

Arbeiten aus dem Paul-Ehrlich-Institut
 11th International Paul-Ehrlich-Seminar, Bad Homburg 2005
 Verlag Chmielorz

Recent Advances in the Use of Genetically Engineered Negative Signaling Molecules to Treat Allergic Diseases¹

Andrew Saxon, Daocheng Zhu, Ke Zhang, Lisa A. Chan, Christopher L. Kepley

Abstract

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 gamma and epsilon 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. In addition, IgE-mediated release from lung tissue was inhibited through GE2. The mechanism of inhibition in mast cells included alterations in IgE-mediated Ca²⁺ mobilization, Syk phosphorylation and the formation of Dok-Grb2-SHIP complex. Proallergic effects of Langerhans-like dendritic cells and B cell IgE switching were also inhibited by GE2. *In vivo*, GE2 was shown to block passive cutaneous anaphylaxis (PCA) 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.

¹ **Abbreviations:** **CBMC** Cord blood-derived mast cells; **Dok** downstream of kinase; **ERK** extracellular signal-related kinase; **Grb2** growth factor receptor-bound protein 2; **ITAM** immunoreceptor tyrosine-based activation motif; **ITIM** immunoreceptor tyrosine-based inhibition motif; **LLDC** Langerhans-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

Summary

The balance between positive and negative signaling controls mast cell and basophil reactivity that is critical in the expression of human allergic diseases. This approach using a human Fc γ -Fc ϵ fusion protein to co-aggregate Fc ϵ RI with the Fc γ RII holds promise as a new therapeutic platform for the immunomodulation of allergic diseases and potentially other mast cell/basophil-dependent disease states.

Introduction

Mast cell and basophil mediator release driven by the ITAM-containing Fc ϵ RI 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 negative signaling in mast cells and basophils via ITIM-containing receptors has opened the way to new approaches that employ negative signaling as way of controlling human allergic reactions [4-5]. Negative regulation of signaling through ITAM-containing receptors by signals from co-cross linked ITIM-containing Fc γ RIIb has been demonstrated in mouse and human cells [6-8]. The ability of the ITIM-containing IgG receptor Fc γ RIIb 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]. Fc γ RIIb was first implicated in the negative regulation of human Fc ϵ RI-mediated signaling by Daëron [6]. It was later extended to peripheral blood basophils [8] and cord blood-derived human mast cells (CBMC) [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 Fc γ -Fc ϵ fusion protein to achieve inhibition of allergic reactivity.

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

The goal of immunotherapy (IT) is to induce mast cell/basophil non-responsiveness 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” co-aggregate Fc γ RIIb receptors with Fc ϵ RI. As antigen-specific IgG levels increase during IT, the serum concentrations increase enough so that monomeric IgG (or complexed to Ag) is able to bind Fc γ RIIb on mast cell and basophils. Upon subsequent allergen exposure, the Fc γ RIIb-bound IgG and the Fc ϵ RI-bound IgE bind the antigen. These Fc ϵ RI-bound IgG complexes then also bind to surface Fc γ RIIb and initiate an inhibitory rather than activating signal.

Daëron *et al.* were the first to demonstrate that Fc ϵ RI/Fc γ RII co-aggregation on human basophils inhibited IgE-mediated degranulation through ITIM signaling [6]. 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 Fc ϵ -Fc γ using IgE with IgG to the same antigen [8]. However, it is not clear if this mechanism occurs *in vivo*.

