Genetically engineered negative signaling molecules in the immunomodulation of allergic diseases

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Purpose of review

This review summarizes current knowledge regarding the control of human mast cell and basophil signaling and recent developments using a new therapeutic platform consisting of a human bifunctional γ and ε heavy chain (Fcγ–Fcε) protein to inhibit allergic reactivity.

Recent findings

Crosslinking of FcγRIIb to FcεRI on human mast cells and basophils by a genetically engineered Fcγ–Fcε protein (GE2) leads to the inhibition of mediator release upon FcεRI challenge. GE2 protein was shown to inhibit cord blood-derived mast cell and peripheral blood basophil mediator release *in vitro* in a dose-dependent fashion, including inhibition of human IgE reactivity to cat. IgE-driven mediator release from lung tissue was also inhibited by GE2. The mechanism of inhibition in mast cells included alterations in IgE-mediated Ca^{2+} mobilization, spleen tyrosine kinase phosphorylation and the formation of downstream of kinase–growth factor receptor-bound protein 2–SH2 domain-containing inositol 5-phosphatase (dok–grb2–SHIP) complexes. Proallergic effects of Langerhan's like dendritic cells and B-cell IgE switching were also inhibited by GE2. *In vivo*, GE2 was shown to block passive cutaneous anaphylaxis driven by human IgE in mice expressing the human FcεRI and inhibit skin test reactivity to dust mite antigen in a dose-dependent manner in rhesus monkeys.

Summary

The balance between positive and negative signaling controls mast cell and basophil reactivity, which is critical in the expression of human allergic diseases. This approach using a human Fcγ–Fcε fusion protein to co-aggregate FcεRI with the FcγRII holds promise as a new therapeutic platform for the immunomodulation of allergic diseases and potentially other mast cell/basophil-dependent disease states.

Keywords

allergic reactions, FcεRI, FcγRIIb, mast cells and basophils, negative signaling transduction

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Abbreviations

CBMC	cord blood-derived mast cell
Dok	downstream of kinase
Grb2	growth factor receptor-bound protein 2
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
LLDC	Langerhan's like dendritic cell
PCA	passive cutaneous anaphylaxis
SHIP	SH2 domain-containing inositol 5-phosphatase
SHP	SH2 domain-containing phosphatase
Syk	spleen tyrosine kinase
NIP	4-hydroxy-3-iodo-5-nitrophenylacetyl
FcεRI	high-affinity receptor for IgE Fc
FcγRII	receptor II for IgG Fc (CD32)

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Introduction

Mast cell and basophil mediator release driven by the immunoreceptor tyrosine-based activation motif (ITAM)-containing FcεRI is a critical component in expression of human immediate hypersensitivity-allergic disease. For years, various pharmacologic approaches have been employed to antagonize the effects of these mediators [1•,2]. More limited success has been achieved with approaches aimed at inhibiting mediator release [3]. A great deal of new information about the role of negative signaling in mast cells and basophils via immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors has opened the way for new approaches that employ negative signaling as a way of controlling human allergic reactions [4,5]. Negative regulation of signaling through ITAM-containing receptors by signals from co-crosslinked ITIM-containing FcγRIIb has been demonstrated in mouse and human cells [6–8]. The ability of the ITIM-containing IgG receptor FcγRIIb to act as a negative regulator of ITAM-containing immunoreceptors was recognized initially through studies of the antigen receptor of B cells and was subsequently extended to studies of T-cell activation through T-cell receptors [9]. FcγRIIb was first implicated in the negative regulation of human FcεRI-mediated signaling by Daeron *et al.* [6]. It was later extended to peripheral blood basophils [8] and cord blood-derived human mast cells (CBMCs) [10••]. Herein we review current progress in understanding the regulation of human mast cells and basophil signaling, and highlight a potential therapeutic approach using a novel human Fcγ–Fcε fusion protein to achieve inhibition of allergic reactivity.

Inhibitory signaling in the control of human basophils and mast-cell function

The goal of immunotherapy is to induce mast cell/basophil nonresponsiveness by injecting suboptimal concentrations of allergen to induce mast cell/basophil inhibition. However, it is not known why mast cells and basophils become desensitized after this treatment. One hypothesis proposes that 'blocking antibodies' leads to co-aggregation of FcγRIIb receptors with FcεRI. During immunotherapy the serum levels of antigen-specific IgG levels increase such that antigen-specific IgG (complexed to Ag or possibly as monomeric IgG) binds to FcγRIIb on mast cells and basophils. Upon subsequent allergen exposure, FcεRI-bound IgE binds to IgG complexed to antigen, leading to co-aggregation of FcεRI and FcγRIIb that initiates an inhibitory rather than an activating signal.

