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The role of IgG sialylation in the attenuation of anaphylactic reactions

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Abstract

Allergen-specific IgE antibodies can induce allergic reactions, e.g. systemic anaphylaxis, by cross-linking the high-affinity FcεRI on mast cells and basophils, leading to cell activation and the release of inflammatory mediators. By contrast, allergen-specific immunotherapy (AIT)-induced IgG antibodies have been demonstrated to provide protection in IgE-mediated allergic reactions through allergen masking as well as cross-linking of FcεRI with the IgG inhibitory receptor FcγRIIB. However, at high antigen doses IgG antibodies themselves can cause anaphylaxis in mice by driving FcγR-induced activation of myeloid cells.

FcγR-dependent effector functions of IgG antibodies are influenced by their subclass as well as their Fc N-glycosylation pattern. In this regard, enrichment of non-galactosylated (agalactosylated) IgG antibodies has been observed in patients suffering from autoimmune diseases and is generally associated with pro-inflammatory immune responses. By contrast, galactosylated plus sialylated IgG antibodies have been linked to disease remission and are assumed to mediate anti-inflammatory immune responses in the context of intravenous immunoglobulin (IVIG, pooled serum IgG from healthy donors), which is used in high concentrations to treat inflammatory autoimmune diseases.

The main aim of this thesis was to investigate mechanisms how different IgG subclass Fc N-glycosylation patterns might modify an IgG-mediated anaphylactic reaction as well as possible influences on IgG-controlled IgE-mediated anaphylaxis.

I could show that enrichment of total serum IgG antibody sialylation with high amounts of IVIG or lower amounts of sialylated antigen-unspecific IgG1 antibodies attenuated IgG-mediated anaphylaxis compared to degalactosylated IgG1 antibodies in mice. Mechanistically, sialylated IgG1 antibodies induced via the C-type lectin receptor SignR1 the up-regulation of FcγRIIB on blood monocytes/ macrophages. Interestingly, sialylated antigen-unspecific IgG2a antibodies failed to mediate this inhibitory mechanism, suggesting an IgG subclass specific effect. Similar mechanisms might also play a role in an observed enhanced capacity of antigen-specific IgG1 antibodies to block IgE-mediated anaphylaxis upon pre-treatment with high amounts of IVIG.

The results suggest that SignR1-dependent up-regulation of inhibitory FcγRIIB via sialylated murine IgG1 antibodies can prevent IgG-mediated anaphylaxis as well as improve the blocking capacity of IgG antibodies in IgE-mediated anaphylaxis.

Accordingly, in humans a correlation between the level of total serum IgG antibody sialylation and the level of FcγRIIB expression on myeloid cells could be shown.

Altogether, the data suggest that enhancing the level of the sialylated fraction of total serum IgG antibodies might alter the ratio of activating to inhibitory FcγRs, and thus, might be an efficient tool to attenuate IgG- and IgE-mediated reactions in patients suffering from severe allergic diseases. Parts of the presented experiments have already been published in the peer-reviewed journal *The Journal of Allergy and Clinical Immunology* (Epp *et al.*, 2018).

Zusammenfassung

Allergen-spezifische IgE Antikörper können allergische Reaktionen, z.B. systemische Anaphylaxie, durch die Kreuzvernetzung des hochaffinen FcεRI auf Mastzellen und Basophilen und deren Freisetzung von Entzündungsmediatoren auslösen. Im Gegensatz dazu können IgG Antikörper, z.B. induziert durch allergen-spezifische Immuntherapien (AIT), IgE-vermittelte allergische Reaktionen sowohl durch Antigenmaskierung wie auch durch Kreuzvernetzung des FcεRI mit dem inhibitorischen IgG Rezeptor FcγRIIB inhibieren. Allerdings wurde in Mausstudien gezeigt, dass bei hohen Antigendosen IgG Antikörper selbst anaphylaktische Reaktionen durch eine FcγR-vermittelte Aktivierung von myeloiden Zellen auslösen können.

FcγR-abhängige Effektorfunktionen von IgG Antikörpern werden sowohl durch deren Subklasse als auch Fc N-Glykosylierungsmuster beeinflusst. Diesbezüglich konnte gezeigt werden, dass Patienten mit Autoimmunerkrankungen vermehrt nicht-galaktosylierte (agalaktosylierte) IgG Antikörper aufweisen, welche daher mit proinflammatorischen Immunantworten assoziiert werden. Im Gegensatz dazu werden galaktosylierte plus sialylierte IgG Antikörper mit einer Verbesserung des Krankheitsbildes in Verbindung gebracht und es wird vermutet, dass diese die anti-inflammatorische Wirkung des intravenösen Immunglobulins (IVIG, gepooltes Serum IgG von gesunden Spendern), das in hohen Dosen zur Behandlung von entzündlichen Autoimmunerkrankungen eingesetzt wird, vermitteln.

