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ARTICLE

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Systematic mutational analysis reveals an essential role of N275 in IgE stability

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Abstract

Therapeutic antibodies have predominantly been IgG-based. However, the ongoing clinical trial of MOv18 IgE has highlighted the potential of using IgE antibodies in cancer therapy. While extensive studies targeting IgG glycosylation resulted in a rational basis for the development of enhanced biotherapeutics, IgE glycosylation remains an area with limited analyses. Previous studies on the role of IgE glycosylation present conflicting data with one study emphasizing the importance of N275 and T277 residues for FcεRI binding whereas another asserts the nonsignificance of IgE glycosylation in receptor interaction. While existing literature underscores the significance of glycans at the N275 position for binding to FcεR1 receptor and initiation of anaphylaxis, the role of other IgE glycosylation sites in folding or receptor binding remains elusive. This study systematically investigates the functional significance of N-linked glycosylation sites in the heavy chain of IgE which validates the pivotal role of N275 residue in IgE secretion and stability. Replacement of this asparagine to non-amine group moieties does not affect IgE function in vitro, yet substitution with aspartic acid compromises antibody yield. The deglycosylated IgE variant exhibits superior efficacy, challenging the conventional importance of glycosylation for effector function. In summary, our study unveils an intricate relationship between N-glycosylation sites and the structural–functional dynamics of IgE antibodies. Furthermore, it offers novel insights into the IgE scaffold, paving the way for the development of more effective and stable IgE-based therapeutics.

KEYWORDS
antibody engineering, HER2/neu receptor, N-glycosylation, recombinant IgE

1 | INTRODUCTION

As of now, over 100 antibodies are under review or approved in the United States and EU for both cancer and noncancer therapy applications. It is noteworthy that the majority of the therapeutic antibodies in the current market belong to the IgG class. IgE has entered the field of therapeutic antibodies with the ongoing clinical trial of MOv18 IgE (Identifier NCT02546921, (Karagiannis et al., 2007a)). The preclinical data of therapeutic IgE has yielded valuable insights into their functional and cytotoxic properties against tumors (Gould et al., 1999; Karagiannis et al., 2009). This has highlighted potential areas where they may exhibit superiority over the IgG molecule. IgE antibody is mainly associated with allergy. However, epidemiological studies suggest an inverse relation between allergy and cancer (Turner et al., 2006) which renders this antibody class promising for the treatment of cancer

due to several desirable properties. For example, IgE shows a higher affinity towards its cognate receptors, lacks inhibitory receptors and has a longer half-life in tissues. Moreover, it can overcome antibody blockade mechanisms and shows enhanced antigen uptake and presentation by antigen-presenting cells (APCs) to cognate T cells (Sutton et al., 2019). Importantly, immune cells such as monocytes, macrophages, mast cells, dendritic cells (DCs), and eosinophils are found in tumors that express IgE receptors and are responsive to IgE-mediated effector function (Leoh et al., 2015). Thus, IgE has enormous potential in combating malignancies. MOv18 IgE antibody is designed to recognize and bind to the tumor-associated antigen folate receptor alpha (FRα) which is a validated target for cancer therapy. The promising early clinical MOv18 IgE results highlight the potential opportunities of IgE-based therapeutics for treating human diseases. Furthermore, preclinical studies of CSPG4 IgE indicate the growth of this antibody class (Chauhan et al., 2023). CSPG4 IgE exhibited enhanced anti-melanoma activity compared to CSPG4 IgG restraining tumor growth, activating immune responses, and notably, without eliciting allergic reactions. The results implied that CSPG4 IgE shows potential as a therapeutic agent for melanoma. Additionally, this class of antibodies can be further investigated for both solid and liquid tumors.

While extensive studies targeting the IgG glycans yielded a rational basis for the development of enhanced biotherapeutics (Golay et al., 2022; Trzos et al., 2023), IgE glycosylation remains an underexplored area. The fundamental structural unit of IgE molecules consists of two identical heavy chains and two identical light chains connected by disulfide bonds. The N-terminal variable regions of both the heavy and light chains form the antigen binding sites, while the C-terminal constant regions of the heavy chains interact with immune receptors to facilitate effector functions. As compared with IgG, IgE has two distinct features: a substantial carbohydrate content (12%) in the epsilon (ε) heavy chain and an additional constant region termed CH4.

The IgE molecule is known for being the most extensively glycosylated antibody, with glycans occupying seven specific asparagine (N) sites: N21, N49, N99, N146, N252, N264, and N275 positions. Notably, at the 275th position, which is conserved across all mammalian IgE, only oligomannose structures are present (Uniprot: P01854; (Plomp et al., 2014)). The IgE N275 position is therefore considered homologous to the IgG N297 residue.

There have been limited analyses of N-glycosylation and its role in IgE function. Site-specific glycosylation analyses of IgE in human serum samples showed that N264 is unoccupied with glycans, N275 is linked to oligomannosidic structures and other sites (N21, N49, N99, N146, and N252) are occupied exclusively with complex type glycans (Plomp et al., 2014). Given that IgE is heavily glycosylated, there is a gap in comprehending the structure and function of its glycans, as the current literature does not specify the role of glycans in the current antitumor IgE antibodies MOv18 IgE (Karagiannis et al., 2003a), Trastuzumab IgE (Karagiannis et al., 2009), and anti-CSPG4 IgE antibody (Chauhan et al., 2023).

While glycosylation is crucial for IgE secretion, precursor glycan processing does not impact secretion or allergic responses. The attachment of tetradecasaccharide (Glc3Man9GlcNAc2) to the Fc chain is the sole requirement for secretion. Inhibitors of glucosidase I or mannosidase II do not affect IgE secretion speed or quantity (Granato & Neeser, 1987). Oligomannose glycans at N275 are hindered by the CH2 hinge domain's asymmetrically bent conformation. IgE has 8.3% Man5GlcNAc2 glycans resulting from Glc3Man9GlcNAc2 precursor trimming. The CH2 domain undergoes a flipping motion, enabling partial trimming to Man5GlcNAc2 during glycan biosynthesis (Arnold et al., 2004). In addition, glycans play a significant role in the water solubility of IgE as the removal of hydrophilic, acidic glycans might increase the exposure of hydrophobic regions thus leading to aggregation (Basu et al., 1993).

In tissues, the half-life of IgE (~2 weeks) is significantly longer than IgG (2–3 days) (Lawrence et al., 2017). Interestingly, sialylation of IgE plays an essential role in prolonging half-life in vivo, removal of sialic acid decreased the half-life of the IgE antibody (Dühring et al., 2023; Shade et al., 2020). This finding aligns with the observation that allergic individuals have higher serum IgE for longer durations and correlates with increased sialic acid content on IgE (Shade et al., 2020).

IgE-based therapeutics have several advantages. For example, a low level of IgE in the serum leads to reduced competition between endogenous and therapeutic IgE for cognate receptor occupancy, thus boosting efficacy. Additionally, local IgE retention in tissues (Oettgen, 2016) combined with lower endogenous IgE levels in the blood, may lead to lower therapeutic doses and reduced administration frequency as compared to IgG-based therapy. The capability of IgE to activate various effector cells like mast cells, monocytes, and macrophages through antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) also presents a potential enhancement of therapeutic effects. Tumor-specific IgE can re-educate tumor-associated macrophages (TAM), thus enhancing antigen presentation and facilitating ADCP (Pellizzari et al., 2020). IgE might play a pivotal role in suppressing tumor by ADCC involving mast cells and ADCP involving macrophages and monocytes (Bracher et al., 2007; Josephs et al., 2017; P. Karagiannis et al., 2009; S. N. Karagiannis et al., 2003b, 2007b, 2008; Nakamura et al., 2020; Wei et al., 2017).

The complex glycans in the constant heavy chain domain (Fab) modulate the interaction of IgE with the antigen as revealed by mutational analyses. Glycosylation at the N275 site has been recently demonstrated to be crucial for the binding of IgE to the high-affinity receptor FcεRI and the initiation of anaphylaxis (Shade et al., 2015). However, the role of N-glycans in the interaction of IgE Fc to its receptor and effector function remains unclear.

