

Apoptosis of mouse mast cells is reciprocally regulated by the IgG receptors Fc γ RIIB and Fc γ RIIIA

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Keywords

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Abstract

Background: Mast cells are important effector cells in allergy. They usually have a long life span and resist cell death induction. Fc γ receptor- and IgG immune complex-mediated apoptosis has been demonstrated in B-lineage cells, but not in mast cells. The aim of the current study was to investigate whether mast cells could respond to apoptosis induction by IgG immune complex aggregation of Fc γ receptors. It is known that mouse mast cells express the low-affinity Fc γ receptors Fc γ RIIB and Fc γ RIIIA, which bind IgG especially in the form of antigen-IgG immune complexes.

Methods: Mouse bone marrow-derived cultured mast cells were examined for surface expression of Fc γ RIIB and Fc γ RIIIA. Apoptosis of such cells from wild-type, Fc γ RIIB^{-/-} or Fc γ RIIIA^{-/-} mice was measured following receptor aggregation by IgG immune complexes.

Results: Our data demonstrate that aggregation of either Fc γ RIIB or Fc γ RIIIA by IgG immune complexes induced apoptosis of mouse bone marrow-derived cultured mast cells. However, mast cells expressing both Fc γ RIIB and Fc γ RIIIA were able to resist cell death induction by IgG immune complexes.

Conclusion: Our findings reveal a fine-tuning system for regulating mast cell apoptosis through aggregating Fc γ receptors by IgG immune complexes. Such apoptosis regulation may have a substantial impact on mast cell homeostasis during allergic inflammation.

Allergic inflammation involves the participation of a wide variety of cell types, including mast cells. In allergy, multivalent antigens bind and crosslink IgE molecules bound to the high-affinity IgE receptor (Fc ϵ RI) expressed on mast cells and basophils, resulting in allergic inflammation through the release of proinflammatory mediator molecules. The number of mast cells is increased in tissue sites of allergic inflammation, and there is a correlation between the number of mast cells in the tissue and the severity of the allergic symptoms. The most commonly used drugs for allergy treatments, such as sodium cromoglycates and antihistamines, target mast

cells or their released products. Of note, mast cells have a long life span and common pathways that normally kill immune cells do not operate efficiently in mast cells. Furthermore, mast cells do not die upon activation but instead demonstrate activation-induced survival (1, 2), making them one of the most long-lived cell types. Thus, research on conditions leading to mast cell apoptosis and the mechanisms involved would provide valuable knowledge for developing novel therapeutic strategies against allergic disorders that involve mast cells.

Mast cells can be activated to express effector functions by many mechanisms that are independent of Fc ϵ RI. In humans, but not in mice, mast cells can be activated to degranulate in response to antigen-IgG immune complexes through the activating IgG receptor Fc γ RI, which is up-regulated by interferon- γ (3). In mice, such IgG-mediated elicitation of mast cell function is achieved through the activating

Abbreviations

BMMC bone marrow-derived cultured mast cell; CTMC connective tissue mast cell; MMC mucosal mast cell; SIT specific allergen immunotherapy.

receptor Fc γ RIIA (3, 4). The intracytoplasmic domains of Fc γ RIIA contain immunoreceptor tyrosine-based activation motifs (ITAM). In addition to Fc γ RIIA, mouse mast cells express the inhibitory IgG receptor Fc γ RIIB, which contains immunoreceptor tyrosine-based inhibition motifs (ITIM) in its intracytoplasmic domain and is critical for down-modulating immune responses (4, 5). In humans, three isoforms of Fc γ R exist: Fc γ RIIA and Fc γ RIIC, which are activating receptors, and Fc γ RIIB, which is an inhibitory receptor. Mice express only the inhibitory isoform, that is, Fc γ RIIB.

When coaggregated on the same cell with receptors bearing ITAM, Fc γ RIIB negatively regulates ITAM-mediated cell activation. Co-crosslinking of the Fc γ RIIB with the activating receptors inhibits the function of the latter on many immune effector cells (5). Fc γ RIIB-deficient mice have increased production of antibodies leading to autoimmunity (6, 7). These mice are more susceptible to allergic stimulations (5). Fc γ RIIB inhibits SCF-induced mast cell survival (8). Therefore, Fc γ RIIB is described as a 'brake' which plays a key role in limiting the duration of immune responses, avoiding the development of autoimmunity or allergy.

Our previous work and that of others demonstrate that, in addition to the immuno-inhibitory effect, Fc γ RIIB can induce cell apoptosis in B-lineage cells, including naive B cells, memory cells, plasmablasts, and terminally differentiated plasma cells (9–12). The apoptotic signal is generated when Fc γ RIIB molecules are crosslinked to themselves. As Fc γ RIIB-mediated inhibition of cell activation has been confirmed in various types of immune effector cells expressing this receptor, we speculated that Fc γ RIIB-mediated cell death might exist in other cell types, for example mast cells, in addition to B-lineage cells. In this study, we provide evidence that Fc γ RIIB crosslinking by IgG immune complexes induces mouse mast cell apoptosis. Furthermore, we unexpectedly observed Fc γ RIIA-mediated apoptosis by IgG immune complexes. A reciprocal regulation loop involving these two receptors was identified.

Methods

Mice

C57BL/6 mice were purchased from Taconic Farms (Ry, Denmark). Fc γ RIIB^{-/-} mice were either purchased from The Jackson Laboratory (a mixed C57BL/6;129 genetic background) or obtained from Prof Ken Smith (on the C57BL/6 background). No differences have been observed in mast cell cultures derived from these two strains of mice under our experimental conditions. Fc γ RIIA^{-/-} mice (on the C57BL/6 background) were purchased from The Jackson Laboratory. All animal procedures were carried out with the approval of the Ethical Committee for Laboratory Animals in Gothenburg.

