



Suppression of mast cell degranulation through a dual-targeting tandem IgE–IgG Fc domain biologic engineered to bind with high affinity to FcγRIIb

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ABSTRACT

Mast cells and basophils play a central role in allergy, asthma, and anaphylaxis, as well as in non-allergic inflammatory, neurological and autoimmune diseases. Allergen-mediated cross-linking of IgE bound to FcεRI leads to cellular activation, and the low-affinity Fc receptor FcγRIIb is a key inhibitor of subsequent degranulation. FcγRIIb, when coengaged with FcεRI via allergen bound to IgE, stimulates ITIM domain-mediated inhibitory signaling that efficiently suppresses mast cell and basophil activation. To assess the therapeutic potential of directed coengagement of FcεRI and FcγRIIb in the absence of FcεRI crosslinking, we developed a fusion protein comprising the coupled Fc domains of murine IgE and human IgG1. As a key functional component of this tandem Fcε–Fcγ biologic, we engineered its IgG1 Fc domain to bind to human FcγRIIb with 100-fold enhanced affinity relative to native IgG1 Fc. Using mast cells from mice transgenic for human FcγRIIb, we show that this tandem Fc binds with high affinity to murine FcεRI and human FcγRIIb on mast cells, triggers phosphorylation of FcγRIIb, and inhibits FcεRI-dependent calcium mobilization. Control tandem Fc biologics containing a native IgG1 Fc domain or lacking binding to Fcγ receptors were markedly less active, demonstrating that the affinity-optimized tandem Fc can inhibit degranulation through stimulation of FcγRIIb signaling as well as through competition with allergen–IgE immune complex for FcεRI binding. We propose that in the context of a fully human tandem Fc biologic, high-affinity coengagement of FcεRI and FcγRIIb has potential as a novel therapy for allergy and other mast cell and basophil-mediated pathologies.

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1. Introduction

Mast cells are strategically localized near blood vessels, nerves, epithelia, and mucous membranes, and play important roles in host immune responses to pathogens and in allergic reactions, as well as in maintaining homeostasis during wound healing [1–3]. Basophils, the circulating complement to tissue mast cells, are also critical contributors to allergic and inflammatory reactions [4,5]. Mast cell and basophil activation is induced by the crosslinking of FcεRI, the high-affinity receptor for the Fcε domain of IgE, mediated by multivalent allergen bound to IgE. FcεRI crosslinking leads to degranulation of secretory vesicles and the release of many inflammatory mediators including vasoactive peptides, proteases,

heparin, TNF, platelet-activating factor, prostaglandins, cytokines and chemokines [1,6,7]. The extraordinary sensitivity and responsiveness of mast cells and basophils to minute amounts of allergen can result in severe reactions including allergic asthma, rhinitis, urticaria pigmentosa and anaphylaxis. Beyond the primary allergic role for these cells, diverse non-allergic inflammatory, neurological and autoimmune pathologies are also associated with their activation [2–5].

Given the central role of mast cells and basophils in allergy and inflammation, much effort has been made to understand not only mechanisms leading to their activation, but also the inhibitory pathways involved in regulating degranulation [4,8–16]. From such work, several potential therapeutic strategies have been developed. For example, cell-stabilizing drugs, β₂-adrenoceptor agonists, corticosteroids, mediator antagonists, phosphodiesterase inhibitors, and ion channel blockers have been shown to be effective in blocking both early and late responses in allergy and asthma [17]. Others have developed inhibitors of kinases known to be involved in mast cell and basophil development and activation, such as Syk, c-kit, and Btk [16,18–20]. In addition, monoclonal antibodies targeting the Th2 cytokines IL-4, IL-13 and IL-5 and their receptors are in development as biologic agents intended to control allergic inflammation

Abbreviations: FcεRI, high-affinity receptor for IgE Fc; FcγRIIb, low-affinity receptor for IgG Fc; FSC, forward scatter; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITAM, immunoreceptor tyrosine-based activation motif; OVA, ovalbumin; SSC, side scatter.

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[17]. The IgE–FcεRI signaling axis has been clinically validated with one biologic; sequestration of soluble IgE by the monoclonal antibody omalizumab is an approved therapy in allergy and asthma [21]. Although efficacious, IgE sequestration by omalizumab does not acutely suppress mast cell stimulatory signaling, likely because IgE is already bound to FcεRI at high affinity (0.1 nM K_d) [22,23] with a slow dissociation rate. However, it has been reported that chronic dosing of omalizumab can reduce FcεRI expression, partially desensitizing mast cells to IgE [24].

An essential mechanism for suppressing the activity of multiple immune cells, including mast cells and basophils, involves FcγRIIb, the low-affinity inhibitory receptor for the Fc domain of both IgG and IgE [25]. FcγRIIb possesses an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail which, when brought into proximity with an activated receptor possessing an immunoreceptor tyrosine-based activation motif (ITAM), inhibits its signaling and suppresses immune cell activation [12,26–29]. FcγRIIb has been detected on mast cells derived from several human and murine progenitor cell types [30,31], as well as on basophil-derived cell lines [32], although its presence on primary human mast cells is controversial and awaits experimental confirmation [33]. In vitro studies, FcγRIIb signaling was shown to inhibit mast cell degranulation and activation through a mechanism involving Dok1, SHIP1, and inhibition of PI3 kinase and Erk [34–36]. In the past decade, several groups have exploited the biology of FcγRIIb to develop mast cell and basophil-specific inhibitors. For example, coengagement of FcεRI and FcγRIIb to inhibit degranulation and allergic responses has been explored using several biologics, including bispecific antibodies [14,37] and IgG1 Fc fusions with IgE Fc, anti-IgE, or allergen [38–42]. Although these approaches have shown promise in in vitro and in vivo models, they may have potential therapeutic limitations; for example, one reported bispecific antibody targets not only the inhibitory receptor FcγRIIb but also the activating receptor FcγRIIa [14], whereas the Fcγ domains of the fusion proteins are identical to native IgG1 Fc and so have very low (>1 μM) affinity for FcγRIIb and lack selectivity with respect to activating receptors such as FcγRIIa.

