From the Institute of Nutritional Medicine
of the University of Lübeck
Director: Prof. Dr. med. Christian Sina

The role of IgG and IgE glycosylation in allergic disease

Dissertation
for Fulfillment of
Requirements
for the Doctoral Degree
of the University of Lübeck
from the Department of Natural Sciences

Submitted by
Lara Dühring
from Buxtehude

Lübeck 2023

First referee:	Prof. Marc Ehlers
Second referee:	Prof. Lars Redecke
Date of oral examination:	06.05.2024
Approved for printing:	08.05.2024

Table of content

Abstract

Allergen-specific IgE antibodies mediate allergic recations, including systemic anaphylaxis, by crosslinking the high affinity FcεRI on mast cells and basophils, which leads to cell degranulation and release of inflammatory mediators, e.g. histamine. In contrast, allergen-specific IgG antibodies, induced during allergen immunotherapy (AIT) can attenuate IgE-mediated allergic reactions by interacting with allergenic proteins thus blocking allergen binding to cell surface IgE and via FcγRIIB-dependent inhibition of effector cells. However, in case of high allergen-doses, also IgG antibodies can induce anaphylaxis in mice through activation of myeloid cells mediated by Fcγ-receptors.

Effector functions of IgG antibodies depend not only on the subclass but also on the *N*-glycosylation pattern at Asn 297 in the Fc part of the antibody. Elevated levels of agalactosylated IgG antibodies have been associated with pro-inflammatory immune responses whereas galactosylated and additionally sialylated IgG antibodies have been associated with disease remission. In the context of treating inflammatory autoimmune diseases with high concentrations of intravenous immunoglobulin (IVIG, pooled serum IgG from healthy donors), it is further assumed that those antibodies can mediate anti-inflammatory immune responses. The idea is, that the sialylated subfraction of the total IgG antibodies of the IVIG can upregulate the inhibitory IgG-receptor FcγRIIB on effector cells, which has an impact on all IgG-mediated reactions. IgE antibodies harbour several *N*-glycosylation sites, but little is known about the function of different IgE glycosylation patterns.

One aim of this thesis was to investigate whether the sialylation level of the total serum IgG antibodies can influence IgG-mediated as well as IgG-controlled IgE-mediated allergic reactions. Furthermore it should be investigated how different IgE glycosylation patterns modify an IgE-mediated anaphylactic reaction.

I could show that application of sialylated allergen-unspecific human IgG4 antibodies can control an allergen-specific IgG-mediated anaphylaxis and the application of allergen-unspecific murine IgG1 can enhance an IgG-dependent inhibition of an IgE-mediated anaphylaxis. The effect of sialylated murine IgG1 antibodies correlated with an upregulation of FcγRIIB on blood basophils that could explain the inhibitory effects mechanistically.

Abstract

Considering the role of IgE glycosylation in allergic disease, I could show that desialylation increases the affinity of IgE antibodies to FcεRI as well as mast cell sensitization *in vitro*. Accordingly, desialylated allergen-specific IgE glycoforms induced a stronger mast cell and basophil activation upon allergen-stimulation compared to sialylated IgE glycoforms, suggesting that sialylated IgE antibodies exhibit inhibitory properties, as described for IgG, IgA and IgM.

Interestingly, during my *in vivo* studies, it turned out that desialylated and sialylated IgE antibodies have similar potentials to sensitize blood basophils and induce an IgE-mediated anaphylaxis. For future experiments, optimized experimental mouse model settings that allow the investigation of other inflammatory conditions and/or consider the release or expression of glycan-binding receptors/molecules might be neccessary to visualize differences between different sialylation levels of IgE antibodies *in vivo* as I could observe *in vitro*.

Altogether, I could show that the sialylation-level of the total serum IgG antibodies correlate the inhibition of an allergic reaction. Furthermore, the data suggest that the glycosylation pattern of (allergen-specific) IgE antibodies could be a potential biomarker to predict the severity of an IgE-mediated allergic reaction.

Zusammenfassung

Allergen-spezifische IgE Antikörper vermitteln allergische Reaktionen, unter anderem eine systemische Anaphylaxie, indem sie den hochaffinen FcεRI auf Mastzellen und Basophilen quervernetzen, was zur Degranulation der Zellen und zur Freisetzung inflammatorischer Mediatoren, wie zum Beispiel Histamin führt. Im Gegensatz dazu können Allergen-spezifische IgG Antikörper, die im Laufe einer Allergen Immuntherapie (AIT) induziert werden, IgE-vermittelte Reaktionen durch Allergenmaskierung und FcγRIIB-vermittelte Inhibition von Effektorzellen attenuieren. Allerdings können im Falle hoher Allergenkonzentrationen auch Allergen-spezifische IgG Antikörper durch FcγR-vermittelte Aktivierung myeloider Zellen, eine Anaphylaxie in Mäusen auslösen.

Effektorfunktionen von IgG Antikörpern hängen nicht nur von der IgG Subklasse, sondern auch von deren *N*-Glykosylierungsmuster an Asn 297 im Fc Teil ab. Ein erhöhtes Vorkommen agalaktosylierter IgG Antikörper ist mit proinflammatorischen Immunantworten assoziiert, wohingegen eine Galaktosylierung und terminale Sialylierung sowohl von Gesamt-Serum-IgG Antikörpern, als auch Antigen-spezifischen IgG Antikörpern mit einer Krankheitsremission bei inflammatorischen Immunerkrankungen einhergeht. Außerdem konnte in Mausexperimenten gezeigt werden, dass sialylierte IgG Antikörper anti-inflammatorische Reaktionen im Kontext der Therapie von inflammatorischen Autoimmunerkrankungen mit hoch konzentriertem intravenösem Immunglobulin (IVIG, gepooltes Serum-IgG gesunder Spender) vermitteln. Die Idee dabei ist, dass die sialylierte Subfraktion der Gesamt-IgG Antikörper des IVIG den inhibitorischen IgG-Rezeptor FcγRIIB auf Effektorzellen hochregulieren kann, was alle IgG-vermittelten Reaktionen beeinflusst. IgE besitzt mehrere *N*-Glykosylierungsstellen, jedoch ist über die Funktion verschiedener IgE Glykosylierungsmuster bisher wenig bekannt.

Das Ziel dieser Arbeit war es, zu untersuchen, ob der Sialylierungsgrad des Gesamt-Serum IgGs eine IgG-vermittelte, sowie eine IgG-kontrollierte IgE-vermittelte allergische Reaktion beeinflussen kann. Weiterhin sollte untersucht werden, wie verschiedene IgE-Glykosylierungsmuster eine IgE-vermittelte anaphylaktische Reaktion modifizieren.

Ich konnte zeigen, dass die Applikation von sialyliertem, Allergen-unspezifischem humanen IgG4 eine IgG-vermittelte Anaphylaxie reduzieren, und die Applikation von sialyliertem, allergen-unspezifischem murinen IgG1 eine IgG-abhängige Inhibierung einer IgE-vermittelten Anaphylaxie verstärken konnte. Der Effekt der sialylierten murinen IgG1 Antikörper korrelierte mit einer Hochregulierung von FcγRIIB auf Blut-Basophilen, was die inhibitorischen Effekte mechanistisch erklären könnte.

Weiterhin konnte ich zeigen, dass eine IgE Desialylierung die Affinität von IgE Antikörpern zu FcεRI und auch die Mastzell-Sensitisierung *in vitro* erhöht. Entsprechend induzierten desialylierte Allergen-spezifische IgE Antikörper *in vitro* eine stärkere Mastzell- und Basophil-Aktivierung nach Allergen-Stimulation, verglichen mit sialylierten IgE-Glykoformen, was vermuten lässt, dass sialylierte IgE Antikörper, wie auch für IgG, IgA und IgM beschrieben, inhibitorische Eigenschaften besitzen.

In meinen *in vivo* Untersuchungen zeigten desialylierte und sialylierte IgE Antikörper vergleichbare Potentiale, Blut-Basophile zu sensitisieren und eine IgE-vermittelte Anaphylaxie auszulösen. Optimierte Bedingungen in experimentellen Mausmodellen, die eine Untersuchung anderer inflammatorischer Konditionen erlauben und/oder die Expression bzw. Freisetzung Zucker-bindender Rezeptoren oder löslicher Moleküle berücksichtigen, könnten nötig sein, um die Wirkungsunterschiede zwischen verschiedenen Sialylierungsleveln von IgE Antikörpern, die ich *in vitro* beobachten konnte auch *in vivo* nachzuweisen. Dies soll Gegenstand zukünftiger Experimente sein.

Zusammenfassend konnte gezeigt werden, dass der Sialylierungsgrad der Gesamt-Serum IgG Antikörper mit der Inhibierung einer allergischen Reaktion korreliert. Weiterhin lassen die Daten vermuten, dass der Sialylierungsgrad (allergen-spezifischer) IgE Antikörper ein potenzieller Biomarker dafür sein könnte, den Schweregrad einer IgE-vermittelten allergischen Reaktion vorherzusagen.

1 Introduction

1.1 Allergic diseases

Every day our body encounters various foreign organisms and substances. With each contact the immune system has to decide whether this contact is harmless and can be tolerated (e.g., internal molecules from tissue-surfaces or environmental substances like pollen, foods, drugs or animal hair) or potentially threatening (e.g., pathogenic bacteria, viruses or parasites) and needs to be eliminated. To avoid severe damage for the host, it must be further ensured that the immune system does not overreact, otherwise the host would possibly experience excessive immune responses against either self or foreign antigens, which are referred to as hypersensitivity reactions. These processes lead to a well-balanced immune response between tolerance and protection under physiological conditions, whereas a disruption of this balance, however, can result in pathological conditions such as autoimmune or allergic diseases (Dispenza, 2019; Greenberger, 2019; Murphy and Weaver, 2018).

Allergic diseases are defined as abnormal, adaptive immune responses against otherwise harmless substances – referred to as allergens – in predisposed individuals (Murphy and Weaver, 2018). Over the last years an alarming rise in the prevalence of allergic diseases has been reported with the outcome that today every 1 out of 3 people suffers from allergies, with the tendency probably still increasing (Akarsu et al., 2022). Considering the impact of allergic diseases of the patients' quality of life and their burden on healthcare costs, the induction of immunological tolerance to successfully treat allergic diseases is still not sufficiently understood and demands further research to explore novel therapeutic strategies (Akarsu et al., 2022; Lee et al., 2022; Tabynov et al., 2022; Zissler and Schmidt-Weber, 2020).

Hypersensitivity reactions have been categorized into four types by the immunologists Coombs and Gell (1963) according to their pathophysiology/ mechanism of tissue injury, which are associated with different immunological mechanisms. This classification is widely accepted (Figure 1) and has been further extended over the last decades. Type I (= immediate hypersensitivity) reactions are generally mediated by immunoglobulin (Ig)E antibodies activating mast cells and basophils to rapidly release inflammatory mediators such as histamine. However, there are non-IgE-mediated mechanisms with a delayed onset of symptoms, classified as type II-IV hypersensitivity reactions. Unlike type I hypersensitivity reactions type II and type III reactions are mediated by IgM and IgG antibodies. In type II reactions those antibodies are described to bind self antigens on circulating blood cells such as erythrocytes, platelets and neutrophils leading to cytotoxic responses in the host. Cytolytic destruction of the

target cells in type II reactions is caused either by activation of the complement system or by cell-bound IgG or IgM antibodies engaging Fc-receptors on effector cells such as neutrophils or macrophages. Type III reactions are characterized by the formation of soluble immune complexes of either IgG or IgM antibodies with self- or foreign antigens, causing organ damage by deposition in the tissue (e.g., small arteries, renal glomeruli) and subsequent recruitment of inflammatory cells as well as complement activation. Type IV hypersensitivity reactions are mediated by antigen-sensitized T-cells instead of antibodies accompanied by the release of inflammatory cytokines. Furthermore, there are non-immunological mechanisms, which can lead to a direct activation of effector cells, e.g. via cold or sunlight. However, this thesis focuses on type I/ immediate hypersensitivity reactions that, in severe cases, can lead to potentially lifethreatening systemic anaphylaxis (Averbeck et al., 2007; Dispenza, 2019; Greenberger, 2019; Murphy and Weaver, 2018).

Types of hypersensitivity reactions

Immunologic mechanism				Non-immunologic mechanism
Type I	**Type II**	**Type III**	**Type IV**	
Immediate reaction	Delayed reaction			
	Complement-/ FcγR-mediated		Cell-mediated	
Antibody-mediated hypersensitivity	Antibody-mediated cellular cytotoxicity	Immune complex-mediated	T-cell mediated	
IgE/ IgG (?) antibodies	IgG/ IgM antibodies	IgG/ IgM antibodies	T-helper/ cytotoxic T-cells	
allergy (pollen/ drug/ food), anaphylaxis	hemolytic anemia, transfusion reaction	Arthus reaction	contact dermatitis	Direct activation of effector cells by cold, sunlight or drugs

Figure 1: Types of hypersensitivity reactions according to Coombs and Gell (1963).

Modified according to Dispenza (2019), Finkelman (2016) and Greenberger (2019).

1.1.1 Pathomechanisms of immediate hypersensitivity reactions

An immune response to a certain allergen can be divided into two phases – the sensitization phase and the elicitation phase (Averbeck et al., 2007). In the sensitization phase, allergens enter the body for the first time, e.g., through the skin, through inhalation (e.g. pollen or house-dust mite allergens) or the consumption of certain foods or injections (drugs, insect stings) (Figure 2). Tissue resident antigen presenting cells (APCs), such as dendritic cells (DCs) or Langerhans cells (LCs), capture and process these allergens, migrate to the lymph nodes and prime naïve $CD4^+$ T helper (Th0) cells by presenting peptides of the allergen via major histocompatibility complex class II molecules (MHC II). Upon allergen recognition, Th0 cells differentiate and proliferate into certain subsets (e.g., Th1, Th2, Th17, regulatory T-cells (Treg)) with characteristic cytokine profiles and transcription factors thereby mediating different types of immune responses. Immediate hypersensitivity reactions are classically mediated by IgE antibodies. Through activation of the transcription factors STAT6 and GATA3 the T-cell polarization is driven from naïve Th0 cells towards the pro-allergic Th2 phenotype which is accompanied by the production of cytokines such as interleukine (IL)-4, IL-5 and IL-13. Secretion of these cytokines subsequently induces class-switching to IgE in activated B cells and leads to the production of allergen-specific IgE antibodies (Gould et al., 2003; Gould and Sutton, 2008a; Murphy and Weaver, 2018; Oettgen, 2010; Shamji et al., 2021).

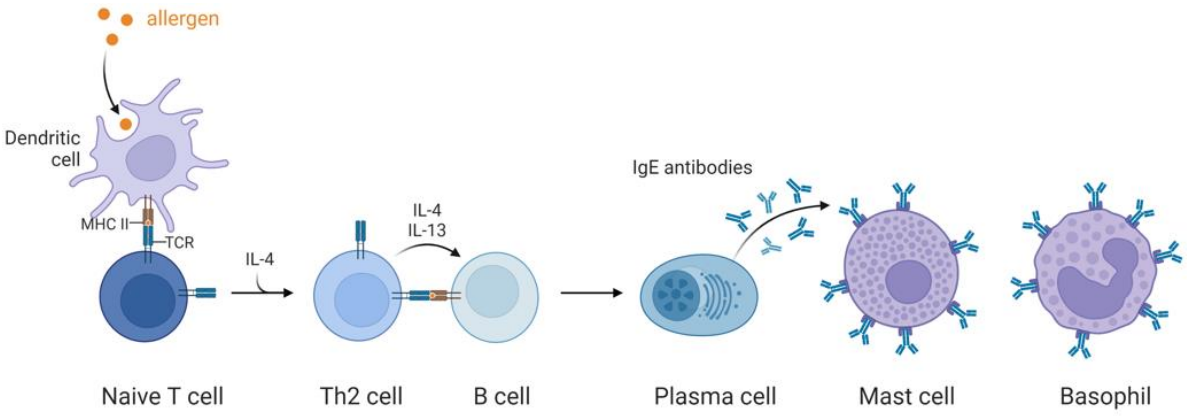

Figure 2: Allergic sensitization.

Differentiation of naïve $CD4^+$ T helper cells (Th0) to pro-allergic Th2 cells is facilitated by allergen uptake, processing and subsequent presentation by DCs. Secretion of cytokines such as IL-4 and IL-13 by Th2 cells induces B cell class switching to IgE and maturation to IgE producing plasma cells. IgE antibodies subsequently bind to effector cells such as mast cells and basophils via the high affinity IgE Fc receptor FcεRI. This figure was created with BioRender.

IgE antibodies are the least abundant immunoglobulins in circulation with particulary low serum levels of ~150 ng/ mL in healthy individuals (compared to IgG with ~10 mg/ mL) and a

rather short half-life (1-3 days, compared to 3 weeks for IgG antibodies). However, IgE titers can be elevated up to 10 times in atopic individuals, increasing the risk of developing allergies. Furthermore, binding of IgE antibodies to the high affinity IgE Fc receptor FcεRI on mast cells and basophils stabilizes the IgE-receptor complex, elevating the tissue half-life of IgE to several weeks because of restricted diffusion from the receptor and rebinding to other cell receptors in the proximity (Gould et al., 2003; Kanagaratham et al., 2020; Oettgen, 2010; Shamji et al., 2021).

Interestingly, the severity of an IgE-mediated allergic response can be attenuated by allergen-specific IgG antibodies via two general mechanisms. First, circulating allergen-specific IgG antibodies compete with IgE antibodies for allergen binding, thus preventing crosslinking of FcεRI by cell-bound IgE on effector cells and consequently inhibiting their activation. Furthermore, it has been discovered that allergen-specific IgG antibodies can inhibit IgE-mediated cell activation by crosslinking FcεRI with the inhibitory IgG Fc receptor FcγRIIB on effector cells, which abrogates degranulation an downstream cytokine synthesis (Epp et al., 2018a; Kanagaratham et al., 2020; Petry et al., 2021a; Strait, 2006).

Nevertheless, in the case of cell activation upon crosslinking of allergen-specific IgE antibodies, mast cells and basophils release preformed vasoactive mediators such as histamine, proteases (tryptase, chymase) and lysosomal enzymes as well as *de novo* synthesized lipid mediators such as leukotrienes, prostaglandins and platelet-activating factor (PAF) in the "early phase response" within minutes. A few hours after allergen-exposure, newly synthesized cytokines such as IL-1, TNF-α and IL-8 are secreted in the "late phase response". Those mediators induce smooth muscle constriction, elevate the vascular permeability and increase the recruitment of effector cells to the site of inflammation. Depending on the route and location of allergen-exposure the ensuing immediate symptoms can vary from skin reactions (rashes, itching), edema, increased mucus secretion and sneezing or (chronic) asthma to potentially life-threatening hypotension and anaphylaxis (Dispenza, 2019; Gould et al., 2003; Gould and Sutton, 2008a; Murphy and Weaver, 2018).

1.1.2 Antibody-mediated anaphylaxis

The "International Consensus on anaphylaxis (ICON)" defines the term as "a serious, generalized or systemic, allergic hypersensitivity reaction that can be life-threatening or fatal", with the most common triggers being foods, drugs and instect stings. Anaphylaxis is characterized by a rapid onset and life-treatening airway, breathing, and/or circulating problems

which is usually associated with skin and mucosal changes (Reber et al., 2017). The lifetime prevalence of anaphylaxis is currently estimated at 0.05-2 % in the USA and ~3 % in Europe and several studies have noted a rise in the incidence, particulary in the hospitalizations and Emergeny Room (ER) visits due to anaphylaxis (Yu and Lin, 2018). Unfortunately, only little progress has been made in recent years concerning prophylaxis and more importantly therapy/ treatment of anaphylaxis. Treatment options are still limited to general avoidance of the allergen, allergen immunotherapy (AIT) and medications such as antihistamines, epinephrine and intravenous fluids to manage anaphylaxis symptoms (Finkelman, 2007; Finkelman et al., 2016; Peavy and Metcalfe, 2008).

The classical pathway of anaphylaxis is mediated by allergen-specific IgE antibodies bound to FcεRI on mast cells and basophils as the main drivers of IgE-mediated anaphylactic reactions, as described above and in Figure 3 (Feyerabend et al., 2011; Finkelman, 2007; Reber et al., 2013; Shamji et al., 2021).

However, it becomes more and more accepted that a second pathway of anaphylaxis exists, likely mediated bei allergen-specific IgG antibodies. First evidence for this pathway has been provided by groups that could induce active systemic anaphylaxis in mast cell-, FcεRI- or IgE deficient mice (Dombrowicz et al., 1993; Finkelman, 2007; Jacoby et al., 1984; Oettgen et al., 1994). Furthermore, human cases of drug-induced anaphylaxis (e.g., by monoclonal antibodies, antibiotics, analgesics or dextran) have been described in patients lacking allergen-specific IgE antibodies and basophil or mast cell activation (measured by serum tryptase levels). In these cases, the severity of the anaphylactic reaction was associated with elevated levels of allergen-specific IgG antibodies in the sera of the patients, further indicating an IgE-independent mechanism. Since then, IgG-mediated anaphylaxis has been described in various experimental mouse models of active and passive anaphylaxis. It requires high allergen doses and is mediated by myeloid cells such as neutrophils, macrophages or basophils activated through Fcγ-receptors (Figure 3) (Finkelman, 2007; Finkelman et al., 2016; Jönsson et al., 2011; Marat V. Khodoun et al., 2011; Mancardi et al., 2013).

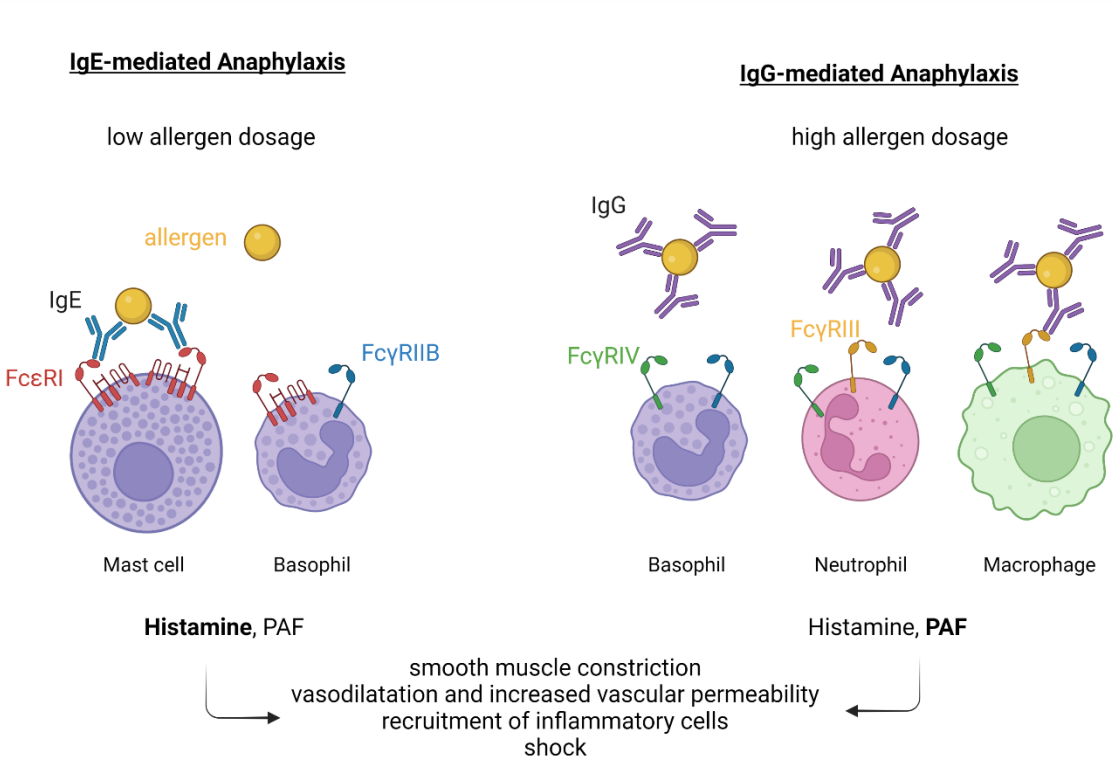

Figure 3: Pathways of anaphylaxis.

IgE-mediated anaphylaxis is initiated by allergen-specific IgE antibodies binding the high affinity IgE Fc receptor FcεRI, expressed on mast cells and basophils. Subsequent allergen exposure leads to receptor crosslinking and the release of vasoactive mediators (e.g., histamine) which induces anaphylaxis. Furthermore, a second pathway of anaphylaxis has been proposed mediated by allergen-specific IgG antibodies forming soluble immune complexes and induced by FcγRIII and/ or FcγRIV-mediated activation of myeloid cells (e.g., macrophages, neutrophils, basophils) when allergen doses are high. Upon activation these cells predominantly release the vasoactive mediator platelet-activating factor (PAF) which causes anaphylaxis symptoms. Both pathways are clinically indistinguishable. This figure was created with BioRender.

Although both pathways in general show the same symptoms, there are certain features to distinguish IgG- from IgE-mediated anaphylaxis. Whereas in IgE-mediated anaphylaxis allergen crosslinking of FcεRI through receptor-bound IgE results in the release of histamine and mast cell proteases, in IgG-mediated anaphylaxis, circulating antigen-specific IgG antibodies form immune complexes with the allergen. Those complexes bind to effector cells expressing the activating IgG Fc-receptors FcγRIII and FcγRIV such as monocytes/ macrophages, neutrophils and basophils thus driving IgG-mediated anaphylactic reactions (Beutier et al., 2017; Bruhns and Jönsson, 2015). Furthermore, it has been demonstrated that PAF rather than histamine is the main mediator of IgG-mediated allergic reactions, since blocking of PAF protected mice from IgG-mediated anaphylaxis. Also, PAF levels positively correlated with anaphylaxis severity in humans. However, PAF is a mediator also secreted by

platelets and other effector cells showing an increased expression of FcεRI in allergic individuals, such as activated mast cells and monocytes/ macrophages (Bruhns and Jönsson, 2015; Finkelman et al., 2016; Gill et al., 2015; Jiao et al., 2013; Marat V. Khodoun et al., 2011). Considering these findings, it is possible that in humans PAF in addition to histamine is associated with IgE-mediated anaphylaxis. However, investigation of the FcγR profile of human immune effector cells indicates the possibility of their participation in an alternative IgG-mediated pathway of systemic anaphylaxis, especially in cases of human drug-induced anaphylaxis, where antigen-doses are high due to intravenous administration of large amounts of the drug. Mouse models of IgG-mediated anaphylaxis also required higher antigen doses for anaphylaxis induction compared to models of IgE-mediated anaphyalxis (Beutier et al., 2017; Bruhns, 2012; Finkelman et al., 2016; Gill et al., 2015; Jiao et al., 2014; Jönsson et al., 2011).

In summary, besides the classical IgE-mediated pathway an alternative pathway of anapyhlaxis has been proposed in several studies, mediated by allergen-specific IgG antibodies that activate myeloid cells via activating FcγRs to release e.g., PAF. Since the symptoms of both pathways of anaphylaxis are indistinguishable in mice (and potentially in humans), it is of great importance to understand the underlying pathomechanisms of each of the two pathways to avoid adverse reactions and explore novel therapeutic approaches.

1.2 Allergen Immunotherapy

The treatment of allergic dieseases is often still limited to the reduction of allergic symptoms with antihistamines, corticosteroids or epinephrine. Using these antisymptomatic medications a certain relief for the patients can be achieved, but they do not eliminate the cause of the disease. By contrast, allergen immunotherapy (AIT) is a disease modifying therapy for IgE-mediated allergic reactions (e.g. alleric rhinitis or allergic asthma) with the main goal to restore immunological tolerance in case the body comes into contact with the respective allergen. It is antigen driven, antigen specific and long lasting, often providing a lifelong reduction of symptoms. Furthermore, by treating patients suffering from allergic rhinitis with AIT, there is a chance to prevent the onset of allergic asthma. Although AIT is practiced for over 100 years, the underlying cellular and immunological mechanisms have been discovered only in the last 20 years with general, therapy guiding biomarkers still lacking (Lee et al., 2022; Zissler and Schmidt-Weber, 2020).

Introduction

In conventional AIT protocols the induction of immunological tolerance is achieved by repeated subcutaneous (SCIT), sublingual (SLIT) or oral (OIT) administration of increasing allergen-doses. There are several immunological mechanisms which have been identified to be critical for induction of tolerance during AIT: Since IgE-mediated allergic diseases are classical type 2 immune responses, dominated by Th2 cells producing the cytokines IL-4 and IL-13 promoting the generation of allergen-specific IgE antibodies, one mechanism of AIT is the suppression of type 2 immunity and the shift from Th2 towards non- Th2 responses (Th1, Treg). Several studies showed for example an attenuated type 2 inflammation during AIT, measured by decreasing levels of IL-4, IL-5 and IL-13, whereas type 1 cytokines such as Interferon-γ (IFNγ) increased after three years of AIT, suggesting a successful switch from type 2 towards type 1 immunity. Furthermore, the induction of regulatory cells, such as regulatory T- and B cells has been observed during AIT, indicating upcoming immune tolerance. These cells produce cytokines such as IL-10 and TGF-β, which act anti-inflammatory by inhibiting $CD4^+$ T cell proliferation and subsequently IgE production. Furthermore, these cytokines induce B cell class switching from IgE to IgG and IgA. In particular, during AIT, increasing amounts of allergen-specific IgG4 and IgA2 antibodies have been described (Akarsu et al., 2022; Epp et al., 2018a; Kanagaratham et al., 2020; Lee et al., 2022; Tabynov et al., 2022; Zissler and Schmidt-Weber, 2020).

It is also possible that an allergic disease resolves by developing a natural tolerance through increasing levels of allergen-specific IgG antibodies, especially IgG4, which observed in many children that naturally overcome food allergies to milk, egg or soy and in beekeepers, both having prolonged/ repeated exposure to a certain allergen. Here, the individuals are positive for allergen-specific IgE antibodies, but do not develop clinical symptoms of allergic disease (Akarsu et al., 2022; Müller, 2005).

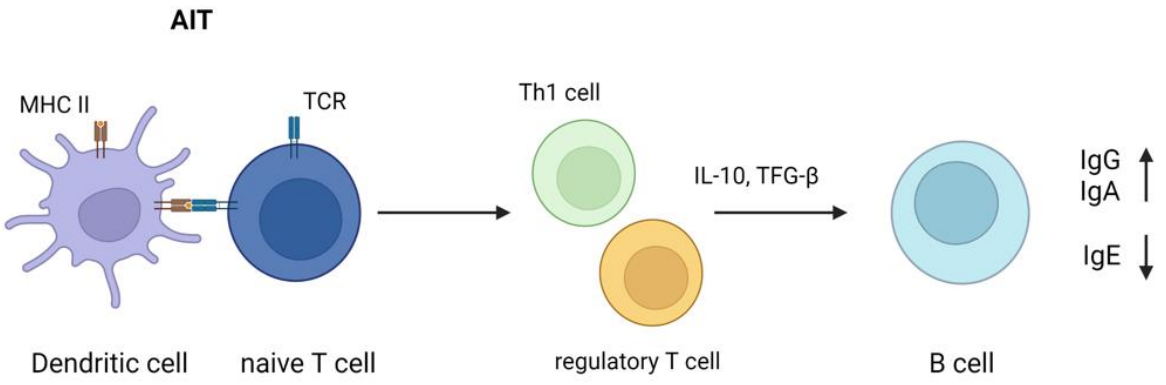

Figure 4: Mechanims of allergen-specific immunotherapy.

The induction of immunological tolerance during AIT is accompanied by the differentiation of naïve T cells into Th1 and regulatory T cells that produce anti-inflammatory cytokines such as TGF-β and IL-10. These cytokines facilitate B cell class switching to IgG and IgA and are associated with a longterm decrease of IgE production. This figure was created with BioRender.

However, the induction of allergen-specific IgG antibodies during AIT is not considered a general biomarker for therapy success, since it does not always correlate with the improvement of clinical symptoms. One explanation could be that the IgG antibodies are directed against another epitope of the allergen than the disease-causing IgE antibodies, therefore being unable to prevent allergen-binding to IgE and inhibition of IgE-mediated responses. Furthermore, it has been shown that IgG-dependent inhibition of IgE-mediated reactions not only depends on the quantity but also the affinity of the induced IgG antibodies. In experimental mouse models of anaphylaxis it has been shown that high-affinity IgG antibodies are required for allergen-blocking, whereas FcγRIIB-dependent inhibition was also mediated by IgG antibodies with low-affinity to the allergen (Akarsu et al., 2022; Epp et al., 2018a; Kanagaratham et al., 2020; Petry et al., 2021a; Zha et al., 2018).

In summary, the mechanisms of AIT- as well as IgG-induced inhibition of IgE-mediated allergic reactions have only been partially understood and require further research.

In the following section I will give an overview about the structure of IgE antibodies, and their binding to IgE Fc receptors as well as other IgE associated proteins. Furthermore, I will provide an introduction to IgE glycosylation including evidence from several studies for its role in allergic diseases.

1.3 IgE antibodies

Antibodies are part of the humoral immune system, mediating the specificity for antigens that come into contact with our immune system. They are produced by so called plasma cells (terminally differentiated B cells) and implement a variety of immune responses (Murphy and Weaver, 2018; Schroeder and Cavacini, 2010; Shamji et al., 2021; Vidarsson et al., 2014a).

Antibodies consist of two identical heavy (H) and light chains (L) linked by disulfide bonds, which gives the molecule a Y-shaped structure (Figure 5). Furthermore, each chain contains constant (C) and variable (V) domains. Thereby, each light chain contains one constant domain, whereas the heavy chains consist of either three or four constant domains, depending on the antibody isotype. Recognition of antigens is mediated by the fragment antigen-binding region (Fab) that is formed by one complete L-chain and the variable and C_H1 parts of one heavy chain,

corresponding to the amino-terminal variable and constant domains of both the heavy and the light chains. Each antibody has two Fab fragments which can each bind one antigen. The carboxy-terminal parts of the heavy chains form the fragment crystallizable (Fc), defining the isotype of an antibody, i.e., IgM, IgD, IgG, IgA or IgE. The Fab and the Fc parts of the antibody are linked by a flexible hinge region. Antibody isotypes differ in their size and serum half-lives as well as in FcR binding, their ability for complement fixation and their response to antigen and thus, their effector functions (Murphy and Weaver, 2018; Schroeder and Cavacini, 2010; Shamji et al., 2021; Vidarsson et al., 2014a).

The ability of antibodies to recognize a wide variety of different antigens is given by certain genetic mechanisms occurring during different stages of B-cell development:

- First, through differential organization and arrangement of several gene segments, forming the variable domain of the heavy and the light chains. The variable domains of the light chain are arranged by variable- (V) and joining- (J) gene segments. To build the heavy chains a diversity- (D) segment is added to the V- and J-gene segements.
- In a second step, random addition and removal of nucleotides at the intersections of the V(D)J segments leads to further diversity of the antibody repertoire. Those mechanisms involve DNA rearrangement and take place during early B-cell development.
- Third, a process called somatic hypermutation, which requires the help of T cells in the germinal center takes place after B-cell exposure to an antigen. Thereby, the variable domain genes of B cells undergo point mutations, which can result in the generation of antibodies with an elevated affinity to a certain antigen, referred to as affinity maturation (Gould et al., 2003; Murphy and Weaver, 2018; Schroeder and Cavacini, 2010).

Aside from affinity maturation, during an immune response, B cells also undergo class switch recombination, resulting in the different Ig isotypes. Thereby, a rearranged V(D)J region can be combined with any of the heavy chain constant domains (Cμ, Cδ, Cγ, Cα and Cε) (Gould et al., 2003; Murphy and Weaver, 2018; Schroeder and Cavacini, 2010).

IgE antibodies are the least abundant isotype in circulation with a rather short serum half-life, but and increased tissue half-life due to engagement with the high affinity IgE Fc receptor FcεRI as described in section 1.1.1. In contrast to IgG antibodies, which can be divided into four subclasses (IgG1, 2a, 2b and 3 in mice and IgG1-IgG4 in humans) there is only one IgE antibody isotype. IgE antibodies harbour, alongside with IgM antibodies, four constant domains (Cε1-Cε4) with Cε3 and Cε4 beeing homologous to Cγ2 and Cγ3 of IgG. The Cε2 domain is the most distinguishing feature of IgE since it gives the native molecule a compact, highly bent structure,

compared to the extended, flexible form of IgG. Furthermore, molecular analysis showed that the IgE Fc can either engage in an open or a closed conformation by rotating relative to Cε4 which dictates their interaction with the two IgE Fc receptors FcεRI and CD23/ FcεRII (Engeroff et al., 2020; Gould and Sutton, 2008a; Jabs et al., 2018).

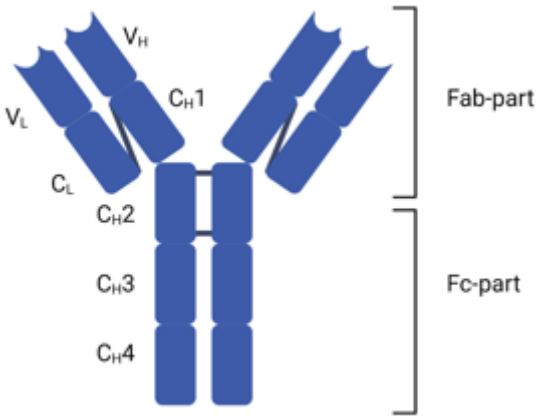

Figure 5: IgE antibody structure.

IgE antibodies consist of two identical heavy (H) and two identical light (L) chains. The light chains are composed of a variable (V_L) and a constant (C_L) domain. The heavy chains consist of one variable (V_H) and 4 constant domains (C_H1-C_H4). Antigen-binding is mediated by the Fab fragments (fragment antigen binding) whereas the IgE effector function is mediated by the Fc fragment (fragment crystallizable). This figure was created with BioRender.

Interaction with IgE Fc receptors

Two receptors have been described that interact with the Fc portion of IgE antibodies. Both differ in their type/ molecular structure, their affinity to IgE as well as their expression profile on immune cells (Gould et al., 2003; Gould and Sutton, 2008a; Murphy and Weaver, 2018).

The first receptor, FcεRI belongs to the immunglobulin superfamily and is also referred to as the "high affinity" IgE receptor, binding monomeric IgE with an affinity of ($K_d = 10^{10}$-10^{11} M^{-1}). It consists of an extracellular IgE-binding α-chain, a signal-amplifying β-chain, and a signal transducing γ-chain. The β- and γ-chain both hold an intracellular immunoreceptor tyrosine-based activating motif (ITAM). FcεRI is highly expressed as an $\alpha\beta\gamma_2$ tetramer on mast cells and basophils (2x 10^5 molecules per cell) and as an $\alpha\gamma_2$ trimer, lacking the amplifying β-subunit, on human APCs, monocytes/ macrophages, eosinophils, platelets and smooth muscle cells. Allergen crosslinking of the tetrameric form of FcεRI on mast cells and basophils initiates the pro-allergic singaling cascade, activating these cells to release preformed mediators (e.g., histamine, tryptase) and *de novo* synthesize lipidmediators (e.g., prostaglandins, leukotrienes)

(Gould et al., 2003; Gould and Sutton, 2008a; Murphy and Weaver, 2018). Furthermore, the release of inflammatory cytokines such as IL-4, IL-5 and IL-13 results in leucocyte recruitment which further elevates the allergic response. However, several studies have shown that IgE binding to FcεRI stabilizes the IgE-receptor complex, enhances FcεRI-expression and can prolong mast cell survival. For example, in patients with seasonal allergic rhinitis, FcεRI is upregulated on mast cells and basophils which correlates with elevated serum IgE concentrations (Gould and Sutton, 2008; Shamji et al., 2021; Sutton and Davis, 2015).

