



Modulation of regulatory T cells by intranasal allergen immunotherapy in an experimental rat model of airway allergy



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ABSTRACT

Allergic airway diseases such as asthma and allergic rhinitis are increasing in prevalence worldwide. The theory of an altered Th1/Th2 balance in allergic diathesis has recently been termed a “procrustean paradigm” as it failed to explain many preclinical findings. Regulatory T cells (Treg) have now been shown to be critical in T-cell homeostasis and in the maintenance of peripheral tolerance to allergens. Allergen specific immunotherapy (SIT) has been shown to induce regulatory T cells in allergic patients. Among various types of SIT, intranasal immunotherapy had not been studied in detail for the treatment of allergic airway diseases. So, there was a need to study the contribution of regulatory T cells and their mechanistic pathways following intranasal immunotherapy *in vivo*. It had been previously shown that intranasal allergen immunotherapy using *Alstonia scholaris* pollen extract abrogates allergic airway inflammation with decline in IgE and Th2 cytokine levels. The present study for the first time offers a multi-targeted approach towards attenuation of airway allergy by the generation of CD4 + CD25 + Foxp3 + T cells and other subsets of Treg cells like Tr1 cells, Th3 cells, CTLA4 + Treg cells, and also modulation of various Treg cell surface molecules like GITR, OX40, CD39 and CD73 by intranasal immunotherapy in the same animal model. This animal experiment will thus help to chart out newer molecular targets for treating allergic asthma or rhinitis.

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1. Introduction

Allergic asthma is a Th-2 mediated disorder which is characterized by production of allergen-specific IgE, reversible airflow obstruction, airway hyper-responsiveness (AHR) to a wide variety of specific or non-specific stimuli, chronic airway inflammation and airway remodeling [1,2]. This abnormal behavior of airway cells is due to dysregulated immune homeostasis in the airways [3]. There exists compelling evidences that regulatory T cells (Tregs) are essential in the maintenance of immune homeostasis in the airways [4]. Subsets of Treg cells include naturally occurring, thymic derived CD4 + CD25 + Treg cells, inducible CD4 + CD25 + T cells, IL-10 producing Treg cells (Tr1 cells), and TGF β producing Th3 type Treg cells. Naturally occurring CD4 + CD25 + Treg

cells have been shown to express a variety of cell surface molecules that include CD25, CD45RB^{low}, CD62L, CTLA-4, GITR, OX-40 and most specifically the transcription factor Foxp3. Foxp3 functions as a master switch gene in the development and function of Treg cells [5]. The development of an allergic response to common inhaled allergens has been postulated to occur as a consequence of impairment in the numbers, function, or both of allergen specific Treg cells [6–8].

Allergen-specific immunotherapy (SIT) has been shown to improve allergen dysfunction and redirect the immune system away from allergic response and this has been shown to be associated with increased or restored Treg functions [9,10]. But the various pathways of Treg mediated suppression of allergic inflammation in SIT needs further elucidation.

In our previous study, we have developed a therapeutic intranasal immunotherapy rat model using *Alstonia scholaris* pollen and have shown that it effectively attenuates allergic airway inflammation [11]. In the present work, we have studied the effect of intranasal immunotherapy on Foxp3 expression in CD4 + CD25 + regulatory (Treg) cells

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and other surface molecules on Treg cells as a mechanism for achieving peripheral T-cell tolerance using the same animal model of *Alstonia scholaris* pollen allergy.

2. Materials and methods

2.1. Reagents and chemicals

DAPI, fetal bovine serum, poly-L-lysine, RPMI-1640, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) and Histopaque-1077 were purchased from Sigma-Aldrich, USA. Monoclonal primary antibodies specific to anti-CD25 (SC 664), anti-Foxp3 (SC 31738), anti-TGF β (SC 146), anti-GITR (SC 34906), anti-OX40 (SC 10943), anti-CTLA4 (SC 70894), anti-CD39 (SC 18771), anti-CD73 (SC 14684) and β -actin (SC 47778) were purchased from Santa Cruz Biotechnology, Santa Cruz, California, USA and monoclonal primary antibodies specific to anti-IL10 (555083), anti-CD4 (554835) was purchased from BD Biosciences. All other chemicals were purchased from local suppliers and were of the highest purity grade.

2.2. Preparation of *Alstonia scholaris* pollen extract

Fresh pollen samples were collected from the flowering plants of *Alstonia scholaris* during the flowering season (September to November). The pollens were then dried in hot air oven (35–40 °C) and extracted in phosphate buffered saline as described previously [11]. The protein content of pollen extract was determined following Lowry's method.

2.3. Animals and animal grouping

Albino Wistar rats of same age group, and body weight 100–120 g was obtained from a recognized laboratory animal breeder. Rats were housed in polypropylene cages at an ambient temperature of 25–30 °C and 45–55% relative humidity with a 12 h each of dark and light cycle. All the rats were given a 15 day period of acclimatization before starting the experiment. Rats were fed pellet diet and water ad libitum. Animal housing, care and the conduct of experimental procedures were done in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals guidelines in India.

The animals were grouped into four groups, as detailed in our previous study [11]. To summarize:

Sensitization phase

Group-I (normal group/vehicle control group): Three consecutive i.p. injections of saline (1 ml) at 0, 7 and 14 day intervals.

Group-II (alum group/adjuvant control group): Three consecutive i.p. injections of aluminium hydroxide (20 μ g/g) in 1 ml of saline at 0, 7 and 14 day intervals.

Group III (*Alstonia* group): Three consecutive i.p. injections of *Alstonia scholaris* pollen extract (1 μ g/g) and aluminium hydroxide (20 μ g/g) in 1 ml of saline were administered at 0, 7 and 14 day intervals.

Challenge phase

On day 50, 72 and 48 h before the sacrifice, rats from Group III were given intranasal (i.n.) challenges with *Alstonia scholaris* pollen extract (0.6 μ g/g) in 50 μ l of saline. Groups I and II received 50 μ l of intra nasal saline challenge [11].

Immunotherapy protocol

Group IV (intranasal immunotherapy group): Three consecutive i.p. injections of *Alstonia scholaris* pollen extract (1 μ g/g) and aluminium hydroxide (20 μ g/g) in 1 ml of saline were administered at 0, 7 and 14 day intervals. These rats further received immunotherapy on days 19, 22, 26, 33, 36 and 40 with the following increasing dose of *Alstonia scholaris* pollen extract (0.04 μ g/g, 0.2 μ g/g, 1 μ g/g, 5 μ g/g, 25 μ g/g, 40 μ g/g and 40 μ g/g) in 1 ml of saline via intranasal route and then on day 50, 72 and 48 h before the sacrifice, rats were given intranasal (i.n.) challenges

with *Alstonia scholaris* pollen extract (0.6 μ g/g) in 50 μ l of saline as detailed before [11].

2.4. In situ-immunofluorescence analysis

The lungs were dissected out from the rat and fixed in 10% formalin and embedded in paraffin for in situ immunofluorescence. Five-micrometer paraffin sections were cut on poly-L-lysine coated slides and incubated overnight at 37 °C. The slides were deparaffinised with xylene (2 $\times$ 3 min) after which it was passed thrice through dehydration steps with 100%, 95%, 70%, and 50% ethanol each for 5 min. The slides were then washed with ddH₂O until they were ready for antigen retrieval. After this step, the slides were dipped in the antigen retrieval buffer (1% trypsin, 0.5 M calcium chloride, pH 7.8) [11]. They were then washed with ice-cold PBS and then the sections were dipped in the blocking solution for 2 h at room temperature (2% BSA in PBS) followed by overnight incubation at 4 °C in anti-FOXP3 primary antibody solution (1:200 dilutions in PBS with 1% BSA) in a humidified chamber. This is followed by washing of tissue sections thrice (15 min per wash) with 5–10 ml of PBS followed by incubation with TRITC fluorescence-conjugated secondary antibody solution (dilution range 1:400 in TBS containing 1% BSA) for 2 h at room temperature in darkness.

The sections were again washed thrice (15 min per wash) with PBS for removal of excess antibody from the slide with PBS. Excess buffer was blotted from the slide with an absorbent wipe. The cover slips were mounted on slides using 2 μ l of mounting media with DAPI to visualize the nuclei. Immunofluorescent images were captured with a Nikon Trinocular phase-contrast microscope Model E-200 fitted with a DPI digital camera and appropriate fluorescence filters. The acquisition software used was NIS Elements (Nikon) [11].

2.5. Isolation of splenic lymphocyte

Briefly, the whole spleen were dissected out from the rat, then it was mechanically minced and passed through an 80-gauge wire mesh and incubated in petri dishes with PBS for 1 h at 37 °C in 4% CO₂ environment. The non-adherent cells were collected and washed with PBS. Resulting cell suspensions were layered on Histopaque 1077 density gradient and subjected to density gradient centrifugation for 20 min at 1700–1800 rpm. Lymphocyte layers were carefully removed from the buffy layer interface. Cells from this layer were washed thrice and suspended in RPMI-1640 for further analysis. Purity of isolated lymphocytes was characterized by identifying CD3 marker on the cells in the isolated populations by flow cytometry [11,12].

2.6. Labeling and gating of cells for the flowcytometric analysis

Multiple staining was done for the flowcytometric analysis. Isolated splenic lymphocytes were washed and resuspended in PBS. For analyzing cell surface protein expression on T-cells, 1 $\times$ 10⁷ cells/ml were incubated with 5 μ l of diluted (1:250) primary monoclonal antibodies i.e. anti-CD4. The cells were centrifuged, washed, resuspended in PBS, and then incubated with secondary FITC-conjugated secondary antibody for 30 min in the dark. Then the cells were incubated with 5 μ l of diluted (1:250) primary monoclonal antibodies anti-CD25. The cells were again centrifuged, washed, resuspended in PBS, and then incubated with PE-conjugated secondary antibody for 30 min in the dark. After this step, cells were treated with paraformaldehyde (0.3% in PBS) at 4 °C for 45 min. Then, the cell suspension was washed with cold PBS and permeabilized with Triton X-100 (0.5%) for 1 h at 4 °C. After centrifugation, the pellet was washed twice with cold PBS and resuspended in 200 ml PBS. After permeabilization cells were then incubated with 5 μ l of diluted (1:250) monoclonal anti-Foxp3 at a concentration of 1:250 for 30 min at 4 °C. Finally, the cells were again centrifuged, washed, resuspended in PBS, and then incubated with secondary PerCP-conjugated secondary antibody for 30 min in the dark. Similar tagging procedure was followed

for other cell surface proteins like IL10, TGF β , GITR, OX40, CTLA4, CD39 and CD73.

For analysis, desired cell population on dot plot SSC versus FSC were gated and analyzed for CD4 and CD25 co-expression. Double positive cells from dot plot CD4 + FITC (FL1) versus CD25 + PE (FL2) were again gated and analyzed for Foxp3 expression in the next dot plot i.e. SSC versus Foxp3 conjugated with PerCP (FL3). The population of cells in the lower right quadrant was the desired population i.e. CD4 + CD25 + Foxp3 + Cells.

Similarly, for CD4 + CD25 + IL10 + Cells, double positive cells from dot plot CD4 + FITC (FL1) versus CD25 + PE (FL2) were again gated and analyzed for IL10 expression in the next dot plot i.e. SSC versus IL10 conjugated with PerCP (FL3). The population of cells in the lower right quadrant was the desired population i.e. CD4 + CD25 + IL10 + Cells.

For CD4 + CD25 + TGF β + Cells, double positive cells from dot plot CD4 + FITC (FL1) versus CD25 + PE (FL2) were again gated and analyzed for TGF β + expression in the next dot plot i.e. SSC versus TGF β + conjugated with PerCP (FL3). The population of cells in the lower right quadrant was the desired population i.e. CD4 + CD25 + TGF β + Cells.

For CD4 + Foxp3 + GITR + Cells, double positive cells from dot plot CD4 + FITC (FL1) versus Foxp3 + PerCP (FL3) were again gated and analyzed for GITR expression in the next dot plot i.e. SSC versus GITR conjugated with PE (FL2). The population of cells in the lower right quadrant was the desired population i.e. CD4 + Foxp3 + GITR + Cells.

For CD4 + CD25 + OX40 + Cells, double positive cells from dot plot CD4 + FITC (FL1) versus CD25 + PE (FL2) were again gated and analyzed for OX40 expression in the next dot plot i.e. SSC versus OX40 conjugated with PerCP (FL3). The population of cells in the lower right quadrant was the desired population i.e. CD4 + CD25 + OX40 + Cells.

For CD4 + CD25 + CTLA + Cells, double positive cells from dot plot CD4 + FITC (FL1) versus CD25 + PE (FL2) were again gated and analyzed for CTLA4 expression in the next dot plot i.e. SSC versus CTLA4 conjugated with PerCP (FL3). The population of cells in the lower right quadrant was the desired population i.e. CD4 + CD25 + CTLA + Cells.