The present study reports a systematic analysis of the functionality of all N-linked glycosylation sites present in the heavy chain of the IgE molecule. Although altering all asparagine residues to non-amine group moieties had no impact on its in vitro binding function, the conversion of asparagine to aspartic acid compromised the antibody's secretion yield. This situation contrasted with other glycovariants, where modifications typically target a conserved glycosylation site (N275). Notably, the deglycosylated variant exhibited enhanced efficacy compared to the wild

type (WT), highlighting the importance of glycosylation in this context. Furthermore, this study introduces a new FcεR1 activation assay for the functional exploration of therapeutic IgE antibodies. ADCC is a pivotal mechanism of action for therapeutic IgE which is mediated by mast cells in tumor cell elimination (Leoh et al., 2015). in vitro, ADCC analysis typically involves testing antibodies using human donor-derived effector cells. However, PBMC-based assays display high variability across different donors. In this study, a customized functional assay was developed and optimized which is mechanistically relevant to the PBMCs-based assessment for IgE therapeutics. The assay utilized Jurkat Lucia NFAT cells with constitutive expression of FcεR1 receptors as effector cells. This stable cell line expresses firefly luciferase under the control of the nuclear factor of activated T cells (NFAT) response elements, offering a robust and precise method. Upon stimulation, calcium influx activates calcineurin, initiating NFAT translocation to the nucleus, and inducing luciferase gene expression, which is detectable in the supernatant.

Overall, our study presents novel insights into IgE scaffold design suitable for IgE therapeutic antibody development in the future.

2 | RESULTS AND DISCUSSION

2.1 | Role of conserved asparagine(N) residues in the IgE antibody expression

To investigate the role of N-linked glycosylation in IgE function, Trastuzumab was chosen as the model antibody framework. This humanized monoclonal antibody targets the human epidermal growth factor receptor 2 (HER2/neu) and is used for the treatment of early-stage breast cancer. The sequence encoding the variable regions of the heavy (V_H) and light chain (V_L) of the Trastuzumab IgG1 was cloned upstream of the IgE constant heavy chain domain (CH_1) domain and CL domain, respectively, thus generating the recombinant human Trastuzumab IgE antibody (Figure 1m). Similarly, an IgE anti-digoxigenin antibody was generated as isotype control. To evaluate the functional role of N-linked glycosylation systematically, Trastuzumab IgE glycovariants were generated replacing all seven asparagine residues with glutamine (N to Q substitution), yielding variants IgE N21Q, IgE N49Q, IgE N99Q, IgE N146Q, IgE N252Q, IgE N264Q, and IgE N275Q (Figure 1b–h).

The glycovariants were expressed in Expi 293 cells and affinity purified using HiTrap ™ Protein L column followed by gel filtration chromatography (GFC) to remove the free light chains. Subsequently, quality control analysis using size exclusion chromatography (SEC) revealed >95% purity which was unaffected by freeze–thaw cycles, thus demonstrating the stability of the purified antibodies (Supporting Information S1: Figure 1 and Supporting Information S1: Table 2). In addition, analysis of the GFC eluates on SDS PAGE under non-reducing conditions revealed pure, intact Trastuzumab IgE antibodies migrating at the expected size of ~170 kDa (Figure 2a).

While comparable yields of IgE WT and IgE glycovariants were obtained, recovery of the IgE N275Q molecule after GFC purification failed (Figure 2a). Given the confirmed essential role of N275, two additional IgE variants targeting this conserved amino acid were

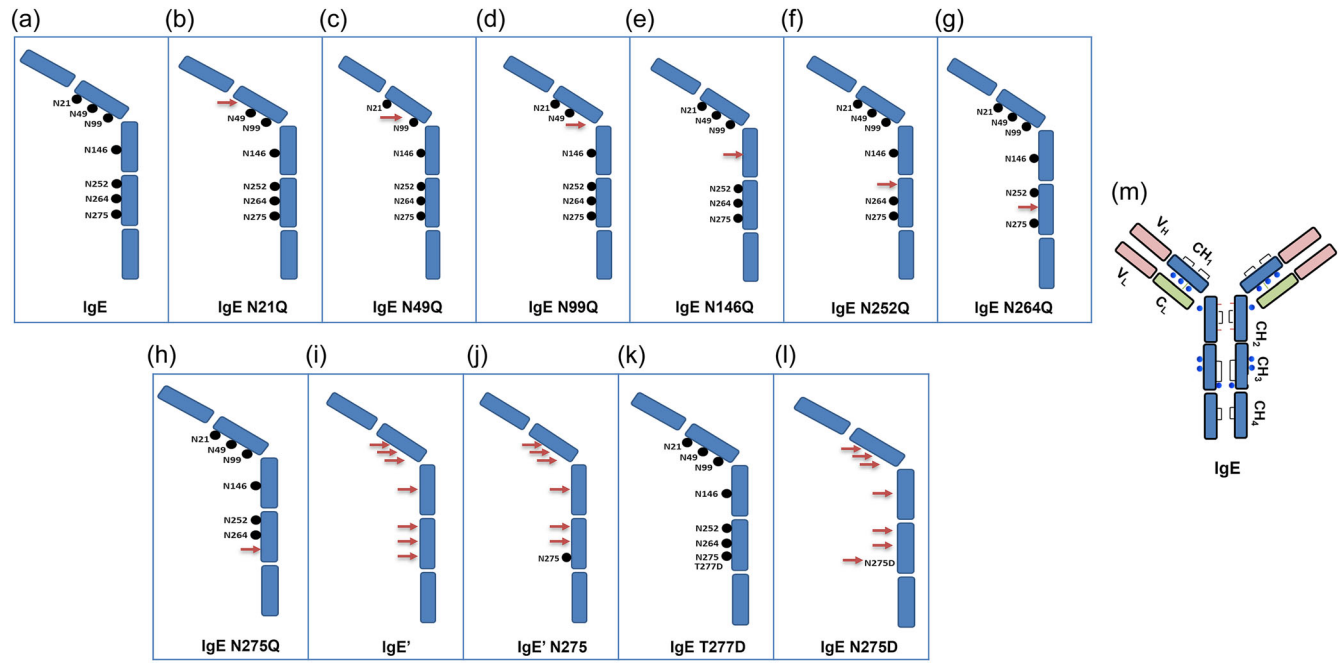


FIGURE 1 Schematic diagram showing the engineered Trastuzumab IgE variants. The modified IgE antibodies were generated by sequential substitution of the Asn residues (N) in Trastuzumab IgE heavy chain with Gln (Q). The mutation targeting N residue is numbered from the N-terminal of the heavy chain. An unmodified IgE is shown in (m) with different domains in the light and heavy chain. The blue, pink and green color show constant heavy chain (HC), variable and constant light chain (LC) respectively. The red arrow indicates the site of mutation, and the black dot shows the position of the N residue in IgE constant heavy chain domain (CH). The half antibody is for representation purpose, while all the antibodies are produced in their full length form.

