Immunoglobulin e (IgE) is important for resistance to parasitic infections (Dombrowicz et al., 1996; Gould et al., 2003; Matsumoto et al., 2013) and protection against venom toxins (Arnold et al., 2007; Marichal et al., 2013; Palm et al., 2013). Yet IgE is also responsible for triggering allergic reactions, one of the most common chronic conditions worldwide (Dorrington and Bennich, 1978; Arnold et al., 2007; Pawankar et al., 2013; Plomp et al., 2014). These diseases include asthma and atopic dermatitis, as well as allergies to food, dust mites, insect venom, pollen, and pet dander. Allergic reactions manifest as localized wheal and flare irritations, can have respiratory symptoms, including sneezing, rhinitis, and asthma, and in extreme cases can be life threatening in the form of anaphylaxis. Although IgE is the least abundant Ig in circulation, with a short serum half-life, it persists for weeks bound to the surface of mast cells by the high-affinity IgE receptor, FcεRI, in tissues (Gould et al., 2003). Cross-linking of mast cell–bound IgE by allergens activates the cells and results in release of mediators that induce vasoconstriction, vascular permeability, and smooth muscle contraction (Gould et al., 2003; Galli and Tsai, 2012).

IgE is the most heavily glycosylated monomeric Ig in mammals, with seven N-linked glycosylation consensus sequences (N-X-S/T) distributed across each heavy chain of human IgE (Arnold et al., 2007). The importance of glycosylation in Ig biology is increasingly appreciated. For example, the single glycan on IgG at N297 is essential for structural integrity of the constant fragment (Fc), and without it IgG cannot engage FcεRI receptors (Feige et al., 2009). However, the precise role 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 (Cδ3) 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.
FcεRI binding and effector functions (Nettleton and Kochan, 1995; Sayers et al., 1998; Björklund 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.

Figure 1. N-linked glycosylation is essential for IgE-mediated allergic inflammation. (a and b) Quantified vascular leakage and representative ear images after PCA with PBS, WT, or PNG–poly-mIgE specific for OVA or peanut extracts (*n* = 4 ears; 2 independent experiments; a) or monoclonal mIgE specific for OVA, DNP, or TNP (*n* = 8; 2, 3, and 3 independent experiments for OVA, DNP, and TNP; b). Bar, 1 cm. (c) Histograms and mean fluorescent intensity (MFI) of WT or PNG-αOVA-mIgE binding to mBMMCs determined by A647-OVA and mFcεRI (*n* = 3; 2 independent experiments). (d) β-Hexosaminidase activity after OVA stimulation of LAD2 cells sensitized with WT or PNG-treated αOVA-hIgE (*n* = 3; 5 independent experiments). (e) Quantified OVA-induced PCA in mFcεRI−/− or hFcεRI−mFcεRI−/− mice sensitized by WT or PNG-αOVA-hIgE (*n* = 4; 2 independent experiments). (f) FACS of WT or PNG-αOVA-hIgE to hFcεRI−HeLa cells assessed by A647-OVA and α-hFcεRI-PE (*n* = 2; 2 independent experiments). Means and SEM are plotted; ***, *P* < 0.001; ****, *P* < 0.0001; ***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05; ns, not significant.
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 FcεRI interactions (Dombrowicz et al., 1993; Gould et al., 2003). To determine the contribution of glycosylation to interactions with mouse FcεRI (mFcεRI), mouse BM-derived mast cells (mBMMCs) were sensitized in vitro with native or deglycosylated α-OVA-mlgE overnight. When ligand–receptor interactions were analyzed by flow cytometry using Alexa Fluor 647–OVA (A647-OVA), we found that although α-OVA-mlgE bound to the mast cells, PNG-α-OVA-mlgE did not (Fig. 1 c). Indeed, WT-mlgE specific for OVA, DNP, or TNP, but not PNG-mlgE, was able to activate mBMMCs upon antigen stimulation (not depicted). Together, these results demonstrate that mlgE 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 (α-OVA-hlgE, PNG-α-OVA-hlgE) and sensitized human LAD2 mast cells in vitro with these preparations. OVA stimulation resulted in dose-dependent degranulation of α-OVA-hlgE–sensitized mast cells, as assessed by β-hexosaminidase release. Consistent with our results, PNG-α-OVA-hlgE was incapable of instigating degranulation upon OVA stimulation (Fig. 1 d). In parallel, we administered α-OVA-hlgE or PNG-α-OVA-hlgE intradermally to transgenic mice expressing human FceRI while lacking mFcεRI (hFcεRI+/mFcεRI−/−; Dombrowicz et al., 1996). These transgenic mice have a broad cellular distribution of hFcεRI expression, whereas mFcεRI is restricted to mast cells and basophils in the WT mice. hlgE is unable to elicit PCA in mFcεRI−/− mice, which served as an injection control group. α-OVA-hlgE triggered robust vascular leakage in the ears of hFcεRI+/mFcεRI−/− mice upon OVA challenge (Fig. 1 e). The response was significantly diminished in PNG-α-OVA-hlgE–treated ears (Fig. 1 e), confirming N-linked glycosylation is also essential for the in vivo activity of human IgE.