For CD4 + Foxp3 + CD39 + Cells, double positive cells from dot plot CD4 + FITC (FL1) versus Foxp3 + PerCP (FL3) were again gated and analyzed for CD39 expression in the next dot plot i.e. SSC versus CD39 conjugated with PE (FL2). The population of cells in the lower right quadrant was the desired population i.e. CD4 + Foxp3 + CD39 + Cells.

For CD4 + Foxp3 + CD73 + Cells, double positive cells from dot plot CD4 + FITC (FL1) versus Foxp3 + PerCP (FL3) were again gated and analyzed for CD73 expression in the next dot plot i.e. SSC versus CD73 conjugated with PE (FL2). The population of cells in the lower right quadrant was the desired population i.e. CD4 + Foxp3 + CD73 + Cells.

The percent expression of each protein was flowcytometrically assessed in FACS Calibur Instrument (BD Biosciences, USA) using Cell Quest Pro software. For each sample, 40,000 events were acquired and analyzed.

2.7. Immunoblot analysis

Splenic lymphocytes were isolated on Histopaque 1077 and were lysed in NP-40 lysis buffer (mM MgCl₂, 4 mM CaCl₂, 10mMTris, pH 7.5, and 0.4% NP-40 with protease inhibitors). Briefly, 100 μ g of protein was loaded on to each well and were separated on 10% polyacrylamide gels and transferred to PVDF membranes (millipore) in blot buffer (192 mM glycine; 25 mM Tris-base; 20% (v/v) methanol; 0.04% SDS). Membranes were incubated in 5% nonfat skimmed milk in TBST blocking solution (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5% (w/v) skimmed milk powder; 0.02% sodium azide; 0.1% Tween 20) for 1 h followed by incubation with monoclonal anti-Foxp3 (1:5000), on a rocking platform overnight at 4 °C [13]. Membranes were washed thrice in TBST washing solution (20 mMTris-HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20) and then incubated with AP conjugated secondary antibody (1:15,000).

Bands were developed by alkaline phosphatase methods (substrate buffer: 100 mMTris-HCl, pH 9.5; 100 mM NaCl; 5 mM MgCl₂, BCIP staining solution: 20 mg/ml in 100% di-methyl formamide and NBT staining solution: 50 mg/ml in 70% di-methyl formamide). For each staining solution procedure used the substrate buffer containing 80 μ l BCIP solution and 60 μ l NBT solution per 10 ml is mixed freshly for each staining. Band detection and pixel intensity count were done by using ImageJ 1.50b (NIH, USA) software. Bar graphs are provided adjacent to immunoblot figures.

2.8. Statistical analysis

Data of protein expression level by flowcytometry and Immunoblot were analyzed using factorial one-way ANOVA. Benferroni's post-hoc test was applied to the data: a value of $p < 0.001$ following this post-hoc procedure was considered statistically significant. All results were evaluated statistically by applying the GraphPad Prism software (Prism 4 for Windows, Version 4.03).

3. Results

3.1. Allergen sensitization-challenge attenuates Foxp3 + regulatory T cells which is increased following intranasal immunotherapy

CD4 + CD25 + Foxp3 + regulatory T cells have been shown to play a critical role in control of immune responses [14]. Various studies have shown that subcutaneous immunotherapy (SIT) increases CD4 + CD25 + Foxp3 + regulatory T cells (Treg) both locally [15] and systemically [16].

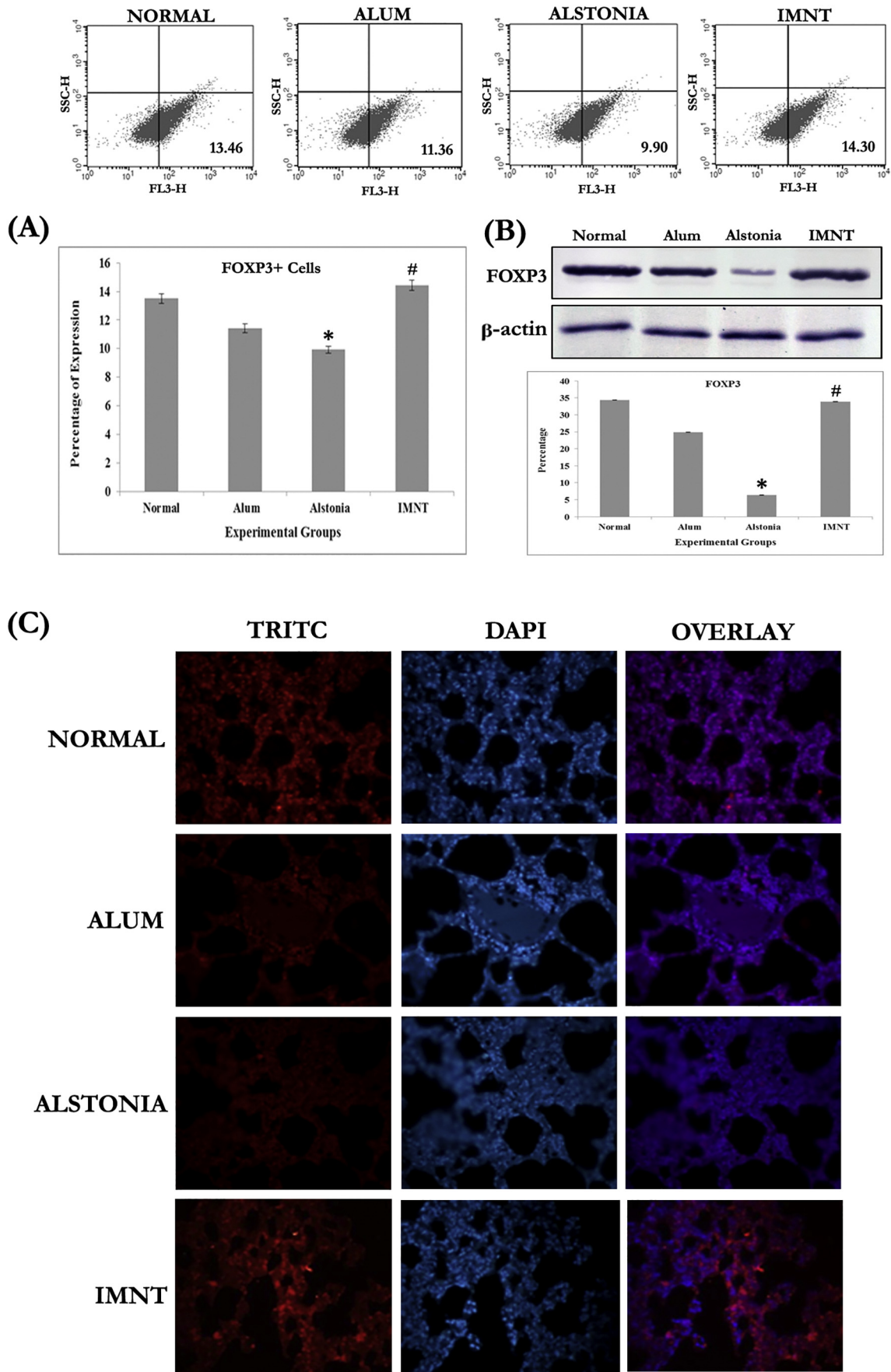
Flowcytometric analysis [$F(3,20)=251.47$] revealed (Fig. 1A) a significant decrease ($p < 0.001$) in CD4 + CD25 + Foxp3 + Treg cells after *A. scholaris* sensitization and challenge (9.925 ± 0.244) as compared to normal group (13.511 ± 0.336) and adjuvant (alum) group (11.433 ± 0.318). Intranasal immunotherapy with *A. scholaris* pollen extract significantly increased ($p < 0.001$) the frequency of CD4 + CD25 + Foxp3 + Treg cells as compared to the Alstonia group (14.446 ± 0.318).

Immunofluorescence imaging studies corroborated with the data of flowcytometry. The Alstonia group exhibiting much lower expression of Foxp3 than the normal or the adjuvant (alum) group as is evident from the low fluorescence intensity observed. In contrast specific allergen immunotherapy via intranasal route caused increased expression of Foxp3 in splenic lymphocytes as is shown by marked increase in intensity of fluorescence (Fig. 1C).

Similar results from immunoblot experiments (Fig. 1B) [$F(3,20)=3081.3$] further strengthened our finding that there was significant decrease ($p < 0.001$) in Foxp3 expression in Alstonia group (6.435 ± 0.056) as compared to normal group (34.425 ± 0.056) and adjuvant (alum) group (24.901 ± 0.056) while intranasal immunotherapy significantly increased Foxp3 expression (33.933 ± 0.058).

3.2. Intranasal allergen immunotherapy increases CD4 + CD25 + IL10 + Tr1 cells

Apart from CD4 + CD25 + Foxp3 + Treg cells, interleukin-10 (IL 10) producing Type 1 regulatory T cells (Tr 1) also play important role in the control of allergic inflammation [17]. IL10 expression in CD4 + CD25 + T cells by flowcytometric analysis (Fig. 2) [$F(3,20)=2445.7$] revealed a significant decrease ($p < 0.001$) in Alstonia group (1.876 ± 0.054) as compared to normal group (2.716 ± 0.058) and adjuvant (alum) group (2.566 ± 0.067). Intranasal immunotherapy with *A. scholaris* pollen extract significantly increased ($p < 0.001$) the IL10 expression in CD4 + CD25 + T cells as compared to the Alstonia group (4.765 ± 0.064).



3.3. CD4 + CD25 + TGF β + T cells are attenuated following *Alstonia scholaris* sensitization and challenge which is reversed by intranasal allergen immunotherapy

Transforming growth factor β (TGF β) is a suppressive cytokine which is involved in the control of atopic conditions. Experimental studies have further shown that antigen induced tolerance requires cell-cell contact with TGF β + Foxp3 + Tregs [18]. TGF β expression in CD4 + CD25 + T cells by flowcytometric analysis (Fig. 3) [F(3,20)=1589.3] revealed a significant decrease ($p < 0.001$) in *Alstonia* group (2.216 ± 0.082) as compared to normal group (5.045 ± 0.105) and adjuvant (alum) group (3.78 ± 0.090). Intranasal immunotherapy with *A. scholaris* pollen extract significantly increased ($p < 0.001$) the TGF β expression in CD4 + CD25 + T cells as compared to the *Alstonia* group (5.621 ± 0.091).

3.4. Treg mediated suppression is partly accomplished by CTLA4 dependent mechanism following intranasal immunotherapy

There are now increasing evidences that cytotoxic T lymphocyte antigen 4 (CTLA4) plays a major role in regulating T cell tolerance [19,20]. Treg cells expressing CTLA4 can downregulate CD80 and CD86 on antigen presenting cells and thus prevent unnecessary immune stimulation [21].

Flowcytometric analysis revealed (Fig. 4) [F(3,20)=1134.8] a significant decrease ($p < 0.001$) in CD4 + CD25 + CTLA4 + T cells after *A. scholaris* sensitization and challenge (1.065 ± 0.098) as compared to normal group (2.543 ± 0.067) and adjuvant (alum) group (1.768 ± 0.070). Intranasal immunotherapy with *A. scholaris* pollen extract significantly increased ($p < 0.001$) the frequency of CD4 + CD25 + CTLA4 + T cells as compared to the *Alstonia* group (3.635 ± 0.079).

3.5. Intranasal immunotherapy increases GITR + Treg cells after its diminution in allergic airway disease

Glucocorticoid induced tumor necrosis factor receptor (GITR) is a crucial marker of which enhances Treg proliferation and thus mediates immune tolerance [22].

Flowcytometric analysis revealed (Fig. 5) [F(3,20)=2519.6] a significant decrease ($p < 0.001$) in Foxp3 + GITR + Treg cells in *Alstonia* group (0.945 ± 0.066) as compared to normal group (2.615 ± 0.044) and adjuvant (alum) group (1.645 ± 0.061). Intranasal immunotherapy significantly increased ($p < 0.001$) the frequency of Foxp3 + GITR + Treg cells as compared to the *Alstonia* group (3.973 ± 0.078).

3.6. Allergen sensitization-challenge increases OX40 expression on Treg cells which decreases following intranasal immunotherapy

OX40 is a recently identified cell surface molecule which is constitutively expressed by CD4 + CD25 + Treg cells [23]. Stimulation of OX40 causes selective abrogation of Foxp3 + Treg cells by causing marked downregulation of Foxp3 gene expression and thus this molecule is a potent negative regulator of Foxp3 + Tregs [24].

Flowcytometric analysis revealed (Fig. 6) [F(3,20)=24,069] a significant increase ($p < 0.001$) in OX40 expression in CD4 + CD25 + T cells

in *Alstonia* group (4.11 ± 0.014) as compared to normal group (1.103 ± 0.010) and adjuvant (alum) group (2.16 ± 0.037). Intranasal immunotherapy significantly decreases ($p < 0.001$) the OX40 expression in CD4 + CD25 + T cells as compared to the *Alstonia* group (1.423 ± 0.010).