GE2, a human Fc γ -Fc ϵ fusion protein inhibits human basophil and mast cell function in vitro

Realizing the therapeutic potential for regulating Fc ϵ RI signaling, we developed a molecule that utilizes Fc ϵ RI co-aggregation to ITIM-containing receptors [11]. This fusion protein (GE2) consists of the human IgG1 γ Hinge-CH γ 2-CH γ 3 region linked to the human IgE CH ϵ 2-CH ϵ 3-CH ϵ 4 region. The human gamma 1 hinge-CH2-CH3 region was placed ahead of the ϵ heavy chain CH2 through CH4 domains with a 17 amino-acid linker containing a bglIII site and (Gly₄Ser)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 Fc γ RII (CH γ 2-CH γ 3) and Fc ϵ RI (CH ϵ 2-CH ϵ 3) [13-14]. The first constant domains (CH γ 1 or CH ϵ 1) were deleted as these domains are associated with BiP (immunoglobulin heavy chain binding protein) and do not contain light chains [15]. The proposed binding and mechanism for GE2's inhibitory effects on human mast cells and basophils is shown in Figure 1.

Human basophils purified from the peripheral blood, known to express Fc γ RIIb were passively sensitized with 10 μ g/ml of chimeric human anti-NIP IgE. One hour later, doses of GE2 ranging from 0.01 to 10 μ g/ml were added and compared to a

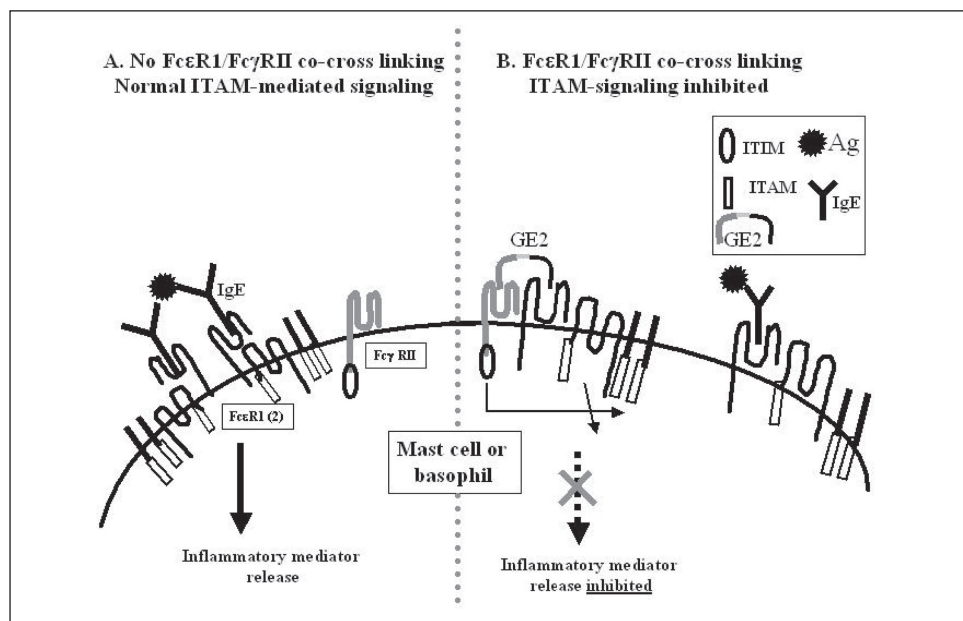


Fig. 1. Schematic showing the proposed mechanism for GE2 effect on human basophils and mast cells. Panel A shows antigen cross-linking two Fc ϵ RI and inducing activation with enhancement of Syk and other downstream signaling molecules. Fc γ RII is unoccupied. Panel B shows GE2 co-crosslinking Fc γ RIIb to Fc ϵ RI that results in lower Syk kinase levels such that the cell is inhibited in its ability to function via Fc ϵ RI activation.

human IgE myeloma control. After another hour's 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 subjects were incubated with GE2 and then challenged with purified cat antigen, Fel d 1, GE2 again showed a dose response with 78% inhibition of Fel d 1 specific release at 10µg/ml of GE2. 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. Unstimulated cord blood mast cells express the ITIM-containing receptor, FcγRII, but not FcγRI or FcγRIII. Co-aggregation of FcεRI and FcγRII significantly inhibited IgE-mediated histamine release, cytokine production, and Ca²⁺ mobilization. Taken together, these differing approaches show that GE2 can inhibit IgE-mediated mediator release in passively or natural sensitized human basophils and mast cells [16].