Das Hauptziel dieser Arbeit war es Mechanismen zu untersuchen, wie das IgG Fc N-Glykosylierungsmuster verschiedener IgG Subklassen eine IgG-vermittelte anaphylaktische Reaktion beeinflusst, und in wie fern diese eine IgG-kontrollierte IgE-vermittelte anaphylaktische Reaktion beeinflussen.

Es konnte in Mausexperimenten gezeigt werden, dass die Anreicherung von sialylierten IgG Antikörpern im Serum durch hohe Dosen IVIG oder geringere Dosen sialylierter antigen-unspezifischer IgG1 Antikörper eine IgG-vermittelte Anaphylaxie im Vergleich zu degalaktosylierten antigen-unspezifischen IgG1 Antikörpern abschwächt. Mechanistisch betrachtet, induzierten sialylierte IgG1 Antikörper über den C-Typ Lektin Rezeptor SignR1 eine Hochregulation des FcγRIIB auf Monozyten/ Makrophagen im Blut. Interessanterweise konnten sialylierte antigen-unspezifische IgG2a Antikörper diese inhibitorischen Mechanismen nicht vermitteln, was auf eine IgG Subklassen spezifische Funktion hindeutet. Ähnliche Mechanismen könnten auch für eine beobachtete verstärkte Inhibition

einer IgE-vermittelte Anaphylaxie durch antigen-spezifische IgG1 Antikörper bei einer Vorbehandlung mit hohen Dosen an IVIG von Bedeutung sein.

Die Ergebnisse deuten darauf hin, dass eine SignR1-abhängige Hochregulation des inhibitorischen FcγRIIB durch sialylierte murine IgG1 Antikörper sowohl eine IgG-vermittelte Anaphylaxie verhindern als auch die Fähigkeit von IgG Antikörpern eine IgE-vermittelte Anaphylaxie zu blockieren, verbessern kann. Dementsprechend konnte in ersten humanen Blutanalysen eine Korrelation zwischen dem Level an sialyliertem Gesamt-IgG und dem Expressionslevel des FcγRIIB auf myeloiden Zellen gezeigt werden.

Insgesamt weisen die Daten darauf hin, dass ein erhöhter Anteil an sialylierten IgG Antikörpern im Serum eine Verschiebung des Verhältnisses von aktivierenden zu hemmenden FcγRs bewirken und somit ein effizientes Mittel zur Abschwächung von IgG- und IgE-vermittelten allergischen Reaktionen bei Patienten sein könnte. Teile der gezeigten Ergebnisse wurden bereits in der „peer-review“ Zeitschrift *The Journal of Allergy and Clinical Immunology* veröffentlicht (Epp *et al.*, 2018).

1 Introduction

1.1 Allergic diseases

In our everyday life, we encounter a number of pathogens, whereby our immune system initiates reactions to protect us against these environmental threats (e.g. bacteria, viruses or parasites). At the same time, it is equally important for our immune system to prevent excessive responses towards harmless stimuli, such as the body's own tissues or environmental substances like food, pollen or drugs. Any imbalance between immune protection and tolerance can lead to autoimmune or allergic diseases (Warrington *et al.*, 2011; Murphy *et al.*, 2012; Nicholson, 2016).

The term allergy or allergic disease refers to an abnormal adaptive immune response that is directed towards innocuous and non-infectious substances - referred to as allergens - in predisposed individuals. Concerningly, the prevalence of allergic diseases, including atopic dermatitis, rhinitis, allergic asthma, food allergies as well as insect and drug allergies is large and steadily increasing in developed countries. The considerable impact on patients' quality of life as well as the increasing burden to healthcare costs are demanding further therapeutic strategies (Ono, 2000; Kay, 2001; De Monchy *et al.*, 2013; Ring *et al.*, 2014).

