE (Fc ϵ RI) (13, 14), but also for IgG (Fc γ RIIA) (15–17). Fc ϵ RI are high-affinity receptors, which bind monomeric IgE, whereas Fc γ RIIA are low-affinity IgG receptors, which bind immune complexes with a high avidity. In mast cells, Fc ϵ RI (18) and Fc γ RIIA (19) are constitutively associated with FcR γ and FcR β , which contain ITAMs. Mouse mast cells also express Fc γ RIIB. Fc γ RIIB are low-affinity IgG receptors, which negatively regulate IgE-induced (20) and IgG-induced (21) mast cell activation when they are coaggregated by immune complexes with Fc ϵ RI or Fc γ RIIA, respectively. Fc γ RIIB contain an ITIM (22), which mediates the recruitment of the inositol phosphatase SHIP1 (23, 24). SHIP1 is a major inhibitor of Fc ϵ RI signaling in mast cells (25).

On the basis of the above considerations, a good mast cell model should 1) be representative of mature-differentiated tissue mast cells, and 2) respond not only to IgE, but also to IgG Abs. The only available source of significant numbers of homogeneous nontransformed mouse mast cells is bone marrow-derived mast cells (BMMC). BMMC are often considered as an in vitro equivalent of mucosal-type mast cells. They, however, are immature cells whose physiological in vivo equivalent is not known. They can indeed reconstitute not only mucosal-type mast cells, but also serosal-type mast cells, when injected i.v. into mast cell-deficient mice (26). BMMC may correspond to precursors of the mature tissue mast cells, which initiate allergies and inflammatory diseases. BMMC express Fc ϵ RI, Fc γ RIIA, and Fc γ RIIB (15). They release mediators when sensitized by IgE and challenged with Ag and they have been used extensively for studying Fc ϵ RI signaling. They, however, do not or hardly respond to IgG immune complexes. For the above two reasons, BMMC may not be a suitable model for studying either IgE-induced allergic reactions or IgG-induced mast cell-dependent inflammation.

Peritoneal mast cells are mature serosal-type mast cells. They represent <5% of cells recovered in peritoneal washings from nor-

mal mice. They vigorously degranulate not only when sensitized with IgE and challenged with Ag, but also when challenged with preformed soluble IgG immune complexes (27). They can be separated from other peritoneal cells by techniques based on centrifugation through high-density medium (28), but in very low numbers ($\leq 1 \times 10^5$ /mouse) only, and with a variable purity. We found recently that significant numbers of mast cells can be generated in culture from mouse peritoneal cells (29), which might be useful for studying mast cell-dependent IgE- and IgG-induced tissue inflammation. They indeed respond both to IgE and to IgG Abs and we show here that this property is due to mild SHIP1-dependent negative regulation. We also show that they contain and release massive amounts of preformed vasoactive granular mediators and proteases, but secrete no or small amounts of newly formed proinflammatory molecules, including eicosanoids, chemokines, and cytokines. This novel in vitro model thus unravels unique properties of serosal-type mature mouse mast cells.

Materials and Methods

Mice

C57BL/6 mice, purchased from IFFA-CREDO or from Charles River Laboratories, were used as donors of bone marrow and peritoneal cells. BALB/c mice, purchased from IFFA-CREDO, were used for immunizations. SHIP1^{-/-} mice, generated by Dr. G. Krystal (The Terry Fox Laboratories, Vancouver, British Columbia, Canada), and SHIP1^{+/+} littermate controls were provided by Dr. M. Huber (Max Plank Institut für Immunbiologie, Freiburg, Germany). Bone marrow and peritoneal cells from RFc γ RIIB^{-/-}, RFc γ RIIA^{-/-}, and wild-type (wt) littermate control mice (generation 8 on C57BL/6 background) were provided by Dr. J. V. Ravetch (Rockefeller University, New York, NY). Mice were used at 6–9 wk of age.

Cells

Femoral bone marrow cells were collected and cultured in Opti-MEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 μ g/ml streptomycin (complete Opti-MEM), and 4% supernatant of X63 transfectants secreting murine IL-3 (a gift of Dr. P. Dubreuil, Institut de Cancérologie et d'Immunologie, Marseille, France). Cultures were passaged every 3 days by resuspending the pelleted cells in fresh culture medium at a concentration of 3×10^5 /ml. Peritoneal cells were collected from the same mice injected with 2 ml of RPMI 1640 i.p. They were seeded at 1×10^6 /ml in complete Opti-MEM supplemented with 4% supernatant of CHO transfectants secreting murine SCF (a gift from Dr. P. Dubreuil). Twenty-four hours later, nonadherent cells were removed and fresh culture medium was added to adherent cells. Three days later, nonadherent cells and adherent cells recovered with trypsin-EDTA were harvested, pelleted, and resuspended in fresh culture medium at a concentration of 3×10^5 /ml. The same procedure was repeated twice a week. Age-matched cultures (3–9 wk old) were used for experiments. Culture reagents were obtained from Invitrogen Life Technologies.

Abs and Ags

The rat anti-mouse Fc γ RIIB/IIIA mAb 2.4G2 was affinity purified from culture supernatants on protein G-Sepharose (Amersham Biosciences). The mouse anti-Ly-17.2 mAb K9.361 (30) and the mouse IgE anti-DNP mAb

³ Abbreviations used in this paper: SCF, stem cell factor; BMMC, bone marrow-derived mast cell; wt, wild type; MAR, mouse anti-rat; GAM, goat anti-mouse; GAR, goat anti-rabbit; RAM, rabbit anti-mouse; PCMC, peritoneal cell-derived mast cell; PAR, protease-activated receptor; TX100, Triton X-100; mMCP, murine MCP; LT, leukotriene.

