A single glycan on IgE is indispensable for initiation of anaphylaxis

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.1084/jem.20142182

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:23474065

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
A single glycan on IgE is indispensable for initiation of anaphylaxis

Kai-Ting C. Shade,1 Barbara Platzer,2,3 Nathaniel Washburn,4 Vinidhra Mani,1 Yannic C. Bartsch,1 Michelle Conroy,1 Jose D. Pagan,1 Carlos Bosques,1 Thorsten R. Mempel,1 Edda Fiebiger,2,3 and Robert M. Anthony1

Immunoglobulin e (IgE) antibodies are the primary mediators of allergic diseases, which affect more than 1 in 10 individuals worldwide. IgE specific for innocuous environmental antigens, or allergens, binds and sensitizes tissue–resident mast cells expressing the high-affinity IgE receptor, FcεRI. Subsequent allergen exposure cross-links mast cell–bound IgE, resulting in the release of inflammatory mediators and initiation of the allergic cascade. It is well established that precise glycosylation patterns exert profound effects on the biological activity of IgG. However, the contribution of glycosylation to IgE biology is less clear. Here, we demonstrate an absolute requirement for IgE glycosylation in allergic reactions. The obligatory glycan was mapped to a single N-linked oligomannose structure in the constant domain 3 (Ce3) of IgE, at asparagine–394 (N394) in human IgE and N384 in mouse. Genetic disruption of the site or enzymatic removal of the oligomannose glycan altered IgE secondary structure and abrogated IgE binding to FcεRI, rendering IgE incapable of eliciting mast cell degranulation, thereby preventing anaphylaxis. These results underscore an unappreciated and essential requirement of glycosylation in IgE biology.

Abbreviations used: CD, circular dichroism; DNP, dinitrophenol; EndoF1, endoglycosidase F1; mIgE, polyclonal mouse IgE; peptide–cutaneous anaphylaxis; PNG, asparagine–glycosidase F; poly–N–glycosidase F; TNP, trinitrophenol.

Copyright © 2015 Shade et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/.

1Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129
2Division of Gastroenterology, Hepatology, and Nutrition, Boston Children’s Hospital and 3Department of Pediatrics, Harvard Medical School, Boston, MA 02115
4Momenta Pharmaceuticals, Cambridge, MA 02142
FcεRI binding and effector functions (Nettleton and Kochan, 1995; Sayers et al., 1998; Bjoerklund et al., 1999; 2000; Hunt et al., 2005). However, these findings have been contradicted (Basu et al., 1993; Young et al., 1995), supported by studies using a functional aglycosylated IgE derived from Escherichia coli (Helm et al., 1988; Henry et al., 2000). Therefore, we sought to determine whether glycosylation was required for the in vivo activity of IgE.

We conducted a systematic analysis of all glycosylation sites on mouse and human IgE, which revealed a single glycan in the IgE Ce3 domain to be essential for triggering anaphylaxis. This site was occupied almost exclusively by oligomannose glycans, whereas complex antennary glycans were found at the other sites throughout mouse and human IgE. Selective enzymatic removal of the oligomannose glycan altered secondary structure of IgE, prevented binding to FcεRI on mast cells, and importantly, attenuated anaphylaxis in vivo.

Together, the findings herein identify the IgE oligomannose glycan essential for in vivo activity and structural integrity of this Ig class.
RESULTS AND DISCUSSION

Enzymatic deglycosylation attenuates IgE

To generate polyclonal mouse IgE (poly-mIgE), we immunized mice with OVA or extract from the common food allergen peanuts in alum. The mice were bled and IgG depleted from the serum. All N-linked glycans were removed from the poly-mIgE by treatment with the endoglycosidase peptide-N-glycosidase F (PNG). This treatment did not reduce recognition of peanuts or OVA by poly-mIgE, as determined by ELISA (unpublished data). Next, we injected poly-mIgE intradermally into the ears of mice in a model of passive cutaneous anaphylaxis (PCA), highly dependent on the interactions between IgE, FceRI \( + \) mast cells, and allergens (Dombrowicz et al., 1993), and the next day intravenously challenged with appropriate allergens and Evan’s blue dye. Poly-mIgE elicited robust anaphylaxis to peanuts or OVA, as measured by vascular leakage of the blue dye. However, after treatment with PNG, poly-mIgE induced significantly decreased anaphylaxis to either allergen (Fig. 1 a). Next, we treated three monoclonal mIgEs specific for model allergens OVA, dinitrophenol (DNP), or trinitrophenol (TNP) with PNG. Enzymatic deglycosylation and antigen binding were verified by lectin blotting and ELISA assays, respectively (unpublished data). Although mIgE specific for OVA, DNP, or TNP triggered strong allergen-specific anaphylactic responses in vivo, PNG treatment significantly attenuated vascular leakage (Fig. 1 b).