3.7. Inhibitory function of Tregs following intranasal allergen immunotherapy is partly achieved by purinergic mechanisms

Accumulation of adenosine in the immediate pericellular zone is a putative mechanism of Treg mediated suppression [25]. The generation of this extracellular adenosine is regulated by ectonucleotidases CD39 & CD73, and their coexpression is associated with Foxp3 + Treg cells [26].

Flowcytometric analysis revealed (Fig. 7) [F(3,20)=379.09] a significant decrease ($p < 0.001$) in Foxp3 + CD39 + Treg cells in *Alstonia* group (9.043 ± 0.631) as compared to normal group (18.453 ± 0.526) and adjuvant (alum) group (14.836 ± 0.526). Intranasal immunotherapy significantly increased ($p < 0.001$) the frequency of Foxp3 + CD39 + Treg cells as compared to the *Alstonia* group (18.371 ± 0.628).

Similarly, Flowcytometric analysis also revealed (Fig. 8) [F(3,20)=111.48] a significant decrease ($p < 0.001$) in Foxp3 + CD73 + Treg cells in *Alstonia* group (11.04 ± 0.635) as compared to normal group (15.66 ± 0.548) and adjuvant (alum) group (13.345 ± 0.555), which were significantly increased ($p < 0.001$) following intranasal allergen immunotherapy as compared to the *Alstonia* group (16.646 ± 0.581).

4. Discussion

The present study clarifies the role of Tregs during intranasal immunotherapy and for the first time unearths the various mechanisms of Treg induced immunosuppression following intranasal immunotherapy.

We have previously established a sensitization-challenge and intranasal immunotherapy rat model of pollen allergy using *Alstonia scholaris* pollen extract [11]. In our present work we have used this established model to study the modulation of Treg cells by intranasal immunotherapy.

Previous investigators had shown that Foxp3 protein expression within Treg cells is significantly decreased in asthmatic patients [27–29]. So, activation and expansion of CD4 + CD25 + Foxp3 + Tregs and other subtypes of Treg cells represent a novel approach to induce antigen specific tolerance. Subcutaneous immunotherapy being an invasive method, there has been a longstanding interest in the use of lesser painful treatment options like intranasal immunotherapy for the treatment of pollinosis [30–33], but there are paucity of studies which have had investigated the modulation of Treg cells by intranasal allergen specific immunotherapy. We have shown in our study that allergen sensitization and challenge causes reduction in CD4 + CD25 + Foxp3 + Treg cells which is brought to normal by intranasal immunotherapy. Though the role of Treg cells in immune tolerance is well known and we have found similar results in our model, but the mechanism of suppression by these cells still remain contentious despite various postulates [34]. We studied the various mechanisms of Treg mediated suppression following intranasal immunotherapy in our model.

Fig. 1. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on Foxp3 expression in splenic lymphocytes: (A) Flow cytometric analysis of Foxp3 and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 40,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in Foxp3 expression in Tregs in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). Foxp3 expression in Tregs increased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal n = 6 per group). (B) Expression of Foxp3 protein was analyzed by immunoblotting using anti-Foxp3 antibody. Beta-actin was used as loading control. Bands were analyzed densitometrically and pixel intensities of each band are displayed. Results demonstrated that there was a significant (*) decrease in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). Foxp3 expression increased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal n = 6 per group). (C) Study of Foxp3 expression by in-situ immunofluorescent technique shows representative immunofluorescent stained photomicrographs with TRITC tagged Foxp3 (Red) and DAPI stained nuclei which appears blue in normal, alum, *Alstonia* and immunotherapy paraffin embedded lung tissue. In their *Alstonia* group there is marked down regulation of Foxp3 in lung lymphocytes compared to normal and adjuvant control group. After intranasal immunotherapy there is marked up-regulation of Foxp3. Magnification 40 $\times$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

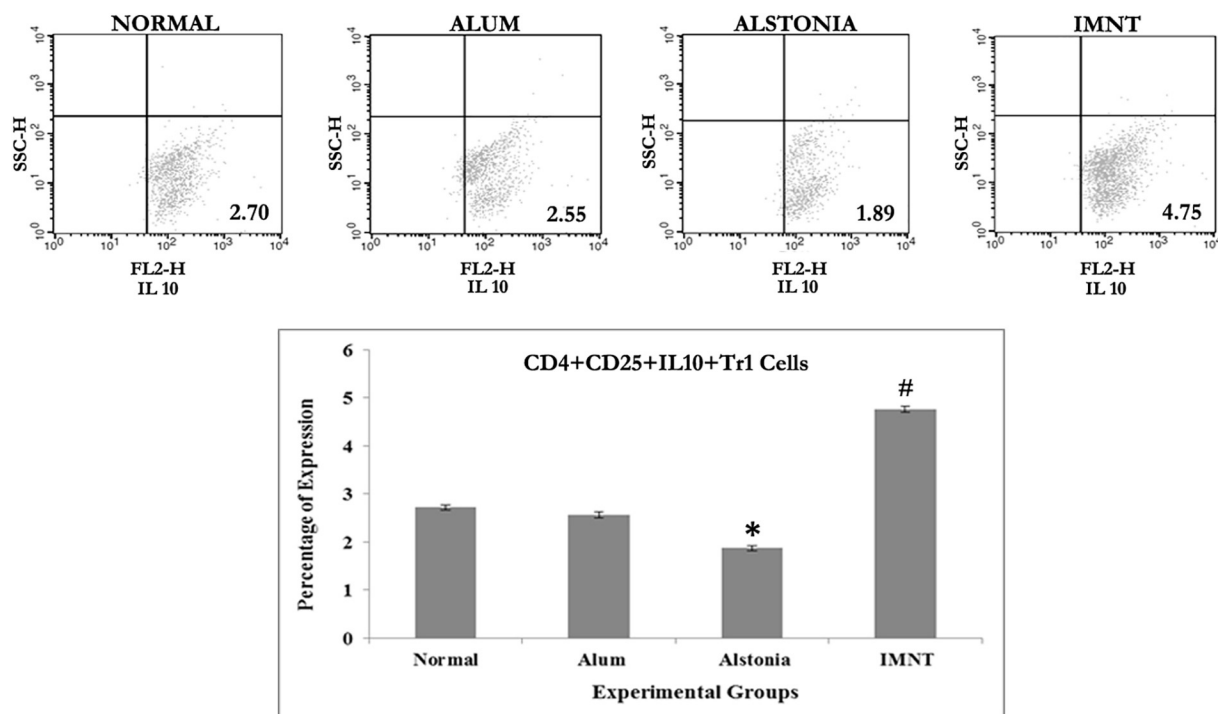


Fig. 2. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on Interleukin 10 (IL10) expression in splenic regulatory T cells (Tregs): Flow cytometric analysis of IL 10 and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 40,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in IL 10 expression in Tregs in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). IL 10 expression in Tregs increased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal $n = 6$ per group).

Apart from the CD4 + CD25 + Foxp3 + Treg we have found decrease in CD4 + CD25 + IL10 + Treg cells following allergen sensitization and challenge in our model and following intranasal

immunotherapy there is an increase in IL10 producing Treg cells. The experimental model result was consistent with low levels of IL10 in bronchoalveolar lavage fluid of adult asthmatic patients as

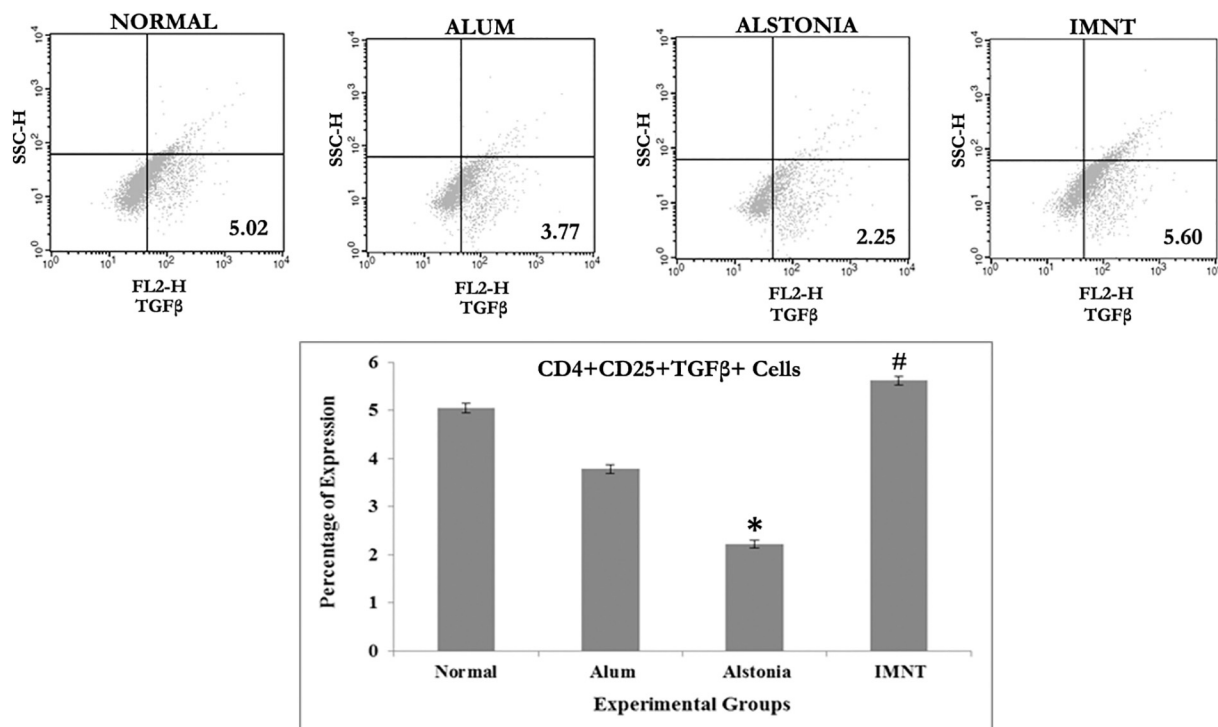


Fig. 3. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on membrane bound TGF β expression in splenic regulatory T cells (Tregs): Flow cytometric analysis of membrane bound TGF β and its percent positive cells are represented in bar diagrams. Percentage of the expression refers to the positive cells out of 40,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in membrane bound TGF β expression in Tregs in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). Membrane bound TGF β expression in Tregs increased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal $n = 6$ per group).

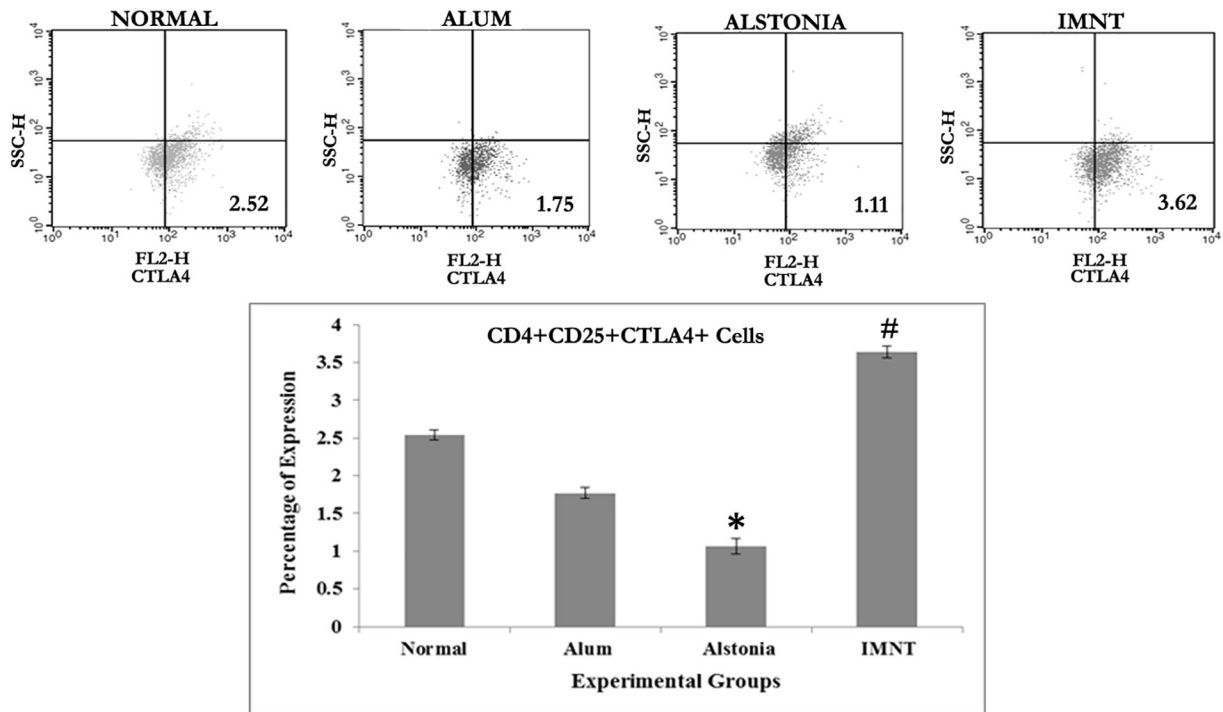


Fig. 4. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on CTLA 4 expression in splenic regulatory T cells: Flow cytometric analysis of CTLA 4 and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 40,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in CTLA 4 expression in Tregs in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). CTLA 4 expression in Tregs increased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal $n = 6$ per group).

compared with healthy controls [35]. Furthermore, it has been shown that specific allergen immunotherapy (SIT) induce CD4 + CD25 + Treg cells and after successful completion of

immunotherapy for one year, the intracellular IL10 positive Tcells increases which was almost exclusively localized to CD4 + CD25 + cells [36].