The second receptor, known as the "low affinity" IgE receptor CD23/ FcεRII is a single chain type II integral membrane protein of the C-type lectin superfamily. The single chain binds IgE with an affinity of $K_d = 10^6 – 10^7\ M^{-1}$ but the molecule commonly exists as a homo-trimer, remarkably elevating the affinity of IgE for this receptor. CD23 was first identified on B cells as an important regulator of IgE production but is expressed on several other cell types such as various APCs as well as lung and gut epithelial cells, where it contributes e.g., to allergen trafficking and presentation by binding IgE immune complexes. The latter is for example investigated in the context of food allergies as well as a potential target for allergic airway inflammation in asthma. Membrane-bound CD23 consists of a "lectin head" domain that is spaced from the membrane by an N-terminal stalk domain which is susceptible for proteolytic cleavage by the protease ADAM10 and leads to the release of soluble CD23. This mechanism has been demonstrated to be elevated in the absence of IgE thus leading to an upregulation of IgE production by IgE^+ B cells that result in enhanced allergic inflammation. Conclusively, CD23 can act as a positive as well as a negative regulator of IgE synthesis. Noteworthy, CD23 binds IgE at the junction of Cε3 and Cε4 and not to its carbohydrate residues. Furthermore, binding to CD23 is accompanied by conformational changes in the structure of the IgE Fc as described above, which prevents IgE binding to FcεRI and vice versa. Thus, the engagement of IgE with its receptors is tightly regulated by mechanisms of allosteric competition (Engeroff et al., 2020; Gould and Sutton, 2008a; Jabs et al., 2018; Sutton and Davies, 2015).

1.3.1 IgE antibody glycosylation

To date, it is widely accepted that the effector functions of antibodies are influenced not only by their subclass but also by their Fc *N*-glycosylation pattern. Especially IgG antibody Fc glycosylation has been extensively investigated. IgG antibodies have one distinct glycosylation site at asparagine (Asn) 297 located in both C_H2 domains of the antibody Fc part (Figure 6A). The attached glycan consists of a biantennary heptasaccharide core structure containing

mannose and N-acetylglucosamine (GlcNAc). This core structure can be modified by the addition of branching residues such as fucose or a bisecting GlcNAc and further elongated by terminal galactose and sialic acid molecules, which leads to a variety of different possible glycosylation patterns (Figure 6B, Bartsch et al., 2020; Collin and Ehlers, 2013; Kaneko et al., 2006; Nimmerjahn and Ravetch, 2008).

According to different studies the glycan structure at the Fc part of the IgG antibody is essential for the induction of antibody-dependent effector functions since enzymatic cleavage of the Fc-glycan resulted in decreased FcγR-binding and complement-activation (Allhorn et al., 2008; Dekkers et al., 2018; Nandakumar et al., 2007).

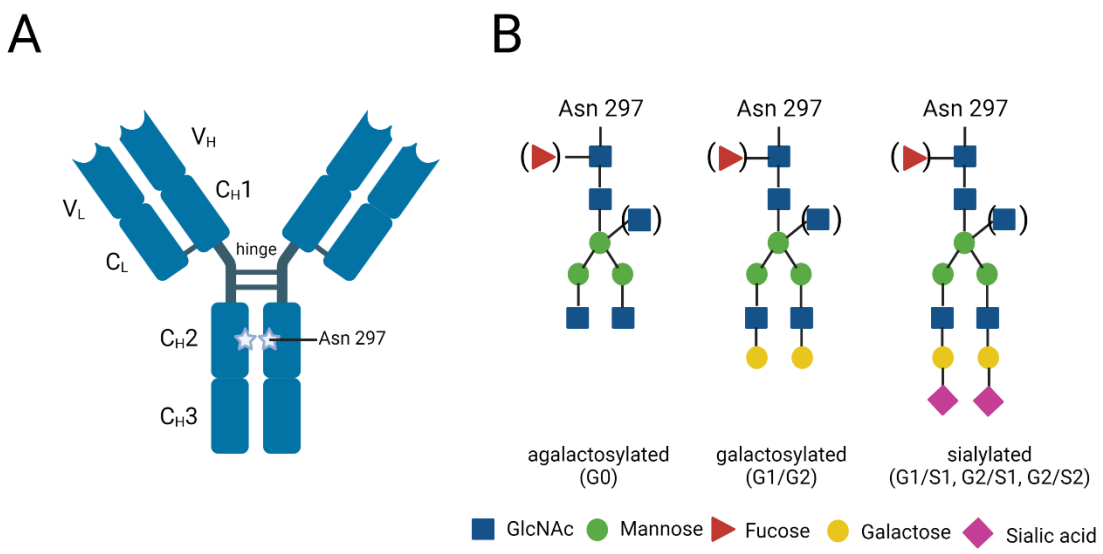

Figure 6: IgG structure and Fc glycosylation.

(A) *IgG antibodies harbour one conserved Fc glycosylation site at Asn 297 in the C_H2 domain of each heavy chain.* ***(B)*** *The core structure consists of a biantenarry heptasaccharide composed of 4 N-acetylglucosamine molecules (GlcNAc, blue) and 3 mannose residues (green). The core saccharide can be extended by fucose (red), bisecting GlcNAc, galactose (G, yellow) and sialic acid (S, pink). This figure was created with BioRender.*

It has been shown that due to age- and gender-dependent differences as well as altered IgG glycosylation upon vaccination, the IgG glycosylation profile varies among healthy individuals. (Alter et al., 2018; Dall'Olio et al., 2013; Hess et al., 2013; Selman et al., 2012; Yamada et al., 1997). Furthermore, the IgG glcosylation pattern could be linked to certain diseases, since

several human studies reported alterations in the IgG glycosylation pattern of patients suffering from inflammatory autoimmune diseases. For example, in patients with systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis or inflammatory bowel disease, increased amounts of non-galactosylated (agalactosylated; G0) IgG antibodies have been observed and correlated with disease progression and severity. On the other hand, elevated levels of galactosylated and terminal sialylated IgG antibodies have been associated with disease remission and also have been detected in woman during pregnancy. These observations led to the hypothesis that pro-inflammatory effector mechanisms are associated with non-galactosylated IgG antibodies, whereas galactosylated and terminal sialylated IgG antibodies mediate less or even anti-inflammatory immune responses (Ackerman et al., 2013; Collin and Ehlers, 2013; Decker et al., 2016; Dekkers et al., 2018; Nimmerjahn et al., 2007; Ohmi et al., 2016; Scherer et al., 2010; Trbojević Akmačić et al., 2015; Vučković et al., 2015).

This hypothesis was further supported by elucidating the therapeutic effect of IVIG (pooled serum IgG from healthy donors). Anti-inflammatory effects of IVIG have been observed in patients with autoimmune diseases and linked to IgG antibody Fc glycosylation. In fact, since neuraminidase-treatment abrogated this protective effect in several experimental mouse models, the anti-inflammatory effect of IVIG could be attributed to its sialylated subfraction (Kaneko et al., 2006; Samuelsson et al., 2001; Schwab et al., 2014). Furthermore, it has been suggested that IVIG mediates anti-inflammatory effects via enhancing the amount of total sialylated IgG antibodies. This, in turn led to an upregulation of the inhibitory FcγRIIB on immune effector cells by binding of sialic acid to the type II Fc receptor specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin-related 1 (SIGN-R1) or its human homolog dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN), which both belong to the sugar binding C-type lectin superfamily (Anthony et al., 2011, 2008).

In contrast to IgG antibodies, IgE is the most heavily glycosylated among all immunoglobulin classes with 7 *N*-linked glycosylation sites distributed across the heavy chains of human IgE and 9 across murine IgE, respectively (Figure 7A; Arnold et al., 2004a; Shade et al., 2019, 2015, 2020). However, role of glycosylation in IgE biology as well as in the context of allergic disease is still poorly understood. Systematic analysis of all glycosylation sites on human and mouse IgE revealed that a single glycan at Asn 394 (human IgE) or Asn 384 (murine IgE), respectively, on the Cε3 domain is essential for initiating anaphylaxis. This glycosylation site is occupied by an oligomannose glycan, whereas the other glycosylation sites hold complex, antennary

structures as described above in the context of IgG glycosylation (Figure 7B; Plomp et al., 2014; Shade et al., 2019, 2020, 2015a).

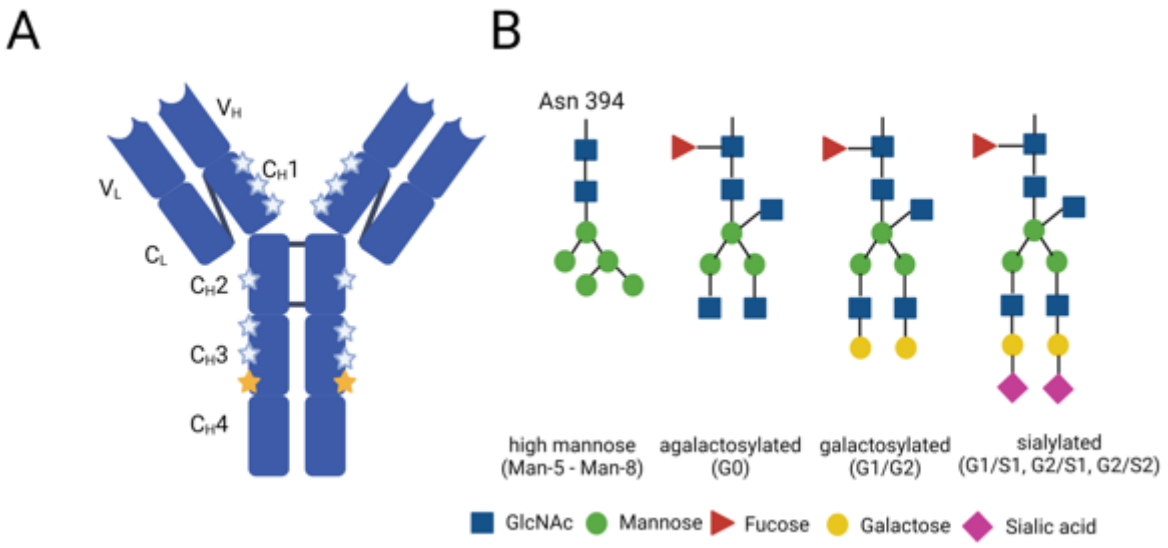

Figure 7: Structure and glycosylation of IgE antibodies.

(A) *IgE antibodies have 7 (human IgE) or 9 (murine IgE) N-linked glycosylation sites distributed across each heavy chain of the antibody.* ***(B)*** *Asn 394 (human IgE)/ Asn 384 (murine IgE, yellow star) holds an oligomannose glycan that is essential for IgE folding and binding to FcεRI. The other glycosylation sites hold complex antennary glycans composed of GlcNAc (blue), mannose (green) and fucose (red) that can be elongated by galactose (yellow) and sialic acid (pink). This figure was created with BioRender.*

Selective enzymatic removal of the oligomannose glycan altered the secondary structure of the IgE molecule in a way that prevents binding to FcεRI on mast cells and basophils and attenuates anaphylaxis *in vivo*. Subsequently, this oligomannose structure was identified to be ortholog to the IgG glycan at Asn 297 which dictates IgG effector function as described above (Shade et al., 2019, 2015; Wurzburg et al., 2000). Noteworthy, the oligomannose structure is the only glycan on IgE which is not exposed since it was found to occupy the cavity between the two IgE Fc parts as described for the key glycan attached to the IgG Fc (Jabs et al., 2018; Sondermann et al., 2000; Wurzburg et al., 2000). The remaining *N*-linked glycosylation sites of human IgE, aside from Asn 383, which is unoccupied, have been reported to hold complex antennary glycans that are almost exclusively fucosylated and highly sialylated (Arnold et al., 2004a; Plomp et al., 2014a; Shade et al., 2015, 2020a; Wu et al., 2016). Mutation of the complex glycan structures suggested a minimal role in IgE-FcεRI-binding (Nettleton and Kochan, 1995; Sayers et al., 1998; Shade et al., 2015; Young et al., 1995), whereas mutation of the glycosylation site of Cε1 in the Fab portion of the antibody led to a slight reduction of IgE-

mediated effector cell degranulation, suggesting a possible role in antigen-binding (Shade et al., 2015). However, genetic disruption removes the entire glycan from the IgE molecule, paying no regard to the possible roles that are dependent on the heterogeneity of the complex glycans and, compared to IgG, this role is poorly understood in the context of allergic disease.

Studies using a group of β-galactoside binding lectins, named galectins, have suggested a potential role of the complex type glycans in IgE-mediated effector functions. Upon the 15 different galectins characterized in mammals, two of them have found to be IgE-binding proteins (Dumic et al., 2006; Liu, 2005; Niki et al., 2009). Galectin-3, first named ε-binding protein, was originally identified in rat basophilic leukemia (RBL) cells by its capacity to bind IgE (Liu et al., 1985; Robertson et al., 1990). Furthermore, heterogenous recognition of IgE glycoforms by galectin-3 could be observed since neuraminidase-treatment (removal of terminal sialic acids) drastically increased galectin-3 binding capacity to different myeloma IgE antibodies which have been demonstrated to be abberantly high sialylated (Baenziger et al., 1974) suggesting that galectin-3 predominantly recognizes IgE antibodies with low sialylation levels (Robertson et al., 1990). Interestingly, this feature of galectin-3 was further used to characterize heterogenous IgE glycoforms of patients with hyper-IgE syndrome or atopic dermatitis varying in their extend of sialylation (Robertson and Liu, 1991). Effector functions of Galectin-3 in the context of allergic disease have been extensively studied over the years. Galectin-3 is expressed on the surface of mast cells and basophils and has been shown to activate these cells by binding FcεRI, IgE or both (Dumic et al., 2006; Frigeri et al., 1993; Robertson and Liu, 1991). Furthermore, there is evidence that galectin-3 is involved in several processes in allergic inflammation such as development of a Th2 phenotype, eosinophil recruitment, airway remodeling and increased expression of inflammatory mediators. In an *in vivo* model it could be demonstrated that bronchoalveolar lavage (BAL) fluid of ovalbumin-challenged mice contained significantly elevated levels of galectin-3 compared to control mice (Dumic et al., 2006; Gao et al., 2013; Zuberi et al., 2004).

The second IgE-binding galectin, galectin-9, on the other hand has been associated with several anti-allergic effects. There is evidence that galectin-9 suppresses mast cell degranulation and survival (Kojima et al., 2014), regulates the immune balance to reduce atopic dermatitis (Kim et al., 2020), suppresses food allergy (Kivit et al., 2012; Sziksz et al., 2012) and attenuates asmathic reactions in guinea pigs and passive cutaneous anaphylaxis in mice, likely by interfering with IgE-antigen complex formation by targeting the glycan structures of the IgE antibody (Niki et al., 2009).

A recent study from Shade *et al.* has suggested IgE sialylation as a potential regulator of allergic disease. It could be demonstrated that the sialic acid content of the total serum IgE from peanut-allergic individuals is increased compared to non-atopic individuals and that the removal of sialic acid from IgE attenuates effector cell activation and anaphylaxis in several experimental models of allergic disease including *in vivo* models such as passive systemic or passive cutaneous anaphylaxis (Shade et al., 2020a). Noteworthy, these findings are in contrast to the consensus that antibody sialylation is associated with anti-inflammatory effects of IgG (Anthony et al., 2011; Bartsch et al., 2020; Epp et al., 2018a; Petry et al., 2021a), IgA (Steffen et al., 2020) and IgM (Colucci et al., 2015) antibodies.

Taken together, it is well established that the effector functions of IgG antibodies are influenced by their glycosylation pattern, for example by modulating the affinity of the IgG antibodies for classical activating or inhibitory IgG FcγRs (type I FcRs) or facilitating the differential interaction with sugar-binding receptors, especially of the C-type lectin family (type II FcRs). Thereby, IgG sialylation is associated with anti-inflammatory effects and disease remission in several autoimmune and infectious diseases as well as in models of IgG-mediated anaphylaxis. The role of IgE glycosylation for IgE biology and allergic disease is less clear. A mandatory oligomannose glycan that is essential for maintaining the correct secondary structure of the IgE molecule and its ability to bind FcεRI has been identified. However, there is only a small body of evidence about the role of the remaining complex type glycans on IgE and the impact of IgE glycosylation on IgE-mediated allergic reactions.

1.4 Aim of the thesis

Allergen-specific IgE antibodies mediate allergic recations, including systemic anaphylaxis, by crosslinking the high affinity FcεRI on mast cells and basophils, which leads to cell degranulation and subsequent release of mediators such as histamine. In contrast, allergen-specific IgG antibodies, induced for example during allergen immunotherapy (AIT) can attenuate IgE-mediated allergic reactions by interacting with allergenic proteins thereby blocking allergen binding to cell surface IgE and via FcγRIIB-mediated inhibition of effector cells. However, in case of high allergen-doses, also IgG antibodies can induce anaphylaxis in mice through activation of myeloid cells mediated by Fcγ-receptors.

It is widely accepted that the IgG effector functions are affected by their subclass and also their Fc *N*-glycosylation pattern. Thereby, IgG Fc glycosylation has been demonstrated to modulate

the affinity of the antibody to activating and inhibitory classical FcγRs as well as sugar-binding receptors of the C-type lectin family. However, there is only little knowledge about the role of IgE glycosylation in anaphylactic reactions.

One aim of this thesis was to investigate whether the sialylation level of the total serum IgG antibodies can influence IgG-mediated as well as IgG-controlled IgE-mediated allergic reactions. Furthermore it should be investigated how different IgE glycosylation patterns modify an IgE-mediated anaphylactic reaction.

First, the potential of antigen-unspecific sialylated human IgG4 antibodies to attenuate IgG-mediated allergic reactions should be investigted in a murine IgG-mediated passive systemic anaphylaxis model. Furthermore, the differential regulation of the inhibitory FcγRIIB as an underlying mechanism on a cellular level should be elucidated.

Second, sialylated antigen-unspecific murine IgG1 antibodies should be investigated regarding their protective effect in IgG-controlled IgE-mediated anaphylaxis.

Third, differently glycosylated murine IgE antibodies should be generated *in vitro*. Subsequently, these antibodies should be compared regarding their binding capacity for the high affinity IgE Fc receptor FcεRI, their ability to sensitize and activate effector cells of IgE mediated allergic reactions as well as their serum half-life. Furthermore, the obtained results should be verified in an IgE-mediated anaphylaxis mouse model.

In summary, the results obtained during this project regarding the role of the glycosylation of IgG and IgE antibodies in the context of allergic disease might contribute to the exploration of new diagnostic and therapeutic approaches for IgG- as well as IgE-mediated allergic reactions.

2 Materials and Methods

2.1 Materials

2.1.1 Mice

C57BL/6 mice were originally purchased from Jackson Laboratory (Bar Harbour, ME; C57BL6/J) and used for our own breeding colonies or additionally purchased from Janvier Labs

(Le Genest-Saint-Isle, France). Mice were bred and maintained in a pathogen-free facility at the University of Lübeck. For all experiments 8-12 weeks old female mice were used. The animal test proposals (V241 – 25487/2018 (48-6/18), V241 - 44603/2018 (79-8/18), V 242 - 12783/2020 (23-3/20), V 242 - 29438/2020 (44-6/20), IX 552 – 79310/2022 (74-8/22)) were approved by local authorities of the Animal Care and Use Committee (Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung, Kiel, Germany).

2.1.2 Cell Lines

IgE anti-TNP (clone IgEL a2) hybridoma cell line (ATCC-TIB-142) (Rudolph et al., 1981).

Human mast cell line LAD2 (S. Kirshenbaum et al., 2019).

2.1.3 Antibodies

Table 1: In vivo application

Antibody	Clone	Company
Human IgG4 (from IVIG)	polyclonal	Biotest AG (Langen, Germany)
Mouse anti-TNP IgG1	H5	InVivo (Henningsdorf, Germany)
Mouse IgG1 isotype control	MOPC-21	BioXCell (Lebanon, NH, USA)

Table 2: ELISA antibodies

Epitope	Reactivity	Clone	Conjugate	Company
IgE	mouse	polyclonal	- HRP	Bethyl Labs (Montgomery, TX, USA)
IgG-Fc	human	HP-6017	- HRP	Biozol (Eching, Germany)
IgG4	human	HP-6025	HRP	Biozol (Eching, Germany)

Table 3: Antibodies for flow cytometry

Epitope	Reactivity	Clone	Conjugate	Company
FcεRI	human	AER-37	BV605	BioLegend (San Diego, CA, USA)
B220	mouse	RA3-6B2	BV786	BioLegend (San Diego, CA, USA)

CD3	mouse	17A2	APC-Cy7	BioLegend (San Diego, CA, USA)
CD19	mouse	6D5	APC-Cy7	BioLegend (San Diego, CA, USA)
CD49b	mouse	HMα1	FITC	BioLegend (San Diego, CA, USA)
CD11b	mouse	M1/70	BV421	BioLegend (San Diego, CA, USA)
FcεRI	mouse	MAR-1	AF700	BioLegend (San Diego, CA, USA)
FcγRIIB	mouse	AT130-2	APC	Fisher Scientific (Schwerte, Germany)
Gr-1	mouse	RB6-8C5	Biotin BV605	BioLegend (San Diego, CA, USA)
Ly6G	mouse	1A8	PerCP-Cy5.5	BioLegend (San Diego, CA, USA)
IgE	mouse	RME-1	AF488	BioLegend (San Diego, CA, USA)

2.1.4 Enzymes

Table 4: List of used enzymes

α-2,6-sialyltransferase (human)	Roche (Basel, Switzerland)
β-1,4-galactosyltransferase	Roche (Basel, Switzerland)
Endoglycosidase S (EndoS)	provided by Matthias Collin, University Lund (Sweden)
Sialidase A + 5x reaction buffer B	Agilent (Santa Clara, CA, USA)

2.1.5 Proteins

Table 5: List of used proteins

Protein	Conjugate	Company
α1-acid glycoprotein (Orosomucoid) from bovine plasma	-	Sigma-Aldrich (St. Louis, MO, USA)
Recombinant murine galectin-3	-	R&D Systems (Minneapolis, MN, USA)
Human FcεRIα	-	Sino Biological Europe (Eschborn, Germany)
Murine FcεRIα	-	Sino Biological Europe (Eschborn, Germany)
Ovalbumin Grade VI	-	Sigma-Aldrich (St, Louis, MO, USA)
Sambucus nigra lectin (SNA)	HRP	Biomol (Hamburg, Germany)
Streptavidin	BV605	BioLegend (San Diego, CA, USA)

2.1.6 Adjuvants

Table 6: List of used adjuvants

Adjuvant	Company

Incomplete Freund´s Adjuvant (IFA)	Sigma-Aldrich (St, Louis, MO, USA)
M. Tuberculosis Des. H37 Ra (non-viable)	BD Bioscience (San Diego, CA, USA)
Alhydrogel adjuvant 2 % (Alum)	InvivoGen (Toulouse, France)

2.1.7 Media and Supplements

Cell culture

Table 7: List of cell culture media and supplements

Fetal calf serum (FCS)	Life Technologies (Darmstadt, Germany)
L-Glutamine	Life Technologies (Darmstadt, Germany)
Penicillin (10,000 IU mL^{-1})/Streptomycin (10 mg mL^{-1})	Life Technologies (Darmstadt, Germany)
Penicillin (10,000 IU mL^{-1})/Streptomycin (10 mg mL^{-1})/Amphotericin B (25 µg mL^{-1})	Lonza (Basel, Switzerland)
Primatone	Sigma-Aldrich (St. Louis, MO, USA)
RPMI1640 (L-Glutamine)	Life Technologies (Darmstadt, Germany)
Recombinant human stem cell factor (rhSCF)	PeproTech (Hamburg, Germany)
StemPro-34 + Nutrient Supplement	Life Technologies (Darmstadt, Germany)

Media composition

Table 8: Cell culture media composition

Hybridoma culture medium	RPMI1640 (L-Glutamine) + 1% Penicillin-Streptomycin + 10% FCS
Hybridoma production medium	RPMI1640 (L-Glutamine) + 1% Penicillin-Streptomycin + 0,03% Primatone
LAD2 culture medium	StemPro-34 medium + 2,5% StemPro-34 Nutrient supplement + 2 mM L-Glutamine + 1% Penicillin-Streptomycin + 100 ng mL^{-1} rhSCF

2.1.8 Chemicals

Table 9: List of used chemicals

2-(N-morpholino)ethanesulfonic acid (MES)	Merck (Darmstadt, Germany)
Acetic acid	Carl Roth (Karlsruhe, Germany)
Acetonitrile Rotisolv HPLC Gradient	Carl Roth (Karlsruhe, Germany)
Aminobenzamide (2-AB)	Sigma-Aldrich (St, Louis, MO, USA)
Ammonia (25%)	Carl Roth (Karlsruhe, Germany)
Ammonium chloride (NH_4Cl)	Sigma-Aldrich (St. Louis, MA, USA)
Bovine Serum Albumin (BSA)	GE Healthcare (Little Chalfont, GB)
Bromphenol blue	AppliChem (Darmstadt, Germany)
Calcium chloride ($CaCl_2$)	Sigma-Aldrich (St, Louis, MO, USA)
Carbonate-bicarbonate	Sigma-Aldrich (St, Louis, MO, USA)
Cellulose	Merck (Darmstadt, Germany)
Citric acid	Sigma-Aldrich (St, Louis, MO, USA)
CMP-sialic acid	Millipore (Billerica, MA, USA)
CnBr activated Sepharose 4B	GE Healthcare (Little Chalfont, GB)
Coomassie brilliant blue R-250	GE Life Science (Little Chalfont, GB)
Dimethyl sulfoxide (DMSO)	Merck (Darmstadt, Germany)
Ethanol	Carl Roth (Karlsruhe, Germany)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (St. Louis, MA, USA)
Fixable viability dye (eFluor780)	Thermo Fisher (Waltham, MA, USA)
Formic acid	Fluka (St, Louis, MO, USA)
Gelantine	Sigma-Aldrich (St. Louis, MA, USA)
Glycine	Merck (Darmstadt, Germany)
Graphite (Carbograph)	Grace (Columbia, MD, USA)
HEPES	Life Technologies (Darmstadt, Germany)
Hydrogen chloride (HCl)	Merck (Darmstadt, Germany)
Intratect® (IVIG)	Biotest AG (Langen, Germany)
Isopropanol	Otto Fischar (Saarbrücken, Germany)
Ketamin 10 mg/ml	WDT (Garbsen, Germany)
Manganese(II) chloride	Sigma-Aldrich (St. Louis, MA, USA)
Methanol	AppliChem (Darmstadt, Germany)
PageRuler Protein Ladder, 10- 250 kDa	ThermoFisher Scientific (Waltham, MA, USA)
p-nitrophenyl N-acetyl-β-D-glucosaminide	Sigma-Aldrich (St. Louis, MA, USA)
Potassium chloride (KCl)	Carl Roth (Karlsruhe, Germany)
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth (Karlsruhe, Germany)

Potassium phosphate, dibasic (K_2HPO_4)	Sigma-Aldrich (St, Louis, MO, USA)
Protein G Sepharose	GE Life Sciences (Little Chalfont, GB)
Reaction buffer B, 5x	Prozyme (Hayward, CA, USA)
Rompun 2% (Xylazine)	Bayer (Lerverkusen, Germany)
Sodium acetate (NaOAc)	Sigma-Aldrich (St, Louis, MO, USA)
Sodium chloride (NaCl)	Carl Roth (Karlsruhe, Germany)
Sodium cyanoborohydride	Merck (Darmstadt, Germany)
Sodium hydrogen carbonate ($NaHCO_3$)	Carl Roth (Karlsruhe, Germany)
Sodium hydroxide (NaOH)	Merck (Darmstadt, Germany)
Sulfuric acid	Sigma-Aldrich (St, Louis, MO, USA)
TMB substrate reagent set	BD Biosciences (Heidelberg, Germany)
TNP-BSA	Biocat (Heidelberg, Germany)
TNP-Ficoll	BioSearch Technol. (Petaluma, CA, USA)
TNP-OVA	BioSearch Technol. (Petaluma, CA, USA)
Trifluoroacetic acid	Merck (Darmstadt, Germany)
Tris-HCl	Sigma-Aldrich (St, Louis, MO, USA)
Trisma Base (Tris)	Sigma-Aldrich (St. Louis, MA, USA)
Triton X-100	Sigma-Aldrich (St. Louis, MA, USA)
Trypan blue	Sigma-Aldrich (St. Louis, MA, USA)
Tween 20	Sigma-Aldrich (St. Louis, MA, USA)
UDP-galactose	Millipore (Billerica, MA, USA)

2.1.9 Buffers and Solutions

Antibody purification

Table 10: List of buffers and solutions used for antiody purification

Activation buffer	1 mM HCl in ddH_2O
Blocking buffer	0,1 M Tris-HCl in ddH_2O, adjusted to pH 8
Coupling buffer	0,1 M $NaHCO_3$, adjusted to pH 8,3 0,5 M NaCl in ddH_2O
Elution buffer	0,1 M Glycine in ddH_2O, adjusted to pH 2,8

High pH wash buffer	0,1 M Tris-HCl 0,5 M NaCl in ddH_2O, adjusted to pH 8
Low pH wash buffer	0,1 M Acetic acid 0,5 M NaCl in ddH_2O, adjusted to pH 4

SDS-PAGE

Table 11: List of buffers and solutions used for SDS-PAGE

Coomassie destain solution	40% Methanol 10% Acetic acid in ddH_2O
SDS loading buffer	0.25 M Tris (pH 6.8) 0.5 M DTT 25% Bromphenol blue 50% Glycerol 10% SDS in ddH_2O
SDS running buffer	25 mM Tris 192 mM Glycin 0.1% SDS in ddH_2O, adjusted to pH 8.3

ELISA

Table 12: List of buffers and solutions used for ELISA

Coating buffer	50 mM Carbonate/ bicarbonate buffer in ddH_20
Blocking buffer	2,5% milk powder in 1x PBS
ELISA buffer	1 g Gelantine 30 g BSA 3 mM EDTA in 1x PBS
Wash buffer	PBST (0,5 mL L^{-1} Tween 20 in 1x PBS)

Lectin buffer	10 mM HEPES 0,1 mM $CaCl_2$ 0,15 M NaCl 0,1% Tween 20
Sulfuric acid	4,2% sulfuric acid in ddH_2O

Flow cytometry

Table 13: List of buffers and solutions used for flow cytometry

Erylysis buffer	150 mM NH_4Cl 100 µM EDTA 1 mM $NaHCO_3$ in ddH_2O, adjusted to pH 7.2

Mast cell assay

Table 14: List of buffers and solutions used for the mast cell activation assay

Blocking buffer	1% BSA in 1x PBS
Buffer	0.1% BSA in 1x PBS
Triton X-100 solution	0.1% Triton X-100 in ddH_2O
Substrate buffer	50 mM tri-sodiumcitrate in ddH_2O (pH 8,48) 50 mM citric acid in ddH_2O (pH 2,22), adjusted to pH 4,5
Gycine buffer (Stop-Solution)	0.4 M glycine in ddH_2O, adjusted to pH 10.7

2.1.10 Consumables

Table 15: List of the used consumables

Amicon Ultra Centrifugal Filters, 10K; 100K	Merck (Darmstadt, Germany)
Cell Culture Flasks for suspension cells	Greiner Bio-one (Kremsmünster, Austria)
Costar Assay plates, 96 well	Corning (Kennebunk, ME, USA)
ELISA reagent reservoir	Thermo Fisher (Waltham, MA, USA)
FACS tubes (5 mL)	Sarstedt (Sarstedt, Germany)

Falcon tubes (15, 50 mL)	Greiner Bio-one (Kremsmünster, Austria)
Filter Stericup 0,22 µm	Merck (Darmstadt, Germany)
Gravity Flow Column (10, 20 mL)	G-Biosciences (St. Louis, MO, USA)
HPLC vial ND11	Carl Roth (Karlsruhe, Germany)
Maxi Column	G Biosciences (St, Louis, MO USA)
Micro inserts HPLC vials	Carl Roth (Karlsruhe, Germany)
Mini-Protean® TGX™ Protein Gel, 10%	Bio-Rad-Laboratories (Hercules, CA, USA)
Needle (26G, 30G)	Braun (Melsungen, Germany)
pH indicator	Merck (Darmstadt, Germany)
Pipette tips (10, 200, 1000 µL)	Sarstedt (Nümbrecht, Germany
Reaction tubes (0.5, 1.5, 2.0 mL)	Sarstedt (Nümbrecht, Germany)
Serological pipettes (5, 10, 25, 50 mL)	Sarstedt (Nümbrecht, Germany)
Serum MiniCollect	Greiner Bio-one (Kremsmünster, Austria)
Single-use syringes (1 mL)	Braun (Melsungen, Germany)
Suspension culture plate (24-well, 48-well)	Greiner Bio-one (Kremsmünster, Austria)
Vial lid ND11 PTFE Septum	Carl Roth (Karlsruhe, Germany)
XBridge BEH Glycan 1.7µm column	Waters Corporation (Milford, MA, USA)

2.1.11 Equipment

Table 16: List of used laboratory equipment

Analytical scale	Sartorius (Göttingen, Germany)
Autoclave VX-75	Systec (Linden, Germany)
Centrifuge 5424R	Eppendorf (Hamburg, Germany)
Centrifuge 5810R	Eppendorf (Hamburg, Germany)
Concentrator plus (speed-vac)	Eppendorf (Hamburg, Germany)
Direct heat CO_2 Incubator	Integra (Fernwald, Germany)
Dual-Pump KP-22	Fischer Analytics (Weiler, Germany)
Flow cytometer Attune NxT	Thermo Scientific (Waltham, MA, USA)
Gel chamber	Biometra (Göttingen, Germany)
Gelsystem PowerPac Basic	Bio-Rad-Laboratories (Hercules, CA, USA)
Hemocytometer	Sigma-Aldrich (St. Louis, MA, USA)
HPLC Ultimate 3000 system	Thermo Fisher (Waltham, MA, USA)
Incubator AutoFlow NU-5510	NuAir (Plymouth, MN, USA)
Microplate-Photometer Spectra Max iD3	Molecular Devices (San José, CA, USA)
Microscope Primovert	Zeiss (Oberkochen, Germany)
NanoDrop™ One	Thermo Scientific (Waltham, MA, USA)

pH-meter FiveEasy F20	Mettler-Toledo (Columbus, OH, USA)
Pipetboy Accu 2	Integra Bioscience (Zizers, Switzerland)
Pipette (single-channel)	Eppendorf (Hamburg, Germany)
Pipwtte (multi-channel)	Eppendorf (Hamburg, Germany)
Pipette Multistep	Eppendorf (Hamburg, Germany)
Plate shaker Polymax 1040	Heidolph (Schwabach, Germany)
Sterile hood NU-437-600E	Integra (Fernwald, Germany)
Tailveine restrainer for mice	Braintree Scientific (Braintree, USA)
Thermometer Physitemp BAT-12	Science Products (Hofheim, Germany)
Thermomixer Compact	Eppendorf (Hamburg, Germany)
Tube Rotator	VWR (Radnor, PA, USA)
Vacuum pump MZ2C	Vacuubrand (Wertheim, Germany)
Vortex-Genie 2	Scientific Industries (Bohemia, NY, USA)
Water bath	

2.1.12 Software

Table 17: List of the used software

Chromeleon Software V6.9	Thermo Fisher (Waltham, MA, USA)
GraphPad Prism v. 6.04	GraphPad Software (San Diego, CA, USA)
FlowJo v10.0.7	Treestar (Ashland, OR, USA)
Microsoft Office 365	Microsoft (Albuquerque, NM, USA)

2.2 Methods

2.2.1 Cell culturing

Media and growth conditions

The IgEL a2 hybridoma cell line was maintained in RPMI 1640 medium supplemented with 10% FCS/ 1% Penicillin-Strepromycin (100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin).

The LAD2 mast cell line was maintained in StemPro-34 medium supplemented with 2,5% StemPro-34 Nutrient Supplement, 2 mM L-Glutamine, 1% Penicillin-Strepromycin (100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin) and 100 ng mL^{-1} rhSCF.

Cells were placed in a CO_2 incubator with 5% CO_2 at 37 °C and 100% relative air humidity for cultivation and were frequently examined for appearance, growth behaviour and possible contaminations.

Subculturing of cell lines

All work was performed under sterile conditions. As IgEL a2 cells grow in suspension they were splitted by withdrawing a portion of the cell suspension and addition of fresh cell culture medium to the culture flask approximately every 2-3 days when 70-80% confluent.

LAD2 cells were cultured according to the protocol described by Rådinger (Rådinger et al., 2010).

Freezing of cells

For cryo-perservation cells were harvested and counted. Cells were frozen in portions of $5x10^7$ cells mL^{-1}. After centrifugation (300x g at 4 °C for 5 min), the supernatant was discarded and the cell pellet was resuspended in freezing medium (RPMI 1640 medium for hybridoma cells, StemPro-34 medium for LAD2 cells; each supplemented with 10% of the cryoprotective agent DMSO). 1 mL aliquots of the cell suspension were transferred into cryo-tubes, freezed using a cryo container and stored at -80 °C.

Thawing of cells

The frozen hybridoma cells were taken from the -80 °C storage and thawed in a 37 °C water bath. As soon as they were thawed, cells were suspended in fresh cell culture medium and immediately centrifuged at 300x g for 5 minutes to remove the DMSO from the freezing medium. The supernatant was discarded, and the cell pellet was resuspended in fresh culture medium and transferred into a culture flask. Cells were incubated overnight, and the medium was exchanged the next day.

LAD2 cells were thawed according to the protocol described by Rådinger (Rådinger et al., 2010).

2.2.2 Antibody production

To produce anti-TNP IgE antibodies, IgEL a2 cells were expanded as described in 2.2.1. Eventually, cells were harvested and washed with 1x PBS by centrifugation at 300x g for 5 minutes. The cell pellet was resuspended in hybridoma production medium (FCS-free RPMI 1640 medium supplemented with 0.03% Primatone/ 1% Penicillin-Streptomycin). Cells were incubated for up to 14 days before antibodies were harvested. Therefore, cells were centrifuged at 800x g for 5 minutes. The supernatant was sterile filtered (0.22 µm filter), supplemented with 1% Penicillin-Streptomycin + Amphotericin B to avoid contamination and stored at 4 °C until further use.

The anti-TNP IgG1 antibodies (clone H5) were purchased from InVivo (Henningsdorf, Germany).

2.2.3 Antibody purification

Purification of anti-TNP IgE antibodies from cell culture supernatants was achieved by affinity chromatography. Therefore TNP-Sepharose was prepared. For the purification of human IgG4 an anti-human IgG4 sepharose column already existed in our lab.

Generation of TNP-Sepharose for antibody purification

The coupling of TNP-BSA to cyanogen bromide (CNBr)-activated sepharose was performed according to the protocol of Kavran and Leahy (Kavran and Leahy, 2014). In brief, 6 mg TNP(34)BSA were dialyzed against coupling buffer using a 10 kDa centrifugal filter. Furthermore, 0.75 mg (CNBr)-activated sepharose were resuspended in 15 mL of 1 mM HCl (activation buffer) and transferred into a column. After 2 hours of activation at 4 °C the TNP-BSA (2,5 mL) was added to the sepharose and incubated at 4 °C on a tube rotator overnight for coupling. To remove unbound TNP-BSA, the TNP-coupled sepharose was washed with coupling buffer and centrifuged at 1000x g for 5 minutes. Uncoupled binding sites of the sepharose preparations were blocked with 0.1 M Tris-HCl (pH 8) for 2 hours on a tube rotator at room temperature. Finally, the sepharose preparation was washed six times, alternating between high and low pH wash buffers. The column was stored in 20% ethanol in PBS at 4 °C until further use.

