European Journal of Immunology Clinical·Basic·Translational

Leukocyte and lymphoid organ ontogeny

Basic

Research Article

FcγR-dependent apoptosis regulates tissue persistence of mucosal and connective tissue mast cells

Tongqian Wu[1,2], Shirong Yan[1,2], Yu-Wen Yeh[3], Yu Fang[1,2] and Zou Xiang[3,4]

[1] Center for Clinical Laboratory, Affiliated Hospital of Guizhou Medical University, Guiyang, 550004, P. R. China
[2] School for Clinical Laboratory, Guizhou Medical University, Guiyang, 550004, P. R. China
[3] Department of Health Technology and Informatics, Faculty of Health and Social Sciences, Hong Kong Polytechnic University, Hong Kong, P. R. China
[4] Department of Microbiology and Immunology, Mucosal Immunobiology and Vaccine Research Center, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden

Rodent mast cells can be divided into two major subtypes: the mucosal mast cell (MMC) and the connective tissue mast cell (CTMC). A decade-old observation revealed a longer lifespan for CTMC compared with MMC. The precise mechanisms underlying such differential tissue persistence of mast cell subsets have not been described. In this study, we have discovered that mast cells expressing only one receptor, either FcγRIIB or FcγRIIIA, underwent caspase-independent apoptosis in response to IgG immune complex treatment. Lower frequencies of CTMC in mice that lacked either FcγRIIB or FcγRIIIA compared with WT mice were recorded, especially in aged mice. We proposed that this paradigm of FcγR-mediated mast cell apoptosis could account for the more robust persistence of CTMC, which express both FcγRIIB and FcγRIIIA, than MMC, which express only FcγRIIB. Importantly, we reproduced these results using a mast cell engraftment model, which ruled out possible confounding effects of mast cell recruitment or FcγR expression by other cells on mast cell number regulation. In conclusion, our work has uncovered an FcγR-dependent mast cell number regulation paradigm that might provide a mechanistic explanation for the long-observed differential mast cell subset persistence in tissues.

Keywords: apoptosis · connective tissue mast cell · Fcγ receptor · mast cell · mucosal mast cell

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Mast cells have been traditionally regarded as important effector cells in allergic pathology since the identification of IgE and mast cell-associated histamine more than half a century ago [1]. In allergy, plurivalent antigens bind and crosslink IgE molecules bound to the high-affinity IgE receptor (FcεRI) expressed on mast cells, resulting in cell degranulation and release of proinflammatory mediator molecules. Three major categories of mast cell mediators have been described: (1) preformed granule-associated mediators such as histamine and serotonin; (2) newly generated lipid mediators, such as leukotrienes and prostaglandins; and (3) de novo synthesized cytokines including chemokines. IgE-mediated activation of mast cells initiates the early phase of allergic responses, resulting in pathologies including greater levels of epithelial permeability, mucous production, smooth muscle

Correspondence: Dr. Zou Xiang and Dr. Yu Fang
e-mail: xiang.y.zou@polyu.edu.hk; yu.fang@gmc.edu.cn

contraction, vasodilatation, and neurogenic inflammation. The immediate response is followed by the recruitment of a variety of other immune cells that participate in the late phase of the reaction, further exacerbating allergic pathology [2]. In recent decades, the roles of mast cells have been extended beyond allergy and have encompassed a much broader spectrum in both health and disease. Accumulating evidence has also implicated mast cells in host defense against infection and envenomation, tissue repair, inflammatory disorders other than allergy, and tumor development [3].

Mast cells are derived from BM progenitors that migrate via the blood to almost all tissues, where they further differentiate and mature into different phenotypes depending on the local microenvironment. Mast cells are enriched in the skin, around blood vessels, and in mucosal membranes such as the respiratory and gastrointestinal tracts. Tissue-specific distribution of mast cells is dependent on various mediators. The number of mast cells is increased at sites of allergic inflammation, and there is a correlation between mast cell density in the tissue and the severity of allergic symptoms [2, 4]. Mast cells are highly heterogeneous and can be divided into two major subtypes: mucosal mast cells (MMC) and connective tissue mast cells (CTMC) in rodents, corresponding to tryptase-containing mast cells (MC_T) and both tryptase- and chymase-containing mast cells (MC_{TC}) in humans, respectively [5, 6]. Overall, mast cells are believed to be long-term tissue-resident cells with a slow turnover. Yet, an interesting observation was made back in the 1980s, which revealed a longer lifespan for rodent CTMC than MMC in tissues [7]. However, the mechanisms that account for mast cell tissue persistence have not been documented.

In addition to IgE-mediated activation, mast cells also express Fc receptors that bind IgG, which are referred to as Fcγ receptors (FcγR). FcγR exists in multiple isoforms that are categorized, either in terms of affinity as high-affinity and low-affinity receptors, or in terms of signaling pathways they induce, as activating and inhibitory receptors. For mice, there are two high-affinity IgG receptors, FcγRI (CD64) and FcγRIV, and two families of low-affinity IgG receptors, FcγRII (CD32) and FcγRIII (CD16). FcγRII has three subtypes, that is, FcγRIIA, FcγRIIB, and FcγRIIC. FcγRIII has two subtypes, that is, FcγRIIIA and FcγRIIIB. While all the other FcγR have been classically defined as activating receptors, FcγRIIB is an inhibitory receptor [8]. However, our previous work has demonstrated that FcγRIIIA can suppress immune responses by acting as an inhibitory receptor [9].

Altogether, three FcγR subtypes, that is, FcγRIIA (human), FcγRIIB (both human and mouse), and FcγRIIIA (mouse), are constitutively expressed by mast cells. These low-affinity FcγR respond to stimulation with IgG immune complexes, but not monomeric IgG molecules. FcγRIIB is a single-chain molecule with an intracytoplasmic domain that contains the immunoreceptor tyrosine-based inhibition motif [10]. FcγRIIIA requires one IgG-binding α-chain and a signal-transducing adaptor molecule composed of two Fc receptor common γ-chains, which contain the ITAM. The two γ-chains are required for the expression and signaling of the entire receptor complex. Similar to FcγRIIB, FcγRIIA is a single-chain receptor; but contrary to FcγRIIB, FcγRIIA is an activating receptor which signals through ITAM located in the intracellular domain of its α-chain. Mouse FcγRIIIA is predicted to be the counterpart of human FcγRIIA in terms of functionality and sequence similarity in the extracellular portion [10]. It is well documented that FcγRIIB plays an important role in dampening the allergic responses by bridging this inhibitory receptor to the high-affinity IgE receptor, FcεRI, through the IgG-allergen-IgE complex [11].

The major impact of the antibody on immune regulation depends on the interaction of the antigen–antibody immune complexes, through the Fc domain of the antibodies, with Fc receptors expressed on various types of immune cells including mast cells. Studies addressing the roles of FcγR have focused on their pleiotropic functions which include the release of inflammatory mediators, phagocytosis, antibody-dependent cellular cytotoxicity, and chemotaxis [10, 12]. Engagement of FcγR either promotes or inhibits these processes. It was first discovered on naïve B cells that homotypic crosslinking of FcγRIIB, which is the only FcγR expressed on B cells, without aggregating the BCR results in B-cell apoptosis [13, 14]. We previously confirmed that antibody-forming plasma cells, or effector B cells, also undergo apoptosis upon FcγRIIB crosslinking, which is a critical mechanism for regulating humoral immune responses [15]. We also showed that this killing mechanism through homotypic aggregation of FcγRIIB by IgG immune complexes induces mast cell apoptosis [16]. It has been classically documented that CTMC express both FcγRIIB and FcγRIIIA, while MMC only express FcγRIIB [17-19]. Using in vitro cultured cell models, we previously reported that MMC undergo apoptosis in response to the aggregation of FcγRIIB, the only IgG receptor expressed on these cells, by antigen-IgG immune complexes. In contrast, CTMC resist this type of apoptosis induction, probably because of their expression of both FcγRIIB and FcγRIIIA [16]. Given that there exist natural IgG immune complexes under normal physiological conditions [20], it is likely that the distinct lifespan profiles of mouse CTMC and MMC can be regulated by differential cell apoptosis through the aggregation of FcγR by IgG immune complexes. In this study, we provide further evidence demonstrating the in vivo relevance of the FcγR-mediated cell death induction in the regulation of mast cell survival in tissues.

