

IgG-dependent activation of human mast cells following up-regulation of Fc γ RI by IFN- γ

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It has been reported that Fc γ RI is up-regulated on human mast cells (huMC) by IFN- γ and aggregation of this receptor using mouse F(ab')₂ specific for receptor-bound, mouse anti-CD64 F(ab')₂ results in activation. To determine whether huMC can similarly be stimulated by aggregation of Fc γ RI-bound human IgG, IFN- γ -treated, CD34⁺-derived, cultured huMC were sensitized with human immunoglobulins and activation was evaluated following addition of antibodies specific for each IgG isotype. Degranulation was also examined following simultaneous IgG- and IgE-dependent aggregation of Fc γ RI and Fc ϵ RI. Activation of IFN- γ -treated huMC sensitized with 100 ng/ml IgG₁ resulted in 40% β -hexosaminidase (β -hex) release; minimal degranulation was observed using IgG₂, IgG₃ or IgG₄. IgG₁-dependent activation led to PGD₂ and LTC₄ generation as well as elevated cytokine production, most notably TNF- α . Preincubation of cells with F(ab')₂ from CD64-specific clones 10.1 and 32.2 reduced IgG₁-mediated β -hex release by 46% and 74%, respectively. While IgG-dependent cell stimulation induced half-maximal degranulation by 11 min, IgE-dependent activation resulted in half maximal responses within 1 min. Simultaneous activation of huMC via Fc γ RI and Fc ϵ RI led to additive degranulation using suboptimal concentrations of IgG₁ and IgE. Activation of huMC thus may occur via monomeric IgG and Fc γ RI thereby providing a novel paradigm for huMC recruitment into inflammation.

Key words: Mast cell / Fc receptor / Degranulation / Cytokine / IgG

Received	23/2/01
Revised	23/5/01
Accepted	28/8/01

1 Introduction

It has been reported that aggregation of the high affinity IgG receptor (Fc γ RI) on IFN- γ -treated human mast cells (huMC) using murine F(ab')₂ fragments specific for receptor bound mouse anti-Fc γ RI F(ab')₂ fragments results in mast cell activation as evidenced by degranulation as well as elevated arachidonic acid production and mRNA expression for cytokines such as TNF- α , GM-CSF, IL-3, IL-6 and IL-13 [1, 2]. The presence of Fc γ RI on huMC thus may provide a novel link by which mast cells recruited into sites of inflammation may become activated through IgG-dependent mechanisms, in addition to IgE-dependent mechanisms, in such diseases as scleroderma, inflammatory bowel disease (IBD), fibrosis, vasculitis, synovium inflammation [3–11].

However, for this to be possible, it must be demonstrated that human IgG will bind to Fc γ RI on the surface

of huMC and mediate huMC activation and mediator release if cross-linked. To explore this possibility, we exposed IFN- γ -treated huMC to monomeric IgG₁, IgG₂, IgG₃ or IgG₄ and examined whether these immunoglobulins could mediate mast cell degranulation and mediator production following addition of the appropriate anti-IgG antibodies. We also examined whether simultaneous activation of huMC through IgG- and IgE- dependent mechanisms would promote a larger degree of activation than that observed if mast cells were activated through only a single class of immunoglobulin.

As will be shown, IFN- γ -treated huMC are activated following sensitization with IgG followed by the addition of anti-IgG, thus aggregating Fc γ RI. Cross-linking receptor-bound IgG leads to mast cell degranulation, increased PGD₂ and LTC₄ production and TNF- α release. Simultaneous activation via IgG- and IgE-dependent mechanisms led to additive degranulation using suboptimal sensitizing concentrations of IgG₁ and IgE. This is the first report of IgG-dependent activation of human mast cells and may be relevant to understanding the recruitment of mast cells in non-IgE-mediated inflammation.

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Abbreviations: huMC: Human mast cell(s) β -hex: β -Hexosaminidase NP: 4-Hydroxy-3-nitrophenylacetyl