General purification process

TNP-coupled sepharose was washed with 50 mL 1x PBS (flow by gravity). Next, the cell culture supernatant was loaded onto the TNP-BSA column and IVIG (diluted 1:10 in 1x PBS) was loaded to the anti-human IgG4 column. The flow-through was collected for a second purification step. Then, the sepharose was washed with at least 50 mL 1x PBS before antibodies were eluted in 1 mL fractions with 0.1 M glycine (pH 2.9). The pH of the eluate was adjusted to pH 7 with 1 M Tris-HCl (pH 8.9) immediately. The antibody concentration was determined by UV-Spectrometry (NanoDrop) and the buffer was exchanged to 1x PBS using 10 kDa centrifugal filters. Purified antibodies were stored at -80 °C until further use. The columns were washed with 0.1 M glycine to clear them from any remaining bound antibodies followed by 50 mL 1x PBS to restore a physiological pH. The columns were stored in 20% ethanol in 1x PBS at 4 °C.

2.2.4 *In vitro* desialylation of proteins

For desialylation, glycoproteins (anti-TNP IgE abs, α1-acid glycoprotein (AGP)) were incubated in 5x Reaction Buffer B with α-2,6-sialidase A (50 mU/ 1 mg protein) for 48 hours at 37 °C on a shaker at 350 rounds per minute (rpm). The reaction was stopped by dilution in 1x PBS followed by exchanging the 5x Reaction Buffer B to 1x PBS using centrifugal filters. After glycan-modification the anti-TNP IgE abs were purified again using the TNP-coupled sepharose as described in 2.2.3 to remove the enzymes used for desialylation.

2.2.5 *In vitro* galactosylation and sialylation of antibodies

For galactosylation, antibodies were incubated with human β-1,4-galactosyltransferase (transferase to antibody mass ratio = 1:100) and the substrate UDP-galactose (substrate to antibody mass ration = 1.2:1) in 100 mM MES buffer (pH 7.2) supplemented with 20 mM Manganese(II) chloride at 37 °C and 350 rpm for 24 hours. For further sialylation, human α-2,6-sialyltransferase (transferase to antibody mass ratio = 1:10) and the substrate CMP-sialic acid (substrate to antibody mass ratio = 1:2) were added to the galactosylated antibodies for an additional 8 hours at 37 °C and 350 rpm. Reactions were stopped by dilution in 100 mM MES buffer and subsequent buffer exhange to 1x PBS using centrifugal filters. After glycan-modification the anti-TNP IgE abs were purified again using the TNP-coupled sepharose as described in 2.2.3 to remove the enzymes used for galactosylation and sialylation.

2.2.6 Antibody Fc *N*-glycosylation analysis by HPLC

Fc *N*-glycans of the enriched IgG4 antibodies were cleaved through incubation of 100 µg antibodies with recombinant endoglycosidase S (EndoS) from *Streptococcus pyogenes* (Collin, 2001) overnight at 37 °C and 350 rpm. Afterwards, samples were stored at -20 °C until further analysis.

Glycan analysis was performed via high-performance liquid chromatography (HPLC) on a Dionex Ultimate 3000 using an Xbridge XP BEH Glycan column. The method was established by Yannic Bartsch, a former PhD student of our laboratory and is described in detail in his dissertation (Bartsch, 2019). In brief, glycans from EndoS-digested antibodies were first purified by graphitized carbon columns to separate the glycans, which were retained in the column from proteins, salts, and other contaminations. Glycan elution was achieved by 25% acetonitrile/ 0.1% triflouroacetic acid. Next, eluted glycans were vacuum dried with a vacuum centrifuge.

Graphite purified glycans were labeled with flourophore 2-aminobenzamide (2-AB) for detection by a flourescence detector. Excessive labeling reagent was removed by cellulose chromatography. Labeled glycans were eluted by H_2O and again vacuum dried using a vacuum centrifuge. Finally, glycans were reconsituted in 80% acetonitrile. 5 µL of the sample were transferred to the HPLC machine and separated by hydrophilic interaction liquid chromatography. More hydrophobic glycans had a longer retention time. The resulting chromatograms were analyzed using the software Chromeleon 6. All identified peaks were previously confirmed by MALDI-TOF analysis (Bartsch, 2019).

2.2.7 SDS-PAGE

SDS-PAGE was performed to analyze IgE antibody integrity and -glycosylation, since it could not be performed by HPLC analysis in our laboratory. Therefore, 5 µl of each IgE antibody glycoform were diluted in 5x sodium dodecyl sulfate (SDS)-loading buffer and heat denturated for 5 minutes at 95 °C. The denaturated samples were resolved by SDS-polyacrylamid gel electrophoresis (SDS-PAGE) using pre-casted 10% polyacrylamide gels (Mini-PROTEAN® TGX™). The gel was placed into a gel chamber filled up with SDS-running buffer. The comb was removed and 10 µl of the samples were loaded onto the gel and ran against a protein molecular weight standard (PageRuler Protein ladder). Electrophoresis was performed at 80 V

for 10 minutes, followed by 120 V for up to 1 hour by electrical current at 75 mA. Proteins were stained with Coomassie-Brilliant Blue for 1 hour. Background staining of the gels was reduced by either repeated washing steps with fresh destaining solution for 1 hour or ddH_2O overnight at 4 °C. Afterwards the gels were documented photographically.

2.2.8 ELISA

Anti-human IgG4 ELISA

The anti-human IgG4 ELISA (enzyme-linked immunosorbent assay) was conducted to verifiy the IgG subclass after purification from IVIG.

Therefore, mouse anti-human IgG-Fc (clone HP-6017) was diluted to a final concentration of 4 µg mL^{-1} in coating buffer and 35 µl were transferred to each well of a 96-well plate. Plates were incubated either for 1 hour at room temperature on a plate shaker or overnight at 4 °C. All following steps were performed at room temperature. Next, residual binding sites were blocked by incubating the wells with 150 µL ELISA buffer for 1 hour on a plate shaker. In the meantime, samples were diluted in ELISA buffer as indicated. After blocking, wells were washed three times with 200 µL PBST and 35 µL of each sample was added per well. All samples were measured in duplicates. Plates were again incubated for 1 hour on a plate shaker. After washing the plates three times with 200 µL PBST again, bound antibodies were detected using 50 µL of a mouse anti-human IgG4 antibody (clone HP-6025) or mouse anti-human IgG-Fc antibody (clone HP-6017) conjugated to horseradish peroxidase (HRP) diluted in ELISA buffer. Plates were incubated for 1 hour at room temperature for detection. After washing the plates three times with 200 µL PBST, 100 µL of freshly prepared TMB-substrate per well was added with a multichannel pipette. The reaction was stopped using 50 µL/ well 4.2% sulfuric acid and absorbance was measured at 450 nm wavelength using an ELISA plate reader.

TNP-specific ELISA

The anti-TNP antibody ELISA was conducted to verify IgE antibody reactivity after purification from cell culture supernatants and glycan modification, as well as comparing serum half-lives of different anti-TNP IgE glycoforms.

Therefore, TNP-Ficoll was diluted to a final concentration of 10 µg mL^{-1} in coating buffer and 100 µL were transferred to each well of a 96-well plate. Plates were incubated either for 1 hour at room temperature on a plate shaker or overnight at 4 °C. All following steps were performed at room temperature. Next, residual binding sites were blocked by incubating the wells with 200 µL blocking buffer for 1 hour on a plate shaker. In the meantime, samples were diluted in blocking buffer as indicated. After blocking, wells were washed three times with 200 µL PBST and 100 µL of each sample was added per well. All samples were measured in duplicates. Plates were again incubated for 1 hour on a plate shaker. After washing the plates three times with 200 µL PBST again, bound antibodies were detected using 100 µL of a polyclonal goat anti-mouse IgE antibody conjugated to horseradish peroxidase (HRP) diluted in blocking buffer. Plates were incubated for 1 hour at room temperature for detection. After washing the plates three times with 200 µL PBST, 100 µL of freshly prepared TMB-substrate per well was added with a multichannel pipette. The reaction was stopped using 50 µL/ well 4.2% sulfuric acid and absorbance was measured at 450 nm wavelength using an ELISA plate reader.

SNA-Lectin ELISA

SNA (*Sambucus nigra*)-lectin ELISA was established and performed in addition to SDS-PAGE to verify the (de)sialylation of IgE antibodies and the glycoprotein (asialo)-α1-acid glycoprotein ((asialo)-AGP) as SNA has a particulary high affinity to sialic acid (Sauer et al., 2014).

Therefore, anti-TNP IgE antibodies were diluted to a final concentration of 1 µg mL^{-1} and AGP glycoforms of 2 µg mL^{-1} in coating buffer and 100 µL were transferred to each well of a 96-well plate. All samples were measured in duplicates. Plates were incubated either for 1 hour at room temperature on a plate shaker or overnight at 4 °C. All following steps were performed at room temperature. Next, residual binding sites were blocked by incubating the wells with 200 µL blocking buffer for 1 hour on a plate shaker. After blocking, wells were washed three times with 200 µL PBST. Coated antibodies and glycoproteins were detected using 100 µl of SNA-lectin conjugated to horseradish peroxidase (HRP) diluted in lectin buffer. Plates were incubated for 1 hour at room temperature for detection. After washing the plates three times with 200 µL PBST, 100 µL of freshly prepared TMB-substrate per well was added with a multichannel pipette. The reaction was stopped using 50 µL/ well 4.2% sulfuric acid and absorbance was measured at 450 nm wavelength using an ELISA plate reader.

FcεRIα ELISA

FcεRIα ELISA was performed to assess the binding capacity of different anti-TNP IgE glycoforms to either the murine or the human form of the high-affinity IgE-receptor.

Therefore, murine and human FcεRIα were diluted to a final concentration of 5 µg mL^{-1} in coating buffer and 50 µL were transferred to each well of a 96-well plate. Plates were incubated overnight at 4 °C. All following steps were performed at room temperature. Next, wells were washed three times with 200 µL PBS. Then, residual binding sites were blocked by incubating the wells with 200 µL blocking buffer for 1 hour on a plate shaker. In the meantime, samples were diluted in blocking buffer as indicated. After blocking, wells were washed three times with 200 µL PBS and 50 µL of each sample was added per well. All samples were measured in duplicates. Plates were again incubated for 2 hours on a plate shaker. After washing the plates three times with 200 µL PBS again, bound antibodies were detected using 50 µL of a polyclonal goat anti-mouse IgE antibody conjugated to horseradish peroxidase (HRP). Plates were incubated for 1 hour at room temperature for detection. After washing the plates three times with 200 µL PBS, 50 µL of freshly prepared TMB-substrate per well was added with a multichannel pipette. The reaction was stopped using 50 µL/ well 4.2% sulfuric acid and absorbance was measured at 450 nm wavelength using an ELISA plate reader.

Galectin-3 ELISA

Galectin-3 ELISA was performed to assess the binding capacity of different anti-TNP IgE glycoforms to galectin-3, a well described IgE-binding protein (Liu, 2005).

Therefore, recombinant murine galectin-3 was diluted to a final concentration of 10 µg mL^{-1} in coating buffer and 100 µL were transferred to each well of a 96-well plate. Plates were incubated overnight at 4 °C. All following steps were performed at room temperature. Next, wells were washed three times with 200 µL PBST. Then, residual binding sites were blocked by incubating the wells with 200 µL blocking buffer for 1 hour on a plate shaker. In the meantime, samples were diluted in blocking buffer as indicated. After blocking, wells were washed three times with 200 µL PBST and 100 µL of each sample was added per well. All samples were measured in duplicates. Plates were again incubated for 1 hour on a plate shaker. After washing the plates three times with 200 µL PBST again, bound antibodies were detected using 100 µL of a polyclonal goat anti-mouse IgE antibody conjugated to horseradish peroxidase (HRP). Plates were incubated for 1 hour at room temperature for detection. After washing the plates three times with 200 µL PBST, 100 µL of freshly prepared TMB-substrate

per well was added with a multichannel pipette. The reaction was stopped using 50 µL/ well 4.2% sulfuric acid and absorbance was measured at 450 nm wavelength using an ELISA plate reader.

2.2.9 Passive systemic anaphylaxis (PSA)

IgG-mediated anaphylaxis

IgG-mediated passive systemic anaphylaxis (PSA) was induced through intravenous (i.v.) injection of allergen-specific IgG antibodies, followed by i.v. challenge with the appropriate allergen. Here, mice were sensitized with 200 µg anti-TNP IgG1 antibodies in 200 µL 1x PBS on day 0 and challenged intravenously 30 minutes later with 20 µg TNP-OVA in 200 µL 1x PBS. The severity of the anaphylactic reaction was assessed by measuring the body core/ rectal temperature after allergen-challenge.

IgE-mediated anaphylaxis

IgE-mediated passive systemic anaphylaxis was induced in two different setups. In the 24-hour model, mice were sensitized by intravenous injection of 5 µg anti-TNP IgE antibodies in 200 µL 1x PBS on day 0 and challenged intravenously with 1 µg TNP-OVA in 200 µL 1x PBS on day 1. In the 6-hour model mice were sensitized by intravenous injection of 10 µg anti-TNP IgE antibodies in 200 µL 1x PBS on day 0 and challenged with 1 µg TNP-OVA in 200 µL 1x PBS 6 hours later. The severity of the anaphylactic reaction was assessed by measuring the body core/ rectal temperature after allergen-challenge.

Additional treatment regimens in PSA

Administration of sialylated IgGs

As a treatment approach in IgG-mediated anaphylaxis mice received 1 mg sialylated antigen-unspecific human IgG4 antibodies 24 hours prior to the induction of the anaphylactic response by TNP-specific IgG antibodies and the TNP-OVA challenge.

As a treatment approach in IgG-controlled IgE-mediated anaphylaxis mice received 1 mg sialylated antigen-unspecific murine IgG1 antibodies 1 hour before sensitization of the mice

with TNP-specific IgE antibodies and the TNP-OVA challenge 24 hours later. TNP-specific IgG1 antibodies (1.4 µg) to control IgE-mediated anaphylaxis were administered i.v. 90 minutes before TNP-OVA challenge.

Anti-ASGP-R treatment

To block the hepatic asialo-glycoprotein receptor, mice were treated with the ASGP-R ligand asialo-α1-acid glycoprotein (asialo-AGP). Therefore, mice were injected intravenously with 1 mg asialo-AGP 1 hour prior to the sensitization of mice with 10 µg TNP-specific IgE antibodies and the TNP-OVA challenge 6 hours later.

2.2.10 Sample collection

For collection of serum and whole blood mice were anesthesized with Ketamine/ Xylazine (80 mg/kg Ketamine, 10 mg/kg Xylazine, i.p.). Blood was collected via heart puncture with 26G needles. Afterwards, mice were sacrificed by cerebral dislocation.

Blood was collected in tubes containing 10 mM EDTA in PBS for whole blood analysis by flow cytometry or in serum gel tubes for antibody analysis by ELISA. Serum tubes were centrifuged at 4.000x g for 5 minutes and the supernatant was transferred into new tubes and stored at -20 °C until further analysis.

2.2.11 Flow cytometry analysis

Murine blood basophils

For the analysis of murine whole blood, 400 µl EDTA blood were incubated with 5 mL erylysis buffer for 5 minutes at room temperature in 50 mL falcon tubes. Afterwards, erylysis was stopped by adding 20 mL 1x PBS and the cells were centrifuged at 300x g for 5 minutes at 4 °C. Cells were washed by resuspension in 20 mL 1x PBS and centrifugation at 300x g for 5 minutes for a second time. Next, cells were resuspended in 200 µl 1x PBS and transferred into a 96-well plate.

Cells were pelleted by centrifugation of the 96-well plate at 300x g for 5 minutes at 4 °C. The supernatant was discarded by inverting the plate. For the staining of cell surface markers, the cells were resuspended in 50 µl of an antibody master mix. Two staining protocols were applied:

Cells were either stained for anti-mouse CD49b (1:100) and anti-mouse FcεRIα (1:100) for the identification of basophils and anti-mouse FcγRIIB (1:200) to investigate the FcγRIIB surface expression on murine basophils or for anti-mouse CD49b (1:100) and anti-mouse IgE (1:100) for the identification of IgE-positive basophils and the allergen TNP-BSA (1:100) to investigate the loading of murine basophils with anti-TNP IgE antibodies. In addition, all cells were stained with fixable viability dye (1:500). Cells were stained for 30 minutes on ice in the dark. Next, cells were washed twice by adding 150 µl of 1x PBS per well and centrifugation at 300x g for 5 minutes at 4 °C. The supernatant was discarded by inverting the plate and cells were resuspended in 200 µl 1x PBS and measured with the flow cytometer Attune NxT. Evaluation was performed with the software FlowJo.

Additional administrations in the analysis of murine blood basophils

IVIG administration

As a treatment approach mice received 20 mg IVIG intraperitoneally 24 hours prior to the analysis of murine basophils for their surface expression of FcγRIIB.

Administration of sialylated murine IgGs

As a treatment approach mice received 200 µg sialylated murine anti-TNP IgG1 antibodies (clone H5) i.v. 24 hours prior to the analysis of murine basophils for their surface expression of FcγRIIB.

Administration of murine anti-TNP IgE antibodies

To investigate the loading of murine blood basophils with different IgE glycoforms, mice received 10 µg murine anti-TNP IgE glycoforms i.v. 6 hours prior to the blood cell analysis.

Anti-ASGP-R treatment

To block the hepatic ASGP-R, mice were treated with the ASGP-R ligand asialo-AGP. Therefore, mice were injected intravenously with 1 mg asialo-AGP 1 hour prior to the administration of the IgE antibodies and the blood cell analysis 6 hours later.

Mast cells

For the analysis of the sensitization of LAD2 cells with murine anti-TNP IgE antibodies 2.5x 10^5 cells were incubated with 0.2 µg different anti-TNP IgE glycoforms over night.

The next day, cells were harvested, transferred into 15 ml falcon tubes and pelleted by centrifugation at 200x g for 5 minutes at 4 °C. Cells were washed twice with 1x PBS and resuspended in 200 µl 1x PBS. Next, cells were transferred into a 96-well plate and pelleted by centrifugation of the 96-well plate at 200x g for 5 minutes at 4 °C.

To analyze the loading of the cells with anti-TNP IgE antibodies, cells were stained with a master mix containing fixable viability dye (1:500) and cell surface markers anti-human FcεRIα (1:100) and either anti-mouse IgE (1:200) or the allergen TNP-BSA (1:200). Cells were stained for 30 minutes on ice in the dark. Next, cells were washed twice by adding 150 µl of 1x PBS per well and centrifugation at 200x g for 5 minutes at 4 °C. The supernatant was discarded by inverting the plate and cells were resuspended in 200 µl 1x PBS and measured with the flow cytometer Attune NxT. Evaluation was performed with the software FlowJo.

2.2.12 Mast cell activation assay

LAD2 mast cell line

Activation and degranulation of the human mast cell line LAD2 was measured by the release of the granule component β-hexosaminidase. The assay was performed according to Kuehn *et al.* (Kuehn et al., 2010).

In brief, 2.5x10^5 cells per 0.5 mL culture medium were sensitized with 0.02 µg anti-TNP IgE antibodies over night at 37 °C in a 24-well plate. Following sensitization, cells were washed three times with 1x PBS containing 0.1 % BSA to remove excess IgE. Then, 2- 5x10^4 cells were stimulated with 0.2 µg TNP-OVA in a total volume of 240 µL for 30 minutes on a plate shaker at 37 °C. In addition, for total β-hexosaminidase release, cells were incubated with 0.05% Triton

X-100 solution, lysing the cells. After stimulation cells were centrifuged at 400x g for 12 minutes at 4 °C. Subsequently, 100 µL of cell free supernatant was transferred into a 96-well ELISA plate and incubated with 50 µL of the β-hexosaminidase substrate p-nitrophenyl N-acetyl-β-D-glucosaminide (PNAG, 1.36 mg mL^{-1}) in citrate buffer for 90 minutes at 37 °C. The reaction was stopped by adding 50 µL 0.4 M glycine buffer into each well and absorbance was measured at 405 nm wavelength using an ELISA plate reader and the percentage of β-hexosaminidase release was calculated as the percentage of total release induced by Triton X-100 treatment.

$CD34^+$ differentiated human mast cells

This assay was performed in a cooperation project with the Fraunhofer Institute for toxicology and experimental medicine (ITEM) Hanover in the research group of Dr. Susann Dehmel. For experiments using human differentiated mast cells, blood was received from Blutspendedienst NSTOB. Therefore, all of the patients or their next of kin, caretakers, or guardians gave written informed consent to the Blutspendedienst NSTOB. Fraunhofer received only anonymized samples from healthy donors. Blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphocyte Separation as described before (Fuss et al., 2009). Human mast cells were differentiated from $CD34^+$ hematopoietic progenitor cells isolated from PBMCs using the MACS human $CD34^+$ isolation kit according to the manufacturer's instructions (Miltenyi Biotec, CD34 MicroBead Kit, human). Differentiation into mast cells was performed as described (Yin et al., 2017) and verified by expression of FcεRIα (clone AER-37; BioLegend; Alexa Fluor® 488) and CD117 (clone 104D2; BioLegend; PerCP/Cy5.5) on viable $CD45^+$ (clone HI30; BioLegend; Alexa Fluor® 700) cells using flow cytometry (Cytoflex S, Beckman Coulter). To assess mast cell degranulation, the differentiated cells (3-5x10^4 cells/per well) were incubated with 200 ng mL^{-1} of the anti-TNP IgE glycoforms at 37 °C and 5% CO_2 for 20 h. Subsequently, cells were centrifuged, supernatants discarded, pellets washed, and cells re-suspended in HEPES buffer, followed by stimulation with 200 ng mL^{-1} of TNP-OVA per well in a total volume of 100 µL at 37 °C without CO_2 for 30 min. The β-hexosaminidase assay was performed as described (Kuehn et al., 2010). The absorbance of cell supernatants and lysates was measured in the TECAN Infinite F200 PRO microplate reader at 405 nm with a 620 nm reference filter and i-control 1.10 software. The percentage of degranulation was calculated as follows (Kuehn et al., 2010):

$$\%\, release = \frac{2 \times supernatant}{\left(\left(\frac{1}{2}\right) \times supernatant\right) + 4 \times lysates}$$

2.2.13 Human blood basophil activation assay

This assay was performed in a cooperation project with the Forschungszentrum Borstel in the research group of Prof. Dr. med. Uta Jappe. The method used in this study was modified from a protocol that has been described in detail recently (Behrends et al., 2021). In brief, basophils in fresh heparinized blood were incubated with 10 or 50 µg mL^{-1} of the anti-TNP IgE glycoforms at 37 °C for 1 h. Subsequently, the samples were analyzed for binding of the different IgE glycoforms to basophils by flow cytometry or further stimulated with 100 ng mL^{-1} of TNP-OVA for 30 min to analyze basophil activation by flow cytometry. After staining, red blood cells were lysed and removed from the samples before fixation. Basophils were identified with fluorophore-conjugated anti-FcεRIα (clone AER-37 (CRA-1); BioLegend; PE/Cy7) and anti-CD203c (clone NP4D6; BioLegend; PE) Abs. Murine IgE binding was analyzed with an anti-murine IgE Ab (RME-1; Biolegend; FITC), and basophil activation identified with an anti-CD63 Ab (clone H5C6; BioLegend; APC). Positive controls included the bacterial peptide fMLP (N-formyl-l-methionyl-l-leucyl-phenylalanine), and a mix of two activating anti-human IgE Abs (goat polyclonal anti-hu IgE ; 10 µg mL-1; Abcam and goat polyclonal anti-hu IgE ε-chain specific; 10 µg mL^{-1}; Sigma Aldrich). All measurements were conducted on an LSRII instrument (BD Biosciences, San Jose, California, USA; 4 lasers, 18 bandpass (BP) filters). Analyses were performed utilizing the FCSExpress7 program (DENOVO™ Software, Glendale, CA, USA). The blood donor gave written informed consent for the analysis of blood samples. The ethics committee of the University of Lübeck approved the ethic proposal (Reference no. 13-086).

2.2.14 Analysis of serum IgE titer after immunization of mice with ovalbumin and different adjuvants

Immunization protocol

Mice were immunized intraperitoneally with 100 µg of ovalbumin (OVA) plus the adjuvants enriched complete Freund´s adjuvant (eCFA) and Alum. Enriched CFA was prepared by adding

100 mg heat-killed Mtb.H37 RA to 20 mL incomplete Freund´s adjuvant (IFA) to reach a final concentration of 5 mg of Mtb mL^{-1}. Alum was purchased ready to use from InvivoGen (Toulouse, France). Mice were immunized in a total volume of 200 µL; therefore, 100 µL of the adjuvant solution and 100 µL of an OVA-PBS solution (1 mg mL^{-1}) were mixed before injection. Blood serum was taken on day 12 for further analysis. Immunizations were performed by Hanna Lunding, a former PhD student in our lab.

Analysis of serum IgE titers by IgE-sandwich ELISA

This ELISA was established to capture IgE antibodies from the sera of mice, which were immunized with OVA and different adjuvants to assess the potential of these adjuvants to induce IgE antibodies.

Therefore, a polyclonal goat anti-mouse IgE antibody was diluted to a final concentration of 10 µg mL^{-1} in coating buffer and was transferred to each well of a 96-well plate. Plates were incubated for 1 hour at room temperature or overnight at 4 °C. All following steps were performed at room temperature. Next, residual binding sites were blocked by incubating the wells with 200 µL blocking buffer for 1 hour on a plate shaker. In the meantime, samples were diluted in blocking buffer as indicated. After blocking, wells were washed three times with 200 µL PBST and 100 µL of each sample was added per well. All samples were measured in duplicates. Plates were again incubated for 1 hour on a plate shaker. After washing the plates three times with 200 µL PBST again, bound antibodies were detected using 100 µL of a polyclonal rat anti-mouse IgE antibody conjugated to horseradish peroxidase (HRP) diluted in blocking buffer. Plates were incubated for 1 hour at room temperature for detection. After washing the plates three times with 200 µL PBST, 100 µL of freshly prepared TMB-substrate per well was added with a multichannel pipette. The reaction was stopped using 50 µL/ well 4.2% sulfuric acid and absorbance was measured at 450 nm wavelength using an ELISA plate reader.

2.2.15 Statistical analysis

Statistical analysis was conducted using GraphPad Prism, Version 6. Data are represented as means +/- standard error of the mean (SEM). Continuous measurements of the body temperature in passive systemic anaphylaxis were analyzed using two-tailed t-tests for comparison of two groups and two-way ANOVA with Turkey´s post-test correction for

comparison of more than two groups. For analysis of the expression levels of the different cell surface receptors/ bound IgE antibodies, the median fluorescence intensity (MFI) of each population was calculated using FlowJo and is presented as the mean with +/- standard error of the mean (SEM). One out of at least two independent experiments is shown. P-values below 0.05 were considered significant. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

3 Results

3.1 Immunomodulatory mechanisms in IgG-mediated anaphylaxis

To date, many studies have validated, that not only IgE antibodies, but also allergen-specific IgG antibodies have the potential to induce severe systemic anaphylaxis in mice, given that antigen doses are high (Epp et al., 2018a). Also it is known that there are IgG subclass specific differences in the anaphylactic potential of IgG antibodies, as murine IgG2 antibodies have a higher anaphylactic potential than murine IgG1 antibodies in different mouse models of IgG-mediated passive systemic anaphylaxis (Beutier et al., 2017; Epp et al., 2018a; Jönsson et al., 2011).

Other studies have demonstrated, that the i.v. administration of high amounts of serum IgG from healthy donors (intravenous immunoglobulin; IVIG) has protective effects in mouse models of autoimmune diseases such as rheutmatoid arthritis (Anthony et al., 2011, 2008) due to up-regulation of the inhibitory FcγRIIB and down-regulation of activating FcγRIV on immune effector cells in a SIGN-R1 (specific ICAM-3-grabbing non-integrin-related 1)-dependent manner. Moreover, this effect could be assigned to the sialylated subfraction of the IVIG (Anthony et al., 2011, 2008; Hirose et al., 2015).

In this project, I used a murine IgG-mediated passive systemic anaphylaxis (PSA) model:

- to investigate the potential of high amounts of sialylated allergen-unspecific human IgG4 and murine IgG1 antibodies to attenuate systemic anaphylaxis,
- to analyze the influence of IVIG or sialylated murine IgG antibodies on the expression of the inhibitory FcγRIIB on blood basophils as effector cells of systemic anaphylaxis,

considering the impact of the glycosylation pattern of IgG antibodies on antibody-mediated anaphylaxis.

3.1.1 Sialylated, allergen-unspecific human IgG4-dependent attenuation of murine IgG-mediated anaphylaxis

Here, I continued the work of the former PhD student in our lab, Janina Petry, who could show, that IVIG also attenuates murine IgG1-mediated PSA (Petry et al., 2021a). What had not been investigated by now is, whether the protective effect of IVIG could be, aside from the mandatory sialylation of IgG Fc *N*-glycans, also mediated by a distinct IgG antibody subclass.

Therefore, I generated sialylated human IgG4 antibodies and analyzed them, regarding their protential to attenuate murine IgG1-mediated passive systemic anaphylaxis in mice. Human IgG4 antibodies were purified from IVIG using an anti-human IgG4 coupled sepharose column in collaboration with two other PhD students from our laboratory, Selina Lehrian and Jana Sophia Buhre. Finally, the purified IgG4 antibodies were analyzed concerning their enrichment by ELISA. Compared to IVIG, there was a clear enrichment of human IgG4 subclass antibodies after the purification process (Figure 8).

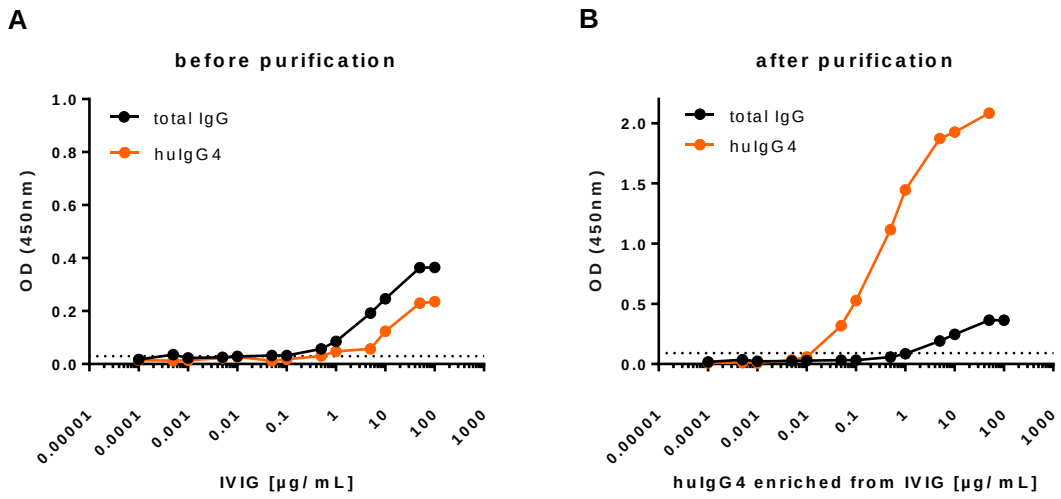

Figure 8: Purification of human IgG4 from IVIG.

*Enrichment of human IgG4 antibodies from IVIG was verified by ELISA. ELISA plates coated with 4 µg/mL of mouse-anti-human IgG Fc were incubated with serial dilutions of (**A**) IVIG (before purification) and (**B**) enriched human IgG4 antibodies from IVIG (after purification). Detection was performed using HRP-coupled goat-anti-human IgG-Fc ("total IgG", black) or mouse-anti-human IgG4 antibodies ("huIgG4", orange). Optical density at 450 nm was measured using an ELISA plate reader. The dotted line shows the background signal of the plate. ELISA were performed by Selina Lehrian in our laboratory.*

Next, the human IgG4 antibodies were additionally sialylated *in vitro* to study their impact on murine IgG1-mediated anaphylaxis. Therefore, the enriched human IgG4 antibodies were galactosylated and subsequently sialylated ("sial") using β-1,4-galactosyltransferase and α-2,6-sialyltransferase, respectively. The successful *in vitro* sialylation was analyzed by HPLC. Therefore, Fc *N*-glycans were cleaved by recombinant endoglycosidase S (EndoS) from *Streptococcus pyogenes* (a gift from Mattias Collin, (Lund, Sweden; (Collin, 2001)) and purified as described in section 2.2.6. Figure 9 shows the glycan distribution of the additionally sialylated IgG4 antibodies enriched from IVIG. After *in vitro* sialylation the IgG4 antibodies contained 98.1% terminal sialic acid residues. Only a small fraction of Fc *N*-glycans (2%) was still terminal galactosylated. No agalactosylated glycans were detected.

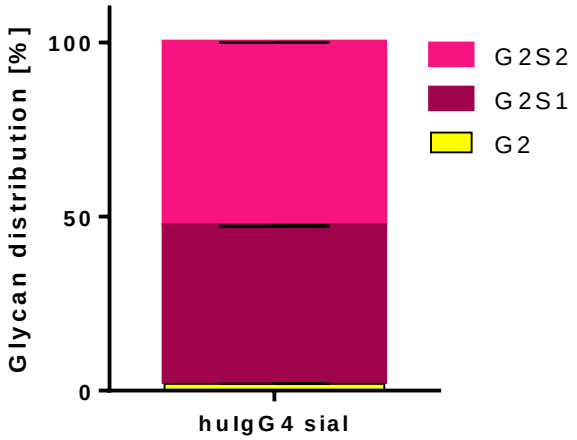

Figure 9: Fc N-glycosylation of purified human IgG4 antibodies.

Glycan distribution of the human IgG4 antibodies enriched from IVIG after in vitro galactosylation and subsequent sialylation ("sial"). G2 = bi-galactosylated, S1 mono-sialylated, S2 = bi-sialylated. The HPLC analysis of the human IgG4 antibody glycosylation was performed by Carsten Kern in our laboratory.

Murine IgG1-mediated passive systemic anaphylaxis (PSA) was conducted using an anti-TNP murine IgG1 antibody (clone H5). PSA can be induced in mice through intravenous (i.v.) injection of allergen-specific IgG antibodies, followed by challenging the mice i.v. with the appropriate allergen. Here, for induction of IgG1-mediated PSA, mice were sensitized with 200 µg anti-TNP IgG1 antibodies, followed by a challenge with 20 µg TNP-OVA 30 minutes later. The severity of the anaphylactic reaction was assessed by measuring the decrease in body core/ rectal temperature after allergen challenge (Figure 10A). In addition, to investigate the potential of sialylated human IgG4 antibodies to attenuate murine IgG1-mediated anaphylaxis, mice were treated i.v. with 1 mg enriched sialylated human IgG4 antibodies 23.5 hours before sensitization with murine anti-TNP IgG1 antibodies as indicated in Figure 10A.

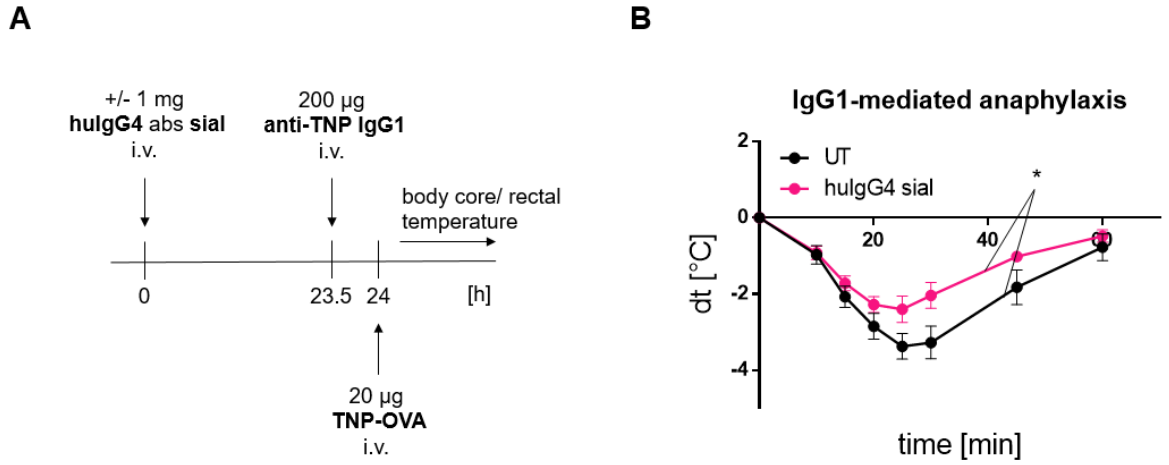

Figure 10: Attenuation of murine IgG1-mediated PSA in mice by sialylated human IgG4 antibodies.

(A) *Experimental setup of the IgG1-mediated passive systemic anaphylaxis model. Mice were sensitized i.v. with 200 µg anti-TNP IgG1 antibodies and subsequently challenged i.v. with 20 µg TNP-OVA 30 minutes later. The severity of the anaphylactic reaction was measured as the decrease in body core/ rectal temperature after allergen challenge. To investigate the effect of total IgG sialylation on anaphylaxis severity, mice were treated i.v. with 1 mg of sialylated allergen-unspecific human IgG4 antibodies 23.5 hours prior to sensitization.* ***(B)*** *The effect of the sialylated human IgG4 (pink, "huIgG4 sial") on the anaphylactic reaction was compared to mice not treated with IgG4 (black, "UT" (untreated)). Data are presented as mean +/- SEM, n = 4-5 for all groups. *P < 0.05 by two-tailed t-test.*

As anticipated, also enriched sialylated allergen-unsopecific human IgG4 antibodies had the potential to significantly attenuate murine IgG-mediated PSA (Figure 10B). Hence, the protective effect in IgG-mediated PSA, described for the sialylated subfraction of IVIG can also be mediated by a distinct, sialylated IgG subclass.

It has been described, that one protective, antigen-unspecific effect of IVIG might be caused by the interaction of the terminal sialic acid with the type II Fc-receptor SIGN-R1, which leads to the up-regulation of the inhibitory FcγRIIB on effector cells (Anthony et al., 2008, 2011; Kaneko et al., 2006). Furthermore, IgG Fc-sialylation leads to structural changes, which reduces the affinity for type I Fc-receptors (Kaneko et al., 2006). Hence, the data show, that sialylated allergen- unspecific human IgG4 antibodies also have the potential to attenuate IgG-mediated systemic anaphylaxis in mice.

3.1.2 Sialylated murine IgG1-dependent attenuation of murine IgG-mediated anaphylaxis is associated with FcγRIIB upregulation on blood basophils

In this section, I continued the work of Dr. Janina Petry from our laboratory, who could show, that the application of sialylated allergen-unspecific IgG1 leads to an up-regulation of FcγRIIB on blood monocytes/ macrophages, but not on neutrophils (Petry et al., 2021a). Since basophils are considered drivers of IgE-, as well as murine IgG-mediated anaphylaxis (Finkelman et al., 2016; Gould and Sutton, 2008b) I established a protocol for a flow cytometric analysis of murine blood basophils, in collaboration with Johann Rahmöller, a former MD student of our laboratory, and analyzed the capacity of high doses of IVIG or lower doses of sialylated murine IgG1 antibodies (without any antigen) to modify the expression level of the inhibitory FcγRIIB.

