IL-4 selectively enhances FcγRIII expression and signaling on mouse mast cells

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Abstract:

Fc receptors for IgG (FcγR) are widely expressed in the hematopoietic system and mediate a variety of inflammatory responses. There are two functional classes of FcγR, activation and inhibitory receptors. Since IgG immune complexes (IgG IC) bind each class with similar affinity, co-expression of these receptors leads to their co-ligation. Thus, expression levels of this antagonistic pair play a critical role in determining the cellular response. Murine mast cells co-express the activation receptor FcγRIII and the inhibitory receptor FcγRIIb and can be activated by IgG IC. Mast cell activation contributes to allergic and other inflammatory diseases—particularly those in which IgG IC may play important roles. Using mouse bone marrow-derived mast cells, we report that IL-4 selectively increases FcγRIII expression without altering FcγRIIb. This enhanced expression could be induced by Stat6 activation alone, and appeared to be mediated in part by increased FcγRIIIα protein synthesis without significant changes in transcription. The increase in FcγRIII expression was functionally significant, as it was matched by enhanced FcγR-mediated degranulation and cytokine production. Selective regulation of mast cell FcγR by interleukin-4 could alter inflammatory IgG responses and subsequently disease severity and progression.

Keywords: Mast cell | FcγR | IL-4 | CD16

Article:

1. Introduction

Mast cells have long been appreciated as effector cells in allergy and asthma. Recently, their role in inflammation has diversified, as mast cells are now implicated in a variety of inflammatory processes including host resistance to bacterial infections [1], mouse models of multiple

sclerosis[2], rheumatoid arthritis [3], and inflammation in cardiovascular disease [4]. Mast cell responses are characterized by the production of vasoactive mediators, proteases, lipid-derived factors, and cytokines [5]. The high affinity receptor for IgE, FcεRI, is the most thoroughly studied mediator of mast cell function. However, recent studies have emphasized the importance of the IgG Fc receptors FcγRIIb and FcγRIII [6], [7], [8], [9], [10].

Murine FcγR are widely expressed in the hematopoietic system. These receptors fall into two distinct classes: activation and inhibitory receptors. Activation receptors include the high affinity FcγRI, and the low affinity FcγRIII. Aggregation of FcγRI or FcγRIII can mediate a variety of inflammatory processes, including degranulation, phagocytosis, antibody-dependent cellular cytoxicity, cytokine production, and release of inflammatory arachidonic acid metabolites [11], [12]. Signal transduction through these receptors is mediated by immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the Fc receptor γ chain, through recruitment and activation of tyrosine kinases such as lyn and syk [11], [12].

In contrast to the activation receptors, murine FcγRIIb mediates inhibitory IgG signaling. While ITAM sequences elicit pro-inflammatory FcγR signaling, FcγRIIb possesses immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that preferentially interact with tyrosine and inositol phosphatases. Crosslinkage of FcγRIIb with an activation receptor such as the B cell receptor, T cell receptor, FcεRI, FcγRI, or FcγRIII can inhibit pro-inflammatory signaling [13], [14], [15]. The contrasting functions of FcγRIIb and FcγRIII, together with their shared ligand binding and co-expression on monocytes, macrophages, neutrophils, and mast cells, allow control of IgG-mediated signaling. Mice deficient in FcγRIIb have been used to demonstrate the importance of balancing this dual expression. These animals are prone to developing autoimmune conditions such as collagen-induced arthritis and Goodpasture's syndrome [11], [12]. It appears that the activation and inhibitory Fcγ receptors have evolved as a paired antagonistic signaling system, allowing changes in expression levels to alter the stimulus transduced by IgG immune complexes.

Murine mast cells express both FcγRIIb and FcγRIII [12]. While FcγRIIb is a single chain transmembrane protein, FcγRIII is expressed as either a trimeric complex, via association with paired FcεRIγ subunits, or a tetrameric complex, through association with FcεRIβ and γ subunits. γ subunit association is required for FcγRIII expression and signaling. In contrast, β chain amplifies FcγRIII signaling, but is not obligatory for expression [16]. The balance between FcγRIIb and FcγRIII expression on mast cells, is critical in determining the mast cell response to IgG complexes, as recently demonstrated using a mouse model of multiple sclerosis. Mast cell deficient mice reconstituted with FcγRIII−/− mast cells greatly reduced disease severity compared to mice reconstituted with wild type mast cells. In contrast, reconstitution with FcγRIIb−/− mast cells severely augmented disease pathology [17].

Mast cell-mediated inflammation is most frequently studied in the context of T helper cell type 2 (Th2) immunity. Interleukin-4 (IL-4) is a Th2 cytokine that is critical in controlling allergic responses. Best known for inducing Th2 development and B cell isotype switching to IgE, IL-4 is also a mast cell growth factor and has been shown to induce FcεRI expression on in vitro-cultured human mast cells [18], [19], [20]. Due to these activities, IL-4 is associated with the

pro-inflammatory effects of the Th2 response. Our studies of IL-4 have led to a more balanced view of its role in inflammation. We recently demonstrated that IL-4 inhibits mast cell FcεRI and Kit expression and signaling. Further, IL-4 was found to combine with IL-10 to induce mast cell death [21], [22], [23]. It appears that IL-4 may initially enhance inflammatory responses, but balance these effects with a delayed inhibition of mast cell function and survival, thus arguing for IL-4-mediated homeostasis in the Th2 response. These studies prompted us to explore IL-4 effects on the balance of mast cell FcγRIIb/RIII expression and function. Given their identical ligand binding properties and co-expression on mast cells, altering the ratio of these surface receptors could greatly affect mast cell function and consequently, inflammatory disease.