Daeron *et al.* [6] were the first to demonstrate that FcεRI/FcγRII co-aggregation on human basophils inhibited IgE-mediated degranulation through ITIM signaling. We extended these studies in a more biologically relevant model system, which showed that antigen-induced basophil histamine release and IL-4 production could be inhibited by co-aggregation of Fcε–Fcγ using IgE with IgG to the same aggregated antigen [8]. It is not clear, however, if this mechanism occurs *in vivo*.

GE2, a human Fcγ–Fcε fusion protein, inhibits human basophil and mast cell function *in vitro*

Realizing the therapeutic potential for regulating FcεRI signaling, we developed a molecule that utilizes FcεRI co-aggregation to ITIM-containing receptors [11]. This fusion protein (GE2) consists of the human IgG_1 γ hinge–CHγ2–CHγ3 region linked to the human IgE CHε2–CHε3–CHε4 region. The human γ1 hinge, CH2–CH3 region was placed ahead of the ε heavy chain CH2–CH4 domains with a 17-amino-acid linker containing a BgIII site and $(Gly_4Ser)_3$ positioned between the two Fc regions. This linker facilitates chain pairing and minimizes refolding and aggregation problems encountered when the two chains are expressed individually [12]. GE2 was characterized as the predicted ∼140 KDa dimer and contained the binding sites for FcγRII (CHγ2–CHγ3) and FcεRI (CHε2–CHε3) [13,14]. The first constant domains (CHγ1 and CHε1) were deleted as these domains associated with BiP (immunoglobulin heavy chain binding protein). GE2 does not contain light chains [15]. The proposed binding and mechanism for GE2's inhibitory effects on human mast cells and basophils is shown in Fig. 1.

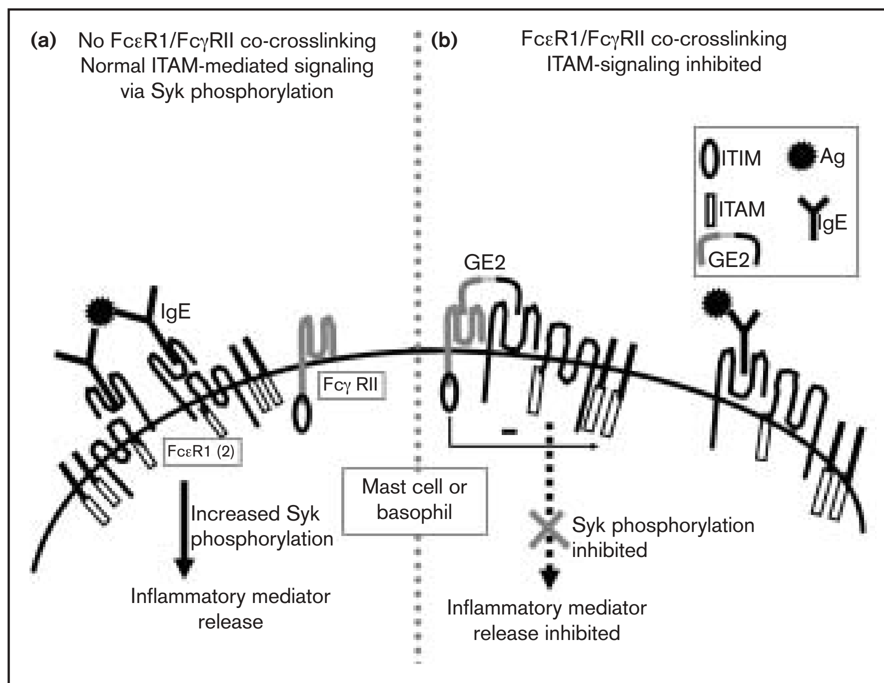
Human basophils purified from the peripheral blood, known to express FcγRIIb were passively sensitized with 10 μg/ml of chimeric human anti-4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP) IgE. One hour later, doses of GE2 ranging from 0.01 to 10 μg/ml were added and compared with a human IgE myeloma control. After another hour of incubation, the cells were activated with antigen NIP-BSA. At 10 μg/ml of GE2, there was an average of 84% inhibition while 1 μg/ml of GE2 inhibited histamine release by about 59%. Myeloma IgE at 10 μg/ml only decreased the release by 19%. Similarly, when naturally sensitized basophils obtained from cat-allergic individuals were incubated with GE2 and then challenged with purified cat antigen, Fel D1, GE2 again showed a dose response with 78% inhibition of Fel D1 specific release at 10 μg/ml of GE2 [16••]. Importantly, GE2 itself did not cause degranulation when it was incubated with basophils sensitized *in vivo* to cat. GE2's effects on basophil mediator release were also time dependent; as the time between GE2 addition and the stimulus for release was decreased, so was GE2's ability to inhibit. In addition, we have shown that GE2 can inhibit mast cell/basophil function from human lung tissue that had bound IgE *in vivo*. Recently, we demonstrated that CBMC, IgE-mediated responses could be inhibited with GE2 (Fig. 2). Therefore, blood basophils and CBMCs express the ITIM-containing FcγRIIb isoform which, when co-aggregated to FcεRI, inhibits IgE-mediated release. Taken together, these differing approaches show that GE2 can inhibit IgE-mediated mediator release in passively or natural sensitized human basophils and mast cells.