To create a potent and selective mast cell and basophil inhibitor acting through coengagement of FcγRIIb and FcεRI while avoiding potential stimulatory effects mediated by crosslinking of FcεRI, we have engineered a tandem Fcε–Fcγ biologic incorporating an IgG1 Fc domain with optimized affinity and selectivity for FcγRIIb. We have previously shown that introduction of S267E and L328F amino acid substitutions into the Fc domain of human IgG1 increases its affinity for FcγRIIb by 100-fold or more [43,44]. This IgG1 Fc domain has proven immunosuppressive in human in vitro and transgenic mouse models when incorporated into an anti-CD19 antibody currently under investigation in clinical trials [43,45]. In this report, we describe the incorporation of the identical engineered Fcγ domain into a tandem mouse Fcε–human Fcγ biologic and show, in mast cells derived from mice transgenic for human FcγRIIb, that its inhibition of mast cell degranulation requires high-affinity engagement of FcγRIIb. We demonstrate using idiotype control tandems, as well as mast cells derived from wildtype mice, that this tandem Fcε–Fcγ biologic blocks degranulation through FcγRIIb inhibitory signaling and also through competition with IgE for binding to FcεRI. This

work demonstrates that amino acid engineering of the Fc domain can overcome biologically imposed limitations on the design of new therapeutic strategies, and suggests that a similar fully human tandem Fcε–Fcγ biologic could be useful in the treatment of mast cell and basophil-mediated diseases.

2. Materials and methods

2.1. Fcε–Fcγ tandem Fcs and other reagents

DNA sequences encoding tandem Fcε–Fcγ biologics were constructed as follows. First, DNA encoding the fusion of a standard antibody signal peptide, the murine IgE Fc region (positions 229–547, comprising Ce2–Ce3–Ce4), and 21 amino acids of human IgG1 (positions 216–236, comprising the final portion of the CH1 domain and the hinge region with C220S substitution) of human IgG1 was generated by gene synthesis (Integrated DNA Technologies, Coralville, Iowa). Positions are numbered according to the EU index [46]. The synthesized DNA was subcloned into the vector pTT5 (National Research Council, Canada) encoding a human IgG1 heavy chain. Additional subclonings into similar vectors containing S267E/L328F (optimized for high affinity to FcγRIIb [43]) and G236R/L328R (optimized to eliminate binding to FcγRIIb [45]) amino acid substitutions previously introduced by site-directed mutagenesis (QuikChange, Stratagene, Cedar Creek, TX) were performed in parallel. Resulting tandem Fcε–Fcγ constructs were transfected into HEK293E cells (National Research Council, Canada) for expression, and tandem Fcε–Fcγ biologics (as described in Table 1) were purified using protein A affinity chromatography (GE Healthcare).

2.2. Mast cell differentiation from mouse bone marrow

C57BL/6 mice transgenic for human FcγRIIb and null for mouse FcγRIIb (*Fcgr2b*^{−/−} *hFCGR2B*⁺) as described [45] were provided by Jeffrey Ravetch of the Rockefeller University. Mast cells were differentiated from human FcγRIIb transgenic and wildtype C57BL/6 mice as previously described [47]. In brief, bone marrow was flushed out of mouse femurs, cells were washed, and red blood cells were eliminated using ACK lysis buffer (Invitrogen, Carlsbad, CA). Bone marrow was cultured at 1 × 10⁶ cells/ml in mast cell media (RPMI 1640 supplemented with 10% FBS, L-glutamine, penicillin, streptomycin, HEPES, sodium pyruvate, nonessential amino acids, 2-mercaptoethanol and 30 ng/ml (final concentration) of murine IL-3) for 4 weeks, with medium changed twice weekly. After 4 weeks, mast cell purity was assessed by flow cytometry (staining for FcεRI and c-kit) and by toluidine-blue staining [47]. Mast cells were used up to 8 weeks after initiation of differentiation.

2.3. Mast cell β-hexosaminidase release assay

Mast cells were sensitized overnight with 1 μg/ml of anti-ovalbumin (OVA) IgE (clone E-C1, Chondrex, Redmond, WA) or FITC-labeled anti-OVA IgE (labeled using FITC Antibody Labeling Kit, Pierce, Rockford, IL) in the absence or presence of other biologics, washed and resuspended in Tyrode's buffer (10 mM

Table 1
Binding affinities of tandem Fc proteins to FcγRIIb and FcεRI receptors.

Name	Description	Fc domain 1	Fc domain 2	FcγRIIb (SPR)		FcεRI (cell surface)	
				K _D (nM)	Fold	IC ₅₀ (μg/mL)	Fold
XENP8138	IgG1 control with native affinity for FcγRIIb	Mouse IgE	Human IgG1	1200	1	0.97	1
XENP8142	Engineered for high affinity to FcγRIIb	Mouse IgE	hulG1 S267E/L328F	12	100	0.43	2.3
XENP8146	IgG1 control lacking binding to Fcγ receptors	Mouse IgE	hulG1 G236R/L328R	n.b. ^a		0.88	1.1

^a No detectable binding.

HEPES, 130 mM NaCl, 6.2 mM D-glucose, 3.0 mM KCl, 1.4 mM CaCl₂, 1.0 mM MgCl₂ and 0.1% BSA). Cells (5×10^4 /well in 200 μ l) were plated in triplicate in 96-well plates and stimulated for 60 min with 1 μ g/ml OVA (Sigma–Aldrich, St. Louis, MO) or anti-FITC IgG clone 4.4.20 [48] (constructed by gene synthesis and subcloned into pcDNA3.1Zeo as a full-length chimeric antibody), followed by harvest of supernatants and cell pellets. For measurement of β -hexosaminidase activity, 50 μ l of supernatant was incubated with 50 μ l 3.7 mM p-nitrophenol-N-acetyl- β -D-glucosaminide (Sigma–Aldrich, St. Louis, MO) for 1 h, followed by quenching of the enzymatic reaction by addition of 100 μ l 2 M NaOH and measurement of absorbance at 405 nm. Activity was calculated as the percent of maximal β -hexosaminidase in untreated cell pellets lysed in water by 3 freeze–thaw cycles.

2.4. Flow cytometry

Mast cells were washed in PBS, resuspended in PBS supplemented with 3% FCS, and stained for 1 hr with antibodies against Fc ϵ RI (clone MAR-1, eBioscience, San Diego, CA), c-kit (clone YB5.B8, BD Pharmingen, San Diego, CA) and Fc γ RIIb (clone 2B6, described previously [49], constructed by gene synthesis and subcloned into pcDNA3.1Zeo as full-length chimeric antibody). Labeled cells were washed and examined using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA).