To determine whether hlgE glycosylation was important for hFcεRI binding, HeLa cells engineered to express hFcεRI (hFcεRI−/HeLa) were sensitized with α-OVA-hlgE, incubated with A647-OVA, and analyzed by flow cytometry. Although α-OVA-hlgE bound to hFcεRI−HeLa cells was detected by A647-OVA, PNG–α-OVA-hlgE 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 α-OVA-mlgE 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-α-OVA-mlgE domain mutants promoted robust vascular leakage after OVA challenge similar to WT (Fig. 2 a), Ce1-α-OVA-mlgE glycosylation mutants exhibited slightly enhanced vascular leakage. In contrast, anaphylaxis elicited by the Ce3-α-OVA-mlgE mutant was significantly attenuated. WT-, Ce1-, Ce2-, and Ce4-α-OVA-mlgE activated mBMMCs upon OVA stimulation, but Ce3-α-OVA-mlgE did not (not depicted). α-OVA-hlgE domain–specific glycosylation mutants were also generated and tested for mast cell degranulating activity (Fig. 2 b). WT- and Ce2-α-OVA-hlgE domain mutants triggered robust degranulation upon OVA stimulation. Although degranulation was slightly reduced in mast cells sensitized with Ce1-α-OVA-hlgE, mutation of Ce3 glycosylation sites completely abolished OVA-specific degranulation. Importantly, Ce1-α-OVA-hlgE mutants bound to FceRI−HeLa cells, as determined by A647-OVA flow cytometry, but Ce3-α-OVA-hlgE 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 mlgE, individual glycosylation site mutants were generated by conversion of N361 or N384 to Q. Both WT- and N361Q-α-OVA-mlgE elicited a robust anaphylactic response in vivo, but N384Q-α-OVA-mlgE did not (Fig. 3 a). Moreover, a reciprocal mutant, in which all N-linked glycosylation sites were disrupted except N384 (N384Q-only-α-OVA-mlgE), promoted a strong PCA upon OVA administration (Fig. 3 a). To confirm the importance of N384 glycosylation for mlgE function, the glycosylation consensus sequence was
disrupted by mutation of the third-position amino acid (T386A). Indeed, T386A-αOVA-mIgE could not initiate anaphylaxis (Fig. 3a). Neither N384Q- nor T386A-αOVA-mIgE was able to activate mBMMCs in vitro (not depicted). Collectively, our data reveal glycosylation specifically at N384 in the Cε3 domain of mIgE is key for initiation of anaphylaxis.