GE2 functions to inhibit mast cell and basophil signaling

The mechanisms of inhibition through FcεRI–FcγRII co-aggregation highlight the differences between the human and rodent systems. First, rodent studies using cell lines implicate SH2 domain-containing inositol 5-phosphatase (SHIP) more so than SH2 domain-containing phosphatase (SHP-1) in FcγRII-mediated inhibitory signaling [17–19]. In contrast, our data with peripheral blood basophils implicate SHP-1 in the FcγRII-mediated inhibitory signaling [11,20]. Second, in rodents, co-aggregation of FcεRI to FcγRII does not prevent FcεRI-mediated activation of spleen tyrosine kinase (Syk) [7]. However, we have shown in peripheral blood basophils [8,11] that FcγRII appears to regulate some part of Syk function as has been demonstrated in other human cells [21•]. Third, while FcεRI–FcγRII co-aggregation in rodent and human systems inhibits the Ca^{2+} response to FcεRI stimulation, we have demonstrated that the kinetics of this inhibition is distinct in peripheral blood basophils [8] and CBMCs [10] compared with rodent cells [17,18]. Finally, in results not observed in rodent systems, co-aggregation in human systems increased the tyrosine phosphorylation of the adapter protein downstream of kinase (Dok), growth factor receptor-bound protein 2 (Grb2), and SHIP.

Figure 1. Schematic showing the proposed mechanism for the genetically engineered Fcγ–Fcε protein (GE2) effect on human basophils and mast cells

(a) Antigen crosslinking two FcεRIs and inducing activation with enhancement of spleen tyrosine kinase (Syk) and other downstream signaling molecules. FcγRII is unoccupied. (b) GE2 co-crosslinking FcγRIIb to FcεRI, which results in the inhibition of signaling events downstream of the FcεRI immunoreceptor tyrosine-based activation motif (ITAM), such that the cell is inhibited in its ability to function via FcεRI activation. ITIM, immunoreceptor tyrosine-based inhibition motif.