2.5. Fc ϵ RI and Fc γ RIIb binding affinities

Surface plasmon resonance measurements were performed in HBS-EP running buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20, GE Healthcare) using a Biacore 3000 instrument (GE Healthcare). A protein A chip/tandem Fc capture format with Fc γ RIIb (R&D Systems) as analyte was utilized. A protein A (Pierce Biotechnology) CM5 biosensor chip (GE Healthcare) was generated using a standard primary amine coupling protocol. Tandem Fc ϵ –Fc γ biologics at 100 nM were immobilized on the protein A surface for 0.5 min at 10 μ l/min. Fc γ RIIb in semi-log serial dilutions (starting at 1 μ M, 6 concentrations total) was injected over tandem Fc-bound surface for 1.5 min at 20 μ l/min followed by a 3 min dissociation phase. After each cycle, the surface was regenerated by injecting glycine buffer (10 mM, pH 1.5). Data were processed by zeroing time and response before the injection of receptor and by subtracting appropriate nonspecific signals (response of reference channel and injection of running buffer). Equilibrium constants were calculated by global fitting of binding data with a conformational change model using BIAevaluation software (GE Healthcare). For determining the binding of labeled Fc ϵ –Fc γ tandems to Fc ϵ RI, wildtype mast cells were incubated overnight with 1 μ g/ml of IgE and increasing amounts of tandem Fc molecules. After incubation, cells were washed and Fc ϵ RI-bound IgE was detected by flow cytometry using a labeled anti-IgE antibody. IC₅₀ values were calculated using a standard four-parameter sigmoidal dose–response fit of the data using Prism software (GraphPad Software, La Jolla, CA).

2.6. Fc γ RIIb activation assay

Mast cells were sensitized overnight with 1 μ g/ml anti-OVA IgE in the presence or absence of the indicated tandem Fc ϵ –Fc γ biologics. Cells were washed in mast cell media and stimulated with 1 μ g/ml of OVA for 3 min and lysed for 20 min in lysis buffer (0.15 M NaCl, 0.05 M Tris pH 8.0, 1 mM EDTA, 1% NP-40) in the presence of 0.2 M Na₃VO₄, 0.1 M NaF, and 0.1 M PMSF (Sigma–Aldrich, St. Louis, MO) plus a mixture of other protease inhibitors (Roche Applied Sciences, Mannheim, Germany). Lysates were centrifuged 10 min at 12,000 rpm; then postnuclear lysates

were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose membranes by western blotting. Membranes were probed with anti-phosphotyrosine 292 of Fc γ RIIb (clone EP926Y, Epitomics, Burlingame, CA) and HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA).

2.7. Calcium mobilization

Mast cells were sensitized overnight with 1 μ g/ml anti-OVA IgE in the presence or absence of 1 μ g/ml of the indicated tandem Fc ϵ –Fc γ biologics. Sensitized cells were washed, resuspended in HBSS, loaded with Fluo-4 NW calcium dye (Molecular Probes, Eugene, OR) for 45 min at 37 °C, and stimulated with 1 μ g/ml OVA for 2 min. Calcium mobilization was monitored and analyzed using the NovoSTAR plate reader (BMG Labtech, Cary, NC).

3. Results

3.1. Phenotype and function of mast cells differentiated from bone marrow of human Fc γ RIIb transgenic mice

To develop a model suitable for assessing the activity of tandem mouse Fc ϵ –human Fc γ biologics, we first determined the expression level and functionality of Fc γ RIIb on mast cells derived from human Fc γ RIIb-transgenic mice. These mice were generated through introduction of a large bacterial artificial chromosome into mice that do not express endogenous mouse Fc γ RIIb [45,50], so human Fc γ RIIb expression level and cellular distribution is predicted to be regulated primarily by the gene's associated promoter and other regulatory elements. These mice are known to express a high level of functional human Fc γ RIIb on B cells [45]; however, Fc γ RIIb expression on mast cells and basophils has not yet been described. Fig. 1A shows that after 4 weeks of differentiation of bone marrow precursor cells in the presence of murine IL-3, a homogeneous c-kit⁺ Fc ϵ RI⁺ population comprising >95% of gated live cells (gate P1 on the FSC/SSC plot) is detectable in cultures from both wildtype and transgenic mice. Notably, this population is positive for human Fc γ RIIb when derived from transgenic but not wildtype C57BL/6 mice. Both C57BL/6 and human Fc γ RIIb-transgenic mast cells possessed metachromatic granules detectable upon staining with toluidine blue, an indicator of the presence of heparin, histamine, β -hexosaminidase and other mast cell mediators (data not shown).

We next assessed whether the human Fc γ RIIb present on transgenic mast cells was functional, as defined by two criteria: (1) constitutive overexpression of this receptor did not perturb Fc ϵ RI-mediated activation and degranulation in transgenic relative to wildtype mast cells, and (2) upon coengagement with Fc ϵ RI, the transgenic human receptor was capable of inhibiting mast cell degranulation mediated by allergen-IgE crosslinking of Fc ϵ RI. We therefore assayed for β -hexosaminidase release (an accepted marker of mast cell degranulation) in mast cells derived from Fc γ RIIb-transgenic and wildtype mice. Mast cells were sensitized overnight with FITC-labeled or unlabeled mouse IgE in the presence or absence of FITC-labeled or unlabeled anti-human Fc γ RIIb antibody, and degranulation in all groups was assessed after addition of anti-FITC antibody to artificially crosslink Fc ϵ RI and Fc γ RIIb. As shown in Fig. 1B, β -hexosaminidase release after stimulation with anti-FITC antibody was minimal in transgenic and wildtype control groups in which Fc ϵ RI was loaded with unlabeled IgE (columns 1–6). In contrast, anti-FITC antibody-mediated crosslinking of FITC-labeled IgE loaded onto Fc ϵ RI did stimulate degranulation of mast cells (columns 7–12). As expected, this artificial FITC-dependent crosslinking of Fc ϵ RI similarly activated mast cells derived from Fc γ RIIb transgenic and wildtype mice (columns 7–8). In the