2. Results and discussion

2.1. IL-4 selectively enhances FcγRIII expression

To assess the effects of IL-4 on FcγRIIb and FcγRIII expression, we cultured BMMC[1] in IL-3 alone or IL-3 + IL-4. After 3 days of culture, there was a slight increase in FcγR expression as assessed by flow cytometry with FITC-2.4G2, an antibody that recognizes both FcγRIIb and FcγRIII (Fig. 1A). To determine if this increase in FcγR expression was due to an increase in FcγRIIb, these BMMC were stained with K9.361, an antibody specific for FcγRIIb. No change in FcγRIIb expression was detected after the addition of IL-4 in culture for 3 days as compared to cells cultured in IL-3 alone (Fig. 1B). This led us to explore the possibility that IL-4 upregulated FcγRIII surface expression without altering FcγRIIb expression. In order to test this directly, we harvested BMMC from FcRγ$^{-/-}$mice that lack FcγRIII but express FcγRIIb, and from Fcγ$^{-/-}$ mice that lack FcγRIIb but express FcγRIII [9], [24]. In concordance with K9.361 staining, the addition of IL-4 did not significantly alter FcγRIIb surface expression. In contrast, culture in IL-3 + IL-4 consistently increased FcγRIII surface expression more than 2-fold over BMMC cultured in IL-3 alone (Fig. 1C). Because BMMC basal surface levels of FcγRIIb is greater than FcγRIII expression [25], an IL-4-mediated increase in FcγRIII could be expected to yield only a slight increase in staining total FcγR expression, consistent with the data shown in Fig. 1A.

IL-4 elevated FcγRIII expression from days 2 through at least day 14 of culture. This effect required a minimum of 1 ng/ml of IL-4, with maximal effects at 10 ng/ml. FcγRIIb expression showed little or no change during this culture period (Figs. 2A and B). Thus, IL-4 selectively enhanced mast cell FcγRIII surface expression without significantly altering the expression of FcγRIIb for up to 14 days.

2.2. IL-4 modestly alters Fc*γ*RIII*α* mRNA expression and upregulates protein expression

We investigated the mechanism of IL-4-mediated FcγRIII regulation by first assessing FcγRIIIα mRNA changes through RNase protection assay (RPA) analysis. The addition of IL-4 to BMMC cultured in IL-3 for 30 min to 72 h did not significantly alter FcγRIIIα mRNA expression, though a moderate increase was noted after 96 h of culture (Figs. 3A and B). This finding corroborates a related study in which we found that IL-4 downregulates FcεRI surface expression without significantly altering mRNA expression of FcεRI subunits, including the FcεRIγ chain known to pair with FcγRIII (Gillespie et al., submitted). Since IL-4 increased surface FcγRIII by day 2,

and mRNA levels do not increase until day 4, it did not appear that IL-4 regulates FcγRIII expression via transcriptional control of the FcγRIIIα or FcεRIγ chains.