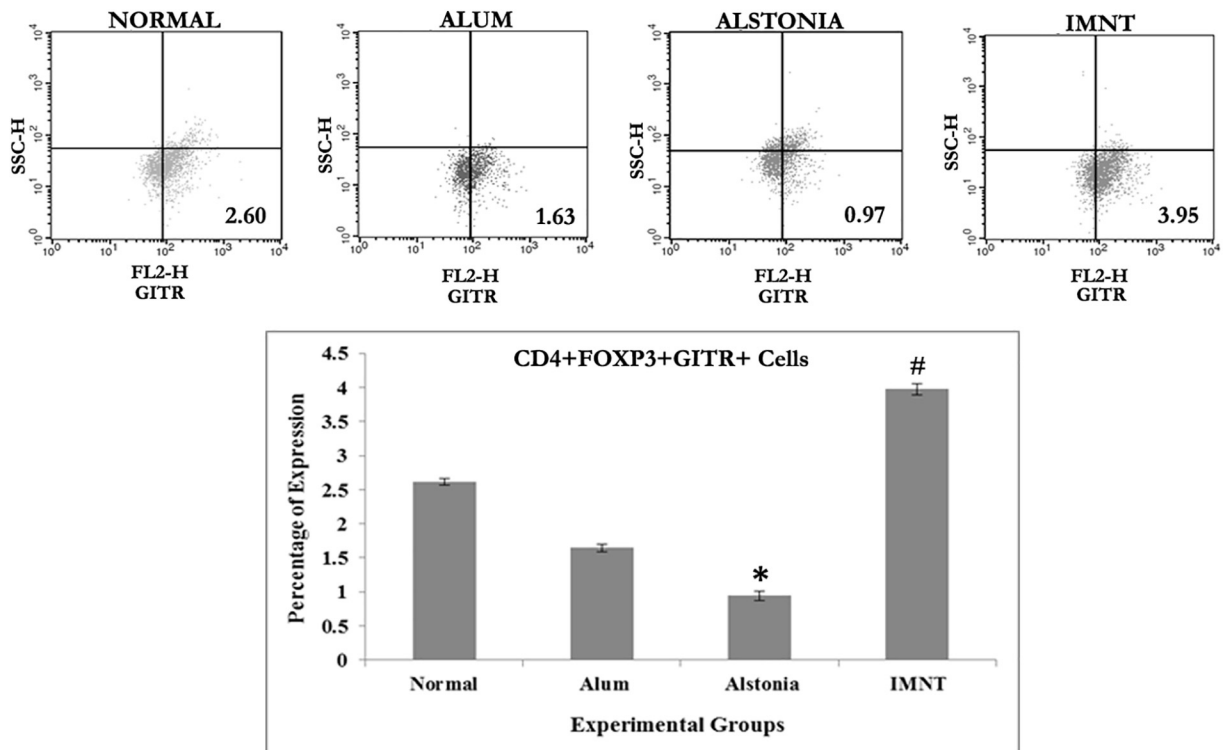


Fig. 5. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on GITR expression in splenic regulatory T cells: Flow cytometric analysis of GITR and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 40,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in GITR expression in Tregs in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). GITR expression in Tregs increased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal $n = 6$ per group).

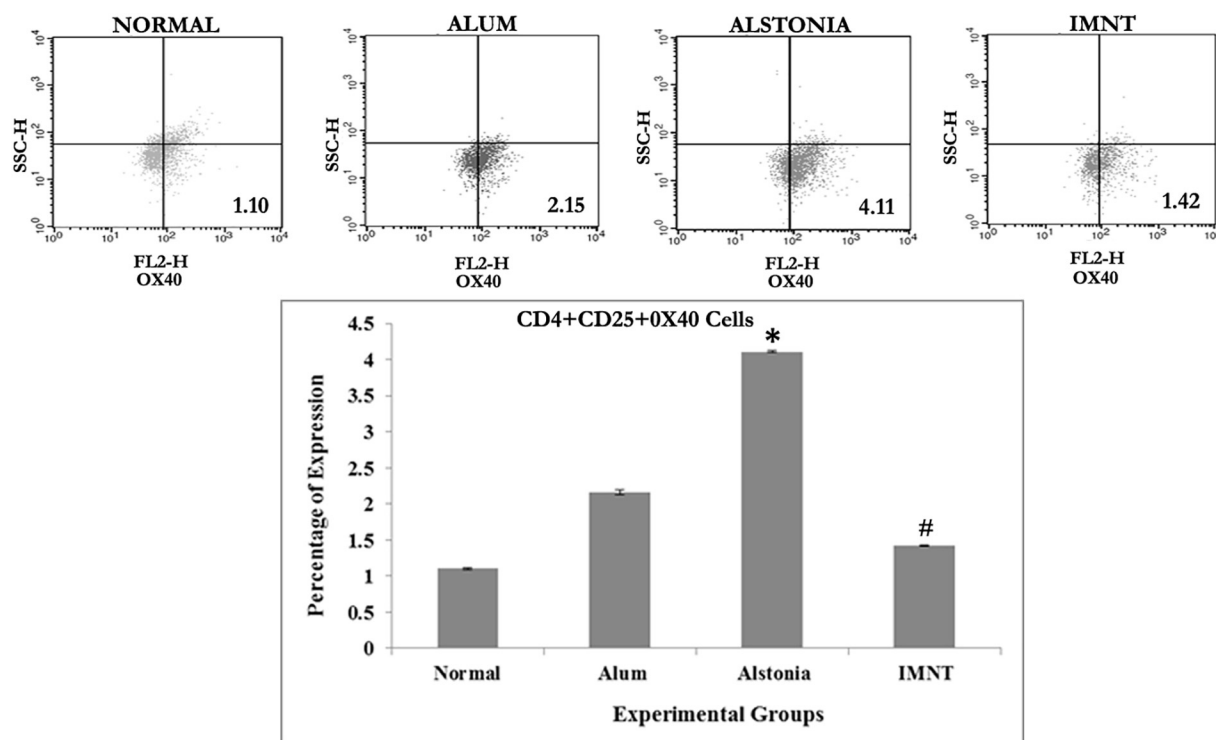


Fig. 6. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on OX40 expression in splenic regulatory T cells: Flow cytometric analysis of OX40 and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 40,000 cells analyzed. Results demonstrated that there was a significant (*) increase in OX40 expression in Tregs in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). OX40 expression in Tregs decreased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal $n = 6$ per group).

Similarly to IL10, transforming growth factor β (TGF β) is also involved in the control of atopic state. We have found that there is a reduction in CD4 + CD25 + TGF β + Treg cells *Alstonia scholaris* pollen

sensitization and challenge. As previous experimental work with TGF β knock-out mice had shown a greater susceptibility to bronchial hyper-reactivity and bronchial inflammation resembling asthma [37], so in

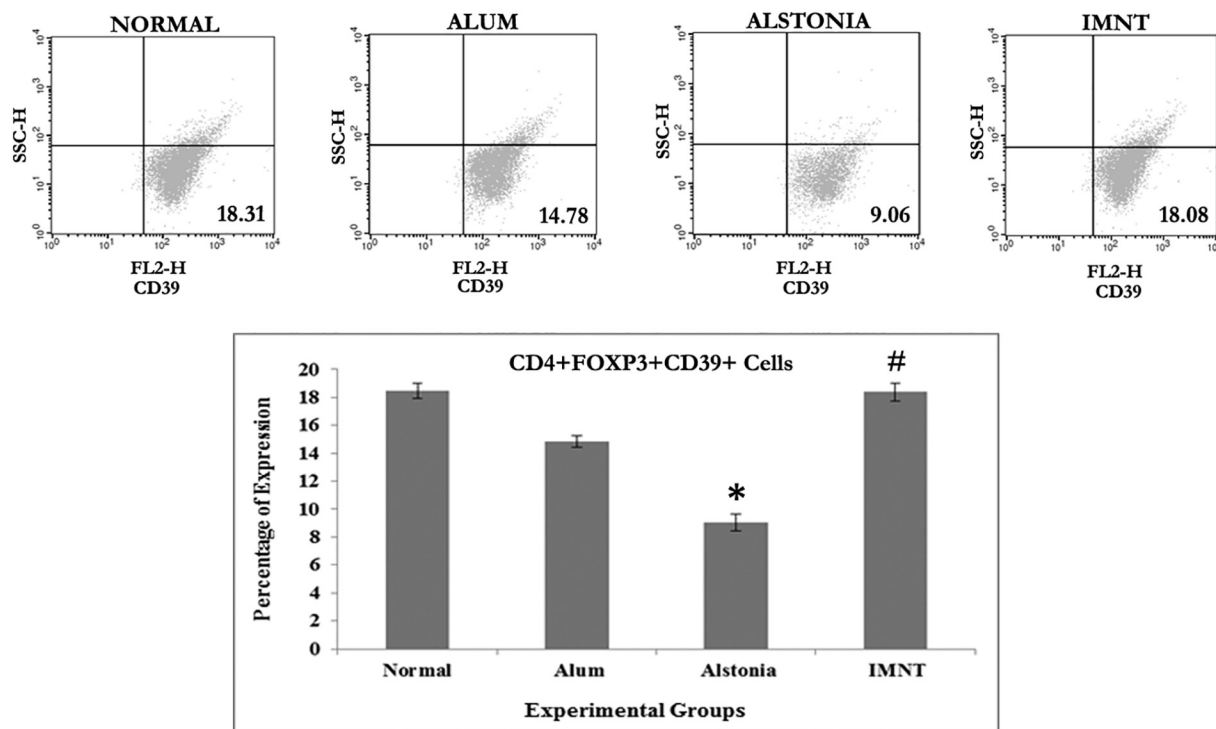


Fig. 7. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on CD39 expression in splenic regulatory T cells: Flow cytometric analysis of CD39 and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 40,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in CD39 expression in Tregs in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). CD39 expression in Tregs increased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal $n = 6$ per group).

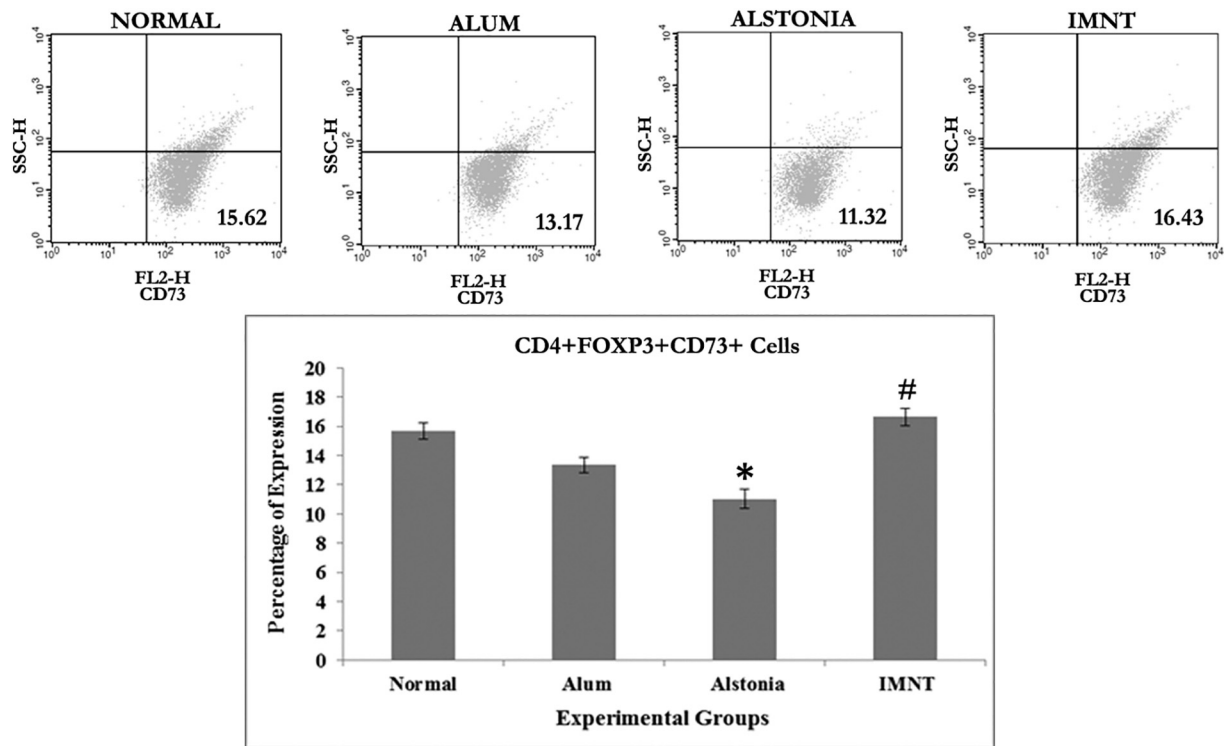


Fig. 8. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on CD 73 expression in splenic regulatory T cells: Flow cytometric analysis of CD 73 and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 40,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in CD 73 expression in Tregs in the *Alstonia* group compared with that of normal control and adjuvant control ($p < 0.001$). CD 73 expression in Tregs increased significantly (#) following intranasal immunotherapy. Column values are represented as mean $\pm$ SD (animal $n = 6$ per group).

our model the reduction in TGF β + Treg cells correlates with the establishment of allergic airway inflammation. Intranasal allergen immunotherapy caused rise in CD4 + CD25 + TGF β + Treg cells showing that TGF β is needed for the maintenance of immune tolerance. This theory was proven in an earlier work where immune tolerance was induced by repeated aerosolized OVA exposure caused CD4 + T cells to express both surface and secreted form of TGF β and furthermore the cell surface TGF β was responsible for the more effective inhibition of allergic response [18]. The autocrine action of IL10 and TGF β is important in the induction of immune tolerance by allergen immunotherapy and both these cytokines are critical factors in class switching from inflammatory IgE to non-inflammatory IgG4 and IgA respectively [38,39].