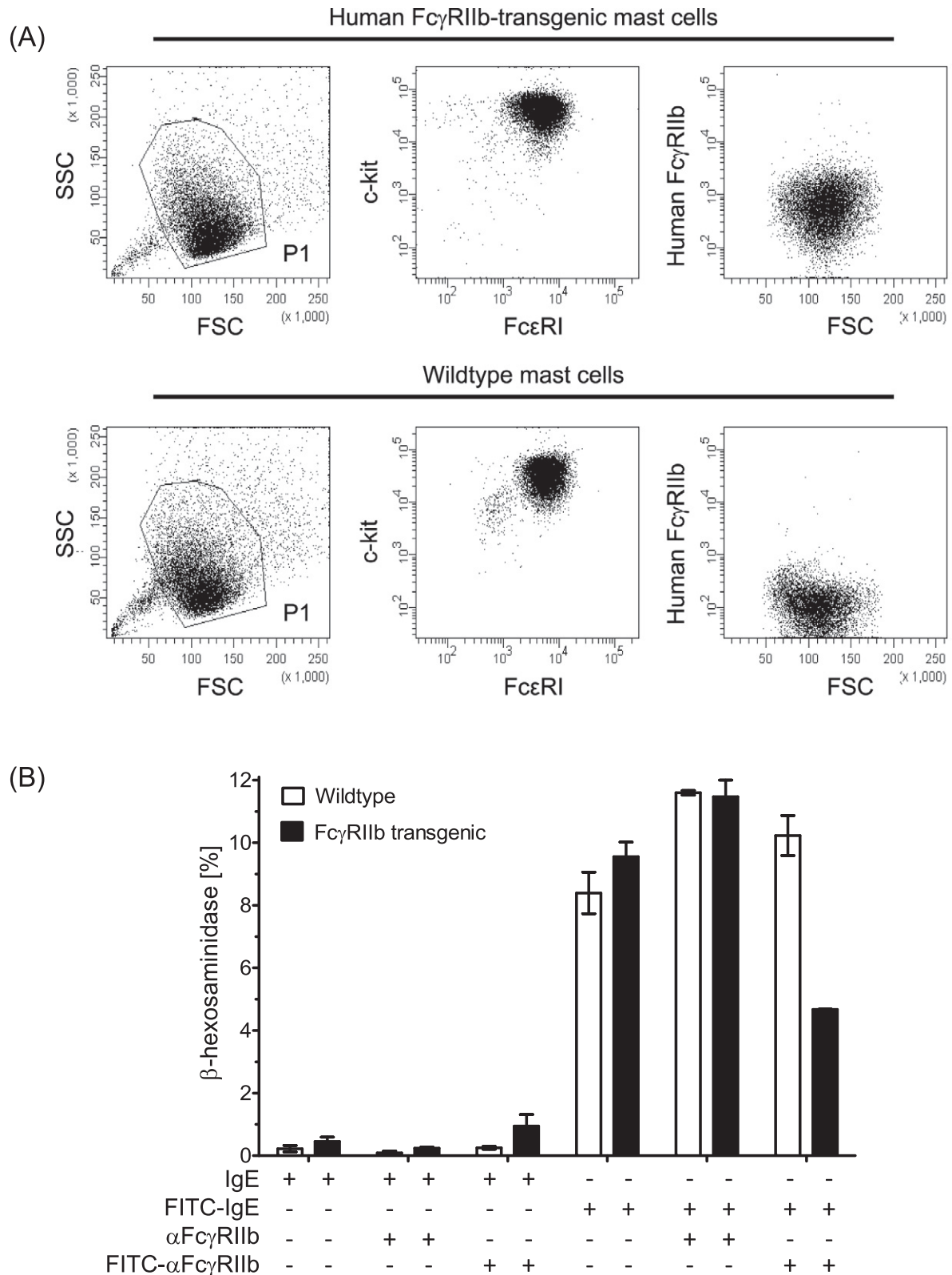


Fig. 1. Expression and function of human FcγRIIb on mast cells derived from FcγRIIb-transgenic mice. (A) Bone marrow cells from wildtype C57BL/6 mice and mice overexpressing human FcγRIIb were cultured in the presence of murine IL-3 for 4 weeks. Dot plots were gated for live cells based on forward and side scatter (left panels), and mast cell differentiation was assessed by flow cytometry using antibodies against c-kit and FcεRI (center panels). Expression of human FcγRIIb was assessed by staining with a labeled FcγRIIb-specific antibody (right panels). Results shown are representative of at least three independent experiments. (B) Mast cells derived from FcγRIIb-transgenic or wildtype mice were sensitized overnight with 1 μg/ml of unlabeled or FITC-labeled IgE in the presence or absence of 1 μg/ml of unlabeled or FITC-labeled anti-FcγRIIb antibody. After sensitization, mast cells were washed, resuspended in Tyrode's buffer, stimulated with 1 μg/ml of anti-FITC antibody, and β-hexosaminidase release was measured in the supernatants. Results are expressed as the percentage of β-hexosaminidase activity relative to supernatants from lysed cells (defined as maximal release). Results shown are representative of at least two independent experiments. In (B), points represent duplicates; error bars represent mean ± SEM.

presence of a selective anti-human Fc γ RIIb antibody that crosslinks Fc γ RIIb in the absence of Fc ϵ RI binding, degranulation of transgenic or wildtype mast cells was not suppressed (columns 9–10). However, coengagement of Fc γ RIIb and Fc ϵ RI via FITC crosslinking did suppress degranulation of mast cells derived from human Fc γ RIIb transgenic but not from wildtype mice (columns 11–12). These results in an artificial allergen-independent system show that human Fc γ RIIb can inhibit mast cell degranulation only when coengaged with mouse Fc ϵ RI, suggesting that coengagement of inhibitory and stimulatory receptors, rather than simple crosslinking of Fc γ RIIb alone, may also be required to suppress allergen-IgE immune complex-mediated degranulation. These findings also demonstrate that mast cells derived from mice transgenic for human Fc γ RIIb are phenotypically and functionally similar to those derived from wildtype mice, suggesting that this is an appropriate model to test the effects of tandem Fc ϵ –Fc γ biologics in vitro and ultimately in mouse models of allergy.

3.2. Structure and characterization of mouse Fc ϵ –human Fc γ tandem Fc biologics

In previous efforts to therapeutically target mast cell Fc ϵ RI and Fc γ RIIb with Fc ϵ –Fc γ fusions, the low (above 1 μ M Kd) affinity of human IgG1 Fc for Fc γ RIIb coupled with the high ($\sim$ 0.1 nM Kd) affinity of human IgE Fc for Fc ϵ RI [51] resulted in bispecific molecules with dramatically different binding affinities for these two cell surface receptors targeted for coengagement [38,42]. To more efficiently target this inhibitory pathway, we reasoned that a similar biologic that improved on the >10,000-fold lower binding affinity of the Fc γ –Fc γ RIIb interaction would augment recruitment of Fc γ RIIb to Fc ϵ RI and more potently suppress mast cell activation.