Results

Mast cells initiate apoptosis after treatment with IgG immune complexes in an FcγR-dependent manner

We have previously reported that mouse BM-derived cultured mast cells (BMMC) can be induced to undergo apoptosis by IgG immune complexes aggregating either FcγRIIB or FcγRIIIA [16]. This was demonstrated using mast cells lacking either FcγRIIB or FcγRIIIA following receptor aggregation. However, in the presence of both FcγRIIB and FcγRIIIA as for cells derived from the WT mice, mast cells resist such apoptosis induction [16]. In the

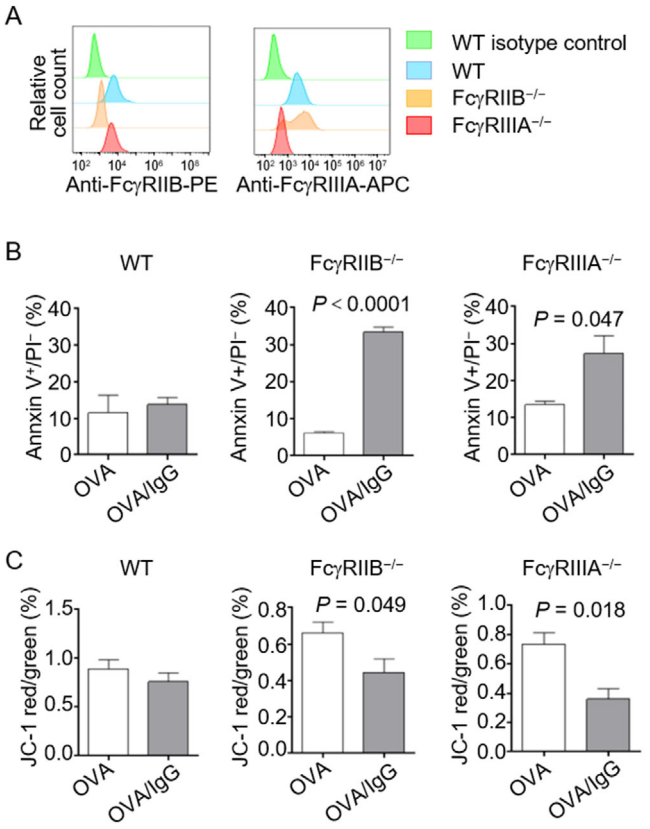

Figure 1. BM-derived cultured mast cells (BMMC) respond to induction of apoptosis after treatment with IgG immune complexes in an FcγR expression-dependent manner. BMMC were cultured from WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ strains of mice. (A) Cell surface expression of FcγRIIB and FcγRIIIA by BMMC was analyzed by flow cytometry using a specific anti-FcγRIIB antibody (clone AT130-2) (left panel) or a specific anti-FcγRIIIA antibody (clone 275003) (right panel). (B to C) BMMC were treated with OVA only or immune complexes composed of OVA and anti-OVA IgG (OVA/IgG) for 16 h. Mast cell apoptosis was analyzed using flow cytometry by measuring the levels of Annexin V and PI binding (B) or measuring the reduction in mitochondrial membrane potential (C). JC-1, a membrane-permeable lipophilic cationic fluorochrome that exerts enhanced green fluorescence and reduced red fluorescence when the cells fail to maintain mitochondrial membrane potential, a feature of apoptotic cells. Unpaired two-tailed Student's *t*-test was used for the statistical analysis (B). Data are representative of three separate experiments (A) or expressed as mean ± SEM of three independent primary BMMC cultures.

current study, we tried to validate this finding and extend the study by revealing the in vivo relevance of FcγR-mediated mast cell apoptosis. In the previous work [16], we cultured BMMC in the presence of X63/0 myeloma cell-derived IL-3. In the current study, we cultured BMMC, in the presence of recombinant IL-3, from WT mice or mice that were deficient in either FcγRIIB or FcγRIIIA. BMMC obtained from these three strains of mice demonstrated similar basic features of mast cell biology in terms of morphology (Supporting Information Fig. S1A-C), degranulation (Supporting Information Fig. S1D and E), and cytokine production (Supporting Information Fig. S1F and G). BMMC derived from WT mice expressed both FcγRIIB and FcγRIIIA (Fig. 1A). In contrast to the WT BMMC, cells deficient in either FcγRIIB or FcγRIIIA demonstrated signs of cell death following treatment with IgG immune complexes, as confirmed by both Annexin V binding (Supporting Information Fig. S2A and Fig. 1B) and the reduction in mitochondrial membrane potential (Supporting Information Fig. S2B and Fig. 1C).

Next, we explored whether caspase activity was involved in FcγR-mediated mast cell apoptosis. While WT cells resisted cell death after IgG immune complex treatment as before, efficient cell death was still induced in the presence of a pan-caspase inhibitor in cells lacking FcγRIIB or FcγRIIIA (Fig. 2A). Consistently, no activities of polycaspase (Fig. 2B) or caspase-3/7 (Fig. 2C) could be detected following receptor aggregation by treatment with IgG immune complexes. The caspase detection system was sound, as we could demonstrate that BMMC deprived of IL-3 in culture medium underwent apoptosis with detectable caspase activity (Supporting Information Fig. S3). Thus, our data supported that no caspase activity was involved in FcγRIIB- or FcγRIIIA-mediated mast cell apoptosis.

Mast cell tissue persistence is regulated by the presence of FcγR

We next explored the implication of FcγR-mediated mast cell apoptosis with respect to tissue mast cell persistence in the steady state, as natural IgG immune complexes are present under normal physiological conditions [20]. Because tissue mast cells do not proliferate substantially under steady-state conditions [21, 22], their tissue persistence may be correlated to cell death.

We first focused on the mouse nasal tissue, where both CTMC, which express both FcγRIIB and FcγRIIIA, and MMC, which express only FcγRIIB, are located [23]. We have developed a robust technique to discriminate between MMC and CTMC by flow cytometry (Fig. 3A, upper panels) [23]. This allows quantitative analysis of the proportion of nasal tissue mast cell subsets. In all the three strains of mice, nasal tissues were dominated with MMC when mice were 8 weeks old (Fig. 3A, lower left panel), a time point corresponding to young adulthood. While the mice were aging, progressive accumulation of CTMC at the expense of MMC was observed at 37 weeks of age (Fig. 3A, lower middle panel) and 58 weeks of age (Fig. 3A, lower right panel). This trend was more pronounced in the WT mice compared with either FcγRIIB- or FcγRIIIA-deficient, age-matched mice (Fig. 3A, lower panels). Taken together, these data could possibly suggest the vulnerability of the single receptor-expressing CTMC to apoptosis induction.

Next, we assessed the frequency of peritoneal mast cells in the three strains of mice during aging. Peritoneal mast cells are exclusively CTMC; therefore, the WT peritoneal mast cells express both receptors [24]. At 8-week-old, both FcγRIIB- and FcγRIIIA-deficient mice showed a tendency towards lower frequencies of peritoneal mast cells (Fig. 3B, lower left panel). Such reduced mast cell frequencies in mice that only expressed one of the receptors were significant when they reached 37 and 55 weeks of age (Fig. 3B, lower middle and right panels).