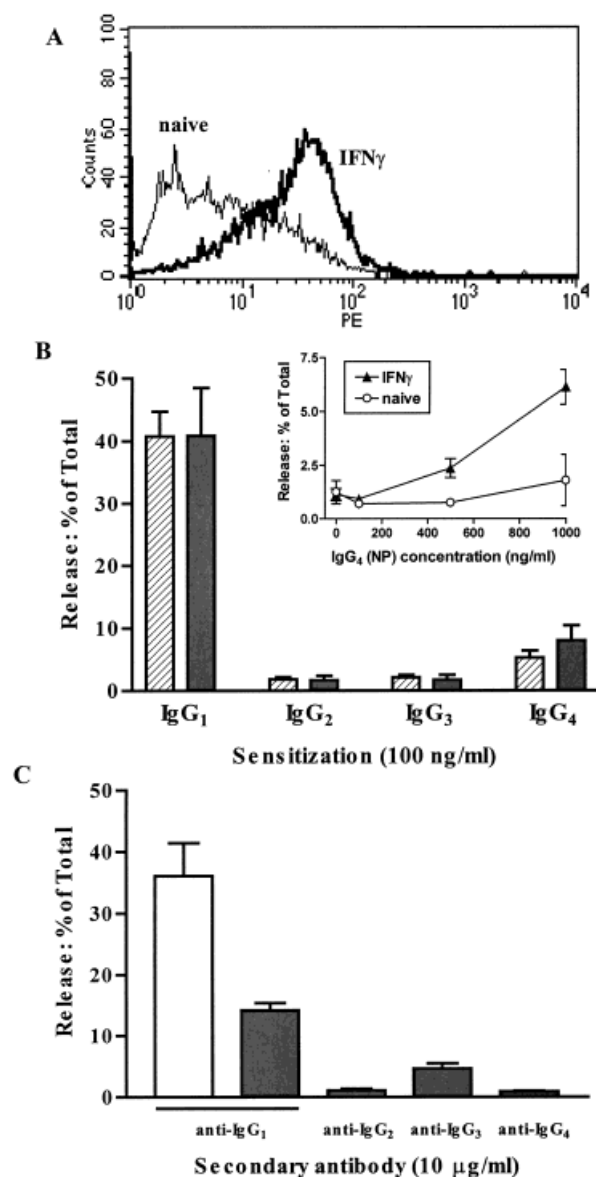
2 Results

2.1 Surface bound IgG mediates huMC degranulation

It has been reported that the high affinity IgG receptor Fc γ RI is up regulated on huMC following treatment with IFN- γ and aggregation of this receptor using F(ab')₂ fragments to cross-link receptor bound anti-CD64 F(ab')₂ activates mast cells as evidenced by degranulation, increased arachidonic acid generation and elevated mRNA expression for cytokines such as TNF- α , GM-CSF, IL-3, IL-6 and IL-13 [1, 2]. We thus first confirmed that huMC cultured in the presence of IFN- γ expressed Fc γ RI at higher levels than that observed on naive (non-IFN- γ cultured) mast cells (Fig. 1A). Pre-treatment of huMC with G-CSF or TGF β , which up-regulates Fc γ RII expression on neutrophils and Fc γ RIII by monocytes [12, 13], did not alter Fc γ RI surface expression in huMC (data not shown). As has been reported following IFN- γ culture [1, 14], the surface expression of Fc γ RII and Fc γ RIII was unaffected by IFN- γ as well as by G-CSF or TGF- β treatment (data not shown).

Fig. 1. IgG-dependent degranulation of human mast cells following Fc γ RI up-regulation by IFN- γ . (A) Cultured huMC were treated with or without IFN- γ (15 ng/ml) for approximately 40 h. Surface expression of Fc γ RI was verified using biotin-conjugated anti-CD64 (clone 10.1) followed by Strep Avidin-conjugated PE. Cell fluorescence was determined using a FACScan. (B) HuMC were cultured with IFN- γ (15 ng/ml) overnight and were subsequently sensitized for an additional 16 h with monomeric IgG₁, IgG₂, IgG₃, or IgG₄ (100 ng/ml) purified from human myelomas. Surface bound IgG was cross-linked using either isotype specific anti-IgG or a common anti-IgG recognizing all four isotypes. β -hex release was determined after stimulation for 30 min as described in Sect.4. Hatched bars represent activation using common anti-IgG; gray bars represent isotype specific activation. Each data point represents the mean and SE from three to five experiments. Inset: HuMC were sensitized overnight with NP-specific IgG₄ in the presence of myeloma IgE to prevent non-specific IgG₄ binding to Fc ϵ RI; aggregation was achieved using 1 ng/ml NP-BSA for 30 min. Closed triangles represent data from IFN- γ treated mast cells; open circles represent data from naive, non-IFN- γ -treated cells. Each data point represents the mean and SE from two to three experiments. (C) HuMC were cultured with or without IFN- γ (15 ng/ml) overnight and were subsequently sensitized with whole IgG (1,000 ng/ml) purified from normal sera (gray bars), or monomeric IgG₁ (positive control; 100 ng/ml) purified from human myelomas. Surface bound IgG was cross-linked using isotype specific anti-IgG and β -hex release was determined after stimulation for 30 min as outlined in Sect. 4. Each data point represents the mean and SE from three to seven experiments.

IFN- γ -treated huMC were then sensitized with 100 ng/ml IgG₁, IgG₂, IgG₃ or IgG₄ purified from human myelomas. This concentration was selected as it is an IgE concentration that results in robust human mast cell activation in our laboratory. Isotype-specific anti-IgG or common anti-IgG was then added to aggregate Fc γ RI-bound IgG. Under these conditions, cells sensitized with IgG₁ myeloma protein released approximately 40% of their granule contents as measured by β -hex release (Fig. 1B). IgG₄ sensitized cells in the presence of anti-IgG released 5–8% of granule contents while less than 3% degranulation was observed following cross-linking of surface bound IgG₂ or IgG₃. Mast cell degranulation was also evaluated utilizing NP-specific IgG₄. Addition of NP-BSA to IFN- γ treated mast cells sensitized with NP-IgG₄ resulted in 6% degranulation (Fig. 1B inset). This ap-



pears to be consistent with the low levels of IgG₄ mediated degranulation observed via sensitization with myeloma IgG₄ protein followed by addition of anti-IgG or anti-IgG₄ antibodies. To verify that IgG from normal sera could also mediate mast cell degranulation, huMC were treated sequentially with purified, whole IgG followed by isotype-specific anti-IgG. In agreement with degranulation mediated via purified myeloma IgG, whole IgG followed by anti-IgG₁ was most effective in mediating β -hex release by IFN- γ -treated mast cells (Fig. 1C). β -hex release (<3%) by naive huMC sensitized with whole IgG was equivalent with that observed by unstimulated cells (data not shown). Thus, both myeloma IgG protein and IgG from normal sera sensitizes IFN- γ -treated huMC so that addition of anti-IgG leads to mast cell degranulation, with IgG₁ being the most effective at mediating activation in this system.

We next determined the conditions that led to maximal activation in the presence of surface bound IgG₁. Similar to our initial experimental design, β -hex release reached a plateau using 100 ng/ml monomeric IgG₁ (Fig. 2A). While naive huMC averaged only 7% β -hex release, IFN- γ -treated mast cells demonstrated nearly 40% degranulation following aggregation of bound IgG₁. Maximal release of β -hex following IgG₁ sensitization also occurred when mast cells were cultured using 15 ng/ml of human, recombinant IFN- γ (Fig. 2B), although significant degranulation was observed by mast cells cultured with as little as 0.1 ng/ml IFN- γ . Finally, 1 μ g/ml of anti-

IgG₁ was confirmed as an optimal concentration of the secondary antibody required to cross-link receptor bound IgG and induce maximal β -hex release (Fig. 2C). Addition of anti-IgG₁ alone to IFN- γ -treated mast cells in the absence of IgG sensitization yielded no degranulation.