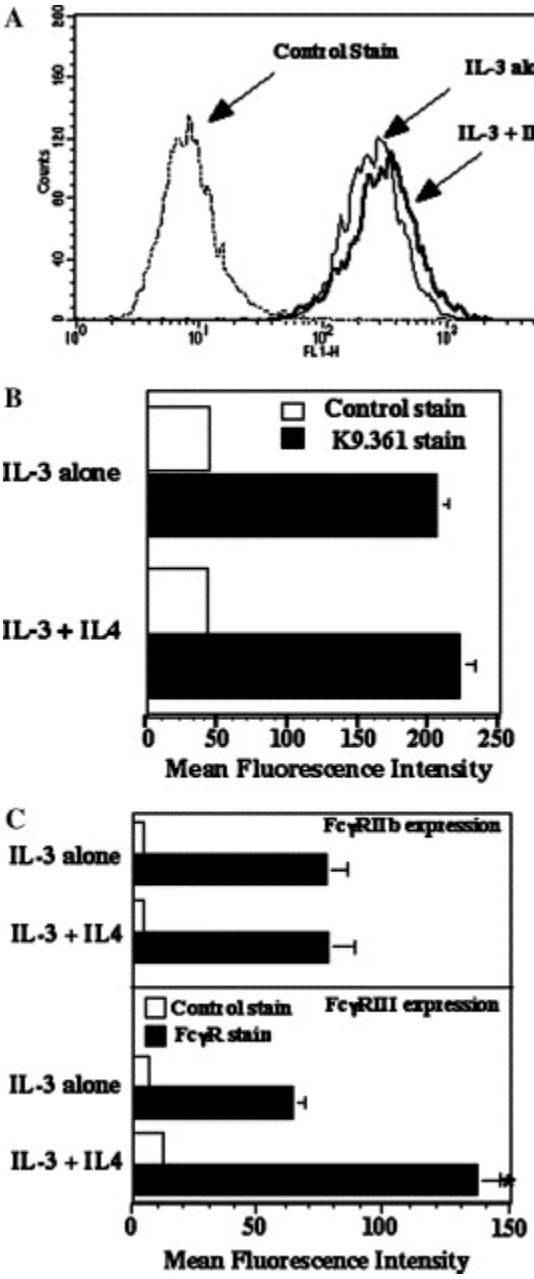

Fig. 1. IL-4 selectively enhances FcγRIII expression. BMMC derived from wild mice were cultured in IL-3 or IL-3 + IL-4 for three days and assessed for FcγR expression by flow cytometry with (A) anti-FcγRII/FcγRIII antibody (2.4G2) or (B) anti-FcγRII antibody (K9.361). (C) BMMC derived from FcRγ$^{-/-}$ (top) or FcγRIIb$^{-/-}$ (bottom) mice were cultured in IL-3 or IL-3 + IL-4 for 3 days and assessed for FcγR expression by flow cytometry using FITC-2.4G2. Data shown are from at least seven independent experiments using 12 different cell populations. *p<0.05 as determined by analysis of variance (ANOVA) and least significant difference (LSD) analysis.

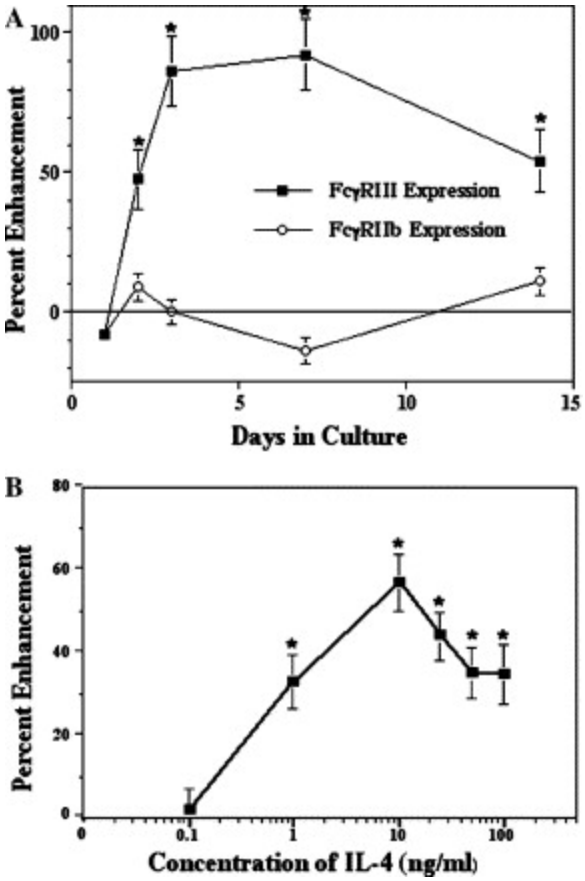

Fig. 2. Kinetics of IL-4-mediated FcγR regulation. (A) BMMC derived from FcRγ$^{-/-}$or FcγRIIb$^{-/-}$ mice were cultured in IL-3 or IL-3 + IL-4. FcγR expression was assessed by flow cytometry on the indicated days by FITC-2.4G2 staining. Percent enhancement and standard error measurements were calculated by comparing mean fluorescence intensities of IL-3 + IL-4 treated BMMC to the same cells cultured in IL-3. Data shown are from nine independent BMMC populations analyzed in at least six separate experiments. $*p<0.05$ as determined by ANOVA and LSD analysis. (B) BMMC derived from FcγRIIb$^{-/-}$ mice were cultured in IL-3 or IL-3 with increasing concentrations of IL-4. Percent enhancement was calculated by comparing the mean fluorescence of cells cultured in IL-3 + IL-4 to cells cultured in IL-3. $*p<0.05$ as determined by ANOVA and LSD analysis.

There are several post-transcriptional mechanisms by which IL-4 could increase FcγRIII surface expression, including increased protein synthesis, increased protein stability, and enhanced protein trafficking from preformed intracellular pools. To determine if the changes in FcγRIII surface expression were matched by an increase in total FcγRIIIα protein levels, we employed 2.4G2 staining of fixed and permeabilized FcγRIIb-deficient BMMC. This technique, modeled after intracellular staining of cytokines, allowed detection of both surface and intracellular FcγRIIIα, since unlabelled 2.4G2 antibody added prior to fixation could block surface but not intracellular FITC-2.4G2 staining (data not shown). These experiments demonstrated an IL-4-mediated increase in total FcγRIIIα protein expression that resembled the enhanced surface expression (Fig. 4A).