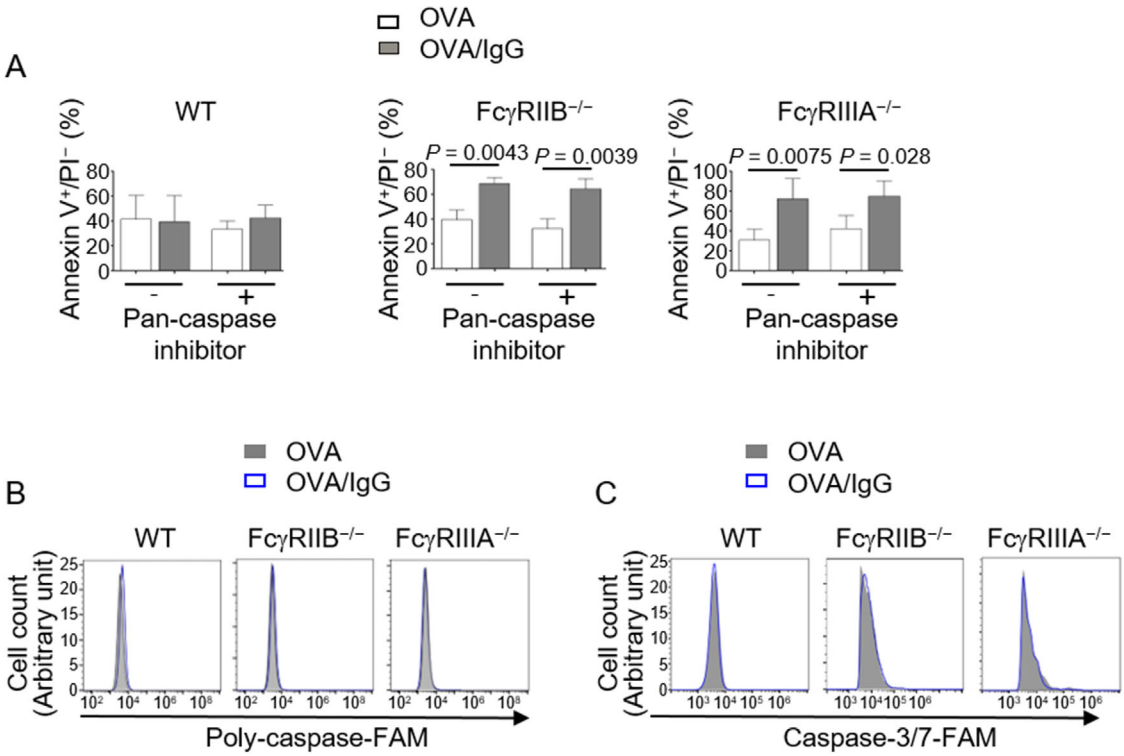

Figure 2. FcγR-mediated apoptosis of BM-derived cultured mast cells (BMMC) does not initiate caspase activities. BMMC were cultured from WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice. Cells were treated with OVA only or immune complexes composed of OVA and anti-OVA IgG (OVA/IgG) for 16 h. (A) Mast cell apoptosis was determined by flow cytometry measuring the levels of Annexin V and PI staining intensity. In some of the experiments, a pan-caspase inhibitor was present during the incubation. (B and C) Expression of polycaspase (B) and caspase-3/7 (C) was measured by flow cytometry based on the use of a carboxyfluorescein (FAM) group as a fluorescent reporter. Unpaired two-tailed Student's *t*-test was used for the statistical analysis (A). Data are shown as means ± SEM (n = 3) (A) or are representative of three separate experiments (B, C).

FcγR regulate the persistence of engrafted peritoneal mast cells in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice

Reduced abundance of CTMC in FcγRIIB- and FcγRIIIA-deficient mice does not necessarily reflect increased levels of cell death. Two obvious confounding elements are the recruitment of mast cells from precursors and cell proliferation. To confirm that apoptosis, rather than precursor recruitment, is the mechanism accounting for reduced numbers of single FcγR-expressing CTMC, we performed adoptive transfer studies using *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice which lack mast cells [25] as hosts. As engraftment of mast cells to the nasal tissue is difficult, we relied on the peritoneal engraftment model to exclude the involvement of differential cell recruitment in our cell number counting. To this end, identical numbers of mast cells cultured from the BM of FcγRIIB$^{-/-}$ and WT mice were separately engrafted into the peritoneal cavity of two groups of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Twelve weeks after the adoptive transfer, mice that received FcγRIIB-deficient BMMC indeed were shown to have lower frequencies of surviving mast cells compared with adoptively transferred WT cells (Fig. 4A).

Next, we transferred identical numbers of BMMC cultured from WT and FcγRIIIA$^{-/-}$ mice into the peritoneal cavity of the same host mouse, as we could distinguish between the adoptively transferred WT cells and FcγRIIIA$^{-/-}$ cells using a specific antibody that recognizes mouse FcγRIIIA (clone 275003). Percentages of peritoneal mast cells were determined at 4, 10, 15, and 25 weeks after adoptive transfer. Progressive loss of FcγRIIIA$^{-/-}$ cells during these investigation time points was observed (Fig. 4B).

In these engraftment assays, cell number regulation could not be affected by altered recruitment of new precursors as the recipient mice did not generate mast cells or mast cell precursors. However, BMMC were still capable of proliferation (Supporting Information Fig. S4). To rule out any confounding issue of cell proliferation, we compared the proliferative capacity of the adoptively transferred WT and FcγRIIIA$^{-/-}$ mast cells, and confirmed their equal proliferation before engraftment and at 4, 10, and 15 weeks after engraftment (Fig. 4C). Taken together, these data support that precursor recruitment and cell proliferation were not a confounding issue in our assessment of cell number regulation as a result of FcγR-mediated apoptosis.

Aggregated IgG induces peritoneal mast cell apoptosis in an FcγR-dependent manner

As mast cell apoptosis may be induced to a low extent by homeostatic levels of IgG immune complexes, it is difficult to detect such apoptosis in vivo. To confirm the effect of IgG immune complexes