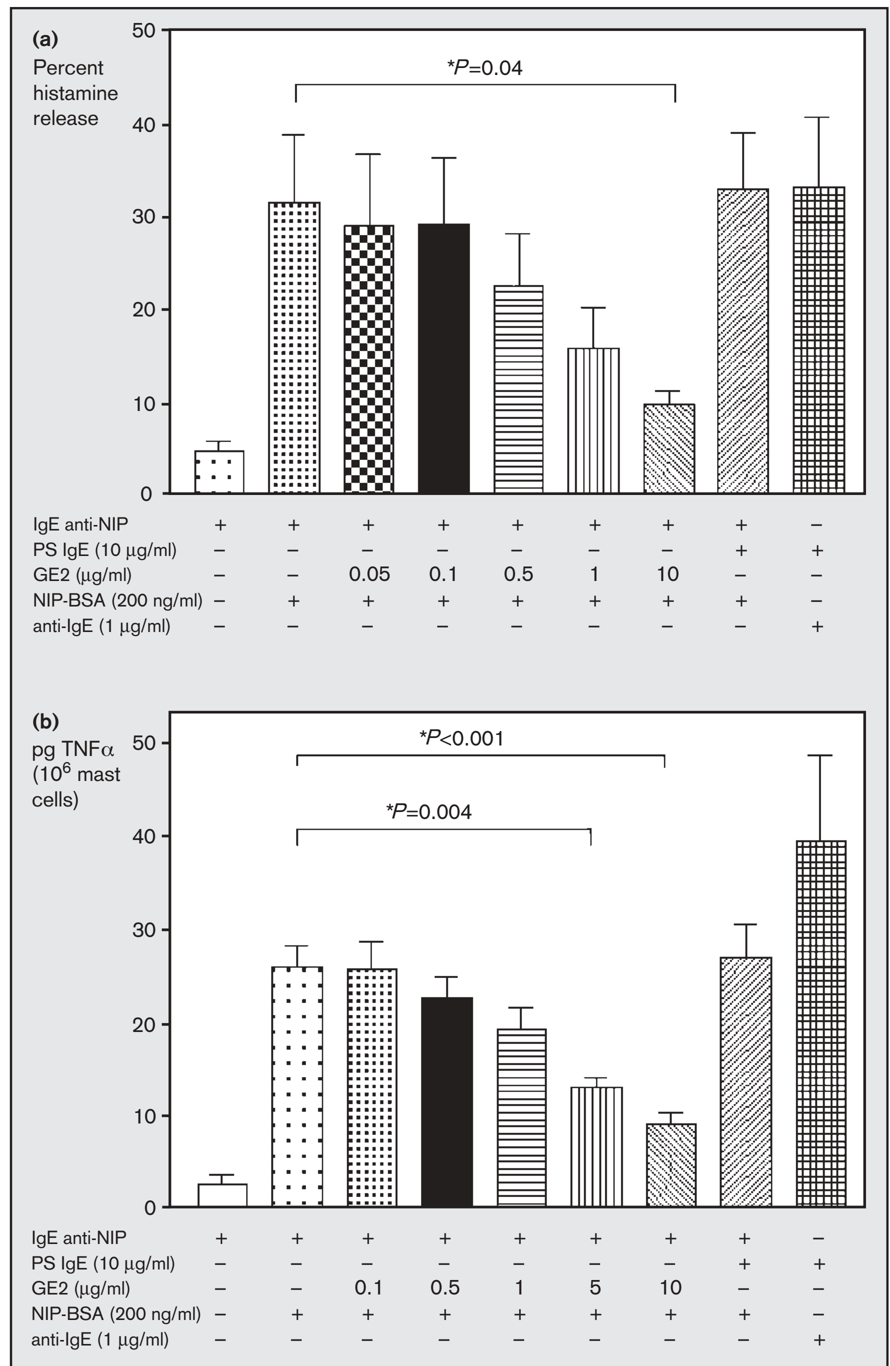
Tyrosine phosphorylation of Dok was associated with increased binding to Grb2. Surprisingly, in nonstimulated cells, there were complexes of phosphorylated SHIP–Grb2–Dok, which were lost upon IgE-receptor activation but retained under conditions of FcεRI–FcγRII co-aggregation. Our results implicate Dok, SHIP, and Grb2 as key intermediates in regulating IgE-mediated degranulation and cytokine production [10••]. They further implicate these signaling intermediates as the true 'gatekeepers' [22] of human mast cell degranulation. Clearly, the results from rodents cannot be directly extrapolated to the situation in humans.

FcεR–FcγR co-aggregation *in vitro* inhibits key proallergic processes in human B cells and Langerhan's like dendritic cells

While basophils and mast cells were the primary targets for which GE2 was designed, other cells express both FcγRII and FcεRs, for example human Langerhan's like dendritic cells (LLDCs) express FcεRI and FcγRII while human B cells express FcγRII and FcεRII (CD23), the low-affinity IgE receptor. Thus we have tested the ability of GE2 to inhibit the function of those cells *in vitro* through receptor co-aggregation. Notably, an antibody to CD23 has been shown to inhibit isotype switching to IgE by human B cells [23•]. We have shown that GE2 is extremely potent at inhibiting various steps involved in IL-4 plus CD40-driven class switch recombination and subsequent IgE production. The resulting inhibition resulted from a combination of inhibition of initiation of ε germ line transcription plus a direct effect on the process of the isotype switch itself. The effects of GE2 on class switch recombination in human B cells were clearly shown to require binding to both CD23 and FcγR. This inhibition of class switch recombination was dependent on CD23 binding and the phosphorylation of extracellular-signal related kinase, and it was mediated via suppression of IL-4-induced STAT6-phosphorylation [24••].