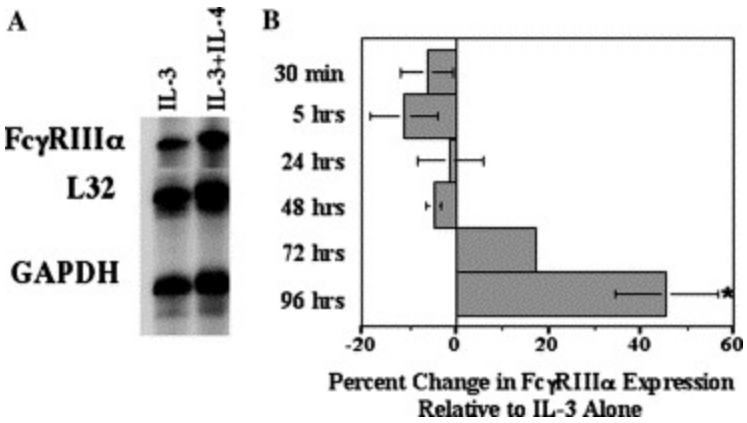

Fig. 3. IL-4 increases FcγRIIIα mRNA protein synthesis with minimal effects on transcription. (A) Wild type BMMC were stimulated for 4 days with the indicated cytokines, and total RNA was subjected to RPA analysis as described in Materials and methods. Data shown are a representative BMMC population harvested after 4 days of culture, the only time point that showed any change in FcγRIIIα mRNA expression. (B) Summary of percent change in FcγRIIIα mRNA in cells cultured in IL-3 + IL-4, as compared to cells cultured in IL-3 alone. Phosphorimaging was used to determine the ratio of FcγRIIIα expression to expression of L32 + GAPDH for each sample. This ratio was used to compare FcγRIIIα expression in BMMC treated with IL-3 + IL-4 to those cultured in IL-3 alone. Data are means and standard errors from 3 to 11 samples from five independent experiments.

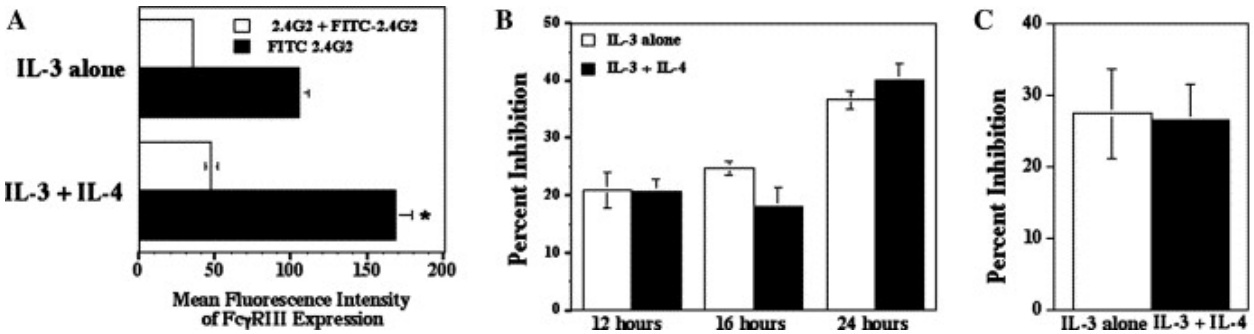

Fig. 4. IL-4 enhances FcγRIII expression without altering protein stability or trafficking. (A) BMMC derived from FcγRIIbα$^{-/-}$ mice were cultured in the indicated cytokines for 3 days, and assessed for total FcγRIIIα expression by intracellular staining with FITC-2.4G2, as described in Materials and methods. Statistical significance was evaluated as described in Fig. 1. *p<0.05 as determined by ANOVA and LSD analysis. (B) BMMC derived from FcγRIIb$^{-/-}$ mice were cultured for three days in IL-3 alone or in IL-3 + IL-4 prior to the addition of cycloheximide (4 μg/ml final) for the times indicated. Intracellular staining with 2.4G2 was used to assess total FcγRIIIα expression. Percent change in expression was determined by comparing mean fluorescence intensities of cells treated with cycloheximide to those in DMSO. Data shown are means and standard errors of nine different BMMC populations from three representative experiments. (C). BMMC derived from FcγRIIb$^{-/-}$ mice were cultured for three days in IL-3 alone or in IL-3 + IL-4 prior to the addition of Brefeldin A (3 μg/ml) for 20 h. Staining with 2.4G2 was used to assess surface FcγRIII expression by flow cytometry. Percent inhibition was determined by comparing mean fluorescence intensities of cells treated with BFA to those treated with media alone. Data shown are means and standard errors of six different cell populations in three independent experiments.

To determine if the increase in FcγRIIIα protein expression was related to changes in protein stability, we employed the translational inhibitor cycloheximide. FcγRIIb-deficient BMMC were stimulated for 3 days with IL-3 alone or with IL-3 + IL-4, after which cycloheximide was added to the cultures. Intracellular 2.4G2 staining during the proceeding 24 h showed that the rate of total FcγRIIIα degradation was unaltered by IL-4 treatment, indicating that IL-4 stimulation had no effect on FcγRIIIα stability (Fig. 4B).

There remained the possibility that IL-4 facilitated recycling of endocytosed FcγRIII, resulting in increased surface expression. Brefeldin A (BFA) prevents surface expression of newly synthesized proteins by inhibiting transport from the endoplasmic reticulum to the golgi, and blocks surface protein recycling by preventing endosomes from fusing with the trans-golgi [26], [27]. In order to determine if IL-4 upregulated FcγRIII surface expression by altering protein transport or recycling, we assessed changes in surface levels of FcγRIII after the addition of BFA to the culture. FcγRIIb-deficient BMMC were cultured in IL-3 alone or IL-3 + IL-4 for 3 days prior to the addition of BFA. Surface FcγRIII expression was measured 20 h later, the latest time point possible without affecting cell viability. Cells cultured in IL-3 + IL-4 had virtually the same sensitivity to BFA as cells cultured in IL-3 alone, as both groups demonstrated a 25% reduction in FcγRIII surface expression compared to cells treated with vehicle alone (Fig. 4C). These data agree with an earlier study by Kubo and co-workers [28] showing that surface FcγRIII is very stable and undergoes little receptor recycling.