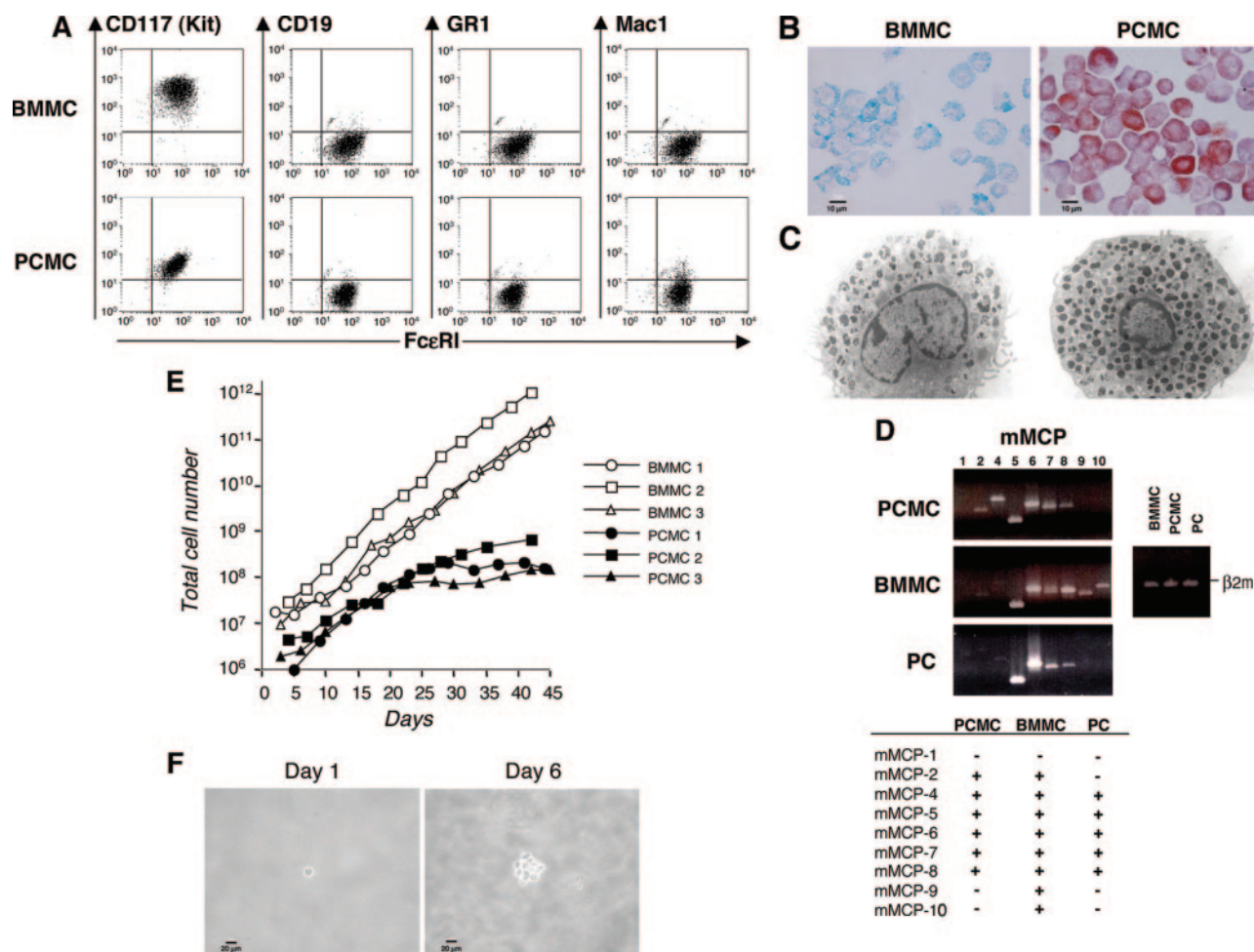


FIGURE 1. Generation and characterization of PCMC. *A*, Phenotype of cultured mast cells. FcεRI, CD117, CD19, GR1, and Mac1 expression was assessed by direct immunofluorescence. Fluorescence was analyzed by flow cytometry. *B*, Morphology of cultured mast cells. PCMCs and BMMCs were cytocentrifuged, stained with Alcian blue/safranin and observed under the microscope. *C*, Ultrastructure of cultured mast cells. PCMC and BMMC were observed by electron microscopy. *D*, Mouse mast cell protease transcripts. RNA extracted from PCMC, BMMC, and peritoneal cells (PC) was analyzed by RT-PCR using primers specific for the indicated proteases. β_2 -Microglobulin transcripts were used as loading controls. The table recapitulates the results shown in agarose gels. *E*, Growth curves of cultured mast cells. PCMC and BMMC were cultured as described in *Materials and Methods*. The figure represents the number of cells that could have been obtained if all cells had been kept in culture for the indicated times. This number was calculated as follows. At each passage, cells were resuspended at 3×10^5 cells/ml. After 3–4 days, the new concentration of viable cells was determined by counting trypan blue-excluding cells under the microscope. The total number of cells (N) was calculated by multiplying this concentration (C) by the volume of culture (V). Cells were again diluted at 3×10^5 cells/ml for the next passage, thus defining the new volume of culture as $V = N/3 \times 10^5$. The various $N = C \times V$, calculated at each passage, were plotted as a function of time to establish growth curves. Three independent experiments (labeled 1–3) are shown. In each experiment, BMMC and PCMC were derived from the bone marrow or the peritoneal cells, respectively, of the same two mice. *F*, SCF-dependent proliferation of peritoneal mast cells. Fifty peritoneal mast cells, individually harvested using a micropipette, were resuspended in SCF-containing culture medium diluted in soft agar at 37°C , layered over cold soft agar that had been previously layered over adherent SCF-secreting CHO transfectants and plated in a petri dish. Only individual cells were observed in the upper layer at day 1. Small clones were observed at day 6 of culture.

2682-I (31) were used as culture supernatants. K9.361, which recognizes the Ly-17.2 alloantigen, encoded by the *Ly-17b* allele of the *fcr2b* gene, was demonstrated as being an FcγRIIB-specific mAb with no cross-reactivities to other FcγRs, including FcγRIIIA (32). The IgE concentration in 2682-I supernatant was $10 \mu\text{g/ml}$ as titrated by ELISA. Allophycocyanin-labeled anti-CD117 Abs, PE-labeled anti-CD19 Abs, PE-labeled anti-GR1 Abs, and PE-labeled anti-Mac1 Abs were obtained from BD Pharmingen. FITC-labeled anti-FcεRI Abs were obtained from e-Bioscience. FITC-labeled mouse anti-rat (MAR) F(ab')₂, FITC-labeled goat anti-mouse (GAM) F(ab')₂, FITC-labeled goat anti-rabbit (GAR) F(ab')₂, rabbit anti-mouse (RAM) F(ab')₂ and intact IgG Abs were obtained from Jackson ImmunoResearch Laboratories. BSA, from Sigma-Aldrich, was dinitrophenylated with dinitrobenzene-sulfonic acid (Eastman Kodak). DNP₁₅-BSA was obtained. Mouse anti-GST serum was raised in BALB/c mice injected once with purified GST in CFA and twice in IFA i.p. IgG were affinity purified from serum on protein G-Sepharose. PE-labeled anti-IL-6 and anti-IL-10 Abs and biotinylated anti-TGFβ1 Abs were obtained from BD Biosciences. PE-labeled anti-IFN-γ, anti-TNF-α, and anti-IL-4 Abs

were obtained from Sérotec. Biotinylated anti-IL-13 Abs were obtained from R&D Systems. FITC-labeled streptavidin was obtained from Molecular Probes. The mouse anti-FcRβ mAb JRK was a gift from Dr. J.-P. Kinet (Harvard Medical School, Boston, MA). Rabbit anti-Lyn, anti-Src homology region 2 domain-containing phosphatase 1 (SHP-1), anti-SHP-2, anti-Gab2, anti-Sos and anti-phospholipase C-γ1 Abs were obtained from Upstate Biotechnology, as well as mouse anti-Vav Abs. Rabbit anti-growth factor receptor-bound protein 2 (Grb2), anti-PLC-γ2, and anti-SHIP1 were obtained from Santa Cruz Biotechnology. Mouse anti-Fyn Abs were obtained from BD Transduction Laboratories. Rabbit anti-ERK and anti-Akt Abs were obtained from Cell Signaling Technology. HRP-labeled GAR and GAM were obtained from Santa Cruz Biotechnology.

Direct immunofluorescence

Cells were incubated for 5 min at 0°C with $10 \mu\text{g/ml}$ 2.4G2. They were then incubated for 15 min at 0°C with $10 \mu\text{g/ml}$ FITC-labeled anti-FcεRI

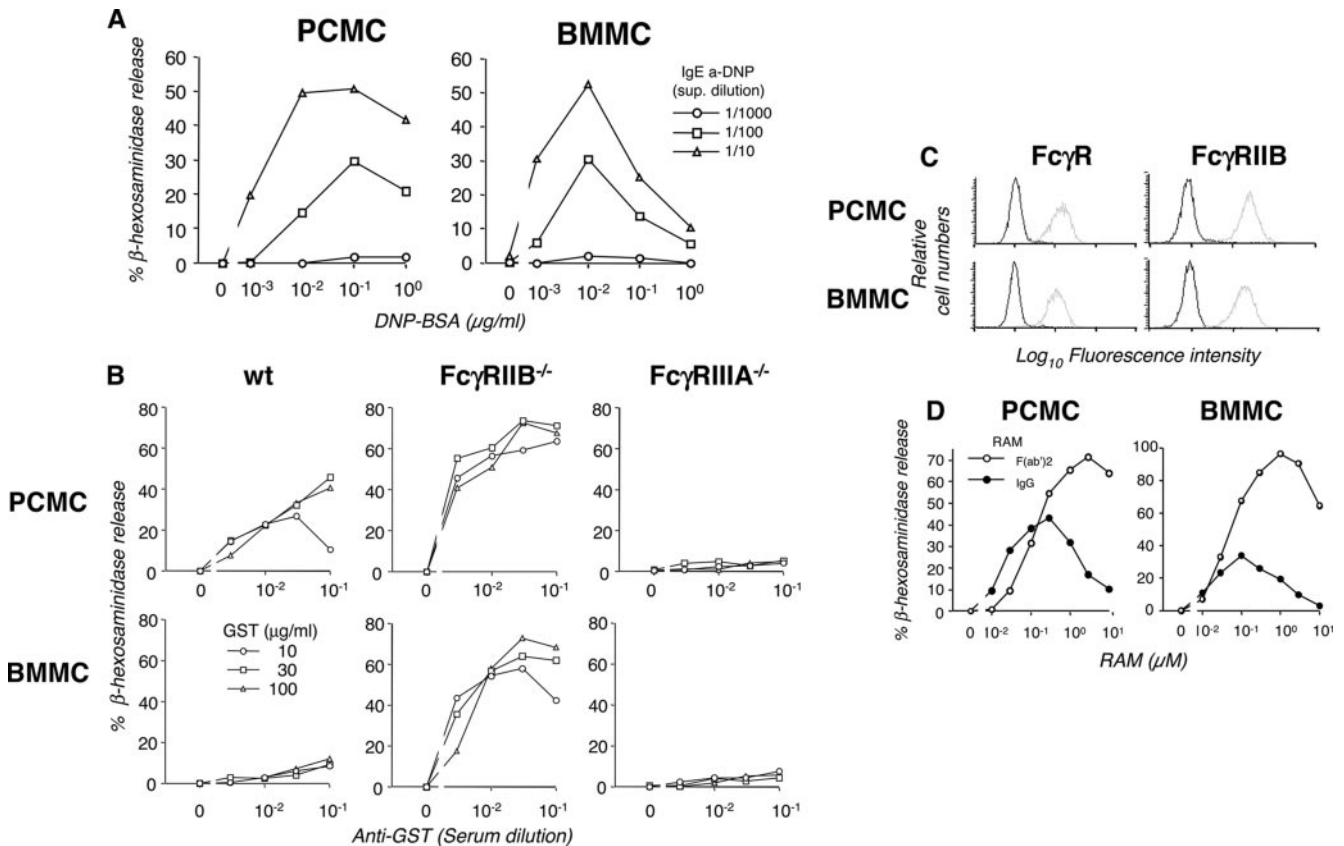


FIGURE 2. IgE- and IgG-induced β -hexosaminidase release by PCMC. **A**, Fc ϵ RI-dependent β -hexosaminidase release. PCMC and BMCM were sensitized with mouse IgE anti-DNP and challenged with DNP-BSA for 10 min at 37°C. The percentage of β -hexosaminidase released was plotted against the concentration of DNP-BSA. **B**, Fc γ R-dependent β -hexosaminidase release. PCMC and BMCM from wt, Fc γ RIIB $^{-/-}$, and Fc γ RIIIA $^{-/-}$ mice were challenged with immune complexes made of GST and mouse anti-GST immune serum for 10 min at 37°C. The percentage of β -hexosaminidase released was plotted against the dilutions of anti-GST serum. **C**, Expression of Fc γ R. Fc γ R expression was assessed by indirect immunofluorescence using 2.4G2 (gray histograms: cells incubated with 2.4G2 and FITC-MAR F(ab')₂; black histograms: cells incubated with FITC-MAR F(ab')₂ only). Fc γ RIIB expression was assessed by indirect immunofluorescence using K9.361 (gray histograms: cells incubated with K9.361 and FITC-GAM F(ab')₂; black histograms: cells incubated with FITC-GAM F(ab')₂ only). Fluorescence was analyzed by flow cytometry. **D**, Fc γ RIIB-dependent inhibition of IgE-induced β -hexosaminidase release. Wt BMCM and PCMC, sensitized with mouse IgE, were challenged with equimolar concentrations of RAM F(ab')₂ or IgG for 10 min at 37°C. The percentage of β -hexosaminidase released was plotted against the concentration of RAM.