In order to gain a better understanding of the underlying immune mechanisms the immunologists Coombs and Gell (1963) categorized hypersensitivity reactions into four different types. This classification has been extended over the years and is widely accepted (Figure 1). Type I (= immediate hypersensitivity) reactions are classically attributed to immunoglobulin (Ig)E-mediated immune responses driven by mast cells and basophils and the rapid release of preformed inflammatory mediators, such as histamine. On the other hand, non-IgE-mediated reactions are historically classified as type II-IV and show a delayed onset of symptoms. Both, type II and III hypersensitivities are described to be mediated by antibodies of the IgG and IgM antibody isotype. In type II reactions, antibodies recognize cell surface antigens (e.g. self-antigens or drugs bound to cell membrane) leading to cell cytotoxicity. Cell lysis is caused by either effector cells, such as macrophages and neutrophils, which become activated due to the engagement of cell bound antibodies with their Fc receptors (FcRs) or directly by the activation of the complement system. On the other hand, in type III reactions tissue injury is induced by the

formation and deposition of soluble immune complexes and the subsequent activation of complement and the recruitment of inflammatory cells. Type IV reactions are not mediated by antibodies; instead, immune reactions are triggered by the activation of allergen-specific T cells and the release of inflammatory cytokines. Furthermore, non-immunological mechanisms leading to a direct activation of mast cells and basophils have been described. However, in this thesis I will focus on type I/ immediate hypersensitivity reactions that can lead to anaphylaxis (Descotes and Choquet-Kastylevsky, 2001; Schnyder and Pichler, 2009; Murphy *et al.*, 2012; Baldo and Pham, 2013).

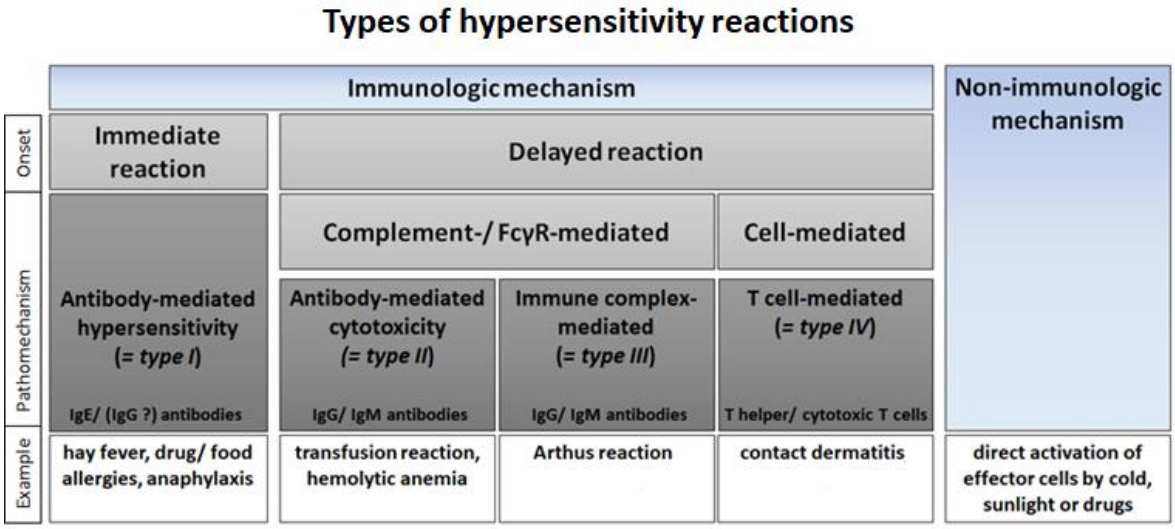

Figure 1: Classification of hypersensitivity reactions based on Gell and Coombs (1963). *Modified according to Descotes and Choquet-Kastylevsky (2001), Finkelman et al. (2016), Pichler and Hausmann (2016).*

1.1.1 Pathomechanisms of immediate hypersensitivity reactions

The development of allergies requires prior sensitization to a certain allergen (Figure 2). Allergens may enter the body through the skin, through inhalation, ingestion via the gastrointestinal tract or injections (e.g. insect stings, drugs). Antigen presenting cells, such as dendritic cells (DCs), sample these allergens and migrate to regional lymph nodes where they process and present peptides in the context of major histocompatibility complex (MHC) class II molecules to naïve CD4+ T helper (Th) cells. Upon recognition, Th cells undergo proliferation and differentiation into different subsets (e.g. Th1, Th2, Th17, regulatory T cells) with characteristic transcription factors, cytokine profiles and distinct roles in orchestrating immune reactions. Type I/ immediate hypersensitivity reactions are classically attributed to IgE-mediated immune responses. Here, polarization of naïve CD4+

Th cells towards Th2 cells through activation of the transcription factors STAT6 and GATA-3 facilitates the secretion of cytokines such as interleukine (IL)-4 and IL-13. In turn, these cytokines favor an immunoglobulin class switching in activated B cells and the production of IgE antibodies (Kay, 2001; Galli *et al.*, 2008; Zhu and Paul, 2008; Stone *et al.*, 2010; Dullaers *et al.*, 2012; Valenta *et al.*, 2015; Oettgen, 2016).