Unstimulated LLDCs derived from CD14-positive monocytes from atopic donors were shown to express FcγRII. When passively sensitized with antigen-specific human IgE and then challenged with antigen, LLDCs were stimulated to produce IL-16, an important proallergic cytokine. IL-16 enhances the production of inflammatory mediators such as histamine, RANTES and monocyte chemotactic protein-1 and is a potent chemoattractant for CD4+ T cells into areas of allergic inflammation. However, when FcεRI and FcγRII were co-aggregated with GE2, IL-16 production was significantly inhibited in a dose-dependent fashion up to 63%. Exposure of LLDCs to GE2 alone did not induce IL-16 production [25•]. These results further extend our studies demonstrating the ability of GE2 to inhibit FcεRI-mediated responses through co-aggregation with FcγRIIb and at the same time show that human LLDCs

Figure 2. FcγRII/FcεRI co-aggregation inhibits FcεRI-mediated mast cell degranulation and tumor necrosis factor α production



4-Hydroxy-3-nitrophenylacetyl-IgE-sensitized cord blood-derived mast cells (CBMCs) were incubated with or without genetically engineered Fcγ–Fcε protein (GE2) or nonspecific IgE (PS). The cells were washed, incubated with or without 200 ng/ml 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP)-BSA or anti-IgE for 45 min (histamine release) or 12 h (cytokine production) and mediator release measured in the supernatants. Results are represented as the standard error of the mean of four (a) or two (b) separate experiments (±SEM). *Indicates values significantly reduced ($P < 0.01$) when comparing cells challenged with or without GE2. (Previously published in Kepley *et al.* [10•].)

can be modulated in a fashion similar to mast cells and basophils.

GE2 inhibits basophil and mast cell function *in vivo*

We have tested the ability of GE2 to function *in vivo* to inhibit allergic reactivity through mast cells and basophils. In the first approach, we employed transgenic mice that express the human FcεRIα and have the murine FcεRIα chain knocked out, hFcεRIαtg mice. The human FcεRIα chain combines with the mouse β and γ chains to form a functional chimeric human/mouse FcεRIα receptor. These animals demonstrate allergic reactivity mediated by passively administered human IgE antibody when challenged with antigen. At the same time, the murine FcγRIIb expressed on these hFcεRIα transgenic animals' mast cells binds human IgG so that GE2 is expected to be functional in them.

GE2 blocked passive cutaneous anaphylaxis at sensitized skin sites in human Fcε–RIα transgenic mice

We utilized passive cutaneous anaphylaxis (PCA) in the hFcεRIαtg mice to test the ability of GE2 to block IgE-driven *in vivo* human FcεRI-mediated mast cell release. hFcεRIαtg mice were locally sensitized at multiple sites with 250 ng of model genetically engineered human IgE antibodies to 4-hydroxy-3-nitrophenylacetyl or dansyl. Varying doses of GE2 or control myeloma IgE was added at the sites. The mice were then challenged intravenously 6 h later with the relevant antigen bound to a carrier and reactivity was assessed. GE2 was able to block PCA reactivity in a dose-dependent fashion, with complete inhibition occurring at 250 ng of GE2.

These experiments were extended using human serum containing high levels of IgE to cat. FcεRIα transgenic mice were locally sensitized at individual skin sites with serum from a highly cat-allergic individual. Four hours later, GE2 or control material was administered to these sites and a further 4 h later, the mice were challenged intravenously with Fel d1 and Evans blue dye. Reactivity was scored 15–20 min later. PCA reactivity was inhibited in a dose-dependent manner by GE2 protein, injected with complete inhibition at 2 μg. Treatment of the cat-allergic serum at 56°C for 30 min, conditions known to abolish IgE binding to FcεRI, abolished PCA reactivity, thereby showing that the Fel d1-specific IgE was responsible for the PCA reactivity. Reactivity was not significantly blocked by administration of equivalent amounts of human IgE or human IgG. Importantly when an intravenous challenge of Fel d1 was given immediately after administration locally of only GE2, there was no local reaction, showing that GE2 itself was not causing the reactions at the injection sites.