Abs and allophycocyanin- or PE-labeled Abs. Cells were washed and fluorescence was analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

Alcian blue/safranin staining

Cells were cytocentrifuged, air-dried, incubated for 20 min with 0.5% Alcian blue in 0.3% acetic acid, rinsed in water, and incubated for 20 min with 0.1% safranin in 1% acetic acid. Cells were examined with a Nikon Eclipse TE 2000-U microscope.

Electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.01 M PBS (pH 7.4) for 1 h at 4°C, postfixed with 2% osmium tetroxide for 1 h, dehydrated with ethanol, and embedded in Epon epoxy resin. Ultra-thin sections (80–100 nm) were stained with uranyl acetate and lead citrate, and examined at 80 kV using a JEOL (JEM-1005) electron microscope.

RT-PCR analysis of murine MCP (mMCP) and SHIP1 transcripts

Total RNA was extracted from cultured mast cells or from cells recovered by peritoneal washing using TRIzol (Invitrogen Life Technologies). Reverse transcription was performed on 1 μg of RNA using the Prostar First-Strand RT-PCR kit (Stratagene Europe). Transcripts were detected by PCR using the oligonucleotides listed in Table I. PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide and visualized under UV light.

Peritoneal mast cell cloning in soft agar

Mast cells, identified by their morphology under the microscope, were individually picked-up from peritoneal cells, resuspended at 37°C in soft agar dissolved in SCF-containing medium, and layered over medium containing soft agar previously layered over adherent SCF-secreting CHO transfectants.

β -Hexosaminidase release

Mast cells, sensitized with IgE anti-DNP, were challenged for 10 min at 37°C with indicated reagents. Nonsensitized mast cells were challenged for 10 min at 37°C with preformed immune complexes made by incubating serum anti-GST or affinity-purified IgG anti-GST with GST at the indicated dilutions or concentrations for 15 min at 37°C immediately before use. Reactions were stopped on ice. Five microliters of supernatant was mixed with 45 μl of β -hexosaminidase substrate (Sigma-Aldrich) and incubated for 2 h at 37°C. A total of 0.2 M glycine (pH 10) was added, and absorbance at 405 nm was measured.

Indirect immunofluorescence

Surface labeling. Cells were incubated for 1 h at 0°C with K9.361 supernatant or 10 $\mu\text{g/ml}$ 2.4G2 in medium containing 5% FCS, washed, and stained with 50 $\mu\text{g/ml}$ FITC-GAM or MAR F(ab')₂ for 30 min at 0°C. Fluorescence was analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

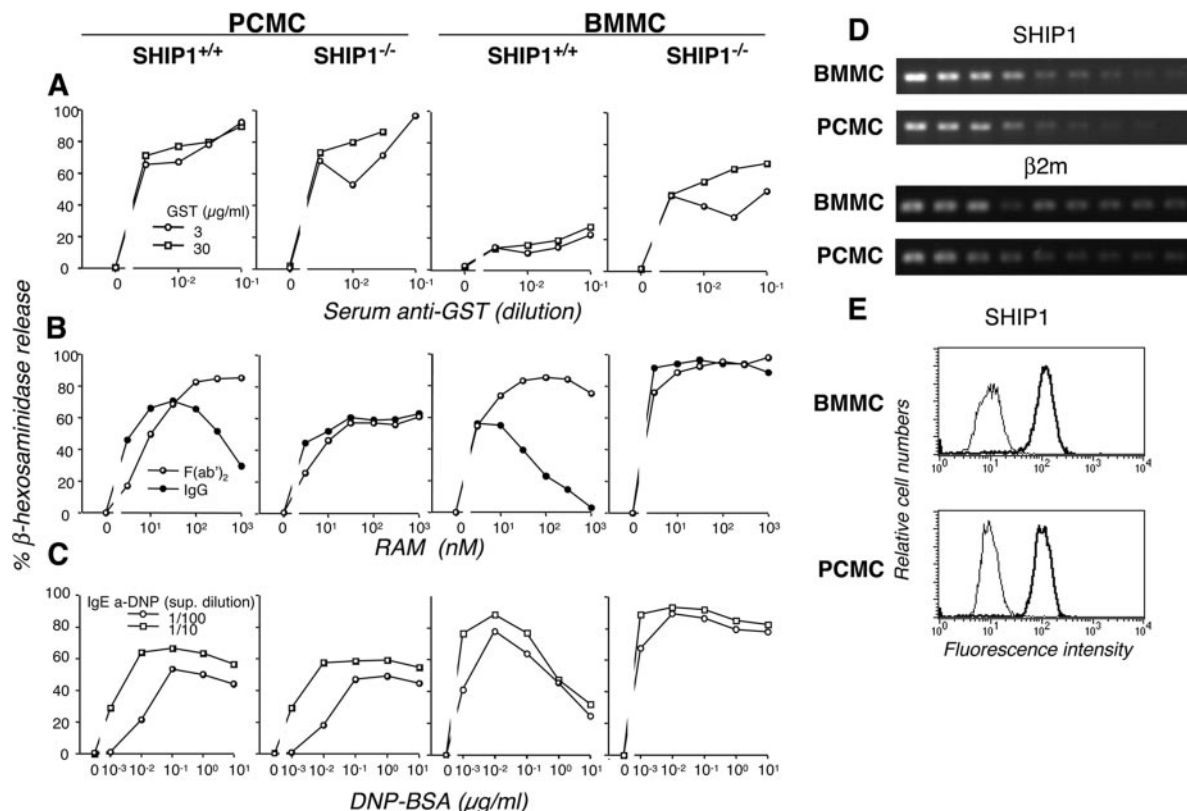


FIGURE 3. Effects of the deletion of SHIP1 in PCMC. *A*, FcγR-dependent β-hexosaminidase release. SHIP1^{+/+} and SHIP1^{-/-} PCMC and BMMC were challenged with GST anti-GST immune complexes for 10 min at 37°C. The percentage of β-hexosaminidase released was plotted against the dilutions of anti-GST serum. *B*, FcγRIIB-dependent negative regulation of IgE-induced β-hexosaminidase release. SHIP1^{+/+} and SHIP1^{-/-} PCMC and BMMC were sensitized with mouse IgE and challenged with equimolar concentration of RAM F(ab')₂ or IgG for 10 min at 37°C. The percentage of β-hexosaminidase released was plotted against the concentration of RAM. *C*, FcεRI-dependent β-hexosaminidase release. SHIP1^{+/+} and SHIP1^{-/-} PCMC and BMMC were sensitized with mouse IgE and challenged with DNP-BSA for 10 min at 37°C. The percentage of β-hexosaminidase released was plotted against the concentration of DNP-BSA. *D*, SHIP1 transcripts. RNA extracted from PCMC and BMMC were analyzed by RT-PCR using primers specific for SHIP1 or β₂-microglobulin. Serial 2-fold dilutions of PCR products were electrophoresed in agarose gel. *E*, Intracellular expression of SHIP1. BMMC and PCMC were fixed, permeabilized, and intracellular SHIP1 was assessed by indirect immunofluorescence with rabbit anti-SHIP1 Abs and FITC-GAR F(ab')₂; (thick histograms: cells incubated with rabbit anti-SHIP1 Abs and FITC-GAR F(ab')₂; thin histograms: cells incubated with FITC-GAR F(ab')₂ only). Fluorescence was analyzed by flow cytometry.

Intracellular labeling. Cells were fixed for 20 min with 3% paraformaldehyde in PBS, permeabilized for 15 min with 0.5% saponin in 2% BSA-PBS, and incubated for 30 min at 0°C with the indicated Abs in saponin-containing BSA-PBS. Cells were washed in PBS and stained with the indicated labeled Abs in saponin-containing BSA-PBS for 30 min at 0°C. Fluorescence was analyzed by flow cytometry.

LTC₄ production

Mast cells, sensitized with IgE anti-DNP, were challenged with DNP-BSA for 20 min at 37°C. LTC₄ was titrated in supernatants by competitive ELISA (Neogen).

MIP-1α secretion

Mast cells, sensitized with IgE anti-DNP, were challenged with DNP-BSA for the indicated periods of time at 37°C. MIP-1α was titrated in supernatants by ELISA (R&D Systems).

TNF-α secretion

Mast cells, sensitized with IgE anti-DNP, were challenged for various periods of time at 37°C with DNP-BSA. TNF-α was titrated in supernatants by a cytotoxicity assay on L929 cells as described previously (33).

Morphological assay for mast cell degranulation

Cultured mast cells or peritoneal cells, sensitized with mouse IgE, were challenged for 5 min at 37°C with RAM F(ab')₂. Reactions were stopped on ice, and cells were stained with toluidine blue.

Histamine release

Mast cells, sensitized with IgE anti-DNP, were challenged for 10 min at 37°C with DNP-BSA in serum-free medium. Reactions were stopped on ice. Histamine was measured in cells and supernatants using a high sampling rate automated continuous flow fluorometric technique (34).

Western blot analysis

Mast cells were lysed in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100 (TX100), 1 mM Na₃VO₄, 5 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF. Proteins were quantified using a Bio-Rad protein assay. Twenty micrograms of proteins were electrophoresed and Western blotted with indicated Abs followed by HRP-GAR or HRP-GAM. Labeled Abs were detected using an enhanced chemoluminescence kit (Amersham Biosciences). When indicated, cells were lysed by being boiled for 5 min at 95°C in 10 mM Tris (pH 7.4) containing 1% SDS. Lysates were passed six times through a gauge-26 needle, centrifuged at 12,000 rpm for 10 min at 4°C, and immediately electrophoresed.