2.2 IgG₁-mediated mast cell degranulation occurs independent of Fc ϵ RI

It has been reported in rodent systems that IgG may bind to Fc ϵ RI, in addition to Fc γ RI, and mediate cell activation [15–17]. Furthermore, preliminary, unpublished data suggests IgE and IgG₄ demonstrate some cross reactivity for surface binding on cultured human mast cells. Thus,

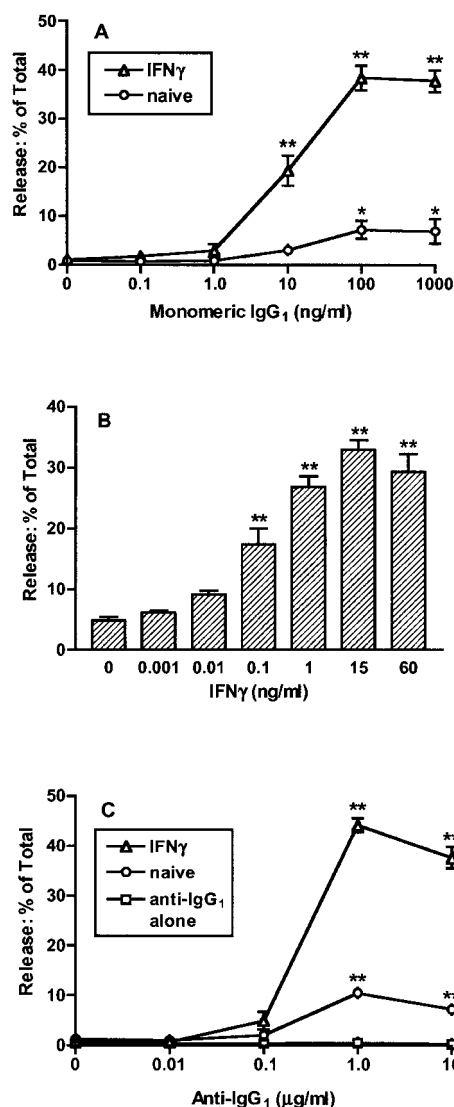


Fig. 2. IgG₁-mediated degranulation of human mast cells. (A) Cultured huMC were treated with or without IFN- γ (15 ng/ml) overnight and were subsequently sensitized with monomeric IgG₁ for an additional 16 h. Surface bound IgG₁ was cross-linked using anti-IgG₁ and β -hex release was determined after stimulation for 30 min as described in Sect. 4. Asterisks represent statistical significance from non-sensitized cells (0 ng/ml IgG₁) at $p \leq 0.05$ (*) or $p \leq 0.01$ (**) using a Tukey-Kramer multiple comparison test. (B) HuMC were cultured overnight with IFN- γ at the concentrations indicated and were subsequently sensitized with IgG₁ (100 ng/ml) for an additional 16 h. Cells were stimulated and degranulation was measured as described above. Double asterisks (**) represents statistical significance from naive cells (0 ng/ml IFN- γ) at $p \leq 0.01$ using a Tukey-Kramer multiple comparison test. (C) HuMC were cultured with or without IFN- γ (15 ng/ml) and IgG₁ (100 ng/ml) as described above; surface bound IgG₁ was aggregated using anti-IgG₁. β -hex release was determined after stimulation for 30 min as outlined in Sect. 4. Double asterisks (**) represents statistical significance from non-stimulated cells (0 μ g/ml anti-IgG₁) at $p \leq 0.01$ using a Tukey-Kramer multiple comparison test. All data (panels A–C) represent means and SE from two to six experiments.

to eliminate the possibility that IgG₁-mediates huMC degranulation via Fc ϵ RI, IgG₁-mediated β -hex release was evaluated in the presence of IgE. In these experiments, we first confirmed the IgE sensitizing concentration that mediates maximal degranulation by huMC in the absence or presence of IFN- γ (Fig. 3A). Fc ϵ RI-mediated degranulation appeared to be similar amongst naive and IFN- γ -treated huMC, suggesting that IFN- γ did not alter Fc ϵ RI-mediated huMC activation.

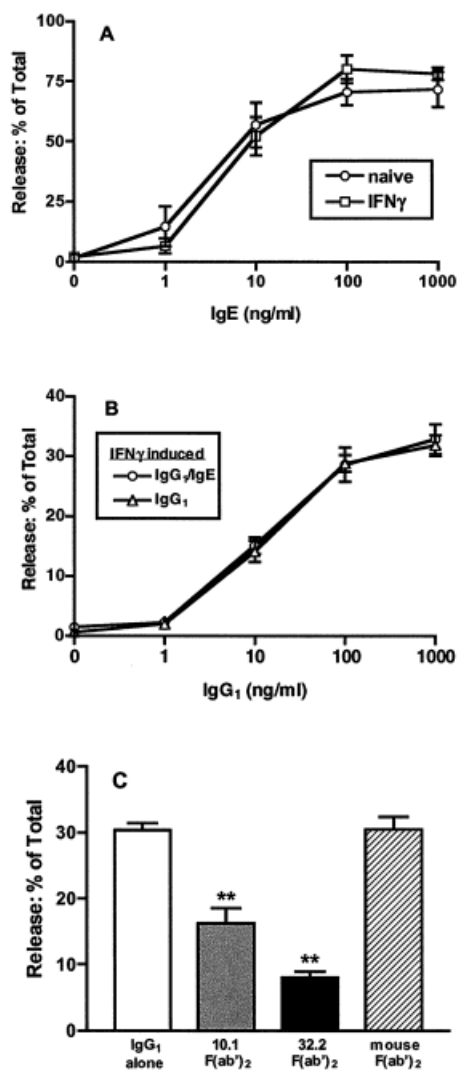
Subsequently, IFN- γ -treated huMC were cultured with 100 ng/ml IgE followed by sensitization with IgG₁. As shown in Fig. 3B, no difference was observed in IgG₁-mediated release between mast cells that were or were not pre-exposed to IgE, arguing further against the activation of huMC by IgG₁ through an interaction with Fc ϵ RI.