Mice were treated with IVIG or sialylated murine IgG1 antibodies (clone H5). The next day, blood basophils were analyzed by flow cytometry. Blood cells were pre-gated on single cells and viable leucocytes. Next, CD49b$^+$ FcεRI$^+$ basophils were identified (Figure 11) and analyzed for their surface expression level of FcγRIIB (Figure 12).

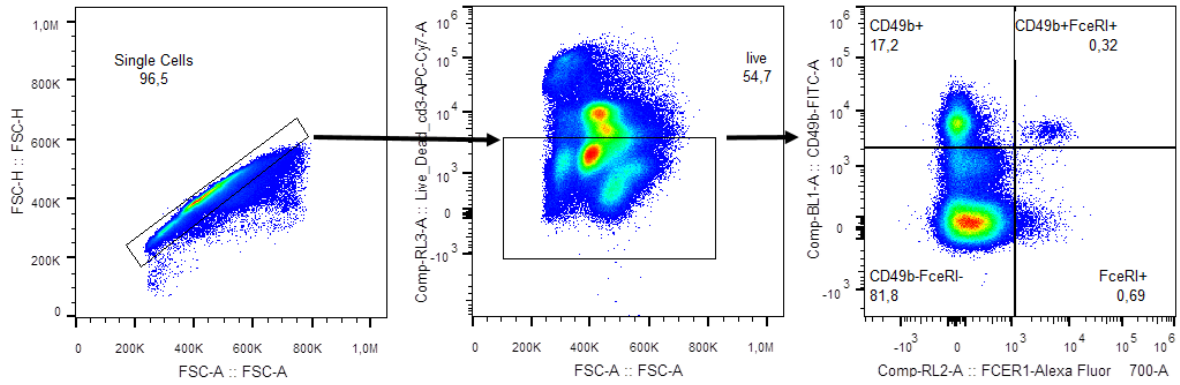

Figure 11: Gating strategy for murine blood basophils.

Mice were treated i.p. with 20 mg IVIG or i.v. with 200 µg sialylated murine IgG1 antibodies (clone H5) and blood was analyzed by flow cytometry 24 hours later. Whole blod cells were pre-gated on single cells and viable leucocytes. Next CD49b$^+$FcεRI$^+$ basophils were identified.

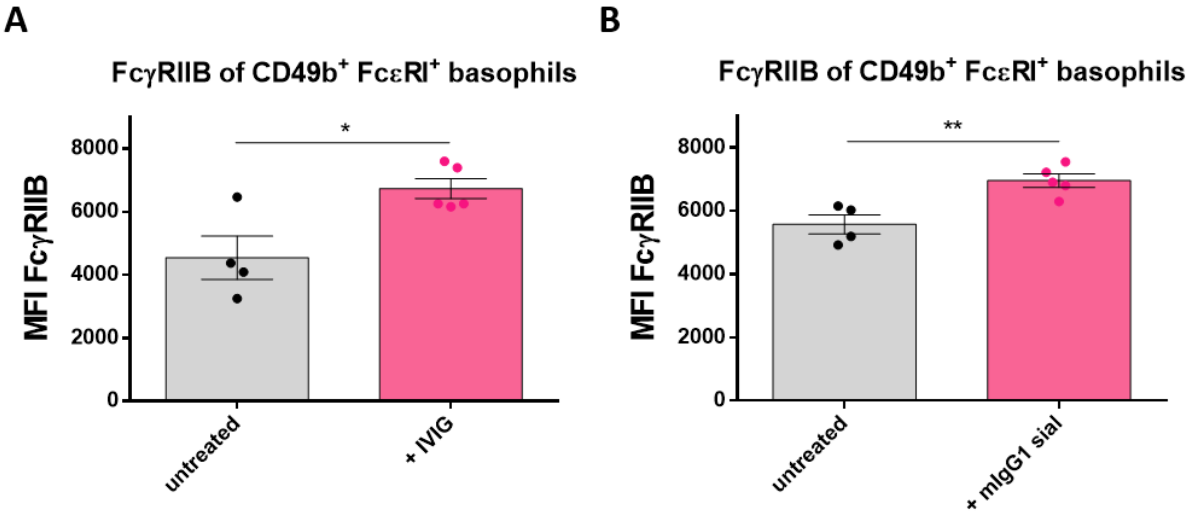

Figure 12: Treatment with IVIG or sialylated murine IgG1 antibodies upregulates FcγRIIB expression on blood basophils.

*Mice were treated **(A)** i.p. with 20 mg IVIG or **(B)** i.v. with 200 µg sialylated murine IgG1 antibodies and were analyzed by flow cytometry 24 hours later. Whole blood cells were pre-gated on single, viable leucocytes. Next CD49b$^+$FcεRI$^+$ basophils were identified and analyzed for their cell surface expression of FcγRIIB. The Median Flourescence Intensity (MFI) of FcγRIIB of untreated or IVIG/mIgG1 sial treated mice in CD49b$^+$ FcεRI$^+$ basophils is shown. Individual symbols represent individual mice. One of at least two independent experiments is shown. *P < 0.05, **P < 0.01 by two-tailed t-test.*

According to the literature, murine blood basophils present high levels of FcγRIIB (Figure 12, (Kerntke et al., 2020). Upon treatment with IVIG (Figure 12A) or sialylated murine IgG1 antibodies (Figure 12B), an up-regulation of FcγRIIB could be observed 24 hours later.

Taken together, in section 3.1 inhibition of IgG1-mediated anaphylaxis in mice by IVIG was demonstrated not only to be dependent on the Fc sialylation of the IgG antibodies, but could also be mediated by a distinct sialylated IgG subclass. Signficiant attenuation of IgG1-mediated PSA occurred in mice, treated with sialylated polyclonal human IgG4 antibodies enriched from IVIG. Furthermore, protection mediated by sialylated human IgG4 or murine IgG1 antibodies could be associated with an up-regulation of the inhibitory FcγRIIB on blood basophils, an effector cell population of IgE- as well as IgG-mediated anaphylaxis (Finkelman et al., 2016).

Parts of the presented experiments were published in Petry J*, Rahmöller J*, Dühring L*, Lilienthal G-M, Lehrian S, Buhre JS, Bartsch YC, Epp A, Lunding H, Moremen KW, Leliavski A, and Ehlers M. Enriched blood IgG sialylation attenuates IgG-mediated and IgG-controlled IgE-mediated allergic reations. *J Allergy Clinic Immunol 2021* 147(2):763-767. doi: 10.1016/j.jaci.2020.05.056. (*these authors contributed equally).

3.2 Sialylated murine allergen-unspecific IgG antibodies as a treatment approach in IgG-controlled IgE-mediated anaphylaxis

Many studies have shown, that allergen-specific IgG antibodies have the potential to inhibit murine IgE-mediated allergic reactions through allergen-masking as well as FcγRIIB-dependent inhibition on effector cells (Epp et al., 2018a; Kanagaratham et al., 2020; Möbs et al., 2012; Petry et al., 2021a; Strait, 2006).

As demonstrated above, sialylated human IgG4 antibodies can attenuate IgG-mediated allergic reactions in an antigen-unspecific manner. Furthermore, treatment of mice with high amounts of IVIG or lower amounts of sialylated, allergen-unspecific murine IgG antibodies leads to an

up-regulation of FcγRIIB on blood basophils, one of the drivers of IgE-mediated allergic reactions (Gould et al., 2003), in a SIGN-R1-dependent manner (Petry et al., 2021a). This mechanism has been shown to enhance the blocking property of allergen-specific IgG antibodies in an IgE-meditaed PSA model in mice (Petry, 2019).

In my project I expanded this hypothesis and investigated, whether sialylated allergen-unspecific murine IgG1 antibodies (clone MOPC-21) also have the potential to increase the attenuation of IgE-mediated PSA, caused by allergen-specific murine IgG1 antibodies (clone H5).

Anti-TNP IgE antibodies were produced by culturing the hybridoma cell line IgEL a2. Purification of anti-TNP IgE antibodies was achieved from cell culture supernatants using selfmade TNP-coupled sepharose columns (s. 2.2.3). Ig isotype and TNP-specificity were frequently analyzed by TNP-reactive ELISA (Figure 13).

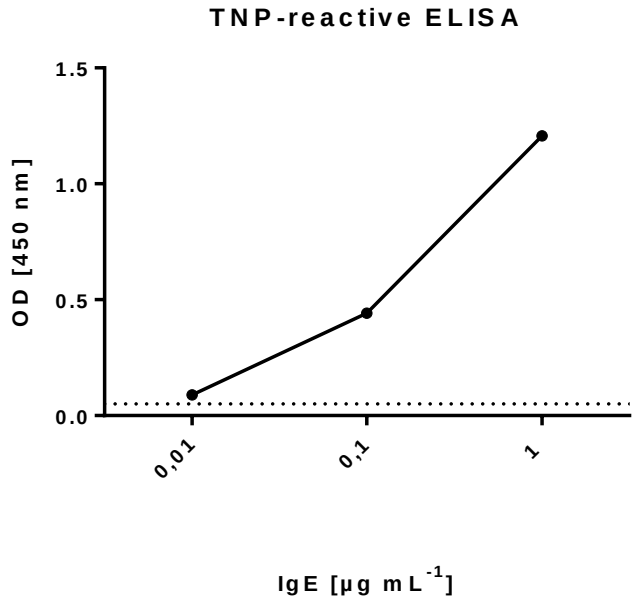

Figure 13: Analysis of genberated murine anti-TNP IgE antibodies.

Ig isotype and TNP-specificity of the anti-TNP IgE antibodies produced by the hybridoma cell line IgEL a2 was analyzed by ELISA. ELISA plates were coated with 10 µg TNP-Ficoll per well and incubated with serial dilutions of purified anti-TNP IgE antibodies. Detection was performed using a HRP-coupled goat anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM.The dotted line shows the background signal of the plate.

For the induction of IgE-mediated passive systemic anaphylaxis, mice were sensitized i.v. with 5 µg anti-TNP IgE antibodies and challenged i.v. with 1 µg TNP-OVA 24 hours later. The therapeutic potential of sialylated allergen-unspecific murine IgG1 antibodies was tested under

preventive treatment conditions. Therefore, mice received 1 mg sialylated allergen-unspecific murine IgG1 antibodies one hour prior to the i.v. sensitization with anti-TNP IgE antibodies.

As shown in Figure 14 the transfer of 1.4 µg allergen-specific IgG1 antibodies (clone H5) 90 minutes prior to the TNP-OVA challenge attenuated IgE-mediated anaphylaxis and demonstrated the inhibitory property of allergen-specific murine IgG1, as previously shown (Epp et al., 2018a; Möbs et al., 2012; Petry et al., 2021a; Strait, 2006). Furthermore, additional treatment with allergen-unspecific sialylated murine IgG1 antibodies (assumed to upregulate FcγRIIB on effector cells) significantly enhanced the inhibitory potential of allergen-specific IgG1 antibodies in IgE-mediated anaphylaxis (Figure 14B).

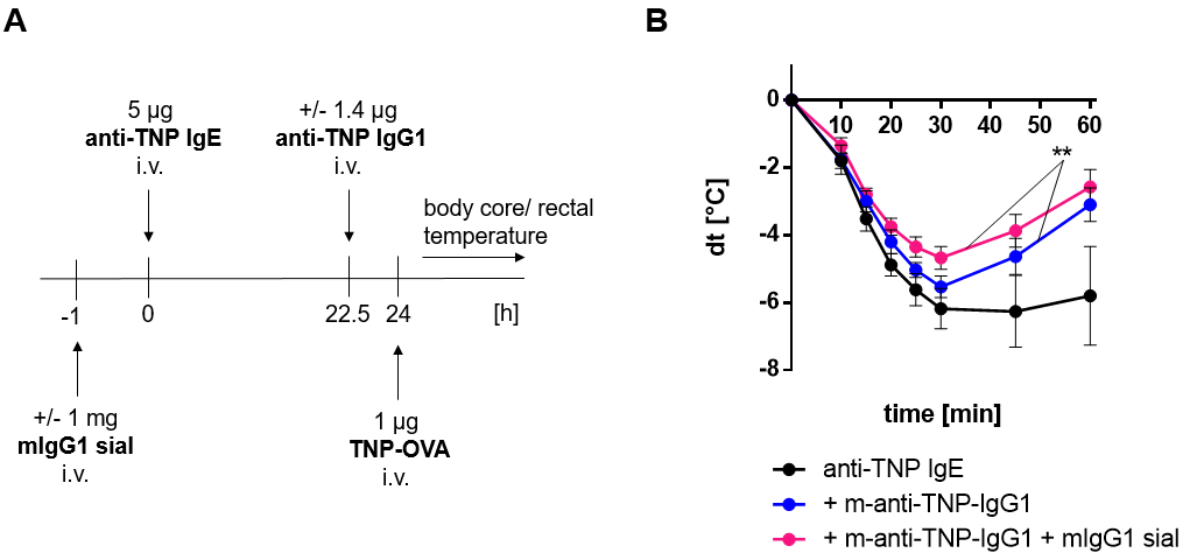

Figure 14: Treatment with allergen-unspecific murine IgG1 antibodies enhances IgG1 dependent attenuation of IgE-mediated anaphylaxis.

(A) *Experimental setup of the IgE-mediated passive systemic anaphylaxis. Mice were sensitized i.v. with 5 µg anti-TNP IgE antibodies and challenged i.v. with 1 µg TNP-OVA 24 hours later. In addition, mice were treated with 1.4 µg anti-TNP IgG1 antibodies and additionally with 1 mg sialylated allergen-unspecific murine IgG1 antibodies (clone MOPC-21), when indicated.* ***(B)*** *The severity of the anaphylactic reaction was measured as the drop of the body core/ rectal temperature upon TNP-OVA challenge. Data are presented as mean +/- SEM, n = 3-5 for all groups. One of at least two independent experiments is shown. **P < 0.01 by two-tailed t-test.*

Thus, treatment with sialylated allergen-unspecific murine IgG1 antibodies increased the protective effect of allergen-specific IgG antibodies likely due to an enhancement of the sialylated serum IgG subfraction, leading to an upregulation of FcγRIIB on corresponding immune cells. These data further confirmed that the total serum IgG pool act as an immunobuffer system with higher proportions of sialylated IgG correlating with higher expression of the inhibitory FcgRIIB.

Parts of the presented experiments were published in Petry J*, Rahmöller J*, Dühring L*, Lilienthal G-M, Lehrian S, Buhre JS, Bartsch YC, Epp A, Lunding H, Moremen KW, Leliavski A, and Ehlers M. Enriched blood IgG sialylation attenuates IgG-mediated and IgG-controlled IgE-mediated allergic reations. *J Allergy Clinic Immunol 2021* 147(2):763-767. doi: 10.1016/j.jaci.2020.05.056. (*these authors contributed equally).

3.3 The role of IgE-glycosylation in IgE-mediated anaphylaxis

IgE antibodies can cause allergic reactions up to severe systemic anaphylaxis by crosslinking the high-affinity IgE-receptor FcεRI on mast cells and basophils, which leads to their activation and the release of inflammatory mediators, such as histamine (Gould and Sutton, 2008). Other than IgG antibodies, which have one distinct *N*-glycosylation site in the Fc-part at Asn 297 (Bartsch et al., 2020; Collin and Ehlers, 2013; Epp et al., 2018a), IgE antibodies are heavily glycosylated with *N*-glycosylation sites in the Fc- as well as in the Fab-part of the antibody (Gould and Sutton, 2008; Plomp et al., 2014; Shade et al., 2019, Figure 7). To date, the role of IgE glycosylation in the context of allergic diseases is poorly understood. Studies showed, that the oligomannose glycan on Asn 394 (human IgE) and Asn 384 (murine IgE), respectively, is crucial for IgE-binding to FcεRI as genetic disruption or enzymatic removal of this glycan alters IgE secondary structure in a way that abrogates IgE-binding to FcεRI and thus prevents anaphylaxis. All other glycosylation sites are of the complex type as described for IgG antibodies (Shade et al., 2015). Noteworthy, in contrast to IgG antibodies, complex type glycans of native IgE antibodies have been demonstrated to be mainly terminal sialylated (Arnold et al., 2004b; Plomp et al., 2014a; Shade et al., 2019, 2015). Furthermore, there is first evidence, that IgE sialylation plays a role in allergic disease (Shade et al., 2020a), but even though first studies have been conducted, there are still many open questions regarding the role of IgE glycosylation in this context. During my doctoral studies, I further investigated the role of IgE glycosylation in allergic disease.

Therefore, I used human mast cells and basophils as well as a murine IgE-mediated passive systemic anaphylaxis model:

- to compare the potential of different IgE glycoforms to bind the high affinity IgE receptor FcεRI, to bind and activate mast cells and basophils and to induce IgE-mediated anaphylaxis

- to investigate the role of the hepatic asialo-glycorprotein receptor concerning the serum half-life of different IgE glycoforms

3.3.1 *In vitro* FcεRIα binding is affected by IgE glycosylation

IgE antibodies are the least prevalent immunglobulins in circulation with half-lifes of only 12 hours and 1-3 days in mouse and human serum, respectively (Gould et al., 2003; Gould and Sutton, 2008a). However, their tissue half-life is greatly increased, because of the high-affinity ($K_d = 10^{11}$ M^{-1}) to the IgE-Fc receptor FcεRI (Sutton and Davies, 2015), which coates the cell surface of mast cells and basophils, as well as monocytes, dendritic cells and eosinophils. Aside from the mandatory oligomannose glycan at Asn 394/384 (Shade et al., 2019, 2015), there is first evidence that IgE sialylation might be a regulator of allergic reactions (Shade et al., 2020a).

As mentioned in section 3.2, I produced murine monoclonal anti-TNP IgE antibodies by culturing the hybridoma cell line IgEL a2. Purification of the IgE antibodies from cell culture supernatants was achieved using selfmade TNP-coupled sepharose columns (s. 2.2.3). Purified native IgE antibodies were *in vitro* desialylated ("desial") as well as *in vitro* galactosylated plus sialylated ("high sial") as described in sections 2.2.4 and 2.2.5 (Figure 15A). Because IgE antibody glycosylation contributes to up to 12% of the antibody molecular weight (Plomp et al., 2014), successful *in vitro* glycan modification could be demonstrated by SDS-PAGE (Figure 15B). Additionally, I established an ELISA using HRP-coupled *sambucus nigra* (SNA) lectin, to confirm successful additional sialylation and complete desialylation of the IgE antibodies (Figure 15C).

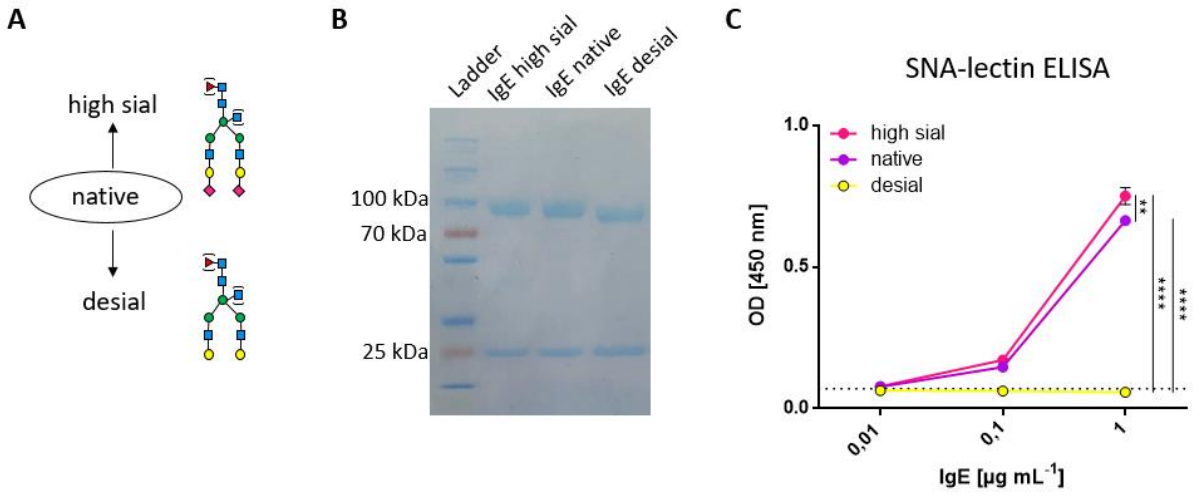

Figure 15: Murine IgE antibody glycosylation and analyzation.

(A) *Different glycoforms of murine anti-TNP IgE antibodies (clone IgEL a2) were produced via in vitro (de)glycosylation approaches starting from the native anti-TNP IgE antibody (intermediate sialylation): Highly sialylated IgE (high sial) and desialylated IgE (desial). IgE glycosylation was verified by (**B**) SDS-PAGE and (**C**) SNA Lectin-ELISA.* ***(B)*** *SDS-PAGE of 5 µg anti-TNP IgE antibodies under reducing conditions showed two bands corresponding to the IgE heavy- and light-chains. Following in vitro galactosylation plus sialylation („high sial", pink) and desialylation („desial", yellow) starting from native IgE („native", purple), the IgE antibody heavy chains migrated at a different molecular weight, compared to the native IgE antibody.* ***(C)*** *ELISA plates coated with serial dilutions of different IgE glycoforms were incubated with HRP-conjugated sambucus nigra (SNA) lectin for detection of terminal sialic acids. Absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM. **P < 0.01; ****P < 0.0001 by two-way ANOVA with multiple comparisons. The dotted line shows the background signal of the plate.*

TNP-specificity of all IgE glycoforms was frequently analyzed by TNP-reactive ELISA (Figure 16).

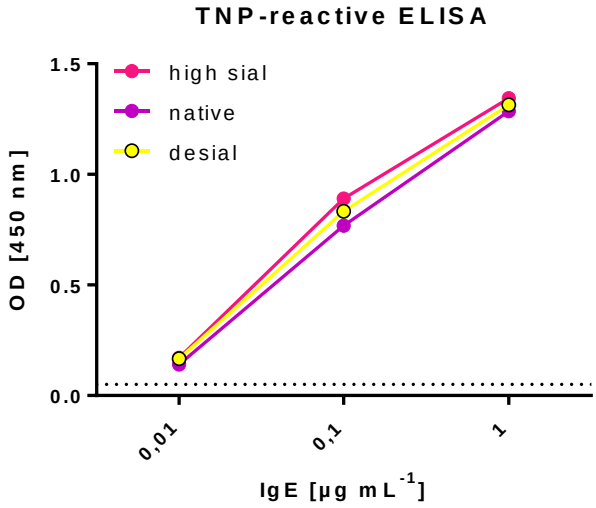

Figure 16: TNP-specificity of different IgE glycoforms.

Antigen-binding of all IgE glycoforms was verified by TNP-reactive ELISA. ELISA plates coated with 10 µg mL^{-1} TNP-Ficoll per well were incubated with serial dilutions of different anti-TNP IgE glycoforms. Detection was performed using a HRP-coupled goat anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM. The dotted line shows the background signal of the plate.

Next, differently glycosylated anti-TNP IgE antibodies were analyzed by ELISA for their potential to bind the α-subunit of the human and murine form of the high affinity IgE receptor FcεRI (FcεRIα, Figure 17). The protocol was modified from Marie Kubiak, a former bachelor student in our laboratory, who established the FcεRIα ELISA.

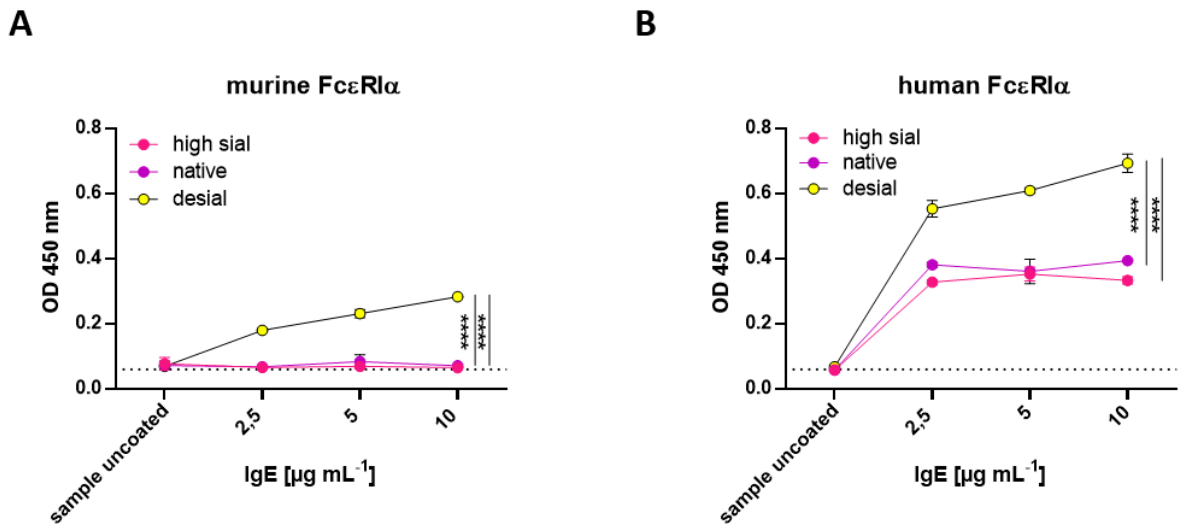

Figure 17: Binding of different IgE glycoforms to FcεRIα.

ELISA plates coated with 5 µg mL^{-1} ***(A)*** *murine or* ***(B)*** *human FcεRIα per well were incubated with serial dilutions of differently glycosylated anti-TNP IgE antibodies. Detection was performed using a HRP-coupled goat anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM. The dotted line shows the background signal of the plate. Experiments were conducted together with Clarissa Pfeufer, a master student in our laboratory. ****P < 0.0001 by two-way ANOVA with multiple comparisons.*

Figure 17 shows, that murine anti-TNP IgE antibodies have the potential to bind both, the murine and the human form of FcεRIα. In general, all IgE glycoforms showed increased binding to the human FcεRIα compared to murine FcεRIα. However, it could be demonstrated that the binding capacity of the IgE antibodies for the IgE receptor FcεRIα depends on the IgE glycosylation pattern. Desialylated IgE antibodies showed a significantly higher binding capacity for FcεRIα compared to sialylated IgE glycoforms. Native sialylated and highly sialylated IgE antibodies showed almost no binding to murine FcεRIα (Figure 17A) and only low binding to human FcεRIα (Figure 17B) in this setting.

Since I presented in Figure 17B, that murine anti-TNP IgE antibodies are able to bind the human form of FcεRIα, in the next step, I investigated whether different murine anti-TNP IgE glycoforms could be used to sensitize and thus potentially activate the human mast cell line LAD2. Therefore 2.5x 10^5 cells were incubated with 0.2 µg of differently glycosylated IgE antibodies over night and analyzed by flow cytometry the next day. All cells were stained with fixable viability dye and antibodies against human FcεRIα. Mast cells were pre-gated on single, viable cells. The loaded IgE glycoforms were either detected using the flourochrome-coupled allergen TNP-BSA or a flourochrome-coupled anti-mouse IgE antibody (Figure 18).

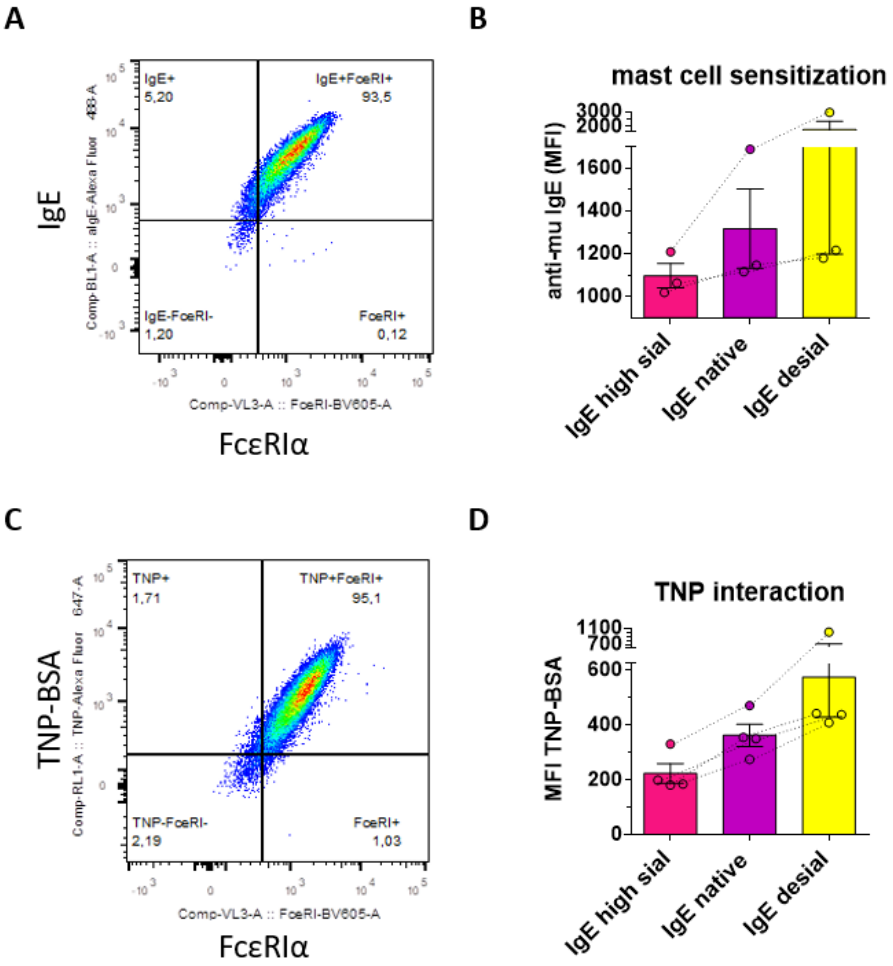

Figure 18: Sensitization of LAD2 mast cells with different IgE glycoforms.

*LAD2 cells were incubated with 0.2 µg anti-TNP IgE glycoforms per 2.5x 10^5 cells over night and analyzed by flow cytometry the next day. Mast cells were pre-gated on single, viable cells. Next, (**A**) FcεRI$^+$ IgE$^+$- and (**C**) FcεRI$^+$ TNP$^+$ - cells were identified. The the median fluorescence intensity of (**B**) IgE and (**D**) TNP are shown. n=3-4 for all groups. The dotted lines indicate which data points belong to the same experiment.*

As shown in Figure 18, LAD2 mast cells could be sensitized with all of the produced IgE glycoforms. Interestingly, the median fluorescence intensity of IgE (Figure 18B) and TNP (Figure 18D) was increased in cells sensitized with desialylated IgE antibodies compared to native and highly sialylated IgE antibodies, which is in line with the data presented in Figure 17B, where desialylated IgE antibodies showed an increased binding capacity for the human FcεRIα, compared to native sialylated and highly sialylated IgE antibodies. Furthermore, in this setting I also observed a slightly higher sensitization potential of native compared to highly sialylated IgE antibodies.

Taken together, the data presented in this section confirmed a successful generation of different anti-TNP IgE glycoforms with conserved affinity to the allergen (Figures 15 and 16) and that

the affinity of the IgE antibodies to FcεRIα depends on the IgE glycosylation pattern (Figure 17). Furthermore, human LAD2 mast cells could be sensitized with differently glycosylated murine IgE antibodies in an IgE-sialylation dependent manner (Figure 18).

3.3.2 The degree of effector cell activation depends on the IgE glycosylation pattern

Human mast cell activation

Mast cell activation can be measured by assessing the release of degranulation compounds, such as the enzyme β-hexosaminidase, following FcεRI crosslinking (Kirshenbaum et al., 2003; Kuehn et al., 2010). The following assay has been established in our labortatory by a former PhD student, Dr. Janina Petry (Petry, 2019). I adapted the protocol and repeated the assay to clarify preliminary results concerning the potential of differently glycosylated anti-TNP IgE antibodies to activate the human mast cell line LAD2. Therefore, LAD2 cells were sensitized with 0.02 µg anti-TNP IgE glycoforms per 2.5x 10^5 cells for 20 hours at 37 °C. The next day, cells were stimulated with 0.2 µg TNP-OVA or 0.1% Triton X-100 (to simulate full degranulation; positive control) for 30 minutes to induce degranulation and β-hexosaminidase release. Finally, the percentage of β-hexosaminidase release was calculated as percentage of total release induced by 0.05% Triton X-100 treatment (Figure 19).

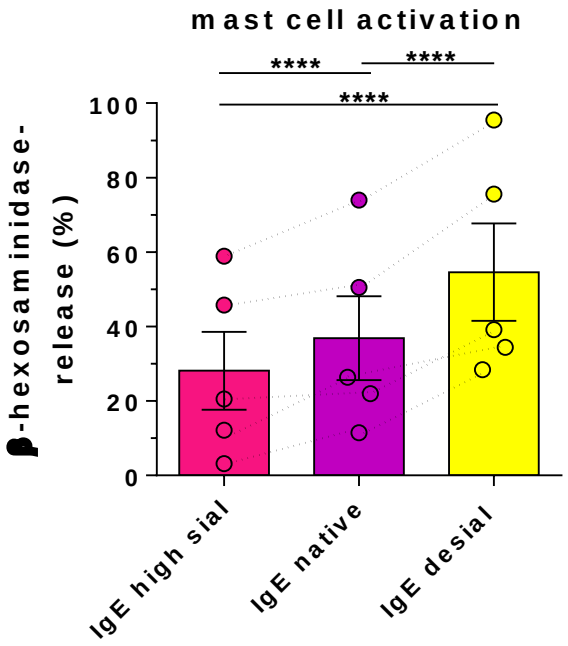

Figure 19: Mast cell activation by differently glycosylated IgE antibodies.

*Mast cell activation was analyzed by β-hexosaminidase release. Therefore, 2.5x 10^5 cells were sensitized with 0.02 µg of differently glycosylated anti-TNP IgE antibodies for 20 hours. Next, cells were stimulated with 0.2 µg TNP-OVA for 30 minutes. The released β-hexosaminidase was calculated as percentage of total release by 0.05% Triton X-100 treatment. Data are presented as mean +/- SEM. ****P < 0.0001 by one-way ANOVA. Pooled data of five independent experiments are shown. The dotted lines indicate which data points belong to the same experiment.*

As anticipated, the produced IgE glycoforms also showed a different potential to activate the human mast cell line LAD2 (Figure 19), likely due to their varying binding capacity to FcεRI on the mast cell surface (Figures 17B and 18). Following sensitization, desialylated IgE antibodies induced a significantly stronger mast cell activation upon allergen stimulation, compared to the sialylated IgE glycoforms. Furthermore, native IgE antibodies with an intermediate sialylation level also induced a significantly higher mast cell activation compared to highly sialylated IgE antibodies, which is in line with the decreased sensitization potential of highly sialylated IgE antibodies presented in Figure 18. A similar trend was shown on $CD34^+$ differentiated human mast cells in a cooperation project with the Fraunhofer ITEM (the research group of Dr. Susann Dehmel, suppl. Figure 32).

Human blood basophil acitvation

Furthermore, in a cooperation project with the Forschungszentrum Borstel (the research group of Prof. Dr. med. Uta Jappe), the sensitization and activation of human blood basophils with different IgE glycoforms was investigated. Therefore, fresh human blood cells were incubated with 10 µg/mL of the anti-TNP IgE glycoforms for 1 h at 37 °C and binding of the different murine IgE glycoforms to basophils was analyzed by flow cytometry (Figure 20). IgE-binding was assessed using a flourochrome-coupled anti-mouse IgE antibody (Figure 20A and B). Basophil activation was induced by subsequent addition of 0.1 µg/mL TNP-OVA and CD63 upregulation, as a common marker of basophil activation, was analyzed after 30 min through flow cytometry (Figure 20C).

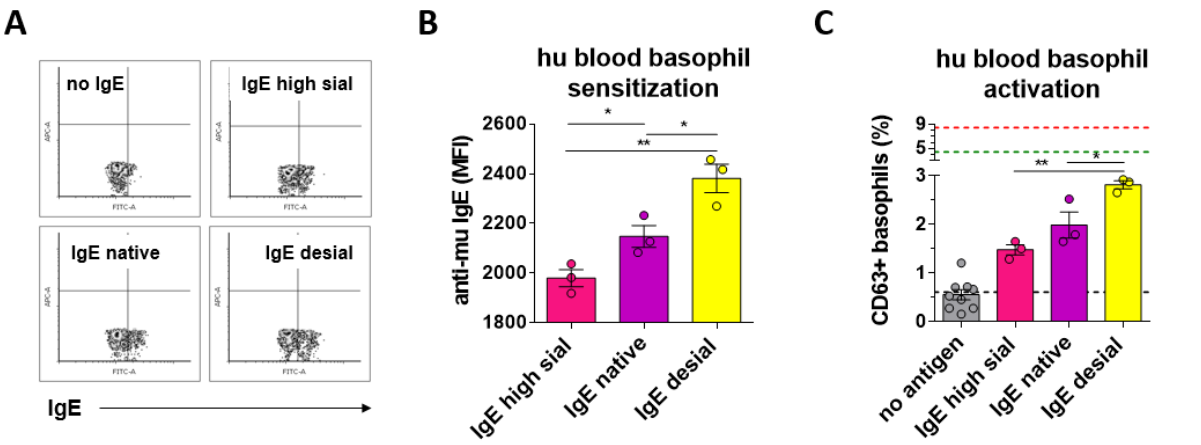

Figure 20: Human basophil sensitization and activation by differently glycosylated IgE antibodies.

(A) *Human blood basophil sensitization and activation was analyzed by flow cytometry. Human blood cells were incubated with 10 µg mL^{-1} of different IgE glycoforms for 1 h. Basophils were identified as FcεRIα^{+} CD203c^{+} cells and IgE-binding was analyzed using an anti-mouse IgE antibody.* ***(B)*** *The Median Flourescence Intensity (MFI) of the labeled anti-mouse IgE antibody on basophils sensitized with different IgE glycoforms is shown.* ***(C)*** *Basophil activation was induced by subsequent addition of 0.1 µg mL^{-1} TNP-OVA and analyzed by CD63 upregulation through flow cytometry 30 min later. Black dotted line: PBS negative control; green dotted line: activation with a mix of two activating anti-human IgE Abs; red dotted line: activation with fMLP (positive controls). Data are represented as mean + SEM, *P < 0.05 and **P < 0.01 by one-way ANOVA. Experiments were conducted by Jochen Behrends and Theresa Walsemann in the group of Prof Jappe.*

Similar to mast cell sensitization and activation (Figures 18 and 19) desialylated IgE antibodies also had a significantly higher capacity to sensitize (Figure 20B) and activate (Figure 20C) human blood basophils compared to sialylated IgE glycoforms. Furthermore, also on blood basophils a decreased sensitization potential of highly sialylated IgE antibodies compared to native IgE antibodies could be observed (Figure 20B) which is in line with the results presented in Figure 18. However, no differences in basophil activation between native and highly sialylated IgE antibodies could be observed in this setting (Figure 20C).

Taken together, the presented *in vitro* data in sections 3.3.1 and 3.3.2 suggest that decreasing levels of IgE sialylation likely leads to an increased ability of IgE antibodies to bind the high affinity FcεRI on effector cells of allergic disease and thus to an increased effector cell activation. Glycan-binding receptors/molecules may additionally be involved in regulating the different potentials of IgE antibodies with varying sialylation levels to sensitize and activate effector cells.