Allergic reactions are highly dependent on IgE and FceRI interactions (Dombrowicz et al., 1993; Gould et al., 2003). To determine the contribution of glycosylation to interactions with mouse FceRI (mFceRI), mouse BM-derived mast cells (mBMMCs) were sensitized in vitro with native or deglycosylated αOVA-mIgE overnight. When ligand–receptor interactions were analyzed by flow cytometry using Alexa Fluor 647–OVA (A647-OVA), we found that although αOVA-mIgE bound to the mast cells, PNG–αOVA-mIgE did not (Fig. 1 c). Indeed, WT-mIgE specific for OVA, DNP, or TNP, but not PNG–mIgE, was able to activate mBMMCs upon antigen stimulation (not depicted). Together, these results demonstrate that mIgE glycosylation is necessary for mast cell binding in vitro and eliciting anaphylaxis to multiple antigens in vivo.

We next generated and enzymatically deglycosylated OVA-specific human IgE (αOVA-hIgE, PNG–αOVA-hIgE) and sensitized human LAD2 mast cells in vitro with these preparations. OVA stimulation resulted in dose–dependent degranulation of αOVA-hIgE–sensitized mast cells, as assessed by β-hexosaminidase release. Consistent with our results, PNG–αOVA-hIgE was incapable of instigating degranulation upon OVA stimulation (Fig. 1 d). In parallel, we administered αOVA-hIgE or PNG–αOVA-hIgE intradermally to transgenic mice expressing human FceRI while lacking mFceRI (hFceRI\(^{-/-}\); Dombrowicz et al., 1996). These transgenic mice have a broad cellular distribution of hFceRI expression, whereas mFceRI is restricted to mast cells and basophils in the WT mice. hIgE is unable to elicit PCA in mFceRI\(^{-/-}\) mice, which served as an injection control group. αOVA-hIgE triggered robust vascular leakage in the ears of hFceRI\(^{-/-}\)/mFceRI\(^{-/-}\) mice upon OVA challenge (Fig. 1 e). The response was significantly diminished in PNG–αOVA-hIgE–treated ears (Fig. 1 e), confirming N-linked glycosylation is also essential for the in vivo activity of human IgE.

To determine whether hIgE glycosylation was important for hFceRI binding, HeLa cells engineered to express hFceRI (hFceRI\(^{-/-}\)-HeLa) were sensitized with αOVA-hIgE, incubated with A647-OVA, and analyzed by flow cytometry. Although αOVA-hIgE bound to hFceRI \( + \)-HeLa cells was detected by A647-OVA, PNG–αOVA-hIgE did not (Fig. 1 f), despite recognizing OVA by ELISA (not depicted). Together, these results indicate that IgE glycosylation is essential for the initiation of anaphylaxis and FceRI interactions.

Mapping glycosylation requirements of IgE

We generated a panel of αOVA-mIgE mutants selectively lacking all glycosylation sites on each constant domain (Ce1–4) by mutating asparagine (N) to glutamine (Q). After confirming that all domain-specific mutants recognized OVA similarly (not depicted), these antibodies were tested in vivo by PCA. Although Ce2- or Ce4-αOVA-mIgE domain mutants promoted robust vascular leakage after OVA challenge similar to WT (Fig. 2 a), Ce1-αOVA-mIgE glycosylation mutants exhibited slightly enhanced vascular leakage. In contrast, anaphylaxis elicited by the Ce3-αOVA-mIgE mutant was significantly attenuated. WT-, Ce1-, Ce2-, and Ce4-αOVA-mIgE activated mBMMCs upon OVA stimulation, but Ce3-αOVA-mIgE did not (not depicted). αOVA-hIgE domain–specific glycosylation mutants were also generated and tested for mast cell degranulating activity (Fig. 2 b). WT- and Ce2-αOVA-hIgE domain mutants triggered robust degranulation upon OVA stimulation. Although degranulation was slightly reduced in mast cells sensitized with Ce1-αOVA-hIgE, mutation of Ce3 glycosylation sites completely abolished OVA-specific degranulation. Importantly, Ce1-αOVA-hIgE mutants bound to FceRI\(^{-/-}\)-HeLa cells, as determined by A647-OVA flow cytometry, but Ce3-αOVA-hIgE did not (Fig. 2 c). Together, these results indicate that Ce1 glycosylation plays a minor role in modulating IgE functions, perhaps by controlling Fab arm flexibility (Arnold et al., 2007), but is not involved in FceRI binding. More importantly, Ce3 glycosylation is required for both mouse and human IgE to bind FceRI and initiate anaphylaxis.