Of the various mechanisms of Treg mediated suppression, one area of research that gathered considerable momentum recently is the Treg mediated suppression of Antigen presenting cells (APC). A prominent phenotypic characteristic of natural Treg cells is that they constitutively express high levels of CTLA 4 which interacts with CD80/CD86 on APCs and causes their downregulation [40]. We have shown that in the setting of allergic airway inflammation there is abatement of CD4 + CD25 + CTLA4 + Treg cells. Intranasal allergen immunotherapy causes rise in CD4 + CD25 + CTLA4 + Treg cells. Work from the Sakaguchi group showed that Treg cells downregulate CD80 and CD86 in an adhesion dependent manner [41]; and this downregulation was annulled if Treg cells were deficient in CTLA-4 [42]. Another study by Pietruchuk et al. [43] have shown that Treg cells in allergic asthma shows reduced expression of Foxp3 and CTLA4 which causes initiation and perpetuation of allergic airway inflammation [43]. Experimental studies involving epicutaneous immunotherapy in peanut sensitized mice has shown that there is increase in the number of CTLA 4 + Tregs following immunotherapy [44]. Schneider et al. [45] have now conclusively shown that CTLA-4 upregulates Lymphocyte function-associated antigen-1 (LFA-1) mediated cell-adhesion and clustering, which contributes to Treg mediated suppression via interacting with CD80/CD86 and ICAM-1 expressed on DCs [45].

Apart from CTLA-4, CD4 + CD25 + Treg cells are also equipped with other cell surface molecules like Glucocorticoid-induced tumor necrosis factor receptor (GITR) and OX40 which functions as regulator of the suppressive functions of Treg cells [41,46,47].

Glucocorticoid-induced tumor necrosis factor receptor (GITR) is a cell surface molecule which is expressed highly on naturally occurring CD4 + CD25 + Treg cells and activated CD4 + T effector cells [48]. For the first time we have shown that there is reduction in Foxp3 + GITR + Treg cells in allergic airway inflammation which was increased with intranasal allergen immunotherapy. Though some workers have shown that conjugation of GITR with GITR-ligand (GITR-L) in the presence of IL2 enhances proliferation of Foxp3 + Treg cells and also increases their suppressor function with minimal effect on effector T cells [22], others have shown that stimulation of CD4 + CD25 + Tregs through GITR breaks immunological tolerance [48,49]. Thus, GITR activation may have varied effects on Treg cells.

Similar to GITR, another member of tumor necrosis factor (TNF) receptor superfamily protein which is constitutively expressed on regulatory T cells is OX40 (also called CD134)-a new costimulatory molecule [50]. T effector cells also can readily express OX40 upon activation [50]. Interestingly, we have found in our model that allergen sensitization and challenge increased CD4 + CD25 + OX40 + Treg cells and this rise was abrogated following intranasal immunotherapy. The recent finding that deliberate stimulation of OX40 in-vivo can break tolerance of peptide antigens [51] is in line with our results and it suggests that the effect of OX40 signaling on a regulatory type of immune response is likely to be profound. It has been demonstrated that OX40 is a potent negative regulator of Foxp3 + Tregs and stimulation of OX40 on CD4 + Foxp3 + Tregs consistently abolished their suppressor activities in-vitro [51].

Suppression by CD4 + CD25 + Treg cells is also accomplished by extra and/or immediate pericellular accumulation of adenosine, which plays a critical and autonomous role in inhibiting effector T cells through several type 1 purinergic (adenosine) receptors [52]. This extracellular

nucleotide catabolism pathway involves various ectonucleotidases like CD39 and CD73. In our model we have shown that in an allergic inflammatory milieu there is reduction in CD25 + Foxp3 + CD39 + Treg cells and CD25 + Foxp3 + CD73 + Treg cells which is augmented following intranasal immunotherapy. Previous study has shown that CD39 together with CD73 are novel surface markers of CD4 + Treg cells and the robust generation of adenosine controlled by these ectoenzymes distinguishes classic Treg cells from other CD4 + T cell population [53].

5. Conclusion

The data presented in this paper provides compelling evidence that *Alstonia scholaris* pollen induced sensitization and challenge causes abatement of CD4 + CD25 + Foxp3 + regulatory T cells which are required for maintenance of immune homeostasis and T cell tolerance. We show here that intranasal allergen immunotherapy causes augmentation of CD4 + CD25 + Foxp3 + regulatory T cells and thus abrogated allergic airway inflammation. Furthermore, we have elaborately delineated various mechanisms of Treg mediated suppression and have conclusively demonstrated that there is increase in IL10 producing Tr1 cells, TGFβ + Treg cells, CTLA + Treg cells, GITR + Treg cells, CD39 + Treg cells and CD73 + Treg cells with decrease in OX40 + Treg cells by intranasal immunotherapy. Exemplification of these diversiform pathways of regulatory T cell mediated suppression following intranasal allergen immunotherapy is a novel finding which have significant therapeutic relevance and paves the way for newer drug designs.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2017.03.017>.

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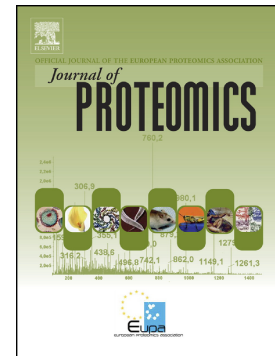
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Recombinant plant-derived human IgE glycoproteomics

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Abstract

The increasing biotechnological interest in human IgE antibodies demands advanced systems which allow their proper expression. However, this is still a challenge due to the complexity of the molecule, particularly regarding the diverse N-glycosylation pattern. Here, we present the expression of recombinant IgE in wild type and glycan-engineered *Nicotiana benthamiana* plants and in-depth N-glycosylation analyses. Mass spectrometric profiling revealed that plant IgE has a site occupancy rate that ranges from non-occupied at glycosite 6 (GS6) to 100 % occupancy at GS1 and 2. Similarly to human cell-derived IgE, plant versions carry complex N-glycans at GS1-5 and oligomannosidic structures at GS7. Computational modelling suggests that spatial position (or orientation) of glycans can impair processing or site occupancy on adjacent glycosites. IgE expressed in glycoengineered and wild type plants carry, respectively, GnGn and plant-typical GnGnXF structures at large homogeneity. This contrasts with the glycan diversity of HEK cell-derived IgE, carrying at least 20 different glycoforms. Importantly, IgE glycoengineering allows the control of its glycosylation, a so far unmet need when using well-established expression systems. This enables the elucidation of possible carbohydrate-dependent IgE functions.

Significance

Targeted glycosylation of recombinant proteins may provide an advantage in therapeutic applications. Despite increasing biotechnological interest in IgE antibodies, knowledge and impact of glycosylation on this antibody class are scarce. With the elucidation of the glycosylation profile and the ability to glyco-engineer recombinant IgE, we provide an important step towards the generation of IgE with other targeted N-glycans. This will facilitate detailed structure–function studies and may lead to the production of IgE with optimized activities.

1. Introduction

Due to recent breakthrough observations demonstrating glycosylation-dependent immunomodulatory effects of antibodies, glycobiology has become of special interest in immunology research. Immunoglobulin E (IgE), the least abundant immunoglobulin class in human serum, is principally known for its role in allergic responses [1], but it is also involved in anti-parasitic immune reactions [2]. In addition, although not yet fully understood, IgE may play an important role in the recognition of cancer by the immune system [3]. Recent studies also point to possible therapeutic applications of monoclonal IgE in the context of cancer [4, 5]. An outstanding feature of IgE antibodies is their complex glycosylation pattern: approximately 12 % of their molecular weight is carbohydrates, making them the most heavily glycosylated immunoglobulin class. This molecule contains seven potential N-glycosylation sites (GS1-7 at Asn21, 49, 99, 146, 252, 264 and 275; note that Asn are numbered from the start of the CH1 domain) in the heavy chain constant region (Fig. 1A). N-glycan profiling of serum IgE (from healthy and hyperimmune donors) revealed that GS7 (Asn275), located in the CH3 domain of the antibody, is exclusively decorated with oligomannosidic structures (OMS, Man5-Man9). While GS6 (Asn264) does not seem to be occupied, all other sites in the CH1-CH3 domains (GS1-5) carry complex-type N-glycans. The majority is bi-antennary, core-fucosylated structures with one or two terminal N-acetylneuraminic acid residues (70-80%); approximately 10-15 % of these N-glycans have bisecting GlcNAc [6, 7]. Notably, significant N-glycan diversity has been observed at each glycosite and between different (patho-)physiological stages [7-9]. While mounting evidence favours N-glycan involvement in the modulation of IgG activity, less is known about this important post-translational modification for other immunoglobulins, like IgE. Early studies have shown that at least precursor N-glycans (i.e. Glc3Man9GlcNAc2) are essential for the secretion of IgE [10]. The impact of fully processed (sialylated) N-glycans, which are the most abundant glycoform in human serum IgE, is still under debate. Researchers are particularly interested in understanding the interaction of IgE with effector cells of the immune system through their Fc receptors: the high-affinity FcεRI and the low-affinity CD23/FcεRII [11, 12]. Partially inconsistent results have been reported for FcεRI binding: while some studies concluded that IgE glycosylation is essential for FcεRI binding and pro-inflammatory effector functions [13-16], others did not find such correlation [17, 18]. A more recent study points to the requirement of an occupied C-terminal glycosite (carrying oligomannosidic N-glycans) for FcεRI binding and subsequent initiation of the IgE-mediated allergic cascade [19]. The IgE glycosylation status also seems to influence binding to FcεRII [20], an evolutionary-related form of the IgG receptor DC-SIGN. Nevertheless, more results are required to draw precise conclusions.

A major obstacle to elucidate N-glycan-dependent IgE activities is the current technological limitations to produce this antibody isotype with targeted N-glycan structures. *In vivo* glycoengineering is being developed in various expression systems as a method to control the composition of glycans and to enhance the pharmacological properties of recombinant proteins, including monoclonal antibodies [21-23]. Plant-based expression platforms have become a very attractive alternative to well-established expression systems, as the turnaround time from DNA to mg quantities of purified recombinant protein can be as short as one to two weeks [24]. An exciting aspect of using plants as production hosts is their ability for extensive glycoengineering (recently reviewed in [25]). Paradoxically, their limited endogenous glycosylation repertoire has turned out to be an advantage for generating proteins with homogeneous N-glycans, in contrast to the large glycome and the resulting N-glycan heterogeneity in mammalian cells, which prevents targeted manipulations. Importantly, wild type (WT) plants generate human-like complex N-glycans that most frequently terminate with GlcNAc residues (GnGn). However, they also synthesize N-glycan structures not present on mammalian proteins, i.e. core α 1,3-fucose and β 1,2-xylose residues (namely, GnGnXF). A central issue in N-glycan engineering is the generation of plants that lack plant-specific glycan residues [26]. Antibodies expressed in such mutant plants are decorated with GnGn structures, the mammalian core structure for further diversification (reviewed in [27]).

Despite the recently discovered potential of IgE as a therapeutic molecule, access to recombinant IgE is scarce. Recombinant expression of functionally active IgE has so far been reported in human and insect cells [4, 19, 28, 29]. However, data on their glycosylation status are incomplete. The expression of monoclonal IgE in HEK cells resulted in a large N-glycan diversity, with many new N-glycan structures in comparison to human serum IgE; on the other hand, insect cell-derived IgE exhibited an overall N-glycan pattern fundamentally different from a typical human serum IgE: instead of human type complex N-glycans (galactosylated and sialylated structures), they carried insect cell-typical structures that terminate with mannose residues [29]. Such an N-glycosylation pattern might have negative effects on the activity of therapeutic IgE. Although plants have proven to be a suitable host for the efficient expression and glycosylation of complex and functionally active human proteins (including antibodies like IgG, IgA and IgM) [27, 30], their applicability for the production of IgE has not been demonstrated yet.