3.3.3 Desialylated IgE antibodies do not induce systemic anaphylaxis *in vivo*

In this study it could be demonstrated, that the produced IgE glycoforms behaved differently *in vitro*. Desialylated IgE antibodies showed increased FcεRI binding activity (Figures 17 and 18), as well as a higher potential to activate human mast cells (Figure 19 and suppl. Figure 30) and basophils (Figure 20) compared to native and highly sialylated IgE antibodies.

In this section desialylated as well as native and highly sialylated IgE antibodies were investigated for their potential to induce allergic reactions *in vivo,* using the IgE-mediated passive systemic anaphylaxis model introduced in 3.2.

To induce passive systemic anaphylaxis, mice were sensitized with 5 µg of different murine anti-TNP IgE glycoforms and challenged 24 hours later with 1 µg TNP-OVA as the allergen.

The severity of the anaphylactic reaction was measured as a drop of body core/ rectal temperature after allergen-challenge (Figure 21A).

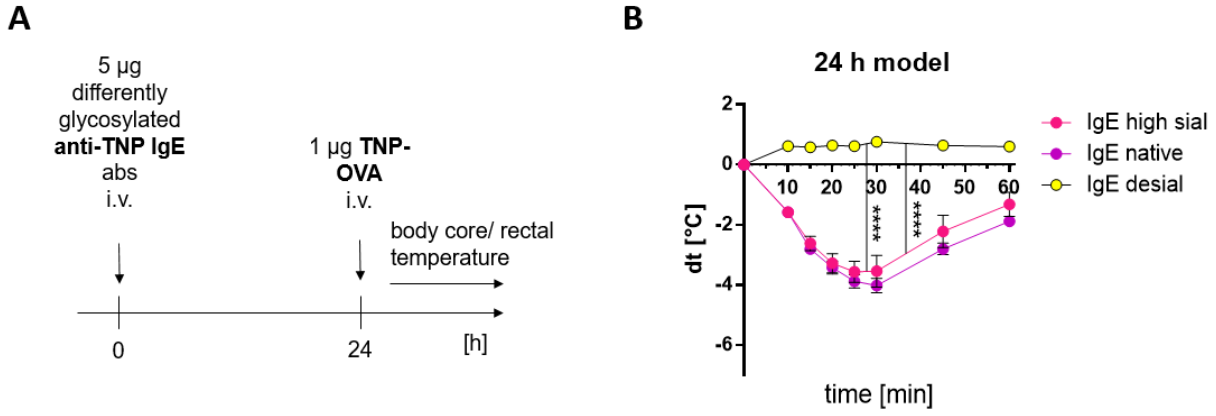

Figure 21: Induction of IgE-mediated passive systemic anaphylaxis with different IgE glycoforms.

(A) *Experimental setup of the IgE-mediated passive systemic anaphylaxis. Mice were sensitized i.v. with 5 µg anti-TNP IgE antibodies and challenged i.v. with 1 µg TNP-OVA 24 hours later.* ***(B)*** *The severity of the anaphylactic reaction was measured as the drop of the body core/ rectal temperature upon TNP-OVA challenge. Data are presented as mean +/- SEM, n = 5 for all groups. One of at least two independent experiments is shown. ****P < 0.0001 by two-way ANOVA with multiple comparisons.*

It could be demonstrated that native and highly sialylated IgE antibodies induce a comparable systemic anaphylactic reaction 24 hours after sensitization. Surprisingly, considering the *in vitro* data presented in 3.3.1 and 3.3.2, desialylated IgE antibodies did not induce anaphylaxis at all (Figure 21B). However, it turned out that desialylated IgE antibodies could not be detected in the blood serum already 6 hours after sensitization (Figure 22A) suggesting a dramatically decreased half-life of this IgE glycoform. Serum levels of native and highly sialylated IgE antibodies were rather comparable (Figure 22B).

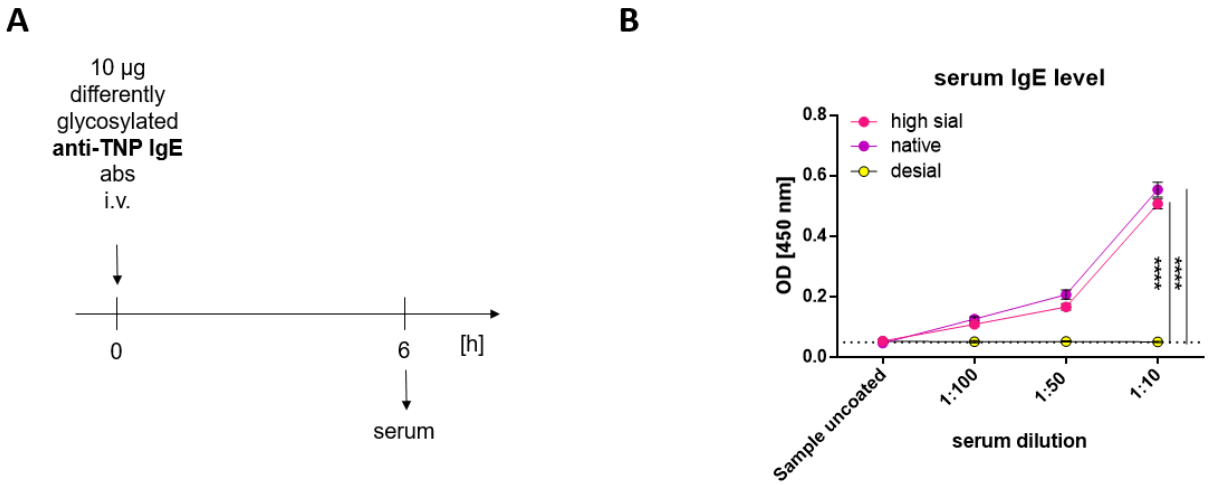

Figure 22: Serum level of differently glycosylated anti-TNP IgE antibodies.

(A) *Experimental setup. Mice were sensitized i.v. with 10 µg anti-TNP IgE antibodies and serum was taken 6 hours later.* ***(B)*** *IgE sera were analyzed by TNP-reactive ELISA. ELISA plates coated with 10 µg mL^{-1} TNP-Ficoll per well were incubated with serial sera dilutions of IgE-sensitized mice. Detection was performed using a HRP-coupled goat anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM. The dotted line shows the background signal of the plate. n=2-5 for each group. ****P < 0.0001 by two-way ANOVA with multiple comparisons.*

Next, the sensitization of murine blood basophils with different IgE glycoforms *in vivo* was investigated by flow cytometric analysis to check whether the desialylated IgE antibodies are possibly bound to FcεRI on effector cells, considering their enhanced receptor binding capacity *in vitro*. Therefore, mice were sensitized with 10 µg highly sialylated or desialylated anti-TNP IgE antibodies and whole blood was taken after 6 hours for flow cytometric analysis (Figure 23). Blood cells were pre-gated on single, viable leucocytes, as shown in Figure 11. Next, CD49b$^+$ IgE$^+$ basophils were identified (Figure 23A) and analyzed for their sensitization with anti-TNP IgE antibodies (Figure 23B).

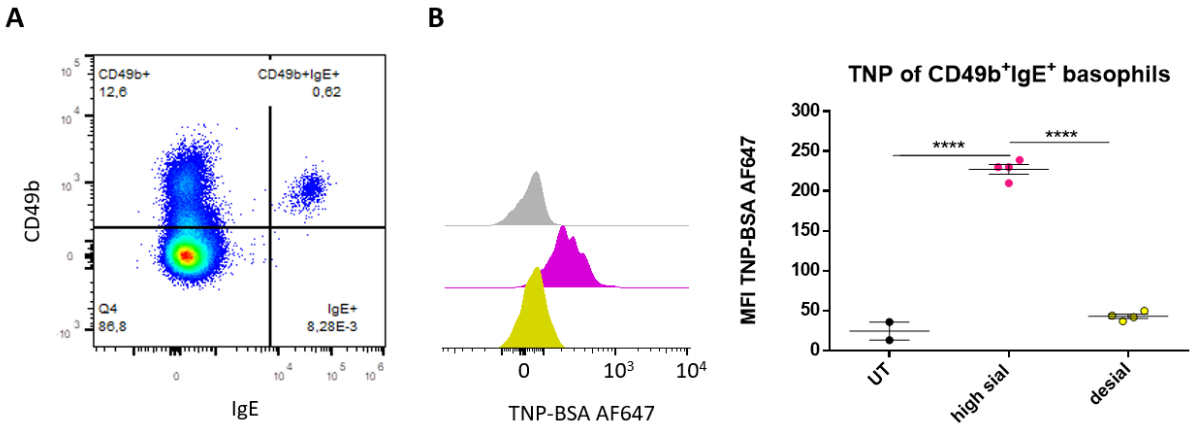

Figure 23: Sensitization of murine blood basophils with anti-TNP IgE antibodies.

(A) *Identification of blood basophils. Mice were sensitized with 10 µg anti-TNP IgE antibodies and blood was taken 6 hours later. Blood cells were pre-gated on single, viable leucocytes (s. Figure 11) and identified as CD49b$^+$IgE$^+$ cells.* ***(B)*** *The Median Flourescence Intensity (MFI) of the labeled allergen TNP-BSA of unsensitized (UT) or anti-TNP IgE-sensitized mice in CD49b$^+$IgE$^+$ basophils is shown including overlay histograms of the allergen binding to surface IgE from representative mice of each group and unsensitized controls (grey). Individual symbols represent individual mice. One of at least two independent experiments is shown. ****P < 0.00001, by one-way ANOVA.*

Figure 23 shows that anti-TNP IgE antibodies could be detected in mice sensitized with highly sialylated IgE antibodies, whereas the fluorescence intensity of the allergen TNP-BSA in mice sensitized with desialylated IgE antibodies is similar to the signal measured in unsensitized controls, indicating that little to no desialylated anti-TNP IgE antibodies bound to FcεRI on basophils. This finding further supports the hypothesis, that desialylated IgE antibodies have a

dramatically reduced half-life *in vivo.* Thus, it is likely that they are not able to sensitize effector cells of IgE-mediated allergic reactions and, consequently, cannot induce systemic anaphylaxis.

Taken together, the results presented in this section showed that surprisingly, desialylated anti-TNP IgE antibodies do not induce systemic anaphylaxis *in vivo* (Figure 21) despite their increased pro-allergic potential *in vitro* (3.3.1 and 3.3.2). Further investigation of the serum IgE levels (Figure 22) and the sensitization of blood basophils (Figure 23) led to the hypothesis that desialylated anti-TNP IgE antibodies have a markedly decreased serum half-life *in vivo* as they could neither be detected in the serum (Figure 22B) nor on blood basophils (Figure 23B) of sensitized mice already 6 hours after sensitization.

3.3.4 The half-life of desialylated IgE antibodies is regulated by asialo-glycoprotein receptors

Asialo-glycoprotein receptors are involved in the natural degradation of erythrocytes and the clearance of desialylated glycoproteins of the serum by binding terminal galactose or N-acetyl-galactosamine residues with high affinity, which leads to the internalization and degradation of the glycoproteins (Grewal, 2010; Gupta, 2012). A prominent example of this receptor family is the hepatic asialo-glycoprotein receptor (ASGP-R) (Grewal, 2010; Rigopoulou et al., 2012). It is described, that inhibition or knock out of this receptor significantly prolongs the half-life of e.g. desialylated IgA antibodies (Rifai et al., 2000; Rigopoulou et al., 2012) or platelets (Ellies et al., 2002).

The results presented in section 3.3.3 led to the assumption that also desialylated IgE antibodies have a decreased half-life *in vivo*, which could be mediated through rapid clearance of this IgE glycoform via the ASGP-R pathway.

To follow up on the hypothesis, I established an *in vivo* mouse model, where asialo-glycoprotein receptors were blocked with an excess of the ligand asialo-α-1-acid glycoprotein (AGP) (Matsumoto et al., 2010) 1 hour before sensitization with differently glycosylated anti-TNP IgE antibodies.

First, I investigated whether desialylated IgE antibodies can be detected in the sera of sensitized mice upon ASGP-R blocking. Therefore, mice were i.v. injected with 1 mg asialo-AGP and sensitized with 10 µg either highly sialylated or desialylated anti-TNP IgE antibodies 1 hour later. Serum was taken 6 hours after sensitization and analyzed by TNP-reactive ELISA (Figure 24).

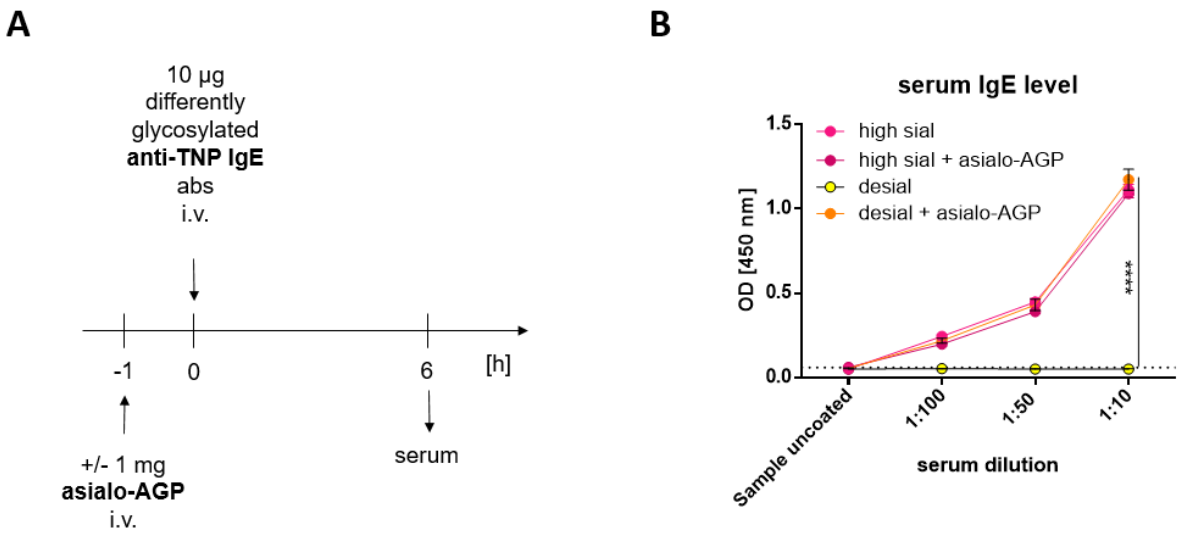

Figure 24: Serum level of differently glycosylated anti-TNP IgE antibodies upon ASGP-R blocking.

(A) *Experimental setup. Asialo-glycoprotein receptors were blocked by i.v. injection of 1 mg asialo-AGP 1 hour before sensitization. Mice were sensitized i.v. with 10 µg anti-TNP IgE glycoforms and serum was taken 6 hours later.* ***(B)*** *IgE sera were analyzed by TNP-reactive ELISA. ELISA plates coated with 10 µg mL^{-1} TNP-Ficoll per well were incubated with serial sera dilutions of IgE-sensitized mice. Detection was performed using a HRP-coupled goat anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM. The dotted line shows the background signal of the plate. n=5 for each group. ****P < 0.0001 by two-way ANOVA with multiple comparisons.*

As anticipated, upon ASGP-R blocking, desialylated anti-TNP IgE antibodies stayed in circulation and could be detected in the serum of sensitized mice after 6 hours. In fact, the serum level of desialylated IgE antibodies was rather comparable to the serum level of highly sialylated IgE antibodies. Noteworthy, the half-life of highly sialylated anti-TNP IgE antibodies is not affected by the ASGP-R as serum levels did not change upon ASGP-R blocking (Figure 24B).

Next, the basophil sensitization with desialylated anti-TNP IgE antibodies upon ASGP-R blocking with asialo-AGP was investigated. Therefore, mice were i.v. injected with 1 mg asialo-AGP and sensitized with 10 µg either highly sialylated or desialylated anti-TNP IgE antibodies 1 hour later. Whole blood was taken 6 hours after sensitization and basophils were analyzed by flow cytometry, as described in section 3.3.3 (Figure 25).

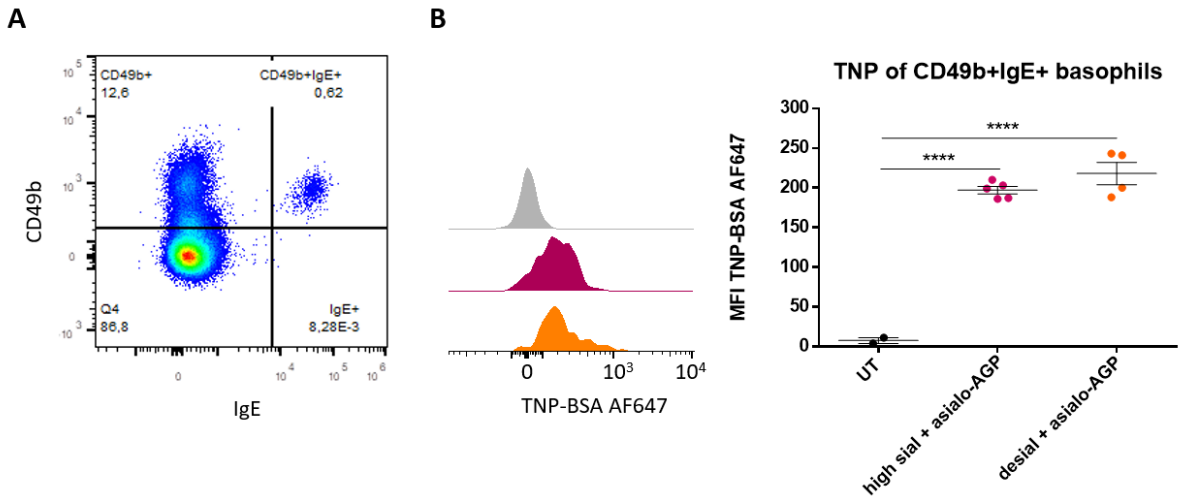

Figure 25: Sensitization of murine blood basophils with anti-TNP IgE antibodies upon ASGP-R blocking.

(A) *Identification of blood basophils. Asialo-glycoprotein receptors were blocked by i.v. injection of 1 mg asialo-AGP 1 hour before sensitization. Mice were sensitized with 10 µg anti-TNP IgE antibodies and blood was taken 6 hours later. Blood cells were pre-gated on single, viable leucocytes (s. Figure 12) and identified as CD49b$^+$IgE$^+$ cells.* ***(B)*** *The Median Flourescence Intensity (MFI) of the labeled allergen TNP-BSA of unsensitized (UT) or anti-TNP IgE-sensitized mice in CD49b$^+$IgE$^+$ basophils is shown including overlay histograms of the allergen binding to surface IgE from representative mice of each group and unsensitized controls (grey). Individual symbols represent individual mice. One of at least two independent experiments is shown. ****P < 0.00001, by one-way ANOVA.*

It could be demonstrated in Figure 25 that, upon ASGP-R blocking, desialylated IgE antibodies also have the potential to sensitize basophils, since a comparable signal of the allergen TNP-BSA could be detected in mice sensitized with highly sialylated and desialylated anti-TNP IgE antibodies (Figure 25B).

Considering that upon ASGP-R blocking the serum half-life of desialylated IgE antibodies could be increased (Figure 24) and effector cells could be sensitized with this IgE glycoform (Figure 25) in the next step, the potential of desialylated IgE antibodies to induce IgE-mediated PSA was investigated (Figure 26). Therefore, the ASGP-R was blocked by i.v. injection of 1 mg asialo-AGP 1 hour before sensitization with 10 µg either higly sialylated or desialylated anti-TNP IgE antibodies. PSA was induced 6 hours later by challenging the mice with 1 µg TNP-OVA. The severity of the anaphylactic reaction was measured as a drop of body core/ rectal temperature after allergen-challenge (Figure 26A).

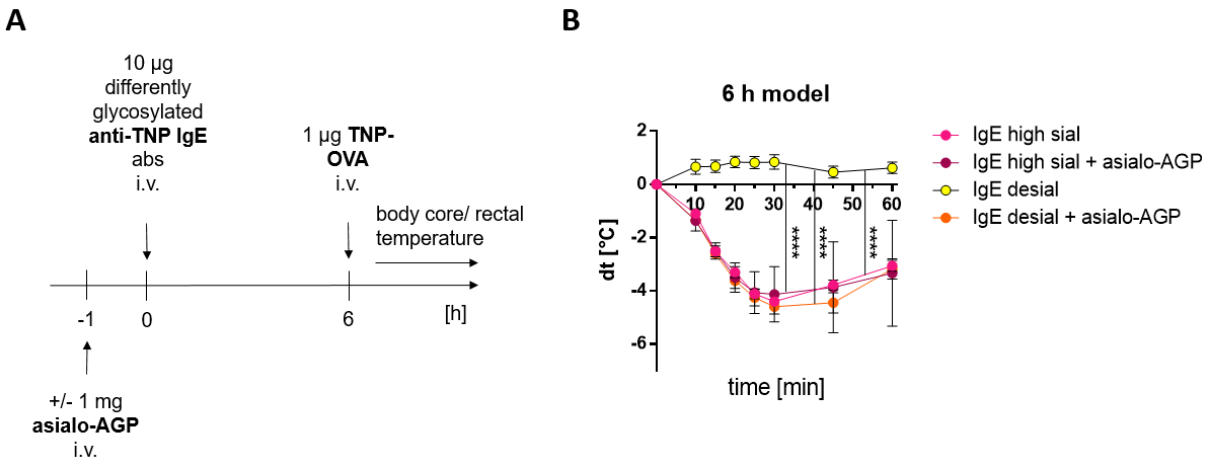

Figure 26: Upon ASGP-R blocking, desialylated IgE antibodies induce systemic anaphylaxis in vivo.

(A) *Experimental setup of the IgE-mediated passive systemic anaphylaxis. The ASGP-R was blocked by i.v. injection of 1 mg asialo-AGP 1 hour before sensitization. Mice were sensitized i.v. with 10 µg anti-TNP IgE antibodies and challenged i.v. with 1 µg TNP-OVA 6 hours later.* ***(B)*** *The severity of the anaphylactic reaction was measured as the drop of the body core/ rectal temperature upon TNP-OVA challenge. Data are presented as mean +/- SEM, n = 3-5 for all groups. One of at least two independent experiments is shown. ****P < 0.0001 by two-way ANOVA with multiple comparisons.*

As anticipated, when the ASGP-R was blocked with asialo-AGP, desialylated IgE antibodies induced PSA and the anaphyalactic potential of highly sialylated IgE antibodies was not affected by ASGP-R blocking. Surprisingly, the anaphylactic reaction induced by desialylated IgE antibodies was rather comparable to the reaction induced by highly sialylated IgE antibodies (Figure 26B), considering that desialylated IgE antibodies showed an increased anaphylactic potential *in vitro* compared to native- and highly sialylated IgE antibodies (sections 3.3.1-3.3.2, Figures 18-20).

Taken together, in sections 3.3.3 and 3.3.4 it could be demonstrated that desialylated IgE antibodies have a dramatically reduced serum half-life compared to highly sialylated IgE antibodies (Figures 21-23). This could be attributed to rapid clearance of desialylated IgE antibodies via the ASGP-R pathway *in vivo* (Figures 24-26). After ASGP-R-blocking with an excess of the ligand asialo-AGP, the half-life of desialylated IgE antibodies could be prolonged resulting in a comparable serum appearance of desialylated and highly sialylated IgE antibodies 6 hours after sensitization (Figure 24). Furthermore, upon ASGP-R blocking also desialylated IgE antibodies had the potential to sensitize blood basophils (Figure 25) and induce an IgE-mediated passive systemic anaphylaxis (Figure 26) comparable to the reaction mediated by highly sialylated IgE antibodies. Other experimental mouse model settings, inflammatory conditions and/or the release or expression of glycan-binding receptors/molecules might be neccessary to visualize differences between different sialylation levels of IgE antibodies *in vivo*.

The experiments presented in sections 3.3.1 - 3.3.4 were published in:
Dühring L*, Petry J*, Lilienthal G-M, Bartsch YC, Kubiak M, Pfeufer C, Lehrian S, Buhre JS, Lunding HB, Kern C, Behrends J, Walsemann T, Gädert L, Sommer C, Krüger L, Blanchard V, Dehmel S, Jappe U, Rahmöller J, Ehlers, M. Sialylation of IgE reduces FcεRIα binding and mast cell and basophil activation in vitro and increases IgE half-life in vivo. *Allergy 2023.* doi: 10.1111/all.15665. Epub ahead of print. (*these authors contributed equally).

3.3.5 IgE-binding by galectin-3 depends on the IgE glycosylation pattern

Galectins are a family of 15 β-galactoside binding lectins in mammals with various intra- and extracellular functions, including modulation of immune responses (Liu, 2005). Galectin-3, former known as ε- or IgE-binding protein, has been identified by its binding-capacity of IgE (Liu et al., 1985). Further, it could be demonstrated that galectin-3 also binds FcεRI (Frigeri et al., 1993) and increases effector cell activation by crosslinking IgE, FcεRI or both (Dumic et al., 2006; Zuberi et al., 2004).

In this section, I investigated in a galectin-3-reactive ELISA whether galectin-3 has different binding affinities for the three produced IgE glycoforms (Figure 27).

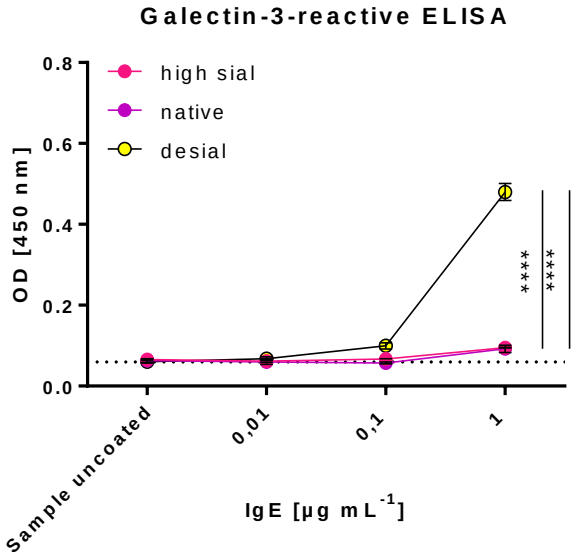

Figure 27: Binding of different IgE glycoforms to galectin-3.

*ELISA plates coated with 10 µg mL^{-1} murine galectin-3 per well were incubated with serial dilutions of the different anti-TNP IgE glycoforms. Detection was performed using a HRP-coupled goat anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM. The dotted line shows the background signal of the plate. ****P <*

0.0001 by two-way ANOVA with multiple comparisons.One of at least two independent experiments is shown.

In line with the literature (Robertson et al., 1990; Robertson and Liu, 1991), it could be demonstrated that galectin-3 strongly binds to desialylated IgE antibodies, whereas highly sialylated and native IgE antibodies showed only little galectin-3 binding activity (Figure 27).

In this section, it could be demonstrated that the interaction of the IgE-binding protein galectin-3 with IgE antibodies depends on the IgE glycosylation pattern. Galectin-3 showed increased binding capacity to desialylated IgE antibodies and almost no binding to native or highly sialylated IgE antibodies (Figure 27). This finding is in line with the literature, considering that galectins bind terminal β-galactoside sugars (Dumic et al., 2006; Liu, 2005). Those exist to a greater extend in the non-sialylated IgE glycoform compared to the sialylated IgE glycoforms (native, high sial), where most of the glycosylation sites contain terminal sialic acids, which were shown to inhibit galectin-3 binding to these IgE glycoforms (Robertson et al., 1990; Robertson and Liu, 1991). It is tempting to hypothesize that galectin-3 might be one of the regulators of different IgE glycoforms *in vivo*. Thus, future studies will investigate the impact of galectin-3 on effector cell sensitization and systemic anaphylaxis induced by different IgE glycoforms.

3.4 Immunization with different adjuvants induces varying amounts of IgE antibodies

It is well established in our laboratory that immunization with ovalbumin (OVA) as a foreign antigen in combination with different adjuvants leads to the production of OVA-specific IgG antibodies with different sialylation levels, depending on the (inflammatory) stimulus given by the adjuvant (Bartsch, 2019; Bartsch et al., 2020; Lunding, 2021). Thereby, the stimulus given by aluminium-based adjuvants such as alum was less inflammatory than the stimulus given by oil-based adjuvants such as enriched complete Freunds adjuvant (eCFA, containing 5 mg/mL *Mtb* in incomplete Freunds adjuvant (IFA)). Therefore, immunization of mice with OVA-eCFA leads to higher antigen-specific IgG titers with elevated levels of IgG antibody agalctosylation compared to mice immunized with OVA-alum. This finding could be linked to the differential regulation of the sialyltransferase St6gal1 in antibody-producing plasma cells during the germinal center reaction, depending on the adjuvant used for immunization (Bartsch, 2019; Bartsch et al., 2020; Lunding, 2021).

Results

During AIT similar protocols are used to restore tolerance to a certain allergen. Therefore, increasing amounts of the allergen over time, combined with an adjuvant (e.g. alum) are used to trigger an immune response in allergic patients resulting e.g. in the production of allergen-specific IgG antibodies (Lee et al., 2022; Shamji et al., 2021; Tabynov et al., 2022). These IgG antibodies on the one hand can mask the allergen in circulation before it can crosslink allergen-specific IgE antibodies on mast cells and basophils and on the other hand inhibit the IgE-FcεRI signaling on these cells by crosslinking FcεRI with the inhibitory IgG receptor FcγRIIB (Kanagaratham et al., 2020; Shamji et al., 2021). It should be noted that although elevated allergen-specific IgG titers during AIT are associated with increasing tolerance to the allergen, it is not considered a general biomarker for AIT success (Zissler and Schmidt-Weber, 2020).

Based on these findings, as a side project I investigated whether the immunization with OVA in combination with different adjuvants also affects the induction of IgE antibodies considering that the choice of adjuvant for AIT could be crucial regarding its success. Therefore, mice were immunized intraperitoneally with 100 µg OVA in combination with either alum or eCFA and blood was sampled on day 12 for further analysis (Figure 28A). The immunizations were performed by Dr. Hanna Lunding, a former PhD student in our laboratory.

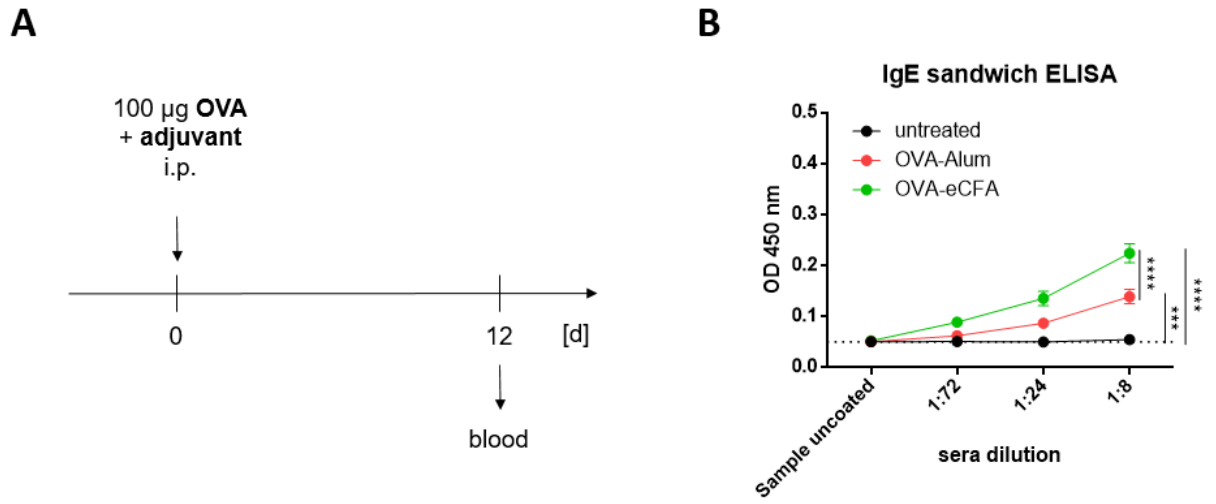

Figure 28: The amount of IgE antibodies induced upon immunization depends on the used adjuvant.

(A) *Experimental setup of the OVA-immunization model. Mice were immunized i.p. with 100 µg OVA plus adjuvant. Blood serum was sampled on day 12 after immunization for further analysis. The immunizations were performed by Dr. Hanna Lunding, a former PhD student in our laboratory.* ***(B)*** *IgE antibodies induced upon OVA immunization were measured by IgE sandwich ELISA. ELISA plates coated with 10 µg mL^{-1} of a polyclonal goat anti-mouse IgE antibody were incubated with serial sera dilutions of immunized mice. Detection was performed using a HRP-coupled goat anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM. The dotted line shows the background signal of the plate. n=2-4 for each group. ***P < 0.001, ****P < 0.0001 by two-way ANOVA with multiple comparisons.*

Results

Figure 28 shows that increased levels of IgE in the serum of mice can be detected upon an immunization with OVA-eCFA 12 days after immunization compared to an immunization with OVA-alum. No IgE was detected in untreated mice with this setting.

These results are in line with previous findings of our group regarding the induction of allergen-specific IgG antibodies where an immunization with OVA-eCFA also induced higher titers of allergen-specific IgG antibodies than an immunization with OVA-alum (Bartsch et al., 2020; Lunding, 2021). Furthermore, it could be demonstrated that alum induces allergen-specific IgE antibodies early during AIT (Jensen-Jarolim et al., 2021; Tabynov et al., 2022; Zubeldia et al., 2019). Future studies will investigate whether different adjuvants also induce different IgE glycosylation patterns.

4 Discussion

Allergen-specific IgE antibodies can mediate allergic reactions by binding the high affinity IgE Fc receptor FcεRI on mast cells and basophils. Crosslinking of the receptor upon allergen exposure leads to activation of these cells and the release of vasoactive mediators such as histamine, which are responsible for the symptoms of allergic disease. In severe cases, IgE-mediated allergic reactions can result in potential life-threatening systemic anaphylaxis. It could be demonstrated that allergen-specific IgG antibodies, induced during AIT, are able to inhibit IgE-mediated anaphylaxis through allergen-masking as well as crosslinking FcεRI with the inhibitory IgG Fc receptor FcγRIIB. Nevertheless, when allergen doses are high, also allergen-specific IgG antibodies can induce systemic anaphylaxis in mice trough FcγR-mediated activation of monocytes/ macrophages, neutrophils and basophils (Beutier et al., 2017; Finkelman et al., 2016; M. V. Khodoun et al., 2011).

4.1 Immunomodulatory mechanisms in IgG-mediated anaphylaxis

To date, it is well established that the effector functions of IgG antibodies depend on the IgG subclass. Three different IgG subclasses have been identified in mice (IgG1, IgG2a, IgG2b and IgG3) varying in their affinity for FcγRs which modulate immune responses by inducing activating or inhibitory signals. Three activating FcγRs have been described in mice, FcγRI, FcγRIII and FcγRIV. Signalling of these receptors is counteracted by the inhibitory FcγRIIB, thus modulating the activation threshold of effector cells (Bruhns and Jönsson, 2015; Vidarsson et al., 2014a). In the context of anaphylaxis, IgG2 antibodies have been demonstrated to induce a stronger anaphylactic reaction compared to IgG1 antibodies which could be assigned to their differential interaction with FcγRs. IgG1 antibodies only bind one activating FcγR (FcγRIII) and have a relatively high affinity for FcγRIIB, whereas increased binding affinity to activating FcγRs was described for IgG2 antibodies as well as a decreased affinity for FcγRIIB, compared to IgG1 antibodies. Thus, the IgG subclass clearly contributes to the severity of the anaphylactic reaction. IgG1-induced anaphylaxis is mediated by FcγRIII expressed on monocytes/ macrophages, neutrophils and basophils and can be attenuated by FcγRIIB due to an increasing activation threshold of effector cells when crosslinked with activating FcγRs (Beutier et al., 2017; Epp et al., 2018a; Petry et al., 2021a).

IgG effector functions can be further modulated by the IgG Fc *N*-glycosylation pattern. Increased amounts of agalactosylated IgG antibodies have been described in patients with inflammatory autoimmune diseases. Simultaneously, the therapeutic effect of IVIG in several

murine disease models could be attributed to its sialylated subfraction. Thus, agalactosylated IgG antibodies have been associated with more pro-inflammatory immune responses, whereas terminal galactosylation and sialylation has been demonstrated to suppress inflammation and is observed e.g., during pregnancy and disease remission (Anthony et al., 2008; Collin and Ehlers, 2013; Kaneko et al., 2006).

Most data on IgG glycosylation are reported for inflammatory autoimmune diseases but at the time I started my thesis, the laboratory had found that IgG glycosylation also plays a role in IgG-mediated anaphylaxis. Furthermore, they identified a receptor by which protective effects of sialylated IgG antibodies could be mediated. First of all, a reduced anaphylactic potential of sialylated TNP-specific IgG1 antibodies was observed compared to agalactosylated IgG1 antibodies in a 24-hour PSA model (Epp et al., 2018a). It could be demonstrated that this effect was not due to a reduced allergen affinity or serum half-life of the sialylated antibodies and that FcγRs are required to induce PSA in our mouse model since no anaphylaxis could be induced in FcγR-deficient mice, regardless of the IgG glycosylation pattern (Epp et al., 2018a; Petry et al., 2021a). Hence FcγRs are absolutely required to induce IgG-mediated anaphylaxis.

It has been demonstrated that IgG antibody glycosylation introduces conformational changes in the antibody Fc part resulting in an altered binding affinity to FcγRs and thus modulating antibody effector functions. Without terminal galactose and sialic acid, the IgG Fc part engages in an open conformation that is essential for the interaction with type I receptors, the classical FcγRs. In contrast, terminal galactosylation and sialylation forces the IgG Fc into a closed conformation, reducing the affinity to type I Fc receptors but increasing the affinity for sugar-binding type II Fc receptors for example of the c-type lectin superfamily (Fiebiger et al., 2015; Karsten et al., 2012; Pincetic et al., 2014; Schwab et al., 2014; Sondermann et al., 2000).

Hence, also in IgG-mediated anaphylaxis glycan-dependent differences were assumed to be mediated by sugar-binding type II Fc receptors. Indeed, the reduced potential of sialylated IgG1 antibodies to induce PSA could be demonstrated to be a consequence of the interaction of the sialic acid residues of the Fc glycan with the type II Fc receptor SIGN-R1 (since depletion of SIGN-R1 with anti-SIGN-R1 blocking antibodies abrogated the protective effect of IVIG) as well as sialylated allergen-specific IgG antibodies in IgG-mediated anaphylaxis (Epp et al., 2018a; Petry et al., 2021a).

The anti-inflammatory effects of the sialylated subfraction of IVIG were first described in mouse models of inflammatory autoimmune diseases and a pathway was discovered how these protective effects are mediated. Thereby, sialylated IgG antibodies bind to SIGN-R1 on splenic

marginal zone macrophages which leads to the secretion of IL-33 and in turn to the proliferation of IL-4 producing basophils inducing the upregulation of the inhibitory receptor FcγRIIB on effector cells (Anthony et al., 2011, 2008).