Protease activity secretion

Mast cells, sensitized with IgE anti-DNP, were challenged for the indicated periods of time at 37°C with DNP-BSA. Reactions were stopped on ice. Proteolytic activity was measured in supernatants using an enzymatic assay. Briefly, 100 μl were incubated for 30 min at 37°C with 10 μl of 0.2 M Tris (pH 7.8), 0.02 M CaCl₂ and 10 μl of 0.4% resorufin-labeled casein (Roche Diagnostic). A total of 100 μl of 5% trichloroacetic acid was

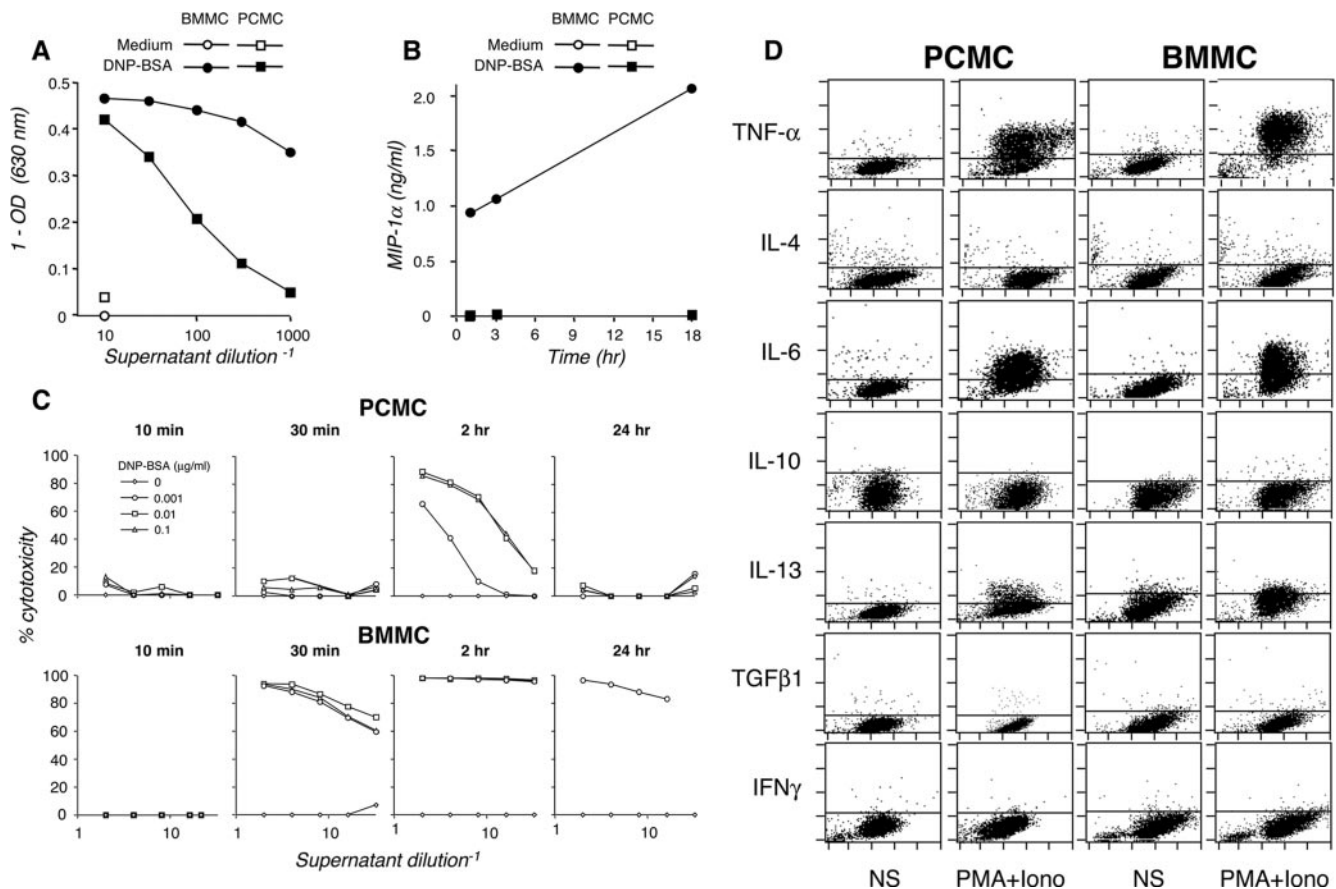


FIGURE 4. LTC₄, MIP-1 α , TNF- α , IL-6, and IL-13 production by PCMC. **A**, Fc ϵ RI-dependent LTC₄ production. PCMC and BMMC, sensitized with mouse IgE anti-DNP, were challenged with medium or DNP-BSA for 20 min at 37°C. LTC₄ was titrated by ELISA. The figure represents the OD of serial dilutions of supernatants. **B**, Fc ϵ RI-dependent MIP-1 α production. PCMC and BMMC, sensitized with mouse IgE anti-DNP, were challenged with medium or DNP-BSA for 1, 3, or 18 h at 37°C. MIP-1 α was titrated in supernatants by ELISA. The figure represents the concentration of MIP-1 α as a function of time. **C**, Fc ϵ RI-dependent TNF- α secretion. PCMC and BMMC, sensitized with mouse IgE anti-DNP, were challenged with the indicated concentrations of DNP-BSA for 10 min, 30 min, 2 h, or 24 h at 37°C. TNF- α secreted in supernatants was titrated by cytotoxicity on L929 cells. The figure represents the percentage of cytotoxicity induced by serial dilutions of supernatants. **D**, PMA plus ionomycin-induced (PMA + Iono) cytokines synthesis. PCMC and BMMC were challenged with PMA + ionomycin (right columns) or with medium alone (NS, left columns) for 2 h at 37°C. Cells were fixed, permeabilized, stained with Abs or reagents indicated in *Material and Methods*, and analyzed by flow cytometry. Log₁₀ fluorescence intensity was plotted against forward scatter (FSC).

added, and plates were incubated for 10 min at 37°C before being centrifuged for 5 min. Eighty-microliter supernatants were mixed with 120 μ l of 0.5 M Tris (pH 8.8). The absorbance was read at 570 nm.

Results

High numbers of homogeneous mature serosal-type mast cells can be generated in culture from mouse peritoneal cells

Peritoneal cells from two C57BL/6 mice were cultured in SCF-containing medium as described in *Materials and Methods*. BMMC were generated in parallel from bone marrow cells of the same two mice cultured in IL-3-containing medium. One month-old cultures of both types consisted of Fc ϵ RI⁺, Kit⁺, CD19⁻, GR1⁻, and Mac1⁻ homogeneous cells (Fig. 1A). This phenotype is characteristic of mast cells. Approximately 1×10^{10} and 1×10^8 mast cells could be recovered, after 1 mo, from bone marrow and from peritoneal cell cultures, respectively. Large numbers of pure mast cells can therefore be generated by culturing mouse peritoneal cells with SCF. These cells will be referred to as peritoneal cell-derived mast cells (PCMC).

PCMC and BMMC contained granules stained with Alcian blue/safranin. PCMC granules were much more intensely colored in red than BMMC granules (Fig. 1B), indicating a higher heparin content. When examined by electron microscopy, more numerous

granules were seen in PCMC. They were larger, more homogeneous, and had a higher density than BMMC granules (Fig. 1C). These staining and morphological properties are characteristic features of mature mast cells. PCMC and BMMC contained mast cell-specific protease transcripts. Both expressed mMCP-2, mMCP-4, mMCP-5, mMCP-6, mMCP-7, and mMCP-8. BMMC, but not PCMC, expressed mMCP-9 and mMCP-10. The same mMCP transcripts as in PCMC, except mMCP-2, were found in peritoneal cells (Fig. 1D). PCMC therefore retain most properties of mature serosal-type peritoneal mast cells.

BMMC and PCMC proliferated at similar rates during the first three weeks. Differing from BMMC, however, which grew steadily for 2–3 mo, PCMC started to proliferate more slowly during the fourth week of culture, and they stopped proliferating after about one month (Fig. 1E). Noticeably, typical mast cells, which were observed in small numbers at the onset of cultures, rapidly increased in numbers within the first days of culture, suggesting that PCMC could result from an expansion of differentiated peritoneal mast cells. To test this possibility, individual mast cells, identified by their size and morphology among the peritoneal cells harvested from C57BL/6 mice, were isolated by micromanipulation and cultured in soft agar layered over adherent SCF-producing