We next attempted to block CD64-mediated activation by adding two different anti-CD64 monoclonal antibodies prior to IgG₁ sensitization of huMC. Antibodies from clone 10.1 and clone 32.2 partially inhibited IgG₁-mediated degranulation by 46% and 74%, respectively, while F(ab')₂ fragments of an irrelevant murine IgG did not affect IgG₁ sensitization (Fig. 3C). As Fc ϵ RI expression appears unaffected by IFN- γ culture, IgG₁ and IgE do not demonstrate cross reactivity for huMC sensitization, and anti-CD64 antibodies inhibit IgG₁-mediated degranulation, IgG₁-mediated activation of IFN- γ -treated huMC appears to occur through Fc γ RI.

Fig. 3. IgG₁-mediated mast cell degranulation is independent of Fc ϵ RI. (A) Cultured huMC were incubated in the absence or presence of IFN- γ (15 ng/ml) overnight and were subsequently sensitized with chimeric IgE anti-NP for an additional 16 h. Surface bound IgE was cross-linked using NP-BSA and β -hex release was determined after stimulation for 30 min as described in Sect. 4. Data represent mean $\pm$ SE from four to five experiments. (B) HuMC were cultured with IFN- γ (15 ng/ml) overnight. Human IgG₁ (100 ng/ml), with or without 100 ng/ml IgE, was incubated with huMC for an additional 16 h. Surface bound IgG was cross-linked using anti-IgG₁ and β -hex release was determined after stimulation for 30 min as described in Sect. 4 ($n=3$ experiments). (C) HuMC were cultured with IFN- γ (15 ng/ml) overnight. F(ab')₂ anti-CD64 monoclonal antibodies (clone 10.1 or 32.2) or mouse IgG were incubated with huMC for 30 min followed by human IgG₁ for an additional 16 h. Surface bound IgG₁ was cross-linked using anti-IgG₁ and β -hex release was determined after stimulation for 30 min as described in Sect. 4. Double asterisks (**) represent statistical significance from cells sensitized with IgG₁ alone at $p \leq 0.01$ using a Tukey-Kramer multiple comparison test. Data represent means and SE from four to nine experiments.

2.3 IgG-dependent mediator production by huMC

The above experiments demonstrate that IgG₁ sensitized mast cells release granule contents as evidenced by β -hex release following IgG₁ cross-linking by anti-IgG. To determine if Fc γ RI aggregation would induce generation of arachidonic acid metabolites, we next activated IFN- γ -treated mast cells using IgG₁ and anti-IgG₁, and measured PGD₂ and LTC₄ generation. As seen in Fig. 4A, elevated PGD₂ generation was observed by huMC via both IgG₁- and IgE-dependent mechanisms, although in these experiments, significantly more PGD₂ was observed following aggregation of surface bound IgG₁ when Fc γ RI and Fc ϵ RI stimuli were adjusted to induce a similar percent of degranulation. LTC₄ production was also increased following Fc γ RI and Fc ϵ RI aggregation,



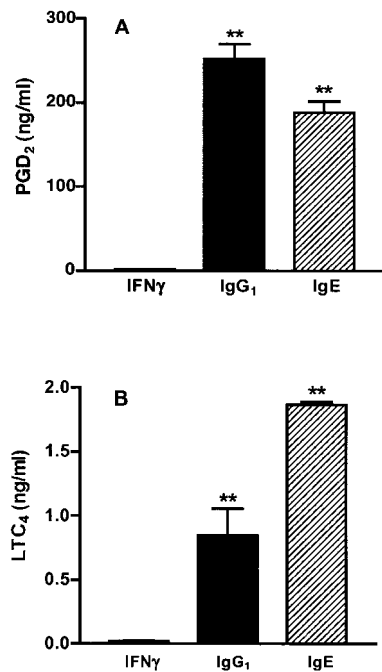


Fig. 4. Arachidonic acid metabolite generation follows Fc γ RI aggregation on huMC. huMC (2×10^4 cells) were cultured in the absence or presence of IFN- γ (15 ng/ml) overnight, and subsequently sensitized with monomeric IgG₁ or IgE anti-NP at 100 ng/ml for an additional 16 h. Surface bound IgG₁ or IgE was cross-linked using anti-IgG₁ or NP-BSA, respectively, at concentrations determined to result in approximately equivalent degranulation. The huMC were pelleted by centrifugation after stimulation (30 min) and PGD₂ (A) or LTC₄ (B) generation was determined in the supernatant using an enzyme immunoassay. Data represent mean and SE from four experiments. Both PGD₂ and LTC₄ generation by Fc ϵ RI and Fc γ RI stimulated groups were statistically elevated over IFN- γ treated cells at $p \leq 0.01$ (**) using Dunnett's test. IgG₁ and IgE sensitized cells were statistically different at $p \leq 0.05$ using a two-tailed, unpaired t -test.

although LTC₄ generation was significantly greater following Fc ϵ RI aggregation than that that observed following IgG₁ cross-linking (Fig. 4B). Thus, activation of huMC via monomeric IgG₁ results in the generation of both PGD₂ and LTC₄.

It has been reported that cytokine mRNA is up-regulated when IFN- γ -treated huMC are stimulated for 4 h using anti-CD64 specific F(ab')₂ [1, 2]. As can be seen in Fig. 5A–D, activation of huMC via Fc ϵ RI consistently up-regulated message for GM-CSF, IL-3, IL-13 and TNF- α . Aggregation of receptor bound IgG₁ on IFN- γ -treated huMC also mediated significant up-regulation of these cytokine messages but to a lesser degree than IgE in all cases except TNF- α , where cross-linking of IgG₁ led to

gene transcription that was statistically greater than that observed following IgE-mediated activation. The marked TNF- α gene expression was verified as TNF- α protein secretion was also elevated following cross-linking of surface bound IgG₁ (Fig. 5E).

2.4 Simultaneous activation of huMC through Fc γ RI and Fc ϵ RI

The consequences of activation of huMC simultaneously through Fc γ RI and Fc ϵ RI have not been studied. To determine if simultaneous activation of mast cells through both receptors would lead to greater degranulation, we first examined degranulation kinetics mediated by IgG₁ and IgE to determine the ideal time at which maximal release occurs. As shown in Fig. 6A, the plateau of degranulation mediated by either immunoglobulin occurred by 30 min. While Fc ϵ RI aggregation resulted in a peak response within a minute (half maximum <30 s), β -hex release due to Fc γ RI cross-linking did not near its plateau for more than 15 min (half maximum release $\approx$ 11 min).