An obligate IgE Ce3 glycan

To dissect the importance of the two N-linked glycosylation sites in Ce3 of mIgE, individual glycosylation site mutants were generated by conversion of N361 or N384 to Q. Both WT- and N361Q-αOVA-mIgE elicited a robust anaphylactic response in vivo, but N384Q-αOVA-mIgE did not (Fig. 3 a). Moreover, a reciprocal mutant, in which all N-linked glycosylation sites were disrupted except N384 (N384Q-only-αOVA-mIgE), promoted a strong PCA upon OVA administration (Fig. 3 a). To confirm the importance of N384 glycosylation for mIgE function, the glycosylation consensus sequence was
disrupted by mutation of the third-position amino acid (T386A). Indeed, T386A-αOVA-mlgE could not initiate anaphylaxis (Fig. 3 a). Neither N384Q- nor T386A-αOVA-mlgE was able to activate mBMMCs in vitro (not depicted). Collectively, our data reveal glycosylation specifically at N384 in the Cε3 domain of mlgE is key for initiation of anaphylaxis.

We hypothesized that N384 was required for mlgE binding to FceRI on mast cells. To test this in vivo, equal amounts of A488- and A568-labeled WT-αOVA-mlgE (A488-WT/ A568-WT) or A488-WT-αOVA-mlgE and A568-N384Q-αOVA-mlgE (A488-WT/A568-N384Q) were injected intradermally into mouse ears and assessed by flow cytometry the next day. Dermal mast cells (CD45+ c-Kit+ CD11b−) from WT mice bound WT-αOVA-mlgE regardless of fluorophore conjugate but preferentially bound WT- over N384Q-αOVA-mlgE when both were administered (Fig. 3 b and Fig. S1). Dermal mast cells from mFcεRI−/− mice showed minimal binding of WT-αOVA-mlgE, confirming the requirement

Figure 2. Glycosylation of IgE Cε3 is required for anaphylaxis and FceRI binding. (a) Quantified OVA-specific PCA by PBS, WT, or αOVA-mlgE domain mutants (n = 8; 3 independent experiments). Bar, 1 cm. (b) β-Hexosaminidase activity after OVA-induced degranulation in LAD2 cells sensitized with WT or αOVA-mlgE glycomutants (n = 3; 3 independent experiments). (c) WT-αOVA-hlgE or Cε1- and Cε3-αOVA-hlgE glycosylation mutant binding to hFcεRI−/−HeLa cells assessed by A647-OVA and α-hFcεRI-PE FACS (n = 2; 2 independent experiments). Mean and SEM are plotted; ****, P < 0.0001; ***, P < 0.001; *, P < 0.05; ns, not significant.
Figure 3. A single N-linked glycosylation site is indispensable for anaphylaxis and hFcεRI binding. (a) Quantified OVA-induced PCA with PBS, WT, or αOVA-mlgE Cε3 domain glycomutants (n = 8; 3 independent experiments). (b) Scatter plots of CD45+c-Kit+CD11b− mast cells recovered from WT or mFcεRI−/− ears injected with A488-WT and A568-WT αOVA-mlgE or A488-WT and A568-N384Q αOVA-mlgE the previous day. Percentage of A488 and A568 double-positive mast cells are shown (n = 2; 2 independent experiments). (c) Histograms and MFI of WT or N384Q αOVA-mlgE binding to
of FceRI engagement in vivo (Fig. 3 b and Fig. S1). Furthermore, N384Q-αOVA–mlgE was not bound by mast cells when injected alone, indicating the mutation and not competition with WT-αOVA–mlgE prevented mast cell engagement (not depicted). In parallel, we primed mBMMCs with WT- or N384Q-αOVA–mlgE and detected A647-OVA bound to WT- but not N384Q-αOVA–mlgE–sensitized cells (Fig. 3 c). These results demonstrate that the N384 glycan is crucial for stable mlgE interactions with mFceRII on mast cells in vitro and in vivo.