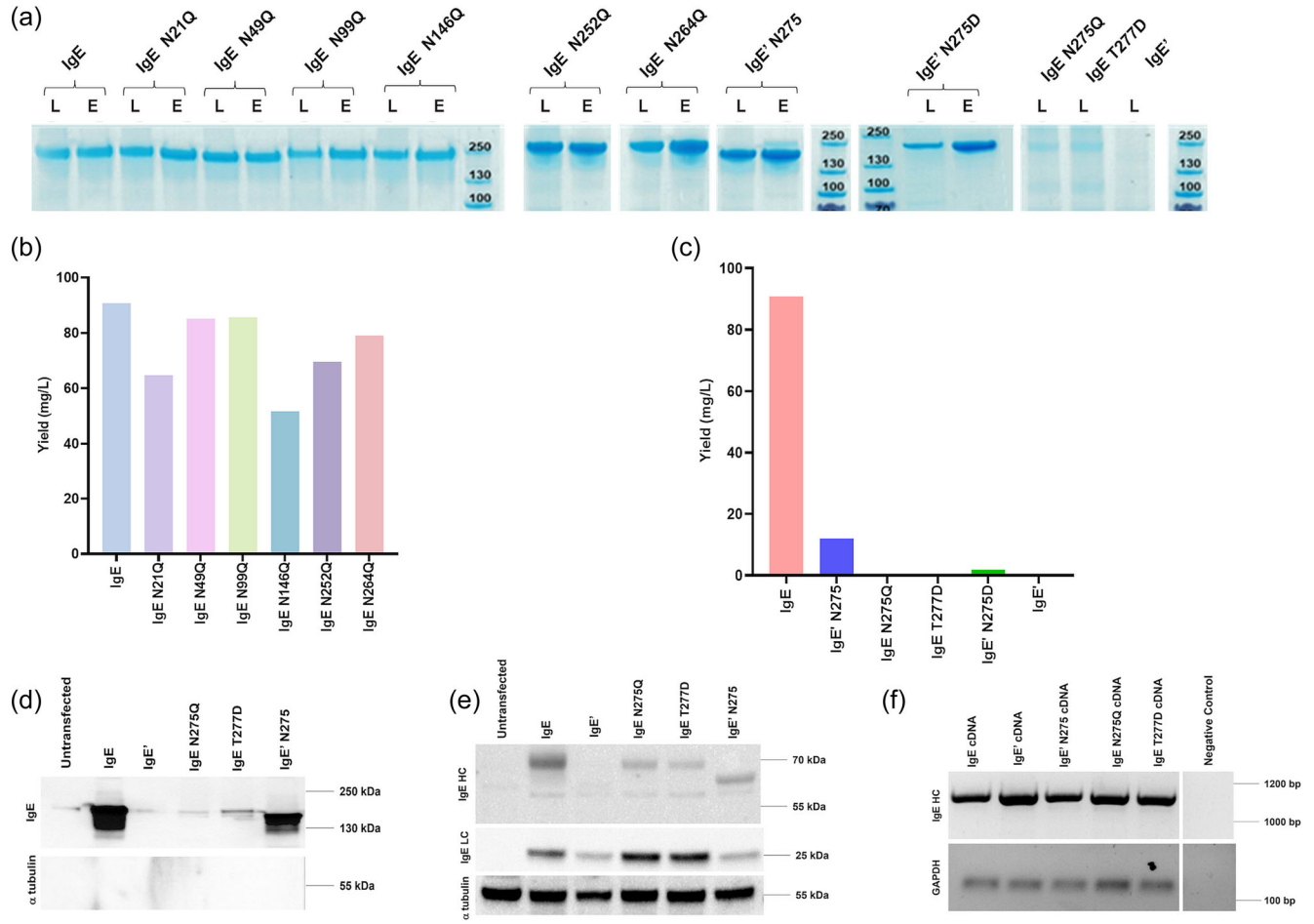
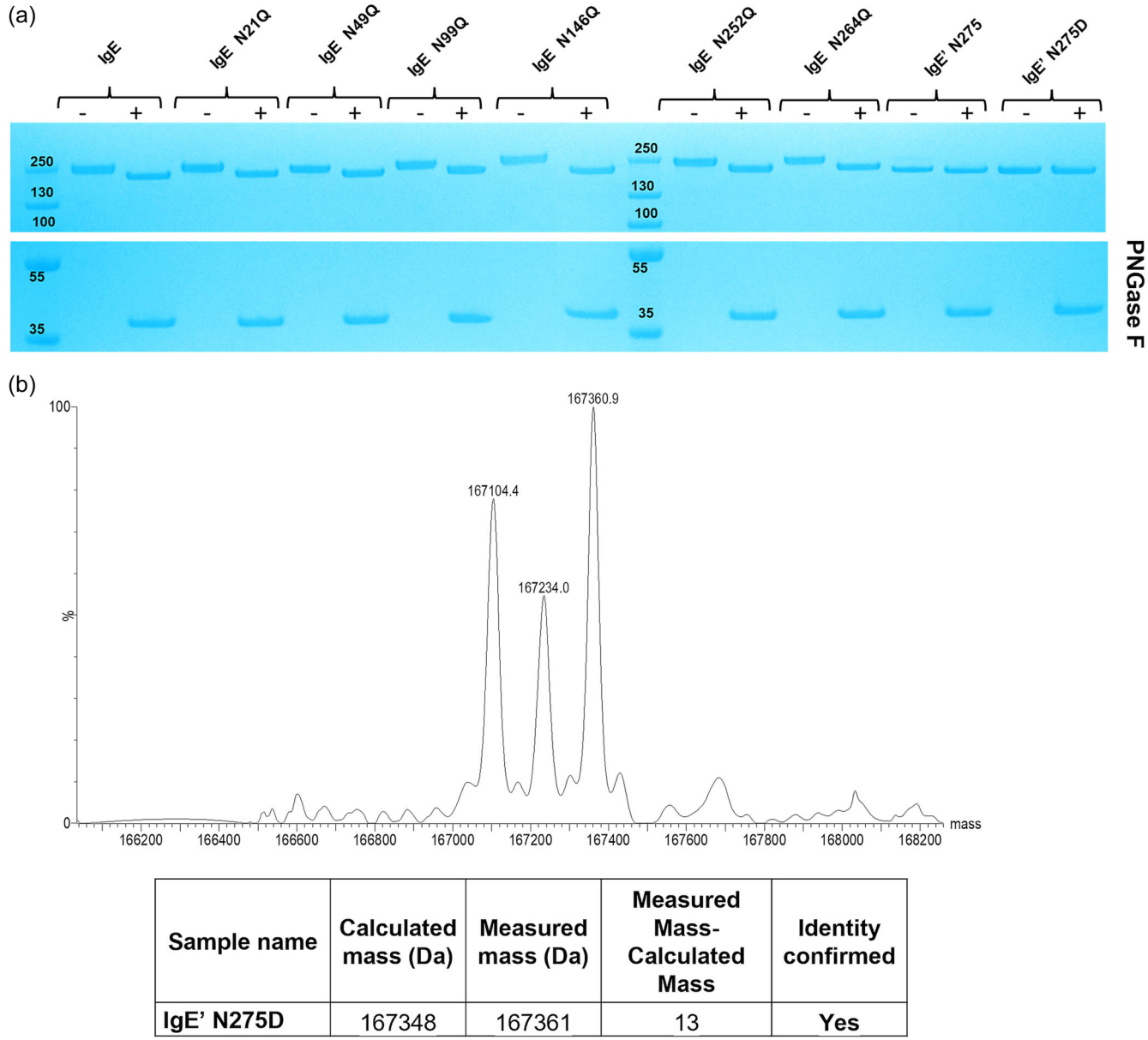


FIGURE 2 Expression analyses of engineered Trastuzumab IgE variants. (a) Nonreducing polyacrylamide gel electrophoresis of IgE WT and its glycovariants expressed in Expi 293 after gel filtration chromatography. Glycovariants such as IgE N275Q, IgE T277D and IgE' could not be recovered from affinity chromatography due to low expression levels (L: Load; E: Eluate). (b) Targeting the non-conserved Asn275 residue with the mutation results in a yield of the secreted antibodies comparable to that of the IgE WT. (c) Targeting the Asn275 residue directly (N275Q, E') or the glycosylation motif (T277D) with the mutation drastically reduces antibody secretion. Restoration of the N275 alone (E'N275) or a different amino acid residue at 275 position (E'N275D) is insufficient to restore antibody yield. (d) Western blot analysis using the supernatants and total cell lysate (e) from cells expressing the recombinant variants shows low amounts of heavy chain protein indicating intracellular stability of the heavy chain. The antibodies used for staining the blots are shown on the left of the panels. (f) RT-PCR analysis using samples prepared from cell lysate shows similar levels of mRNAs of Asn275 variants. The genes targeted by the primers are shown on the left of the panel.

designed. IgE' wherein all seven C_H N→Q substitutions resulted in a deglycosylated IgE antibody (Figure 1i) and IgE'N275 where except for the conserved N275 amino acid in the C_{H3} domain all six N were substituted to Q (Figure 1j). Remarkably, similar to IgE N275Q, the IgE' antibody was not recovered from the supernatant. Additionally, the presence of N275 residue was not sufficient to restore the antibody production levels since low amounts of IgE' N275 (12 mg/L) were recovered as compared with the IgE WT (90.75 mg/L). To evaluate if the nature of amino acid substitution at the N275 position plays any role, a new variant IgE' N275D was generated wherein the conserved N275 residue in IgE' molecule was replaced with an aspartic acid residue (Figure 1l). Interestingly, low amounts of monomeric Trastuzumab IgE' N275D were recovered (1.85 mg/L) after GFC purification (Figure 2a,c). This highlights the crucial involvement of the N275 residue, along with its associated glycosylation, in the secretion and stability of Trastuzumab IgE. It implies that while N275 residue-mediated glycosylation is necessary, it alone is not adequate for ensuring IgE antibody stability. This underscores the potential contribution of other asparagine residues in the constant region to achieve optimal levels of IgE production.