Here, we focused on elucidating the N-glycosylation status of recombinant IgE antibodies produced in two different expression systems. An in-depth glycoproteomic analysis of monoclonal IgE targeted to growth factor receptor HER2/neu (namely, HER2-IgE), produced in mammalian cells (HEK293) and in *Nicotiana benthamiana* plants, was performed by liquid-

chromatography electrospray ionization-mass spectrometry (LC-ESI-MS/MS). We were able to obtain glycopeptides from IgE heavy chain covering all seven potential N-glycosylation sites by using a combination of three different proteases. Resulting spectra revealed information on site occupancy, site-specific glycosylation and overall glycosylation profiles.

2. Materials and methods

2.1. Cloning of HER2-IgE for plant based expression

MagnICON® vectors (courtesy of Icon Genetics, GmbH [31]) carrying the cDNA of HER2-IgE heavy and light chains [4] were generated in two steps: first, a 1287-bp cDNA fragment encoding the Ig epsilon chain constant region CH1-CH4 (UniProtKB - P01854) was codon-optimized and synthesized for *N. benthamiana* with flanking Bsmbl restriction sites (Invitrogen™ GeneArt™, <https://www.thermofisher.com>). To facilitate cloning of variable regions, Bsal sites were also introduced. The Bsmbl-Bsmbl fragment was cloned into the Bsal sites of the magnICON® TMV-based viral vector resulting in pICHα26211:IgEHc. Similarly, a 324-bp codon-optimized fragment containing the cDNA of the kappa chain constant region (UniProtKB - P01834) was amplified with primer pair LcFW/LcREV (Supplementary Table 1) that introduces Bsal restriction sites and flanks the fragment with Bsmbl restriction sites. The constant domain of the light chain was cloned into a PVX-based vector, resulting in pICHα31160:IgELc. In a second step, the Bsal restriction sites between the viral promoter and the constant region were used to insert the variable region. The cDNA encoding the variable domains of the light chain and heavy chain of trastuzumab (PDB ID: 1n8z [32]) were amplified with primers that introduce flanking Bsal restriction sites (LcHER2FW/LcHER2REV and HcHER2FW/HcHER2REV, respectively; Supplementary Table 1) and subsequently cloned into pICHα31160:IgELc and pICHα26211:IgEHc plasmids, resulting in the plant expression vector pICHα31160:HER2-IgELC (or PVXαHER2-IgELC) and pICHα26211:HER2-IgEHC (or TMVαHER2-IgEHC) (Supplementary Fig. 1).

2.2. Expression and purification of HER2-IgE

Nicotiana benthamiana WT and ΔXT/FT plants [26] were grown in a growth chamber at 22°C with a 16 h light/ 8h dark photoperiod. Leaves of 4–5 week old plants were used for agro-infiltration. Agrobacteria (strain GV3101 pMP90) transformed with either PVXαHER2-IgELC or TMVαHER2-IgEHC vectors were grown in liquid culture at 29 °C for 24 h, harvested by gentle centrifugation (5 min at 3000 g) and resuspended in infiltration buffer (10 mM MES pH 5.6; 10 mM MgSO₄) as previously described [33]. The two recombinant bacteria strains were mixed and

diluted to a final OD₆₀₀ of 0.1 for infiltration into WT (^{WT}HER2-IgE) and Δ XT/FT (Δ ^{XF}HER2-IgE) plant leaves. 4-5 days after infiltration, leaves were harvested and flash-frozen in liquid nitrogen. Total soluble proteins were extracted in 1.5 M NaCl, 45 mM Tris, 1 mM EDTA, and 40 mM ascorbic acid (2:1 buffer/fresh weight) and recombinant IgE was purified by affinity chromatography (Protein A SepharoseTM Fast Flow, GE Healthcare). ^{WT}HER2-IgE and Δ ^{XF}HER2-IgE were eluted with 0.1 M Glycine/HCl (pH 2.7), neutralized with 1 M Tris (pH 9) and dialysed against PBS.

Cloning and expression of HER2-IgE in HEK293 cells (^{HEK}HER2-IgE) was described earlier [28]. Briefly, the heavy and light chains of HER2-IgE were cloned in pViro-2-hygro-mcs (InvivoGen) using the Polymerase Incomplete Primer Extension (PIPE) cloning method. Subsequently, HER2-IgE was transiently expressed in Expi293TM cells (Thermo Fischer Scientific) using the ExpiFectamineTM 293 transfection kit (Thermo Fischer Scientific), according to the manufacturer's instructions. ^{HEK}HER2-IgE was purified using a HiTrap KappaSelect (GE Healthcare) pre-packed column. Antibodies were eluted with 0.2 M Glycine/HCl (pH 2.3), neutralized with 1 M Tris (pH 9) and dialysed against PBS. Serum IgE (^{serum}IgE, obtained from a polysensitised allergic patient after informed consent was given) was purified by affinity chromatography using sepharose-coupled anti-IgE monoclonal antibody [34]. IgE was eluted using 5 M MgCl₂ and subsequently exchanged for PBS with Amicon Ultra Centrifugal filter tubes (Merck Millipore, Darmstadt, Germany). Purified IgE samples were reduced with 2-mercaptoethanol at 70 °C for 5 min and analysed by SDS-PAGE on a 12 % gel that was subsequently stained with Coomassie Brilliant Blue R 250 (Carl Roth GmbH + Co. KG).

2.3. Glycopeptide mass spectrometry analysis

SDS-PAGE protein bands corresponding to the heavy chain (at ~75 kDa) were excised and S-alkylated with iodoacetamide. Subsequently, the samples were proteolytically digested in 25 mM NH₄HCO₃ with either trypsin (Promega), proteinase K (Sigma Aldrich) or a combination of trypsin and chymotrypsin (Roche). This procedure allowed the extraction of glycopeptides covering all seven glycosites: GS1: NIPSNATSVTL; GS2: DTGSLNGTTM; GS3: VAHTPSSTDWVDNK; GS4: TINIT; GS5: GTVNLTW; GS6: ASGKPVNHSTR, and GS7: NGTLTVTSTLPVGTR. GS1 and 2 were obtained by the combined trypsin and chymotrypsin digestion; GS3, 5, 6 and 7 are tryptic peptides, and GS4 was obtained by digestion with proteinase K. After digestion, the peptides were extracted by adding 50 μ L 25 mM NH₄HCO₃ and then 50 μ L 100% ACN with two additional washes with 50 μ L 5% formic acid. The pooled extracts were dried in a centrifugal vacuum concentrator. The peptide mixture was analysed using a Dionex Ultimate 3000 system

directly linked to a Q-TOF instrument (maXis 4G ETD, Bruker) equipped with the standard ESI source (end plate offset 500 V; capillary 4500 V; dry gas (nitrogen) 5.0 L/min; dry temp 200°C) in the positive ion, data dependent acquisition mode. MS-scans were recorded (range: 150-2200 m/z, spectra rate: 0.5 Hz). Instrument calibration was performed using an ESI calibration mixture (Agilent). For peptide separation, a Thermo BioBasic C18 separation column (5 µm particle size, 150 x 0.32 mm) was used, with the solvent system recently described [30]. For the relative quantification of the different glycoforms, peak areas of EIC (Extracted Ion Chromatograms) of the first four isotopic peaks were summed. All observed charge states and adducts (ammonium), as well as the formation of formylated glycopeptides, were considered. MS/MS spectra were used for the verification of the glycopeptides by detection of oxonium ions HexNAc ($m/z = 204.1$), Hex+HexNAc ($m/z = 366.1$) and the unique Y1 ion (peptide+HexNAc). N-glycosylation site occupancy was deduced from the ratio of deamidated to unmodified peptide upon N-glycan release with 0.15 mU of PNGase A (Europa Bioproducts) overnight at 37 °C. For serum IgE, due to technical reasons, the summed peak areas of all glycoforms were compared to the area of the unglycosylated peptide. This method gave very similar values when compared to the standard approach

2.4. Molecular Modelling

A molecular model of human IgE-Fc was produced using both the bent (PDB ID 1O0V [35]) and extended (PDB ID 4J4P [36]) crystal structures of IgE-Fc. As representative of complex N-glycans, we modelled GnGn structures ($\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$) in GS4 and 5; and as representative of OMS in GS7, Man5 structures ($\text{Man}_5\text{GlcNAc}_2$) were modelled. Glycan structures were modelled using the glycan fragment database [37]. The energy was minimized to relieve steric clashes using the molecular operating environment (MOE; Chemical Computing Group, Inc. 2014) and the Amber99 force field. For GS5, additional GnGn structures were generated in the extended model of IgE-Fc to show that the N-glycan is free to take a broad range of conformations.

3. Results

3.1. Expression of monoclonal IgE in mammalian cells and *Nicotiana benthamiana* plants

For recombinant IgE expression, we used a monoclonal antibody targeted to the growth factor receptor HER2/neu (namely, HER2-IgE [4]). This antibody variant is analogous to the clinically approved IgG1 trastuzumab, the standard of care in HER2-overexpressing breast cancer. The heavy and the light chain of HER2-IgE were cloned into viral-based expression vectors

(PVX α HER2-IgELC, TMV α HER2-IgEHC) and transiently expressed in *Nicotiana benthamiana* wild type (WT) and the glycosylation mutant plants Δ XT/FT, which synthesize human type bi-antennary complex N-glycans lacking plant-specific xylose and core fucose residues [26]. Plant leaves were harvested 4-5 days post-infiltration and IgE was purified by immune-affinity (^{WT} HER2-IgE, $^{\Delta XF}$ HER2-IgE). Similarly, appropriate HER2-IgE constructs were transiently expressed in HEK293 cells and immune-affinity purified (^{HEK} HER2-IgE). As control, human serum IgE (^{serum} IgE) obtained from a polysensitised allergic donor was included in our analyses. SDS-PAGE analysis of the four samples under reducing conditions exhibited two major bands corresponding to the size of the IgE heavy and light chains (75 and 25 kDa, respectively) (Fig. 1B). Compared to the human versions, plant-derived ^{WT} HER2-IgE and $^{\Delta XF}$ HER2-IgE exhibit a slightly reduced molecular mass of the heavy chain, most probably due to differences in glycosylation. In some samples, a weak signal at 55 kDa appeared, corresponding to a degradation product of the heavy chain. Importantly, SDS-PAGE analysis of plant-derived HER2-IgE under non reducing conditions indicates correct assembly into heterodimers (Supplementary Fig. 2).

3.2. Glycosite occupancy

IgE heavy chains carry three glycosites on the CH1 domain (GS1-3), one on the CH2 domain (GS4) and three on the CH3 domain (GS5-7) (Fig. 1A). In order to analyse all seven glycopeptides, the IgE heavy chains were proteolytically digested with different enzymes. GS1 and 2 were obtained by combination of trypsin and chymotrypsin digestion; GS3, 5, 6 and 7 are tryptic peptides, and GS4 was obtained by digestion with proteinase K. Apart from our limitation of not finding GS4 upon proteinase K digestion, we find occupancy rates for serum IgE similar to those previously reported by Plomp *et al.* [7]. Remarkably, the overall pattern of site occupancy was similar for all four samples tested (^{WT} HER2-IgE, $^{\Delta XF}$ HER2-IgE, ^{HEK} HER2-IgE, ^{serum} IgE) (Fig. 2). The degree of occupation of GS1 and 2 was found to be virtually 100 % in all four samples. Differences between plant- and human-derived IgE site occupancies were observed for GS3 and 5. While these sites are efficiently occupied in human cell-derived variants (75-90 %), decreased levels were detected in the plant versions (18-48 %). Just as previously reported for human serum IgE [7], no IgE variant was glycosylated at GS6 (Fig. 2 and Supplementary Table 2).

3.3. Site specific N-glycan profiling

The glycosylation status of the different IgEs was analysed regarding the relative abundance of different glycoforms at each glycosite by LC-ESI-MS/MS (Fig. 3 and Supplementary Tables 3

and 4). The MS spectra of all four IgE samples exhibited the presence of complex N-glycans at GS1-5, oligomannosidic structures (OMS) at GS7 and unglycosylated GS6 (Fig. 3 and Supplementary Tables 3 and 4). Complex glycosylation of plant-derived IgE is relatively homogenous in both WT and Δ XT/FT plants. GS1-5 showed a single dominant glycoform, namely GnGnXF or GnGn, for WT plants (^{WT} HER2-IgE) and Δ XT/FT plants ($^{\Delta XF}$ HER2-IgE), respectively. This glycoform accounts for up to 75 % (Fig. 3 and Supplementary Table 3). In addition, some OMS appeared (up to 27 %) and $^{\Delta XF}$ HER2-IgE also carried traces of fucosylated structures (GnGnF, up to 11.5 %), most probably due to incomplete RNAi-based downregulation of α 1,3-fucosyltransferase expression [26].