We hypothesized that N384 was required for mIgE binding to FcεRI on mast cells. To test this in vivo, equal amounts of A488- and A568-labeled WT-αOVA-mIgE (A488-WT/ A568-WT) or A488-αOVA-mIgE and A568-N384Q-αOVA-mIgE (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-mIgE regardless of fluorophore conjugate but preferentially bound WT- over N384Q-αOVA-mIgE when both were administered (Fig. 3b and Fig. S1). Dermal mast cells from mFcεRI−/− mice showed minimal binding of WT-αOVA-mIgE, confirming the requirement
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–mIgE was not bound by mast cells when injected alone, indicating the mutation and not competition with WT–αOVA–mIgE prevented mast cell engagement (not depicted). In parallel, we primed mBMMCs with WT– or N384Q–αOVA–mIgE and detected A647-OVA bound to WT– but not N384Q–αOVA–mIgE–sensitized cells (Fig. 3 c). These results demonstrate that the N384 glycan is crucial for stable mIgE interactions with mFceRI on mast cells in vitro and in vivo.

Three Cε3 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 in vitro (Fig. 3 d). Furthermore, N394Q–αOVA–hIgE was unable to elicit vascular leakage by PCA in hFceRI+/mFceRI−/− mice (Fig. 3 e). We next examined the role of N394 glycan in hFceRI binding. Flow cytometry of hFc RI−/−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 hFcRI (Fig. 3 f). Furthermore, although WT–αOVA–hIgE bound and saturated immobilized hFcRI in vitro, N394Q–αOVA–hIgE did not (Fig. 3 g). These results identify the glycans at N394 and N384 of hIgE and mIgE, respectively, as essential for interacting with FceRI 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 (Cε1–4) of recombinant mouse and human αOVA–IgG by glycoprofiling mass spectroscopy (Plomp et al., 2014). Eight of nine N-linked glycosylation consensus sequences in αOVA–mIgE were primarily occupied by highly processed complex biantennary glycans (Fig. 4 a and Table S1). Similarly, all but one site on αOVA–hIgE was primarily occupied by complex biantennary glycans containing predominantly complex antennary glycans (Fig. 4 b and Table S2). Interestingly, the glycosylation sites identified as essential for initiating anaphylaxis, N384 of mIgE 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 glycans unaffected, unlike PNG which removes all N-linked glycans. Thus, we treated mIgE 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–mIgE (Fig. 4 c). Furthermore, EndoF1–αOVA–mIgE did not bind to mFceRI 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 mIgE is required for initiating anaphylaxis, interacting with mast cells, and binding mFceRI.

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 hFcRI 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 FceRI by altering conformation of IgE. The glycans is likely not involved in glycan–glycan or glycan–protein interactions with FceRI, consistent with structural studies of IgE and FceRI (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 mBMMCs determined by A647-OVA and mFceRI (n = 3; 2 independent experiments). (d) OVA-induced degranulation assayed by β-hexosaminidase in LAD2 cells sensitized with WT or αOVA–hIgE glycosylation mutants (n = 3; 4 independent experiments). (e) OVA-induced PCA vascular leakage in hFceRI+/mFceRI−/− mice by PBS, WT, or N394Q αOVA–hIgE (n = 3; 2 independent experiments). (f) Binding of WT or αOVA–hIgE mutants to hFcRI−/−HeLa cells as assessed by A647-OVA (n = 2; 2 independent experiments). (g) Binding of increasing concentrations of WT or N394Q αOVA–hIgE to immobilized hFcRIα in vitro (2 independent experiments.) Mean and SEM are plotted; ****, P < 0.0001; ***, P < 0.001; **, P < 0.01; ns, not significant.
Figure 4. Removal of the IgE oligomannose glycan abrogates anaphylaxis. (a and b) αOVA-mlgE (a) and αOVA-hlgE (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-specific for OVA, DNP, or TNP (n = 8; 2 independent experimental conditions).
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 using the Biotin-XX Microscale Protein Labeling kit (Molecular Probes), WT- and N384Q-αIgE-hIgE 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).