Cell culture and characterization

Bone marrow-derived cultured mast cells (BMMCs) were maintained at 37°C in 5% CO₂ in RPMI 1640 medium containing 4 mM L-glutamine supplemented with 10% FCS,

50 μ M 2-ME, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cell culture reagents were purchased from Sigma-Aldrich, Steinheim, Germany. To support mast cell differentiation, the medium was further supplemented with 10% conditioned medium from X63/0 myeloma cells transfected with an IL-3 expression vector as a source of IL-3 (from Dr. J. Hallgren-Martinsson, Uppsala University, Uppsala, Sweden). We also differentiated bone marrow cells into mast cells with a connective tissue type (CTMC) or mucosal type (MMC) as reported (13). Essentially, CTMC were obtained by culturing mouse bone marrow cells in RPMI 1640 medium supplemented with 25 ng/ml SCF and 1 ng/ml murine rIL-4 (Immunotools) in addition to the regular additives. Alternatively, MMC were produced in DMEM containing IL-3 (5% supernatant of X63/0 myeloma cells) supplemented with 25 ng/ml recombinant murine SCF, 5 ng/ml recombinant murine IL-9, and 1 ng/ml recombinant human TGF- β 1 (PeproTech, Rocky Hill, NJ, USA) in addition to regular additives.

Mast cell differentiation was examined by measuring cell surface expression of cKit and Fc ϵ RI using PE-conjugated rat anti-mouse cKit (CD117)(Clone ACK4; ImmunoTools, Friesoythe, Germany) and Alexa Fluor 647-conjugated armenian hamster anti-mouse Fc ϵ RI alpha (Clone MAR-1; eBioscience, San Diego, CA, USA). Mast cell differentiation was also confirmed by toluidine blue staining. To this end, cell cultures were cytocentrifuged, air-dried, incubated for 15 min with 0.5% toluidine blue in 0.5 M HCL, and rinsed in water. Cell surface expression of Fc γ RIIA was measured by FITC-conjugated rat anti-mouse Fc γ RIIA (Clone 275003; mouse IgG2a, R&D Systems, Minneapolis, USA). In some experiments, the surface expression of Fc γ RIIA by Fc γ RIIB-deficient cells was also measured by an Alexa Fluor 488-conjugated rat anti-mouse Fc γ RIIB/IIIA antibody (Clone FCR-4G8; CALTAG Laboratories, Carlsbad, CA, USA).

Mast cell degranulation assay

Bone marrow-derived cultured mast cells were sensitized with 1 μ g/ml IgE anti-DNP (Clone SPE-7; Sigma-Aldrich) for 1 h and were subsequently washed and challenged with 100 μ g/ml DNP-₃₀₋₄₀-HSA (Sigma-Aldrich) for 30 min at 37°C in serum-free medium. The supernatant was collected for β -hexosaminidase measurement as a readout for mast cell degranulation. Briefly, the supernatant was mixed with an identical volume of 4-nitrophenyl N-acetyl- β -D-glucosaminide (Sigma-Aldrich), the substrate of β -hexosaminidase, and incubated for 1 h at 37°C. The reaction was stopped by the addition of an equal volume of 0.2 M glycine (Sigma-Aldrich) (pH 10). The absorbance at 405 nm was measured using a spectrophotometer.

In vitro crosslinking of Fc γ RIIB

Cells were treated with ovalbumin (OVA) immune complexes generated by incubating mouse IgG1 anti-OVA (Sigma-Aldrich) and OVA (Sigma-Aldrich) for 30 min at 37°C.

Eighty microlitre of cells was mixed with 20 μ l of the immune complex solution in a 96-well plate and incubated at 37°C in 5% CO₂ for 16 h in the cell culture medium. The final concentrations of IgG and OVA were 280 μ g/ml and 130 μ g/ml, respectively. In some experiments, cells were incubated with 10 μ g/ml anti-Fc γ RIIIA antibody (Clone 275003; mouse IgG2a, R&D Systems) to block Fc γ RIIIA before the addition of the immune complex.

Apoptosis assays

Apoptosis was measured by two methods. Most of the analyses were carried out by flow cytometry determining the profile of Annexin V (BD Biosciences) and 7-amino-actinomycin D (7-AAD; Sigma-Aldrich) staining. In some experiments, apoptosis was also measured by flow cytometry analyzing the propidium iodide (PI, Sigma-Aldrich) intercalation following fixation and permeabilization of the cells by ethanol.

Real-time RT-PCR

RNA was prepared with the RNeasy kit (Qiagen, Hilden, Germany) and cDNA was synthesized using the SuperScript cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Primers specific for the amplification of mouse *fcgr2b*, *fcgr3*, and hypoxanthine phosphoribosyltransferase (HPRT) RNA transcripts were designed using the online Universal ProbeLibrary Assay Design Center (Roche Applied Science, Mannheim, Germany). The *fcgr2b* primer sequences were 5'-CAG CGACCCTGTAGATCTGG-3' and 5'-CCCCTTCAGAAA CACCA-3' (77-nt amplicon). The *fcgr3* primer sequences were 5'-CAAGCCTGTACCATCACTG-3' and 5'-GAG GCACATCACTAGGGAGAA-3' (94-nt amplicon). The HPRT primer sequences were 5'-TCCTCCTCAGACCG CTTT-3' and 5'-CCTGGTTCATCATCGCTAATC-3' (90-nt amplicon). The probes for *fcgr2b* and *fcgr3* were the Universal ProbeLibrary probe numbers 50 and 88, respectively. The probe for HPRT was 5'-RED-CGTCATGCCGACCCGC AGT-BHQ2-3'. Cycling was performed under the following conditions: denaturation at 95°C for 10 min, followed by 45 cycles of PCR (95°C for 10 s, 60°C for 30 s, and 72°C for 1 s) and finished with a final extension at 40°C for 10 s. PCR was performed in duplicate using a final concentration of 1 μ M both for the probe and for each of the primers. The amplification process was conducted in the LightCycler 480 system (Roche Diagnostics). A calibrator cDNA sample was used in all of the measurements for internal calibration. The abundance of *fcgr2b* and *fcgr3* mRNA was assessed relative to that of HPRT mRNA by the LightCycler Relative Quantification software (Roche Diagnostics).