Glycosylation at asparagine residue typically requires the presence of NXT/S motif in the polypeptide (Breitling & Aebi, 2013). The above results show that N-linked glycosylation at the conserved N275 residue plays an important role in antibody production. Therefore, disruption of glycosylation at the IgE N275 residue by mutating the NXT/S motif would phenocopy the IgE N275Q mutation. As expected, the replacement of the threonine residue at amino acid position 277 to aspartic acid (IgE T277D) resulted in non-recovery of the antibody (Figure 2a,c) demonstrating that glycosylation of the N275 residue is critical for overall IgE stability and secretion.



Sample name	Calculated mass (Da)	Measured mass (Da)	Measured Mass-Calculated Mass	Identity confirmed
IgE' N275D	167348	167361	13	Yes

FIGURE 3 Determination of the glycosylation status of engineered Trastuzumab IgE variants. (a) The antibodies were treated with PNGase and the migration was detected using SDS PAGE. – shows a sample without PNGase F treatment and + shows a sample with PNGase F treatment. (b) Intact mass of IgE' N275D.

To investigate the N-glycosylation status of the IgE variants, the recombinant antibodies were incubated with PNGase F and were analyzed using nonreducing SDS-PAGE. The analysis revealed effective removal of glycans from the IgE molecules leading to faster migration of the proteins (Figure 3a). However, IgE' N275 and IgE' N275D failed to show any shift in migration upon electrophoresis, thus confirming the absence of glycans from the molecules, respectively. Indeed, mass spectrometry analysis revealed the absence of glycosylation on the IgE' N275D molecule (Figure 3a,b). IgE WT and its glycovariants were analyzed for global glycan profiles using the HILIC-ESI-QToF mass spectrometry method where glycans were identified based on the fluorescence traces, glycan units (GU), and mass. The data indicate that a single-site mutation does not result in a significant change in the overall glycan composition. However, specific glycan components exhibit a more than two-fold decrease in the IgE glycovariants relative to the IgE WT molecule (Figure 4; Table S1) suggesting that the mutation affects glycan composition at specific positions. The observed glycosylation pattern could be specific to Expi 293 cells as post-translation modifications are cell specific. However, the glycan profile might also be altered due to the conformational changes arising from the removal of neighboring glycosylation sites. This type of observation has been documented in IgA engineering (Lohse et al., 2016). Additionally, our observations indicate that IgE' N275 exhibits only complex glycan components

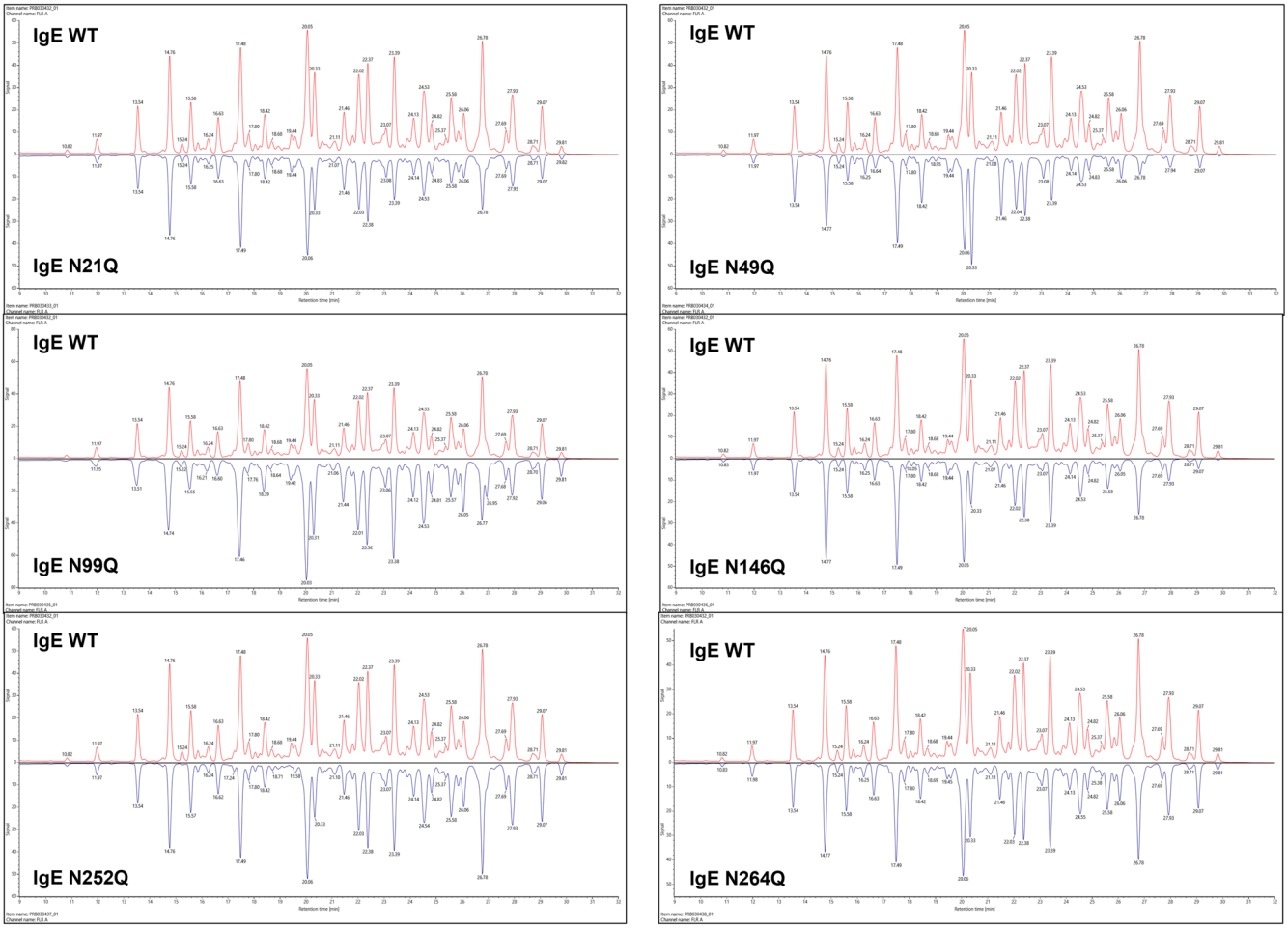


FIGURE 4 Comparison of global glycan profile of IgE WT versus single site mutation glycovariants. Trastuzumab IgE and its variants were analyzed for global glycan profiles using the HILIC-ESI-QToF mass spectrometry method where glycan components were identified based on the fluorescence traces, glycan units (GU) and mass.