Addressing the role of SIGN-R1 in IgG-mediated anaphylaxis it could be demonstrated that treatment of mice with an anti-SIGN-R1 blocking antibody depleted this receptor on splenic monocytes/ macrophages but not on their blood counterpart. Accordingly, in IgG1-mediated anaphylaxis SIGN-R1 expression on splenic monocytes/ macrophages was shown to be required for the anti-inflammatory effects of sialylated IgG1 antibodies. Furthermore, flow cytometry analysis revealed that mice sensitized with sialylated IgG1 antibodies or IVIG showed an increased expression of FcγRIIB on monocytes 24 hours later, whereas the expression level of FcγRIII remained unaltered (Petry et al., 2021a). In accordance with these results, it was shown that the protective effect of sialylated IgG1 antibodies in IgG-mediated anaphylaxis is also a matter of time. The reduced anaphylactic potential of these antibodies could not be observed when mice were challenged 30 minutes after sensitization instead of 24 hours (Petry et al., 2021a). This result suggested that as described for autoimmune diseases (Anthony et al., 2008; Schwab et al., 2014) also in IgG1-mediated anaphylaxis SIGN-R1 was essential for sialylation-dependent attenuation of the anaphylactic reaction only under preventive treatment conditions. On the other hand, disease severity after anaphylaxis onset was also determined by FcγRIIB, since FcγRIIB deficiency increased anaphylaxis independent of the timepoint of antigen challenge (Petry et al., 2021a).

In my thesis, I attempted to answer two open questions in this project: First, given the described protective effect of the sialylated subfraction of IVIG in IgG1-mediated systemic anaphylaxis, I investigated whether this effect could also be mediated by sialylated allergen-unspecific human IgG antibodies of a distinct subclass. Treatment of mice with sialylated human IgG4 antibodies 24 hours bevor PSA induction indeed revealed that sialylated human IgG4 antibodies significantly attenuated IgG1-mediated PSA (Figure 10B).

Despite the appreciated beneficial therapeutic effect of IVIG in inflammatory diseases the therapeutic efficacy and potency of patients treated with IVIG is still limited. In an attempt to create a a novel drug candidate with consistent increased anti-inflammatory activity based on IVIG, Washburn and colleagues introduced a tetra sialylated IVIG drug candidate with maximum Fc sialylation, therby avoiding unwanted alterations to the IVIG mixture. This tetra sialylated IVIG showed 10-fold higher anti-inflammatory activity across different animal models compared to regular IVIG (Washburn et al., 2015). Considering the results I described

above, for future improvements of IVIG therapy, also the IgG subclass should be considered. Enrichment of (sialylated) IgG4 in IVIG treatment could proof beneficial since it is described that IgG4 has enhanced affinity to FcγRIIB compared to activating FcγRs and impaired effector functions compared to the other IgG subclasses (van der Neut Kolfschoten et al., 2007; Vidarsson et al., 2014b). Accordingly, sialylated IgG4 antibodies could modulate the immune response on the one hand by increasing the expression of FcγRIIB in a sialic acid/ SIGN-R1-dependent manner and simultaneously minimize the risk for unwanted side effects because of decreased Fc-mediated effector functions. However, the enrichment of IgG4 in IVIG displays a great challenge, since IgG4 is least abundant human IgG subclass with only 4% of total serum IgG compared to 60% for IgG1 antibodies (Vidarsson et al., 2014b). An alternative treatment opportunity for IVIG would be the use of sialylated humanized monoclonal IgG4 antibodies, since they can be produced and purified in high amounts in cell culture systems. Another advantage of this approach is the high reproducibility of the batches produced in cell culture systems compared to IVIG, which consists of pooled serum IgG of healthy donors and thus potentially underlies great batch differences in e.g., the level of IgG sialylation, depending on the constitution of the recruited donors.

However, it could be demonstrated in our lab that high amounts of IVIG as well as lower amounts of sialylated murine IgG1 antibodies attenuate IgG1-mediated anaphylaxis in an allergen-unspecific manner by enriching the total blood IgG sialylation (Petry et al., 2021a). Second, upon sensitization with high amounts of IVIG or lower amounts of sialylated murine IgG antibodies an upregulation of the inhibitory FcγRIIB on murine blood monocytes, but not on neutrophils could be demonstrated (Petry et al., 2021a). An effect on basophils, another well described effector cell population in murine IgG-mediated anaphylaxis (Finkelman, 2007; Finkelman et al., 2016; Marat V. Khodoun et al., 2011), could not be clarified yet. During my studies, I could extend this knowledge by demonstrating that sensitization of mice with high amounts of IVIG or lower amounts of sialylated murine IgG1 antibodies also leads to an upregulation of FcγRIIB on blood basophils 24 hours after sensitization (Figure 12). Several studies investigating the mechanisms in IgG-mediated anaphylaxis focus on the identification of immune effector cells involved in the reaction. With my findings, I provided further evidence to elucidate the role of different effector cell types in murine IgG-mediated anaphylaxis.

Altogether, the data further support that in the context of IgG-mediated anaphylaxis sialylated human or murine IgG antibodies mediate an upregulation of the inhibitory FcγRIIB on effector cells, likely by enganging with the type II Fc receptor SIGN-R1. In addition, the protective

effect of the sialylated subfraction of IVIG in murine IgG-mediated anaphylaxis could also be mediated by sialylated human IgG4 antibodies, giving new opportunities to design anti-inflammatory drugs to increase therapy potency and efficacy.

However, the pathway initiated by binding of sialylated IgG antibodies to the type II Fc receptor SIGN-R1 promoting the upregulation of FcγRIIB on blood monocytes/ macrophages and basophils thus attenuating the anaphylactic potential of IgG antibodies remains unclear. The involvement of an IL-33 and/ or IL-4-mediated pathway as described for the therapeutic effect of IVIG in mouse models of rheumatoid arthritis by Anthony and colleagues could not be demonstrated so far. Treating mice with antibodies directed against IL-4 and/ or the IL-33 receptor did not abrogate the attenuation of IgG1-mediated anaphylaxis by IVIG (data not shown) suggesting that these cytokines are not involved in the suppression of anaphylactic reactions by sialylated IgG antibodies. Evidence therefore comes from a study from Schwab and colleagues who described that IVIG-induced protection of immunothrombocytopenia (ITP) depends on sialic acid and SIGN-R1, but excluded a role of IL-33 or IL-4 producing basophils in this setting (Schwab et al., 2014).

Together, the suppressive effects of IVIG as well as sialylated murine IgG1 antibodies in IgG1-mediated anaphylaxis, shown by my colleague Dr. Janina Petry, could be further confirmed and extended. The conducted experiments elucidated that the protective effect of the sialylated subfraction of IVIG could also be mediated by sialylated human IgG4 antibodies, thereby suggesting alternative treatment options with high potential for IgG-mediated allergic reactions. Furthermore, the observation that treatment of mice with IVIG or sialylated murine IgG1 antibodies resulted in an upregulation of FcγRIIB on monocytes/ macrophages could be extended to basophils, as another effector cell population of IgG-mediated anaphylaxis.

However, the existence of human IgG-mediated anaphylaxis is still a matter of debate. Human cases of anaphylaxis without detectable allergen-specific IgE antibodies or mast cell activation have been described. In contrast, anaphylaxis severity in these patients positively correlated with serum levels of allergen-specific IgG antibodies, supporting an IgE-independent mechanism of human anaphylaxis (Finkelman et al., 2016). Furthermore, human effector cells have the potential to induce an alternative IgG-mediated pathway of anaphylaxis. The activation of human monocytes/ macrophages, basophils and neutrophils results in the release of PAF, which has been associated with IgG-mediated anaphylaxis in mice. Human basophils contain more granules and human monocytes/ macrophages have been described to have lower expression levels of the inhibitory FcγRIIB than their murine counterparts which makes both

cell types theoretically more effective in the induction of anaphylaxis (Bruhns and Jönsson, 2015; Finkelman et al., 2016; Nimmerjahn et al., 2015).

Several murine studies described that IgG-mediated anaphylaxis requires higher amounts of allergen compared to IgE-mediated anaphylaxis (Finkelman, 2007). In humans, exposure to high amounts of allergen may occur during parenteral administration of drugs. Thus, it has been discussed that an alternative, IgG-mediated pathway might be induced in cases of human drug induced anaphylaxis. This notion could be strongly supported for the first time by Jöhnsson and colleagues, who conducted a study with 86 patients with suspected anaphylaxis to neuromuscular-blocking agents (NMBA) during general anesthesia and 86 matched controls. They found that concentrations of anti-NMBA IgG, FcγR activation, PAF-release as well as activation of neutrophils correlated with the severity of the anaphylactic reaction and demonstrated that neutrophils underwent degranulation and NETosis early after anaphylaxis onset and that anti-NMBA IgG from the plasma of anaphylactic patients induced neutrophil activation *ex vivo* in the presence of NMBA. Furthermore, they observed neutrophil activation in patients without evidence of classical IgE-mediated anaphylaxis (Bruhns and Chollet-Martin, 2021; Jönsson et al., 2019). Furthermore, during AIT, increasing levels of allergen-specific IgG antibodies are induced by treatment with increasing amounts of allergen, thus, providing another setting where IgG-mediated allergic reactions might occur (Epp et al., 2018a; Kanagaratham et al., 2020; Shamji et al., 2021).

However, AIT is the only causal treatment option for patients suffering from severe allergic reactions today, that has the potential to provide long lasting disease improvement by modulating the immune response towards the allergen. Treatment success, thereby, has been associated with increasing amounts of allergen-specific IgG antibodies, especially of the IgG4 subclass. These antibodies have been demonstrated to inhibit IgE-mediated allergic reactions by allergen masking as well as FcγRIIB-dependent inhibition of effector cells which has been shown in several studies of murine IgE-mediated anaphylaxis. However, the induction of allergen-specific IgG4 antibodies during AIT cannot be considered a general biomarker for therapy success since the levels of allergen-specific IgG antibodies did not correlate with disease improvement in all patients. One explanantion for this phenomenon could be that allergen-specific IgG4 antibodies are very efficiently induced during AIT even when they recognize a different epitope of the allergen, compared to the allergen-specific IgE antibodies and thus fail to inhibit IgE-mediated reactions (Möbs et al., 2012; Strait, 2006; Zissler and Schmidt-Weber, 2020). However, based on the results obtained in our lab over the last years

about IgG-mediated anaphylaxis (Epp et al., 2018a; Petry et al., 2021a), which have been further supported by the results presented in this thesis, it is reasonable to hypothesize that the efficiency of AIT depends not only on the amounts and the affinity of allergen-specific IgG antibodies but also on their glycosylation pattern. Here, treatment of mice with IVIG as well as sialylated IgG1 antibodies induced an upregulation of the inhibitory FcγRIIB on basophils (Figure 12), one of the main drivers of IgE-mediated anaphylaxis. Indeed, I could demonstrate in a mouse model of IgG-controlled IgE-mediated anaphylaxis that the blocking properties of allergen-specific IgG antibodies could be further enhanced by sialylated allergen unspecific murine IgG1 antibodies, most likely due to an upregulation of FcγRIIB on effector cells, especially basophils. Accordingly, the modulation of cell activation by increased expression of the inhibitory FcγRIIB or reduced expression of activating FcγRs could be a novel approach to overcome limitations of therapeutic strategies in IgE-mediated anaphylaxis. Transferring the obtained results to a human system, the substitution of sialylated IgG antibodies during AIT would be desireable to induce an upregulation of FcγRIIB on effector cells to attenuate potential adverse allergic reactions mediated by IgE antibodies.

4.2 Immunomodulatory mechanisms in IgE-mediated anaphylaxis

Despite a growing body of evidence for an alternative IgG-mediated pathway of anaphylaxis, most anaphylactic reactions are mediated by allergen-specific IgE antibodies. In contrast to IgG, little is known about the role of IgE glycosylation in IgE-mediated anaphylaxis. Already 30 years ago Robertson and colleagues observed the differential recognition of IgE glycoforms by galectin-3 (former known as ε-binding protein) and described the existence of heterogenous IgE glycoforms in human serum varying in their sialylation level (Robertson and Liu, 1991). They further suggested potential differences in FcεRI-binding or mast cell activation depending on the IgE glycosylation pattern. Therefore, investigating the role of IgE glycosylation in the context of allergic disease might provide an answer to the ongoing questions why not all people develop allergies or even anaphylaxis in the presence of allergen specific IgE antibodies and why AIT is not successful in all patients undergoing this therapy.

To further clarify the role of IgE glycosylation in IgE-mediated anaphylaxis, I produced differently glycosylated anti-TNP IgE antibodies with preserved affinity for the allergen (Figure 15 & 16) and investigated their potential to bind the high affinity IgE Fc receptor FcεRI (Figure 17), considering that it is well described that IgG glycosylation modulates the antibody affinity for activating and inhibitory FcγRs. Thereby, an agalactosylated IgG Fc glycan favors a conformation that increases the binding capacity to (activating) high affinity FcγRs whereas terminal sialylation is correlated with an increased affinity for the low affinity inhibitory

receptor FcγRIIB (Nimmerjahn et al., 2015, 2007; Vidarsson et al., 2014b). Indeed, I could show that desialylated IgE antibodies have an increased affinity for FcεRI compared to sialylated IgE glycoforms (Figure 17). Thus, desialylation might lead to conformational changes in the IgE Fc that favor an open conformation which is associated with increased FcεRI binding rather than a closed conformation that favors binding to CD23 (Engeroff et al., 2020; Jabs et al., 2018).

The sensitization of effector cells upon first allergen-exposure is a key mechanism for the development of allergic reactions. Thus, IgE glycan-dependent sensitization of mast cells and basophils could dictate the severity of the allergic response, making IgE glycosylation a potential marker to predict the risk of developing anaphylaxis. In my studies, I demonstrated that desialylated IgE antibodies also show an increased potential to sensitize human mast cells (Figure 18 and suppl. Figure 30) and basophils (Figure 20) compared to sialylated IgE glycoforms. Interestingly, also differences in effector cell sensitization between native and highly sialylated IgE antibodies could be observed (Figure 18B & 18D, Figure 20B) suggesting that the IgE sialylation level might be a potential, possibly dynamic regulator of the severity of an allergic response. Given the increased binding capacity of desialylated IgE antibodies for FcεRI and and the increased potential to sensitize mast cells and basophils, also differences in effector cell activation can be assumed. Therefore, I measured the β-hexosaminidase release of LAD2 cells sensitized with different IgE glycoforms upon allergen stimulation. As anticipated, mast cells sensitized with desialylated IgE antibodies exhibited a higher degree of activation, compared to cells sensitized with native and highly sialylated IgE antibodies. Interestingly, also differences in mast cell activation between native and highly siyalylated IgE antibodies have been observed (Figure 19). Similar obervations have been made on human differentiated $CD34^+$ mast cells (suppl. Figure 30) in a cooperation project with the Fraunhofer ITEM (research group of Dr. Susann Dehmel) and on human blood basophils (Figure 20C) in a cooperation project with the Forschungszentrum Borstel (research group of Prof. Dr. Uta Jappe). This effect is most likely caused by the increased binding activity of desialylated IgE antibodies for FcεRI (Figure 17), but as mentioned above, also glycan-binding proteins might be involved in the sensitization and activation of effector cells in allergic diseases. For example the IgE-binding protein galectin-3 might contribute to the increased activation of cells sensitized with desialylated IgE glycoforms since galectin-3 is highly expressed on mast cells (Chen et al., 2006) and has been proven to be associated with IgE-mediated activation of basophils (Schroeder et al., 2019).

Discussion

Galectin-3, upon all galectins, is the only chimera-type lectin consisting of one carbohydrate-recognition domain (CRD) and a proline- and glycine-rich N-terminal domain that allows the formation of galectin-3 oligomers at high concentrations. Those oligomers can bind and crosslink IgE bound to FcεRI as well as FcεRI alone thus modulating IgE-mediated allergic reactions (Dumic et al., 2006; Liu, 2005). The role of galectin-3 in allergic responses is not fully understood yet, but many studies describe galectin-3 to be associated with proinflammatory effects in allergic inflammation. For example, Zuberi and colleagues reported a critical role for galectin-3 in a murine model of OVA-induced asthma. They demonstrated the expression of large amounts of galectin-3 in peribronchial inflammatory cells and significantly higher amounts of galectin-3 contained in the bronchoalveolar lavage fluid from OVA challenged mice compared to control mice. By comparing galectin-3 deficient mice with wild type animals they found that OVA-sensitized galectin-3 deficient mice developed fewer eosinophils and a significantly lower airway hyperresponsiveness after airway OVA challenge compared to similarly treated wild type mice. Furthermore, it has been demonstrated that galectin-3 deficient mice to develop a lower Th2 but an increased Th1 response, suggesting a regulatory role of galectin-3 in T cell responses (Gao et al., 2013; Zuberi et al., 2004). This finding makes galectin-3 also a promising target for novel AIT protocols since one goal of AIT is the restoration of immune tolerance by shifting T cell responses from Th2 to Th1/Treg immunity (Lee et al., 2022; Zissler and Schmidt-Weber, 2020). On the other hand, Bambuskova and colleagues reported galectin-3 to be a negative regulator of mast cell degranulation since FcεRI-activated cells with galectin-3 knockdown exhibited upregulated signal transduction and calcium response (Bambouskova et al., 2016). This study is contradicted by the finding of Chen and colleagues that galectin-3-deficient mast cells exhibit impaired mediator release and defective JNK expression (Chen et al., 2006). Altogether, there is evidence that galectin-3 is involved in both positive and negative regulation of allergic inflammation.

In my thesis, I confirmed the differential recognition of the IgE glycoforms by galectin-3 suggested by Robertson and Liu (Robertson and Liu, 1991), since galectin-3 strongly bound the desialylated IgE glycoforms, but there was little to no binding of sialylated IgE antibodies (Figure 27). Future experiments will clarify the role of soluble galectin-3 in the context of mast cell and basophil activation by differently glycosylated IgE antibodies.

In summary, with the data presented here, I confirmed the hypothesis of Robertson and Liu that the IgE glycosylation pattern affects FcεRI-binding and effector cell activation (Robertson and Liu, 1991). Considering the *in vitro* results, IgE glycosylation might have a dramatic impact on

the development and severity of anaphylactic reactions as well as it might function as a potential biomarker for the estimation of the probability to develop anaphylaxis. Furthermore, these data suggest that the role of IgE glycosylation in IgE-mediated anaphylaxis underlies the same principles that are described and widely accepted for IgG antibody glycosylation, namely that desialylated IgE antibodies can be associated with proinflammatory responses in allergic inflammation whereas sialylated IgE antibodies seem to act less or even anti-inflammatory. Nevertheless, most IgE antibodies have been described to be highly sialylated (Björklund et al., 1999; Plomp et al., 2014a). However, due to low serum IgE levels, data from IgE glycan analysis rely on studies which used myeloma IgE or samples from patients with hyper-IgE syndrome. It must be considered, that in these non-physiological conditions there can be an altered IgE glycosylation, e.g., extensive sialylation, which might not reflect the IgE glycosylation pattern under physiological conditions.

To transfer and confirm the obtained *in vitro* data about the role of IgE glycosylation in IgE-mediated allergic reactions into an *in vivo* system, I used a mouse model of IgE-mediated passive systemic anaphylaxis (Figure 21A). Interestingly, only mice sensitized with sialylated IgE antibodies experienced a profound anaphylactic reaction upon allergen challenge 24 hours after sensitization, whereas mice sensitized with desialylated IgE antibodies hardly developed anaphylaxis (Figure 21B). Possible explanations for this observation could be that there is either an unknown inhibitory mechanism mediated by desialylated IgE antibodies or cleavage of the sialic acid residues from the IgE antibody decreases the IgE serum half-life. Since the former would be constrastive to the presented *in vitro* data, I first investigated whether desialylation affects the IgE serum half-life. Indeed, my studies revealed that desialylated IgE antibodies could not be detected in the serum of sensitized mice already 6 hours after sensitization whereas the half-life of the sialylated IgE glycoforms was rather comparable (Figure 22B).

IgE is the least abundant antibody subclass in circulation, because it is highly bound by FcεRI on the surface of effector cells (Gould et al., 2003). Also desialylated IgE antibodies seem to have a higher potential to sensitize mast cells because of increased FcεRI binding (Figure 17), it is reasonable to believe that desialylated IgE antibodies could not be detected in the serum of sensitized mice because they are fully bound to the surface of mast cells and basophils. Therefore, I investigated the occupation of blood basophils of mice sensitized with differently glycosylated anti-TNP IgE antibodies by detecting the antibodies on the basophil surface using the allergenTNP-BSA 6 hours after sensitization (Figure 23). Only in mice that received sialylated anti-TNP IgE antibodies, TNP^+ basophils were detected 6 hours after IgE application,

whereas in mice that received desialylated IgE antibodies no TNP^+ basophils could be detected (Figure 23B). This observation further supports the hypothesis that IgE desialylation reduces the IgE serum half-life.

The removal of exogenously administered glycoproteins is controlled by the asialo glycoprotein-receptor (ASGP-R) in the liver (Ashwell and Morell, 2006; Grewal, 2010; Hudgin et al., 1974; Morell et al., 1971; van den Hamer et al., 1970). It was the first identified mammalian lectin and is part of the c-type lectin receptor family. The ASGP-R functions as a hepatocyte receptor for glycoproteins lacking terminal sialic acid on their glycan chains, therefore called asialo-glycoproteins (Ashwell and Harford, 1982; Ashwell and Kawasaki, 1978; Grewal, 2010), and its activitiy remains an important factor considered in the design of clinical treatments to assure therapeutic levels of glycoproteins in circulation (Drickamer, 1999; Grewal, 2010; Lohse et al., 2016; Stockert, 1995). Ligands of mammalian ASGP-Rs are a wide range of exogenously administered glycoproteins that carry terminal galactose or N-acetyllactosamine residues on their glycan chains leading to rapid capture and internalization, but also some sialylated glycoproteins have been demonstrated to be bound by the ASGP-R. Other lectins with asialo-glycoprotein binding activity are the kupffer-cell receptor, the macrophage galactose receptor and galectins, as mentioned above (Grewal, 2010; Park et al., 2005). The ASGP-R is localized at the vascular site of the hepatocyte cell surface where it removes potential deleterious glycoproteins from circulation (Ashwell and Harford, 1982; Wahrenbrock and Varki, 2006; Weigel, 1994). *In vitro* studies of ASGP-R complexes involved competitive blocking with an excess of asialo-glycoproteins to identify potential ligands of the ASGP-R and determine carbohydrate structure specifications (Grewal, 2010). Using sialic acid free preparations of asialo-ceruloplasmin with exposed terminal galactose residues the ASGP-R was first identified to be a galactose-binding receptor (Ashwell and Morell, 2006; van den Hamer et al., 1970). Other high-affinity ligands of the ASGP-R system are asialofetuin, which has a glycan pool of twothirds triantennary and onethird biantennary glycan structures at three glycosylation sites (Ellies et al., 2002; Morelle et al., 2009; Yet et al., 1988) and asialo-α1-acid glycoprotein (AGP), a glycoprotein with five tri- and tetra-antennary *N*-linked glycans (Baenziger and Fiete, 1979; Bider et al., 1995; Matsumoto et al., 2010; Nakano, 2004; Yik et al., 2002). The use of these blocking agents in competitive binding studies revealed changes in the activity or half-lifes of glycoproteins of the blood coagulation cascade (Ellies et al., 2002; Rotundo et al., 1998; Smedsrød and Einarsson, 1990; Vostal and McCauley, 1991) and in half-lifes of γ-glutamyltransferase , alkaline phosphatase and immunoglobulins (Blom et al., 1998; Mortensen and Huseby, 1997; Rifai et al., 2000; Thornburg et al., 1980; Tomana et al., 1985).

α-2,3-linked sialic acid can mask the underlying ASGP-R ligands on glycoproteins, hence reducing the expression of sialic acid linkages *in vivo* as well as neuraminidase treatment of exogenously administered glycoproteins might unmask ligand identity and thereby facilitate investigations of ASGP-R function in biology and disease (Grewal, 2010).

For example, Ellies and colleagues demonstrated that when the St3Gal-IV sialyltransferase is limiting or absent, ASGP-R ligands appear on a subset of regulatory and prothrombotic components of the mammalian blood coagulation cascade including platelets and von-Willebrandt factor. Furthermore, these proteins are subject to clearance during pathologic conditions of rapid desialylation such as bloodstream infection with *Streptococcus pneumoniae* by NanA neuraminidase activity expressed by the bacterium (Ellies et al., 2002; Grewal et al., 2008). Another study by Rifai and colleagues addressed the differential clearance of the two IgA antibody isotypes (IgA1 and IgA2) from the circulation by the ASGP-R. They demonstrated that the major pathway of IgA clearance is through ASGP-R uptake in the liver since it could be blocked by excess of the artificial ASGP-R ligand galactose-ficoll in wild type and suppressed in ASGP-R-deficient mice. Furthermore, they showed that altered *N*- but not *O*-glycosylation affects recognition by the ASGP-R. In contrast to IgG antibodies, where the *N*-glycan is buried in the Fc-part, IgA *N*-glycans are exposed and available for ASGP-R recognition. Interestingly, only a small percentage of IgA1 was cleared by this pathway which could be attributed to the altered *N*-glycosylation compared to IgA2. IgA1 is highly sialylated which leads to a slow removal by the ASGP-R and the microheterogenity of the carbohydrate structure might explain why only a small percentage of IgA1 is rapidly cleared by the ASGP-R. In addition to a lower level of sialylation IgA2 also has additional *N*-linked glycans which increase the avidity of IgA2 to the ASGP-R (Rifai et al., 2000). In a study addressing IgA nephropathy, it could be demonstrated that disease recurrence can be predicted by altered IgA glycosylation. Decreased amounts of serum galactose deficient IgA1 could be observed which does not underly ASGP-R clearance. The lack of galactose rather facilitates self-polymerization of the IgA1 antibodies which leads to increased pathologic IgA deposits in the kidney (Berthelot et al., 2015).

Since my obtained *in vivo* data on IgE glycosylation in systemic anaphylaxis (Figures 21-23) suggest a reduced half-life of desialylated IgE antibodies and the evidence from other studies that asialo-glycoproteins, including immunoglobulins, are removed from the serum by the hepatic ASGP-R, it is reasonable to belive that desialylated IgE antibodies in circulation underlie the same clearance mechanism. Furthermore, as described for IgA, the *N*-glycans of

IgE antibodies are exposed (Jabs et al., 2018) which potentially increases the recognition by the ASGP-R in case the glycans carry terminal galactose residues. To prove this hypothesis, I established an *in vivo* mouse model were the ASGP-R is blocked with an excess of the ligand asialo-AGP (Grewal, 2010; Matsumoto et al., 2010) (Figure 24A). As anticipated, upon ASGP-R blocking also desialyated IgE antibodies could be detected in the serum of sensitized mice at comparable levels to the sialylated IgE glycoforms 6 hours after sensitization (Figure 24B). The obtained *in vitro* results suggested a higher capacity of desialylated IgE glycoforms to bind FcεRI and activate effector cells compared to sialylated IgE glycoforms (Figures 17-20). Therefore, I investigated the loading of blood basophils with desialylated IgE antibodies compared to highly sialylated IgE antibodies upon ASGP-R blocking 6 hours after sensitization (Figure 25). As expected, also desialylated IgE antibodies were able to sensitize basophils, when the ASGP-R is blocked, but the loading was rather comparable to basophils sensitized with highly sialylated IgE antibodies (Figure 25B). An explanation of this observation might be that in this setting there is an excess of IgE antibodies so the basophil loading is saturated and therefore, no differences between the IgE glycoforms could be observed. In future experiments the ideal amount of IgE to sensitize the mice will be titrated to check, whether desialylated IgE glycoforms also have an increased potential to bind FcεRI *in vivo* when ASGP-R clearance is prevented. Also, glycan-binding proteins such as galectins must be considered as potential regulators of effector cell sensitization with IgE antibodies in an *in vivo* model. Hence, it would be interesting to compare serum half-life and basophil loading of different IgE glycoforms for example in galectin-3-deficient mice compared to wild type animals upon ASGP-R blocking.

Given that ASGP-R blocking with asialo-AGP prolongs the half-life of desialylated IgE antibodies it is not surprising that under these conditions desialylated IgE antibodies also induce systemic anaphylaxis in mice 6 hours after sensitization (Figure 26). However, in this setting the reaction was rather comparable to the reaction induced by highly sialylated IgE antibodies despite the increased anaphylactic potential of desialylated IgE antibodies *in vitro* (Figures 17-20). This observation was expected considering the comparable loading of blood basophils upon ASGP-R blocking (Figure 25B).

It must be considered in this context that the *in vivo* model of IgE-mediated passive systemic anaphylaxis might not be ideal to resolve the differences in effector cell sensitization and activation mediated by sialylated and desialylated IgE antibodies that were observed in the presented *in vitro* experiments, since anaphylaxis is the strongest possible form of an allergic

reaction accompanied by systemic inflammation and effector cell activation. Thus, delicate differences in effector cell activation mediated by differently glycosylated IgE antibodies might be masked since the stimulus in this model is so strong that the threshold of maximized systemic effector cell activation is reached irrespective of the glycosylation pattern of the IgE antibodies used for sensitization. For future experiments alternative mouse models of IgE-mediated allergy that for example focus on a distinct organ, e.g. a murine model of passively induced asthma, in combination with ASGP-R blocking to overcome differences in IgE half-life due to differences in the glycosylation pattern, or consider more strongly different genetic and environmental factors that influence the inflammatory immune status and/or the expression and release of potentially important carbohydrate-binding proteins (Duan et al., 2019; Gao et al., 2013; Shade et al., 2015) should be considered to further investigate the potential of differently glycosylated IgE antibodies *in vivo*.

Altogether, the presented results regarding the role of IgE glycosylation in allergic disease suggest that desialylated IgE antibodies have a higher anaphylactic potential compared to sialylated IgE antibodies which is counteracted *in vivo* by a dramatically reduced half-life. The latter could be attributed to a rapid clearance from the serum by the hepatic ASGP-R. This findings are in line with several studies describing that on the one hand, a lack of sialic acid is associated with increased effector functions and pathogenicity of IgG, IgA and IgM antibodies (Anthony et al., 2011; Bartsch et al., 2020; Colucci et al., 2015; Kaneko et al., 2006; Petry et al., 2021a; Steffen et al., 2020; Xie et al., 2020) and on the other hand, studies addressing the clearance of asialo-glycoproteins, including immunoglobulins, by the ASGP-R system (Ellies et al., 2002; Grewal et al., 2008; Rifai et al., 2000). Furthermore, my conducted experiments provide an explanation why the majority of the IgE antibodies in circulation carry terminal sialic acids (Björklund et al., 1999; Plomp et al., 2014b) since I could show that sialic acid protects the IgE antibodies from clearance through the ASGP-R pathway.

It must be noted that the results obtained in this study are contrary to a recent study published by Shade and colleagues where it is suggested that IgE sialylation is a potential regulator of allergic disease (Shade et al., 2020b). By comparing the total IgE glycosylation of patients allergic to peanut to a group of non-atopic patients they found higher levels of IgE sialylation in allergic individuals and suggested that differences in total IgE glycosylation are predictive of allergic disease (Shade et al., 2020b). However, the cohort used for total IgE glycan analysis was not sex- and age-matched since in the allergic group only 5 out of 21 patients were male whereas in the non-atopic group only 5 out of 18 patients were female. Furthermore, the average

age in allergic individuals was 25.47 years with the oldest member being 52 compared to 36.44 years in the non-atopic group with the oldest member being 69. As described in the introduction of this thesis, the total antibody glycosylation varies between men and woman and during life with woman generally having higher total levels of sialylated antibodies than men and a decrease of antibody sialylation with increasing age (Dall'Olio et al., 2013; Ercan, 2020; Ercan et al., 2017). Therefore, the cohort chosen in this study might not be ideal to predict the risk of developing allergic disease based on the total IgE glycosylation measured in the two groups. Using mouse models of passive systemic (PSA) and passive cutaneous anaphylaxis (PCA) Shade and colleagues made the same observations I presented in this thesis, namely that anaphylaxis is attenuated in mice sensitized with desialylated IgE. However, they concluded that there must be an inhibitory effect of the desialylated IgE glycan since they did not observe differences in the half-life of sialylated and desialylated IgE antibodies. Here, it should be mentioned that in experiments investigating the half-life of the IgE antibodies mice were injected intraperitoneally whereas e.g., the PSA model is based on intravenous antibody injection. These differences in application routes might have led to an underestimation of the half-life effect described in my thesis. However, Shade et al. reported decreased effector cell activation mediated by galactose residues of IgE including experiments with a fusion protein with neuraminidase activity which cleaves sialic acid from cell-bound IgE thereby attenuating cell activation which conflicts with the data presented in this thesis, suggesting an increased mast cell activation after sensitization with desialylated IgE antibodies (Shade et al., 2020b).

Taken together, my studies further contribute to the understanding of the role of IgE glycans in allergic disease. They are in line with several publications describing the modulation of effector functions of IgG, IgA and IgM antibodies by their glycosylation pattern. Furthermore, I could propose a pathway for the regulation of the serum half-life of differently glycosylated IgE antibodies that explains the high degree of sialylation of circulating IgE antibodies. However, my results are conflicting with a recent study from Shade and colleagues. Differences can be in part explained by the choice of the cohort for IgE glycan analysis in this study and the decreased serum half-life of desialylated IgE antibodies which might have been underestimated.

In summary, it is safe to conclude that IgE glycosylation plays an important role in the development and severity of allergic reactions and might as well function as a potential biomarker for the prediction of the risk of developing IgE-mediated anaphylaxis. Transferring the obtained results into a human system, the potential induction of IgE antibodies with distinct glycosylation profiles should be considered in the development of novel AIT protocols in

addition to the suggestions made in section 4.1 regarding the induction of allergen-specific IgG antibodies during AIT. Hence, for future AIT therapies, on the one hand, it would be desireable to induce sialylated allergen-specific IgG(4) antibodies or supplement the therapy with IVIG or sialylated allergen-unspecific IgG(4) antibodies to induce an upregulation of the inhibitory FcγRIIB on effector cells, but on the other hand, for IgE antibodies, a desialylated glycosylation pattern would be favourable, since desialylated IgE antibodies would be rapidly cleared from the serum by the ASGP-R, preventing effector cell sensitization an thus allergic reactions upon allergen exposure.

Allergen extracts used for AIT are supplemented with adjuvants that shape the efficacy and type of immune response to the injected allergen. Only 4 adjuvants are used in currently marketed AIT products: aluminium hydroxide (alum), calcium phosphate, microcrystalline tyrosine (MCT) and the Th1 adjuvant monophosphoryl lipid A (MPLA). The first 3 adjuvants are delivery systems with a depot effect but may also have immunomodulatory properties. These first-generation adjuvants are still widely used, especially alum, despite known limitations such as an increased IgE response and induction of unwanted Th2 immunity. MCT is the depot formulation of L-tyrosine which enhances IgG production without inducing a significant IgE response, is biodegradable and has a high local and systemic tolerability. MPLA is an immunomodulatory agent and the only second-generation adjuvant used for AIT these days. Furthermore, multiple adjuvants are currently being studied, including squalene-water-emulsions (SWE), immunomodulatory sequences (ISS), nanoparticles (liposomes, virus-like particles and biodegradable polymers), phosphatidylserine derivates, Advax and montanide (ISA-51) (Jensen-Jarolim et al., 2021; Tabynov et al., 2022; Zubeldia et al., 2019). In a mouse model of wormwood (Artemisia) pollen allergy Tabynov and colleagues used recombinant wormwood allergen Art v 1 formulated with either newer adjuvants (Advax, Advax supplemented with CpG-Oligodexoxynucelotides as a TLR9-agonist (Advax-CpG) or montanide) or traditional adjuvants (alum, SWE). It could be demonstrated, as described before, that aluminium-based adjuvants are less favorable for AIT because of an induction of Th2 immunity as well as exacerbation of IgE antibodies and eosinophilia which increases the risk of adverse effects during therapy. However, promising results were obtained using Advax, Advax-CpG, SWE and montanide since these adjuvants induced a decrease in total and allergen-specific IgE antibodies as well as an increase in IgG isotypes and a shift in the cytokine profile towards Th1 immunity (Advax, Advax-CpG, montanide) or a balanced Th1/Th2 response (SWE). A key goal of allergy research is the identification of biomarkers/correlates of protection that could be used to predict AIT effectiveness. Interestingly, the clinical protection

of mice from wormwood pollen allergy correlated best with levels of total serum IgE rather than allergen-specific IgE (Tabynov et al., 2022).

It is known that the selected adjuvants for vaccination have a strong influence on the IgG antibody glycosylation pattern. Adjuvants providing a strong pro-inflammatory stimulus are associated with the induction of IgG antibodies with low galactosylation and sialylation levels, whereas more tolerogenic conditions favored the production of galactosylated and sialylated IgG antibodies (Bartsch et al., 2020; Oefner et al., 2012). In a previous study in our lab the impact of eCFA (enriched complete Freund adjuvant) providing a strong inflammatory stimulus was compared with alum and MPLA on IgG subclass distribution and Fc glycosylation profiles in OVA-immunized mice. Alum and MPLA induced high levels of total as well as subclass-specific IgG antibody sialylation compared to eCFA that facilitated protection in IgG- as well as IgG-controlled IgE-mediated anaphylaxis (Epp et al., 2018b; Petry et al., 2021b).

Thus, as indicated before, the induction of sialylated IgG antibodies during AIT protocols seems desireable. Furthermore, the observation that IVIG and as well as sialylated murine IgG antibodies induce an upregulation of the inhibitory FcγRIIB on basophils (Figure 12) might contribute to the enhanced blocking property of IgG1 antibodies in murine IgE-mediated anaphylaxis (Figure 14) and provides an explanation for the effective treatment of drug-induced hypersensitivity by IVIG observed in earlier studies (Kito et al., 2012; Scheuerman et al., 2001).

However, little is known about the induction of allergen-specific IgE antibodies during AIT, especially regarding their glycosylation pattern. Therefore, in a preliminary approach, I compared the total IgE induction in mice immunized with OVA and the proinflammatory adjuvant eCFA to the more tolerogenic adjuvant alum in an IgE-sandwich ELISA (Figure 28). Thereby, I could confirm the observation made by other groups that immunization with alum induced an unwanted IgE response and further that this response is even elevated using stronger adjuvants such as eCFA. Moreover, in control mice without immunization no IgE could be detected suggesting that the induced IgE antibodies upon immunization are probably OVA-specific. It would be interesting whether the glycosylation profile of these IgE antibodies follows the same pattern described for IgG antibody glycosylation upon immunization with different adjuvants (Bartsch et al., 2020; Epp et al., 2018b). Assuming this, an immunization/ AIT with an allergen formulated with alum would induce mostly galactosylated and sialylated IgE antibodies, whereas formulation of the allergen with strong adjuvants such as eCFA would induce predominantly low sialylated IgE antibodies. Considering the results about the IgE serum half-life, a formulation of the allergen with strong proinflammatory adjuvants would be

favorable here since despite increased effector functions these antibodies would be rapidly cleared from circulation through the hepatic ASGP-R pathway. Furthermore, low sialylation programming of IgG antibodies upon vaccination with proinflammatory cytokines such as eCFA was associated with Th1 immunity since it was shown to be dependent on IFNγ^+ T_{FH1} and IL-17A$^+$ T_{FH17} cells (Bartsch et al., 2020) which could contribute to the desired shift in cytokine profile from the pro-allergic Th2 milieu to Th1/Treg immunity during AIT.

Allergen-specific IgE glycosylation analysis is still a matter of research and not yet established. The greatest challenge here is to purifiy sufficient amounts of IgE for further analysis since the levels of allergen-specific IgE in circulation are very low. Hence, studies investigating the role of IgE glycosylation are still based on total IgE glycan analysis. Another promising approach for future analysis could be the use of lectin-based assays for IgE glycan analysis. In a study addressing the immunopathology in COVID-19, Ankerhold and colleagues observed an increased afucosylation of SARS-CoV-2 specific IgG antibodies which leads to increased FcγRIII-dependent activation of immune cells using a lectin-based ELISA with the fucose-recognizing *Aleuria aurantia* lectin (Ankerhold et al., 2021). In the context of IgE glycosylation in allergic disease, IgE sialylation could be addressed in a similar setting using the SNA-lectin, introduced in this thesis in the context of the production of differently glycosylated IgE antibodies, to investigate whether glycosylation pattern of IgE antibodies induced in AIT depends on the adjuvant formulated with the allergen.