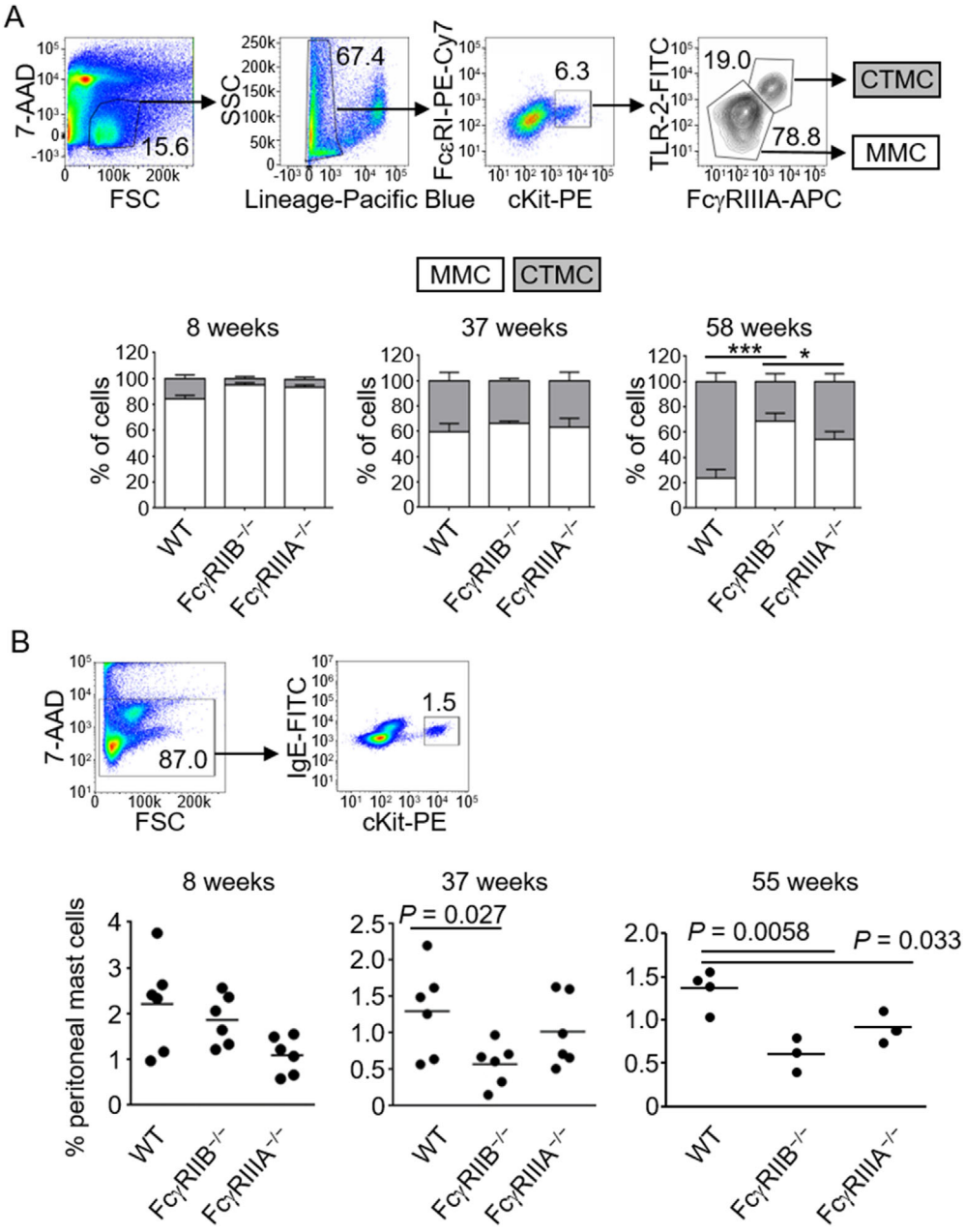

Figure 3. Mast cell tissue persistence is regulated by the expression of FcγR. (A) Mouse nasal tissues from WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice were harvested and digested for flow cytometric analysis. Nasal tissue cells were gated for identifying FcγRIIIA^{+}TLR2^{+} connective tissue mast cells (CTMC) and FcγRIIIA^{-}TLR2^{-} mucosal mast cells (MMC) among the cKit^{+}FcεRI^{+} mast cell population in the nasal tissues; shown is nasal tissue from a representative WT mouse (upper panels). Proportions of nasal CTMC and MMC among all the nasal tissue mast cells from 8-, 37- and 58-week-old mice of the three strains were plotted (lower panels). (B) Mouse peritoneal mast cells were harvested from WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice and analyzed by flow cytometry. Gating strategy is shown for identifying cKit^{+}/IgE^{+} peritoneal mast cells (upper panels). Frequencies of peritoneal mast cells among all the peritoneal cells from 8-, 37-, and 55-week-old mice of the three strains were plotted (lower panels). Data are shown as mean + SEM (n = 5 to 8) (A). Numbers inside or adjacent to outlined areas indicate percent cells in each gate. Each dot represents one individual mouse (B). One-way ANOVA, followed with Tukey's multiple comparison test (A), or unpaired two-tailed Student's *t*-test (B) was used for the statistical analysis. $^{*}p = 0.015$; $^{***}p = 0.0003$ comparing CTMC mean frequencies (A). Data are representative of two separate experiments. 7-AAD, 7-aminoactinomycin D; SSC, side scatter; FSC, forward scatter; lineage, a group of markers including CD3, Ly-6G/Ly-6C, CD19, NK1.1, CD11b, TER119, and B220.

on the induction of mast cell apoptosis in vivo, we delivered heat-aggregated IgG into the peritoneal cavity of the WT, FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice. Consistent with the in vitro assay, mouse peritoneal mast cells developed substantial cell death if they lacked either FcγRIIB or FcγRIIIA after the administration of aggregated IgG (Fig. 5).

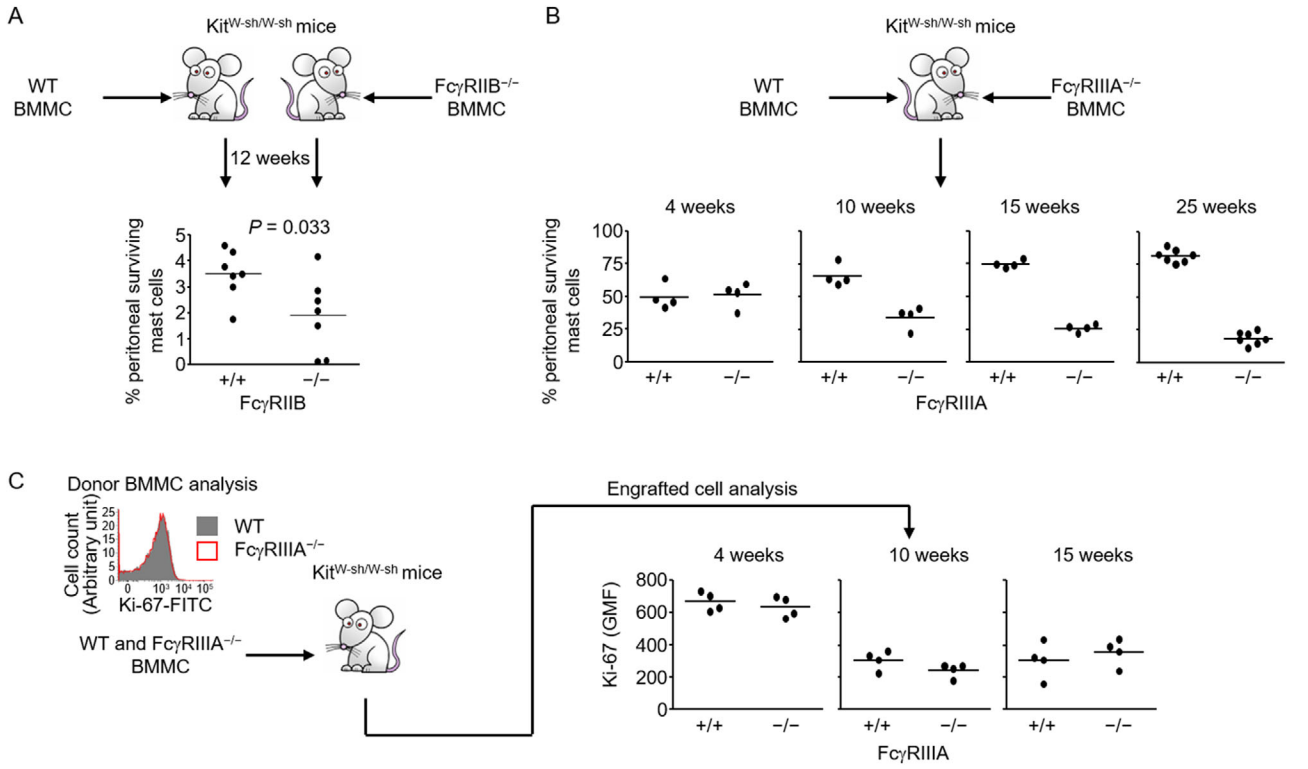

Figure 4. FcγR regulate the persistence of engrafted peritoneal mast cells in $Kit^{W\text{-}sh/W\text{-}sh}$ mice. BM-derived cultured mast cells (BMMC) were cultured from WT, $FcγRIIB^{-/-}$ or $FcγRIIIA^{-/-}$ mice. Identical numbers of BMMC from WT and $FcγRIIB^{-/-}$ mice were separately transferred into the peritoneal cavity of recipient $Kit^{W\text{-}sh/W\text{-}sh}$ mice (A). Identical numbers of BMMC from WT and $FcγRIIIA^{-/-}$ mice were mixed before engraftment into the peritoneal cavity of the same recipient $Kit^{W\text{-}sh/W\text{-}sh}$ mouse (B and C). After various time periods as indicated, frequencies of the engrafted peritoneal mast cells in the recipient mice were analyzed by flow cytometry. The proliferation of donor BMMC and engrafted peritoneal mast cells (lower panels) was determined by measuring Ki-67 expression (C). Each dot represents data from one individual recipient mouse. Unpaired two-tailed Student's *t*-test was used for the statistical analysis (A). The data shown are representative of two separate experiments. GMF, geometric mean fluorescence intensity.