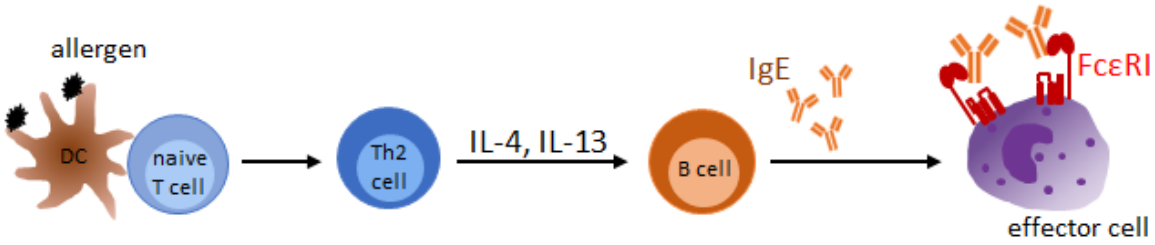

Figure 2: Sensitization phase during an allergic response. *Allergen uptake and presentation by DCs facilitates differentiation of naïve CD4+ T helper (Th) cells to Th2 cells. Subsequent secretion of cytokines such as IL-4 and IL-13 favor an immunoglobulin class switching and the production of IgE antibodies by B cells. IgE antibodies bind to the high-affinity FcεRI on effector cells, such as mast cells and basophils.*

In healthy individuals, serum levels of IgE antibodies are very low (50-200 ng/ mL, compared with 5-10 mg/ mL IgG) and its half-life is rather short (1-2 days, compared with 3 weeks for IgG antibodies). However, in allergic patients IgE serum levels are increased and binding to its high-affinity FcR, FcεRI, on mast cells and basophils stabilizes the IgE-receptor complex on the cell surface. Upon re-encounter of the allergen cross-linking of FcεRI by allergen-specific IgE antibodies leads to an activation of mast cells and basophils (Kay, 2001; Galli *et al.*, 2008; Zhu and Paul, 2008; Stone *et al.*, 2010; Dullaers *et al.*, 2012; Valenta *et al.*, 2015; Oettgen, 2016).

Interestingly, the degree of effector cell activation, and thus, the severity of the allergic response can be modulated by allergen-specific IgG antibodies. Several studies have demonstrated the ability of IgG antibodies to suppress IgE-mediated responses. Thereby, allergen-specific IgG antibodies competing for allergen binding prevent IgE-mediated cross-linking of FcεRI, and thus, suppress the activation of effector cells. In addition, it has been shown that allergen-specific IgG antibodies can inhibit IgE-mediated cell activation in a FcγRIIB-dependent manner. Here, cross-linking of FcεRI to the inhibitory IgG receptor, FcγRIIB, abrogated effector cell degranulation and downstream cytokine synthesis (Strait *et al.*, 2006; Epp *et al.*, 2018; Burton *et al.*, 2017; Zha *et al.*, 2018).

Activation of mast cells and basophils by allergen-specific IgE antibodies causes the release of preformed vasoactive mediators (e.g. histamine, tryptase, heparin) as well as

newly synthesized cytokines (TNF-a, IL-8) and lipid mediators (e.g. leukotrienes and prostaglandins) which elicit smooth muscle constriction, an increased vascular permeability and the recruitment of inflammatory cells. The immediate symptoms of an allergic reaction are diverse and vary depending on the site of the reaction, ranging from skin reactions (redness, itching), edema, hypersecretion of mucus and wheezing up to life-threatening hypotension and anaphylaxis (Kay, 2001; Galli *et al.*, 2008; Zhu and Paul, 2008; Stone *et al.*, 2010; Dullaers *et al.*, 2012; Valenta *et al.*, 2015; Oettgen, 2016).

1.1.2 Antibody-mediated anaphylaxis

Anaphylaxis is defined as a severe and systemic allergic reaction characterized by the rapid onset and progression of symptoms that are potentially life-threatening. Unfortunately, anaphylactic reactions are increasing in frequency and only little progress has been made concerning diagnostic markers and treatment options. In children, food allergens are responsible for most anaphylactic reactions accounting for up to 81% of incidents, whereas in adults drug-induced anaphylaxis is more common (Finkelman, 2007; Simons *et al.*, 2014; Muñoz-Cano *et al.*, 2016; Montañez *et al.*, 2017; Yu and Lin, 2015).

Classically, anaphylaxis is associated with allergen-specific IgE antibodies together with FcεRI-expressing mast cells and basophils as key players, as described above (and in Figure 3). The involvement and importance in anaphylactic reactions of either one of them has been described (Dombrowicz *et al.*, 1993; Feyerabend *et al.*, 2011; Reber *et al.*, 2013; Korosec *et al.*, 2017).