Cell proliferation assay

Cells were stained with PI following fixation and permeabilization by ethanol, and PI intercalation was determined by

flow cytometry. Proliferating cells were represented by the superdiploid population.

Statistical analysis

Data were analyzed for statistical significance using an unpaired *t* test in GraphPad Prism software.

Results

Differential responsiveness to apoptosis induction by BMMC of varying culture durations

We have previously demonstrated that immune complexes made of OVA and anti-OVA IgG (OVA IC) are able to induce plasma cell apoptosis by crosslinking Fc γ RIIB (12). To further investigate whether mast cells would undergo apoptosis following treatment with immune complexes, we incubated BMMC with OVA alone or OVA IC. Apoptosis was measured by flow cytometry analyzing Annexin V and 7-AAD staining profiles. As the routine culture duration to obtain functional BMMC is 3 weeks, we first examined bone marrow cells cultured for 3 weeks in the presence of IL-3. Unlike B-lineage cells, BMMC cultured for 3 weeks did not respond to apoptosis induction as a result of OVA IC treatment (Fig. 1C, upper panels). However, apoptosis was induced by OVA IC in BMMC cultured for 6 weeks (Fig. 1C, lower panels). The percentages of Annexin V⁺/7-AAD⁺ population that represented the apoptotic cells were 15.1 $\pm$ 3.8% following treatment with OVA only and 39.1 $\pm$ 14.5% following treatment with OVA IC.

The contribution of Fc γ RIIB to apoptosis in 6-week-old BMMCs after treatment with IgG immune complexes was then investigated. BMMCs from both Fc γ RIIB^{+/+} and Fc γ RIIB^{-/-} mice were cultured. Six-week-old Fc γ RIIB^{-/-} BMMC resisted apoptosis as a result of treatment with OVA IC, confirmed by two independent apoptosis measurements, Annexin V/7-AAD profiling (Fig. 2A) and PI intercalation (Figs 2B and S1). The data confirmed apoptosis induction through Fc γ RIIB.

The mechanisms underlying the differential responsiveness to OVA IC-mediated apoptosis by the 3- and 6-week-old BMMC cultures were then investigated (Fig. 1). Firstly, BMMC cultured for different periods of time were characterized. In the presence of X-63 conditioned medium containing IL-3, bone marrow cells from both Fc γ RIIB^{+/+} and Fc γ RIIB^{-/-} mice steadily developed mast cell markers, which included surface expression of Fc ϵ RI and cKit, as well as metachromatic granules revealed by toluidine blue staining. However, no substantial differences were observed between 3- and 6-week-old cultures (Fig. S2A–C). Next, antigen- and IgE-dependent aggregation of the high-affinity IgE receptor Fc ϵ RI, the best-studied mast cell activation model by which mast cells participate in immune regulation, was tested. Both the 3- and 6-week-old cultured cells degranulated to a comparable extent upon treatment with IgE followed by the addition of antigen (Fig. S2D).

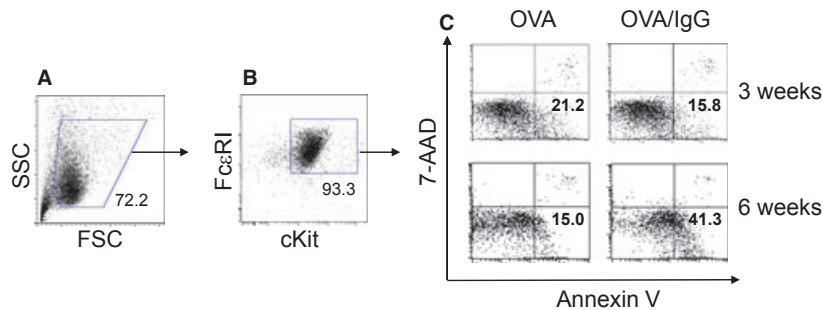


Figure 1 Apoptotic profile of bone marrow-derived cultured mast cells (BMMC) after IgG immune complex treatment. The BMMC derived from C57BL/6 mice had been cultured for 3 or 6 weeks followed by treatment with ovalbumin (OVA) only or immune complexes composed of OVA and anti-OVA IgG for 16 h. Mast cell apoptosis was analyzed by flow cytometry. An example is given to show the gating strategy (A, B). The numbers adjacent to the

boxed areas indicate percentage of cells in each. The debris- and shrunken cell-free compartment as shown in (A) and cKit⁺/FcγRI⁺ mast cells (B) were further analyzed in the apoptosis measurement (C). The apoptotic cells were revealed by the Annexin V⁺/7-AAD⁻ quadrants, with numbers indicating the cell frequency (%). One experiment representing at least six separate experiments is shown.

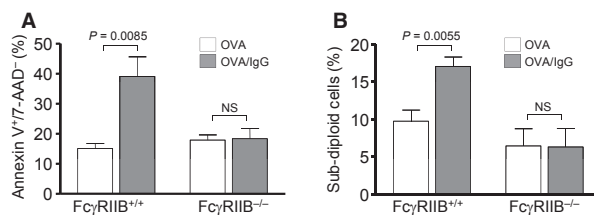


Figure 2 Apoptotic profile of 6-week-old bone marrow-derived cultured mast cells (BMMC) after IgG immune complex treatment. (A) The BMMCs derived from wild-type mice and FcγRIIB-deficient mice were treated and analyzed as explained in Fig. 1. The percentages of apoptotic cells (Annexin V⁺/7-AAD⁻) were plotted. (B) The cells were treated as in (A), but apoptosis was analyzed by PI intercalation following permeabilization of cells. The percentages of the subdiploid population representing the apoptotic cells as shown in Fig. S1 were plotted. Data are expressed as mean + SEM of five separate experiments.