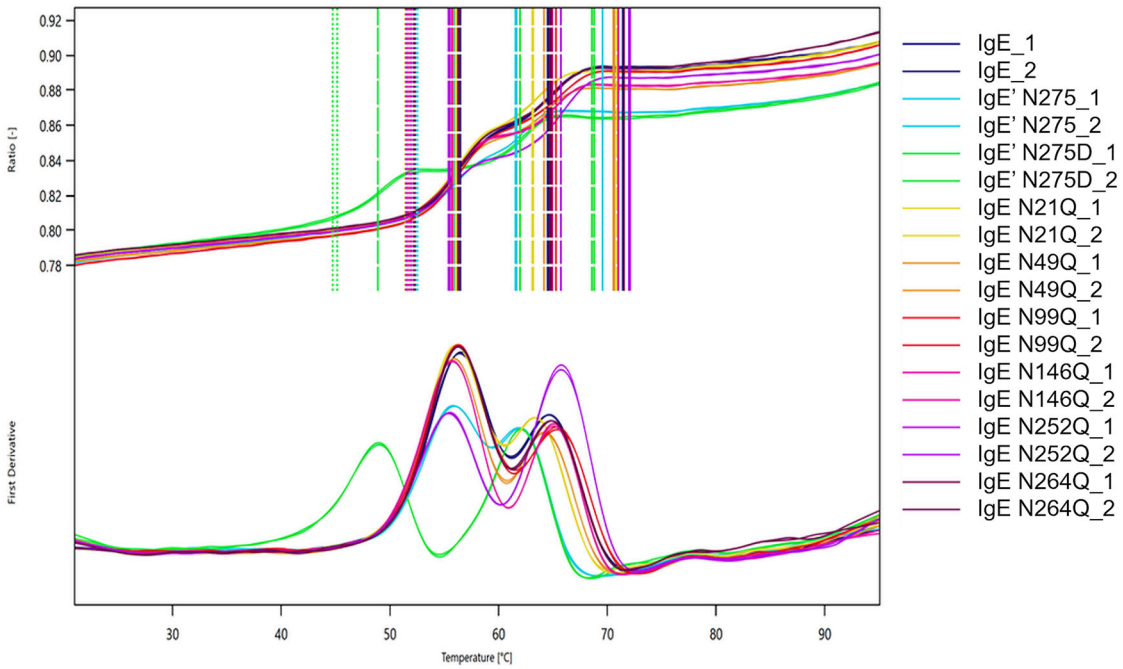
rather than solely mannose, as previously reported in the literature (Montero-Morales et al., 2019; Plomp et al., 2014; Shade et al., 2015) (Supporting Information S1: Table 3 and Supporting Information S1: Figure 3)".

Taken together, the data establishes an essential role of N275 glycosylation in the secretion and assembly of the IgE antibody to its optimal level. In addition, the site-specific N-glycosylation at other asparagine residues in the CH_3 domain of IgE has minimal effect on the antibody yield. Although IgE', IgE N275Q, and IgE' N275D antibodies are nonglycosylated, the variability in their yield might be due to the impact of the mutation at the conserved site on the overall structure of the molecule during antibody maturation and secretion.

For the developability of IgEs as biotherapeutics, the thermostability of the recombinant Trastuzumab IgE and its glycovariants was assessed (Figure 5). Except for IgE' N275D, high thermal stabilities for all glycovariants similar to IgE WT were observed. However, the lower thermostability of the IgE' N275D antibody suggests an important role of glycosylation at the IgE N275 position in mediating thermo tolerance.

2.2 | Amino acid substitution at N275 position determines the expression levels of the antibody

The drastic reduction in recovery of Trastuzumab IgE glycovariants IgE', IgE' N275, IgE N275Q, and IgE T277D from the supernatants could be due to a defect in the assembly, secretion, and stability of the recombinant antibody during assembly or instability of the corresponding mRNAs within the cell. To differentiate between these possibilities, IgE WT, IgE', IgE N275Q, IgE T277D, and IgE' N275 were expressed in Expi 293 cells, and the cellular and secreted proteins were analysed using immunoblotting. The Western blot analysis shows that IgE glycovariants IgE', IgE N275Q, and IgE T277D were undetectable in the supernatant while the IgE' N275 antibody was secreted at low amounts as compared with the IgE WT molecule (Figure 2d). This data is in agreement with the earlier observation showing a significantly reduced yield of the corresponding IgE antibodies after GFC purification (Figure 2a). Interestingly, Western blot analysis using cellular extract reveals impaired antibody assembly primarily due to the instability of the heavy chain polypeptide



	Protein	IgE	IgE N21Q	IgE N49Q	IgE N99Q	IgE N146Q	IgE N252Q	IgE N264Q	IgE' N275	IgE' N275D
Thermostability	Tm1	56.5 °C	56.1 °C	55.9 °C	56.3 °C	55.7 °C	55.4 °C	56.3 °C	56 °C	48.9 °C
	Tm2	64.5 °C	63.2 °C	64.1 °C	65.2 °C	65 °C	65.7 °C	64.7 °C	61.6 °C	62 °C

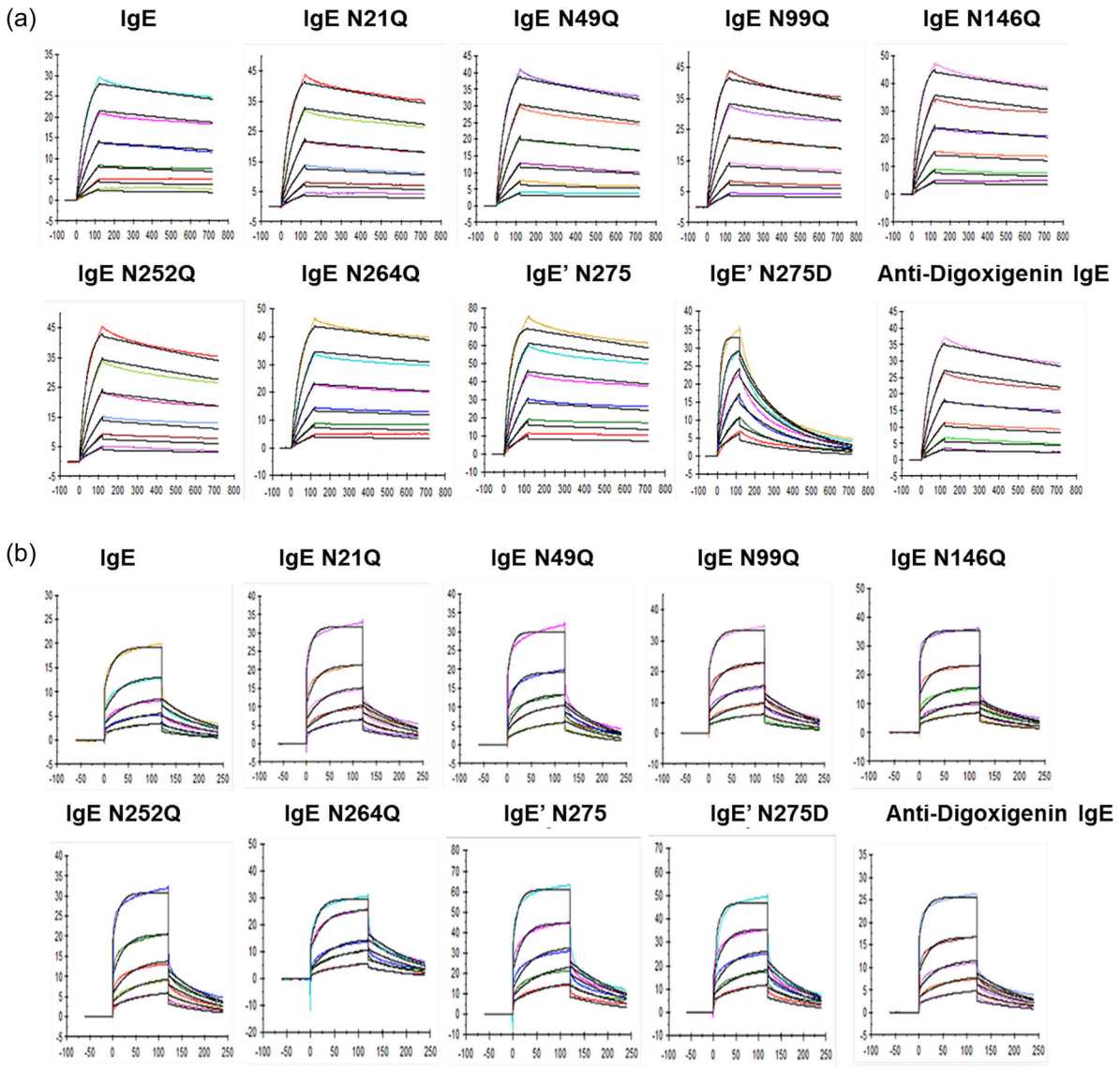
FIGURE 5 Thermostability analysis of the engineered Trastuzumab IgE variants. The plot represents the thermal unfolding curves of IgE and its glycovariants with a temperature range set between 21°C and 95°C, and a heating increment of 0.5°C/min. Measurements were performed in duplicates using two independent capillaries per antibody.