Collectively these data argue that IL-4 enhances FcγRIIIα protein expression by increasing FcγRIIIα protein synthesis, without altering FcγRIIIα mRNA levels, protein stability or surface protein recycling. One possible mechanism by which this could occur is through enhancing translational efficiency. IL-4 signaling may regulate mRNA binding proteins, leading to increased translation without altering mRNA levels. Identifying these binding proteins, while an issue of clear interest, will require significant efforts and is the focus of future study.

The ability of IL-4 to enhance FcγRIII expression is an interesting contrast to IL-4-mediated downregulation of FcεRI. Although these events may have opposing effects on mast cell function, reduced FcεRI expression may be related to the increase in FcγRIII. Our recent study demonstrated that IL-4 diminishes FcεRI expression by decreasing FcεRIβ protein expression without affecting FcεRIα or FcRγ (Gillespie et al., submitted). Since FcγRIII expression requires pairing with FcεRIγ but not FcεRIβ, it is possible that loss of FcεRIβ Results in a de facto increase in FcγRIII expression due to reduced competition for the obligatory γ chain. This theory is supported by the demonstration that FcεRIα-deficient mast cells exhibit increased FcγRIII expression [16]. Further, these changes would likely be mast cell-specific, explaining the differences between our study and those of Te Velde et al., and Pricop et al. [29], [30] who found that IL-4 inhibits FcγRIII expression on monocytes. Therefore IL-4 may be enhancing surface FcγRIII expression both by increasing FcγRIIIα protein synthesis and limiting competition for its expression partner, FcεRIγ.

2.3. The role of Stat6 in IL-4-mediated FcγRIII expression

Because of the importance of Stat6 in IL-4 signaling, we explored the role of Stat6 in IL-4 mediated FcγRIII upregulation. In order to determine if Stat6 was sufficient to upregulate FcγRIII, we infected FcγRIIb-deficient BMMC with a bicistronic retroviral expression vector encoding green fluorescence protein (GFP) alone or GFP and a constitutively active mutant of Stat6, termed Stat6VT [31], [32]. These cells were maintained in IL-3 alone and assessed for FcγRIII expression 5 days post-infection. As shown in Fig. 5, Stat6VT increased FcγRIII approximately surface expression 2-fold, closely matching IL-4-mediated upregulation (Fig. 1C). These data demonstrate that Stat6 activation alone is sufficient to increase FcγRIII expression, data in keeping with IL-4-mediated regulation of FcεRI [, (Gillespie et al.,

submitted)]. Since FcγRIII expression is enhanced without altering FcγRIIIα transcription, it would appear that Stat6-mediated effects are indirect, perhaps by regulating expression of RNA binding proteins as previously suggested.

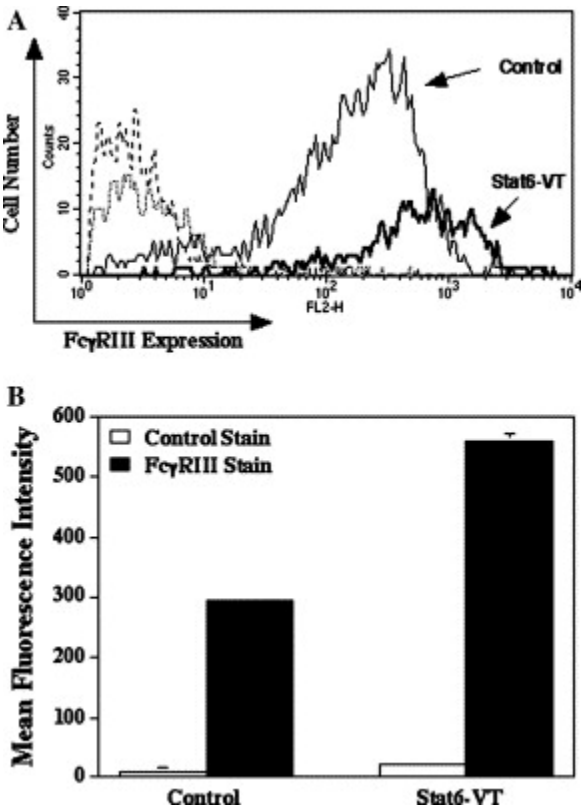

Fig. 5. Stat6 activation is sufficient to enhance FcγRIII expression. (A) BMMC derived from FcγRIIb$^{-/-}$ mice were transfected with bi-cistronic retrovirus expressing GFP alone (control) or GFP and constituitively active Stat6VT as described in Materials and methods. The cells were cultured in IL-3 for 3–5 days and stained with PE-2.4G2 to determine surface FcγRIII expression. Histogram shown is from a representative sample of GFP-positive cells. (B) Average mean fluorescence intensity and standard errors of six samples assessed as in (A).