We have previously shown that two amino acid substitutions in the Fc domain of human IgG1 increase its affinity for human Fc γ RIIb by several hundredfold and, when included in an anti-CD19 antibody, strongly inhibit B cell activation in vitro and in vivo [45]. We therefore incorporated this Fc γ RIIb affinity-enhanced Fc domain into an IgE Fc ϵ –IgG Fc γ fusion biologic (“tandem Fc”) designed to coengage mast cell and basophil Fc ϵ RI and Fc γ RIIb with high affinity, with the goal of suppressing allergen-IgE-mediated crosslinking of Fc ϵ RI and subsequent cell activation and degranulation. As shown in Table 1 and Fig. 2A, we constructed several tandem Fc biologics possessing Fc γ domains binding with native, high, or no affinity to human Fc γ RIIb (XENP8138, XENP8142 and XENP8146, respectively). These tandem Fc proteins contain identical mouse Fc ϵ domains and differ only in the Fc γ substitutions required to either enhance (S267E/L328F) [43] or eliminate (G236R/L328R) [44] binding to human Fc γ RIIb (see Table 1). Binding of these three tandem Fc biologics to mouse Fc ϵ RI on the surface of wildtype murine mast cells (lacking human Fc γ RIIb) was determined based on their ability to compete with mouse IgE for Fc ϵ RI binding (Table 1 and Fig. 2B). Notably, the tandem Fc biologics bound with dramatically different affinities to human Fc γ RIIb, as confirmed by surface plasmon resonance analysis (Fig. 2C). In particular, the Fc γ RIIb-optimized biologic XENP8142 bound with 100-fold higher affinity relative to the native IgG1 biologic XENP8138, with the Fc γ receptor knockout control XENP8146 showing essentially no detectable binding to Fc γ RIIb (Table 1). The binding data for these three Fc domains in the context of a tandem Fc ϵ –Fc γ biologic are consistent with published results for identical Fc domains in the context of a full-length antibody [43,52].

3.3. Increased inhibition of mast cell degranulation by the tandem Fc ϵ –Fc γ biologic with enhanced Fc γ RIIb binding

We first tested the ability of tandem Fc biologics to inhibit mast cell degranulation. Human Fc γ RIIb-transgenic mast cells were

sensitized overnight with mouse anti-OVA IgE in the presence or absence of increasing amounts of tandem Fc biologics with enhanced (XENP8142) or reduced (XENP8146) affinity for Fc γ RIIb; degranulation was then induced by addition of OVA. As shown in Fig. 3A, both XENP8142 and XENP8146 reduced OVA-induced mast cell degranulation in transgenic mast cells in a dose-dependent manner, but the Fc γ RIIb-optimized tandem Fc had $\sim$ 30-fold greater potency than the control tandem Fc lacking binding to Fc γ RIIb, as determined by a sigmoidal dose–response fit of the data. We repeated this study using wildtype mast cells as a control lacking the human Fc γ RIIb receptor target, and also included XENP8138, the tandem Fc with native IgG1 affinity for human Fc receptors. Unlike in transgenic mast cells, degranulation was comparable among all biologics (Fig. 3B), suggesting that the reduced but similar activity was mediated through their shared IgE Fc domains competing for Fc ϵ RI binding with the crosslinked OVA-IgE used to stimulate degranulation.

Having determined that the Fc γ RIIb-optimized XENP8142 more potently inhibited degranulation of transgenic mast cells relative to the XENP8146 control lacking binding to Fc γ RIIb, we next tested how both compared to XENP8138, the tandem Fc possessing a native IgG1 Fc domain. As shown in Fig. 3C, while XENP8142 again strongly inhibited degranulation by $\sim$ 20-fold relative to XENP8146, the inhibitory effect of XENP8138 was similar to that of the receptor knockout control. This demonstrates that mast cell degranulation is most potently suppressed when the tandem Fc has high affinity for Fc γ RIIb. In particular, the low native affinity of IgG1 Fc, in the absence of high avidity generated by allergen-IgE complex formation and crosslinking, did not suppress degranulation more potently than that observed for IgE competition by Fc ϵ RI binding alone.

It has been reported that initiation of productive inhibitory signaling requires coupling of Fc γ RIIb with stimulatory receptors that signal through Src family kinase-dependent pathways [12,26–29,44,53]; in other words, Fc γ RIIb engagement in the absence of an activating receptor target is not effective. For example, high-affinity coengagement of Fc γ RIIb with CD19 (associated with activated lyn), rather than simultaneous but independent engagement of both receptors, was shown to be necessary for the inhibitory effect of the Fc γ RIIb-optimized anti-CD19 antibody XmA5871 on human B cells [43,45]. We inferred that a similar requirement for Fc γ RIIb coupling to an activating receptor exists in mast cells. If so, simultaneous but independent engagement of Fc ϵ RI and Fc γ RIIb using OVA plus anti-OVA IgE as the immune complex, plus an irrelevant Fc γ RIIb-enhanced IgG antibody, should not suppress mast cell degranulation. To test the hypothesis that coengagement is a mechanistic requirement for activity, we compared degranulation in transgenic mast cells sensitized with OVA plus anti-OVA IgE in the presence of either XENP8142 or XENP8146 alone, or XENP8146 combined with XmA5871, which contains the same Fc γ RIIb-enhanced Fc domain as XENP8142. As shown in Fig. 3C, the Fc γ RIIb-enhanced Fc domain in an unrelated antibody, even when mixed with XENP8146 to independently engage Fc ϵ RI, did not suppress degranulation more than XENP8146 alone. This demonstrates that coengagement of Fc ϵ RI and Fc γ RIIb by a single biologic containing both Fc ϵ and Fc γ , rather than simultaneous engagement of these receptors by distinct biologics, is required to suppress the stimulatory Fc ϵ RI signaling pathway involved in mast cell degranulation.

3.4. Phosphorylation of the ITIM of Fc γ RIIb and inhibition of calcium mobilization in tandem Fc-treated mast cells

The earliest event in the Fc γ RIIb signaling cascade is the Src family kinase-mediated phosphorylation of tyrosine residue 292 in the ITIM domain of the cytoplasmic tail of Fc γ RIIb. To explore