Mast cells are still capable of proliferation after maturation (14, 15). Cell proliferation may be a confounding factor for analyzing and comparing apoptosis, even though under our culture conditions, BMMCs after 3 weeks of culture do not proliferate to a great extent. However, to examine the possibility that the apoptosis data were biased by proliferation, the proportion of superdiploid cells representing proliferating cells were measured by PI intercalation in both 3- and 6-week-old cells of both FcγRIIB^{+/+} and FcγRIIB^{-/-} origin. Although 3-week-old cells proliferated to a greater level than the 6-week-old cells, no differences were observed between control and OVA IC treatments (Fig. S3). Apoptosis measurements were therefore not biased by altered proliferation in different treatment groups.

Inhibition of FcγRIIB-mediated apoptosis by FcγRIIIA

In contrast to B cells in which FcγRIIB is the only Fcγ receptor, mouse mast cells may express FcγRIIIA, an activat-

ing IgG receptor, in addition to the inhibitory receptor FcγRIIB (4). We hypothesized that FcγRIIIA may counteract the proapoptotic signaling induced by FcγRIIB and that FcγRIIIA is differentially expressed in 3- and 6-week-old cultures of BMMC. We therefore measured the surface expression of FcγRIIIA on cells cultured for 3 and 6 weeks using an FcγRIIIA-specific antibody (16). As shown in Fig. 3A, the surface expression of FcγRIIIA on both FcγRIIB^{+/+} and FcγRIIB^{-/-} cells was indeed down-regulated after 6 weeks in culture compared to 3 weeks. The down-regulation of FcγRIIIA was also confirmed on FcγRIIB^{-/-} cells using a second antibody (Fig. 3B). Although this antibody (clone FCR-4G8) recognized both FcγRIIB and IIIA, the measurement should only reflect the expression of FcγRIIIA on FcγRIIB-deficient cells. Thus, both antibodies gave consistent results. We also measured the mRNA levels of FcγRIIIA in BMMC derived from FcγRIIB^{+/+} and FcγRIIB^{-/-} mice. In both cell cultures, mRNA expression was reduced at 6 weeks compared with 3 weeks (Fig. 3C). Importantly, FcγRIIB expression at least at the mRNA level was not up-regulated during prolonged culture of BMMC (Fig. 3D). Taken together, these results suggest that BMMC cultured for 3 weeks may have a level of FcγRIIIA expression sufficient to antagonize the proapoptotic signaling induced by FcγRIIB aggregation. In contrast, BMMC cultured for 6 weeks down-regulated the expression of FcγRIIIA, and, as a consequence, treatment with IgG immune complexes most likely aggregated FcγRIIB exclusively, leading to increased apoptosis. To confirm the contribution of FcγRIIIA, we blocked FcγRIIIA on wild-type BMMC cultured for 3 weeks with a specific blocking antibody (16) and then incubated the cells with OVA IC. As predicted, blocking FcγRIIIA restored the responsiveness of these cells to apoptosis induction by OVA IC (Fig. 4A). Incubation with an isotype control antibody did not affect apoptosis induction (data not shown). Furthermore, we cultured BMMC from FcγRIIIA^{-/-} mice and compared their apoptosis profile with that from the wild-type mice. Unlike wild-type BMMC, FcγRIIIA^{-/-} BMMC cultured for

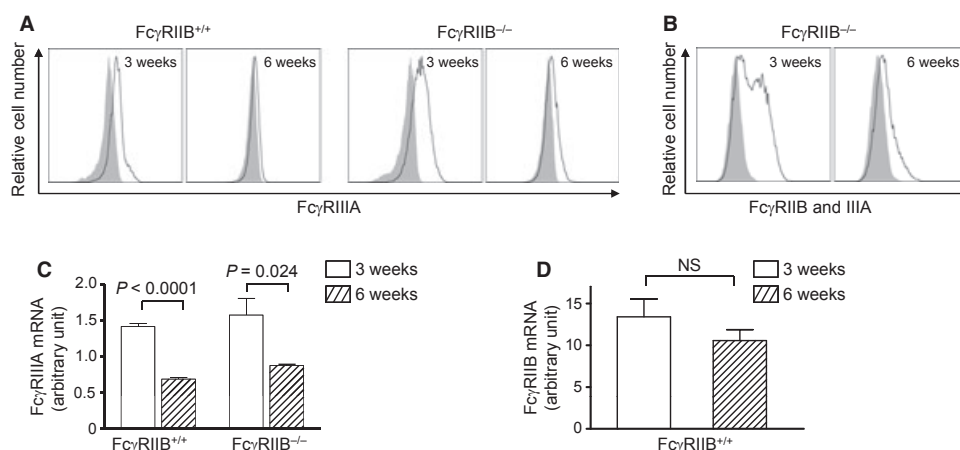


Figure 3 Expression of FcγRIIIA and FcγRIIB by bone marrow-derived cultured mast cells (BMMC) during prolonged culture time. (A and B) Flow cytometry histograms of cell surface staining of FcγRIIIA (A) and FcγRIIB/IIIA (B) on BMMC derived from either wild-type or FcγRIIB-deficient mice for 3 weeks or 6 weeks are shown. The culture time is indicated in the inset. Solid histograms, isotype control antibody; lines, anti-FcγRIIIA-specific antibody (A) or an anti-

body recognizing both FcγRIIB and IIIA (B). (C and D) Relative mRNA expression of FcγRIIIA (C) and FcγRIIB (D) by 3- or 6-week-old BMMC derived from the strains of mice as labeled was evaluated by quantitative real-time PCR analysis. All PCR data shown are normalized to HPRT. One histogram profile representing three separate cultures is shown (A and B), or data are expressed as mean + SEM of four (C) or three (D) experiments carried out in duplicate.