Three Ce3 N-linked glycosylation sites are occupied in αOVA-hIgE. To determine the contribution of these sites to the initiation of anaphylaxis, individual site mutants were generated and examined for degranulation activity. N371Q- or N383Q-αOVA–hIgE exhibited slightly altered ability to activate mast cells upon OVA stimulation compared with WT. Conversely, mutation of either the first position (N394Q-αOVA–hIgE) or third position (T396A-αOVA–hIgE) of the N394 glycosylation site ablated OVA-mediated degranulation (Fig. 3 d). Furthermore, N394Q-αOVA–hIgE was unable to elicit vascular leakage by PCA in hFceRII+/mFceRII−/− mice (Fig. 3 e). We next examined the role of N394 glycan in hFceRII binding. Flow cytometry of hFceRII+/HeLa cells primed with WT-, N371Q-, N383Q-, N394Q-, or T396A-αOVA–hIgE and treated with A647-OVA revealed that WT-, N371Q-, and N383Q- but not N394Q- or T396A-αOVA–hIgE bound hFceRII (Fig. 3 f). Furthermore, although WT-αOVA–hIgE bound and saturated immobilized hFceRII in vitro, N394Q-αOVA–hIgE did not (Fig. 3 g). These results identify the glycan at N394 and N384 of hIgE and mIgE, respectively, as essential for interacting with FceRIII and mast cells and initiating allergen-specific inflammation.

The IgE oligomannose is essential for in vivo activity
We analyzed site-specific glycosylation throughout the four constant domains (Ce1–4) of recombinant mouse and human αOVA-IgE by glycopeptide mass spectroscopy (Plomp et al., 2014). Eight of nine N-linked glycosylation consensus sequences in αOVA–mlgE were primarily occupied by highly processed complex biantennary glycans (Fig. 4 a and Table S1). Similarly, all but one site on αOVA–hIgE contained predominantly complex antennary structures (Fig. 4 b and Table S2). Interestingly, the glycosylation sites identified as essential for initiating anaphylaxis, N384 of mlgE and N394 of hIgE, were occupied by oligomannose glycans, consistent with previous analyses of hIgE glycosylation (Dorrington and Bennich, 1978; Arnold et al., 2007; Plomp et al., 2014).

Endoglycosidase F1 (EndoF1) selectively cleaves N-linked oligomannose and afucosylated hybrid glycans, leaving complex glycan unaffected, unlike PNG which removes all N-linked glycans. Thus, we treated mlgE specific for OVA, DNP, or TNP with EndoF1 and tested these preparations in vivo by PCA. Selective enzymatic removal of oligomannose glycans significantly attenuated vascular leakage compared with WT-mlgE (Fig. 4 c). Furthermore, EndoF1-αOVA–mlgE did not bind to mFceRII or mast cells in vitro or in vivo, respectively (Fig. 4, d and e; and Fig. S1), nor was it able to activate mBMMCs in vitro (not depicted). These results demonstrate that the oligomannose glycan on mlgE is required for initiating anaphylaxis, interacting with mast cells, and binding mFceRIII.

Next, we treated αOVA–hIgE, biotinylated hIgE, or hIgE recovered from human allergic serum with EndoF1 and sensitized mast cells with these preparations. This treatment abolished mast cell activation after cross-linking by OVA, streptavidin, or anti-human light chain, respectively (Fig. 4 f). EndoF1 treatment also ablated hIgE binding to hFceRII in flow cytometry and saturation binding experiments (Fig. 4, g and h). Importantly, EndoF1 treatment did not induce IgE aggregation, as assessed by size exclusion chromatography, in contrast to a previous study reporting dimerization after PNG treatment (Fig. 4 i; Basu et al., 1993).

A previous study has shown that removal of the single N-linked glycan on IgG Fc results in a conformation change that prevents FcγR binding (Feige et al., 2009). Thus, the contribution of the oligomannose glycan to hIgE secondary structure was examined by circular dichroism (CD; Sondermann et al., 2013). Although the UV CD spectra of WT– and EndoF1–buffer control hIgE overlapped, a shift was observed after EndoF1 treatment (Fig. 4 j). This likely reflects small changes in the overall secondary structure of hIgE upon oligomannose removal, resulting in altered hIgE function.