Degranulation was next evaluated in mast cells through IgE-dependent, IgG₁-dependent, or IgG₁- and IgE-dependent mechanisms simultaneously. As seen in Fig. 6B, IgG₁-dependent degranulation complements that observed by IgE-dependent mechanisms, although, as the amount of IgE or IgG₁ used to sensitize huMC increases and degranulation nears maximal levels, simultaneous cell activation through Fc γ RI and Fc ϵ RI appears to have less of an additive effect. Thus, in mast cells sensitized with 1 ng/ml IgE alone (open bar, middle series), degranulation is about 3.5% and simultaneous activation using an additional 5 ng/ml (hatched bars, middle series) or 10 ng/ml (gray bars, middle series) IgG₁ leads to additive degranulation of approximately 13% and 19.5%, respectively. However, when higher concentrations of IgE (5 ng/ml) or IgG₁ (50 ng/ml) are utilized, and degranulation via Fc γ RI or Fc ϵ RI independently approaches 30% or higher, enhancement of β -hex release is only slightly increased when mast cells are simultaneously activated via both IgG₁ and IgE. This suggests that at suboptimal concentrations of IgG or IgE sensitization, simultaneous cross-linking of surface bound IgG and IgE may result in more robust mast cell degranulation and activation under circumstances in which neither would elicit maximal release on their own.

3 Discussion

This work provides further evidence for the functional expression of Fc γ RI on the surface of mast cells and becomes the first report of human mast cell activation

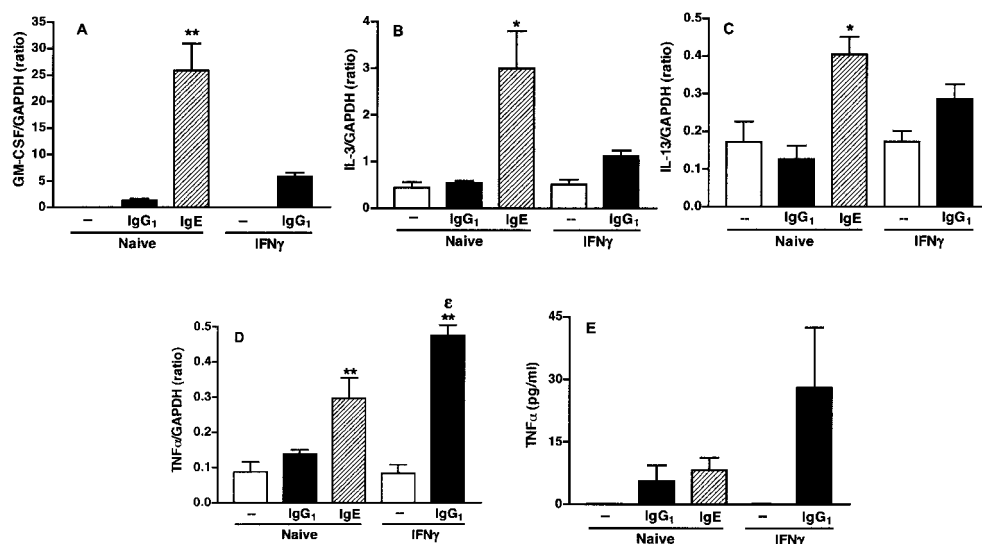


Fig. 5. IgG-dependent cytokine production by huMC. HuMC (3×10^5 cells) were stimulated for 4 h via aggregation of either surface bound monomeric IgG₁ or IgE anti-NP at concentrations determined to result in approximately equivalent degranulation. Cells were pelleted by centrifugation (400×g, 5 min) and the supernatant was reserved for TNF- α measurements using a Quantikine High Sensitivity ELISA (E). Total mRNA was isolated from huMC and GM-CSF (A), IL-3 (B), IL-13 (C), TNF- α (D) and GAPDH gene expression was evaluated via Taqman analysis as described in Sect. 4. GM-CSF (A) and IL-3 (B) gene transcription following Fc ϵ RI aggregation was determined to be statistically greater than naive, unsensitized groups and IFN- γ -treated, IgG₁ sensitized cells at $p \leq 0.01$ (**) or $p \leq 0.05$ (*) using a Tukey-Kramer multiple comparison test. IL-13 production (C) by IgE stimulated cells was also greater than naive, unsensitized cells ($p \leq 0.05$) but not statistically different from IFN- γ -treated, IgG₁ sensitized huMC. GM-CSF, IL-3 and IL-13 gene expression by IFN- γ treated, IgG₁ sensitized cells was statistically greater than naive, IgG₁ sensitized cell at $p \leq 0.01$ or $p \leq 0.05$ when compared directly using a one-tailed, unpaired *t*-test. TNF- α gene expression (D) following Fc γ RI (IFN- γ -treatment) or Fc ϵ RI stimulation was determined to be statistically greater than IFN- γ -treated, unsensitized cells and naive, unsensitized cells, respectively, at $p \leq 0.01$ (**) using a Tukey-Kramer multiple comparison test. IFN- γ -treated, IgG₁ sensitized huMC also demonstrated higher TNF- α gene expression than naive, IgG₁ sensitized cells at $p \leq 0.01$ (**) and IgE sensitized cells at $p \leq 0.05$ (ε).

via monomeric, whole molecule IgG thereby providing a novel paradigm for involvement by human mast cells in non-IgE inflammatory reactions. Fc γ RI (CD64) is the only Fc gamma receptor which binds monomeric IgG with high affinity ($K_a \sim 10^8 - 10^9$ M⁻¹) and is commonly identified on the surface of such cells as macrophages and monocytes, as well as IFN- γ -activated PMN and eosinophils (reviewed in [18]). Mast cells are frequently co-located in regions of inflammation, and as such, are hypothesized to be effector cells of numerous inflammatory pathologies. IgG₁-mediated activation of IFN- γ -treated mast cells resulted in marked degranulation as well as generation of arachidonic acid metabolites and cytokines.