Four glycan species were detected at GS1-5 of ^{serum} IgE: mono- and bi-sialylated bi-antennary N-glycans with core fucose (ANaF and NaNaF), and bisected structures (ANaFbi and NaNaFbi). Notably, our studies do not allow the discrimination of bisected bi-antennary structures from tri-antennary structures (e.g. NaNaFbi from GnNaNaF); however, due to previous results obtained from serum IgE analysis [7, 9], we anticipate the presence of bisected glycoforms. These bisected structures dramatically increase at GS5 (up to 76 %) (Fig. 2 and Supplementary Table 3). In contrast to ^{serum} IgE, in ^{HEK} HER2-IgE the glycosylation of GS1-5 is extremely heterogeneous (Fig. 2 and Supplementary Table 4). In GS1-4, up to 20 different glycoforms were detected. They mainly consist of galactosylated tri- and tetra-antennary N-glycans with 1-4 sialic acid residues. N-glycans at GS5 differ from GS1-4 in that they generally consist of bisected bi-antennary structures terminating with GlcNAc, galactose or sialic acid residues. No branched structures were detected on that site. Overall, the glycan diversity in ^{HEK} HER2-IgE is significantly increased in comparison to the plant- and serum-derived IgEs.

Regarding GS7, all four IgE samples carry almost exclusively oligomannosidic structures, ranging from Man5 to Man9. While Man5-7 are the most abundant glycoforms in ^{HEK} HER2-IgE and ^{serum} IgE, in plants we observe an even distribution of Man5-9. Surprisingly, GS7 of plant-derived HER2-IgE is also decorated with hybrid N-glycans, Man5Gn and Man4Gn (up to 20 %).

3.4. IgE Modelling

To assess a possible impact of the structural conformation of human IgE on its glycosylation, a molecular model based on previously published Fc structural information was built. IgE-Fc, which harbours GS4-7, was crystallographically observed in an extended and a bent structure (Fig. 4) [35, 36]. In the bent structure (Fig. 4B), which has computationally been identified as the energetically favoured one (PDB ID: 4J4P [36]), the CH2 domain pair folds back onto the CH3-CH4 domains, both in free and complex forms (PDB IDs: 1O0V [35] and 2QWR [38]).

Glycosylation modelling of GS4, 5 and 7 suggests that further processing of the OMS at GS7 may be sterically hampered by the CH2 domains in the bent conformation (Fig. 4B). Considering the distance at the amino acid sequence level, GS6 is in close proximity to GS5 and GS7 (12 and 11 amino acids apart, respectively). In the 3D structure, GS5 and GS6 are on the same beta strand, which confers them relatively close physical proximity. By contrast, GS6 and GS7 are oriented in different directions (Fig. 4B). These spatial orientations might have an impact on glycan accessibility and processing. Moreover, the potential conformational diversity of complex N-glycans at GS5 (visualized by a superposition of several GnGn structures on Fig. 4A) suggests that GS5 glycans are on the surface and could interfere with the glycosylation of GS6.

4. Discussion

Despite the increasing knowledge about the important role of IgE antibodies in various immunological processes, their low serum concentration renders them the least characterized antibody isotype. Their powerful physiological role in allergic sensitization in combination with their potential therapeutic use has intensified research interest in human IgE antibodies with tailored specificities. However, due to the complexity of the molecule, recombinant expression is challenging and has mainly been restricted to mammalian cells. While such cells seem to be well suited for the expression of functionally active IgE [4, 19, 28], their usability for targeted glycosylation is hindered by the large glycan diversity in mammalian cells. Here, we pursued the expression of recombinant IgE in plants with a special focus on its glycosylation status in comparison with a HEK cell-derived version. We aimed to assess the use of plants for the production of IgE with a targeted glycosylation profile. ~~elucidation of the glycosylation status of recombinant IgE produced in human cells and plants, in comparison with human serum-derived polyclonal IgE.~~ As a model, we used anti-HER2/neu IgE that retained the functions of its IgG counterpart (trastuzumab) on restricting proliferation of HER2/neu-expressing tumour cells and activating effector cells to kill tumour cells (HER2-IgE) [4].

We found that the method developed in [7] proves to be a valuable tool for in-depth glycoproteomic analysis of monoclonal HER2-IgE. We showed that the type of glycans (complex or oligomannosidic) present at each N-glycosylation site of HER2-IgE is consistent with previous studies using serum IgE [7, 9]; namely, GS7 at the CH3 domain exhibits OMS, while complex glycans are found on GS1-5. Albeit known from other proteins, including IgM and IgD [39], the presence of complex and oligomannosidic N-glycans on a single protein is a peculiarity. To date, the biological relevance, as well as the processes that drive this differential glycan processing, are poorly understood. One model refers to different accessibility of the glycosylation sites either by glycosylation from adjacent glycosites (IgD [6]) or as a consequence of protein

oligomerization (IgM [40]). For IgE, it was proposed that access of OMS to glycosylation-processing enzymes is blocked by the CH2 domain [6]. Molecular modelling suggests that, indeed, processing of the OMS towards complex structures at GS7 may be sterically hampered by the CH2 domain, at least in the bent conformation. Moreover, our modelling studies imply inaccessibility of GS6 due to sterical hindrance of complex glycans present on the adjacent GS5. Our studies suggest that such structures can take a broad range of conformations, influencing the properties of the environment. Further studies are required to confirm these findings. According to sequence alignments, it was suggested that GS7 of IgE is the equivalent of the single IgG glycosite (Asn297) and Asn402 of IgM antibodies [7]. Notably, the IgG-Fc glycosite is occupied by complex-type N-glycans, and the impact of the glycan composition on binding to FcγR is well known [41]. Glycosites with OMS in IgE and IgM are located on the CH3 domain, surrounding the proposed Fc receptor binding sites. This suggests that they may have critical impact on the ability of the antibody to bind to the corresponding Fc receptors and to subsequently induce downstream (effector) functions. Indeed, one study refers to the absolute requirement of a glycosylated GS7 for IgE binding to the FcεRI and subsequent downstream activities [19]. Still, to what extent particular OMS impact this activity is not known. Unexpectedly, significant differences were found when comparing the composition of complex N-glycans of serum- and HEK cell-derived IgE. The most striking alteration was the presence of large amounts of galactosylated tri- and tetra-antennary N-glycans carrying 1-4 sialic acid residues in ^{HEK}HER2-IgE. As a consequence, the number of glycoforms increased fivefold in ^{HEK}HER2-IgE compared to ^{serum}IgE. A similar observation was made by Shade *et al.* [19], who also expressed a monoclonal IgE in HEK cells. The differences in glycosylation between ^{HEK}HER2-IgE and ^{serum}IgE should be interpreted with caution, as the latter is naturally polyclonal and it is derived from a single (allergic) donor. Notably, we also observed the absence of glycans at GS6 on ^{HEK}HER2-IgE. This is in accordance with serum- derived IgE, however contradicts previous results reporting full occupancy of this site on HEK cell-derived monoclonal IgE [19]. Interestingly, in plant-derived IgE (^{WT}HER2-IgE and ^{ΔXF}HER2-IgE), complex N-glycan profiles of GS1-5 are very similar. However, for ^{HEK}HER2-IgE and ^{serum}IgE, distinct complex N-glycans are observed in GS5 (located at the CH3 domain), compared to the similar profiles on GS1-4 (located at CH1 and CH2 domains). This was also previously observed for healthy serum IgE [7]. Little is known about HEK cell-specific glycosylation. Monoclonal IgG produced in such cells exhibit a similar glycosylation profile as serum IgG, with AAF, AGnF and GnGnF being the three major structures (i.e. G2F, G1F and G0F [42]). An exception is the presence of unusually high amounts of bisecting structures in some variants (up to 40 % [43]).

At this moment, it cannot be ruled out that the observed HEK cell-derived glycosylation is restricted to our monoclonal HER2-IgE antibody, although it could also be a general characteristic of the expression system. The fact that another recombinant IgE antibody with a different specificity expressed in HEK cells also carries a large amount of branched structures (with and without sialic acids) [19] points to a peculiarity of HEK cells. Yet, more data on HEK cell-derived recombinant proteins is needed before drawing general statements on the glycosylation pattern of these cells.

Essentially, we show that plants are able to express IgE and that its glycosylation status is similar to human cell-derived IgE, regarding both N-glycan type and site occupancy. In plants, GS1-5 are occupied by complex type N-glycans, GS6 is not occupied and OMS are detected in GS7. Some differences in the level of glycosylation occupancies are observed for GS3 and 5. At the moment, we cannot explain the decreased site occupancy in plant-derived samples, although this has also been observed for other recombinant plant-produced proteins [44]. Overall, it seems that the general molecular mechanisms of plant and mammalian N-glycosylation processing are highly conserved, an unexpected fact given the large phylogenetic distance between the two phyla.

Importantly, we were able to generate a glycan engineered HER2-IgE in plants that is decorated with GnGn structures which feature large homogeneity (Δ^{XF} HER2-IgE). This common eukaryotic core glycoform can be used for further targeted diversification towards glycans usually found on serum IgE, or towards novel structures, to possibly modify or even enhance the activities of the antibody. Using the recently developed plant-based expression system that relies on the versatile combination of glycan engineered plants with transient expression modules [25], it should be possible to generate IgE antibodies with additional targeted glycosylation profiles and to subsequently trace their functionalities. This approach will allow the reconstruction of complex glycoforms, including sialylation, at great homogeneity [45], a so far unmet need for elucidation of glycan-dependent activities.

5. Conclusion

Although HEK cell -derived and human serum IgE have common glycosylation features, there are unexpected differences. These variations need to be considered when using human cell lines for the expression of recombinant proteins. Plant-based IgE expression points to the ability for targeted glycosylation, which may open new ways for glycan-dependent IgE structure-function studies. This will not only help to better understand basic IgE-based mechanisms on the regulation of the immune system, but may also be used for the development of antibody-based therapies, an emerging field in drug development.

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Figure captions

Fig. 1. (A) Schematic representation of the structure and glycosylation status of human serum IgE. Monomers of IgE consist of two heavy chains (ϵ chain) and two light chains. The ϵ chain contains 4 immunoglobulin-like constant domains (CH1-CH4) and one variable domain (VH), while the light chain contains one constant (CL) and one variable domain (VL). Glycosites (GS) 1-7 on the ϵ chain represent Asn21, 49, 99, 146, 252, 264 and 275. (B) Coomassie brilliant blue stained SDS-PAGE (under reducing conditions) of purified IgE antibodies (~500 ng). IgE samples are derived from (1) *Nicotiana benthamiana* wild type plants (^{WT} HER2-IgE); (2) *Nicotiana benthamiana* glycosylation mutant Δ XT/FT ($^{\Delta XF}$ HER2-IgE); (3) HEK293 cells (^{HEK} HER2-IgE) and (4) human serum (^{serum} IgE, single donor with a hyperimmune condition). HC, LC: heavy and light chain, respectively. (M) Molecular weight indicated in kDa.

Fig. 2. Site-specific glycosylation occupancy (%). IgE expressed in *Nicotiana benthamiana* wild type plants (^{WT} HER2-IgE), glycosylation mutant Δ XT/FT ($^{\Delta XF}$ HER2-IgE), HEK293 cells (^{HEK} HER2-IgE) and human serum (^{serum} IgE) was monitored. Values were obtained using two different methods (see Materials and Methods and Supplementary Table 2). Glycosylation efficiency on GS4 in ^{serum} IgE was not determined, as the glycopeptide resulting from Proteinase K digestion was not found.

Fig. 3: LC-ESI-MS/MS analyses. Relative abundance of glycoforms of IgE expressed in plants (^{WT} HER2-IgE, $^{\Delta XTFT}$ HER2-IgE) and in human cells (^{serum} IgE, ^{HEK} HER2-IgE). Schematic representations of N-glycans detected by mass spectrometry at each glycopeptide (GS) are shown (symbol nomenclature in accordance with the Consortium of Functional Glycomics). Note that for ^{HEK} HER2-IgE more than 20 glycoforms were found and thus were grouped in this figure as explained in Supplementary Table 4. Glycoforms present in less than 5% were group in "Others". For detailed information see Supplementary Tables 3 and 4.

Fig. 4: Molecular model of IgE-Fc. The model contains (CH2, CH3 and CH4 domains) based on the extended and bent crystal structures. The protein backbone is coloured in red shades (Chain A) and blue shades (Chain B) with CH2-CH4 domains coloured from dark to light shades. (A) Extended structure. A wide range of possible GnGn structures is modelled on GS5 in Chain A (red arrow) to exhibit the glycan flexibility and proximity to the unoccupied GS6 (black spheres). (B) Bent structure. The accessibility to the OMS on GS7 (green) is hindered due to the folding of the CH2 domains onto the CH3 and CH4 domains. GS: glycosite. Glycan modelling was performed according to the glycan fragment database [37].