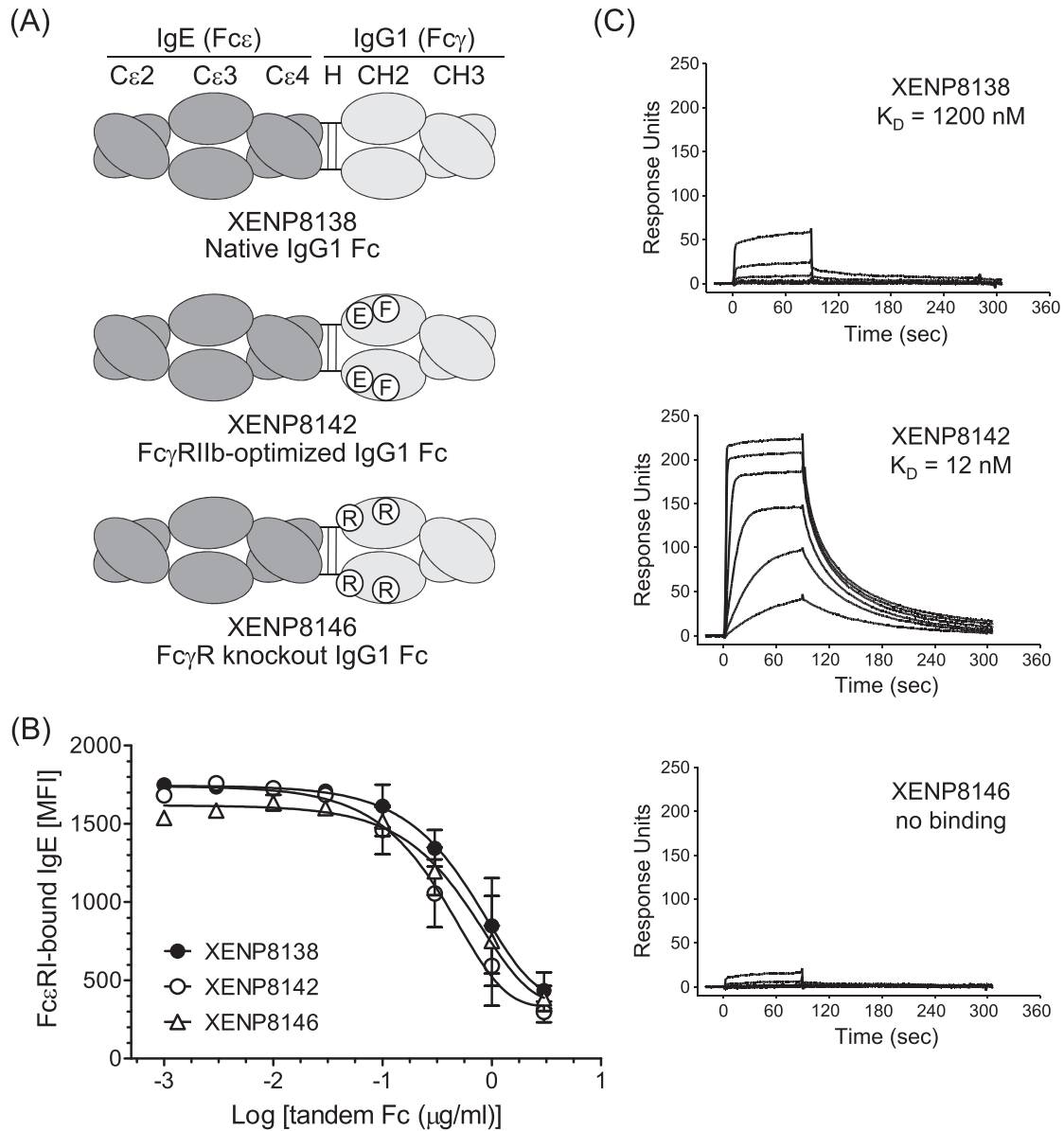


Fig. 2. Characterization of mouse Fcε-human Fcγ tandem molecules. (A) Structure of the tandem Fc molecules, containing the Cε2, Cε3, and Cε4 domains of human IgE Fc coupled to the hinge (H), CH2, and CH3 domains of human IgG1. (B) Competition of native (XENP8138), FcγRIIb-optimized (XENP8142), and Fcγ receptor knockout (XENP8146) forms of tandem Fc biologics with free human IgE for binding to FcεRI on wildtype mast cells. Wildtype mast cells were incubated overnight with 1 μg/ml of IgE and increasing amounts of tandem Fc biologics. After incubation, cells were washed and FcεRI-bound IgE was detected by flow cytometry using a labeled anti-IgE antibody. Dose responses were fitted using non-linear regression of log-transformed data. Results shown are representative of two independent experiments. In (B), points represent duplicates; error bars represent mean ± SEM. All tandem Fcs share identical IgE Fc domains and compete with IgE Fc for binding to FcεRI with similar effectiveness. (C) Binding affinity of tandem Fc biologics for FcεRI and FcγRIIb, measured by surface plasmon resonance.

the effects of tandem Fc biologics on early FcγRIIb signaling events, we assessed the phosphorylation state of Y292 in wildtype and human FcγRIIb-transgenic mast cells treated with different biologics. Mast cells were sensitized overnight with anti-OVA IgE in the presence of XENP8138, XENP8142, or XENP8146 tandem Fcs, or with XENP8146 plus XmAb5871 as a control for simultaneous but independent high-affinity engagement of FcεRI and FcγRIIb. Phosphorylation of Y292 with and without mast cell OVA stimulation was assessed by western blot. As shown in Fig. 4A, mast cells treated with the FcγRIIb-optimized tandem Fc (XENP8142) showed a robust level of Y292 phosphorylation with or without OVA stimulation (lanes 3 and 4). Incubation of mast cells with the tandem Fc lacking affinity for Fcγ receptors (XENP8146) did not stimulate Y292 phosphorylation above the no-allergen control (lanes 5 and

6). Incubation of mast cells with the tandem Fc containing a native IgG1 Fc (XENP8138) also did not induce Y292 phosphorylation in the absence of OVA crosslinking (lane 1), but did show minimal Y292 phosphorylation upon OVA stimulation (lane 2). Finally, simultaneous engagement of anti-OVA IgE-sensitized FcεRI (plus OVA) and FcγRIIb (using the FcγRIIb-optimized antibody control XmAb5871) induced modest Y292 phosphorylation (lanes 7 and 8). Thus, the FcγRIIb-optimized tandem Fcε-Fcγ biologic induces the strongest phosphorylation of the FcγRIIb ITIM, and also inhibits OVA-IgE immune complex-induced degranulation, suggesting that there is a causative link between high-affinity binding of IgG1 to FcγRIIb, ITIM signaling, and subsequent mast cell deactivation.

To further explore the inhibitory effects of FcγRIIb phosphorylation on mast cell signaling, we assessed OVA-induced calcium