Recently, we demonstrated that in cord blood-derived mast cell, IgE-mediated responses could be inhibited with GE2 (Figure 2). Therefore, blood basophils and cord blood-derived mast cells express the ITIM-containing FcγRIIb isoform which, when co-aggregated to FcεRI, inhibits IgE-mediated release.

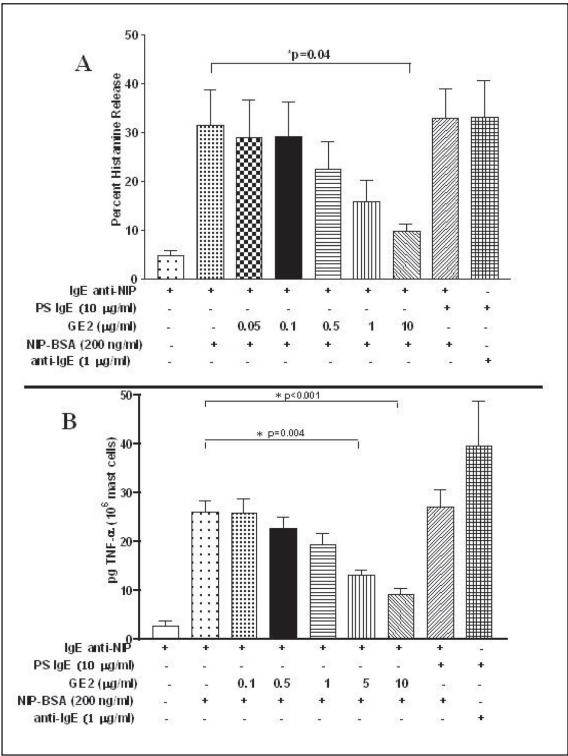


Fig. 2. *FcγRII/FcεRI* co-aggregation inhibits *FcεRI*-mediated mast cell degranulation and *TNF-α* production. NP-IgE-sensitized CBMC were incubated with or without GE2 or non-specific IgE (PS). The cells were washed, incubated with or without 200 ng/ml NIP-BSA or anti-IgE for 45 min (histamine release) or 12 h (cytokine production) and mediator release measured in the supernatants. Results are represented as the standard error of the mean of 4 (A) or 2 (B) separate experiments (±SEM). *Designates values significantly reduced ($P < 0.01$) when comparing cells challenged with or without GE2.

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. Rodent studies using cell lines implicate the phosphatase SHIP more so than SHP-1 in FcγRII-mediated inhibitory signaling [7,21,22]. In contrast, our data with peripheral blood basophils implicate SHP-1 in the FcγRII-mediated inhibitory signaling [23,11]. Second, in rodents, co-aggregation of FcεRI to FcγRII does not prevent FcεRI-mediated activation of Syk [7]. However, we have shown in peripheral blood basophils [8,11] that Syk phosphorylation is down regulated by FcγRII-FcεRI co-aggregation further implicating SHP-1, rather than SHIP, in negative FcγRII signaling [23]. Third, while FcεRI-FcγRII co-aggregation in rodent and human systems inhibits the Ca²⁺ response to FcεRI stimulation, we have demonstrated that the kinetics of this inhibition is distinct in peripheral blood basophils [8] and CBMC [10] as compared to rodent cells [7,21]. Finally, in results not observed in rodent systems, co-aggregation in our model increased the tyrosine phosphorylation of the adapter protein downstream of kinase (Dok), growth factor receptor-bound protein 2 (Grb2), and SH2 domain containing inositol 5-phosphatase (SHIP). Tyrosine phosphorylation of Dok was associated with increased binding to Grb2. Surprisingly, in non-stimulated cells, their 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” [17] of human mast cell degranulation. Clearly, 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 Langerhans-like dendritic cells (LLDC)

While basophils and mast cells were the primary target for which GE2 was designed, other cells express both FcγRII and FcεRs, e.g. human Langerhans-like dendritic cells 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 [18]. 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 epsilon germ line transcription plus a direct effect on the process of 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 (ERK), and it was mediated via suppression of IL-4-induced STAT6-phosphorylation [19].