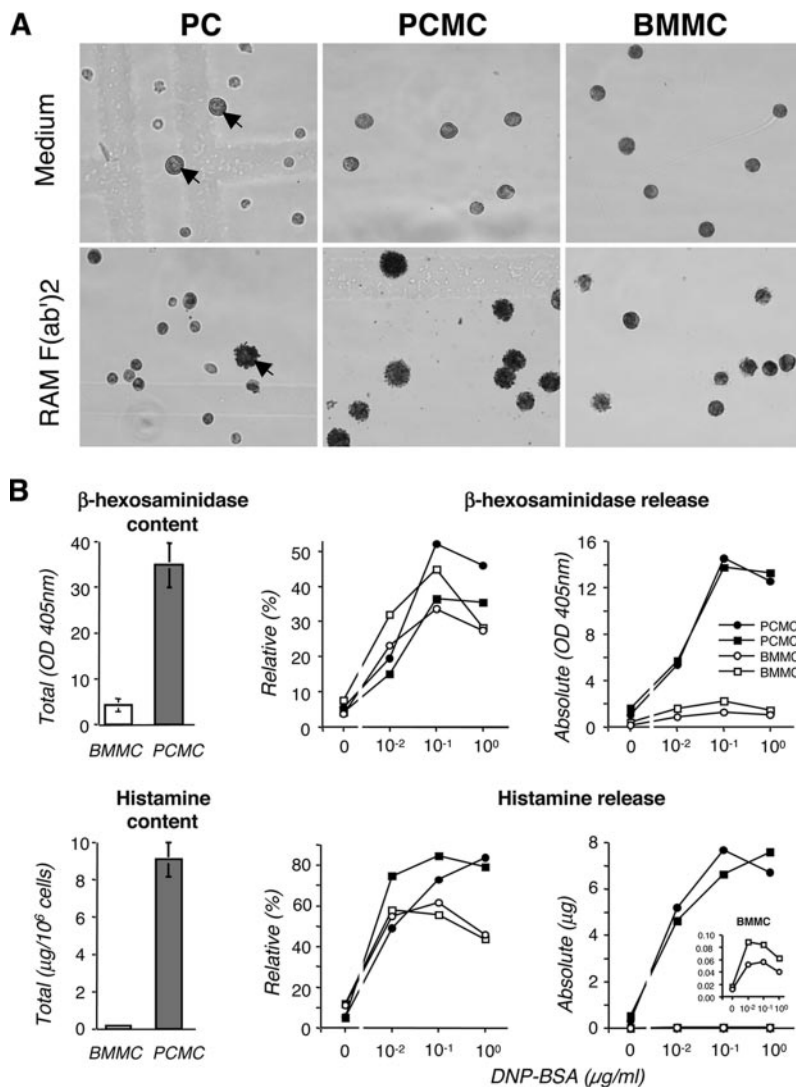


FIGURE 5. Degranulation, β -hexosaminidase, and histamine release by PCMC. *A*, Fc ϵ RI-dependent degranulation. Peritoneal cells (PC), PCMC, and BMMC, sensitized with mouse IgE, were challenged with RAM F(ab')₂ (lower row) or with medium only (upper row) for 5 min at 37°C, stained with toluidine blue, and examined under the microscope. Arrows show mast cells among peritoneal cells. *B*, Fc ϵ RI-dependent β -hexosaminidase and histamine release. PCMC and BMMC, sensitized with mouse IgE anti-DNP, were challenged with the indicated concentrations of DNP-BSA for 10 min at 37°C. A total of 1×10^5 cells were used for β -hexosaminidase release and 1×10^6 cells were used for histamine release. β -Hexosaminidase and histamine were measured in supernatants and in cell lysates. *Left panels*, The mean $\pm$ SD of values measured in BMMC and PCMC lysates. *Right panels*, The relative (percentage) and the absolute amounts of β -hexosaminidase and histamine released by individual cell populations. *Inset*, Histamine released by BMMC with an expanded vertical scale.

CHO transfectants used as feeder cells. Clones developed within 1 wk from single cells (Fig. 1*F*). Differentiated peritoneal mast cells can therefore proliferate in the presence of SCF.

PCMC respond not only to IgE and Ag, but also to IgG immune complexes

BMMC and PCMC expressed comparable levels of Fc ϵ RI (Fig. 1*A*). When sensitized with IgE anti-DNP and challenged with DNP-BSA for 10 min, PCMC and BMMC released similar percentages of β -hexosaminidase. Inhibition observed in excess of Ag was however more pronounced in BMMC than in PCMC (Fig. 2*A*).

When challenged with preformed immune complexes, nonsensitized PCMC dose-dependently released β -hexosaminidase. Under the same conditions, BMMC did not respond or very poorly (Fig. 2*B*, left panel). Immune complex-induced β -hexosaminidase release was enhanced in Fc γ RIIB^{-/-} PCMC. The deletion of Fc γ RIIB enabled BMMC to respond to immune complexes and the responses of Fc γ RIIB^{-/-} BMMC were of the same magnitude as those of Fc γ RIIB^{-/-} PCMC (Fig. 2*B*, middle panel). β -Hexosaminidase release was abrogated in Fc γ RIIA^{-/-} BMMC and PCMC (Fig. 2*B*, right panel). Noticeably, wt BMMC and PCMC expressed comparable levels of Fc γ R as assessed by immunofluorescence with the anti-Fc γ RIIB+IIIA mAb 2.4G2, and comparable levels of Fc γ RIIB as assessed with the anti-allotypic mAb K9.361 (30) (Fig. 2*C*). Fc γ RIIB could therefore negatively regu-

late Fc γ RIIA-dependent cell activation in both BMMC and PCMC, but no difference in the relative expression of activating and inhibitory receptors could explain why it prevented BMMC, but not PCMC, from responding to IgG immune complexes.

To further assess Fc γ RIIB-dependent negative regulation, its ability to inhibit Fc ϵ RI-dependent mast cell activation was investigated in the two cell types. BMMC and PCMC sensitized with mouse IgE were challenged either with RAM F(ab')₂, to aggregate Fc ϵ RI, or with intact RAM IgG, to coaggregate Fc ϵ RI with Fc γ RIIB in wt cells. Fc γ RIIB-dependent inhibition was more pronounced in BMMC than in PCMC (Fig. 2*D*).

SHIP1 is the main intracellular effector of Fc γ RIIB-dependent negative regulation (35). It also controls Fc ϵ RI signaling (36). To investigate the role of SHIP1 in the two types of mast cells, we generated BMMC and PCMC from SHIP1^{-/-} and from wt littermate controls. The deletion of SHIP1 increased immune complex-induced β -hexosaminidase release in BMMC but, surprisingly, not in PCMC (Fig. 3*A*). The deletion of SHIP1, however, abrogated RAM IgG-induced Fc γ RIIB-dependent negative regulation of Fc ϵ RI-dependent β -hexosaminidase release in both BMMC and PCMC (Fig. 3*B*). Inhibition was again more marked in BMMC than in PCMC. As expected, the deletion of SHIP1 increased Ag-induced β -hexosaminidase release by IgE-sensitized BMMC and, as previously reported (36), it abrogated inhibition observed in excess of Ag. It, however, did not detectably affect the response of PCMC challenged under the same conditions

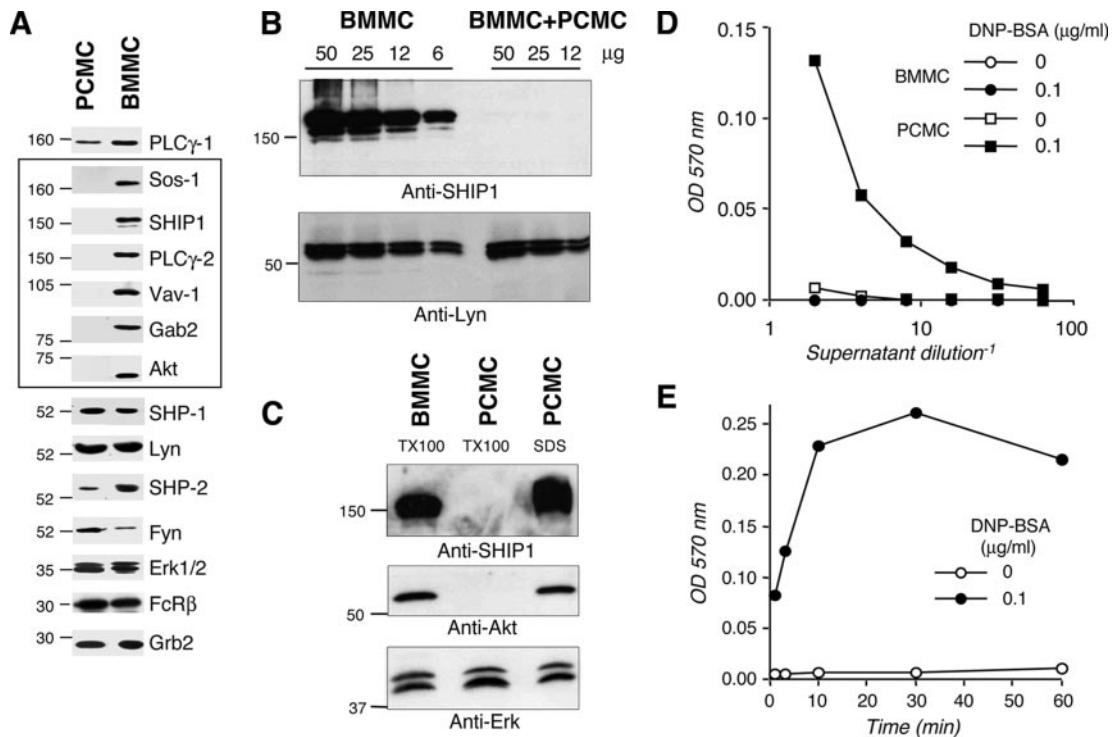


FIGURE 6. Proteolytic activity release by PCMC. **A**, Western blot analysis of intracellular molecules. BMMC and PCMC were lysed in TX100-containing lysis buffer, and lysates were Western blotted with the indicated Abs. This figure shows representative lysates from matched BMMC and PCMC cultures (three independent couples of cells were analyzed in the same experiment), which were Western blotted on the same filter. **B**, Protease activity in PCMC lysates. BMMC were mixed or not with equal numbers of PCMC before they were lysed in TX100-containing lysis buffer. Indicated amounts of proteins were electrophoresed and Western blotted with anti-SHIP1 and anti-Lyn Abs. **C**, Detection of SHIP1 in SDS lysates. BMMC and PCMC were lysed in TX100-containing lysis buffer or by boiling in Tris-SDS, and lysates were Western blotted with anti-SHIP1, anti-Akt, or anti-ERK Abs. **D**, IgE-induced release of proteolytic activity. A total of 1×10^6 PCMC and BMMC, sensitized with mouse IgE anti-DNP, were challenged with DNP-BSA for 10 min at 37°C, and serial 2-fold dilutions of supernatants were analyzed for proteolytic activity. The figure represents the OD as a function of supernatant dilutions. **E**, Kinetics of IgE-induced release of proteolytic activity. PCMC were sensitized with mouse IgE anti-DNP and challenged with DNP-BSA for the indicated periods of time at 37°C. Proteolytic activity was measured in nondiluted supernatants. The figure represents the OD as a function of time.