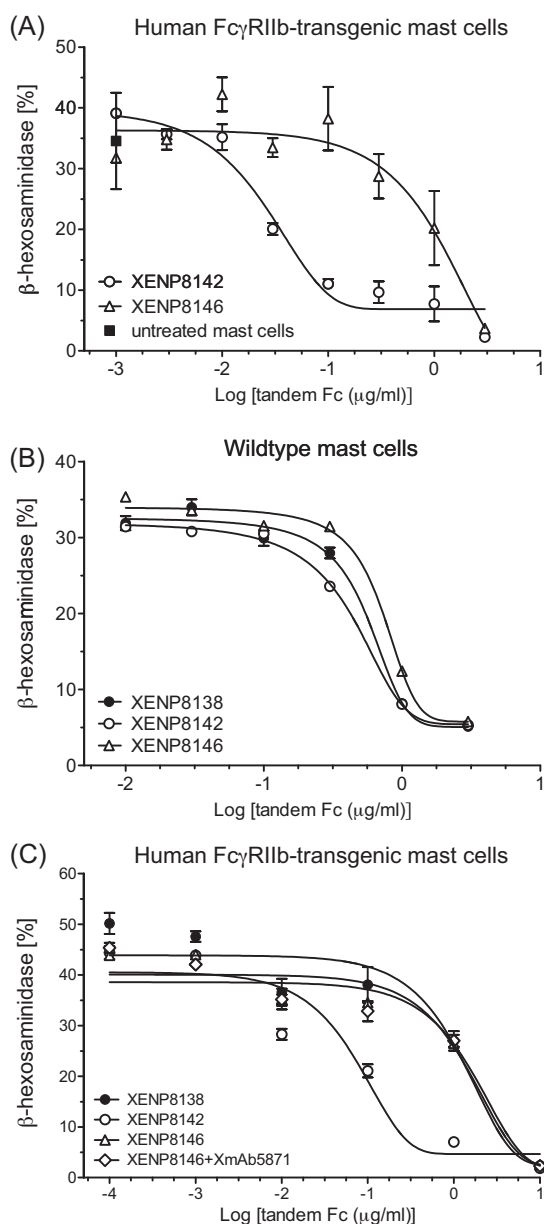


Fig. 3. Suppression of mast cell degranulation by tandem Fc biologics as measured by β -hexosaminidase release. Mast cells from human Fc γ RIIb-transgenic mice (A and C) or wildtype mice (B) were sensitized overnight with 1 μ g/ml of anti-OVA IgE in the presence or absence of 1 μ g/ml of the indicated biologics. Cells were washed and resuspended in Tyrode's buffer and β -hexosaminidase release was measured in the supernatants upon stimulation with 1 μ g/ml OVA. Results are expressed as percentage of β -hexosaminidase relative to supernatants from lysed cells (maximal release). Results shown are representative of at least two independent experiments. In all panels, most points represent triplicates (some duplicates); error bars represent mean $\pm$ SEM.

mobilization as a known activation event downstream of Fc ϵ RI engagement; it has also been shown that mast cell degranulation requires mobilization of calcium from both internal and external stores [54]. We quantified calcium mobilization in both wildtype and transgenic mast cells sensitized overnight with anti-OVA IgE in the presence or absence of Fc γ RIIb-optimized and control tandem Fc biologics, and stimulated with OVA for 2 min. As shown in Fig. 4B, the Fc γ RIIb-optimized XENP8142 suppressed calcium flux in transgenic (top panel) but not wildtype (bottom panel) mast cells. As additional confirmation that this effect required high-affinity engagement of Fc γ RIIb, transgenic mast cells treated with tandem Fcs containing a native IgG1 Fc domain (XENP8138)

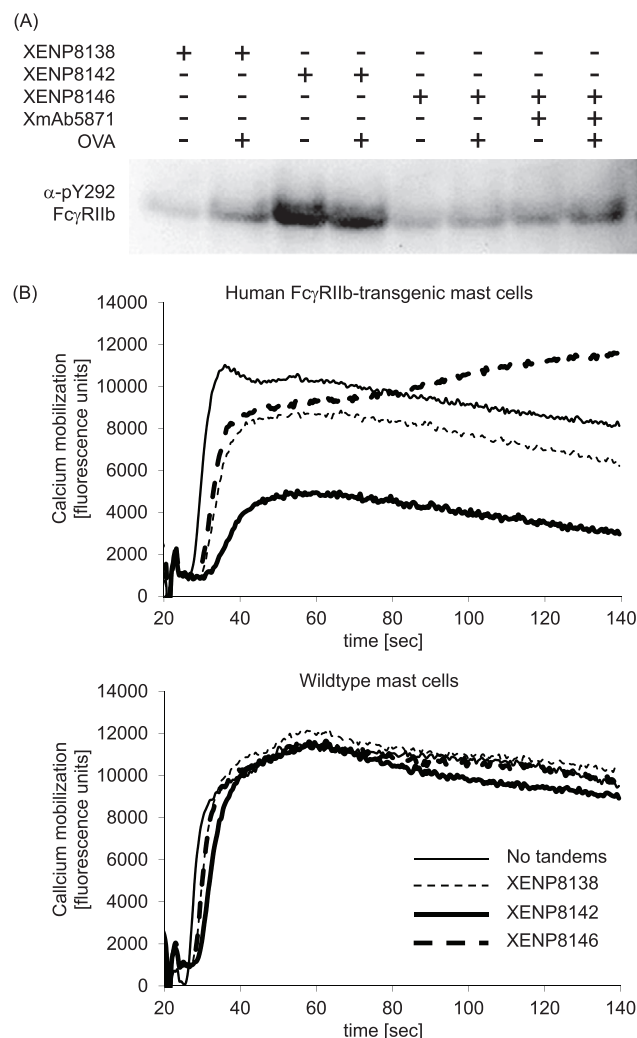


Fig. 4. Phosphorylation of Fc γ RIIb and suppression of Fc ϵ RI-dependent calcium mobilization by tandem Fc biologics. Human Fc γ RIIb transgenic mast cells were sensitized overnight in the presence (+) or absence (-) of 1 μ g/ml anti-OVA IgE and 1 μ g/ml of the indicated biologics. (A) After incubation, cells were stimulated (+) with 1 μ g/ml of OVA or left unstimulated (-). Lysates from 1×10^6 cells were resolved by SDS-PAGE, proteins were transferred to nitrocellulose, and membranes were probed with an anti-pY292 antibody to detect phosphorylation of the ITIM domain of Fc γ RIIb. Results shown are representative of two independent experiments. (B) Calcium mobilization traces were obtained by stimulating mast cells from transgenic (top) or wildtype (bottom) mice with OVA for 2 min after overnight sensitization with anti-OVA IgE in the presence of the indicated biologics, as above. Results shown are representative of two independent experiments.

or lacking binding to Fc γ receptors (XENP8146) mobilized calcium similar to untreated control. These results concur with the degranulation and phosphorylation results in demonstrating that allergen-mediated mast cell activation can be inhibited through high-affinity coengagement of Fc γ RIIb and Fc ϵ RI by a tandem Fc biologic.