Unstimulated LLDC, derived from CD14-positive monocytes from atopic donors, were shown to express FcγRII, an ITIM-containing receptor, but not FcγRI or FcγRIII which are activating (ITAM) receptors. When passively sensitized with anti-

gen-specific human IgE and then challenged with antigen, LLDC were stimulated to produce IL-16, an important pro-allergic cytokine. 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 LLDC to GE2 alone did not induce IL-16 production [20]. 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 LLDC 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, hFcεRIα tg mice. The human FcεRIα chain combines with the mouse β and γ chains to form a functional chimeric human/mouse FcεRI 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 animal mast cells bind human IgG so that GE2 is expected to be functional in them.

A. GE2 blocked passive cutaneous anaphylaxis (PCA) at sensitized skin sites in human FcεRIα transgenic mice. We utilized PCA in the hFcεRIα tg mice to test the ability of GE2 to block IgE-driven *in vivo* human FcεRI-mediated mast cell release. hFcεRIα tg mice were locally sensitized at multiple sites with 250 ng of model genetically engineered human IgE antibodies to 4-hydroxy-3-nitrophenylacetyl (NP) or dansyl. Varying doses of GE2 or control myeloma IgE was added at the sites. The mice were then challenged i.v. 6 hours later with the relevant antigen bound to a carrier and reactivity 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 subject. Four hours later, GE2 or control material was administered to these sites and a further four hours later, the mice were challenged i.v. with Fel d 1 and Evans blue dye. Reactivity was scored 15-20 minutes 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 d 1-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 d 1 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.

B. 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 [24]. We tested whether GE2 could inhibit *D. farinae* (DF) mite skin test reac-

tivity in rhesus monkeys. DF-reactive monkeys underwent graded intradermal injections with GE2 (62.5 to 250 ng) vs. purified human IgE myeloma protein as a control. Five hours later, the sites were then challenged with DF at a dose optimized for reactivity for each animal through previous dose response testing. Animals were simultaneously given i.v. Evans blue dye and skin test reactivity measured 15-20 minutes later. GE2 protein effected complete inhibition at 250 ng and showed complete inhibition in 4 of 5 animals at 125 ng. At 62.5 ng, GE2 still exhibited clear inhibition of reactivity compared to saline or control, while PS 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 non-human 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 gamma 1 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 FcεR1s has been to work on human basophils and cultured mast cells *in vitro* [25].

There are several ITIM-containing receptors that have been identified but their expression has not been investigated on human FcεRI-positive cells [26]. We investigated the expression of several ITIM-containing receptors on peripheral blood basophils, CBMC, KU812 and HMC-1. As seen in Table 1, peripheral blood

	FcγRII	FcγRIIA	FcγRIIB	MAFA	Siglec 5	Sirp-α	CD 22**	CD 81	LIR
Baso-phils	+++	0-+ DV	+++	0-+	0	0	0	0-+ DV	++-++ DV
CBMC	++	0	++	0-+	0-+++ ‡	0-+++	+ -+++ †	+++	+
HMC-1	0-+	0	0-+	0	0	0	0	0	0
KU812	++	0	0-+	0	0	0	0	0	0

Table 1. Detection of ITIM receptors on human FcεRI-positive cells using anti-ITIM receptor antibodies using the methodology as described previously [29]. Peripheral blood basophils (>90%), cord blood-derived mast cell (CBMC), the human mast cell line (HMC-1), and the human basophil cell line (KU812) were tested. The numbers represent approximate fluorescent intensity with 3 being the strongest and 0 representing no reactivity. In human blood basophils we found donor variation (DV) in expression of the FcγRIIA isoform and the leucocyte immunoglobulin-like receptor (LIR). In CBMC, IFN-γ dramatically up-regulated siglec-5 (‡) and down-regulated CD22 (†), while IgE and IL-4 up-regulated the signal-regulatory protein alpha (SIRP-α).