Glycosylation in IgE biology and allergic disease
Here, we demonstrate that glycosylation of IgE is an absolute requirement for initiation of the allergic cascade. This requirement was mapped to a single, highly conserved N-linked site, occupied by oligomannose glycans. Selective removal of this glycan ablated interactions with FceRIII by altering conformation of IgE. The glycan is likely not involved in glycanch–glycan or glycanch–protein interactions with FceRIII, consistent with structural studies of IgE and FceRIII (Garman et al., 2000). This glycosylation site on IgE corresponds to the single site found on IgG Fcs, which governs IgG Fc–mediated effector functions (Garman et al., 2000). Our findings demonstrate a similar functional requirement for glycosylation of IgE, supporting a close evolutionary relationship shared by these Ig classes (Flajnik, 2002). Furthermore, E. coli–derived IgE required refolding in vitro to gain functionality, consistent with
Figure 4. Removal of the IgE oligomannose glycan abrogates anaphylaxis. (a and b) αOVA–mIgE (a) and αOVA–hIgE (b) schematics with N-linked glycosylation consensus sites are shown. Percentages of glycan structures identified by glycopeptide mass spectroscopy at each site are plotted. Glycans are composed of fucose (red), N-acetylglucosamine (GlcNAc; blue), mannose (green), galactose (yellow circles), N-acetylgalactosamine (GalNAc; yellow squares), and sialic acid (pink); representative of two experiments. (c) PCA quantified after PBS, WT, or EndoF1–mIgE specific for OVA, DNP, or TNP (n = 8; 2 independent
glycosylation playing a role in maintenance of the IgE Cε3 structure (Helm et al., 1988; Henry et al., 2000). Recently developed anti-IgE preventive therapies that neutralize circulating IgE or deplete IgE-producing cells have demonstrated some efficacy. However, clinical indications for these treatments have been limited to allergic asthma, chronic idiopathic urticarial, and rhinitis (Galli and Tsai, 2012; Gauvreau et al., 2014; Saini et al., 2015). Thus, the IgE oligomannose may be a potential therapeutic target for both cell-bound and circulating IgE. Furthermore, it is possible that variations in the glycan composition at the conserved site may explain why not all individuals with allergen-specific IgE suffer from allergies.

MATERIALS AND METHODS

Mice. 5–6 wk-old BALB/c female mice were purchased from The Jackson Laboratory. IgE was purified from BALB/c female mice (Bomkowicz et al., 1996) were maintained in the animal facility at Massachusetts General Hospital (MGH). Mice were all housed in specific pathogen–free conditions according to the National Institutes of Health (NIH), and all animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of MGH.

Peanut extract preparation. Unsalted dry-roasted peanuts (Blanched Jumbo Runner cultivar; Planters) were ground to a smooth paste, followed by washing with 20 vol cold acetone, filtered using Whatman paper, and dried. Protein was extracted by agitating the peanut flour overnight with PBS containing protease inhibitor cocktail without EDTA (Roche). The peanut protein extracts were collected as the supernatant after centrifugation at 24,000 g for 30 min.

PCA. Age- and sex-matched mice were randomized allocating to experimental group. 20 ng monoclonal mIgE specific for OVA, DNP (clone SPE-7; Sigma-Aldrich), or TNP (clone IgE-3, BD) or 5 ng polyclonal IgE specific for OVA or peanut extracts was injected intradermally in the BALB/c mice ears, and the next day mice were intravenously challenged with PBS containing 125 µg OVA (Sigma-Aldrich), peanut extract, DNP-HSA (Sigma-Aldrich), or TNP-BSA (conjugation ratio 13; Biosearch Technologies) and 2% Evans blue dye in PBS, 45 min after challenge, the mice were sacrificed and the ears were excised and minced before incubation in N,N-dimethylformamide (EMD Millipore) at 55°C for 3 h. The degree of blue dye in the ears was quantified by absorbance at 595 or 650 nm. For experiments of hIgE, 250 ng oOVA-hIgE was injected intradermally in hFcεRIα+/mFceRIα−/− or mFceRIα−/− mice, followed by intravenous administration of OVA and Evans blue dye 4 h later.