However, IgE antibodies do not appear accountable for all cases of anaphylaxis. Induction of active systemic anaphylaxis in IgE-, FcεRI- or mast cell-deficient mice have provided first evidence of an alternative pathway of anaphylaxis (Jacoby *et al.*, 1984; Oettgen *et al.*, 1994; Dombrowicz *et al.*, 1997; Finkelman, 2007). Accordingly, human cases of drug-induced anaphylaxis (e.g. by analgesics, antibiotics, dextran or therapeutic monoclonal antibodies) without detectable allergen-specific IgE antibodies and without apparent mast cell activation (measured by serum tryptase levels) have been described. Instead, in these patients the severity of the anaphylactic response correlated with serum levels of allergen-specific IgG antibodies, further supporting the notion of an IgE-independent mechanism. Since then, several experimental mouse studies have described an alternative pathway of anaphylaxis mediated by antigen-specific IgG antibodies (when antigen doses are high)

and driven by FcγR-induced activation of myeloid cells, i.e. neutrophils, macrophages and/ or basophils (Figure 3; Kraft *et al.*, 1982; Finkelman, 2007; Steenholdt *et al.*, 2012; Finkelman *et al.*, 2016).

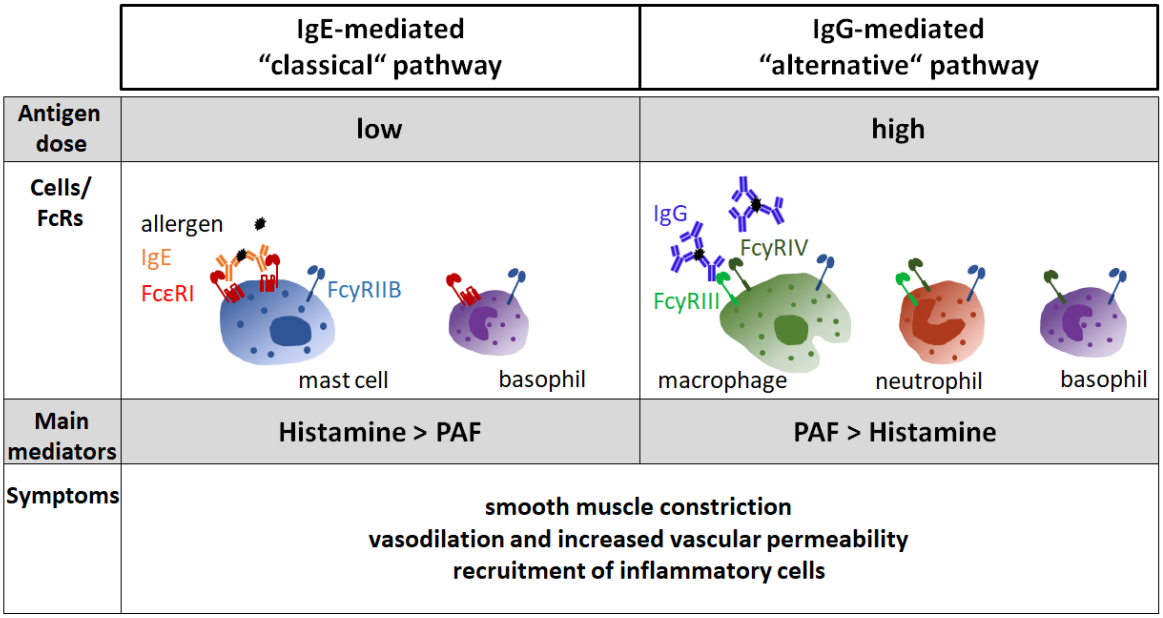

Figure 3: Pathways of anaphylaxis. *During IgE-mediated anaphylaxis allergen-specific IgE antibodies bind to high-affinity FcεRI expressed on mast cells and basophils leading to receptor cross-linking and cell activation upon antigen encounter. The release of preformed vasoactive mediators (e.g. histamine) induces anaphylactic reactions. In addition, an alternative pathway of anaphylaxis has been proposed that is mediated by soluble immune complexes of allergen-specific IgG antibodies and driven by FcγRIII and/ or FcγRIV-induced activation of macrophages, neutrophils and/ or basophils when allergen doses are high. In the course of activation, these cells release the vasoactive mediator platelet-activating factor (PAF), which induces symptoms of anaphylaxis. Clinical symptoms of systemic anaphylaxis induced by either IgE or IgG antibodies are indistinguishable.*

During IgG-mediated anaphylaxis antigen-specific IgG antibodies form immune complexes with the corresponding antigen and bind to activating FcγRs on effector cells. Here, FcγRIII and FcγRIV expressed by monocytes/ macrophages, neutrophils and basophils have been identified as key players of murine IgG-mediated anaphylactic reactions, whereby their relative importance varied depending on the respective model used (Bruhns and Jönsson, 2015; Beutier *et al.*, 2016).