2.4. IL-4 increases Fcγ*R*-mediated inflammatory function on wild type mast cells

Increasing expression of pro-inflammatory FcγRIII might enhance mast cell responses to IgG-mediated stimuli, however co-expression of inhibitory FcγRIIb receptors could block these signals, preventing mast cell activation. With recent data emphasizing the role of mast cell FcγRIII-mediated signaling in inflammatory disease [6], [7], [8], [9], [10], the effect of IL-4 on IgG-mediated activation of wild type mast cells has clinical importance.

We assessed the effects of IL-4 stimulation on the immediate phase of mast cell activation by measuring FcγR-induced release of β-hexosaminidase, a marker of mast cell degranulation [33]. Wild type BMMC expressing both FcγRIIb and FcγRIII were cultured in IL-3 alone or in IL-3 + IL-4 for 3 days, followed by crosslinkage with anti-FcγRIIb/FcγRIII mAb (2.4G2) and goat anti-rat IgG for 60 min. Release of the granule component β-hexosaminidase into culture supernatants was then assessed by enzyme assay. Degranulation was measured as percent specific release of stimulated cells over unstimulated cells. BMMC cultured in IL-3 alone showed no significant β-hexosaminidase release after 2.4G2 crosslinkage, consistent with the

high level of FcγRIIb expression on these cells [25]. However, BMMC cultured in IL-3 + IL-4 demonstrated enhanced β-hexosaminidase release after activation (Fig. 6). These data indicate that selective upregulation of FcγRIII by IL-4 is functionally significant, leading to increased IgG-mediated mast cell degranulation.

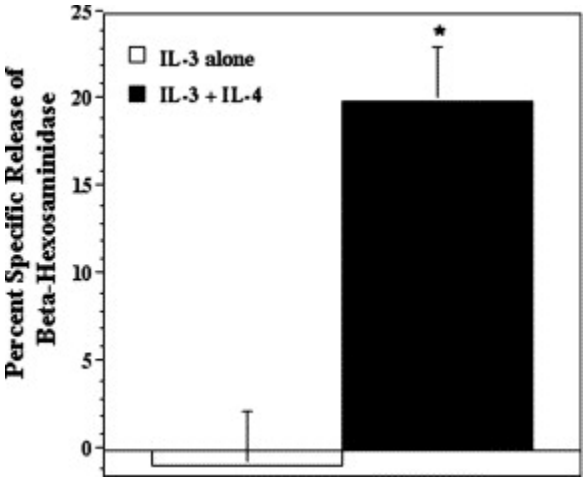

Fig. 6. IL-4 enhances FcγR-mediated β-hexosaminidase release on wild type BMMC. Wild type BMMC were cultured in IL-3 alone or in IL-3 + IL-4 for 3 days. Cells were then stimulated with 2.4G2 and goat anti-rat IgG or were left unstimulated, and assessed for β-hexosaminidase release as described in Materials and methods. Percent specific release was calculated by subtracting β-hexosaminidase release in non-activated cells from that of FcγR XL-stimulated cells as described in Materials and methods. Data shown are means and standard errors from a minimum of six BMMC populations assessed in a minimum of five independent experiments. *p<0.05 when comparing to IL-3 alone, as determined by ANOVA followed by LSD analysis.

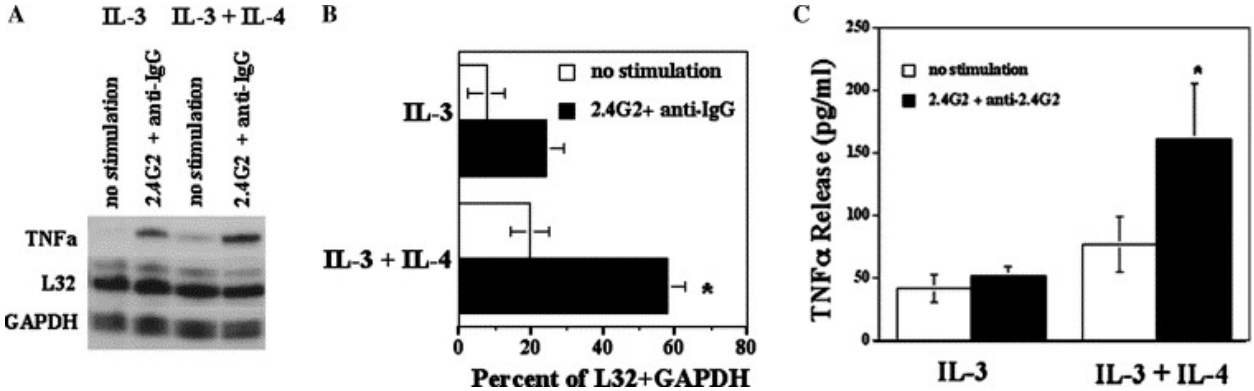

Fig. 7. IL-4 alters FcγR-mediated cytokine production. (A) Wild type BMMC were cultured for 3 days with the IL-3 or IL-3 + IL-4, followed by FcγR XL as described in Fig. 6. Total RNA was subjected to RPA analysis. Data shown are one representative RPA from five independent experiments with similar results using 3–11 independent BMMC populations. (B) Summary of changes in TNFα mRNA expression. Pixel intensities from (A) were obtained by phosphorimager analysis, and the ratio of cytokine gene expression to the sum of the L32 and GAPDH housekeeping gene expression was determined. Data are means and standard errors from 3 to 11 samples from 3 to 5 independent experiments. (C) Wild type BMMC were cultured in the indicated cytokines for 3 days, then stimulated with FcγR XL for 24 h. TNFα release was measured using ELISA as described in Materials and methods. Data shown are means and standard errors from three different BMMC populations assessed in three independent experiments. Each sample was done in triplicate. The lower limit of detection was 15.4 pg/ml. for TNFα. *p<0.05 when compared to cells cultured in IL-3 alone, using ANOVA and LSD analysis.