Immunofluorescence and lectin blotting. Immunofluorescence and lectin blotting 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 non-denaturing conditions at 37°C for 72 h. After transfer to polyvinylidene difluoride membranes, membranes were blocked with 5% dry milk in PBS containing protease inhibitor cocktail without EDTA (Roche). The protein extract proteins were collected as the supernatant after centrifugation at 24,000 g for 30 min.

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 protocols 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 αOVA-hIgE 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. Reconstituted antibodies were generated by transient transfection of the plasmids into HEK293T using Polyethylenimine “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 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 ovalumuzu (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-αIgE-hIgE 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).

PCA. Age- and sex-matched mice were randomized allocating to experimental group. 20 ng monoclonal mlgE 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 protease inhibitor cocktail without EDTA (Roche). The peanut protein extracts were collected as the supernatant after centrifugation at 24,000 g for 30 min.

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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 t-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 × 10^5 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-hIgE, 100 ng biotinylated hIgE, or 25 ng allergen 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-glucosaminide (PNG) digested in a colorimetric assay.

**IgE-FcεRI binding assay and flow cytometry.** nMIMCs 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 t-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml mouse recombinant mouse IL-3 (BioLegend), and 10 ng/ml mouse stem cell factor (PeproTech) for 4–6 wk at 37°C in 5% CO₂. HeLa cells that express human FcεRI-α, β, and -γ chains (hFcεRI-α–HeLa cells) were generated by retroviral transduction. BMMCs or hFcεRI-α–HeLa cells were incubated overnight with 100 ng of mouse or human IgE, respectively. Cells were washed to remove unbound IgE before incubation with A647-OVA (500 ng/ml; Molecular Probes) for OVA-specific IgE or Alexa Fluor 647–streptavidin (1:200 dilution; BioLegend) for biotinylated hIgE for 10 min at 37°C. FcεRI expression of the IgE-sensitized cells was confirmed by staining with PE-labeled anti-hFcεRIα (CRA1-PE clone AER-37; eBioscience) and anti-mFcεRIα (MAR-1-PE; BioLegend). Flow cytometric analysis was performed using the FACSCan II and FACSData software (BD).

**Saturation binding assays.** The extracellular portion of the α chain of hFcεRI (shFcεRIα) was cloned from cDNA of human myeloid dendritic cells into HindIII and BamHI sites of p3xFLAG-CMV-13 (Sigma-Aldrich) to generate shFcεRIα–flag. The plasmid was transiently transfected into HEK293T cells as described above, and shFcεRIα–flag protein was purified from culture supernatants using anti-flag M2 affinity gel (Sigma-Aldrich) per the manufacturer's instructions. 96-well Nunc plates were coated with shFcεRIα–flag (10 ng/µl), blocked with 2% BSA in PBS, and incubated with increasing concentrations of WT- and N394Q-αOVA-hIgE or hIgE (Abcam) and EndoF1-treated hIgE. After 30 min, the wells were washed, and shFcεRIα–flag–bound hIgE was probed by anti-Flag-HRP (Bethyl Laboratories, Inc.) and detected by TMB. The reactions were stopped by 2 M sulfuric acid, and the absorbance measured at 450 nm.

**Size-exclusion chromatography.** IgE monomers and aggregates were resolved in 150 mM sodium phosphate, pH 7.0, containing 1.5 mM Dithio-Bi-ANS (4,4′-Dianilino-1,1′-Biphenyl-5,5′-Disulfonic Acid, Dipotassium Salt) using an HPLC outfitted with a Sepax Zenus-C HP-SEC column 4.6 × 300 mm, 3 µm particle size, and detected by UV at 280 nm. The column was maintained at 30°C and the flow rate at 350 µl/min.