GE2 markedly inhibited skin test reactivity to dust mites in rhesus monkeys

Rhesus monkeys express skin test reactivity and serum IgE directed toward dust mites [26]. We tested whether GE2 could inhibit *Dermatophagoides farinae* mite skin test reactivity in rhesus monkeys. *D. farinae* reactive monkeys underwent graded intradermal injections with GE2 (62.5–250 ng) versus purified human IgE myeloma protein as a control. Five hours later, the sites were then challenged with *D. farinae* at a dose optimized for reactivity for each animal through previous dose–response testing. Animals were simultaneously given Evans blue dye intravenously and skin test reactivity was measured 15–20 min later. GE2 protein effected complete inhibition at 250 ng and showed complete inhibition in four of five animals at 125 ng. At 62.5 ng, GE2 still exhibited clear inhibition of reactivity compared with saline or control, while nonspecific IgE did not show significant inhibitory effects on skin test reaction in any of the doses tested. These results clearly indicate that GE2 protein is able to inhibit naturally occurring dust mite allergen induced allergic skin reactivity in nonhuman primates in a dose-dependent fashion.

Future directions, enhancing FcγRII-related effects and utilization of alternative inhibitory signaling targets

The function of GE2 may be improved by increasing its affinity to FcγRII relative to other FcγRs. This is being undertaken by making mutations in key sites known to alter FcγR binding. Optimizing the linker length between the Fcε and Fcγ may provide enhanced avidity of the molecule. Using the flexible hinge of the γ1 chain as the linker through reversing the order of the GE2 construct also may enhance function. Recently, an approach using bi-specific, murine antibodies to achieve co-aggregation of FcγRII and FcεRIs has also been shown to work on human basophils and cultured mast cells *in vitro* [27•].

There are several ITIM-containing receptors that have been identified, but their expression has not been investigated on human FcεRI-positive cells [28]. We investigated the expression of several ITIM-containing receptors on peripheral blood basophils, CBMCs, the human mast cell line, and the human basophil cell line. As seen in Table 1, peripheral blood basophils and CBMCs express only the ITIM-containing FcγRIIb isoform of FcγRII. Thus, future strategies aimed at harnessing the inherent inhibitory ability of these

Table 1. Immunoreceptor tyrosine-based inhibition motif (ITIM) receptor expression in human mast cells and basophils

	FcγRII	FcγRIIA	FcγRIIB	MAFA	Siglec-5	SIRP-α	CD 22	CD81[a]	LIR
Basophils	+++	0–+ DV	+++	0–+	0	0	0	0–+ DV	++–+++ DV
CBMCs	++	0	++	0–+	0–+++[b]	0–+++[c]	+–+++[d]	+++	+
HMC-1	0–+	0	0–+	0	0	0	0	0	0
KU812	++	0	0–+	0	0	0	0	0	0

Detection of ITIM receptors on human FcεRI-positive cells using anti-ITIM receptor antibodies and utilizing the methodology as described previously [29]. Peripheral blood basophils (>90%), cord-blood derived mast cells (CBMCs), the human mast cell line (HMC-1), and the human basophil cell line (KU812) were tested. The numbers represent approximate fluorescent intensity with +++ being the strongest and 0 representing no reactivity. NKG2, KIR, CD5, 31, 32, 72 were not detected on any of these cell populations. DV, donor variation. In human blood basophils we found donor variation expression of the FcγRIIA isoform and the leukocyte immunoglobulin-like receptor (LIR). [a]The CD81 contains an 'ITIM-like' sequence. [b–d]In CBMCs, IFN-γdramatically upregulated siglec-5 ([b]) and downregulated CD22 ([d]), while IgE and IL-4 upregulated the signal-regulatory protein α (SIRP-α, [c]).

receptors targeted specifically to mast cells and basophils may have therapeutic potential.

Conclusion

Crosslinking of FcεRI and FcγRIIb induces strong inhibitory signaling in CBMCs and peripheral blood basophils. We have shown that this can be accomplished with clear antiallergic effects *in vitro* and *in vivo* with a single human Fcγ–Fcε fusion molecule. The ability of GE2, in addition, to block human B-cell isotype switching to ε and to inhibit proallergic function in LLDCs suggests GE2 may provide broader benefits in the treatment of IgE-mediated diseases than simply through inhibition of mediator release. Thus, the approach of using a chimeric Fcγ–Fcε fusion protein provides a platform for antigen nonspecific immunomodulation of allergic diseases. The work accomplished so far lays a strong foundation for the development of this platform as a human therapeutic intervention. Modifications in the GE2 platform, such as mutations that increase binding to FcγRII or decrease potential binding to FcγRI/III, or alterations in the linker length that increase co-aggregation, are now being explored.

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