(Figure 2e). As compared with IgE WT, the ratio of heavy to light chain in IgE variants IgE', IgE N275Q, and IgE T277D was low due to reduced levels of the heavy chain. This could be due to reduced translation in the endoplasmic reticulum (ER) or misfolding of the overexpressed antibody chains due to a lack of N-linked glycosylation. Indeed, glycosylation is involved in the proper folding of ER-targeted proteins and misfolding may trigger the UPR or ERAD pathway leading to protein degradation (Piirainen & Frey, 2020; Schoberer et al., 2020). Strikingly, expression of IgE' heavy (and light chains) was barely detectable suggesting a severe reduction in translation and protein stability. However, a minimal expression of IgE N275Q and IgE T277D heavy chain was observed (Figure 2e). This shows that the intracellular assembly of the antibodies is impaired resulting in the secretion of trace amounts which could not be recovered from affinity chromatography. Also, the N-linked glycosylation at nonconserved asparagine residues of the IgE CH domain probably aids in the stability of the heavy chain and final assembly of the molecule.

To investigate if the Trastuzumab IgE variants were transcribed optimally, RT-PCR analysis was performed. For this, mRNA was isolated from the lysates of cells expressing IgE WT, IgE', IgE N275Q, and IgE T277D molecules followed by cDNA synthesis and PCR using primers specific to the IgE heavy chain. This analysis shows the presence of comparable levels of heavy chain mRNA in all samples suggesting similar transcript levels in all IgE variants (Figure 2f). Therefore, the low levels of the recombinant antibody variants were likely due to posttranslational events in the ER-Golgi network.

2.3 | Impact of IgE glycosylation status on the target cell binding and effector function

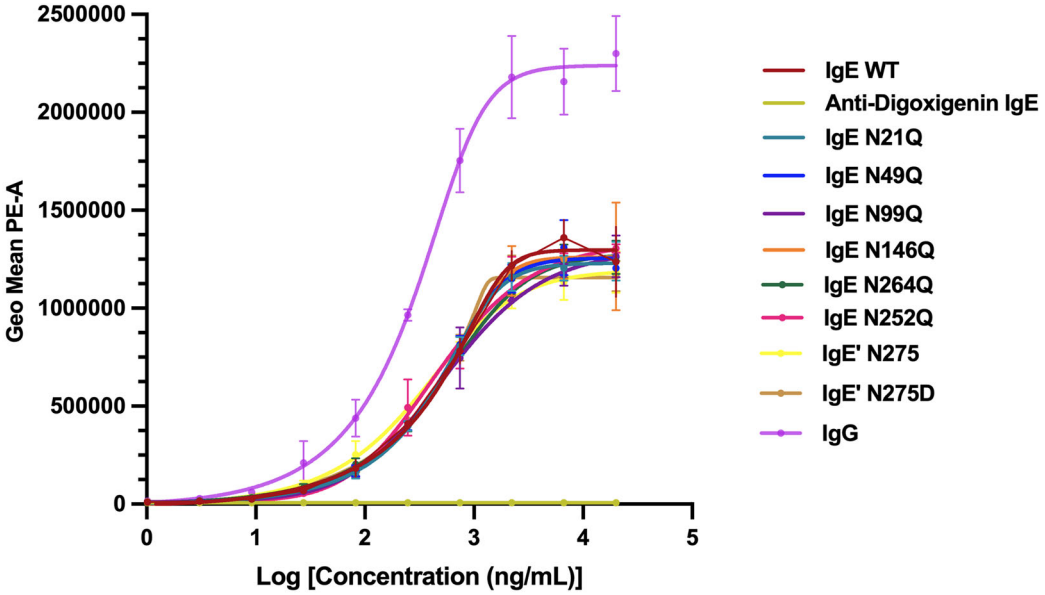
The effector functions of IgE are dependent on binding to its cognate receptors, the high-affinity FcεRI and low-affinity CD23 (FcεRII), via the CH_3 and CH_4 domains (Garman et al., 2000; Holdom et al., 2011; Presta et al., 1994). Therefore, the binding affinity of the recombinant Trastuzumab IgE antibody variants to its receptors was evaluated using surface plasmon resonance assay (SPR) (Figure 6a). IgE' N275D antibody showed a sixfold lower affinity (KD value 7.42 nM) for FcεRI binding than the IgE WT (KD value 1.23 nM). However, other IgE variants show comparable binding to FcεRI. Interestingly, compared with IgE WT, all IgE variants showed marginally higher affinity towards the low-affinity CD23 receptor (Figure 6b). Next, evaluation of cellular binding potency revealed EC50 values comparable to affinities measured with the recombinant target protein (Figure 7), indicating that IgE glycosylation is not essential for Fab-mediated engagement with the target antigen.



Protein	IgE	IgE N21Q	IgE N49Q	IgE N99Q	IgE N146Q	IgE N252Q	IgE N264Q	IgE' N275	IgE' N275D	Anti-digoxigenin IgE
KD (nM) FcεR1	1.23 ± 0.21	1.40 ± 0.13	1.32 ± 0.12	1.29 ± 0.11	1.12 ± 0.09	1.38 ± 0.09	1.02 ± 0.18	0.83 ± 0.08	7.42 ± 1.67	1.33 ± 0.44
KD (nM) CD23	464	203	220	311	199	282	327	195	248	253

FIGURE 6 Sensograms from surface plasmon resonance study. (a) Binding affinity of IgE WT, IgE glycovariants and anti-Digoxigenin IgE towards recombinant FcεR1a Protein. (b) The binding affinity of IgE WT, IgE glycovariants, and anti-Digoxigenin IgE towards recombinant FcεRII (CD23) protein. All the glycovariants have shown similar affinity for FcεR1 and CD23 receptors compared to IgE WT antibody except for IgE' N275D.

For cancer immunotherapy, the therapeutic antibody must elicit robust ADCC which involves the recruitment of cytotoxic immune cells to the cancer cell in an antibody-dependent manner (Bracher et al., 2007; Gould et al., 2003). To test this, Jurkat-Lucia™ NFAT cells (Invivogen) were engineered to constitutively express FcεRI receptor, and these cells were used to evaluate the effector functions of IgE WT and its glycovariants. In this assay, the cells stably express FcεRI receptors. The total number of receptors was quantified using the BD Quantibrite™ Phycoerythrin Fluorescence Quantitation kit which yielded a count of 127996 receptors per cell (Supporting Information S1: Figure 4). In untransfected Jurkat-Lucia™ NFAT cells, endogenous expression of FcεR1 receptors was not detected (Supporting