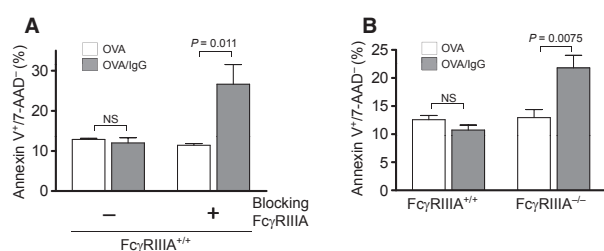


Figure 4 Apoptotic profile of 3-week-old bone marrow-derived cultured mast cells (BMMC) in relation to the presence or absence of FcγRIIIA. (A) 3-week-old BMMCs derived from wild-type mice were incubated with either blocking antibody against FcγRIIIA or medium followed by immune complex treatment and apoptosis analysis as explained in Fig. 1. (B) 3-week-old BMMCs derived from FcγRIIIA^{+/+} and FcγRIIIA^{-/-} mice were treated and analyzed as explained in Fig. 1. Data are expressed as mean + SEM of six separate experiments.

3 weeks showed increased apoptosis following treatment with OVA IC (Fig. 4B), suggesting that FcγRIIIA may indeed antagonize the induction of apoptosis mediated by FcγRIIB.

Although FcγRIIB is expressed by virtually all mouse mast cells, differential expression of FcγRIIIA on subtypes of mouse mast cells is described. In contrast to mouse mast cells of the connective tissue type (CTMC), which have constitutive and functional expression of FcγRIIIA, mucosal mast cells (MMC) demonstrate minimal expression of this receptor (3). We next cultured mouse bone marrow cells and drove their differentiation into CTMC and MMC, as reported (13). As predicted, CTMC expressed a substantial level of FcγRIIIA, which was absent in MMC (Fig. 5A). When these two types of cells were treated with OVA IC, apoptosis was

induced in MMC to a greater level than in CTMC (Fig. 5B). Taken together, these observations on CTMC and MMC are consistent with our finding on BMMC that FcγRIIIA may counteract FcγRIIB-mediated apoptosis.

Reciprocal interactions between FcγRIIB and FcγRIIIA in IgG immune complex-mediated apoptosis

As shown in Figs 1C and 4, wild-type BMMC cultured for 3 weeks resisted OVA IC-induced apoptosis. To our surprise, 3-week-old BMMC derived from FcγRIIB^{-/-} mice developed greater levels of apoptosis upon OVA IC treatment (Fig. 6A). This was an unexpected finding. As the 3-week-old BMMC expressed high levels of FcγRIIIA (Fig. 3A, B), the hypothesis that FcγRIIIA in the absence of FcγRIIB is able to induce apoptosis was suggested. To confirm this, we neutralized FcγRIIIA expression on FcγRIIB^{-/-} cells with a blocking antibody before incubation with OVA IC. As shown in Fig. 6B, blocking FcγRIIIA suppressed IgG immune complex-induced apoptosis of these cells, suggesting that IgG immune complexes could aggregate FcγRIIIA and induce apoptosis in the absence of FcγRIIB. Thus, aggregating either of the two receptors, FcγRIIB or FcγRIIIA, by IgG immune complexes can independently initiate apoptosis, which in turn can be antagonized by the other.

Discussion

Cell apoptosis is one of the key regulatory mechanisms by which the immune system responds to various types of stimuli. Despite recent advances in our understanding of the mechanisms and regulation of mast cell apoptosis (17), detailed mechanisms are not fully understood. In this study,

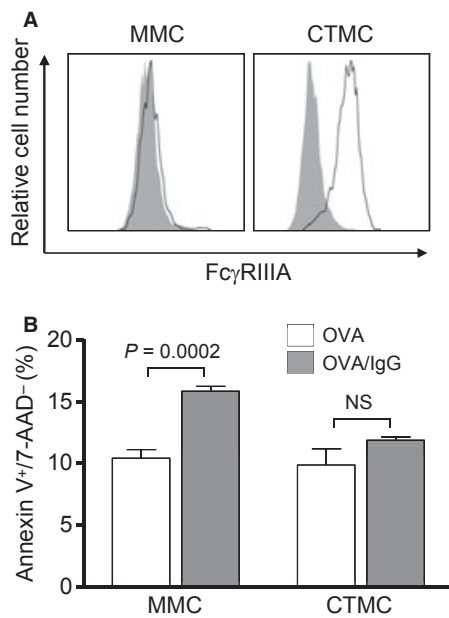


Figure 5 Comparison of mucosal mast cell (MMC) and connective tissue mast cells (CTMC) in terms of Fc γ RIIA expression and apoptosis induction in response to immune complex treatment. (A) Flow cytometry histograms of cell surface staining of Fc γ RIIA are shown. Solid histograms, isotype control antibody; lines, anti-Fc γ RIIA-specific antibody. Histogram profiles representative of four separate cultures are shown. (B) MMC and CTMC were treated and analyzed as explained in Fig. 1. Data are expressed as mean + SEM of five experiments.