The up-regulation of Fc γ RI on huMC following culture with IFN- γ (Fig. 1A), and the inability of TGF- β and G-CSF to increase CD64 surface expression, is consistent with previous results that demonstrated the ability of IFN- γ to increase Fc γ RI expression on huMC [1]. Surface expression of Fc γ RII was unaffected by IFN- γ treatment while Fc γ RIII levels, which demonstrate minimal expression on huMC, also remained unchanged; similar results

were obtained when huMC were cultured with G-CSF and TGF- β (data not shown). In addition to unaltered surface expression levels of Fc γ RII and Fc γ RIII following IFN- γ treatment of huMC, both IgG receptor classes only bind IgG with low affinity (Fc γ RII $K_a < 10^{-7}$ M and Fc γ RIII $K_a \sim 2 \times 10^{-7}$ M). Furthermore, aggregation of Fc γ RII using F(ab')₂ specific antibodies does not induce mast cell degranulation [14]. Consistent with FACS analyses that demonstrated no effect on Fc ϵ RI surface expression following huMC treatment with IFN- γ [1], IFN- γ also had no effect on IgE-mediated β -hex release (Fig. 3A). These observations, combined with the ineffectiveness of robust concentrations of IgE to alter IgG₁-mediated degranulation (Fig. 3B and C) and the partial inhibition of IgG₁ sensitization by anti-CD64 antibodies (Fig. 3C), strongly suggests IgG₁-mediated mast cell activation via Fc γ RI.

Anti-CD64 F(ab')₂ fragments from clones 10.1 and 32.2 reduced IgG₁-mediated degranulation by greater than 40% and 70%, respectively. Incomplete inhibition of Fc γ RI mediated activity using clone 10.1 is consistent

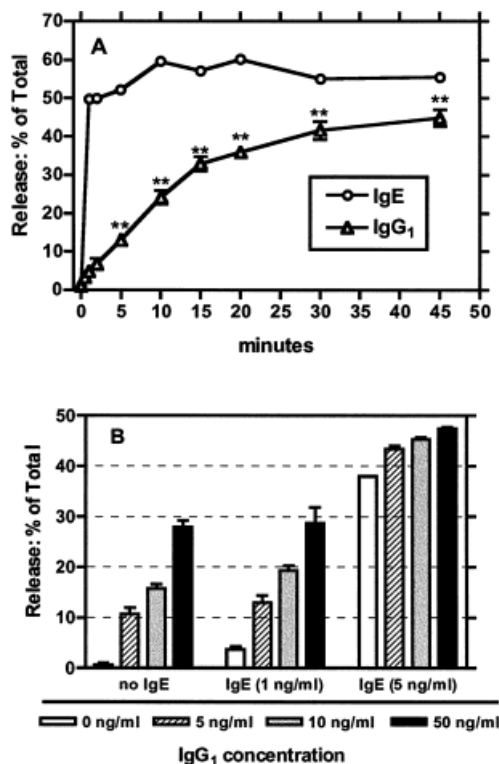


Fig. 6. Fc γ RI- and Fc ϵ RI-mediated degranulation of huMC. (A) HuMC were cultured in the absence or presence of IFN- γ (15 ng/ml) overnight and were subsequently sensitized with human IgG₁ or chimeric IgE anti-NP at 100 ng/ml for an additional 16 h. Surface-bound IgG₁ or IgE was cross-linked using either anti-IgG₁ or NP-BSA for the indicated times and β -hex release was determined as described in Sect. 4. Data represent mean $\pm$ SE following Fc γ RI aggregation from five experiments while data from IgE sensitized huMC is from a single experiment and is representative of previous studies. Double asterisks (**) represent statistical significance from unstimulated, IgG₁-sensitized cells (0 min) at $p \leq 0.01$ using a Tukey-Kramer multiple comparison test. (B) HuMC were cultured with IFN- γ (15 ng/ml) overnight, and subsequently sensitized with between 0–50 ng/ml human IgG₁ and chimeric IgE anti-NP at 0–5 ng/ml for an additional 16 h. Surface-bound IgG₁ and IgE were cross-linked using anti-IgG₁ and NP-BSA and β -hex release was determined after stimulation for 30 min as described in Sect. 4. Data represent mean $\pm$ SE from two to four experiments.

with previous reports in which phagocyte binding to IgG-opsonized erythrocytes was only partially inhibited by preincubation of phagocytes with clone 10.1 antibody [19]. Whereas antibody from clone 10.1 reportedly binds CD64 within the IgG ligand site, clone 32.2 appeared to be more capable at preventing cross-linking of surface bound IgG₁ despite evidence demonstrating its CD64 epitope is distinct from the IgG binding site (reviewed in [18]). The means by which clone 32.2 inhibits IgG₁-

mediated degranulation appears to be more complex and further studies will need to be conducted to elucidate this mechanism.

IgG₁ was the only IgG isotype that mediated significant mast cell activation, albeit cross-linking of surface bound IgG₃ or IgG₄ myeloma proteins, as well as NP-specific IgG₄, demonstrated low level degranulation (Fig. 1B, inset). IgG₃ has an affinity for Fc γ RI similar to that of IgG₁ (K_a 10^8 – $10^9 M^{-1}$) while Fc γ RI binds IgG₄ with much lower affinity [20]; IgG₂ has no specificity for Fc γ RI and accordingly did not mediate activation of huMC. It is unclear why cross-linking of purified, surface bound IgG₃ was unable to elicit degranulation, although others have reported similar inconsistencies in which phagocytes with surface Fc γ RI bind IgG₃-opsinized RBC while experiments designed using IgG₁ were unsuccessful [19]. The inability of myeloma purified IgG₃ to mediate degranulation via Fc γ RI could be due to molecular subtleties in protein structure. Mast cells sensitized with whole IgG resulted in detectable β -hex release following cross-linking with anti-IgG₃, in addition to IgG₁. The difference amongst IgG₁- and IgG₃-mediated degranulation following whole IgG sensitization may reflect the relative concentrations of the IgG isotypes as IgG₁ and IgG₃ account for approximately 66% and 22% of the total IgG typically found in sera from normal donors [21]. Although purified myeloma IgG₄ and NP specific IgG₄ were able to mediate detectable β -hex release, under conditions of whole IgG sensitization, IgG₄ did not mediate cell activation when secondary antibody was added. This may not only reflect the relative concentration of IgG₄ ($\sim 3.5\%$ of total IgG), but also direct competition for Fc γ RI binding in the presence of IgG₁ and IgG₃. β -hex release from naive huMC ($\sim 7\%$ release) is likely due to basal levels of Fc γ RI expression and is consistent with previous flow cytometric analyses as well as histamine release obtained using F(ab')₂ fragments of monoclonal antibodies specific for CD64 [1].