IgE antibodies. To generate recombinant IgE antibodies, the variable and constant regions of heavy and light chains were individually cloned from OVA-specific Tor hybridoma (provided by H. Oettgen, Boston Children’s Hospital, Harvard Medical School, Boston, MA), adapting from the protocol previously described (Morrison, 2002). Once the variable and the constant regions of heavy and light chain were joined by overlapping PCR, the heavy chain was placed under CMV promoter using restriction enzyme sites SalI and XbaI, and the light chain was placed under EF1α promoter using restriction enzyme sites NotI and KpnI in pBUDCE1.4 expression vector (Invitrogen). A similar cloning strategy was used to construct oOVA-hIdgE vector. The N-linked glycosylation sequons of IgE were mutated using the QuickChange II XL Site-Directed Mutagenesis kit (Agilent Technologies), according to the manufacturer’s protocol. Reombinant antibodies were generated by transient transfection of the plasmids into HEK293T using Polyethyleneimine “Max” (Polysciences, Inc.), followed by purification using N-hydroxysuccinimide–activated Sepharose beads (GE Healthcare) coupled to OVA (Sigma-Aldrich). Antibodies generated were verified by immunoblots for size and quantified by IgE ELISA, and the specificity was confirmed by OVA ELISA (see below).

Polyclonal IgE specific for OVA or peanut extracts was prepared by injecting WT BALB/c mice with 10 µg OVA (Sigma-Aldrich) or peanut extracts (preparation) in aluminum hydroxide on days 0, 7, and 14. Mice were bled on days 10, 17, 19, and 21. The sera was separated from the blood by serum gel tubes (BD) and depleted of IgG by incubating with Protein G high-capacity agarose beads (Thermo Fisher Scientific).

Human IgE was purified from de-identified peanut-allergic sera using N-hydroxysuccinimide–activated Sepharose beads coupled to ovalumulub (Xolar; Genentech) after IgG depletion by Protein G high-capacity agarose beads. Purified IgE was verified by Coomassie and immunoblots and quantified by IgE ELISA. Patient sera were collected under protocols approved by the MGH Institutional Review Board.

Fluorescent labeling and glycan digestion. Human IgE (HE1 clone; Abcam) was biotinylated using the Biotin-XX Microscale Protein Labeling kit (Molecular Probes), WT- and N384Q-oOVA-mIgE were conjugated to Alexa Fluor 488 or Alexa Fluor 567 (Molecular Probes) according to the manufacturer’s recommendations. IgE was digested with PNG (New England Biolabs, Inc.) or EndoF1 (Sigma-Aldrich) according to the manufacturer’s instructions under non-denaturing conditions at 37°C for 72 h. All digestions were verified by lectin blot (see below).

Immunofluorescent staining. Immunofluorescent staining were performed as described previously (Anthony et al., 2008). In brief, equal amounts of protein were resolved on 3–8% Tris-Acetate protein gels (Life Technologies) in SDS-PAGE under reducing conditions. After transfer to polyvinylidene difluoride membranes, membranes were blocked with 5% dry milk in PBS containing 0.05% Tween 20 for immunoblotting or with Protein-Free Blocking Buffers (Thermo Fisher Scientific) for lectin blotting. Mouse or human IgE was probed by goat polyclonal anti-mouse or anti-human IgE-HRP (10 ng/ml; Bethyl Laboratories, Inc.), respectively. N-linked glycans were detected by biotinylated Lens culinaris agglutinin (LCA; 5 µg/ml; Vector Laboratories) and α1,3- and α1,6-linked high mannose structures were detected by biotinylated Galanthus nivalis lectin (GNA; 4 µg/ml; Vector Laboratories).

ELISA. Mouse or human IgE was quantified by sandwich ELISA according to instructions from mouse or human IgE ELISA quantitation sets (Bethyl Laboratories, Inc.). Antibodies specific for OVA were verified by 96-well Nunc plates plate coated with 75 µg/ml OVA (Sigma-Aldrich), blocked with 2% BSA in PBS, and probed with goat polyclonal anti-mouse or anti-human IgE-HRP (2 ng/ml; Bethyl Laboratories, Inc.). A similar protocol...
was used for verifying antibodies specific for DNP and TNP, except 5 µg/ml DNP-HSA (Sigma-Aldrich) and TNP-BSA (conjugation ratio 13; Biosearch Technologies) was used for coating. All reactions were detected by 3,3′,5,5′-tetramethylbenzidine (TMB; Thermo Fisher Scientific) and stopped by 2 M sulfuric acid, and the absorbance was measured at 450 nm.