**Glycopeptide mass spectrometry analysis and data analysis.** Site-specific glycosylation was quantified for both the recombinant αOVA-mIgE and αOVA–hIgE using nano LC-MS/MS after enzymatic digestion of the proteins, as previously described (Plomp et al., 2014). Most sites were quantified from the tryptic digest based on the extracted ion current for the most abundant charge state for each of the peptides, except chymotrypsin was used for the analysis of N140 and N168 from αOVA-hIgE and N166, N195, and N207 from αOVA–hIgE. The isolated IgE was prepared for proteolysis by denaturing the protein in 6 M guanidine HCl, followed by reduction with dithiothreitol and alkylation with iodoacetamide. The enzymatic digests were performed in 25 mM ammonium bicarbonate, pH 7.8, overnight (trypsin) or for 4 h (chymotrypsin). The digestion was quenched with formic acid added to 2% wt/wt. The separation was performed on an EasySpray C18 nLC column 0.75 µm × 50 cm (Thermo Fisher Scientific) using water and acetonitrile with 0.1% formic acid for mobile phase A and mobile phase B, respectively. A linear gradient from 1 to 35% mobile phase B was run 120 min. Mass spectra were recorded on a QExactive mass spectrometer (Thermo Fisher Scientific) operated in positive mode and using a top 12 data-dependent method. Glycopeptides were quantified in QualBrowser (Thermo Fisher Scientific) based on the extracted ion area for the most abundant charge state for each glycopeptide. The relative abundance was calculated for all identified glycan species for each site using Excel (Microsoft).

Glycopeptides were quantified based on the extracted ion area for abundant charge state for each glycopeptide. The relative abundance was calculated for all identified glycan species for each site without the use of internal standards making the relative abundances subject to the influence of ionization efficiency. The extracted ion chromatograms for the major and minor species from N394 are shown in Fig. S2 a. Identification of the glycopeptides was based on the presence of the oxonium ions 366.14 (Hex-HexNAc) and 204.08 (HexNAc) common to all glycosylated peptides, as well as the Y1 ion (peptide + HexNAc) which is unique to each site.

In cases where multiple chromatographic peaks were seen for a single mass, higher-energy collisionally activated dissociation (HCD) MS/MS was used for identifying differences between the chromatographically resolved isomers. Fig. S2 c shows the comparison of three neutral glycopeptides having the same apparent molecular weight. The MS² spectrum for the first chromatographic peak shows evidence of a HexNAc–HexNAc structure with a mass of 407.16 D, supporting the assignment of this species as containing terminal N-acetylgalactosamine. The second peak shows evidence of a HexAHexNAc structure at 569.20 not seen in the MS² from the first chromatographic peak. Unique Y ions at 1804.81 and 1950.87 in this second spectrum support the assignment of isomer 2 as containing bisecting N-acetylgalactosamine.

The MS² was also used to differentiate between species of similar mass with different composition. The MS² comparison in Fig. S2 d shows the fragmentation pattern for a trifucosylated glycopeptide (top panel) from hIgE N371 with three sialylated/core-fucosylated glycopeptide (bottom three panels) from the same site. In Fig. S2 d, both oxonium (B) ions and Y ions were useful for elucidating the structure. The comparison of the MS² spectra in the second and third panel from the top in Fig. S2 d suggests the major difference between these species is the location of the sialic acid. The oxonium ion at 495.17 from the MS² spectrum in panel 2 of Fig. S2 d suggests a Galα1–3GalNAc linkage, whereas Gal–NeuAc is more likely the structure in the third panel based on the ion at 657.23. These spectra are representative of the data used to assign likely structures for each of the sites on both mIgE and hIgE.

**Flow cytometry.** Untreated or treated cells that were intradernally injected with fluorescently labeled αOVA-mIgE 16 h prior were separated into dorsal and ventral halves and minced before digestion with Liberase (Roche) and subjected to disruption, to generate single cell suspensions, as previously described.
described (Riol-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 28B; 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 mlgE and hlgE, respectively. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20142182/DC1.