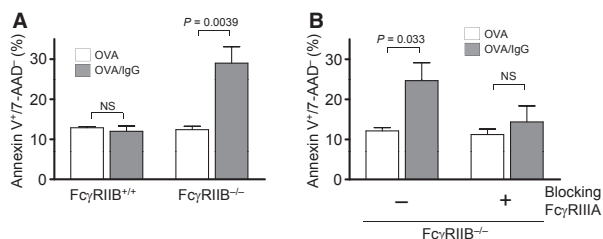


Figure 6 Apoptotic profile of 3-week-old bone marrow-derived cultured mast cells (BMMC) in relation to the presence or absence of Fc γ RIIB. (A) 3-week-old BMMCs derived from Fc γ RIIB^{+/+} and Fc γ RIIB^{-/-} mice were treated and examined as explained in Fig. 1. (B) 3-week-old BMMCs derived from Fc γ RIIB^{-/-} mice were incubated with either blocking antibody against Fc γ RIIA or medium followed by treatment and analysis as explained in Fig. 1. Data are expressed as mean + SEM of six (A) or four (B) separate experiments.

we confirmed that mast cells responded to IgG immune complex- and Fc γ RIIB aggregation-mediated apoptosis, as with B-lineage cells (9–12). However, in terms of Fc γ receptor expression, mast cells differ from B cells, expressing other receptors in addition to Fc γ RIIB. Under certain circumstances, mouse mast cells can express Fc γ RIIA. Our data support that this activating receptor may counteract the

death-promoting effect mediated by Fc γ RIIB. Interestingly, crosslinking of Fc γ RIIA in the absence of Fc γ RIIB by IgG immune complexes also leads to apoptosis. Our data support a model in which balanced expression of Fc γ RIIB and Fc γ RIIA protects the cells from IgG immune complex-mediated apoptosis, providing fine-tuning of mast cell apoptosis and survival.

Based on our observations of the responses of *in vitro*-generated CTMC and MMC to stimulation through Fc γ receptors, it is possible to speculate that CTMCs, which represent a more mature type of mast cells and have a balanced expression of Fc γ R, are able to resist IgG immune complex-mediated apoptosis *in vivo*. In contrast, MMC might be more vulnerable to such apoptosis induction owing to a lack of sufficient Fc γ RIIA. It is reported that IL-4 may up-regulate the *in vitro* expression of Fc γ RIIA (18). If this is also true *in vivo*, we can speculate that a preferential Th2-skewing microenvironment with enriched local production of IL-4 may protect MMC from apoptosis owing to increased expression of Fc γ RIIA, which may counteract Fc γ RIIB-mediated cell death.

We identified regulation of Fc γ RIIA on BMMC dependent on their time in culture, which is not surprising as culture duration-associated phenotypic change in BMMC has been reported previously. For example, the chemokine receptor CCR2 is down-regulated during the culture of BMMC (19). Extended culture of BMMC to longer than 3 weeks also renders these cells incapable of presenting antigen to T cells (20).

Despite the fact that attempts to target Fc γ RIIB expressed on basophils and mast cells for allergy treatment have already been carried out (21, 22), certain controversy exists whether or not human mast cells express Fc γ RIIB, which is not as conclusive as their mouse counterpart. Human mast cells derived from umbilical cord blood express Fc γ RIIB (23, 24). Although human lung mast cells are reported not to express Fc γ RIIB with the less-sensitive technique of immunohistochemistry (25), a functional study indirectly suggests the presence of the receptor on these cells (26). The level of Fc γ RIIB expression on human mast cells may also be donor specific, as it is reported that the promoter polymorphism of Fc γ RIIB alters receptor expression, which is associated with autoimmunity (27, 28). Certainly, more work is needed to characterize the expression of Fc γ RIIB by human tissue mast cells. The expression may be not only donor specific but also tissue specific or maturation specific. Mast cell heterogeneity has been widely observed, and their phenotypes may vary depending on their different anatomical sites (29). In terms of activating Fc γ receptor expression, certain discrepancies between human and mouse mast cells also exist. No evidence of Fc γ RIIA expression has been found on human mast cells. Upon stimulation by interferon- γ , human mast cells, but not their murine counterparts, express the high-affinity receptor Fc γ RI (3). It would be interesting to investigate whether or not human mast cells respond to death induction by aggregating Fc γ RIIB or any of the activating Fc γ receptors. Whether or not any counter-regulatory mechanisms operate by the inhibitory and activating receptors in human mast cells also warrants further investigation.

Specific allergen immunotherapy (SIT) has developed as a promising treatment in IgE-mediated diseases, such as allergic rhinitis, venom anaphylaxis, and asthma. SIT involves the administration of gradually increasing quantities of specific allergens to patients until a dose is reached that inhibits responses to natural exposure. The mechanisms responsible for the efficacy of SIT are not fully understood (30). However, various studies have suggested that Fc γ RIIB may play a critical role (31). Furthermore, it is well documented that, among other findings, SIT is accompanied by increases in allergen-specific IgG, in particular IgG4, and a reduction in mast cell numbers (30). Hitherto, no report is available that provides mechanistic explanations linking the two observations. It is reported that human Fc γ RIIB binds IgG4 (32). It is possible that certain types of human mast cells express Fc γ RIIB. Thus, it may be hypothesized that IgG immune complexes generated as a result of active SIT treatment may aggregate Fc γ RIIB on mast cells, resulting in apoptosis of these cells. Further complexities can be envisaged, with activating Fc γ receptors also participating in the execution and regulation of apoptosis. Furthermore, basophil number is also observed to decrease following the SIT treatment (33). Basophils also express Fc γ RIIB, and it is not known whether these cells can be induced to undergo apoptosis in response to IgG immune complex treatment. Of note, Fc γ RIIB-mediated apoptosis has been observed in eosinophils (34, 35).

The observation that IgG immune complexes can induce apoptosis of mast cells via Fc γ receptors sheds new insights into the apoptosis mechanisms of this enigmatic cell type. A direct inhibition of the survival of these cells by targeting Fc γ receptors may be beneficial in the treatment for allergy as well as other inflammatory diseases.

Author contributions

Yu Fang, Lisa Larsson and Zou Xiang participated in original experimental work and data analysis. Pierre Bruhns

provided intellectual input including experimental design and critical reading of the manuscript. Yu Fang and Zou Xiang wrote the manuscript. Zou Xiang conceived, coordinated, and supervised the study.