While mast cell degranulation is part of the early phase of mast cell activation, cytokine production is an indicator of the late phase response, and is a critical component of inflammatory infiltration and tissue damage [34]. To determine if IL-4 altered FcγR-mediated cytokine production, wild type BMMC were cultured for 3 days in IL-3 alone or IL-3 + IL-4, followed by activation with FcγR crosslinkage. TNFα mRNA and protein levels were measured by RPA and ELISA, respectively. These experiments showed that IL-4 enhanced both FcγR-mediated TNFα mRNA levels and protein secretion (Fig. 7), though this increase was modest in comparison to the effects on beta hexosaminidase release. Together with the degranulation studies, these results demonstrate that IL-4 stimulation enhances the early and late phases of FcγR-mediated pro-inflammatory mediator secretion in mast cells.

The ability of IL-4 to alter FcγR signaling in the context of both pro- and anti-inflammatory IgG receptors could be due solely to the selective upregulation of FcγRIII. However, FcγRIIb expression is still considerable after IL-4 stimulation. It remains possible that enhanced FcγR-mediated mast cell activation is due partly to enhanced FcγRIII expression and partly to inhibition of FcγRIIb signaling. This is the focus of future studies.

The current study demonstrates that IL-4 upregulates FcγRIII surface expression without significantly altering expression of FcγRIIb. IL-4 conveys these changes via alterations in FcγRIIIα protein expression without affecting mRNA synthesis, protein stability, or receptor recycling. Stat6 activation alone induces FcγRIII upregulation, emphasizing the importance of this transcription factor in IL-4-initiated signal transduction. Importantly, modifying the surface ratio of FcγRIIb to FcγRIII is functionally significant, allowing for degranulation and cytokine production after FcγR crosslinkage. Understanding FcγR regulation could be significant to inflammatory disease, as recently demonstrated in a rodent model of multiple sclerosis, where selective FcγRIII expression on mast cells altered disease severity [17]. Similarly, there is recent evidence that FcγR play an important role in a mouse model of rheumatoid arthritis [3]. Manipulation of the FcγRIIb/FcγRIII ratio by cytokines could therefore be important for controlling and treating inflammatory disease.

3. Materials and methods

3.1. BMMC cultures

BMMC were maintained as primary, factor-dependent, multi-clonal populations in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 1 mM Hepes (cRPMI; all materials from Biofluids, Rockville, MD), to which was added 20% volume/volume WEHI-3 cell-conditioned medium (cRPMI/WEHI). BMMC were cultured from bone marrow harvested from femurs of BL6x129 wild type, BL6x129 FcγRIIα-deficient, or FcRγ-deficient mice (Taconic Farms, Germantown, NY), and maintained in cRPMI/WEHI. After 3–4 weeks in culture, these populations were >99% mast cells, as judged by morphology and flow cytometry staining for expression of FcεRI, CD13, Kit, FcγRII/FcγRIII, and T1/ST2 (data not shown). The resulting populations were generally used between weeks 4 and 12.

3.2. Cytokines and reagents

Saponin, cycloheximide, and Brefeldin A were purchased from Sigma Immunochemicals (St. Louis, MO). Murine IL-3 and IL-4 were purchased from R&D Systems (Minneapolis, MN). 2.4G2 (rat anti-mouse FcγRII/RIII), mouse IgE, FITC-conjugated rat anti-mouse CD13, FITC-conjugated rat anti-mouse Kit, PE-conjugated rat isotype control IgG, and FITC-conjugated rat anti-mouse CD4 were purchased from BD PharMingen (San Diego, CA). FITC-conjugated goat F(ab′2) anti-rat IgG was purchased from Southern Biotechnology Associates (Birmingham, AL). FITC-conjugated rabbit anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-conjugated rat anti-mouse T1/ST2 was purchased from Morwell Diagnostics (Switzerland). K9.361 anti-FcγRIIb was the kind gift of Ulrich Hammerling (Memorial Sloan Kettering Cancer Center, NY). *P*-Nitropheno-β-d-acetamido-2-deoxyglucopyranoside was purchased from Sigma (St. Louis, MO).

3.3. Tissue culture conditions for regulation of BMMC Fc*γ*R expression

Cells were washed to remove WEHI-3 CM, and incubated in cRPMI at 37 °C for 4–6 h. Cells were plated at 3×10^5 cells/ml, 200 μl/well in 96-well flat-bottom plates. IL-3 was added to 5 ng/ml, followed by the indicated concentrations of IL-4. Cells were then incubated for the indicated times. Every 4 days half of the media and cytokines were replaced. FcγRIIb/RIII levels were then determined by flow cytometric analysis.