Discussion

Mast cells exert dynamic immune effector and regulatory functions beyond their classically defined role in allergic inflammation. In this respect, mast cell tissue persistence or number regulation is important not only for allergic diseases, but also for a broader spectrum of many different pathophysiological conditions, where mast cells can be either protective or exacerbative. Under homeostatic conditions, the numbers of mast cells are maintained constant in tissues. However, mast cell numbers can increase substantially in various pathological settings, which contributes to mast cell-related symptoms or protective immunity. For example, mast cell numbers in both atopic and nonatopic asthmatic lung tissues are increased compared with healthy controls [26]. Intestinal MMC population expands in rats after nematode infection [27]. In the current study, we revealed an FcγR-mediated mast cell apoptosis mechanism that has been shown to regulate the tissue persistence of MMC and CTMC. We demonstrate that aggregation of FcγRIIB or FcγRIIIA is capable of inducing mast cell apoptosis. Our work provides a mechanistic account of the long observed differential life span of MMC and CTMC in rodent tissues.

Our model of FcγR-mediated mast cell persistence is proposed based on the evidence that there is a clear difference between mast cells from the WT mice and those from mice that are deficient in one of the FcγR. However, if one only focuses on the FcγR-deficient mice, an increased proportion of the CTMC population in both FcγRIIB-deficient mice and FcγRIIIA-deficient mice during aging, at least until 37 weeks, could still be observed (Fig. 3A), suggesting the survival advantage of these single receptor-expressing CTMC. This is a paradoxical finding, because CTMC in these mice only had one FcγR (either FcγRIIIA or FcγRIIB), which would render these CTMC susceptible to FcγR-mediated apoptosis. Therefore, we cannot rule out the presence of other mechanisms that may also operate to differentially regulate the survival or persistence of CTMC versus MMC.

Regulation of mast cell numbers can be achieved through different mechanisms. Mast cell population can be replenished by the recruitment of precursors that differentiate into mature mast cells in tissues [28, 29]. Although mature tissue mast cells are

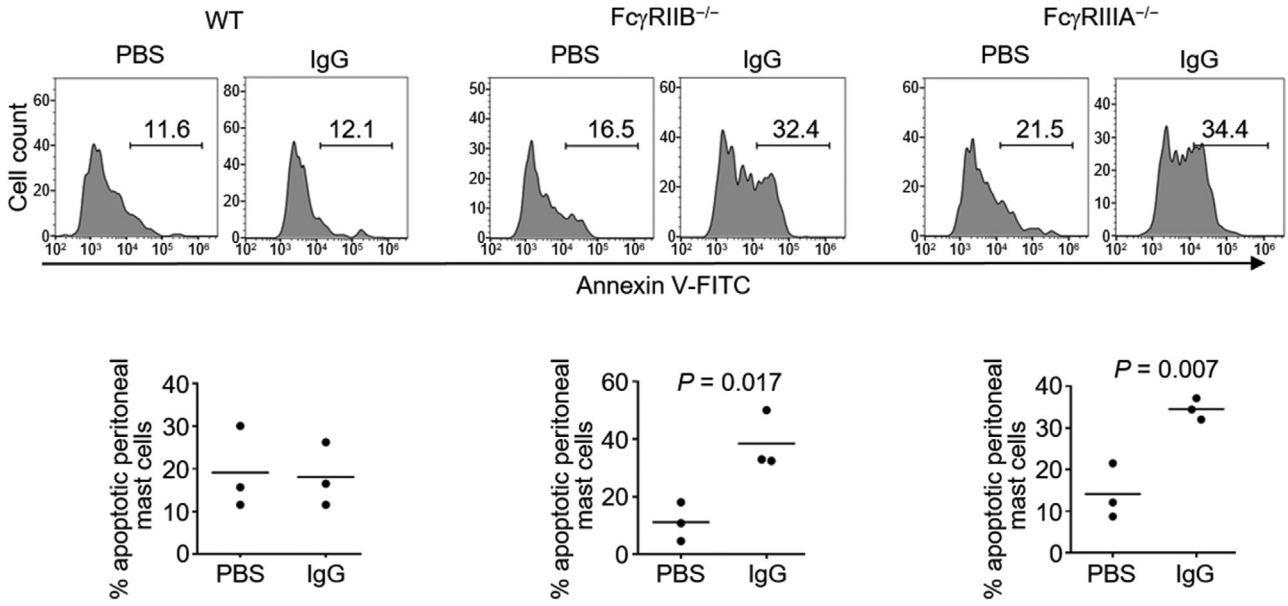

Figure 5. Exogenously delivered aggregated IgG induces peritoneal mast cell apoptosis in an FcγR expression-dependent manner. Each mouse of the WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ strain received intraperitoneally 1 mg heat-aggregated polyclonal IgG purified from normal mouse serum. Mouse peritoneal mast cell apoptosis was examined 16 h after IgG delivery by measuring the levels of Annexin V binding using flow cytometry. Representative histograms of one mouse from each strain (upper panels) and pulled data from a total of three mice in each group (lower panels) are shown. Numbers indicate percent cells that are Annexin V^{+} apoptotic cells (upper panels). Each dot represents one individual mouse. Unpaired two-tailed Student's *t*-test was used for the statistical analysis.

described as not proliferating under normal physiological conditions [21, 22], dysregulation of mast cell biology may induce mast cell proliferation, a typical example of which is an activating mutation of the gene coding for the receptor c-kit, resulting in mastocytosis [30]. Yet, one more cell number regulatory mechanism is cell apoptosis, and various types of Bcl-2 family proteins are implicated in the regulation of mast cell apoptosis [31]. The MMC population in the stomach mucosa is found to be expanded in mice deficient in Bax, whereas CTMC numbers are more stable and less affected by genetic deletion of Bax [32]. On the contrary, Bcl-2 exerts an opposite role in regulating the tissue numbers of MMC, but not CTMC [32]. These findings support a more prominent role for Bcl-2 family members in regulating tissue persistence of MMC compared to CTMC during homeostasis. Forster and colleagues reported mast cell apoptosis mediated through tumor necrosis factor-related apoptosis-inducing ligand receptors [33]. In this study, the authors demonstrated tumor necrosis factor-related apoptosis-inducing ligand receptors are expressed to a greater level in mouse CTMC-like peritoneal mast cells compared with BMMC. Only CTMC-like cells respond to TRAIL-induced apoptosis in the presence of actinomycin D. Furthermore, MMC depend on T-cell-derived cytokines for survival in addition to SCF, whereas CTMC primarily require SCF for their persistence [31]. Therefore, it is possible that T cell-derived cytokines may regulate mast cell apoptosis, and hence, the tissue persistence of MMC.