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Conflict of interest

None.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Propidium iodide (PI) intercalation of bone marrow-derived cultured mast cells (BMMC) after IgG immune-complex treatment.

Figure S2. Characterization of bone marrow-derived cultured mast cells (BMMC) of varying culture durations.

Figure S3. Proliferation profiling of bone marrow-derived cultured mast cells (BMMC) after IgG immune-complex treatment.

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References

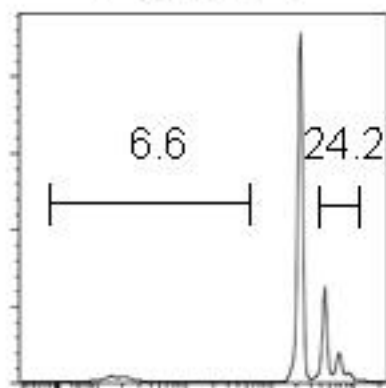
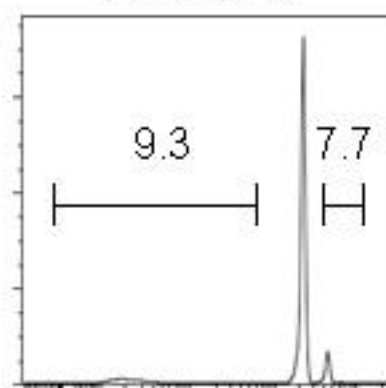
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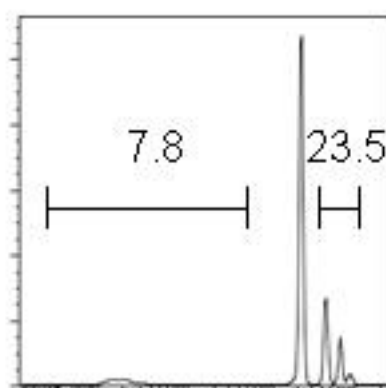
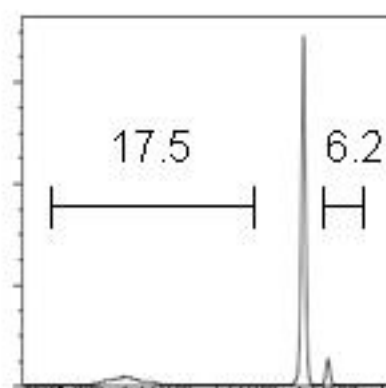
$Fc\gamma RIIB^{+/+}$