** The CD81 contains an "ITIM-like" sequence. NKG2, KIR, CD5, 31, 32, 72 were not detected.

basophils and CBMC express only the ITIM-containing Fc γ RIIb isoform of Fc γ RII. As has been recently reported, we found donor variations in the expression of Fc γ RIIa on peripheral blood basophils [27]. Thus, future strategies aimed at harnessing the inherent inhibitory ability of these receptors targeted specifically to mast cell and basophils may have therapeutic potential.

Conclusion

Crosslinking of Fc ϵ RI and Fc γ RIIb induces strong inhibitory signaling in cord blood-derived human mast cells and peripheral blood basophils. We have shown this can be accomplished with clear antiallergic effects *in vitro* and *in vivo* with a single human Fc γ -Fc ϵ fusion molecule. The ability of GE2, in addition, to block human B cell isotype switching to ϵ and to inhibit proallergic function in LLDC suggests GE2 may provide the broader benefits in the treatment of IgE-mediated diseases rather than simply through inhibition of mediator release. Thus the approach of using chimeric Fc γ -Fc ϵ fusion protein provides a platform for antigen non-specific 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 Fc γ RII or decrease potential binding to Fc γ RI/III or alterations in the linker length that increase co-aggregation are now being explored.

Acknowledgements

Supported by USPHS grants AI-15251 and ongoing support from the Bridges Larson Foundation. CLK was additionally supported by a grant from the American Lung Association and the Food Allergy and Anaphylaxis Network.

References

1. Packard KA, Khan MM. Effects of histamine on Th1/Th2 cytokine balance. *Int Immunopharmacol* 2003; 3: 909-920.
2. Assanasen P, Naclerio RM. Antiallergic-inflammatory effects of H1-antihistamines in humans. *Clin Allergy Immunol* 2002; 17: 101-139.
3. Robbie-Ryan M, Brown MA. The role of mast cells in allergy and autoimmunity. *Curr Opin Immunol* 2002; 14: 728-733.
4. Kinet JP. Allergy and hypersensitivity. *Curr Opin Immunol* 2002; 14: 685-687.
5. Katz HR. Inhibitory receptors and allergy. *Curr Opin Immunol* 2002; 14: 698-704.
6. Daëron M, Malbec O, Latour S, Arock M, Fridman WH. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *J Clin Invest* 1995; 95: 577-585.
7. Malbec O, Fong DC, Turner M, Tybulewicz VL, Cambier JC, Fridman WH, Daëron M. Fc ϵ receptor I-associated lyn-dependent phosphorylation of Fc γ receptor IIB during negative regulation of mast cell activation. *J Immunol* 1998; 160: 1647-1658.
8. Kepley CL, Cambier JC, Morel PA, Lujan D, Ortega E, Wilson BS, Oliver JM. Negative regulation of Fc ϵ RI signaling by Fc γ RII costimulation in human blood basophils. *J Allergy Clin Immunol* 2000; 106: 337-348.
9. Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV. A 13-amino-acid motif in the cytoplasmic domain of Fc γ RIIB modulates B-cell receptor signaling. *Nature* 1994; 368: 70-73.
10. Kepley CL, Taghavi S, Mackay G, Zhu D, Morel PA, Zhang K, Ryan JJ, Satin LS, Zhang M, Pandolfi PP, Saxon A. Co-aggregation of Fc γ RII with Fc ϵ RI inhibits antigen induced secretion and involves SHIP-Grb2-Dok complexes. *J Biol Chem* 2004; 279: 35139-35149.
11. Zhu D, Kepley CL, Zhang M, Zhang K, Saxon A. A novel human immunoglobulin Fc γ -Fc ϵ bifunctional fusion protein inhibits Fc ϵ RI-mediated degranulation. *Nat Med* 2002; 8: 518-521.