4. Discussion

With the growing understanding of the importance of mast cells and basophils in normal physiology and pathophysiology, several therapeutic approaches for suppressing the functions of these cells have been developed. Of most relevance to this report, the established role of Fc γ RIIb as the sole inhibitory Fc receptor for IgG and IgE antibodies has motivated other groups to develop related dual-targeting approaches including anti-Fc ϵ RI-anti-Fc γ RIIb [37]

or anti-Fc γ RII bispecific antibodies [14], IgG1 Fc fusions with anti-IgE ankyrin scaffold proteins [39], fusion proteins containing native IgE Fc combined with native [55,56] or mutated [38] IgG1 Fc domains, and the cat allergen Fel d 1 fused to native IgG1 Fc [40,41]. Our tandem Fc strategy extends such approaches by incorporating in a single biologic the following features: (1) the mechanism exploits actual Fc ϵ –Fc ϵ RI and Fc γ –Fc γ RIIb interactions rather than antibodies targeting these receptors, potentially resulting in more relevant physiological signaling coupled with additional potency due to Fc ϵ competition with IgE–allergen immune complex; (2) the IgG1 domain of the tandem Fc has ~100-fold higher affinity for Fc γ RIIb relative to native IgG1, greatly increasing its ability to recruit Fc γ RIIb, and (3) unlike single-allergen Fc fusion proteins, the tandem Fc should effectively target all mast cells and basophils, and does not entail administering a protein containing a known allergenic domain to patients reactive to this allergen. Of relevance to this third point, a clinical trial of the Fel d 1 fusion protein GFD in cat-allergic patients was recently terminated [57].

Immune complexes of allergen and IgE are known to interact with Fc γ RIIb to suppress mast cell activation through Fc ϵ RI [12,36,58–62]. Fc γ RIIb binds to the Fc domains of IgE and IgG with similar low affinity [63,64]; therefore, mast cells and basophils can in principle be regulated by both allergen–IgG and allergen–IgE immune complexes. Our results demonstrate that the inhibitory signaling driven by multivalent high-avidity coengagement of Fc ϵ RI and Fc γ RIIb can be amplified using a recombinant biologic engineered with high affinity to Fc γ RIIb. The tandem Fc biologic replicates several features of immune complex-mediated mast cell suppression: (1) it stimulates Fc γ RIIb signaling, as measured by phosphorylation of the Fc γ RIIb ITIM; (2) it suppresses calcium flux induced by Fc ϵ RI activation; and (3) it suppresses mast cell degranulation as measured by β -hexosaminidase release.

In mast cells and basophils as well as in other immune cells, ITAM receptor-mediated activation can be negatively regulated by coengagement with the ITIM-bearing Fc γ RIIb, with ITIM phosphorylation of tyrosine 292 being the initial event in the inhibitory signaling cascade [12,28,65]. The inability of the tandem Fcs containing a native Fc γ or Fc γ R knockout Fc to induce this phosphorylation suggests that their primary mechanism of action is via Fc ϵ competition with IgE for binding to Fc ϵ RI, and supports the hypothesis that amplified affinity for Fc γ RIIb is required to activate its signaling upon monovalent binding of IgG Fc. This hypothesis is further supported by our results showing that the native Fc γ and Fc γ R knockout tandem Fcs do not inhibit calcium mobilization upon allergen-induced Fc ϵ RI crosslinking. Our inclusion of these two controls for the affinity-enhanced biologic as well as controls for receptor signaling (including mast cells derived from wildtype vs. human Fc γ RIIb-transgenic mice) demonstrates that the inhibitory effects of Fc γ RIIb engagement are 20–30-fold greater than effects due to competition for Fc ϵ RI by the Fc ϵ domain shared among all the tandem Fc biologics. These data, coupled with the degranulation results, suggest that all tandem Fc biologics containing Fc ϵ can suppress degranulation through competition with OVA–IgE immune complex, but that the Fc γ RIIb affinity-enhanced tandem Fc additionally and primarily acts through induction of inhibitory signaling mediated by Fc γ .

The differences between the expression and function of human and murine Fc γ receptors have been well described [66,67]. Murine mast cells and basophils express Fc γ RIIb, and its crosslinking with Fc ϵ RI inhibits mast cell activation in wildtype mice [68,69]. Fc γ RIIb regulation of mast cells has also been implicated beyond its recognized role in suppression of allergic disease; for example, mice lacking mast cell Fc γ RIIb develop a more rapid and severe pathology in experimental autoimmune encephalomyelitis (EAE), suggesting that mast cells and Fc γ RIIb are potential therapeutic targets in human demyelinating diseases [70,71]. However, a

confounding issue in translating the tandem Fc approach into a human drug is the lack of consensus that human mast cells express Fc γ RIIb. Most published results describe expression in various human cell lines, mainly of basophil origin [72] and in human progenitor-derived mast cells [33], rather than in freshly isolated tissue mast cells. While functional data suggest that Fc γ RIIb is expressed on mast cells in human lung tissue [56], mast cells isolated from human skin after several weeks of culture were reported to express Fc γ RIIa but not Fc γ RIIb [33]. In human cell lines which express Fc ϵ RI and Fc γ RIIb either endogenously or by transfection, coengagement of these receptors reduces mast cell activation [73]; similar results were obtained using progenitor cell-derived human mast cells [55]. To overcome issues related to the source, derivation, purity, and authenticity of human mast cells, in our study we used a transgenic mouse model in which human Fc γ RIIb is expressed on a mouse Fc γ RIIb knockout background. In vitro, mast cells generated from bone marrow of these mice expressed a high level of human Fc γ RIIb but otherwise were phenotypically and functionally indistinguishable from mast cells generated from wildtype mice. These human Fc γ RIIb transgenic mice allow in vitro studies using mast cells with a stable, reproducible, and easily detectable level of human Fc γ RIIb, enabling a thorough evaluation of the inhibitory potential of our mouse Fc ϵ –human Fc γ tandem biologics. In addition, these transgenic mice will facilitate future in vivo studies in which the inhibitory potential of tandem Fcs can be assessed in models of allergy and asthma. We also suggest that the use of tandem Fc biologics in the transgenic mouse will help elucidate the role of mast cells and basophils in other models of autoimmune and inflammatory diseases such as multiple sclerosis and rheumatoid arthritis. Given that the key Fc γ RII-enhanced IgG1 Fc domain is already human, such therapeutic models should also facilitate the translation to clinical assessment through replacement of the mouse IgE Fc with its human counterpart.

In conclusion, our results demonstrate that mast cells can be inhibited through the action of an Fc ϵ –Fc γ fusion protein engineered to engage human Fc γ RIIb with high affinity. This study suggests that an analogous fully human Fc ϵ –Fc γ tandem Fc biologic has potential as a potent and selective inhibitor of cellular activation and degranulation, and thus represents a promising approach in treating mast cell and basophil-mediated pathologies.

Competing interests

All authors are employees of Xencor, Inc., and also hold stock options in Xencor. The authors have no competing interests to declare.

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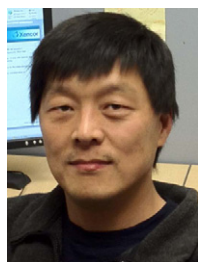
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