$Fc\gamma RIIB^{-/-}$

Relative cell number

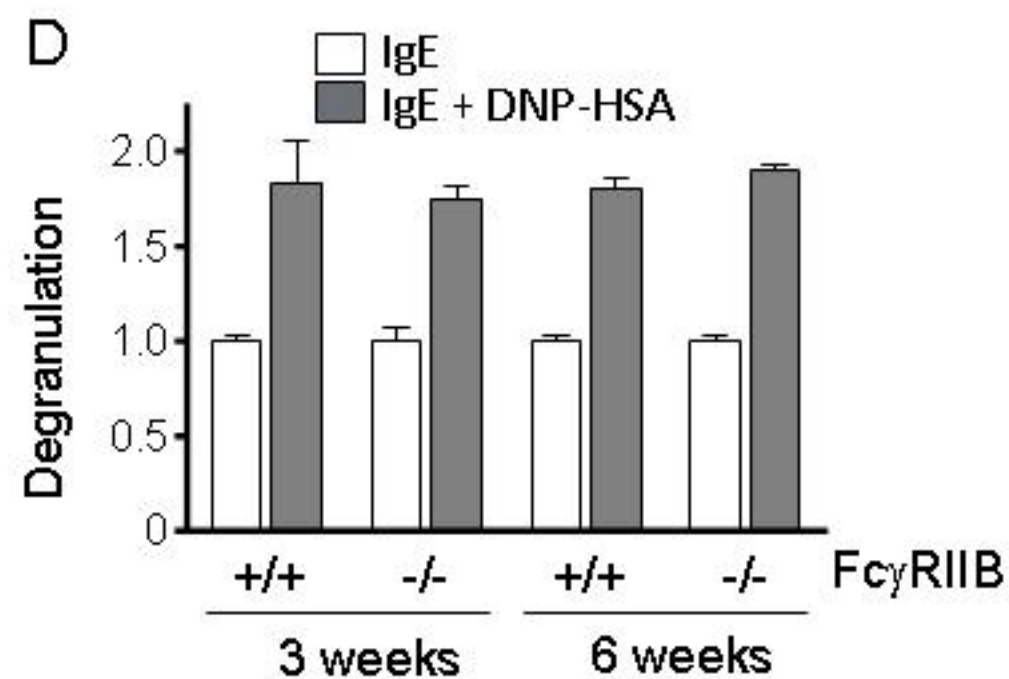
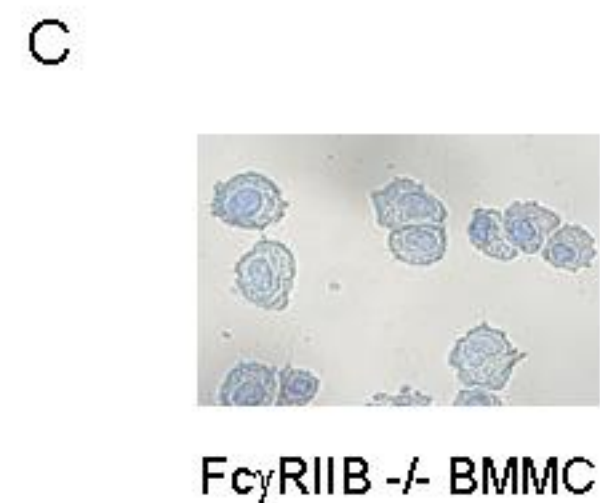
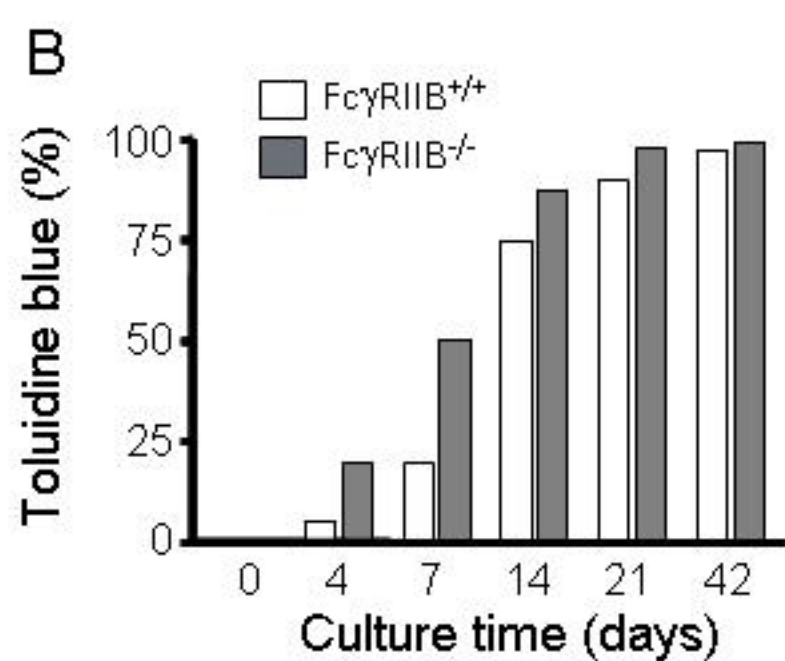
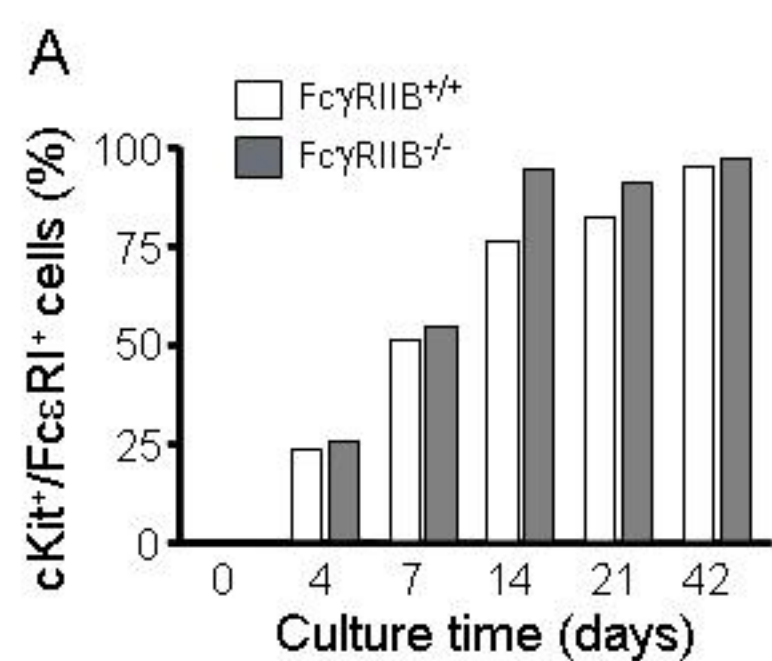


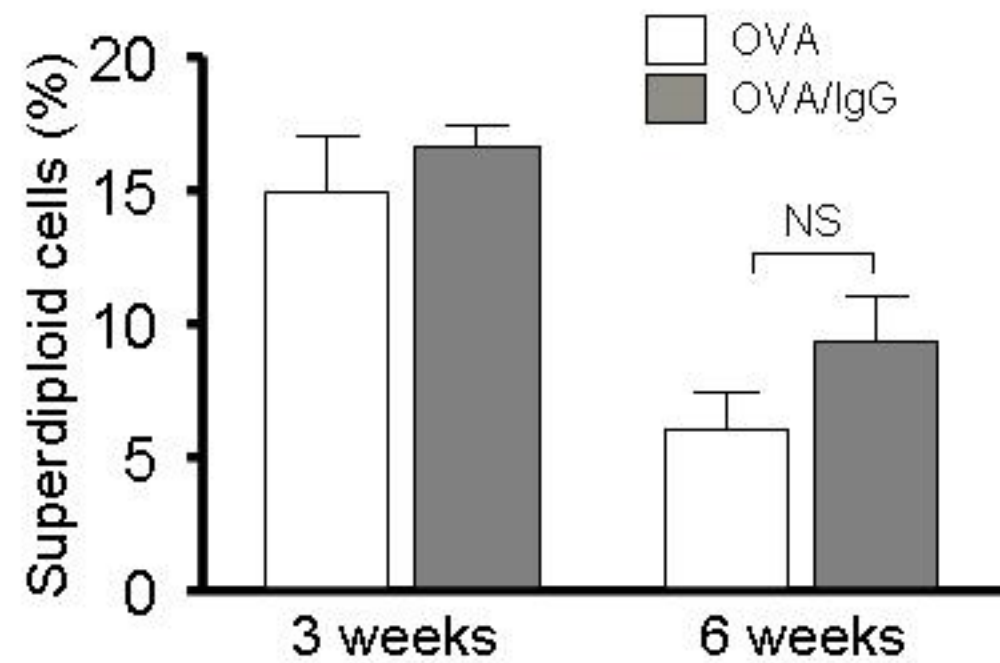
OVA



OVA/IgG

PI



A**B**