Classical programmed cell death is described requiring the involvement of caspases, which are a group of cysteine-dependent aspartate-specific proteolytic enzymes [34]. Caspase activation can be initiated through two main pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway, with the former being regulated through extracellular ligand binding to cell surface death receptors, such as CD95 (Fas) or the TNF receptor, and the latter being controlled by Bcl-2 family proteins [35]. However, abundant evidence also indicates the existence of caspase-independent cell death, which still displays proteolytic degradation features, suggesting the role of alternative proteolytic mechanisms, for example, through exploiting serine proteases, including granzymes, in regulating cell death [36, 37]. Mitochondria-mediated signaling and Bcl-2 family proteins are also implicated in caspase-independent cell death [38]. In the current study, we could clearly detect loss of mitochondrial membrane potential in mast cells following crosslinking of either FcγRIIB or FcγRIIIA by IgG immune complexes. We further confirmed that this cell death induction was not mediated by caspase activation. Caspase-independent cell death has also been described in other mast cell experimental models [39, 40].

Inconsistent expression of FcγR on rodent and human cells has been observed. Despite the fact that it has long been confirmed that rodent mast cells express FcγRIIB, expression of this receptor by human tissue mast cells has lacked sufficient evidence for quite some time. Human mast cells derived from umbilical cord blood express FcγRIIB [41, 42]. Human lung mast cells are reported not to express FcγRIIB [43] using immunohistochemistry, which is a less sensitive technique. Interestingly, FcγRIIB is recently confirmed to be expressed on human gastrointestinal mast cells [44].

Furthermore, a functional study indirectly suggests the presence of FcγRIIB on human mast cells [45]. No experimental evidence of FcγRIIIA expression on human mast cells has been obtained. Human mast cells express the high-affinity receptor FcγRI after treatment with IFN-γ [19]. However, human mast cells express FcγRIIA, which is the human homologue of the mouse FcγRIIIA. It is possible that FcγRIIA and FcγRIIB can exert counter-regulatory roles in determining the tissue persistence of human mast cells, similarly as the FcγRIIIA/FcγRIIB axis in mice. Of course this speculation regarding FcγR-mediated regulation of human mast cell tissue persistence awaits further experimental validation.

In conclusion, we have provided experimental evidence supporting a rheostat role for the FcγRIIB and FcγRIIIA in regulating mouse tissue mast cell persistence. This mechanism is consistent with the long-held observation that CTMC, which express both FcγRIIB and FcγRIIIA, have a longer tissue life span than MMC, which only express FcγRIIB.

Study limitation and future perspectives

In this study, our data support FcγR-mediated mast cell apoptosis as a mechanistic account for the long-observed differential tissue persistence of CTMC and MMC. However, we have not provided direct evidence demonstrating that constitutive IgG immune complex-mediated crosslinking of FcγR, which is assumed to result in cell death, is a critical regulatory machinery underlying mast cell persistence. Transfer of congenically marked FcγRIIB-deficient and FcγRIIIA-deficient BMMC into B cell-deficient mice, which do not have IgG immune complexes, may possibly reveal whether the IgG immune complex-FcγR axis is fundamentally required for the regulation of mast cell tissue persistence. This engraftment model can also reveal whether any FcγR-dependent, but IgG immune complex-independent, mechanisms might also play a role in regulating mast cell tissue persistence.

Materials and Methods

Mice

C57BL/6 mice were purchased from Taconic Farms (Ry, Denmark). FcγRIIB$^{-/-}$ mice were obtained from Dr. Ken Smith, Cambridge. FcγRIIIA$^{-/-}$ mice were purchased from the Jackson Laboratory. Both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice were on the C57BL/6 background. *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were provided by Dr. Gunnar Nilsson (Karolinska Institute, Stockholm, Sweden). All animal procedures were carried out with the approval of the Ethical Committee for Laboratory Animals in Gothenburg, Sweden, and the Ethics Committee of the Guizhou Medical University, China.

Mast cell culture and characterization

BM derived-cultured mast cells (BMMC) were obtained as previously described [46]. Essentially, BM cells were maintained at 37°C in 5% CO_2 in RPMI 1640 medium containing 4 mM L-glutamine supplemented with 10% FBS, and 50 μM 2-ME, 1 mM sodium pyruvate, 0.1 mM MEM nonessential AAs, as well as penicillin and streptomycin. All the cell culture reagents were purchased from Sigma–Aldrich. To support mast cell differentiation, the medium was further supplemented with 10 ng/mL IL-3 (PeproTech). In some experiments, BMMC cultures were polarized to the connective tissue type by using the culture medium described above, plus 25 ng/mL recombinant murine SCF (ImmunoTools) and 1 ng/mL recombinant murine IL-4 (ImmunoTools). Cells were used at 3 weeks of culture. Mast cell differentiation was confirmed by the surface expression of cKit (BV421; clone 2B8; BioLegend) and FcεRI (PE-Cy7; clone MAR-1; BioLegend) using flow cytometry.

BMMC obtained from various strains of mice were stained with 0.5% toluidine blue (Sigma-Aldrich) to visualize mast cell granules. In some experiments, cells were stained with 0.1% safranin (Merck Millipore). Transmission electron microscopic images were obtained using a LEO 912AB Omega transmission electron microscope (Carl Zeiss), as we previously reported [23]. Cell surface expression of FcγRIIB and FcγRIIIA was determined by fluorescent antibodies against FcγRIIB (PE; clone AT130-2; eBioscience) and FcγRIIIA (APC; clone 275003; R&D Systems) using flow cytometry.

Aggregation of mast cell FcγR

For in vitro aggregation of FcγR, BMMC cultured from WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice were treated in a total volume of 100 μL for 16 h with OVA-IgG immunecomplexes prepared by incubating mouse IgG1 anti-OVA (Sigma–Aldrich) and OVA (Sigma–Aldrich) at a molar ratio of 1:1.5 for 30 min at 37°C, as we previously reported [16]. For *in vivo* FcγR aggregation, each mouse of the WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ strain received intraperitoneally 1 mg heat-aggregated polyclonal IgG purified from normal mouse serum. Mouse peritoneal mast cell apoptosis was examined 16 hours after IgG delivery.

Apoptosis assays

Mast cell apoptosis was measured by determining the profile of Annexin V (FITC or APC, BD Biosciences) and PI (Sigma-Aldrich) staining using flow cytometry. Alternatively, apoptosis was analyzed by measuring the reduction in mitochondrial membrane potential by flow cytometry using JC-1 (M8650; Solarbio), a membrane-permeable lipophilic cationic fluorochrome that exerts enhanced green fluorescence and reduced red fluorescence when the cell fails to maintain mitochondrial membrane potential, a feature of the apoptotic cell. Production of polycaspase or

caspase-3/7 was detected by flow cytometry using a Vybrant FAM poly-caspase detection kit (V35117; Invitrogen) and a Vybrant FAM caspase-3 and -7 assay kit (V35118; Invitrogen). In some of the apoptosis assays, a pan-caspase inhibitor (Z-VAD-FMK; G7231; Promega; 20 μM) was applied during the process of FcγR aggregation.

Analysis of nasal tissue mast cells and peritoneal mast cells

Mouse nasal tissue mast cells were analyzed using flow cytometry, as we previously reported [23]. Essentially, lineage-negative (using Pacific Blue-conjugated antibodies against mouse CD3, Ly-6G/Ly-6C, CD19, NK1.1, CD11b, TER119, and B220) cells were gated for the identification of nasal mast cells which expressed FcεRI (PE-Cy7; clone MAR-1; BioLegend) and cKit (PE; clone 2B8; eBioscience). TLR-2 (FITC; clone QA16A01; BioLegend) was used for identifying CTMC, as previously reported [23]. Peritoneal mast cells were identified as positive for cKit (PE; clone 2B8; eBioscience) and surface-bound IgE (FITC; clone R35-72; BD Biosciences). Dead cells were gated out by 7-aminoactinomycin D (7-AAD; Sigma-Aldrich) staining.