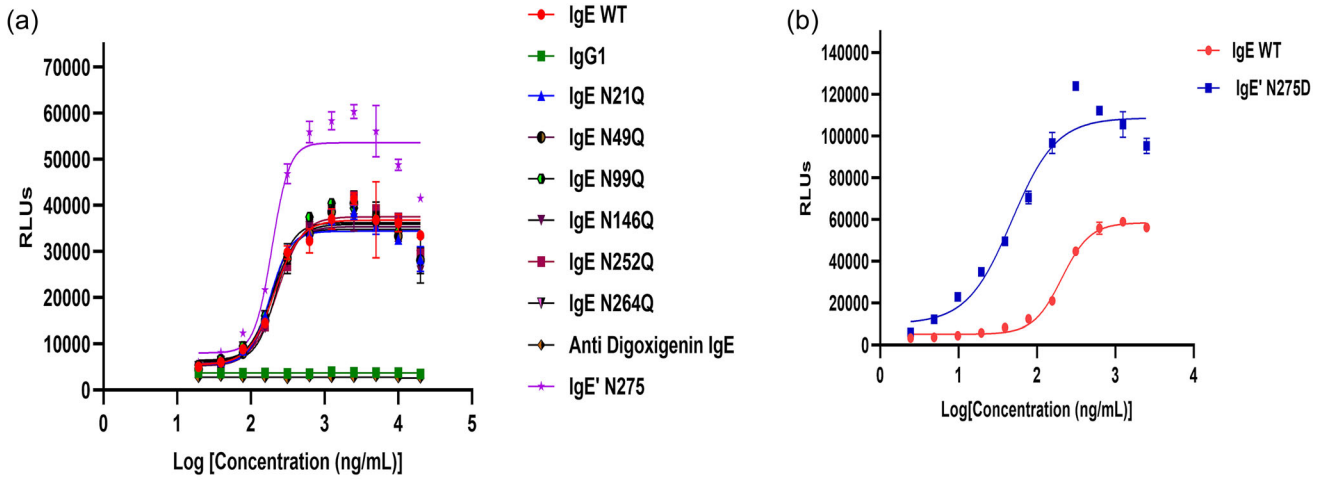
Her2 binding

Protein	IgE	IgE N21Q	IgE N49Q	IgE N99Q	IgE N146Q	IgE N252Q	IgE N264Q	IgE' N275	IgE' N275D	IgG
EC50 (nM)	3.52 ± 0.49	2.86 ± 0.08	2.85 ± 0.03	2.79 ± 0.72	2.76 ± 0.35	3.34 ± 0.74	3.38 ± 0.35	2.27 ± 0.21	2.58 ± 0.04	2.16 ± 0.07

FIGURE 7 Target cell binding and effector function of engineered Trastuzumab IgE variants. Flow cytometry analysis using the different concentrations of IgE WT, IgE glycovariants, anti-Digoxigenin IgE, and Trastuzumab IgG in the presence of SKBR3 cells overexpressing Her2 receptors shows comparable binding affinities. The data from two biological replicates are plotted.

Information S1: Figure 5). Activation of these receptors by an antibody binding to its target antigen initiates downstream signaling processes. This interaction triggers a calcium ion influx, activating calcineurin, which dephosphorylates NFAT. The dephosphorylated NFAT exposes its nuclear localization signal (NLS), allowing it to enter the nucleus and drive the expression of the luciferase gene. This assay is conceptually similar to the existing FcγR assay used for evaluating the functional activity of IgG antibodies. (Hederman et al., 2023; Sankhala et al., 2024) (Supporting Information S1: Figure 2). As shown in Figure 8a,b, except IgE' N275D, IgE WT and its glycovariants had comparable EC50 values. Surprisingly, IgE' N275D showed higher potency as lower concentrations of the purified antibody elicit strong luciferase activity indicating that glycosylation of IgE is dispensable for superior effector function. In addition, the presence of the aspartic acid at the conserved 275 position might induce conformation changes in the CH_3 domain resulting in a stable binding of this molecule with receptors causing prolonged intracellular signaling.

This study addresses the functional importance of the N-linked glycosylation residues in IgE antibodies through structure-function analysis. An earlier study demonstrated an essential role of IgE glycosylation in the FcεR1 interaction and effector function such as initiation of anaphylaxis (Shade et al., 2015). Our analysis shows that glycosylation of the conserved asparagine residue at the 275th position is critical for the intracellular assembly, thermostability, and secretion of the antibody, thereby influencing the overall yield and stability of the molecule. Furthermore, biophysical and cell-based assays using purified recombinant IgE glycovariants demonstrate a minimal impact of N-linked glycosylation on target binding, receptor binding, and effector functions. Surprisingly, compared to the IgE WT, deglycosylated IgE (IgE' N275D) exhibited superior biological function, contradicting the notion that glycans at 275th position are crucial for the biological function of IgE. The flow cytometry-based binding assay of IgE WT and IgE' N275D with Jurkat Lucia NFAT FcεR1 cells show reduced maximum binding capacity of IgE' N275D antibody with the FcεR1 receptors and similar EC50 compared to IgE WT (Supporting Information S1: Figure 6). This aligns with the in vitro binding assay using SPR assay (Figure 6). IgE WT and IgE' N275D exhibit similar target binding affinities in HER2-expressing SKBR3 cells (Figure 7). Interestingly, in the FcεR1 activation assay, we found that IgE' N275D dependent co-engagement of Jurkat Lucia NFAT FcεR1 and SKBR3 cells results in higher luciferase activity implying higher potency and maximal activation efficacy. We hypothesized this could be due to the increased affinity of the target cell-bound antibody with FcεR1 due to conformational changes. This may stabilize the target cell bound IgE' N275D- Jurkat Lucia NFAT FcεR1 complex leading to sustained signaling, thus resulting in increased luciferase production. The literature also indicates that the removal of mannose may result in a slight change in the overall secondary structure, which in turn alters human IgE function (Shade



Protein	IgE	IgE N21Q	IgE N49Q	IgE N99Q	IgE N146Q	IgE N252Q	IgE N264Q	IgE' N275
EC50 (nM)	1.22 ± 0.05	1.22 ± 0.20	1.18 ± 0.02	1.04 ± 0.21	1.37 ± 0.11	1.33 ± 0.10	1.11 ± 0.10	1.21 ± 0.07

Protein	IgE	IgE' N275D
EC50 (nM)	1.44 ± 0.28	0.26 ± 0.03

FIGURE 8 Analysis of the effector function of engineered Trastuzumab IgE variants. SKBR3 cells were incubated with IgE WT, IgE glycovariants, and anti-Digoxigenin IgE in the presence of Jurkat NFAT Lucia cells overexpressing the FcεRI receptor to determine the EC50 values by luciferase assay. The data from two biological replicates are plotted and the experiment was done in triplicates.

et al., 2015). This data represents, to our best knowledge, the first example of a customized Jurkat Lucia NFAT cell line stably expressing the FcεRI receptor to facilitate functional validation of engineered IgE antibodies.

For bioproduction, an IgE molecule with low glycosylation complexity is desirable to minimize batch-to-batch variability and enhance therapeutic efficacy. Our analysis has established two functional antibodies, one which lacks glycosylation (IgE' N275D) and the other with reduced glycans complexity at the Asn 275th position (IgE' N275), as candidate molecules for further development as biotherapeutics. Overall, our study is likely to facilitate the development of IgE-based therapeutics in the future.

3 | MATERIALS AND METHODS

3.1 | Cell lines

Expi 293™ cells, SKBR3 cells, and Jurkat Lucia NFAT FcεR1 cells were cultured and maintained in Expi 293 expression medium (Gibco; cat#A1435101), McCoy's 5 A medium (Gibco; cat#16600082) supplemented with 10% FBS (Gibco; cat# 10099141), and IMDM medium (Gibco; cat#12440053) supplemented with 10% FBS, respectively.

3.2 | Cloning and site-directed mutagenesis

The variable regions of the heavy and light chains of IgE and IgG were synthesized as gene fragments (GeneArt), PCR amplified, and cloned into a pTT5 vector containing the constant regions of IgE and IgG using the Golden Gate method. Mutations in the IgE constant heavy chain were introduced using a Quick Change Lightning Site-directed Mutagenesis kit (Agilent; cat# 210514). All plasmid clones were confirmed using Sanger sequencing. For transfection, plasmid DNA was purified using a Maxi prep kit (MDI membrane technologies, cat# QXEK).