4.3 Summary and Outlook

In this thesis, I addressed the immunomodulatory mechanisms of antibody glycosylation in the context of IgG- as well as IgE-mediated anaphylaxis. The anti-inflammatory effects of sialylated IgG antibodies in IgG-mediated and IgG-controlled IgE-mediated anaphylaxis have been acknowledged. Anti-inflammatory properties of IVIG, mediated by its sialylated subfraction (Anthony et al., 2011, 2008) could also be demonstrated using sialylated allergen-unspecific human IgG4 antibodies (Figure 10). Future research should clarify whether IVIG therapy could be substituted e.g., by therapeutic monoclonal sialylated human IgG4 antibodies to treat allergic-, autoimmune or inflammatory diseases since the studies in our lab, including the results presented in this thesis, showed that lower amounts of sialylated monoclonal IgG antibodies mediate the same protective effects as high amounts of IVIG. Furthermore, there are economic advantages since therapeutic monoclonal antibodies can be produced in high-troughput formats in cell culture systems. Moreover, in contrast to IVIG that consists of pooled

serum IgG from healthy donors, the production of monoclonal therapeutic antibodies is donor-independent with less batch-specific differences e.g., due to donor changes.

The underlying mechanism of the protective effects of sialylated IgG antibodies in IgG-mediated anaphylaxis is the SIGN-R1-dependent upregulation of FcγRIIB on monocytes/ macrophages (Petry et al., 2021b). During my thesis I could extend this finding by demonstrating that FcγRIIB is also upregulated on murine blood basophils after treatment with IVIG or sialylated murine IgG1 antibodies (Figure 12). Furthermore, in a pilot study, total IgG sialylation has been correlated with an upregulation of FcγRIIB on human basophils and neutrophils in our laboratory (Petry et al., 2021b) which is a possible explanation for the increased protective effect of allergen-specific IgG antibodies in IgE-mediated anaphylaxis. These findings are useful considering the ongoing need for further AIT improvements. For example, supplementation of AIT therapy with IVIG, sialylated allergen-unspecific IgG antibodies or even sialylated IgG Fc-parts without antigen-binding activity might elevate the inhibitory potential of allergen-specific IgG antibodies induced during AIT through enhanced FcγRIIB-mediated effector cell inhibition.

My work also contributed to the understanding of the role of IgE glycosylation in allergic disease. Enhanced allergic potential of desialylated IgE glycoforms could be demonstrated *in vitro* and attributed to increased FcεRI-binding and thus effector cell activation (Figures 17-20, suppl. Figure 30) which is in line with studies addressing the role of glycosylation affecting the effector functions of IgG, IgA and IgM antibodies (Bartsch et al., 2020; Colucci et al., 2015; Epp et al., 2018b; Petry et al., 2021b; Steffen et al., 2020). It could be demonstrated that these pro-anaphylactic properties are counteracted *in vivo* by a rather short serum half-life of desialylated IgE antibodies because of rapid clearance through the ASGP-R pathway (Figures 21-26). These results are conflicting to a recent study published by Shade and colleagues who concluded that IgE sialylation is the main modulator of IgE-mediated allergic reactions (Shade et al., 2020b). However, the results obtained in this study are in part questionable because of a non-ideal cohort for human total IgE glycan analysis. Gender as well as age have to be considered in this context because it has been demonstrated that both factors affect the total antibody glycosylation pattern (Dall'Olio et al., 2013; Ercan, 2020; Ercan et al., 2017). In addition, the differences in the serum half-life of distinct IgE glycoforms might have been underestimated here. Nevertheless, conflicting *in vitro* results between this study and the results presented in my thesis cannot be explained so far. Differences in experimental settings, antibody clones or *in vitro* glycosylation protocols might affect the outcome of the experiments.

Thus, the role of IgE-glycosylation in allergic disease is not fully understood and experimental procedures addressing human IgE glycan analysis are still limited to measurements of alterations in total IgE glycosylation. Therefore, establishing a method for allergen-specific IgE glycosylation analysis would be an important asset in clarifying the role of the IgE glycans in allergic disease in the future.

However, fundamental progress has been made during the last years in this area of research with the results obtained in my thesis contributing to the ongoing discussion about the function of the IgE glycans in IgE-mediated allergic reactions. Furthermore, a potential regulatory role of the IgE-binding protein galectin-3 has been suggested, since galectin-3 was found only to bind desialylated IgE antibodies ((Robertson and Liu, 1991), Figure 27). The modulation of allergic responses by galectins or other glycan-binding proteins in allergic disease with respect to the differential binding of IgE glycoforms by these proteins should be subject of future research. There is evidence that galectin-3 has a more proinflammatory role in asthma and atopic dermatitis (Gao et al., 2013; Pasmatzi et al., 2019) and anti-inflammatory properties have been attributed to galectin-9 in mouse models of food allergy as well as atopic dermatitis and systemic anaphylaxis (Kim et al., 2020; Niki et al., 2009; Sziksz et al., 2012). Nevertheless, to my knowledge, there are no studies that correlated these effects with the IgE glycosylation pattern. Furthermore, there are no studies addressing the consequences of the differential abundance of IgE antibodies with varying glycosylation profiles which could be induced for example during the differential programming of B cells in the germinal center reaction. Here, the regulation of the sialyltransferase (SialT) in IgG-producing B cells depends on the inflammatory stimulus upon immunization with different adjuvants (Bartsch et al., 2020). It is reasonable to believe that the programming of IgE^+ B cells underlies the same regulation and should be considered in the development of next generation AIT vaccines. Hence, monitoring the SialT regulation in IgE-producing B cells during novel AIT approaches could be the key to develop improved therapies to prevent life-threatening adverse reactions, such as anaphylaxis, during therapy and contribute to the cure of allergic disease.

A References

Ackerman, M.E., Crispin, M., Yu, X., Baruah, K., Boesch, A.W., Harvey, D.J., Dugast, A.-S., Heizen, E.L., Ercan, A., Choi, I., Streeck, H., Nigrovic, P.A., Bailey-Kellogg, C., Scanlan, C., Alter, G., 2013. Natural variation in Fc glycosylation of HIV-specific antibodies impacts antiviral activity. J. Clin. Invest. 123, 2183–2192. https://doi.org/10.1172/JCI65708

Akarsu, A., Brindisi, G., Fiocchi, A., Zicari, A.M., Arasi, S., 2022. Oral Immunotherapy in Food Allergy: A Critical Pediatric Perspective. Front. Pediatr. 10, 842196. https://doi.org/10.3389/fped.2022.842196

Allhorn, M., Olin, A.I., Nimmerjahn, F., Collin, M., 2008. Human IgG/FcγR Interactions Are Modulated by Streptococcal IgG Glycan Hydrolysis. PLoS ONE 3, e1413. https://doi.org/10.1371/journal.pone.0001413

Alter, G., Ottenhoff, T.H.M., Joosten, S.A., 2018. Antibody glycosylation in inflammation, disease and vaccination. Semin. Immunol. 39, 102–110. https://doi.org/10.1016/j.smim.2018.05.003

Ankerhold, J., Giese, S., Kolb, P., Maul-Pavicic, A., Voll, R.E., Göppert, N., Ciminski, K., Kreutz, C., Lother, A., Salzer, U., Bildl, W., Welsink, T., Morgenthaler, N.G., Grawitz, A.B., Huzly, D., Schwemmle, M., Hengel, H., Falcone, V., 2021. Circulating multimeric immune complexes drive immunopathology in COVID-19 (preprint). Immunology. https://doi.org/10.1101/2021.06.25.449893

Anthony, R.M., Kobayashi, T., Wermeling, F., Ravetch, J.V., 2011. Intravenous gammaglobulin suppresses inflammation through a novel TH2 pathway. Nature 475, 110–113. https://doi.org/10.1038/nature10134

Anthony, R.M., Wermeling, F., Karlsson, M.C.I., Ravetch, J.V., 2008. Identification of a receptor required for the anti-inflammatory activity of IVIG. Proc. Natl. Acad. Sci. 105, 19571–19578. https://doi.org/10.1073/pnas.0810163105

Arnold, J.N., Radcliffe, C.M., Wormald, M.R., Royle, L., Harvey, D.J., Crispin, M., Dwek, R.A., Sim, R.B., Rudd, P.M., 2004a. The Glycosylation of Human Serum IgD and IgE and the Accessibility of Identified Oligomannose Structures for Interaction with Mannan-Binding Lectin. J. Immunol. 173, 6831–6840. https://doi.org/10.4049/jimmunol.173.11.6831

Arnold, J.N., Radcliffe, C.M., Wormald, M.R., Royle, L., Harvey, D.J., Crispin, M., Dwek, R.A., Sim, R.B., Rudd, P.M., 2004b. The Glycosylation of Human Serum IgD and IgE and the Accessibility of Identified Oligomannose Structures for Interaction with Mannan-Binding Lectin. J. Immunol. 173, 6831–6840. https://doi.org/10.4049/jimmunol.173.11.6831

Ashwell, G., Harford, J., 1982. Carbohydrate-Specific Receptors of the Liver. Annu. Rev. Biochem. 51, 531–554. https://doi.org/10.1146/annurev.bi.51.070182.002531

Ashwell, G., Kawasaki, T., 1978. [27] A protein from mammalian liver that specifically binds galactose-terminated glycoproteins, in: Methods in Enzymology. Elsevier, pp. 287–288. https://doi.org/10.1016/0076-6879(78)50029-3

Ashwell, G., Morell, A.G., 2006. The Role of Surface Carbohydrates in the Hepatic Recognition and Transport of Circulating Glycoproteins, in: Meister, A. (Ed.), Advances in Enzymology - and Related Areas of Molecular Biology. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 99–128. https://doi.org/10.1002/9780470122860.ch3

Averbeck, M., Gebhardt, C., Emmrich, F., Treudler, R., Simon, J.C., 2007. CME. Immunologic

A References

Principles of Allergic Disease. JDDG 5, 1015–1027. https://doi.org/10.1111/j.1610-0387.2007.06538.x

Baenziger, J., Kornfeld, S., Kochwa, S., 1974. Structure of the Carbohydrate Units of IgE Immunoglobulin. J. Biol. Chem. 249, 1897–1903. https://doi.org/10.1016/S0021-9258(19)42870-6

Baenziger, J.U., Fiete, D., 1979. Structure of the complex oligosaccharides of fetuin. J. Biol. Chem. 254, 789–795. https://doi.org/10.1016/S0021-9258(17)37874-2

Bambouskova, M., Polakovicova, I., Halova, I., Goel, G., Draberova, L., Bugajev, V., Doan, A., Utekal, P., Gardet, A., Xavier, R.J., Draber, P., 2016. New Regulatory Roles of Galectin-3 in High-Affinity IgE Receptor Signaling. Mol. Cell. Biol. 36, 1366–1382. https://doi.org/10.1128/MCB.00064-16

Bartsch, Y.C., 2019. The role of IL-6 in vaccine-induced IgG Fc glycosylation. University of Lübeck.

Bartsch, Y.C., Eschweiler, S., Leliavski, A., Lunding, H.B., Wagt, S., Petry, J., Lilienthal, G.-M., Rahmöller, J., de Haan, N., Hölscher, A., Erapaneedi, R., Giannou, A.D., Aly, L., Sato, R., de Neef, L.A., Winkler, A., Braumann, D., Hobusch, J., Kuhnigk, K., Krémer, V., Steinhaus, M., Blanchard, V., Gemoll, T., Habermann, J.K., Collin, M., Salinas, G., Manz, R.A., Fukuyama, H., Korn, T., Waisman, A., Yogev, N., Huber, S., Rabe, B., Rose-John, S., Busch, H., Berberich-Siebelt, F., Hölscher, C., Wuhrer, M., Ehlers, M., 2020. IgG Fc sialylation is regulated during the germinal center reaction following immunization with different adjuvants. J. Allergy Clin. Immunol. 146, 652-666.e11. https://doi.org/10.1016/j.jaci.2020.04.059

Behrends, J., Schwager, C., Hein, M., Scholzen, T., Kull, S., Jappe, U., 2021. Innovative robust basophil activation test using a novel gating strategy reliably diagnosing allergy with full automation. Allergy 76, 3776–3788. https://doi.org/10.1111/all.14900

Berthelot, L., Robert, T., Vuiblet, V., Tabary, T., Braconnier, A., Dramé, M., Toupance, O., Rieu, P., Monteiro, R.C., Touré, F., 2015. Recurrent IgA nephropathy is predicted by altered glycosylated IgA, autoantibodies and soluble CD89 complexes. Kidney Int. 88, 815–822. https://doi.org/10.1038/ki.2015.158

Beutier, H., Gillis, C.M., Iannascoli, B., Godon, O., England, P., Sibilano, R., Reber, L.L., Galli, S.J., Cragg, M.S., Van Rooijen, N., Mancardi, D.A., Bruhns, P., Jönsson, F., 2017. IgG subclasses determine pathways of anaphylaxis in mice. J. Allergy Clin. Immunol. 139, 269-280.e7. https://doi.org/10.1016/j.jaci.2016.03.028

Bider, M.D., Cescato, R., Jeno, P., Spiess, M., 1995. High-Affinity Ligand Binding to Subunit HI of the Asialoglycoprotein Receptor in the Absence of Subunit H2. Eur. J. Biochem. 230, 207–212. https://doi.org/10.1111/j.1432-1033.1995.0207i.x

Björklund, J.E.M., Karlsson, T., Magnusson, C.G.M., 1999. N-glycosylation influences epitope expression and receptor binding structures in human IgE. Mol. Immunol. 36, 213–221. https://doi.org/10.1016/S0161-5890(99)00036-X

Blom, E., Mohamoud Ali, M., Mortensen, B., Huseby, N.-E., 1998. Elimination of alkaline phosphatases from circulation by the galactose receptor. Different isoforms are cleared at various rates. Clin. Chim. Acta 270, 125–137. https://doi.org/10.1016/S0009-8981(97)00217-9

Bruhns, P., 2012. Properties of mouse and human IgG receptors and their contribution to disease models. Blood 119, 5640–5649. https://doi.org/10.1182/blood-2012-01-380121

Bruhns, P., Chollet-Martin, S., 2021. Mechanisms of human drug-induced anaphylaxis. J. Allergy Clin. Immunol. 147, 1133–1142. https://doi.org/10.1016/j.jaci.2021.02.013

A References

Bruhns, P., Jönsson, F., 2015. Mouse and human FcR effector functions. Immunol. Rev. 268, 25–51. https://doi.org/10.1111/imr.12350

Chen, H.-Y., Sharma, B.B., Yu, L., Zuberi, R., Weng, I.-C., Kawakami, Y., Kawakami, T., Hsu, D.K., Liu, F.-T., 2006. Role of Galectin-3 in Mast Cell Functions: Galectin-3-Deficient Mast Cells Exhibit Impaired Mediator Release and Defective JNK Expression. J. Immunol. 177, 4991–4997. https://doi.org/10.4049/jimmunol.177.8.4991

Collin, M., 2001. EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG. EMBO J. 20, 3046–3055. https://doi.org/10.1093/emboj/20.12.3046

Collin, M., Ehlers, M., 2013. The carbohydrate switch between pathogenic and immunosuppressive antigen-specific antibodies. Exp. Dermatol. 22, 511–514. https://doi.org/10.1111/exd.12171

Colucci, M., Stöckmann, H., Butera, A., Masotti, A., Baldassarre, A., Giorda, E., Petrini, S., Rudd, P.M., Sitia, R., Emma, F., Vivarelli, M., 2015. Sialylation of N-Linked Glycans Influences the Immunomodulatory Effects of IgM on T Cells. J. Immunol. 194, 151–157. https://doi.org/10.4049/jimmunol.1402025

Dall'Olio, F., Vanhooren, V., Chen, C.C., Slagboom, P.E., Wuhrer, M., Franceschi, C., 2013. N-glycomic biomarkers of biological aging and longevity: A link with inflammaging. Ageing Res. Rev. 12, 685–698. https://doi.org/10.1016/j.arr.2012.02.002

Decker, Y., Schomburg, R., Németh, E., Vitkin, A., Fousse, M., Liu, Y., Fassbender, K., 2016. Abnormal galactosylation of immunoglobulin G in cerebrospinal fluid of multiple sclerosis patients. Mult. Scler. J. 22, 1794–1803. https://doi.org/10.1177/1352458516631036

Dekkers, G., Rispens, T., Vidarsson, G., 2018. Novel Concepts of Altered Immunoglobulin G Galactosylation in Autoimmune Diseases. Front. Immunol. 9, 553. https://doi.org/10.3389/fimmu.2018.00553

Dispenza, M.C., 2019. Classification of hypersensitivity reactions. Allergy Asthma Proc. 40, 470–473. https://doi.org/10.2500/aap.2019.40.4274

Dombrowicz, D., Flamand, V., Brigman, K.K., Koller, B.H., Kinet, J.-P., 1993. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor α chain gene. Cell 75, 969–976. https://doi.org/10.1016/0092-8674(93)90540-7

Drickamer, K., 1999. C-type lectin-like domains. Curr. Opin. Struct. Biol. 9, 585–590. https://doi.org/10.1016/S0959-440X(99)00009-3

Duan, S., Koziol-White, C.J., Jester, W.F., Nycholat, C.M., Macauley, M.S., Panettieri, R.A., Paulson, J.C., 2019. CD33 recruitment inhibits IgE-mediated anaphylaxis and desensitizes mast cells to allergen. J. Clin. Invest. 129, 1387–1401. https://doi.org/10.1172/JCI125456

Dumic, J., Dabelic, S., Flögel, M., 2006. Galectin-3: An open-ended story. Biochim. Biophys. Acta BBA - Gen. Subj. 1760, 616–635. https://doi.org/10.1016/j.bbagen.2005.12.020

Ellies, L.G., Ditto, D., Levy, G.G., Wahrenbrock, M., Ginsburg, D., Varki, A., Le, D.T., Marth, J.D., 2002. Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. Proc. Natl. Acad. Sci. 99, 10042–10047. https://doi.org/10.1073/pnas.142005099

Engeroff, P., Caviezel, F., Mueller, D., Thoms, F., Bachmann, M.F., Vogel, M., 2020. CD23 provides a noninflammatory pathway for IgE-allergen complexes. J. Allergy Clin. Immunol. 145, 301-311.e4. https://doi.org/10.1016/j.jaci.2019.07.045

Epp, A., Hobusch, J., Bartsch, Y.C., Petry, J., Lilienthal, G.-M., Koeleman, C.A.M., Eschweiler, S.,

A References

Möbs, C., Hall, A., Morris, S.C., Braumann, D., Engellenner, C., Bitterling, J., Rahmöller, J., Leliavski, A., Thurmann, R., Collin, M., Moremen, K.W., Strait, R.T., Blanchard, V., Petersen, A., Gemoll, T., Habermann, J.K., Petersen, F., Nandy, A., Kahlert, H., Hertl, M., Wuhrer, M., Pfützner, W., Jappe, U., Finkelman, F.D., Ehlers, M., 2018a. Sialylation of IgG antibodies inhibits IgG-mediated allergic reactions. J. Allergy Clin. Immunol. 141, 399-402.e8. https://doi.org/10.1016/j.jaci.2017.06.021

Epp, A., Hobusch, J., Bartsch, Y.C., Petry, J., Lilienthal, G.-M., Koeleman, C.A.M., Eschweiler, S., Möbs, C., Hall, A., Morris, S.C., Braumann, D., Engellenner, C., Bitterling, J., Rahmöller, J., Leliavski, A., Thurmann, R., Collin, M., Moremen, K.W., Strait, R.T., Blanchard, V., Petersen, A., Gemoll, T., Habermann, J.K., Petersen, F., Nandy, A., Kahlert, H., Hertl, M., Wuhrer, M., Pfützner, W., Jappe, U., Finkelman, F.D., Ehlers, M., 2018b. Sialylation of IgG antibodies inhibits IgG-mediated allergic reactions. J. Allergy Clin. Immunol. 141, 399-402.e8. https://doi.org/10.1016/j.jaci.2017.06.021

Ercan, A., 2020. Sex effect on the correlation of immunoglobulin G glycosylation with rheumatoid arthritis disease activity. Turk. J. Biol. Turk Biyol. Derg. 44, 406–416. https://doi.org/10.3906/biy-2005-7

Ercan, A., Kohrt, W.M., Cui, J., Deane, K.D., Pezer, M., Yu, E.W., Hausmann, J.S., Campbell, H., Kaiser, U.B., Rudd, P.M., Lauc, G., Wilson, J.F., Finkelstein, J.S., Nigrovic, P.A., 2017. Estrogens regulate glycosylation of IgG in women and men. JCI Insight 2. https://doi.org/10.1172/jci.insight.89703

Feyerabend, T.B., Weiser, A., Tietz, A., Stassen, M., Harris, N., Kopf, M., Radermacher, P., Möller, P., Benoist, C., Mathis, D., Fehling, H.J., Rodewald, H.-R., 2011. Cre-Mediated Cell Ablation Contests Mast Cell Contribution in Models of Antibody- and T Cell-Mediated Autoimmunity. Immunity 35, 832–844. https://doi.org/10.1016/j.immuni.2011.09.015

Fiebiger, B.M., Maamary, J., Pincetic, A., Ravetch, J.V., 2015. Protection in antibody- and T cell-mediated autoimmune diseases by antiinflammatory IgG Fcs requires type II FcRs. Proc. Natl. Acad. Sci. 112. https://doi.org/10.1073/pnas.1505292112

Finkelman, F.D., 2007. Anaphylaxis: Lessons from mouse models. J. Allergy Clin. Immunol. 120, 506–515. https://doi.org/10.1016/j.jaci.2007.07.033

Finkelman, F.D., Khodoun, M.V., Strait, R., 2016. Human IgE-independent systemic anaphylaxis. J. Allergy Clin. Immunol. 137, 1674–1680. https://doi.org/10.1016/j.jaci.2016.02.015

Frigeri, L.G., Zuberi, R.I., Liu, F.T., 1993. .epsilon.BP, a .beta.-galactoside-binding animal lectin, recognizes IgE receptor (Fc.epsilon.RI) and activates mast cells. Biochemistry 32, 7644–7649. https://doi.org/10.1021/bi00081a007

Fuss, I.J., Kanof, M.E., Smith, P.D., Zola, H., 2009. Isolation of Whole Mononuclear Cells from Peripheral Blood and Cord Blood. Curr. Protoc. Immunol. 85. https://doi.org/10.1002/0471142735.im0701s85

Gao, P., Simpson, J.L., Zhang, J., Gibson, P.G., 2013. Galectin-3: its role in asthma and potential as an anti-inflammatory target. Respir. Res. 14, 136. https://doi.org/10.1186/1465-9921-14-136

Gill, P., Jindal, N.L., Jagdis, A., Vadas, P., 2015. Platelets in the immune response: Revisiting platelet-activating factor in anaphylaxis. J. Allergy Clin. Immunol. 135, 1424–1432. https://doi.org/10.1016/j.jaci.2015.04.019

Gould, H.J., Sutton, B.J., 2008a. IgE in allergy and asthma today. Nat. Rev. Immunol. 8, 205–217. https://doi.org/10.1038/nri2273

A References

Gould, H.J., Sutton, B.J., 2008b. IgE in allergy and asthma today. Nat. Rev. Immunol. 8, 205–217. https://doi.org/10.1038/nri2273

Gould, H.J., Sutton, B.J., Beavil, A.J., Beavil, R.L., McCloskey, N., Coker, H.A., Fear, D., Smurthwaite, L., 2003. The Biology of IgE and the Basis of Allergic Disease. Annu. Rev. Immunol. 21, 579–628. https://doi.org/10.1146/annurev.immunol.21.120601.141103

Greenberger, P.A., 2019. Drug allergy. Allergy Asthma Proc. 40, 474–479. https://doi.org/10.2500/aap.2019.40.4275

Grewal, P.K., 2010. The Ashwell–Morell Receptor, in: Methods in Enzymology. Elsevier, pp. 223–241. https://doi.org/10.1016/S0076-6879(10)79013-3

Grewal, P.K., Uchiyama, S., Ditto, D., Varki, N., Le, D.T., Nizet, V., Marth, J.D., 2008. The Ashwell receptor mitigates the lethal coagulopathy of sepsis. Nat. Med. 14, 648–655. https://doi.org/10.1038/nm1760

Gupta, G.S., 2012. Animal Lectins: Form, Function and Clinical Applications. Springer Vienna, Vienna. https://doi.org/10.1007/978-3-7091-1065-2

Hess, C., Winkler, A., Lorenz, A.K., Holecska, V., Blanchard, V., Eiglmeier, S., Schoen, A.-L., Bitterling, J., Stoehr, A.D., Petzold, D., Schommartz, T., Mertes, M.M.M., Schoen, C.T., Tiburzy, B., Herrmann, A., Köhl, J., Manz, R.A., Madaio, M.P., Berger, M., Wardemann, H., Ehlers, M., 2013. T cell–independent B cell activation induces immunosuppressive sialylated IgG antibodies. J. Clin. Invest. 123, 3788–3796. https://doi.org/10.1172/JCI65938

Hirose, M., Tiburzy, B., Ishii, N., Pipi, E., Wende, S., Rentz, E., Nimmerjahn, F., Zillikens, D., Manz, R.A., Ludwig, R.J., Kasperkiewicz, M., 2015. Effects of Intravenous Immunoglobulins on Mice with Experimental Epidermolysis Bullosa Acquisita. J. Invest. Dermatol. 135, 768–775. https://doi.org/10.1038/jid.2014.453

Hudgin, R.L., Pricer, W.E., Ashwell, G., Stockert, R.J., Morell, A.G., 1974. The Isolation and Properties of a Rabbit Liver Binding Protein Specific for Asialoglycoproteins. J. Biol. Chem. 249, 5536–5543. https://doi.org/10.1016/S0021-9258(20)79761-9

Jabs, F., Plum, M., Laursen, N.S., Jensen, R.K., Mølgaard, B., Miehe, M., Mandolesi, M., Rauber, M.M., Pfützner, W., Jakob, T., Möbs, C., Andersen, G.R., Spillner, E., 2018. Trapping IgE in a closed conformation by mimicking CD23 binding prevents and disrupts FcεRI interaction. Nat. Commun. 9, 7. https://doi.org/10.1038/s41467-017-02312-7

Jacoby, W., Cammarata, P.V., Findlay, S., Pincus, S.H., 1984. Anaphylaxis in Mast Cell-Deficient Mice. J. Invest. Dermatol. 83, 302–304. https://doi.org/10.1111/1523-1747.ep12340431

Jensen-Jarolim, E., Roth-Walter, F., Jordakieva, G., Pali-Schöll, I., 2021. Allergens and Adjuvants in Allergen Immunotherapy for Immune Activation, Tolerance, and Resilience. J. Allergy Clin. Immunol. Pract. 9, 1780–1789. https://doi.org/10.1016/j.jaip.2020.12.008

Jiao, D., Liu, Y., Lu, X., Pan, Q., Zheng, J., Liu, B., Liu, Y., Wang, Y., Fu, N., 2013. Characteristics of anaphylaxis-inducing IgG immune complexes triggering murine passive systemic anaphylaxis. Allergy 68, 236–245. https://doi.org/10.1111/all.12089

Jiao, D., Liu, Yuan, Lu, X., Liu, B., Pan, Q., Liu, Yanjun, Liu, Yang, Zhu, P., Fu, N., 2014. Macrophages are the dominant effector cells responsible for IgG-mediated passive systemic anaphylaxis challenged by natural protein antigen in BALB/c and C57BL/6 mice. Cell. Immunol. 289, 97–105. https://doi.org/10.1016/j.cellimm.2014.03.018

Jönsson, F., de Chaisemartin, L., Granger, V., Gouel-Chéron, A., Gillis, C.M., Zhu, Q., Dib, F.,

A References

Nicaise-Roland, P., Ganneau, C., Hurtado-Nedelec, M., Paugam-Burtz, C., Necib, S., Keita-Meyer, H., Le Dorze, M., Cholley, B., Langeron, O., Jacob, L., Plaud, B., Fischler, M., Sauvan, C., Guinnepain, M.-T., Montravers, P., Aubier, M., Bay, S., Neukirch, C., Tubach, F., Longrois, D., Chollet-Martin, S., Bruhns, P., 2019. An IgG-induced neutrophil activation pathway contributes to human drug-induced anaphylaxis. Sci. Transl. Med. 11, eaat1479. https://doi.org/10.1126/scitranslmed.aat1479

Jönsson, F., Mancardi, D.A., Kita, Y., Karasuyama, H., Iannascoli, B., Van Rooijen, N., Shimizu, T., Daëron, M., Bruhns, P., 2011. Mouse and human neutrophils induce anaphylaxis. J. Clin. Invest. 121, 1484–1496. https://doi.org/10.1172/JCI45232

Kanagaratham, C., El Ansari, Y.S., Lewis, O.L., Oettgen, H.C., 2020. IgE and IgG Antibodies as Regulators of Mast Cell and Basophil Functions in Food Allergy. Front. Immunol. 11, 603050. https://doi.org/10.3389/fimmu.2020.603050

Kaneko, Y., Nimmerjahn, F., Ravetch, J.V., 2006. Anti-Inflammatory Activity of Immunoglobulin G Resulting from Fc Sialylation. Science 313, 670–673. https://doi.org/10.1126/science.1129594

Karsten, C.M., Pandey, M.K., Figge, J., Kilchenstein, R., Taylor, P.R., Rosas, M., McDonald, J.U., Orr, S.J., Berger, M., Petzold, D., Blanchard, V., Winkler, A., Hess, C., Reid, D.M., Majoul, I.V., Strait, R.T., Harris, N.L., Köhl, G., Wex, E., Ludwig, R., Zillikens, D., Nimmerjahn, F., Finkelman, F.D., Brown, G.D., Ehlers, M., Köhl, J., 2012. Anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcγRIIB and dectin-1. Nat. Med. 18, 1401–1406. https://doi.org/10.1038/nm.2862

Kavran, J.M., Leahy, D.J., 2014. Coupling Antibody to Cyanogen Bromide-Activated Sepharose, in: Methods in Enzymology. Elsevier, pp. 27–34. https://doi.org/10.1016/B978-0-12-420119-4.00003-3

Kerntke, C., Nimmerjahn, F., Biburger, M., 2020. There Is (Scientific) Strength in Numbers: A Comprehensive Quantitation of Fc Gamma Receptor Numbers on Human and Murine Peripheral Blood Leukocytes. Front. Immunol. 11, 118. https://doi.org/10.3389/fimmu.2020.00118

Khodoun, Marat V., Strait, R., Armstrong, L., Yanase, N., Finkelman, F.D., 2011. Identification of markers that distinguish IgE- from IgG-mediated anaphylaxis. Proc. Natl. Acad. Sci. 108, 12413–12418. https://doi.org/10.1073/pnas.1105695108

Khodoun, M. V., Strait, R., Armstrong, L., Yanase, N., Finkelman, F.D., 2011. Identification of markers that distinguish IgE- from IgG-mediated anaphylaxis. Proc. Natl. Acad. Sci. 108, 12413–12418. https://doi.org/10.1073/pnas.1105695108

Kim, H.W., Ju, D.B., Kye, Y.-C., Ju, Y.-J., Kim, C.G., Lee, I.K., Park, S.-M., Choi, I.S., Cho, K.K., Lee, S.H., Kim, S.C., Jung, I.D., Han, S.H., Yun, C.-H., 2020. Galectin-9 Induced by Dietary Probiotic Mixture Regulates Immune Balance to Reduce Atopic Dermatitis Symptoms in Mice. Front. Immunol. 10, 3063. https://doi.org/10.3389/fimmu.2019.03063

Kirshenbaum, A.S., Akin, C., Wu, Y., Rottem, M., Goff, J.P., Beaven, M.A., Rao, V.K., Metcalfe, D.D., 2003. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. Leuk. Res. 27, 677–682. https://doi.org/10.1016/S0145-2126(02)00343-0

Kito, Y., Ito, T., Tokura, Y., Hashizume, H., 2012. High-dose Intravenous Immunoglobulin Monotherapy for Drug-induced Hypersensitivity Syndrome. Acta Derm. Venereol. 92, 100–101. https://doi.org/10.2340/00015555-1168

Kivit, S., Saeland, E., Kraneveld, A.D., Kant, H.J.G., Schouten, B., Esch, B.C.A.M., Knol, J., Sprikkelman, A.B., Aa, L.B., Knippels, L.M.J., Garssen, J., Kooyk, Y., Willemsen, L.E.M., 2012.

A References

Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 67, 343–352. https://doi.org/10.1111/j.1398-9995.2011.02771.x

Kojima, R., Ohno, T., Iikura, M., Niki, T., Hirashima, M., Iwaya, K., Tsuda, H., Nonoyama, S., Matsuda, A., Saito, H., Matsumoto, K., Nakae, S., 2014. Galectin-9 Enhances Cytokine Secretion, but Suppresses Survival and Degranulation, in Human Mast Cell Line. PLoS ONE 9, e86106. https://doi.org/10.1371/journal.pone.0086106

Kuehn, H.S., Radinger, M., Gilfillan, A.M., 2010. Measuring Mast Cell Mediator Release. Curr. Protoc. Immunol. 91. https://doi.org/10.1002/0471142735.im0738s91

Lee, S.P., Shin, Y.S., Kang, S.-Y., Kim, T.-B., Lee, S.M., 2022. Recent Advances in Allergen-Specific Immunotherapy in Humans: A Systematic Review. Immune Netw. 22, e12. https://doi.org/10.4110/in.2022.22.e12

Liu, F.-T., 2005. Regulatory Roles of Galectins in the Immune Response. Int. Arch. Allergy Immunol. 136, 385–400. https://doi.org/10.1159/000084545

Liu, F.T., Albrandt, K., Mendel, E., Kulczycki, A., Orida, N.K., 1985. Identification of an IgE-binding protein by molecular cloning. Proc. Natl. Acad. Sci. 82, 4100–4104. https://doi.org/10.1073/pnas.82.12.4100

Lohse, S., Meyer, S., Meulenbroek, L.A.P.M., Jansen, J.H.M., Nederend, M., Kretschmer, A., Klausz, K., Möginger, U., Derer, S., Rösner, T., Kellner, C., Schewe, D., Sondermann, P., Tiwari, S., Kolarich, D., Peipp, M., Leusen, J.H.W., Valerius, T., 2016. An Anti-EGFR IgA That Displays Improved Pharmacokinetics and Myeloid Effector Cell Engagement *In Vivo*. Cancer Res. 76, 403–417. https://doi.org/10.1158/0008-5472.CAN-15-1232

Lunding, H.B., 2021. Regulation of the alpha2,6-sialyltransferase and IgG Fc glycosylation in immunization-induced germinal center reactions. University of Lübeck.

Mancardi, D.A., Albanesi, M., Jönsson, F., Iannascoli, B., Van Rooijen, N., Kang, X., England, P., Daëron, M., Bruhns, P., 2013. The high-affinity human IgG receptor FcγRI (CD64) promotes IgG-mediated inflammation, anaphylaxis, and antitumor immunotherapy. Blood 121, 1563–1573. https://doi.org/10.1182/blood-2012-07-442541

Matsumoto, K., Nishi, K., Kikuchi, M., Watanabe, H., Nakajou, K., Komori, H., Kadowaki, D., Suenaga, A., Maruyama, T., Otagiri, M., 2010. Receptor-Mediated Uptake of Human α1-Acid Glycoprotein into Liver Parenchymal Cells in Mice. Drug Metab. Pharmacokinet. 25, 101–107. https://doi.org/10.2133/dmpk.25.101

Möbs, C., Ipsen, H., Mayer, L., Slotosch, C., Petersen, A., Würtzen, P.A., Hertl, M., Pfützner, W., 2012. Birch pollen immunotherapy results in long-term loss of Bet v 1–specific TH2 responses, transient TR1 activation, and synthesis of IgE-blocking antibodies. J. Allergy Clin. Immunol. 130, 1108-1116.e6. https://doi.org/10.1016/j.jaci.2012.07.056

Morell, A.G., Gregoriadis, G., Scheinberg, I.H., Hickman, J., Ashwell, G., 1971. The Role of Sialic Acid in Determining the Survival of Glycoproteins in the Circulation. J. Biol. Chem. 246, 1461–1467. https://doi.org/10.1016/S0021-9258(19)76994-4

Morelle, W., Faid, V., Chirat, F., Michalski, J.-C., 2009. Analysis of N- and O-Linked Glycans from Glycoproteins Using MALDI-TOF Mass Spectrometry, in: Packer, N.H., Karlsson, N.G. (Eds.), Glycomics. Humana Press, Totowa, NJ, pp. 3–21. https://doi.org/10.1007/978-1-59745-022-5_1

Mortensen, B., Huseby, N.-E., 1997. Clearance of circulating γ-glutamyltransferase by the asialoglycoprotein receptor. Enzyme forms with different sialic acid content are eliminated at different clearance rates and without apparent desialylation. Clin. Chim. Acta 258, 47–58.

https://doi.org/10.1016/S0009-8981(96)06427-3

Müller, U.R., 2005. Bee venom allergy in beekeepers and their family members. Curr. Opin. Allergy Clin. Immunol. 5, 343–347. https://doi.org/10.1097/01.all.0000173783.42906.95

Murphy, K., Weaver, C., 2018. Janeway Immunologie. Springer Berlin Heidelberg, Berlin, Heidelberg. https://doi.org/10.1007/978-3-662-56004-4

Nakano, M., 2004. Detailed structural features of glycan chains derived from 1-acid glycoproteins of several different animals: the presence of hypersialylated, O-acetylated sialic acids but not disialyl residues. Glycobiology 14, 431–441. https://doi.org/10.1093/glycob/cwh034

Nandakumar, K.S., Collin, M., Olsén, A., Nimmerjahn, F., Blom, A.M., Ravetch, J.V., Holmdahl, R., 2007. Endoglycosidase treatment abrogates IgG arthritogenicity: Importance of IgG glycosylation in arthritis. Eur. J. Immunol. 37, 2973–2982. https://doi.org/10.1002/eji.200737581

Nettleton, M.Y., Kochan, J.P., 1995. Role of Glycosylation Sites in the IgE Fc Molecule. Int. Arch. Allergy Immunol. 107, 328–329. https://doi.org/10.1159/000237017

Niki, T., Tsutsui, S., Hirose, S., Aradono, S., Sugimoto, Y., Takeshita, K., Nishi, N., Hirashima, M., 2009. Galectin-9 Is a High Affinity IgE-binding Lectin with Anti-allergic Effect by Blocking IgE-Antigen Complex Formation. J. Biol. Chem. 284, 32344–32352. https://doi.org/10.1074/jbc.M109.035196

Nimmerjahn, F., Anthony, R.M., Ravetch, J.V., 2007. Agalactosylated IgG antibodies depend on cellular Fc receptors for *in vivo* activity. Proc. Natl. Acad. Sci. 104, 8433–8437. https://doi.org/10.1073/pnas.0702936104

Nimmerjahn, F., Gordan, S., Lux, A., 2015. FcγR dependent mechanisms of cytotoxic, agonistic, and neutralizing antibody activities. Trends Immunol. 36, 325–336. https://doi.org/10.1016/j.it.2015.04.005

Nimmerjahn, F., Ravetch, J.V., 2008. Fcγ receptors as regulators of immune responses. Nat. Rev. Immunol. 8, 34–47. https://doi.org/10.1038/nri2206

Oefner, C.M., Winkler, A., Hess, C., Lorenz, A.K., Holecska, V., Huxdorf, M., Schommartz, T., Petzold, D., Bitterling, J., Schoen, A.-L., Stoehr, A.D., Vu Van, D., Darcan-Nikolaisen, Y., Blanchard, V., Schmudde, I., Laumonnier, Y., Ströver, H.A., Hegazy, A.N., Eiglmeier, S., Schoen, C.T., Mertes, M.M.M., Loddenkemper, C., Löhning, M., König, P., Petersen, A., Luger, E.O., Collin, M., Köhl, J., Hutloff, A., Hamelmann, E., Berger, M., Wardemann, H., Ehlers, M., 2012. Tolerance induction with T cell–dependent protein antigens induces regulatory sialylated IgGs. J. Allergy Clin. Immunol. 129, 1647-1655.e13. https://doi.org/10.1016/j.jaci.2012.02.037

Oettgen, H.C., 2010. Regulation and Biology of Immunoglobulin E, in: Pediatric Allergy: Principles and Practice. Elsevier, pp. 40–51. https://doi.org/10.1016/B978-1-4377-0271-2.00004-3

Oettgen, H.C., Martin, T.R., Wynshaw-Boris, A., Deng, C., Drazen, J.M., Leder, P., 1994. Active anaphylaxis in IgE-deficient mice. Nature 370, 367–370. https://doi.org/10.1038/370367a0

Ohmi, Y., Ise, W., Harazono, A., Takakura, D., Fukuyama, H., Baba, Y., Narazaki, M., Shoda, H., Takahashi, N., Ohkawa, Y., Ji, S., Sugiyama, F., Fujio, K., Kumanogoh, A., Yamamoto, K., Kawasaki, N., Kurosaki, T., Takahashi, Y., Furukawa, K., 2016. Sialylation converts arthritogenic IgG into inhibitors of collagen-induced arthritis. Nat. Commun. 7, 11205. https://doi.org/10.1038/ncomms11205

Park, E.I., Mi, Y., Unverzagt, C., Gabius, H.-J., Baenziger, J.U., 2005. The asialoglycoprotein receptor clears glycoconjugates terminating with sialic acidα2,6GalNAc. Proc. Natl. Acad. Sci. 102, 17125–

17129. https://doi.org/10.1073/pnas.0508537102

Pasmatzi, E., Papadionysiou, C., Monastirli, A., Badavanis, G., Tsambaos, D., 2019. Galectin 3: an extraordinary multifunctional protein in dermatology. Current knowledge and perspectives. An. Bras. Dermatol. 94, 348–354. https://doi.org/10.1590/abd1806-4841.20198426

Peavy, R.D., Metcalfe, D.D., 2008. Understanding the mechanisms of anaphylaxis. Curr. Opin. Allergy Clin. Immunol. 8, 310–315. https://doi.org/10.1097/ACI.0b013e3283036a90

Petry, J., 2019. The role of IgG sialylation in the attenuation of anaphylactic reactions. University of Lübeck.