Even though aggregation of receptor bound IgG₁ mediated gene transcription for GM-CSF, IL-3 and IL-13, only Fc γ RI cross-linking induced TNF- α message which exceeded mRNA expression levels mediated by Fc ϵ RI (Fig. 5). The robust secretion and production of TNF- α following aggregation of Fc γ RI provides a direct link to inflammatory pathology. TNF- α is reported to play a pivotal role in initiating and perpetuating the chronic damage produced in Crohn's disease and rheumatoid arthritis [4, 5, 22]. TNF- α is also key in PMN leukocyte recruitment and adhesion molecule expression (e.g. E-selectin expression, VCAM-1 and ICAM-1) (reviewed in [23]).

Likewise, the arachidonic acid metabolites PGD₂ and LTC₄, which play key roles in inflammation, were mark-

edly elevated following IgG₁-mediated activation (Fig. 4). Preferential generation of PGD₂ or LTC₄ following Fc γ RI or Fc ϵ RI aggregation, respectively, may be due to enhanced activation or membrane translocation of cyclooxygenase or lipoxygenase by one receptor induced pathway compared to the other. Preferential generation of LTC₄ compared with PGD₂ has been previously described in mast cells following stem cell factor treatment and was determined to be due to selective lipoxygenase translocation to a membrane surface [24].

Despite some similarities in signaling pathways, our data thus suggests there is also functional diversity by Fc γ RI and Fc ϵ RI as evidenced by TNF- α production, arachidonic acid metabolite generation, and degranulation kinetics. In part, these differences may be due to the structure of Fc ϵ RI that contains the β -chain as part of its multimeric complex while Fc γ RI does not have this subunit. The Fc ϵ RI β -subunit associates with Lyn tyrosine kinase while the common γ -chain utilized by both Fc γ RI and Fc ϵ RI associates with Syk [25]. The additional tyrosine signaling via the β -chain serves to augment the rate and magnitude of the signal cascade. Cells stimulated via Fc ϵ RI in the absence of the β -chain appear to be less capable of activation as measured by Syk activity and calcium mobilization [26]. Differences in γ -chain association with Fc γ RI or Fc ϵ RI complexes may further contribute to the functional diversity as it was reported that interaction with different FcR subunits alters the properties of γ -chain signaling [27]. Additionally, differences in degranulation kinetics may be in part due to immunoglobulin binding affinities. The affinity of IgE for Fc ϵ RI is approximately 10⁻¹¹ M and that of IgG₁ for Fc γ RI is near 10⁻⁹ M. Conceptually, this allows for differences in receptor on and off rates.

When Fc γ RI and Fc ϵ RI were activated simultaneously via surface bound IgG₁ or IgE, respectively, suboptimal responses by each immunoglobulin were augmented although the additive nature of the responses diminished at higher stimulus strengths (Fig. 6B). As Fc γ RI and Fc ϵ RI reportedly utilize some common signaling molecules such as the γ -chain, Src kinases and Syk tyrosine kinase (reviewed in [25, 28]), mobilization and activation of common intracellular pathways are one possibility for the potential limitations in additive degranulation responses. This diversity amongst homogenous cells following activation of similar signaling pathways suggest there are subtle differences between Fc γ RI and Fc ϵ RI that are receptor specific rather than tissue specific.

The observation that human mast cells can be activated following IFN- γ up-regulation of Fc γ RI provides a novel mechanism by which huMC recruited into sites of inflammation may become activated by IgG-dependent mech-

anisms. This scheme may be relevant to understanding mast cell involvement documented in a number of non-IgE-mediated inflammatory disorders.

4 Materials and methods

4.1 Human mast cells

Human peripheral blood CD34⁺ progenitor cells were collected and processed from normal donors following informed consent via affinity column apheresis as described [29]. Cells were placed in serum-free media (StemPro-34 SFM, Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 50 μ g/ml streptomycin, 100 IU/ml penicillin and the following recombinant human cytokines (PeproTech, Rocky Hill, NJ): 100 ng/ml stem cell factor (SCF), 100 ng/ml IL-6 and 30 ng/ml IL-3 (first week only). Cultures were maintained at 37°C and 5% CO₂, and half of the culture media was replaced every 7 days. Greater than 95% of the cells were confirmed to be mast cells after 8 weeks of culture and greater than 99% to be mast cells after 10 weeks via Kimura staining [30].

4.2 Antibodies

Purified human myeloma IgG₁, IgG₂, IgG₃, IgG₄ and IgE were purchased from Calbiochem (San Diego, CA) and whole IgG from Chemicon (Temecula, CA). These immunoglobulins were centrifuged at 20,000 $\times$ g for 40 min at 4°C to remove aggregated molecules. The immunoglobulin concentration was subsequently determined at A₂₈₀. Human chimeric IgE and IgG₄ directed against 4-hydroxy-3-nitrophenylacetyl (NP) was purchased from Serotec (Raleigh, NC) and was also centrifuged prior to use to remove aggregates. The following mouse anti-human antibodies were purchased from Pharmingen (San Diego, CA): anti-IgG (clone G18–145), anti-IgG₁ (clone JDC-1), anti-IgG₂ (clone G18–21), anti-IgG₃ (clone G18–3), and anti-IgG₄ (clone JDC-14). Mouse IgG F(ab')₂ fragment (Jackson Immuno Research, West Grove, PA), F(ab')₂ fragment and biotinylated whole molecule anti-CD64 from clone 10.1 (mouse IgG₁, Ancell, Bayport, MN), and F(ab')₂ fragment from clone 32.2 (mouse IgG₁, Medarex, Annandale, NJ, respectively) were also purchased.