Nevertheless, IgG-dependent anaphylaxis in humans is debated. Further data supporting the existence of this alternative pathway of anaphylaxis in humans has been provided by a recent study from Muñoz Cano and colleagues, who analyzed patients with lipid transferase protein (LTP, a common food allergen)-induced anaphylaxis. Patients showed elevated levels of allergen-specific IgG1 and IgG3 antibodies as well as increased

expression of activating FcγRs and activation markers on neutrophils (Bruhns and Jönsson, 2015; Muñoz Cano *et al.*, 2016).

Furthermore, it has been demonstrated that in the course of activation monocytes/ macrophages, neutrophils and basophils release the vasoactive mediator platelet-activating factor (PAF). In several murine studies, PAF is reported to be the main mediator of IgG-mediated anaphylaxis, whereas histamine seems to be more important in IgE-mediated anaphylaxis. Blocking of PAF prevented IgG-mediated anaphylaxis in mice. Moreover, in patients it was demonstrated that PAF levels correlated with anaphylaxis severity. However, PAF is also produced by mast cells and monocytes/ macrophages show an increased expression of FcεRI in allergic individuals (Maurer *et al.*, 1994; Vadas *et al.*, 2008; Bruhns and Jönsson, 2015). Both findings may argue that in humans PAF, like histamine, is associated with IgE-mediated anaphylaxis. Nevertheless, human effector cells and their FcγR profile seem to be potential candidates in an alternative pathway of antibody-mediated anaphylaxis. In particular, it has been argued that IgG-mediated anaphylaxis may be of great importance in human cases of drug-induced anaphylaxis. Here, patients often show anaphylactic reactions after the intravenous administration of large doses of the drug. Also in mice, the induction of IgG-mediated anaphylaxis requires high antigen doses (Arias *et al.*, 2011; Jönsson *et al.*, 2011; Bruhns, 2012; Jiao *et al.*, 2014; Gill *et al.*, 2015; Finkelman *et al.*, 2016).

Taken together, besides the classical IgE-mediated pathway several studies have described an alternative pathway of anaphylaxis mediated by antigen-specific IgG antibodies and driven by FcγR-induced activation of myeloid cells. In mice - and potentially in humans - the symptoms of systemic anaphylaxis induced by either IgE or IgG antibodies are indistinguishable, which makes it even more important to further investigate the underlying pathomechanisms in order to avoid such adverse reactions and to improve therapeutic approaches.

1.2 Allergen-specific immunotherapy

Therapeutic approaches, such as epinephrine, corticosteroids or anti-histamines, provide symptomatic relief for patients suffering from severe allergic reactions, although they do not prevent recurring anaphylactic episodes. By contrast, allergen-specific immunotherapy (AIT) is the only treatment regimen to date with the potential to modulate the course of

IgE-mediated allergic diseases and induce lasting improvement. Thereby, repeated subcutaneous or sublingual administration of increasing allergen doses is used, aiming to induce peripheral tolerance. Different mechanisms have been proposed to be critical for tolerance induction by AIT: whereas allergic immune responses are dominated by IL-4- and IL-13-producing Th2 cells that promote the secretion of allergen-specific IgE antibodies, a shift towards Th1 and inducible type 1 regulatory T (Tr1) cells is observed in the course of AIT. For example, differentiation of antigen-specific regulatory T cells could be associated with the remission of cow's milk allergy in children. Furthermore, regulatory T cells produce cytokines such as IL-10 and TGF-β, which not only suppress the production of total and allergen-specific IgE antibodies but contribute to immunoglobulin class switching to IgG and IgA antibodies. In particular, a persistent increase in human IgG4 antibody levels has been observed in the course of AIT (Figure 4; Karlsson et al., 2004; Larché et al., 2006; Sampson et al., 2006; Meiler et al., 2008; Möbs et al., 2012; Burks et al., 2013; Akdis and Akdis, 2015; Jutel et al., 2016; Epp et al., 2018).

The development of natural tolerance provided by increased levels of allergen-specific IgG antibodies, especially IgG4, is also observed in non-allergic individuals with prolonged exposure to a certain allergen. This could be demonstrated in beekeepers as well as children exposed to cat allergens or sensitized to peanut allergens. Here, individuals present allergen-specific IgE antibodies, albeit they do not show any symptoms of allergy (Aalberse *et al.*, 1983; Platts-Mills *et al.*, 2001; Müller, 2005; Santos, *et al.*, 2015b).