(Fig. 3C). The deletion of SHIP1 therefore markedly affected both IgE- and IgG-induced β -hexosaminidase release in BMMC, but not detectably in PCMC, suggesting that SHIP1-dependent negative regulation was more efficient in BMMC than in PCMC. Indeed, Fc γ RIIB-dependent negative regulation, which was abrogated by the deletion of SHIP1 in both cell types, was more efficient in BMMC than in PCMC. This functional difference between the two types of mast cells was not accounted for by a difference in the content of SHIP1. BMMC and PCMC indeed contained comparable amounts of SHIP1 transcripts, as assessed by RT-PCR (Fig. 3D), and comparable amounts of the SHIP1 protein, as assessed by intracellular immunofluorescence (Fig. 3E).

PCMC secrete small amounts of newly formed lipid mediators, chemokines, and cytokines in response to IgE and Ag

When sensitized with IgE anti-DNP and challenged with DNP-BSA for 20 min, BMMC secreted ~ 30 times more LTC $_4$ than PCMC, as assessed by ELISA (Fig. 4A). When sensitized with IgE anti-DNP and challenged with DNP-BSA for longer periods of time, BMMC, but not PCMC, secreted MIP-1 α , as assessed by ELISA (Fig. 4B). When sensitized with IgE anti-DNP and challenged with DNP-BSA for various periods of time, BMMC secreted TNF- α , as assessed by a cytotoxicity assay. PCMC, however, secreted much less TNF- α than BMMC. No TNF- α was detected at 10 min in supernatants from either cell type. Secretion became detectable at 30 min, peaked at 2 h and started to decline at 24 h in BMMC. A moderate secretion was detectable at 2 h only in PCMC (Fig. 4C).

PCMC containing intracellular TNF- α were also detected by immunofluorescence following stimulation by PMA plus ionomycin for 2 h. They were less numerous than BMMC containing TNF- α observed following the same treatment. PMA plus ionomycin-treated PCMC and BMMC also contained IL-6, and lower numbers of cells contained, IL-13, but not IL-4, IL-10, TGF- β 1, or IFN- γ (Fig. 4D).

Therefore, PCMC secreted no or much lower amounts of LTC $_4$, MIP-1 α , and TNF- α . However, PCMC could synthesize the same set of cytokines as BMMC in response to PMA plus ionomycin.

PCMC release large amounts of preformed granular mediators in response to IgE and Ag

When sensitized with IgE and challenged for 5 min with RAM F(ab') $_2$, a robust degranulation was observed in PCMC and in peritoneal mast cells, as assessed morphologically following toluidine blue staining. Degranulated PCMC resembled degranulated peritoneal mast cells. Under the same conditions, BMMC degranulated only weakly (Fig. 5A).

Noticeably, PCMC contained ~ 8 -fold more β -hexosaminidase and ~ 100 -fold more histamine than BMMC (Fig. 5B, left panel). As a consequence, when sensitized with IgE and challenged for 10 min with Ag, PCMC released much higher absolute amounts of β -hexosaminidase and even higher amounts of histamine than BMMC (Fig. 5B, right panel), even though both mast cells released comparable percentages of these mediators (Fig. 5B, middle

panel). Noticeably, both cells released a higher percentage of histamine than of β -hexosaminidase, when challenged identically in the same experiment.

When analyzing TX100 lysates from the two cells, a whole set of high m.w. intracellular proteins, including SHIP1, failed to be detected by Western blotting (Fig. 6A). The discordance between this unexpected observation and the immunofluorescence data shown in Fig. 3E could be best explained if proteolysis occurred during cell lysis despite protease inhibitors present in lysis buffer. To investigate this possibility, equal numbers of PCMC and BMMC were mixed before they were lysed in TX100-containing buffer. The presence of PCMC abrogated the detection of BMMC SHIP1, but not that of molecules observed in PCMC lysates such as Lyn (Fig. 6B). PCMC, but not BMMC, therefore contain a highly efficient protease activity that is released upon cell lysis and hydrolyzes several high-m.w. intracellular proteins. Proteolysis could, however, be prevented if PCMC were lysed in SDS-containing buffer and immediately boiled before electrophoresis. Under these conditions, SHIP1, but also other molecules not seen in PCMC TX100 lysates, such as Akt, were readily detectable, and in similar amounts as in BMMC (Fig. 6C).

On the basis of this observation, we investigated whether proteolytic enzymes contained in PCMC could be released and hydrolyze an exogenous substrate such as casein. Proteolytic activity was indeed detected in supernatants of PCMC sensitized with IgE anti-DNP and challenged with DNP-BSA for 10 min, but not in supernatants of IgE-sensitized, but not challenged PCMC. Under the same conditions, no proteolytic activity was detected in supernatants from IgE-sensitized BMMC, whether they were or not challenged with Ag (Fig. 6D). Proteolytic activity was released from IgE-sensitized PCMC with the same kinetics as β -hexosaminidase, i.e., within the first 10 min after Ag challenge, and it did not increase thereafter (Fig. 6E). Proteolytic enzymes are therefore likely to be contained in PCMC granules and to be released upon degranulation.

Discussion

We characterize here PCMC, a new model of cultured mouse mast cells, which markedly differ from BMMC. Indeed, PCMC consist of mature differentiated mast cells, which retain most of the properties of serosal-type peritoneal mast cells. This, we believe, make them useful for studying immunopathological processes. Serosal-type mast cells are indeed present in tissues involved in allergies and inflammatory diseases. Skin mast cells and synovial mast cells, for instance, both of the serosal type, play critical roles in skin allergies and in the murine model of IgG-induced autoimmune rheumatoid arthritis recently described in K/BxN mice (3), respectively.

Mast cells can be obtained by fractionation techniques from mouse peritoneal cells (28). However, no $>1 \times 10^5$ mast cells can be obtained per mouse, which greatly limits investigations. A few hundred million homogeneous mast cells can be readily generated from the peritoneal cells of two mice and kept in culture for at least 2 mo. These cells are typical mast cells as judged by their expression of Fc ϵ RI and Kit, their morphology, their histamine content, and their functional features. They are mature mast cells as judged by their intense staining with Alcian blue/safranin and by the high number and the dense structure of their granules. They are serosal-type mast cells as judged by their ability to degranulate in response to compound 48/80 (data not shown), by their high content of granular mediators and by the mMCP transcripts they contain (5). These PCMC are likely to result from the expansion of differentiated mast cells present in the peritoneal cavity of normal mice, rather than from the differentiation of Kit⁺ mast cell progenitors, possibly present in peritoneal cells. The number of mast cells rap-

idly increased in a few days, so that the majority of cells in culture had a mast cell morphology after 1 wk. Approximately 1×10^7 cells were obtained after 2 wk and these already consisted of a single population of Fc ϵ RI⁺ cells (data not shown). If, as indicated by the initial slope of growth curves, cells underwent two divisions per week, they originated from $\sim 5 \times 10^5$ cells. This number is compatible with numbers of mast cells contained in peritoneal washings from two adult mice (3–5% of $1\text{--}1.5 \times 10^7$ cells). Supporting this assumption, individual peritoneal mast cells could form clones in SCF-containing soft agar. Likewise, individual mouse peritoneal mast cells were previously reported to form colonies when cultured with SCF and IL-3 in methylcellulose (37). Because PCMC were generated in medium containing supernatant from SCF-secreting CHO transfectants, we investigated whether SCF would be sufficient to generate PCMC. Indeed, a single-cell population of Fc ϵ RI⁺ cells, which released β -hexosaminidase when challenged with preformed GST-IgG anti-GST immune complexes, was obtained when culturing peritoneal cells with recombinant SCF as the sole source of added growth factor (data not shown). Several groups previously reported the in vitro generation of alternatives to BMMC. Mast cells with some degree of differentiation toward the connective tissue type were obtained by culturing mouse bone marrow cells with SCF and a high concentration of IL-4 (38). More interestingly, a few million mast cells were generated from fetal skin, which shared several properties with PCMC (39). Differing from PCMC, however, these mast cells apparently differentiated in cultures from mast cell precursors.

One distinctive property of PCMC is their ability to respond to IgG immune complexes. Importantly, this property is shared with peritoneal mast cells. Peritoneal mast cells have indeed long been known to degranulate in response to IgG Abs (27). Active Abs were found in IgG1, rather than IgG2 fractions of polyclonal mouse Abs (40). All monoclonal IgG1 tested and some IgG2a (41) or IgG2b (42) could activate peritoneal mouse mast cells. As previously observed for peritoneal mast cells (17), immune complex-induced PCMC activation depended on Fc γ RIIIA and, as expected, it was negatively regulated by Fc γ RIIB. Surprisingly, the differential responses of the two cells to IgG immune complexes could not be accounted for by a different ratio of activating/inhibitory Fc γ Rs, revealing that previously unsuspected mechanisms may control Fc γ RIIB-dependent negative regulation. Fc γ RIIIA-dependent cell activation was indeed as efficient in both cells, since responses of similar magnitudes were induced by immune complexes in Fc γ RIIB^{-/-} BMMC and PCMC. Fc γ RIIB-dependent negative regulation, however, was efficient enough to prevent BMMC, but not PCMC, from responding to immune complexes.