4.3 β -hexosaminidase release

For Fc γ RI-mediated degranulation, 1 $\times$ 10⁴ huMC were preincubated with 15 ng/ml human, recombinant IFN- γ (Pharmingen, San Diego, CA) for 24 h. Cells were subsequently incubated overnight with monomeric IgG prior to repeated centrifugation (400 $\times$ g, 5 min) and suspension in Hepes-Tyrodes buffer (10 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na₂HPO₄, 5.6 mM d-glucose, 1.8 mM CaCl₂, 1.3 mM MgSO₄) with 0.025% BSA. Surface bound IgG was cross-linked for 30 min (unless otherwise indicated) using a mouse anti-human IgG. As a positive control, additional cells (non-

IFN- γ treated unless otherwise indicated) were sensitized overnight with IgE anti-NP; Fc ϵ RI aggregation was accomplished using 1 ng/ml NP-BSA (30:1) (Biosearch Technologies, Inc., Novato, CA) for 30 min. Mast cell degranulation was also evaluated following overnight sensitization with NP-specific IgG₄ in the presence of 100 ng/ml myeloma IgE to prevent nonspecific binding of IgG₄ to Fc ϵ RI; aggregation was achieved using 1 ng/ml NP-BSA for 30 min. Reactions were stopped by centrifugation (400 $\times$ g) at 4°C and 50 μ l of supernatant was removed for β -hex measurement. Deionized water was added to the remaining cell pellets; samples were frozen, thawed, and a second 50 μ l was removed to determine the total β -hex content. β -hex samples (50 μ l) were incubated in 0.04 M citric acid with 0.02 M Na₂HPO₄ containing 10 mM p-nitrophenyl N-acetyl- α -D-glucosaminide (pNAG) for 90 min at 37°C. The reaction was developed using 0.4 M glycine and absorbance was determined at 405 nm. Percent release was calculated as follows: [β -hex in supernatant/(β -hex in supernatant + total β -hex in pellet)] $\times$ 100%.

4.4 FACS analysis

The expression of Fc γ RI following IFN- γ incubation was verified using biotin conjugated anti-human CD64 (clone 10.1). Naive or IFN- γ -treated huMC were incubated for 1 h at 4°C with 1 μ g/ml biotinylated anti-CD64 followed by Strep Avidin-conjugated PE (Pharmingen) at 1:800 for 30 min at 4°C under dark conditions. Cell fluorescence was determined using a FACScan and Cell Quest software (Becton Dickinson, San Jose, CA).

4.5 Arachidonic acid metabolite measurement

Cell activation was performed using 2×10^4 huMC as described above (see Sect. 4.3). The huMC were pelleted by centrifugation (400 $\times$ g and 4°C, 5 min) and PGD₂ or LTC₄ generation, and subsequent release into the supernatant (50 μ l), was determined using an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI).

4.6 Taqman real-time PCR analysis

Naive and IFN- γ -treated huMC (3×10^5 cells) were stimulated for 4 h via aggregation of either surface-bound monomeric IgG₁ or chimeric IgE anti-NP. Cells were pelleted by centrifugation (400 $\times$ g, 5 min) and the supernatant was removed for TNF- α ELISA measurements (see below). Total mRNA was isolated from huMC using QIAshredders and RNeasy columns according to the manufacturer's specifications (Qiagen, Valencia, CA). RNA was transcribed to cDNA using Superscript™ First-Strand Synthesis System (Life Technologies) and was analyzed for cytokine gene expression by Taqman™ as described [31] using an ABI Prism Model 7700 Sequence Detection System (Applied Biosystems, Foster

City, CA). The following primers and probes were designed using Primer Express (Applied Biosystems): TNF- α forward 5'-AGAGGGCCTGTACCTCATCTACTC-3', reverse 5'-GTGTGGGTGAGGAGCACATG-3' and fluorogenic probe 5'-6FAM-TCTTCAAGGGCCAAGGCTGCCC-TAMRA-3'; GM-CSF forward 5'-CTTGACCATGATGGCCAGC-3', reverse 5'-TCTGGGTTGCACAGGAAGTTT-3', and fluorogenic probe 5'-6FAM-CAGCACTGCCCTCCAACCCCG-TAMRA-3'; IL-3 forward 5'-CGCGATCTTTTAGTCCAACGT-3', reverse 5'-TTGATGTCCCGAGGCT-CTG-3' and fluorogenic probe 5'-6FAM-CAGCTCGTTCTCTGGGCCTTCTACC-TAMRA-3' and; IL-13 forward 5'-CCTGGAATCCCTGATCAACG-3', reverse 5'-CGCTCAGCATCCTCTGGGT-3' and fluorogenic probe 5'-6FAM-TCAGGCTGCAGTGCCATCGAGAA-TAMRA-3'. GAPDH mRNA expression was measured as an internal control using primers and probes supplied in Taqman Endogenous Control Reagents (Applied Biosystems).

4.7 TNF- α ELISA

Following Fc receptor aggregation for 4 h, supernatants were collected from huMC and frozen at -20°C. TNF- α levels were determined using a Quantikine High Sensitivity ELISA (R & D Systems, Minneapolis, MN).

4.8 Statistics

Statistics were conducted using Graph Pad Prism®, version 3.0a for Macintosh (San Diego, CA). β -hex release, PGD₂ and LTC₄, and TNF- α secretion and gene expression ratios were analyzed by one-way ANOVA. When significant differences were detected ($p \leq 0.05$), test groups were compared to controls using Dunnett's test (equal group sizes) or a Tukey-Kramer multiple comparison test that takes into account unequal sample sizes. Both one- and two-tailed, unpaired *t*-tests were used to perform pair wise comparisons between naive and IFN- γ -induced groups, and between IgG-dependent and IgE-dependent activation, where indicated.

Acknowledgements: This work was supported by the National Institute of Allergy and Infectious Diseases Intramural Research Program.

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