**LAD2 mast cell culture.** Human LAD2 mast cell line (from D. Metcalfe, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD) was cultured in StemPro-34 SFM medium (Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 ng/ml recombinant human stem cell factor (PeproTech). The cells were hemi-depleted each week with fresh medium and maintained at 0.25–5 × 10⁵ cells/ml at 37°C and 5% CO₂.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**IgE-FcεRI binding assay and flow cytometry.** mBMMCs were generated by flushing BM precursors from tibias and femurs of C57BL/6 mice and culturing in RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine, and culturing in RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 ng/ml recombinant mouse IL-3 (BioLegend), and 10 ng/ml mouse stem cell factor (PeproTech). The cells were hemi-depleted each week with fresh medium and maintained at 0.25–5 × 10⁵ cells/ml at 37°C and 5% CO₂.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**IgE-FcεRI binding assay and flow cytometry.** mBMMCs were generated by flushing BM precursors from tibias and femurs of C57BL/6 mice and culturing in RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine, and culturing in RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 ng/ml recombinant mouse IL-3 (BioLegend), and 10 ng/ml mouse stem cell factor (PeproTech). The cells were hemi-depleted each week with fresh medium and maintained at 0.25–5 × 10⁵ cells/ml at 37°C and 5% CO₂.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.

**Human mast cell degranulation assay.** Degranulation was measured as described previously (Kuehn et al., 2010). In brief, LAD2 cells were sensitized with 250 ng αOVA-hlgE, 100 ng biotinylated hlgE, or 25 ng allergic human serum IgE overnight. Upon OVA or streptavidin (Sigma-Aldrich) activation, the level of mast cell degranulation was monitored by the release of β-hexosaminidase in mast cell granules, quantified by the extent of its substrate p-nitrophenyl N-acetyl-β-D-glucosamine (PNGase) digested in a colorimetric assay.
described (Rial-Blanco et al., 2014). Suspension cells were resuspended in PBS and incubated with Zombie Yellow Fixable Viability kit (BioLegend) before incubation with anti-mouse CD16/CD32 (clone 93) in FACS buffer (2 mM EDTA and 0.5% BSA in PBS). Antibodies for surface antigen staining included Alexa Fluor 647 anti-mouse CD117 (e-Kir; clone 2B8; BioLegend), Pacific Blue anti-mouse CD45 (clone 30-F11; BioLegend), PE/Cy7 anti-mouse/human CD11b (clone M1/70; BioLegend), FITC anti-mouse CD8 (clone 53-6.7; BioLegend), PE anti-mouse CD8 (clone 53-6.7; BioLegend), APC anti-mouse CD8 (clone 53-6.7; BioLegend), and PE/CY7 anti-mouse CD8 (clone 53-6.7; BioLegend). Cells were resuspended in FACS buffer after staining and acquired using an LSRII flow cytometer (BD). Data were analyzed using Flowjo version 7.6 software (Tree Star).

Statistical analyses. All statistical analyses were performed using Prism 6 (GraphPad Software), and results are shown as means with SEM. An unpaired Student’s t test was used to compare two unmatched groups. For the comparison between three or more groups, one-way or two-way ANOVA with Bonferroni’s multiple comparisons test was used. Statistical power was not used to determine sample size.

Online supplemental material. Fig. S1 shows the gating strategy for identifying dermal mast cells. Fig. S2 shows glycopeptide mass spectrometry of recombinant mouse and human IgE. Tables S1 and S2, included as separate Excel files, show primary glycopeptide mass spectrometry data from mIgE and hIgE, respectively. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20142182/DC1.

We are grateful to Andrew D. Luster, Frederik Wermeling, and Kate L. Jeffrey for careful reading of the manuscript, Wayne Shreffler for providing de-identified patient samples, Bert Ruiter for providing peanut extract preparations, and Maya Kitaoka for excellent technical support.

This work was supported by National Institutes of Health grants U18AI095261 and 1K22AI091684 to R.M. Anthony, R01CA150975 to T.R. Mempel, K01DK093597 to B. Platzer, and RO1AI075037 to E. Fiebiger; Harvard Digestive Diseases Center and 1K22AI091684 to R.M. Anthony, R01CA150975 to T.R. Mempel, K01DK093597 to B. Platzer, and RO1AI075037 to E. Fiebiger; and a Mizutani Foundation for Glycoscience Research Grant to R.M. Anthony.

The authors declare no competing financial interests.

Submitted: 21 November 2014

Accepted: 11 March 2015

REFERENCES