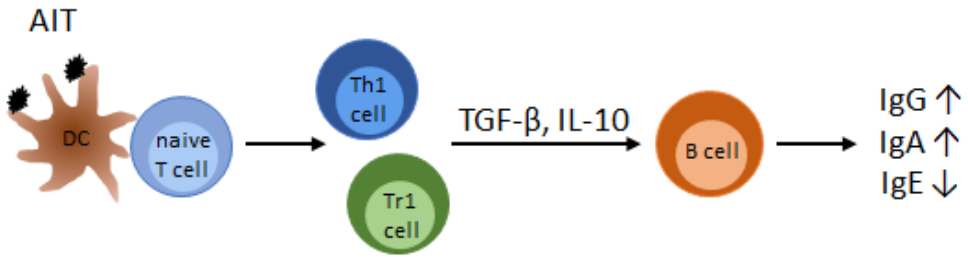

Figure 4: Mechanisms of allergen-specific immunotherapy (AIT). *Tolerance induction by AIT involves the differentiation of Th1 and Tr1 cells, which secrete TGF-β and IL-10. These cytokines, in turn, suppress the production of IgE antibodies and facilitate Ig class switching to IgG and IgA.*

However, the levels of IgG antibodies induced during AIT do not always correlate with the improvement of clinical symptoms. IgG antibodies may be directed to epitopes that differ from those recognized by the allergy-inducing IgE antibodies, thus being unable to block IgE-mediated responses. Furthermore, it has been demonstrated that IgG-dependent inhibition of IgE-mediated anaphylaxis depends not only on the quantity but also the

affinity of the IgG antibodies. Thereby, allergen blocking required high-affinity binding of IgG antibodies, whereas inhibition by FcγRIIB was also mediated by low-affinity IgG antibodies (Denépoux *et al.*, 2000; Larché *et al.*, 2006; Jutel *et al.*, 2016; Epp *et al.*, 2018; Zha *et al.*, 2018).
Together, AIT- and IgG-induced inhibition of IgE-mediated allergic reactions as well as the potential of IgG-mediated allergic reactions, at high antigen titers, have only been partially understood.

In the following section I will provide a brief introduction to the structure of IgG antibodies and their interaction with classical FcγRs embedded in the context of studies providing evidence of their potential role in anaphylactic reactions.

1.3 IgG antibodies

As part of the humoral immune system, antibodies mediate specificity for the wide variety of substances confronting our immune system. They are secreted by terminally differentiated B cells - termed plasma cells - and convey further immune responses (Schroeder and Cavacini, 2010; Murphy *et al.*, 2012; Vidarsson *et al.*, 2014).

Antibodies consist of two identical heavy and two identical light chains that are linked by disulfide bonds forming a Y-shaped structure (Figure 5). Furthermore, each chain comprises variable and constant domains. Antigen recognition is mediated by the two antigen-binding fragments (Fab) that correspond to the amino-terminal variable and constant domains of both the heavy and light chain. On the other hand, the fragment crystallizable (Fc) at the carboxy-terminal part of the heavy chains defines the Ig isotype, i.e. IgM, IgD, IgG, IgA or IgE. The antibody isotypes differ in their size, serum half-life, FcR binding and complement fixation, and thus, their effector functions. The Fab and Fc portions of the antibody are linked by the flexible hinge region (Schroeder and Cavacini, 2010; Murphy *et al.*, 2012; Vidarsson *et al.*, 2014).

Antibodies are able to bind to a wide variety of antigens. Diversity in antigen recognition is given by different mechanisms:

- First, through differential assembly of independent gene segments building the variable domains of the light chain and the heavy chain. The variable domains of

the heavy chain are arranged by so-called variable- (V), diversity- (D) and joining- (J) gene segments, and V- and J-gene segments in case of the light chain.

- Second, the random addition and removal of nucleotides at the intersections of the V(D)J gene segments lead to further diversity of the antibody repertoire.

  Both mechanisms occur during early B cell development and involve DNA rearrangement.

- A third mechanism contributing to antibody repertoire diversity occurs at a later stage. After exposure to the antigen, point mutations are introduced in the variable region by a process called somatic hypermutation, which can lead to increased affinity of the antibody for a certain antigen (Schroeder and Cavacini, 2010; Murphy *et al.*, 2012; Shade and Anthony, 2013; Vidarsson *et al.*, 2014).