Adoptive transfer experiment

BMMC were adoptively transferred into the peritoneal cavity of mast cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. The survival of these engrafted cells was followed after various time lengths as indicated. Mast cell survival in the peritoneal cavity was estimated by the percentages of live FcεRI^{+}cKit^{+} cells. For the cell proliferation assay, both donor cells and engrafted peritoneal mast cells at various time points after engraftment were examined. Cells were first stained for relevant surface markers (FcεRI and cKit) as described above, followed by fixation and permeabilization using the Intracellular Fixation and Permeabilization Buffer Set (BD Biosciences). Cell proliferation was determined by intracellular staining with an antibody against Ki-67 (FITC; clone B56; BD Biosciences), in some assays in parallel with an isotype-matched control antibody, using flow cytometry.

Mast cell activation and measurements

Cells were incubated in culture medium at a concentration of 10^6 cells/mL with various stimuli. In some experiments, cells were incubated with IgE that recognizes DNP (clone SPE-7, Sigma–Aldrich) at a concentration of 1 μg/mL for 1 h, followed by washing and the addition of either 10 μg/mL TNP_{12}-BSA (Santa Cruz) or vehicle control. Cells were then incubated for 30 min for β-hexosaminidase release as a measure of degranulation using an enzymatic colorimetric assay [47] or for 4 h for the measurement of intracellular TNF-α (APC; clone MP6-XT22; BioLegend) and IL-6 (PE; clone MP5-20F3; BioLegend) production using flow cytometry. Alternatively, mast cells were activated with mast cell secretagogues including compound 48/80 and ionomycin (both from Sigma–Aldrich), as previously reported [48].

Statistical analysis

For mouse nasal cell analysis, data were analyzed by one-way ANOVA, followed with Tukey's multiple comparison test. Other experimental data were analyzed for statistical significance by using an unpaired two-tailed Student's *t*-test. A *p*-value of 0.05 or less is considered statistically significant.

Acknowledgements: This paper is dedicated to the memory of Nils Lycke, a great and inspiring mentor of Z.X. and Y.F.'s. The research described in this paper was supported initially by funding from the Mucosal Immunobiology and Vaccine Research Center (MIVAC) at Gothenburg, a center established by Nils Lycke with unwavering passion and commitment. The work was subsequently supported by grants from the Stiftelsen Clas Groschinskys Minnesfond (M12228), Sweden to Z.X., Hong Kong Polytechnic University Research Fund (P0001169) to Z.X., the Hong Kong Research Grants Council General Research Fund (15104418) to Z.X., the National Natural Science Foundation of China (81560266; 81760294) to Y.F., and Guizhou Provincial Innovative Talents Team Program (2019-5610) to Y.F.

Conflict of interest: The authors declare no commercial or financial conflict of interest.

Author contributions: T.W., S.Y., Y.F., Y.W.Y., and Z.X. performed experiments and analyzed the data. Y.F. and Z.X. wrote the manuscript. Y.W.Y. designed the graphical abstract. Z.X. conceived the study.

Ethics approval: All animal procedures were carried out with the approval of the Ethical Committee for Laboratory Animals in Gothenburg, Sweden, the Ethics Committee of the Guizhou Medical University (1503057), China, and the Animal Subjects Ethics SubCommittee of the Research Committee at the Hong Kong Polytechnic University (16-17/30-HTI-R-GRF), Hong Kong.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Peer review: The peer review history for this article is available at https://publons.com/publon/10.1002/eji.202250221

References

1 **Saito, H., Ishizaka, T. and Ishizaka, K.**, Mast cells and IgE: from history to today. *Allergol Int.* 2013. **62**: 3–12.

2 **Galli, S.J., Tsai, M. and Piliponsky, A.M.**, The development of allergic inflammation. *Nature* 2008. **454**: 445–454.

3 **Valent, P., Akin, C., Hartmann, K., Nilsson, G., Reiter, A., Hermine, O., Sotlar, K. et al.**, Mast cells as a unique hematopoietic lineage and cell system: from Paul Ehrlich's visions to precision medicine concepts. *Theranostics* 2020. **10**: 10743–10768.

4 **Hershko, A.Y., Charles, N., Olivera, A., Alvarez-Errico, D. and Rivera, J.**, Cutting edge: persistence of increased mast cell numbers in tissues links dermatitis to enhanced airway disease in a mouse model of atopy. *J Immunol.* 2012. **188**: 531–535.

5 **Vliagoftis, H. and Befus, A.D.**, Rapidly changing perspectives about mast cells at mucosal surfaces. *Immunol Rev.* 2005. **206**: 190–203.

6 **Enerback, L.**, Mast cells in rat gastrointestinal mucosa. 2. Dye-binding and metachromatic properties. *Acta Pathol Microbiol Scand.* 1966. **66**: 303–312.

7 **Enerback, L.**, Mucosal mast cells in the rat and in man. *Int Arch Allergy Appl Immunol.* 1987. **82**: 249–255.

8 **Bruhns, P.**, Properties of mouse and human IgG receptors and their contribution to disease models. *Blood* 2012. **119**: 5640–5649.

9 **Sun, J.B., Holmgren, J., Cragg, M.S. and Xiang, Z.**, Lack of Fc gamma receptor IIIA promotes rather than suppresses humoral and cellular immune responses after mucosal or parenteral immunization with antigen and adjuvants. *Scand J Immunol.* 2017. **85**: 264–271.

10 **Nimmerjahn, F. and Ravetch, J.V.**, Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol.* 2008. **8**: 34–47.

11 **Jonsson, F. and Daeron, M.**, Mast cells and company. *Front Immunol.* 2012. **3**: 16.

12 **Daeron, M.**, Fc receptors as adaptive immunoreceptors. *Curr Top Microbiol Immunol.* 2014. **382**: 131–164.

13 **Pearse, R.N., Kawabe, T., Bolland, S., Guinamard, R., Kurosaki, T. and Ravetch, J.V.**, SHIP recruitment attenuates Fc gamma RIIB-induced B cell apoptosis. *Immunity* 1999. **10**: 753–760.

14 **Tzeng, S.J., Bolland, S., Inabe, K., Kurosaki, T. and Pierce, S.K.**, The B cell inhibitory Fc receptor triggers apoptosis by a novel c-Abl family kinase-dependent pathway. *J Biol Chem.* 2005. **280**: 35247–35254.

15 **Xiang, Z., Cutler, A.J., Brownlie, R.J., Fairfax, K., Lawlor, K.E., Severinson, E., Walker, E.U. et al.**, FcgammaRIIb controls bone marrow plasma cell persistence and apoptosis. *Nat Immunol.* 2007. **8**: 419–429.

16 **Fang, Y., Larsson, L., Bruhns, P. and Xiang, Z.**, Apoptosis of mouse mast cells is reciprocally regulated by the IgG receptors FcgammaRIIB and FcgammaRIIIA. *Allergy* 2012. **67**: 1233–1240.

17 **Katz, H.R., Arm, J.P., Benson, A.C. and Austen, K.F.**, Maturation-related changes in the expression of Fc gamma RII and Fc gamma RIII on mouse mast cells derived in vitro and in vivo. *J Immunol.* 1990. **145**: 3412–3417.

18 **Katz, H.R. and Lobell, R.B.**, Expression and function of Fc gamma R in mouse mast cells. *Int Arch Allergy Immunol.* 1995. **107**: 76–78.

19 **Tkaczyk, C., Okayama, Y., Metcalfe, D.D. and Gilfillan, A.M.**, Fcgamma receptors on mast cells: activatory and inhibitory regulation of mediator release. *Int Arch Allergy Immunol.* 2004. **133**: 305–315.

20 **Panda, S. and Ding, J.L.**, Natural antibodies bridge innate and adaptive immunity. *J Immunol.* 2015. **194**: 13–20.

21 **Combs, J.W., Lagunoff, D. and Benditt, E.P.**, Differentiation and proliferation of embryonic mast cells of the rat. *J Cell Biol.* 1965. **25**: 577–592.