12. Huston JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, Margolies MN, Ridge RJ, Brucoleri RE, Haber E, Crea R, Oppermann H. Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci USA* 1988; 85: 5879-5883.
13. Helm B, Marsh P, Vercelli D, Padlan E, Gould H, Geha R. The mast cell binding site on human immunoglobulin E. *Nature* 1988; 331: 180-183.
14. Hulett MD, Witort E, Brinkworth RI, McKenzie IF, Hogarth PM. Identification of the IgG binding site of the human low affinity receptor for IgG Fc gamma RII. *J Biol Chem* 1994; 269: 15287-15293.
15. Hendershot LM, Kearney JF. A role for human heavy binding protein in the developmental regulation of immunoglobulin transport. *Mol Immunol* 1988; 25: 585-593.
16. Zhang K, Kepley CL, Terada T, Zhu D, Perez H, Saxon A. Inhibition of allergen specific IgE reactivity by a human Ig Fcγ-Fcε bifunctional fusion protein. *J Allergy Clin Immunol* 2004; 114: 321-327.
17. Huber M, Helgason CD, Damen JE, Liu L, Humphries RK, Krystal G. The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation. *Proc Natl Acad Sci USA* 1998; 95: 11330-11335.
18. Rosenwasser LJ, Busse WW, Lizambri RG, Olejnik TA, Totoritis MC. Allergic asthma and an anti-CD23 mAb (IDEC-152): Results of a phase I, single-dose, dose-escalating clinical trial. *J Allergy Clin Immunol* 2003; 112: 563-570.
19. Yamada T, Zhu D, Zhang K, Saxon A. Inhibition of IL-4-induced class switch recombination by a human Ig Fcγ-Fcε chimeric protein. *J Biol Chem* 2003; 278: 32818-32824.
20. Kepley CL, Zhang K, Zhu D, Saxon A. FcεRI-FcγRII co-aggregation inhibits IL-16 production from human Langerhans-like dendritic cells. *Clin Immunol* 2003; 108: 89-94.
21. Ono M, Bolland S, Tempst P, Ravetch JV. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor FcγRIIB. *Nature* 1996; 383: 263-266.
22. Fong DC, Malbec O, Arock M, Cambier JC, Fridman WH, Daëron M. Selective in vivo recruitment of the phosphatidylinositol phosphatase SHIP by phosphorylated FcγRIIB during negative regulation of IgE-dependent mouse mast cell activation. *Immunol Lett* 1996; 54: 83-91.
23. Dustin LB, Plas DR, Wong J, Hu YT, Soto C, Chan AC, Thomas ML. Expression of dominant-negative Src-homology domain 2-containing protein tyrosine phosphatase-1 results in increased Syk tyrosine kinase activity and B cell activation. *J Immunol* 1999; 162: 2717-2724.
24. Schelegle ES, Gershwin LJ, Miller LA, Fanucchi MV, van Winkle LS, Gerriets JP, Walby WF, Omlor AM, Buckpitt AR, Tarkington BK, Wong VJ, Joad JP, Pinkerton KB, Wu R, Evans MJ, Hyde DM, Plopper CG. Allergic asthma induced in Rhesus monkeys by house dust mite (*Dermatophagoides farinae*). *Am J Pathol* 2001; 158: 333-341.
25. Tam SW, Demissie S, Thomas D, Daëron M. A bispecific antibody against human IgE and human FcγRII that inhibits antigen-induced histamine release by human mast cells and basophils. *Allergy* 2004; 59: 772-780.
26. Unkeless JC, Jin J. Inhibitory receptor, ITIM sequences and phosphatases. *Curr Opin Immunol* 1997; 9: 338-343.
27. Su K, Wu J, Edberg JC, Li X, Ferguson P, Cooper GS, Langefeld CD, Kimberly RP. A promoter haplotype of the immunoreceptor tyrosine-based inhibitory motif-bearing FcγRIIb alters receptor expression and associates with autoimmunity. I. Regulatory FCGR2B polymorphisms and their association with systemic lupus erythematosus. *J Immunol* 2004; 172: 7186-7191.