3.3 | IgE production, purification, and biochemical characterization

Expi 293™ cells at the cell density 4*10^6 cells/mL in 100 mL volume were transfected using plasmids encoding Trastuzumab IgE heavy chain and kappa light chain in 1:1 ratio, as per manufacturer's protocol (ExpiFectamine™ 293 Transfection Kit; Cat# A14524). Antibodies were purified from the supernatant using fast protein liquid chromatography (FPLC) on an AKTA Pure™ system with a 5-mL HiTrap ™ Protein L column (Cytiva; cat# 17547815). GFC was performed using a HiLoad 16/600 200 pg superdex preparative column (Cytiva, cat# 28989335). Analytical size exclusion chromatography was performed using TSK gel SuperSW3000 4.6 mm x 30 cm column and 0.4 M perchlorate in 50 mM sodium phosphate buffer (pH 6.3) at a flow rate of 0.35 mL/min on a Shimadzu HPLC. The purified antibody yield was quantified using a spectrophotometer at 280 nm and the final values were extrapolated as mg/L and protein quality was analysed using SDS-PAGE. The intact mass of the IgE' N275D molecule was determined using mass spectrometry.

The thermal unfolding curves of Trastuzumab IgE WT and its glycovariants were generated using the Prometheus NT.48

instrument with a temperature range set between 21°C and 95°C, and a heating increment of 0.5°C/min.

For the SPR assay, 1 μg/mL of FcεR1 receptor (R&D systems; cat# 6678) was immobilized on the CM5 sensor chip (GE Healthcare; cat#BR100530), and IgE glycovariants were passed over it at a flow rate of 50 μL/min with 120 s contact time and 600 s dissociation time. Regeneration was performed by glycine (pH 1.5) at a flow rate of 30 μL/min. The CD23 receptor (R&D systems; cat#123-FE) was immobilized at 5 μg/mL and IgE glycovariants were passed over it with 120 s contact time and 120 s dissociation time at a flow rate of 50 μl/min. Regeneration was done using glycine (pH 2) at flow rate of 30 μL/min.

3.4 | Flow cytometry analysis

For this assay, HER2-expressing SKBR3 cells were used as target cells for Trastuzumab IgE binding. Cells were seeded at 50,000 cells/100 μL of cell staining buffer in 96 well plates and incubated with varying concentrations (from 19.53 ng/mL to 20 ug/mL) of Trastuzumab IgE and the glycovariants. After incubation for 1 h at 4°C, the plate was washed twice with cell staining buffer, treated with 0.4 μg/mL of phycoerythrin (PE) conjugated mouse anti-human IgE secondary antibody (Thermo Scientific; cat# MA1-10375) and incubated for 1 h at 4°C. Thereafter, the cells were washed and resuspended in 200 μL of cell staining buffer. The samples were analyzed in ACEA NovoCyte and the data was analyzed using FlowJO software. A secondary antibody alone was added in one well to check for any nonspecific binding. PE-conjugated anti-human IgG (Abcam; 1:1000) was used for the detection of Trastuzumab IgG. The experiment was repeated twice, and the data was plotted using GraphPad Prism.

3.5 | FcεR1 activation assay

In this assay, HER2-expressing SKBR3 cells were seeded at the density of 50,000 cells/100 μL of McCoy's 5 A medium in 96 well plates and treated with different concentrations of Trastuzumab IgE antibodies and its glycovariants (from 19.53 ng/mL to 20 ug/mL) and for IgE' N275D (1.2 ng/mL to 2.5 μg/mL). Jurkat Lucia NFAT FcεR1 effector cells were added to the medium at a density of 150,000 cells/100 μL and the plate was incubated at 37°C for overnight. Next, the supernatant was mixed with QUANTI-Luc™ reagent (Invivogen; cat# rep-qlc2) in a ratio of 1:1, and luminescence was measured using a luminometer. The assay was done in triplicates and the data was analyzed using GraphPad Prism.

3.6 | RNA isolation and cDNA preparation

mRNA was isolated using RNeasy (+) mini kit (Qiagen; cat# 74104) and cDNA was prepared using 1 μg of mRNA using iScript cDNA Synthesis kit (BioRad; cat# 1708891) as per manufacturer's protocol.

3.7 | Western blot analysis

Cells transfected with plasmid constructs were harvested at 4000 rpm and the supernatant was filtered and collected for small-scale antibody purification. The cell pellet was washed twice with 1× PBS, resuspended in 1 mL of RIPA lysis buffer followed by centrifugation to collect the cell lysate in a separate tube and the cellular proteins were separated under reducing conditions in SDS PAGE, transferred to nitrocellulose membrane using Transblot turbo and the blot incubated in blocking buffer (5% skimmed milk in 1× TBST) for overnight. The membrane was next incubated with mouse anti-IgE mAb (Abcam; cat# ab7294) at 1:1000 and Peroxidase AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG, $F(ab')_2$ fragment specific (pAb) (Jackson ImmunoResearch; Dilution: 1:2500; cat# 109-036-097) for 1 h at RT. Post incubation, the membrane was washed thrice using 1× PBST and developed using the enhanced chemiluminescent kit (ThermoFisher; cat#34075). Anti-alpha tubulin was used as a loading control. The primary and secondary antibodies were (pAb) Rabbit alpha-tubulin (2144) (CST) (1:1000) and (pAb) Goat Anti-Rabbit IgG H&L (HRP) (ab6721) (1:5000) respectively.

3.8 | SDS PAGE mobility shift assay

Ten micrograms of Trastuzumab IgE and glycovariants were mixed with GlycoBuffer 2 in a 20 μL total reaction volume. Five microliters PNGase F (NEB; cat# P0704S) was added to the mixture, mixed gently, and incubated at 37°C overnight. The samples were separated using SDS PAGE to assess the deglycosylation.

3.9 | Jurkat Lucia NFAT FcεR1 stable cell line generation

Jurkat-Lucia™ NFAT (Invivogen; cat# jktl-nfat) was used to establish a cell line constitutively expressing the FcεR1 receptor. The DNA encoding the alpha, beta, and gamma regions of the FcεR1 high-affinity receptor was PCR amplified from the synthesized gene fragments and cloned into the pLVX-IRES-Puro plasmid. Self-cleaving peptides (P2A and T2A) were used in the construct to generate multiple polypeptides from the same transcript. The cloning was performed using the Golden Gate DNA assembly method. HEK293TN cells were used for pseudotyping the lentiviruses for transduction at 48 and 72 h. The production of viruses was confirmed qualitatively using Lenti-X™ GoStix™ Plus (Takara; cat# 631280). Viral transduction was done based on the TransDux™ MAX Lentivirus Transduction kit protocol (System Biosciences; cat# LV860A-1). Puromycin-based selection was done to obtain the positive recombinant clones. The cells were sorted using FITC conjugated Anti-human FcεR1 detection antibody (BioLegend; cat# 334608) to obtain receptor-expressing clones.

3.10 | HILIC-ESI-QToF mass spectrometry

The sample preparation was done using Glycoworks RapidFlour-MS N glycan Kit (Waters; cat#176003603). Fifteen micrograms of the antibody samples were treated with RapiGest SF and incubated at 90°C for 3 min. Post incubation the samples were treated with 1.2 µL of PNGase F and incubated at 50°C for 5 min. Post incubation each sample was labeled with RapidFluor-MS. The labeled samples were cleaned using HILIC plates where glycans were eluted in solid phase extraction (SPE) elution buffer. Ten microliters of each sample was loaded and data was acquired on Waters™ Acquity ™ Premier equipped with an FLR detector and Waters™ Xevo G3 QTof (Electrospray Ionization). The data was analyzed using UNIFI Version 3.6.0.21

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualization, planning, and analysis of the experiments. Shikha Kumari conducted all experiments and collected the data. Shikha Kumari drafted the initial manuscript, which was subsequently edited by Sanjay Ghosh and Achim Doerner The manuscript underwent a thorough review and received approval from all named authors.

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CONFLICT OF INTEREST STATEMENT

The authors are affiliated with IBAB, Merck Healthcare KGaA, or Syngene International Limited, and confirm that there are no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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