Figure 1

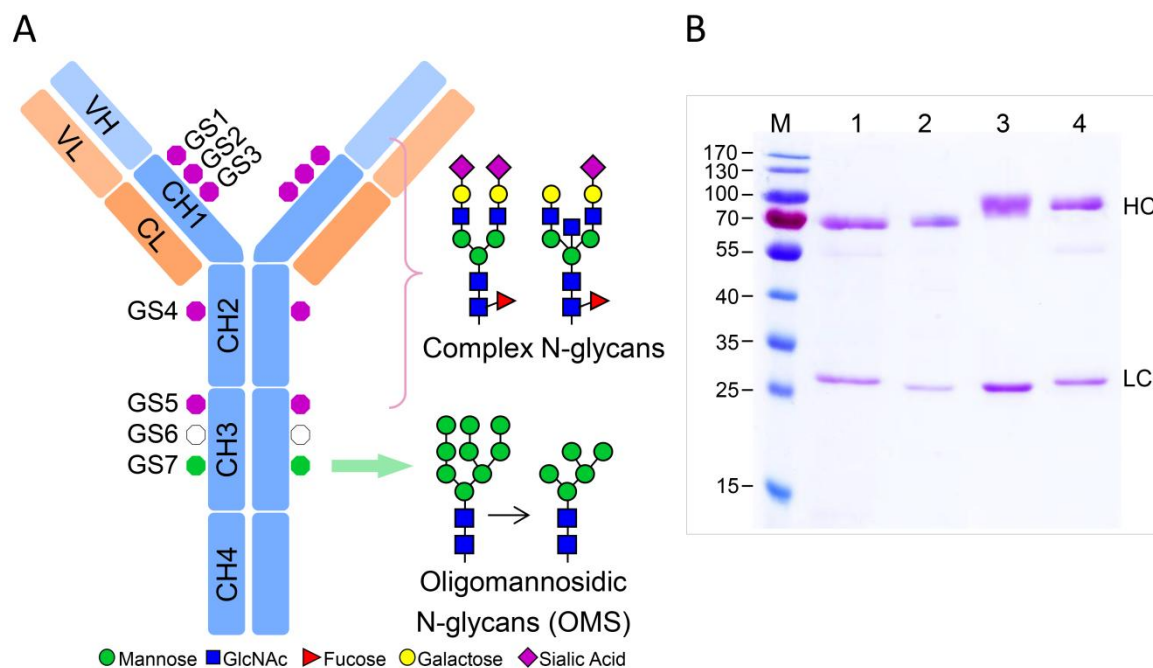


Figure 2

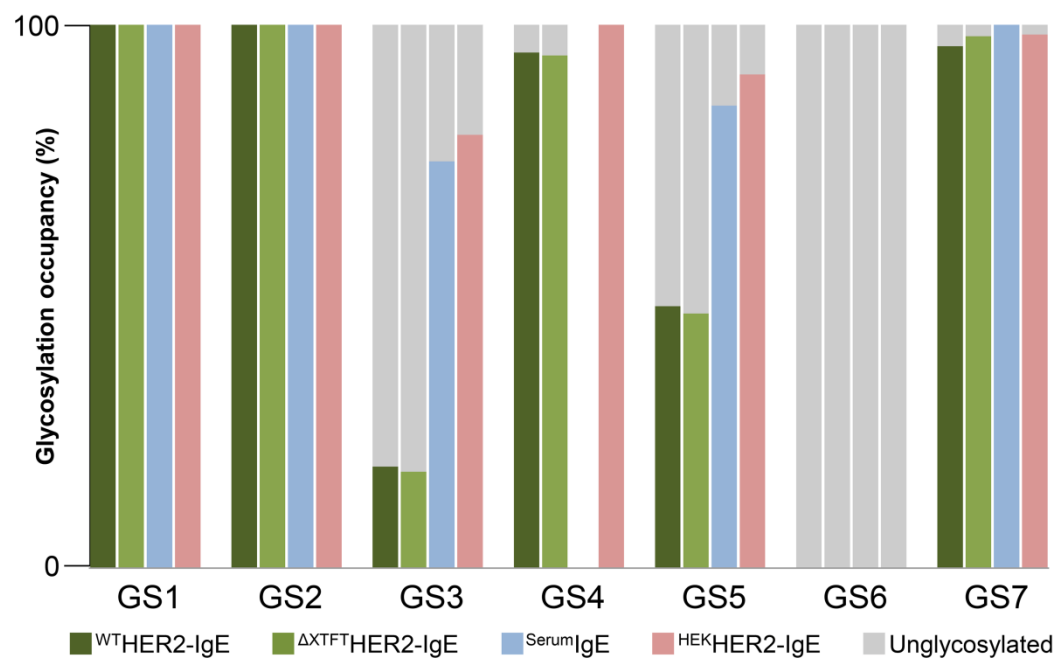


Figure 3

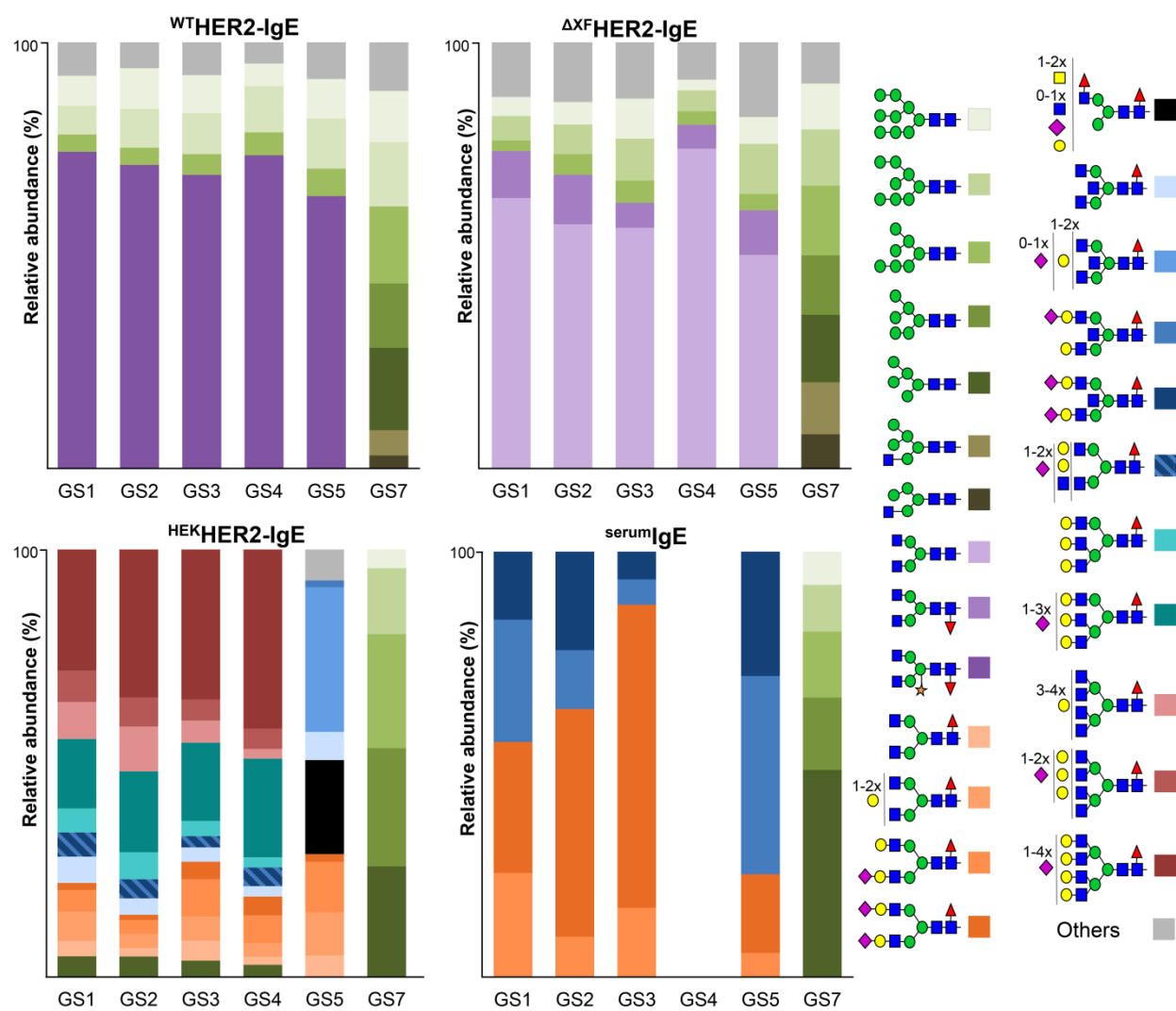
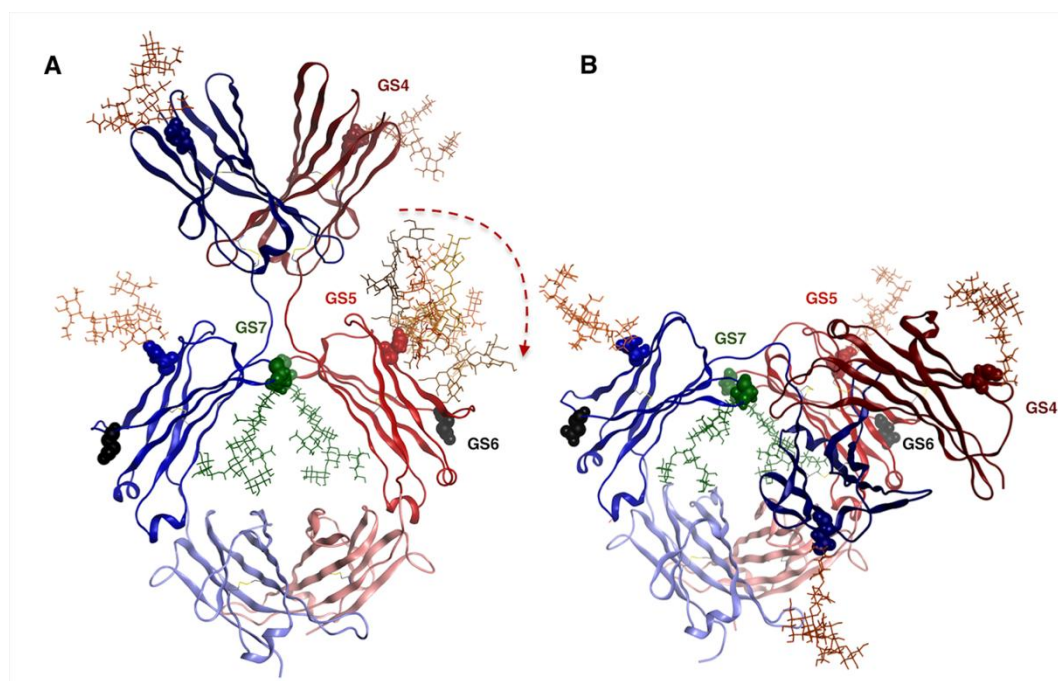


Figure 4

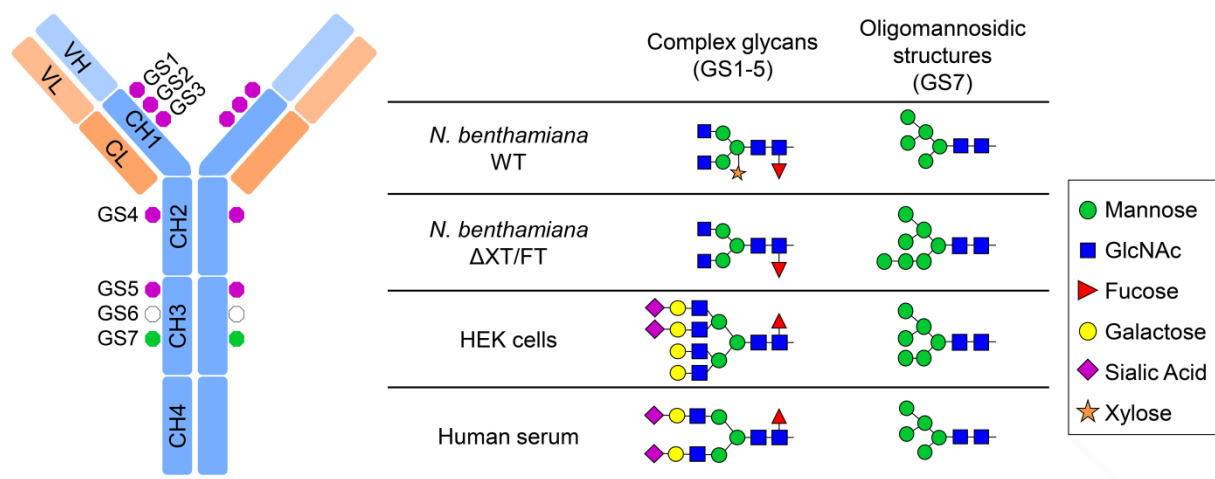


Conflict of interest

The authors declare no conflict of interest.

ACCEPTED MANUSCRIPT

Graphical abstract



“Highlights”

- IgE glycosylation can be engineered towards targeted structures
- HEK cell- and serum-derived IgE differ in their glycosylation pattern
- Molecular modeling suggests sterical hindrance as an important factor in N-glycan processing

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