One likely reason for this difference is that PCMC use less efficiently the lipid phosphatase SHIP1 than BMMC. Indeed, the deletion of SHIP1 differentially affected BMMC and PCMC, although both cell types contained similar amounts of this phosphatase. SHIP1 was shown to negatively regulate Fc ϵ RI signaling (25) and, recently, to account for the inhibition of IgE-induced responses of BMMC in excess of Ag (36). Little or no inhibition of β -hexosaminidase or histamine release was observed in IgE-sensitized PCMC stimulated by an excess of Ag. As expected, the deletion of SHIP1 increased both IgE- and immune complex-induced β -hexosaminidase release, and abrogated inhibition in excess of Ag in BMMC. Noticeably, however, SHIP1 deletion had no detectable effect on IgE- or immune complex-induced β -hexosaminidase release in PCMC. The finding that SHIP1 deletion did not affect immune complex-induced responses in PCMC is intriguing. Immune complexes indeed coengage Fc γ RIIIA and Fc γ RIIB in PCMC since they induced a higher release of β -hexosaminidase in Fc γ RIIB^{-/-} than in wt PCMC. The possibility that Fc γ RIIB-dependent

negative regulation might not depend on SHIP1 in PCMC was excluded by the observation that, like in BMMC, the deletion of SHIP1 abrogated Fc γ RIIB-dependent negative regulation of IgE-induced release of β -hexosaminidase in PCMC. SHIP1 is therefore also used by Fc γ RIIB in PCMC, although, for an unknown reason, less efficiently than in BMMC. Whatever the reason, these results indicate that differentiation-dependent regulatory mechanisms, which control Fc γ RIIB-dependent SHIP1-mediated negative regulation of cell activation determine the ability of mast cells to respond to IgG immune complexes. This may apply to human basophils, which express Fc γ RIIA and Fc γ RIIB and which do not respond to Fc γ RII aggregation (22). Supporting the possibility that a differential use of SHIP1 may determine biological responses of basophils, anti-IgE-induced histamine release by basophils from different donors was inversely correlated with the extent of SHIP1 phosphorylation, although basophils from nonresponders, moderate responders, and high responders contained similar amounts of SHIP1 (43). Noticeably, we found that SHIP1 phosphorylation was of a lower magnitude in PCMC than in BMMC, when challenged with IgE and Ag (data not shown).

Another distinctive feature of PCMC is that their biological responses differ quantitatively and qualitatively, from BMMC responses. With a few exceptions, Fc ϵ RI aggregation triggered the same responses in BMMC and PCMC. These responses, however, markedly differed by their relative intensities. BMMC and PCMC released comparable percentages of β -hexosaminidase and comparable percentages of histamine. PCMC, however, contained almost 10-fold higher amounts of β -hexosaminidase and, like peritoneal mast cells, $\sim$ 100-fold higher amounts of histamine than BMMC. Consequently, PCMC released much more granular mediators than BMMC within the first minutes of activation via Fc ϵ RI. By contrast, PCMC produced much lower amounts of lipid mediators during the first half hour of stimulation, and no MIP-1 α during the first hours. They also secreted much less TNF- α than BMMC. Noticeably, no TNF- α was detected in supernatants of either cell type at 10 min, when degranulation was completed, and no TNF- α was detected by intracellular immunofluorescence in nonstimulated cells, indicating that this cytokine is not stored in BMMC or PCMC granules. TNF- α , however, became detectable intracellularly, several hours following stimulation. Fewer numbers of PCMC than BMMC contained intracellular TNF- α , even following PMA plus ionomycin stimulation, indicating that PCMC not only secreted, but also synthesized less cytokines than BMMC. Altogether these data indicate that early responses are more robust than late responses in PCMC, whereas late responses are more robust than early responses in BMMC.

Another biological response was unique to PCMC. Indeed, Fc ϵ RI aggregation triggered a release of proteolytic activity in PCMC, but not in BMMC. The responsible proteases were not identified. They hydrolyzed cleavage sites that are rare in low m.w. proteins such as casein, and more frequent in high m.w. proteins such as SHIP1. They are present in resting cells because proteolysis was observed in PCMC lysates before stimulation. Noticeably, proteases released in PCMC supernatants did not account for the low amounts of TNF- α found in these supernatants. The amount of TNF- α secreted by IgE-sensitized BMMC in response to Ag was indeed not lower when these were mixed with equals numbers of IgE-sensitized PCMC (data not shown). Proteases were released concomitantly with β -hexosaminidase and histamine upon Fc ϵ RI aggregation, and their concentration in supernatants did not increase with time after 10 min. These data altogether suggest that PCMC-specific proteases are preformed and released upon degranulation. A variety of proteases were reported to be stored in mast cell granules, and their expression pattern were re-

ported to vary with the tissue where mast cells are located (5). PCMC expressed several mMCP transcripts. None of these, however, was selectively expressed in PCMC.

The various molecules that are sequentially produced and released by activated mast cells are thought, altogether, to account for the clinical expression pattern of allergies and inflammatory diseases. Vasoactive granular mediators, especially histamine, account for the local and systemic manifestations of the immediate allergic reaction (44). Histamine was also shown to mediate IgG immune complex-induced, Fc γ RIIA-dependent inflammation in the K/BxN model of autoimmune arthritis (45). It was recently reported to act as a T cell chemotactic factor via type 1 histamine receptors (46). Mast cell proteases induce a variety of biological effects (47), most of which are triggered via the activation of protease-activated receptor-2 (PAR-2) (48). PAR-2 is expressed by neutrophils, endothelial cells, vascular smooth muscle cells, neurons and glial cells, enterocytes, keratinocytes, and many tumor cells. PAR-2 activation is involved in the control of blood pressure and plasma extravasation, in neutrophil infiltration and proliferation, in the induction of pain and, by stimulating the phagocytosis of melanosomes by keratinocytes, in the control of skin pigmentation. PAR-2 also induces keratinocytes to proliferate and to secrete cytokines. Interestingly, PAR-2 is up-regulated in asthma and rheumatoid arthritis. Lipid mediators account for the late-phase reaction which develops locally. Cytokines and chemokines account for the chronic inflammatory reaction, which is responsible for most of the long-lasting clinical symptoms of allergic diseases. Among other cytokines, TNF- α , which induces bronchial hyper-responsiveness (49), airway infiltration by neutrophils and eosinophils (50), activation of airway smooth muscles (51) and myofibroblasts (52), and which up-regulates the expression of adhesion molecules (53), was recognized as playing a major role in asthma-associated remodeling and pulmonary inflammation, especially in asthma refractory to corticosteroid therapy (54). TNF- α also critically contributes to the pathogenesis of rheumatoid arthritis (55, 56).

Although poor releasers of granular mediators, BMMC are potent secretors of proinflammatory chemokines and cytokines. Physiological BMMC equivalents may exist in the bone marrow and, transiently, in the circulation, but not in peripheral tissues. They therefore cannot account for tissue inflammation. If, as discussed above, PCMC result from an expansion of pre-existing peritoneal mast cells and are representative of serosal-type mast cells, these mast cells cannot either account for tissue inflammation. They are indeed poor secretors of cytokines. Other cells, which are known to converge to allergic sites and which infiltrate tissues, are required for inflammation to be generated. The massive amounts of vasoactive mediators and proteases that are released by PCMC within minutes should greatly facilitate the subsequent constitution of an inflammatory infiltrate. One may therefore speculate that serosal-type mast cells function as promoters rather than as effectors of inflammation in allergies and autoimmune diseases.

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Disclosures

The authors have no financial conflict of interest.