3.4. Flow cytometric analysis

To detect FcγRIIb expression, wild type BMMC were washed with FACS buffer [phosphate-buffered saline (PBS)/3%FCS/0.1% sodium azide] in 96-well "V" bottom plates, and resuspended in unlabelled K9.361 ascites diluted 1:100, or in FACS buffer alone for 30 min at 4 °C. Cells were then washed twice with FACS buffer and incubated for 30 min at 4 °C with 10 μg/ml FITC-rabbit anti-mouse IgG, washed twice, and analyzed in the presence of propidium iodide to establish a live cell gate. Flow cytometry was performed using a Becton–Dickinson FACScan (Becton–Dickinson, San Jose, CA). In some of these experiments, mouse IgG was employed as a control primary antibody in place of FACS buffer alone. This resulted in background staining not significantly different than FACS buffer alone. To detect FcγRIII expression, FcγRIIb-deficient BMMC were washed with FACS buffer and incubated for 30 min at 4 °C with 10 μg/ml FITC-2.4G2, either in the presence of unlabelled 2.4G2 to serve as a negative control, or without unlabelled 2.4G2. Cells were then washed twice, and analyzed by flow cytometry in the presence of propidium iodide. We also assessed FcγRIIb expression by 2.4G2 staining of BMMC derived from FcRγ chain-deficient mice, which lack expression of FcγRIII. These results recapitulated our observations with K9.361 staining of WT BMMC. The percent inhibition of FcγR expression was calculated by comparing mean fluorescent intensities (MFI) of populations cultured with IL-3 alone to those cultured with IL-3 and other stimuli using the following equation:

$$\{[(\text{MFI of IL-3 cultured BMMC}) - (\text{MFI of comparison culture})]/(\text{MFI of IL-3 culture})\} \times 100.$$

To detect expression of CD13, Kit, and T1/ST2, BMMC were incubated with unlabelled 2.4G2 ascites for 10 min at 4 °C to block nonspecific binding, followed by FITC-labeled antibodies at

10 μg/ml for 30 min at 4 °C. Cells were then washed twice and analyzed by flow cytometry in the presence of propidium iodide.

3.5. 2.4G2 staining of fixed and permeabilized cells

To detect intracellular FcγRIIIα expression, FcγRIIb deficient BMMC (3×10^5 cells/ml) were washed twice with 1× PBS and fixed with 4% paraformaldehyde/PBS for 20 min at room temperature. Cells were washed with and resuspended in FACS buffer, then stored overnight at 4 °C. Cells were then resuspended in 100 μl of staining buffer (PBS, 0.1% BSA, 0.01 M Hepes, and 0.5% Saponin) and incubated at room temperature for 10 min, prior to 2.4G2 staining as described above. Samples were analyzed by flow cytometry using a forward scatter versus side scatter gate.

3.6. RNase protection assay

For each sample, 5×10^6 BMMC (cultured at 5×10^6 cells/ml) were starved for 4 h in cRPMI, then stimulated with IL-3 (5 ng/ml) alone or IL-3 + IL-4 (20 ng/ml) for the indicated time points. To detect cytokine mRNA expression after 2.4G2 crosslinkage, cells were incubated for 10 min with 10 μg/ml 2.4G2 at 4 °C, washed, and incubated with 2.5 μg/ml goat F(ab′2) anti-rat IgG for 90 min at 37 °C. Total RNA was harvested using TRIzol Reagent (InVitrogen, Carlsbad, CA) and subjected to RPA analysis using the RiboQuant System (PharMingen, San Diego, CA). Pixel intensity was determined using a Typhoon Phosphorimager 445si System equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

3.7. Retroviral infection

FcγRIIb deficient BMMC cultures were infected with retrovirus expressing a bicistronic construct consisting of GFP alone or GFP and the constitutively active Stat6 mutant termed Stat6VT as described previously [31], [32]. FcγRIII surface expression was assessed on the GFP-positive population by flow cytometry analysis using phycoerythrin-coupled 2.4G2 antibody.

3.8. *β*-Hexosaminidase assay

For each sample, 6×10^4 BMMC were cultured with cytokines for the indicated times as described above, then stimulated by 2.4G2 crosslinkage in a volume of 50 μL as described above for RPA measurements. The supernatant was collected 60 min after activation at 37 °C and stored at −20 °C. The pellet was resuspended in PBS/1% NP40, incubated at 0 °C for 30 min, vortexed for 5 min, and clarified by centrifugation at 10,000 rpm for 20 min at 4 °C. β-Hexosaminidase activity in cellular supernatants and pellets was determined as described previously [33]. Percent release was calculated by dividing the amount of β-hexosaminidase activity in the supernatant by the sum of β-hexosaminidase activity in the supernatant and pellet. Specific release was determined by subtracting the percent release of unstimulated cells from cells stimulated by 2.4G2 crosslinkage. Non-specific release averages ranged from 7.9 to 15.11% on day 4.

3.9. ELISA

For each sample, 5×10^5 BMMC were cultured for 4 days with the indicated cytokines in duplicate wells. Equal numbers of BMMC were then stimulated by 2.4G2 crosslinkage in 200 μL cRPMI/IL-3 for 24 h at 37 °C as described above for RPA measurements, with the exception that an extra wash step was included following the incubation with goat anti-rat IgG. Cytokine release was measured using OptEIA ELISA kits (BD Pharmingen, San Diego, CA).

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