Petry, J., Rahmöller, J., Dühring, L., Lilienthal, G.-M., Lehrian, S., Buhre, J.S., Bartsch, Y.C., Epp, A., Lunding, H.B., Moremen, K.W., Leliavski, A., Ehlers, M., 2021a. Enriched blood IgG sialylation attenuates IgG-mediated and IgG-controlled-IgE-mediated allergic reactions. J. Allergy Clin. Immunol. 147, 763–767. https://doi.org/10.1016/j.jaci.2020.05.056

Petry, J., Rahmöller, J., Dühring, L., Lilienthal, G.-M., Lehrian, S., Buhre, J.S., Bartsch, Y.C., Epp, A., Lunding, H.B., Moremen, K.W., Leliavski, A., Ehlers, M., 2021b. Enriched blood IgG sialylation attenuates IgG-mediated and IgG-controlled-IgE-mediated allergic reactions. J. Allergy Clin. Immunol. 147, 763–767. https://doi.org/10.1016/j.jaci.2020.05.056

Pincetic, A., Bournazos, S., DiLillo, D.J., Maamary, J., Wang, T.T., Dahan, R., Fiebiger, B.-M., Ravetch, J.V., 2014. Type I and type II Fc receptors regulate innate and adaptive immunity. Nat. Immunol. 15, 707–716. https://doi.org/10.1038/ni.2939

Plomp, R., Hensbergen, P.J., Rombouts, Y., Zauner, G., Dragan, I., Koeleman, C.A.M., Deelder, A.M., Wuhrer, M., 2014a. Site-Specific N-Glycosylation Analysis of Human Immunoglobulin E. J. Proteome Res. 13, 536–546. https://doi.org/10.1021/pr400714w

Plomp, R., Hensbergen, P.J., Rombouts, Y., Zauner, G., Dragan, I., Koeleman, C.A.M., Deelder, A.M., Wuhrer, M., 2014b. Site-Specific N-Glycosylation Analysis of Human Immunoglobulin E. J. Proteome Res. 13, 536–546. https://doi.org/10.1021/pr400714w

Rådinger, M., Jensen, B.M., Kuehn, H.S., Kirshenbaum, A., Gilfillan, A.M., 2010. Generation, Isolation, and Maintenance of Human Mast Cells and Mast Cell Lines Derived from Peripheral Blood or Cord Blood, in: Coligan, J.E., Bierer, B.E., Margulies, D.H., Shevach, E.M., Strober, W. (Eds.), Current Protocols in Immunology. John Wiley & Sons, Inc., Hoboken, NJ, USA, p. im0737s90. https://doi.org/10.1002/0471142735.im0737s90

Reber, L.L., Hernandez, J.D., Galli, S.J., 2017. The pathophysiology of anaphylaxis. J. Allergy Clin. Immunol. 140, 335–348. https://doi.org/10.1016/j.jaci.2017.06.003

Reber, L.L., Marichal, T., Mukai, K., Kita, Y., Tokuoka, S.M., Roers, A., Hartmann, K., Karasuyama, H., Nadeau, K.C., Tsai, M., Galli, S.J., 2013. Selective ablation of mast cells or basophils reduces peanut-induced anaphylaxis in mice. J. Allergy Clin. Immunol. 132, 881-888.e11. https://doi.org/10.1016/j.jaci.2013.06.008

Rifai, A., Fadden, K., Morrison, S.L., Chintalacharuvu, K.R., 2000. The N-Glycans Determine the Differential Blood Clearance and Hepatic Uptake of Human Immunoglobulin (Ig)a1 and Iga2 Isotypes. J. Exp. Med. 191, 2171–2182. https://doi.org/10.1084/jem.191.12.2171

Rigopoulou, E.I., Roggenbuck, D., Smyk, D.S., Liaskos, C., Mytilinaiou, M.G., Feist, E., Conrad, K., Bogdanos, D.P., 2012. Asialoglycoprotein receptor (ASGPR) as target autoantigen in liver autoimmunity: Lost and found. Autoimmun. Rev. 12, 260–269. https://doi.org/10.1016/j.autrev.2012.04.005

A References

Robertson, M.W., Albrandt, K., Keller, D., Liu, F.T., 1990. Human IgE-binding protein: a soluble lectin exhibiting a highly conserved interspecies sequence and differential recognition of IgE glycoforms. Biochemistry 29, 8093–8100. https://doi.org/10.1021/bi00487a015

Robertson, M.W., Liu, F.T., 1991. Heterogeneous IgE glycoforms characterized by differential recognition of an endogenous lectin (IgE-binding protein). J. Immunol. 147, 3024.

Rotundo, R.F., Rebres, R.A., Mckeown-Longo, P.J., Blumenstock, F.A., Saba, T.M., 1998. Circulating cellular fibronectin may be a natural ligand for the hepatic asialoglycoprotein receptor: Possible pathway for fibronectin deposition and turnover in the rat liver. Hepatology 28, 475–485. https://doi.org/10.1002/hep.510280227

Rudolph, A.K., Burrows, P.D., Wabl, M.R., 1981. Thirteen hybridomas secreting hapten-specific immunoglobulin E from mice with Iga or Igb heavy chain haplotype. Eur. J. Immunol. 11, 527–529. https://doi.org/10.1002/eji.1830110617

S. Kirshenbaum, A., Yin, Y., Sundstrom, J.B., Bandara, G., D. Metcalfe, D., 2019. Description and Characterization of a Novel Human Mast Cell Line for Scientific Study. Int. J. Mol. Sci. 20, 5520. https://doi.org/10.3390/ijms20225520

Samuelsson, A., Towers, T.L., Ravetch, J.V., 2001. Anti-inflammatory Activity of IVIG Mediated Through the Inhibitory Fc Receptor. Science 291, 484–486. https://doi.org/10.1126/science.291.5503.484

Sauer, A.-K., Liang, C.-H., Stech, J., Peeters, B., Quéré, P., Schwegmann-Wessels, C., Wu, C.-Y., Wong, C.-H., Herrler, G., 2014. Characterization of the Sialic Acid Binding Activity of Influenza A Viruses Using Soluble Variants of the H7 and H9 Hemagglutinins. PLoS ONE 9, e89529. https://doi.org/10.1371/journal.pone.0089529

Sayers, I., Cain, S.A., Swan, J.R.M., Pickett, M.A., Watt, P.J., Holgate, S.T., Padlan, E.A., Schuck, P., Helm, B.A., 1998. Amino Acid Residues That Influence FcεRI-Mediated Effector Functions of Human Immunoglobulin E. Biochemistry 37, 16152–16164. https://doi.org/10.1021/bi981456k

Scherer, H.U., van der Woude, D., Ioan-Facsinay, A., el Bannoudi, H., Trouw, L.A., Wang, J., Häupl, T., Burmester, G.-R., Deelder, A.M., Huizinga, T.W.J., Wuhrer, M., Toes, R.E.M., 2010. Glycan profiling of anti-citrullinated protein antibodies isolated from human serum and synovial fluid. Arthritis Rheum. 62, 1620–1629. https://doi.org/10.1002/art.27414

Scheuerman, O., Nofech-Moses, Y., Rachmel, A., Ashkenazi, S., 2001. Successful Treatment of Antiepileptic Drug Hypersensitivity Syndrome With Intravenous Immune Globulin. Pediatrics 107, e14–e14. https://doi.org/10.1542/peds.107.1.e14

Schroeder, H.W., Cavacini, L., 2010. Structure and function of immunoglobulins. J. Allergy Clin. Immunol. 125, S41–S52. https://doi.org/10.1016/j.jaci.2009.09.046

Schroeder, J.T., Adeosun, A.A., Do, D., Bieneman, A.P., 2019. Galectin-3 is essential for IgE-dependent activation of human basophils by A549 lung epithelial cells. J. Allergy Clin. Immunol. 144, 312-315.e1. https://doi.org/10.1016/j.jaci.2019.03.001

Schwab, I., Mihai, S., Seeling, M., Kasperkiewicz, M., Ludwig, R.J., Nimmerjahn, F., 2014. Broad requirement for terminal sialic acid residues and FcγRIIB for the preventive and therapeutic activity of intravenous immunoglobulins in vivo: Immunomodulation. Eur. J. Immunol. 44, 1444–1453. https://doi.org/10.1002/eji.201344230

Selman, M.H.J., de Jong, S.E., Soonawala, D., Kroon, F.P., Adegnika, A.A., Deelder, A.M., Hokke, C.H., Yazdanbakhsh, M., Wuhrer, M., 2012. Changes in Antigen-specific IgG1 Fc N-glycosylation Upon Influenza and Tetanus Vaccination. Mol. Cell. Proteomics 11, M111.014563.

https://doi.org/10.1074/mcp.M111.014563

Shade, K.-T., Conroy, M.E., Anthony, R.M., 2019. IgE Glycosylation in Health and Disease, in: Ravetch, J.V., Nimmerjahn, F. (Eds.), Fc Mediated Activity of Antibodies, Current Topics in Microbiology and Immunology. Springer International Publishing, Cham, pp. 77–93. https://doi.org/10.1007/82_2019_151

Shade, K.-T.C., Conroy, M.E., Washburn, N., Kitaoka, M., Huynh, D.J., Laprise, E., Patil, S.U., Shreffler, W.G., Anthony, R.M., 2020a. Sialylation of immunoglobulin E is a determinant of allergic pathogenicity. Nature 582, 265–270. https://doi.org/10.1038/s41586-020-2311-z

Shade, K.-T.C., Conroy, M.E., Washburn, N., Kitaoka, M., Huynh, D.J., Laprise, E., Patil, S.U., Shreffler, W.G., Anthony, R.M., 2020b. Sialylation of immunoglobulin E is a determinant of allergic pathogenicity. Nature 582, 265–270. https://doi.org/10.1038/s41586-020-2311-z

Shade, K.-T.C., Platzer, B., Washburn, N., Mani, V., Bartsch, Y.C., Conroy, M., Pagan, J.D., Bosques, C., Mempel, T.R., Fiebiger, E., Anthony, R.M., 2015. A single glycan on IgE is indispensable for initiation of anaphylaxis. J. Exp. Med. 212, 457–467. https://doi.org/10.1084/jem.20142182

Shamji, M.H., Valenta, R., Jardetzky, T., Verhasselt, V., Durham, S.R., Würtzen, P.A., van Neerven, R.J.J., 2021. The role of allergen-specific IgE, IgG and IgA in allergic disease. Allergy 76, 3627–3641. https://doi.org/10.1111/all.14908

Smedsrød, B., Einarsson, M., 1990. Clearance of Tissue Plasminogen Activator by Mannose and Galactose Receptors in the Liver. Thromb. Haemost. 63, 060–066. https://doi.org/10.1055/s-0038-1645687

Sondermann, P., Huber, R., Oosthuizen, V., Jacob, U., 2000. The 3.2-Å crystal structure of the human IgG1 Fc fragment–FcγRIII complex. Nature 406, 267–273. https://doi.org/10.1038/35018508

Steffen, U., Koeleman, C.A., Sokolova, M.V., Bang, H., Kleyer, A., Rech, J., Unterweger, H., Schicht, M., Garreis, F., Hahn, J., Andes, F.T., Hartmann, F., Hahn, M., Mahajan, A., Paulsen, F., Hoffmann, M., Lochnit, G., Muñoz, L.E., Wuhrer, M., Falck, D., Herrmann, M., Schett, G., 2020. IgA subclasses have different effector functions associated with distinct glycosylation profiles. Nat. Commun. 11, 120. https://doi.org/10.1038/s41467-019-13992-8

Stockert, R.J., 1995. The asialoglycoprotein receptor: relationships between structure, function, and expression. Physiol. Rev. 75, 591–609. https://doi.org/10.1152/physrev.1995.75.3.591

Strait, R.T., 2006. IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and Fc RIIb cross-linking. J. Clin. Invest. 116, 833–841. https://doi.org/10.1172/JCI25575

Sutton, B.J., Davies, A.M., 2015. Structure and dynamics of IgE-receptor interactions: FcεRI and CD23/FcεRII. Immunol. Rev. 268, 222–235. https://doi.org/10.1111/imr.12340

Sziksz, E., Vannay, Á., Haczku, A., 2012. Galectin-9: a suppressor of food allergy? Allergy 67, 293–295. https://doi.org/10.1111/j.1398-9995.2011.02774.x

Tabynov, Kairat, Babayeva, M., Nurpeisov, Tair, Fomin, G., Nurpeisov, Temirzhan, Saltabayeva, U., Renu, S., Renukaradhya, G.J., Petrovsky, N., Tabynov, Kaissar, 2022. Evaluation of a Novel Adjuvanted Vaccine for Ultrashort Regimen Therapy of Artemisia Pollen-Induced Allergic Bronchial Asthma in a Mouse Model. Front. Immunol. 13, 828690. https://doi.org/10.3389/fimmu.2022.828690

Thornburg, R.W., Day, J.F., Baynes, J.W., Thorpe, S.R., 1980. Carbohydrate-mediated clearance of immune complexes from the circulation. A role for galactose residues in the hepatic uptake of IgG-antigen complexes. J. Biol. Chem. 255, 6820–6825. https://doi.org/10.1016/S0021-9258(18)43646-0

A References

Tomana, M., Phillips, J.O., Kulhavy, R., Mestecky, J., 1985. Carbohydrate-mediated clearance of secretory IgA from the circulation. Mol. Immunol. 22, 887–892. https://doi.org/10.1016/0161-5890(85)90074-4

Trbojević Akmačić, I., Ventham, N.T., Theodoratou, E., Vučković, F., Kennedy, N.A., Krištić, J., Nimmo, E.R., Kalla, R., Drummond, H., Štambuk, J., Dunlop, M.G., Novokmet, M., Aulchenko, Y., Gornik, O., Campbell, H., Pučić Baković, M., Satsangi, J., Lauc, G., 2015. Inflammatory Bowel Disease Associates with Proinflammatory Potential of the Immunoglobulin G Glycome: Inflamm. Bowel Dis. 1. https://doi.org/10.1097/MIB.0000000000000372

van den Hamer, C.J.A., Morell, A.G., Scheinberg, I.H., Hickman, J., Ashwell, G., 1970. Physical and Chemical Studies on Ceruloplasmin. J. Biol. Chem. 245, 4397–4402. https://doi.org/10.1016/S0021-9258(19)63808-1

van der Neut Kolfschoten, M., Schuurman, J., Losen, M., Bleeker, W.K., Martínez-Martínez, P., Vermeulen, E., den Bleker, T.H., Wiegman, L., Vink, T., Aarden, L.A., De Baets, M.H., van de Winkel, J.G.J., Aalberse, R.C., Parren, P.W.H.I., 2007. Anti-Inflammatory Activity of Human IgG4 Antibodies by Dynamic Fab Arm Exchange. Science 317, 1554–1557. https://doi.org/10.1126/science.1144603

Vidarsson, G., Dekkers, G., Rispens, T., 2014a. IgG Subclasses and Allotypes: From Structure to Effector Functions. Front. Immunol. 5. https://doi.org/10.3389/fimmu.2014.00520

Vidarsson, G., Dekkers, G., Rispens, T., 2014b. IgG Subclasses and Allotypes: From Structure to Effector Functions. Front. Immunol. 5. https://doi.org/10.3389/fimmu.2014.00520

Vostal, J.G., McCauley, R.B., 1991. Prothrombin plasma clearance is not mediated by hepatic asialoglycoprotein receptors. Thromb. Res. 63, 299–309. https://doi.org/10.1016/0049-3848(91)90133-H

Vučković, F., Krištić, J., Gudelj, I., Teruel, M., Keser, T., Pezer, M., Pučić-Baković, M., Štambuk, J., Trbojević-Akmačić, I., Barrios, C., Pavić, T., Menni, C., Wang, Y., Zhou, Y., Cui, L., Song, H., Zeng, Q., Guo, X., Pons-Estel, B.A., McKeigue, P., Leslie Patrick, A., Gornik, O., Spector, T.D., Harjaček, M., Alarcon-Riquelme, M., Molokhia, M., Wang, W., Lauc, G., 2015. Association of Systemic Lupus Erythematosus With Decreased Immunosuppressive Potential of the IgG Glycome. Arthritis Rheumatol. 67, 2978–2989. https://doi.org/10.1002/art.39273

Wahrenbrock, M.G., Varki, A., 2006. Multiple Hepatic Receptors Cooperate to Eliminate Secretory Mucins Aberrantly Entering the Bloodstream: Are Circulating Cancer Mucins the "Tip of the Iceberg"? Cancer Res. 66, 2433–2441. https://doi.org/10.1158/0008-5472.CAN-05-3851

Washburn, N., Schwab, I., Ortiz, D., Bhatnagar, N., Lansing, J.C., Medeiros, A., Tyler, S., Mekala, D., Cochran, E., Sarvaiya, H., Garofalo, K., Meccariello, R., Meador, J.W., Rutitzky, L., Schultes, B.C., Ling, L., Avery, W., Nimmerjahn, F., Manning, A.M., Kaundinya, G.V., Bosques, C.J., 2015. Controlled tetra-Fc sialylation of IVIg results in a drug candidate with consistent enhanced anti-inflammatory activity. Proc. Natl. Acad. Sci. 112. https://doi.org/10.1073/pnas.1422481112

Weigel, P.H., 1994. Hypothesis: Galactosyl and N-acetylgalactosaminyl homeostasis: A function for mammalian asialoglycoprotein receptors. BioEssays 16, 519–524. https://doi.org/10.1002/bies.950160713

Wu, G., Hitchen, P.G., Panico, M., North, S.J., Barbouche, M.-R., Binet, D., Morris, H.R., Dell, A., Haslam, S.M., 2016. Glycoproteomic studies of IgE from a novel hyper IgE syndrome linked to PGM3 mutation. Glycoconj. J. 33, 447–456. https://doi.org/10.1007/s10719-015-9638-y

Wurzburg, B.A., Garman, S.C., Jardetzky, T.S., 2000. Structure of the Human IgE-Fc Cε3-Cε4

A References

Reveals Conformational Flexibility in the Antibody Effector Domains. Immunity 13, 375–385. https://doi.org/10.1016/S1074-7613(00)00037-6

Xie, M.M., Bertozzi, C.R., Wang, T.T., 2020. Immunoglobulin E sialylation regulates allergic responses. Immunol. Cell Biol. 98, 617–619. https://doi.org/10.1111/imcb.12368

Yamada, E., Tsukamoto, Y., Sasaki, R., Yagyu, K., Takahashi, N., 1997. [No title found]. Glycoconj. J. 14, 401–405. https://doi.org/10.1023/A:1018582930906

Yet, M.G., Chin, C.C., Wold, F., 1988. The covalent structure of individual N-linked glycopeptides from ovomucoid and asialofetuin. J. Biol. Chem. 263, 111–117. https://doi.org/10.1016/S0021-9258(19)57364-1

Yik, J.H.N., Saxena, A., Weigel, P.H., 2002. The Minor Subunit Splice Variants, H2b and H2c, of the Human Asialoglycoprotein Receptor Are Present with the Major Subunit H1 in Different Hetero-oligomeric Receptor Complexes. J. Biol. Chem. 277, 23076–23083. https://doi.org/10.1074/jbc.M202748200

Yin, Y., Bai, Y., Olivera, A., Desai, A., Metcalfe, D.D., 2017. An optimized protocol for the generation and functional analysis of human mast cells from CD34 + enriched cell populations. J. Immunol. Methods 448, 105–111. https://doi.org/10.1016/j.jim.2017.06.003

Young, R.J., Owens, R.J., Mackay, G.A., Chan, C.M.W., Shi, J., Hide, M., Francis, D.M., Henry, A.J., Sutton, B.J., Gould, H.J., 1995. Secretion of recombinant human IgE-Fc by mammalian cells and biological activity of glycosylation site mutants. Protein Eng. Des. Sel. 8, 193–199. https://doi.org/10.1093/protein/8.2.193

Yu, J.E., Lin, R.Y., 2018. The Epidemiology of Anaphylaxis. Clin. Rev. Allergy Immunol. 54, 366–374. https://doi.org/10.1007/s12016-015-8503-x

Zha, L., Leoratti, F.M.S., He, L., Mohsen, M.O., Cragg, M., Storni, F., Vogel, M., Bachmann, M.F., 2018. An unexpected protective role of low-affinity allergen-specific IgG through the inhibitory receptor FcγRIIb. J. Allergy Clin. Immunol. 142, 1529-1536.e6. https://doi.org/10.1016/j.jaci.2017.09.054

Zissler, U.M., Schmidt-Weber, C.B., 2020. Predicting Success of Allergen-Specific Immunotherapy. Front. Immunol. 11, 1826. https://doi.org/10.3389/fimmu.2020.01826

Zubeldia, J., Ferrer, M., Dávila, I., Justicia, J., 2019. Adjuvants in Allergen-Specific Immunotherapy: Modulating and Enhancing the Immune Response. J. Investig. Allergol. Clin. Immunol. 29, 103–111. https://doi.org/10.18176/jiaci.0349

Zuberi, R.I., Hsu, D.K., Kalayci, O., Chen, H.-Y., Sheldon, H.K., Yu, L., Apgar, J.R., Kawakami, T., Lilly, C.M., Liu, F.-T., 2004. Critical Role for Galectin-3 in Airway Inflammation and Bronchial Hyperresponsiveness in a Murine Model of Asthma. Am. J. Pathol. 165, 2045–2053. https://doi.org/10.1016/S0002-9440(10)63255-5

B Supplements

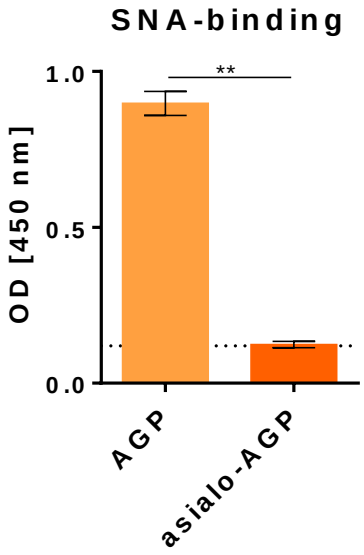

Figure 29: Desialylation of α1-acid glycoprotein (AGP).

*AGP-desialylation was verified by SNA-lectin ELISA. ELISA plates coated with 2 µg mL^{-1} AGP-glycoforms were incubated with HRP-conjugated sambucus nigra (SNA) lectin for detection of terminal sialic acids. Absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM. **P < 0.01 by two-tailed t-test. The dotted line shows the background signal of the plate.*

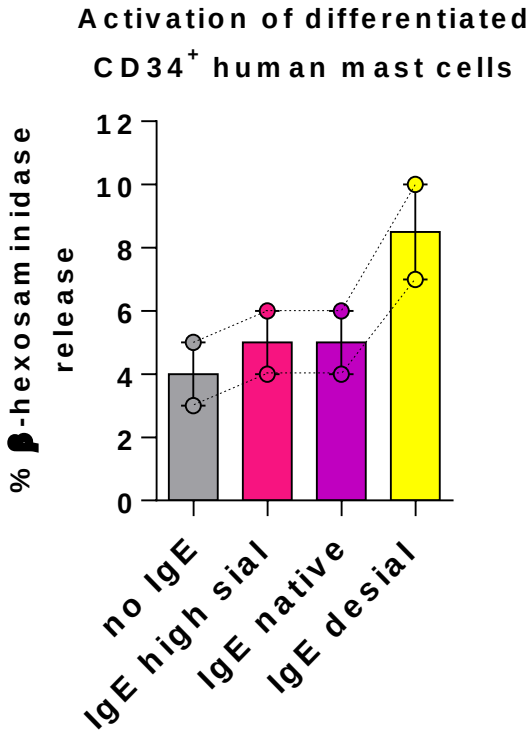

Figure 30: Activation of differentiated human CD34$^+$ mast cells.

Human mast cells were differentiated from CD34$^+$ blood cells. Cells were sensitized with 0.2 µg mL^{-1} of the different anti-TNP IgE glycoforms and stimulated with 0.2 µg mL^{-1} TNP-OVA 20 h later. Mast cell activation was analyzed by β-hexosaminidase release. The figure includes data of two independent experiments (data of each experiment are connected by a dotted line). Experiments were conducted by Leonie Gädert and Charline Sommer from the research group of Dr. Susann Dehmel at the Fraunhofer ITEM.

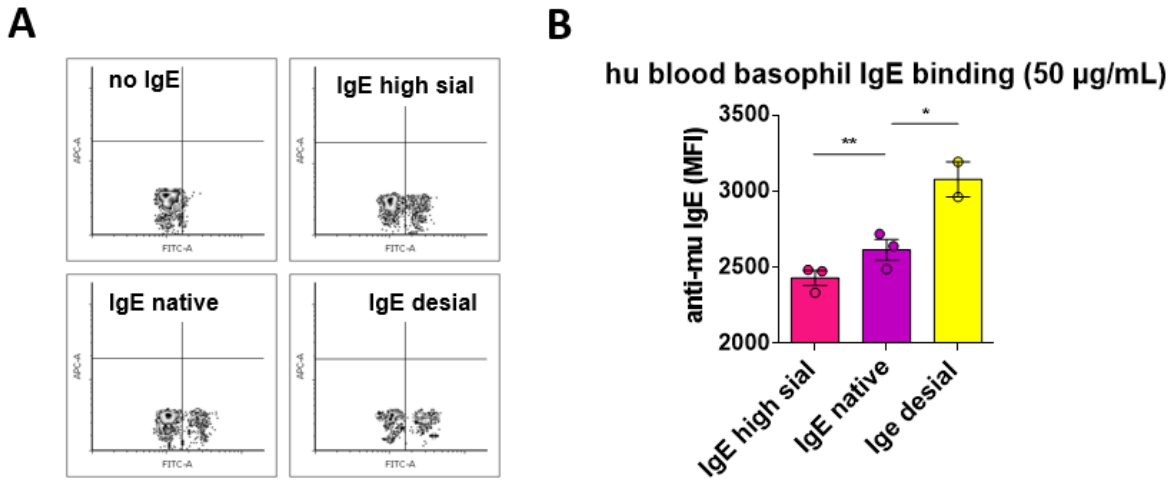

Figure 31: Sensitization of human blood basophils with different IgE glycoforms.

(A) *Human blood basophil sensitization was analyzed by flow cytometry. Human blood cells were incubated with 50 µg mL^{-1} of different IgE glycoforms for 1 h. Basophils were identified as FcεRIα^{+} CD203c^{+} cells and IgE-binding was analyzed using an anti-mouse IgE antibody.* ***(B)*** *The Median Flourescence Intensity (MFI) of the labeled anti-mouse IgE antibody on basophils sensitized with different IgE glycoforms is shown. Data are represented as mean + SEM, *P < 0.05 and **P < 0.01 by one-way ANOVA. Experiments were conducted by Jochen Behrends and Theresa Walsemann from the research group of Prof. Dr. Uta Jappe at the Research Center Borstel.*

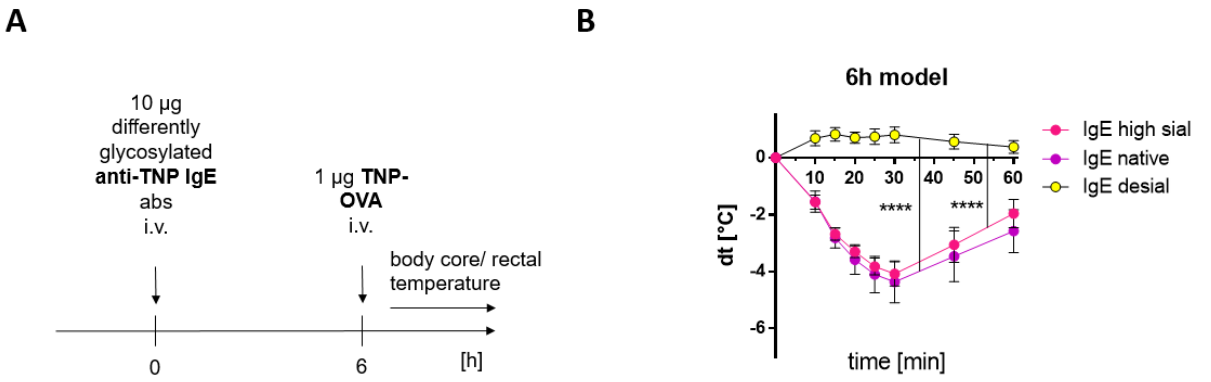

Figure 32: Sialylated IgE glycoforms induce systemic anaphylaxis 6 hours after sensitization.

(A) *Experimental setup of the IgE-mediated passive systemic anaphylaxis. Mice were sensitized i.v. with 5 µg anti-TNP IgE antibodies and challenged i.v. with 1 µg TNP-OVA 6 hours later.* ***(B)*** *The severity of the anaphylactic reaction was measured as the drop of the body core/ rectal temperature upon TNP-OVA challenge. Data are presented as mean +/- SEM, n = 5 for all groups. One of at least two independent experiments is shown. ****P < 0.0001 by two-way ANOVA with multiple comparisons.*

B Supplements

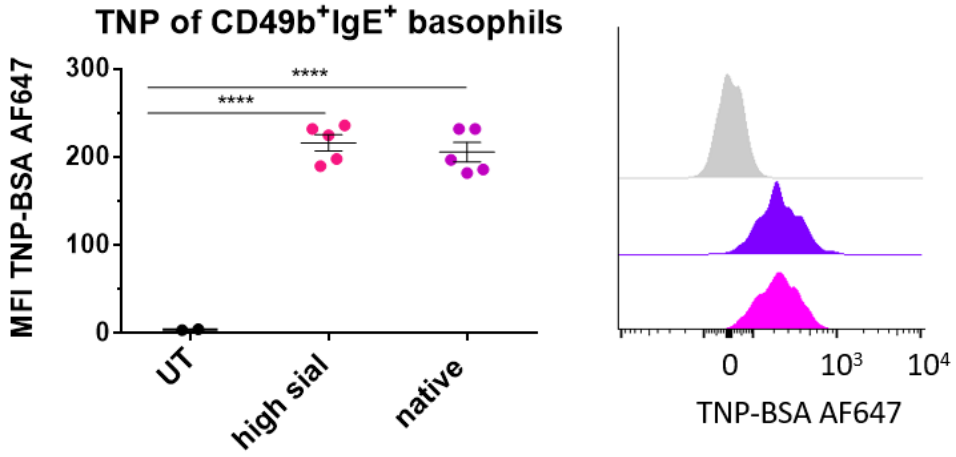

Figure 33: Sensitization of blood basophils with anti-TNP IgE antibodies.

*Mice were sensitized with 10 µg anti-TNP IgE antibodies and blood was taken 6 hours later. Blood cells were pre-gated on single, viable leucocytes (s. Figure 12) and identified as CD49b$^+$IgE$^+$ cells (s. Figures 23 and 25). The Median Flourescence Intensity (MFI) of the labeled antigen TNP-BSA of untreated or anti-TNP IgE-sensitized mice in CD49b$^+$IgE$^+$ basophils is shown including overlay histograms of the allergen binding to surface IgE from representative mice of each group including unsensitized controls (grey). Individual symbols represent individual mice. One of at least two independent experiments is shown. ****P < 0.00001, by one-way ANOVA.*

C List of figures

D List of tables

E Abbreviations

2-AB	2-aminobenzamide
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
AGP	α1-acid glycoprotein
AIT	Allergen immunotherapy
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASGP-R	Asialo-glycoprotein receptor
Asn	Asparagine
BAL	Bronchoalveolar lavage
BSA	Bovine Serum Albumin
C	Constant
CD	Cluster of differentiation
CMP	Cytidine monophosphate
COVID-19	Coronavirus disease 2019
CRD	Carbohydrate recognition domain
D	Diversity
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
DMSO	Dimethylsulfoxide
eCFA	enriched complete Freund adjuvant
EDTA	Ethylenediamintetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EndoS	Endoglycosidase S
Fab	Antigen-binding fragment
FACS	Flourescence activated cell sorting
Fc	Fragment crystallizable
FcR	Fc receptor
FCS	Fetal calf serum
fMLP	N-formyl-1-methionyl-1-leucyl-phenylalanine
G	Galactose
G0	Degalactosylated
GlcNAc	N-acetylglucosamine

E Abbreviations

H	Heavy
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
i.p.	Intraperitoneal
i.v.	Intravenous
ICON	International consensus on
IFA	Incomplete Freund adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukine
ISS	Immunomodulatory sequences
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IVIG	Intravenous Immunglobulin
J	Joining
L	Light
MACS	Magnetic activated cell sorting
MALDI-TOF	Matrix assisted laser ionization/ desorption – time of flight
MCT	Microcrystalline tyrosine
MES	2-(N-morpholino)ethanesulfonic acid
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
MPLA	Monophosphoryl lipid A
NMBA	Neuromuscular blocking agent
OD	Optical density
OIT	Oral immunotherapy
OVA	Ovalbumin
PAF	Platelet-activating factor
PBS	Phosphate Buffered Saline
PBMC	Peripheral blood mononuclear cells
PBST	PBS tween
PCA	Passive cutaneous anaphylaxis
PNAG	p-nitrophenyl N-acetyl-β-D-glucosaminide
PSA	Passive systemic anaphylaxis

E Abbreviations

RBL	Rat basophilic leukemia
rhSCF	recombinant human stem cell factor
S	Sialic acid
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCIT	Subcutaneous immunotherapy
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIGN-R1	Specific ICAM-3 grabbing non-integrin-related 1
SLIT	Sublingual immunotherapy
SNA	*Sambucus nigra*
STAT	Signal transducer and activator of transcription
SWE	Squalene water emulsion
TGF	Transforming growth factor
Th	T helper cell
TMB	3,3',5,5'-Tetramethylbenzidin
TNF	Tumor necrosis factor
TNP	2,4,6 Trinitrophenyl
UDP	Uridine diphosphate
V	Variable
wt	Wild type

F Acknowledgements

At this point, I want to thank the people, who supported me during my time as a PhD student with their time and effort.

First of all, I would like to thank my supervisor, Prof. Dr. Marc Ehlers, for giving me the opportunity to conduct this work in his laboratory. Thank you for many constructive discussions about experiments and results, new input and ideas and not least a piece of advice whenever I needed one.

A special thank goes to Leonie Gädert, Charline Sommer and Dr. Susan Dehmel from the Fraunhofer ITEM for supporting my data with the experiments on human differentiated mast cells and to Theresa Walsemann, Jochen Behrends and Prof. Dr. Uta Jappe from the Forschungszentrum Borstel for conducting the experiments on human blood basophils.

In addition, I would like to thank all my current and former colleagues. Janina Petry and Johann Rahmöller, thank you for welcoming me in the group and training me on this project. You have always been there for me offering a helping hand and answering every question with great patience. Carsten Kern, thank you for your support and contribution to this work with the HPLC analyses of the antibodies. In addition, I want to thank my former student Clarissa Pfeufer who contributed with great diligence to this work during her internship at our laboratory. Thank you for your hard work and always being positive, I really enjoyed working with you.

I am beyond greatful to my colleagues Hanna Lunding, Jana Buhre and Selina Lehrian who have been with me the whole time. You supported me in my experiments, we shared anger and frust but more importantly progress and success with each other. We mastered all the challenges that came with the corona pandemic but most importantly we could always laugh together. You made the laboratory a great place to work every day.

Finally, I want to thank my family and friends: My friends who accompanied and encouraged me throughout my doctoral program. My parents for always keeping me grounded and making home a safe place to gather new energy. My sister who knows me so well and always finds the right words and especially Basti for his love and support that gave me the strength and stamina to accomplish this project. I owe every accomplishment to you.

G Curriculum Vitae

Lara Dühring

born February 10th, 1995

in Buxtehude, Germany

Education

Oct. 2017 – Sept. 2019	**M.Sc. Microbiology and Biochemistry**
	University of Rostock, Germany
	Final term dissertation title: Investigations on the influence of *Streptococcus gallolyticus* on patient-derived carcinoma cells
Oct. 2014 – Jun. 2017	**B.Sc. Biological Sciences**
	University of Rostock, Germany
	Final term dissertation title: Influence of mechanoreceptors of the wing on structure and success of the *Drosophila* courtship song
Jul. 2005 – Jun. 2013	**Higher Education Entrance Qualification**
	Gymnasium Athenaeum, Stade

Work Experience

Jan. 2019 – Mar. 2019	**University of Rostock, Germany**
	Student assistant, department of medical microbiology
Sept. 2018 – Oct. 2018	**Elbeklinikum Buxtehude, Germany**
	Internship, department of molecular cell biology

Languages

German	Native language
English	Fluently spoken

G Curriculum Vitae