Discussion

Kalinke: Thanks a lot for the very interesting presentation. I would like to start right away with one question. At least in the mouse system, it is an ongoing debate how abundant the Fc-gamma receptor expression is.

Kepley: The mouse system is completely different.

Kalinke: I was wondering, when you add reagent consisting of the allergen plus the constant part, is there a dose-dependent inhibitory effect or would you induce the mast cells to degranulate? Because imagining that the Fc-gamma receptor was expressed comparably low as the Fc-epsilon receptor, there should be a threshold

dilution where you just induce mast cell degranulation. This aspect could be of significance with respect to pharmacological considerations.

Kepley: We try to make a faster and a safer form of immunotherapy, which is a problem now. We cannot do those kinds of dose-responses and kinetics, as you have said, in the mouse system because it is totally different. We are looking at those dose and kinetic issues at the moment.

Valenta: I have three questions. First, you showed that the Fel d 1 construct did not induce any histamine release. If so, how could it co-cross-link then? Second, are you not worried about the induction of rheumatoid factors when you inject a construct that bears a Fc-gamma region into a patient? And third, how would you figure out how to administer this construct to patients?

Kepley: The first question, why does the construct not induce mediator release just by itself? We think it is because of an IgG inhibitory motif, and this inhibitory motif is causing some steric hindrance or it is actually binding to the ITIM receptor to inhibit degranulation. Your second question was regarding whether we are worried to induce basically rheumatoid factors when you inject this construct. The answer is, we cannot predict that. Seeing that it is a human Fc part of the IgG, we hopefully predict that it would not induce the response. But if you go beyond that and think: What if it did induce the response, what if it did make more IgGs to that allergen chimeric protein, it might not be such a bad thing, you might get more ITIM coaggregation. Third answer: As we do most things in the US, we will do that subcutaneously, but this is now in the hands of a pharmaceutical company and we do not have any say that kind of decision.

Umetsu: I have a question regarding the gamma-Fd1 construct. It is clear that it can inhibit mast cell degranulation. I was wondering if you looked at the use of this as an immunotherapeutic agent to induce sort of a protective immune response. Do you know what kind of immune response it might induce, say over a couple of weeks' time? Does it induce protective antibodies?

Kepley: Until we start doing it in humans we really do not know what will happen. In the mouse studies they focused more on the PCA response and the immediate-type reactions. They did not really focus on those issues like the IgG levels and that kind of thing. It might not be such a bad thing if you do induce IgG responses – that is what immunotherapy does. So you might actually get more coaggregation of the ITIM receptors. That is something you cannot predict until you start giving it to people.

Lund: I was just wondering, there are other ways to blunt the activation of basophils and mast cells in the absence of IgG molecules. That is simply by preincubating the isolated cells in low amounts of antigen. Do you have any idea if those two ways share the same mechanism or do depend for sure on the Fcγ receptor? I would be talking about two different mechanisms here, do they have a common pathway?

Kepley: I propose the following model: If you give a mast cell low doses of antigen that do not induce degranulation or cytokine production, it is a well-known phenom-

enon that you can desensitize it. I recently published that you are actually inducing syk deficiency. So what I would be proposing to look at syk levels before and after immunotherapy to see that the blunting response, that you see after immunotherapy on mast cells and basophils and has been demonstrated by several different labs, is somehow due to decreases in syk. I do not know whether this answers your question but that is my bias.

Skov: Have you investigated whether this effect of the desensitization of the basophils is allergen-specific?

Kepley: I feel that syk deficiency will be a non-specific phenomenon. The studies looking until you reach maintenance dose indicate that right after you start doing the shots it is not allergen-specific. I am among experts, so they can probably tell me, but I am not aware if it would initially be an antigen-specific response when you are doing the updosing. It is a good question but I do not know the answer for now.