References

- Bach, J. F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *N. Engl. J. Med.* 347: 911–920.
- Nadler, M. J., S. A. Matthews, H. Turner, and J.-P. Kinet. 2000. Signal transduction by the high-affinity immunoglobulin E receptor FcεRI: coupling form to function. *Adv. Immunol.* 76: 325–355.
- Lee, D. M., D. S. Friend, M. F. Gurish, C. Benoist, D. Mathis, and M. B. Brenner. 2002. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* 297: 1689–1692.
- Robbie-Ryan, M., M. B. Tanzola, V. H. Secor, and M. A. Brown. 2003. Cutting edge: both activating and inhibitory Fc receptors expressed on mast cells regulate experimental allergic encephalomyelitis disease severity. *J. Immunol.* 170: 1630–1634.
- Miller, H. R., and A. D. Pemberton. 2002. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology* 105: 375–390.
- Triggiani, M., A. Oriente, G. de Crescenzo, and G. Marone. 1995. Metabolism of lipid mediators in human basophils and mast cells. *Chem. Immunol.* 61: 135–147.
- Galli, S. J., J. R. Gordon, and B. K. Wershil. 1991. Cytokine production by mast cells and basophils. *Curr. Opin. Immunol.* 3: 865–873.
- Kaplan, A. P. 2001. Chemokines, chemokine receptors and allergy. *Int. Arch. Allergy Immunol.* 124: 423–431.
- Gurish, M. F., and J. A. Boyce. 2002. Mast cell growth, differentiation, and death. *Clin. Rev. Allergy Immunol.* 22: 107–118.
- Ihle, J. N., J. Keller, S. Oroszlan, L. E. Henderson, T. D. Copeland, F. Fitch, M. B. Prystowsky, E. Goldwasser, J. W. Schrader, E. Palaszynski, et al. 1983. Biologic properties of homogeneous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, p cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. *J. Immunol.* 131: 282–287.
- Galli, S. J., K. M. Zsebo, and E. N. Geissler. 1994. The kit ligand, stem cell factor. *Adv. Immunol.* 55: 1–96.
- Welle, M. 1997. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *J. Leukocyte Biol.* 61: 233–245.
- Turner, H., and J. P. Kinet. 1999. Signalling through the high-affinity IgE receptor FcεRI. *Nature* 402: B24–B30.
- Dombrowicz, D., V. Flamand, K. K. Brigman, B. H. Koller, and J.-P. Kinet. 1993. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor α chain gene. *Cell* 75: 969–976.
- Benhamou, M., C. Bonnerot, W. H. Fridman, and M. Daéron. 1990. Molecular heterogeneity of murine mast cell Fcγ receptors. *J. Immunol.* 144: 3071–3077.
- Daéron, M., C. Bonnerot, S. Latour, and W. H. Fridman. 1992. Murine recombinant FcγRIII, but not FcγRII, trigger serotonin release in rat basophilic leukemia cells. *J. Immunol.* 149: 1365–1373.
- Hazenbos, L. W., J. E. Gessner, F. M. A. Hofhuis, H. Kuipers, D. Meyer, I. A. F. M. Heijnen, R. E. Schmidt, M. Sandor, P. J. A. Capel, M. Daéron, et al. 1996. Impaired IgG-dependent anaphylaxis and Arthus reaction in FcγRIII (CD16) deficient mice. *Immunology* 5: 181–188.
- Blank, U., C. Ra, L. Miller, K. White, H. Metzger, and J. P. Kinet. 1989. Complete structure and expression in transfected cells of high affinity IgE receptor. *Nature* 337: 187–189.
- Kurosaki, T., I. Gander, U. Wirthmueller, and J. V. Ravetch. 1992. The β subunit of the FcεRI is associated with the FcγRIII on mast cells. *J. Exp. Med.* 175: 447–451.
- Daéron, M., O. Malbec, S. Latour, M. Arock, and W. H. Fridman. 1995. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *J. Clin. Invest.* 95: 577–585.
- Takai, T., M. Ono, M. Hikida, H. Ohmori, and J. V. Ravetch. 1996. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature* 379: 346–349.
- Daéron, M., S. Latour, O. Malbec, E. Espinosa, P. Pina, S. Pasmans, and W. H. Fridman. 1995. The same tyrosine-based inhibition motif, in the intracytoplasmic domain of FcγRIIB, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. *Immunology* 3: 635–646.
- Ono, M., S. Bolland, P. Tempst, and J. V. Ravetch. 1996. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor FcγRIIB. *Nature* 383: 263–266.
- Fong, D. C., O. Malbec, M. Arock, J. C. Cambier, W. H. Fridman, and M. Daéron. 1996. Selective in vivo recruitment of the phosphatidylinositol phosphatase SHIP by phosphorylated FcγRIIB during negative regulation of IgE-dependent mouse mast cell activation. *Immunol. Lett.* 54: 83–91.
- Huber, M., C. D. Helgason, J. E. Damen, L. Liu, R. K. Humphries, and G. Krystal. 1998. The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation. *Proc. Natl. Acad. Sci. USA* 95: 11330–11335.
- Wershil, B. K., and S. J. Galli. 1994. The analysis of mast cell function in vivo using mast cell-deficient mice. *Adv. Exp. Med. Biol.* 347: 39–54.
- Vaz, N. M., and A. Prouvost-Danon. 1969. Behaviour of mouse mast cells during in vitro anaphylaxis. *Progr. Allergy* 13: 111–173.
- Sterk, A. R., and T. Ishizaka. 1982. Binding properties of IgE receptors on normal mouse mast cells. *J. Immunol.* 128: 838–843.
- Malbec, O., M. Malissen, I. Isnardi, R. Lesourne, A.-M. Mura, W. H. Fridman, B. Malissen, and M. Daéron. 2004. Linker for activation of T cells integrates positive and negative signaling in mast cells. *J. Immunol.* 173: 5086–5094.
- Holmes, K. L., R. G. E. Palfree, U. Hammerling, and H. C. Morse. 1985. Alleles of the Ly-17 alloantigen define polymorphism of a murine IgG Fc receptor. *Proc. Natl. Acad. Sci. USA* 82: 7706–7710.
- Liu, F. T., J. W. Bohn, E. L. Ferry, H. Yamamoto, and C. A. Molinaro. 1980. Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation and characterization. *J. Immunol.* 124: 2728–2737.
- Schiller, C., I. Janssen-Graalfs, U. Baumann, K. Schwerter-Strumpf, S. Izui, T. Takai, R. E. Schmidt, and J. E. Gessner. 2000. Mouse FcγRII is a negative regulator of FcγRIII in IgG immune complex-triggered inflammation but not in autoantibody-induced hemolysis. *Eur. J. Immunol.* 30: 481–490.
- Latour, S., C. Bonnerot, W. H. Fridman, and M. Daéron. 1992. Induction of tumor necrosis factor-α production by mast cells via FcγR: role of the FcγRIIIγ subunit. *J. Immunol.* 149: 2155–2162.
- Siraganian, R. P. 1974. An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal. Biochem.* 57: 383–394.
- Ono, M., H. Okada, S. Bolland, S. Yanagi, T. Kurosaki, and J. V. Ravetch. 1997. Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. *Cell* 90: 293–301.
- Gimborn, K., E. Lessmann, S. Kuppig, G. Krystal, and M. Huber. 2005. SHIP down-regulates FcεRI-induced degranulation at supraoptimal IgE or antigen levels. *J. Immunol.* 174: 507–516.
- Takagi, M., T. Nakahata, T. Kubo, M. Shiohara, K. Koike, A. Miyajima, K. Arai, S. Nishikawa, K. M. Zsebo, and A. Komiyama. 1992. Stimulation of mouse connective tissue-type mast cells by hemopoietic stem cell factor, a ligand for the c-kit receptor. *J. Immunol.* 148: 3446–3453.
- Karimi, K., F. A. Redegeld, B. Heijdra, and F. P. Nijkamp. 1999. Stem cell factor and interleukin-4 induce murine bone marrow cells to develop into mast cells with connective tissue type characteristics in vitro. *Exp. Hematol.* 27: 654–662.
- Yamada, N., H. Matsushima, Y. Tagaya, S. Shimada, and S. I. Katz. 2003. Generation of a large number of connective tissue type mast cells by culture of murine fetal skin cells. *J. Invest. Dermatol.* 121: 1425–1432.
- Ovary, Z. 1965. PCA reaction and its elicitation by specific immunoglobulin species and fragments. *Fed. Proc.* 24: 94–97.
- Daéron, M., J. Couderc, M. Ventura, and G.-A. Voisin. 1982. Anaphylactic properties of mouse monoclonal IgG2a antibodies. *Cell. Immunol.* 70: 27–40.
- Hirayama, N., T. Hirano, G. Kohler, A. Kurata, K. Okumura, and Z. Ovary. 1982. Biological activities of anti-trinitrophenyl and anti-dinitrophenyl mouse monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* 79: 613–615.
- Gibbs, B. F., A. Rathling, D. Zillikens, M. Huber, and H. Haas. 2006. Initial FcεRI-mediated signal strength plays a key role in regulating basophil signaling and deactivation. *J. Allergy Clin. Immunol.* 118: 1060–1067.
- Makabe-Kobayashi, Y., Y. Hori, T. Adachi, S. Ishigaki-Suzuki, Y. Kikuchi, Y. Kagaya, K. Shirato, A. Nagy, A. Ujike, T. Takai, et al. 2002. The control effect of histamine on body temperature and respiratory function in IgE-dependent systemic anaphylaxis. *J. Allergy Clin. Immunol.* 110: 298–303.
- Binstadt, B. A., P. R. Patel, H. Alencar, P. A. Nigrovic, D. M. Lee, U. Mahmood, R. Weissleder, D. Mathis, and C. Benoist. 2006. Particularities of the vasculature can promote the organ specificity of autoimmune attack. *Nat. Immunol.* 7: 284–292.
- Bryce, P. J., C. B. Mathias, K. L. Harrison, T. Watanabe, R. S. Geha, and H. C. Oettgen. 2006. The H1 histamine receptor regulates allergic lung responses. *J. Clin. Invest.* 116: 1624–1632.
- Hallgren, J., and G. Pejler. 2006. Biology of mast cell tryptase: an inflammatory mediator. *FEBS J.* 273: 1871–1895.
- Ossovskaya, V. S., and N. W. Bunnett. 2004. Protease-activated receptors: contribution to physiology and disease. *Physiol. Rev.* 84: 579–621.
- Thomas, P. S., D. H. Yates, and P. J. Barnes. 1995. Tumor necrosis factor-α increases airway responsiveness and sputum neutrophilia in normal human subjects. *Am. J. Respir. Crit. Care Med.* 152: 76–80.
- Lukacs, N. W., R. M. Strieter, S. W. Chensue, M. Widmer, and S. L. Kunkel. 1995. TNF-α mediates recruitment of neutrophils and eosinophils during airway inflammation. *J. Immunol.* 154: 5411–5417.
- Amrani, Y., H. Chen, and R. A. Panettieri, Jr. 2000. Activation of tumor necrosis factor receptor 1 in airway smooth muscle: a potential pathway that modulates bronchial hyper-responsiveness in asthma? *Respir. Res.* 1: 49–53.
- Thomas, P. S. 2001. Tumour necrosis factor-α: the role of this multifunctional cytokine in asthma. *Immunol. Cell Biol.* 79: 132–140.
- Thornhill, M. H., S. M. Wellicome, D. L. Mahiouz, J. S. Lanchbury, U. Kyan-Aung, and D. O. Haskard. 1991. Tumor necrosis factor combines with IL-4 or IFN-γ to selectively enhance endothelial cell adhesiveness for T cells: the contribution of vascular cell adhesion molecule-1-dependent and -independent binding mechanisms. *J. Immunol.* 146: 592–598.
- Berry, M. A., B. Hargadon, M. Shelley, D. Parker, D. E. Shaw, R. H. Green, P. Bradding, C. E. Brightling, A. J. Wardlaw, and I. D. Pavord. 2006. Evidence of a role of tumor necrosis factor α in refractory asthma. *N. Engl. J. Med.* 354: 697–708.
- Feldmann, M., and R. N. Maini. 2003. Lasker Clinical Medical Research Award: TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. *Nat. Med.* 9: 1245–1250.
- Hsu, H. C., Y. Wu, and J. D. Mountz. 2006. Tumor necrosis factor ligand-receptor superfamily and arthritis. *Curr. Dir. Autoimmun.* 9: 37–54.