22 **Galli, S.J., Nakae, S. and Tsai, M.**, Mast cells in the development of adaptive immune responses. *Nat Immunol.* 2005. **6**: 135–142.

23 **Fang, Y., Zhang, T., Lidell, L., Xu, X., Lycke, N. and Xiang, Z.**, The immune complex CTA1-DD/IgG adjuvant specifically targets connective tissue mast cells through FcgammaRIIIA and augments anti-HPV immunity after nasal immunization. *Mucosal Immunol.* 2013. **6**: 1168–1178.

24 **Tsai, M., Shih, L.S., Newlands, G.F., Takeishi, T., Langley, K.E., Zsebo, K.M., Miller, H.R. et al.**, The rat c-kit ligand, stem cell factor, induces the development of connective tissue-type and mucosal mast cells in vivo. Analysis by anatomical distribution, histochemistry, and protease phenotype. *J Exp Med.* 1991. **174**: 125–131.

25 **Grimbaldeston, M.A., Chen, C.C., Piliponsky, A.M., Tsai, M., Tam, S.Y. and Galli, S.J.**, Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am J Pathol.* 2005. **167**: 835–848.

26 **Amin, K., Ludviksdottir, D., Janson, C., Nettelbladt, O., Bjornsson, E., Roomans, G.M., Boman, G. et al.**, Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. *Am J Respir Crit Care Med.* 2000. **162**: 2295–2301.

27 **Newlands, G.F., Miller, H.R., MacKellar, A. and Galli, S.J.**, Stem cell factor contributes to intestinal mucosal mast cell hyperplasia in rats infected with *Nippostrongylus brasiliensis* or *Trichinella spiralis*, but anti-stem cell factor treatment decreases parasite egg production during *N. brasiliensis* infection. *Blood* 1995. **86**: 1968–1976.

28 **Dahlin, J.S., Malinovschi, A., Ohrvik, H., Sandelin, M., Janson, C., Alving, K. and Hallgren, J.**, Lin- CD34hi CD117int/hi FcepsilonRI+ cells in human blood constitute a rare population of mast cell progenitors. *Blood* 2016. **127**: 383–391.

29 **Hamey, F.K., Lau, W.W.Y., Kucinski, I., Wang, X., Diamanti, E., Wilson, N.K., Gottgens, B. et al.**, Single-cell molecular profiling provides a high-resolution map of basophil and mast cell development. *Allergy* 2021. **76**: 1731–1742.

30 **Metcalfe, D.D. and Mekori, Y.A.**, Pathogenesis and pathology of mastocytosis. *Annu Rev Pathol.* 2017. **12**: 487–514.

31 **Ekoff, M. and Nilsson, G.**, Mast cell apoptosis and survival. *Adv Exp Med Biol.* 2011. **716**: 47–60.

32 **Maurer, M., Tsai, M., Metz, M., Fish, S., Korsmeyer, S.J. and Galli, S.J.**, A role for Bax in the regulation of apoptosis in mouse mast cells. *J Invest Dermatol.* 2000. **114**: 1205–1206.

33 **Forster, A., Grotha, S.P., Seeger, J.M., Rabenhorst, A., Gehring, M., Raap, U., Letard, S. et al.**, Activation of KIT modulates the function of tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R) in mast cells. *Allergy* 2015. **70**: 764–774.

34 **McIlwain, D.R., Berger, T. and Mak, T.W.**, Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol.* 2015. **7**: a008656.

35 **Hardwick, J.M. and Soane, L.**, Multiple functions of BCL-2 family proteins. *Cold Spring Harb Perspect Biol.* 2013. **5**: a008722.

36 **Bhadra, K.**, A mini review on molecules inducing caspase-independent cell death: a new route to cancer therapy. *Molecules* 2022. **27**: 6401.

37 **Schrader, K., Huai, J., Jockel, L., Oberle, C. and Borner, C.**, Non-caspase proteases: triggers or amplifiers of apoptosis? *Cell Mol Life Sci.* 2010. **67**: 1607–1618.

38 **Pradelli, L.A., Beneteau, M. and Ricci, J.E.**, Mitochondrial control of caspase-dependent and -independent cell death. *Cell Mol Life Sci.* 2010. **67**: 1589–1597.

39 **Yoshikawa, H. and Tasaka, K.**, Caspase-dependent and -independent apoptosis of mast cells induced by withdrawal of IL-3 is prevented by Toll-like receptor 4-mediated lipopolysaccharide stimulation. *Eur J Immunol.* 2003. **33**: 2149–2159.

40 **Burgener, S.S., Brugger, M., Leborgne, N.G.F., Sollberger, S., Basilico, P., Kaufmann, T., Bird, P.I. et al.**, Granule leakage induces cell-intrinsic, granzyme B-mediated apoptosis in mast cells. *Front Cell Dev Biol.* 2021. **9**: 630166.

41 **Kepley, C.L., Taghavi, S., Mackay, G., Zhu, D., Morel, P.A., Zhang, K., Ryan, J.J. et al.**, Co-aggregation of FcgammaRII with FcepsilonRI on human mast cells inhibits antigen-induced secretion and involves SHIP-Grb2-Dok complexes. *J Biol Chem.* 2004. **279**: 35139–35149.

42 **Okayama, Y., Hagaman, D.D., Woolhiser, M. and Metcalfe, D.D.**, Further characterization of FcgammaRII and FcgammaRIII expression by cultured human mast cells. *Int Arch Allergy Immunol.* 2001. **124**: 155–157.

43 **Valent, P., Ashman, L.K., Hinterberger, W., Eckersberger, F., Majdic, O., Lechner, K. and Bettelheim, P.**, Mast cell typing: demonstration of a distinct hematopoietic cell type and evidence for immunophenotypic relationship to mononuclear phagocytes. *Blood* 1989. **73**: 1778–1785.

44 **Burton, O.T., Epp, A., Fanny, M.E., Miller, S.J., Stranks, A.J., Teague, J.E., Clark, R.A. et al.**, Tissue-specific expression of the low-affinity IgG receptor, FcgammaRIIb, on human mast cells. *Front Immunol.* 2018. **9**: 1244.

45 **Zhang, K., Kepley, C.L., Terada, T., Zhu, D., Perez, H. and Saxon, A.**, Inhibition of allergen-specific IgE reactivity by a human Ig Fcgamma-Fcepsilon bifunctional fusion protein. *J Allergy Clin Immunol.* 2004. **114**: 321–327.

46 **Wu, T., Ma, L., Jin, X., He, J., Chen, K., Zhang, D., Yuan, R. et al.**, S100A4 is critical for a mouse model of allergic asthma by impacting mast cell activation. *Front Immunol.* 2021. **12**: 692733.

47 **Xiang, Z. and Nilsson, G.**, IgE receptor-mediated release of nerve growth factor by mast cells. *Clin Exp Allergy.* 2000. **30**: 1379–1386.

48 **Xiang, Z., Ahmed, A.A., Moller, C., Nakayama, K., Hatakeyama, S. and Nilsson, G.**, Essential role of the prosurvival bcl-2 homologue A1 in mast cell survival after allergic activation. *J Exp Med.* 2001. **194**: 1561–1569.

Abbreviations: **BMMC**: bone marrow-derived mast cell · **MMC**: mucosal mast cell · **CTMC**: connective tissue mast cell

Full correspondence: Dr. Zou Xiang, Department of Health Technology and Informatics, Faculty of Health and Social Sciences, Hong Kong Polytechnic University, Hong Kong, P. R. China
e-mail: xiang.y.zou@polyu.edu.hk
Dr. Yu Fang, Center for Clinical Laboratory, Affiliated Hospital of Guizhou Medical University, Guiyang, P. R. China
e-mail: yu.fang@gmc.edu.cn

Received: 18/10/2022
Revised: 29/4/2023
Accepted: 2/5/2023
Accepted article online: 3/5/2023