In the course of an immune response, B cells not only undergo affinity maturation but also class switch recombination, leading to different Ig isotypes conferring different immune responses. Thereby, a rearranged variable domain can be associated with different heavy chain constant domains (i.e. Cμ, Cδ, Cγ, Cα and Cε; Murphy *et al.*, 2012).

IgG antibodies are the most abundant Ig isotype in the serum and have the longest serum half-life due to the interaction with the neonatal Fc receptor, FcRn. Four different IgG antibody subclasses have been identified in humans (IgG1-4) and mice (IgG1, 2a, 2b and 3). Moreover, despite sharing 90% sequence homology, the different subclasses substantially differ in their effector functions by means of their ability to activate complement as well as due to distinct affinities for different FcRs (Schroeder and Cavacini, 2010; Murphy *et al.*, 2012; Vidarsson *et al.*, 2014; Lilienthal *et al.*, 2018).

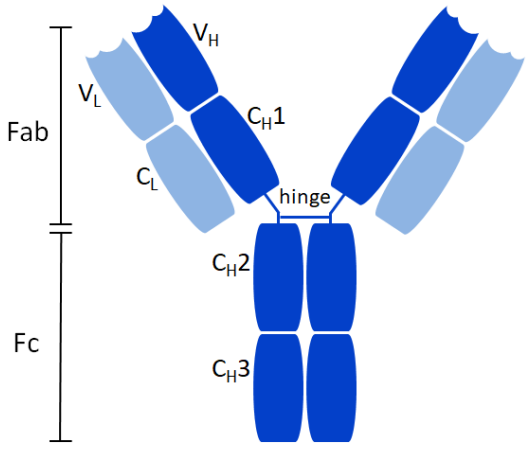

Figure 5: IgG antibody structure. *IgG antibodies consist of two identical heavy (H, dark blue) and two identical light chains (L, light blue). The light chains comprise a variable (V_L) and constant (C_L) domain. The heavy chains comprise a variable (V_H) and several constant (C_H1- C_H3) domains. Antigen recognition is*

mediated by the two antigen-binding fragments (Fab) that are linked by the hinge region to the fragment crystallizable (Fc) mediating antibody effector functions.

Interaction with classical FcγRs

Specific receptors binding the Fc portion of IgG antibodies are expressed on the surface of immune effector cells. The so-called FcγRs are categorized into activating and inhibitory receptors (Table 1). Different activating FcγRs have been recognized in humans (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB) and mice (FcγRI, FcγRIII and FcγRIV). Signal induction of most activating receptors is mediated by the common gamma chain (FcRγ chain), which is associated with cytoplasmic signaling subunits called immunoreceptor tyrosine-based activation motif (ITAM). Upon receptor cross-linking by IgG immune complexes, a signaling cascade is initiated by Src family kinase-mediated phosphorylation of the ITAMs, inducing effector mechanisms such as cytokine and chemokine production, clearance of pathogens by phagocytosis or cell migration. On the other hand, FcγRIIB is the only classical inhibitory FcγR and is conserved among humans and mice. Here, signals are transmitted through an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic region. Co-ligation with ITAM-bearing FcRs and subsequent phosphorylation of the ITIM leads to the recruitment of SHIP phosphatase, which interferes with activating signals by modulating the activation threshold of the effector cells (Nimmerjahn and Ravetch, 2008; Bruhns *et al.*, 2009; Murphy *et al.*, 2012; Shade and Anthony, 2013; Bruhns and Jönsson, 2015).

Beside activating and inhibitory FcγRs, one can also distinguish between high- and low-affinity FcγRs (Table 1). Humans have one high-affinity receptor, FcγRI, whereas in mice FcγRI and FcγRIV bind IgG2 antibodies with high affinity. All other receptors bind IgG antibodies with low to intermediate affinity. Thus, the degree of cell activation is regulated by the affinity of a certain IgG antibody subclass for activating FcγR in relation to the inhibitory FcγRIIB and their expression pattern (Nimmerjahn and Ravetch, 2008; Bruhns *et al.*, 2009; Shade and Anthony, 2013; Bruhns and Jönsson, 2015).

Table 1: Mouse FcγRs. *Mice express three different activating and one inhibitory FcγR. These receptors differ in their affinities (presented as K_A [M^{-1}]) to IgG subclass antibodies and in their expression profile on effector cells involved in murine IgG-mediated anaphylactic reactions* (Bruhns and Jönsson, 2015; Beutier *et al.*, 2016).

	FcγRI	**FcγRIIB**	**FcγRIII**	**FcγRIV**
Function	activation	inhibition	activation	activation
IgG1	-	$3x10^6$	$3x10^5$	-
IgG2a	**$3x10^7$**	$4x10^5$	$7x10^5$	**$3x10^7$**
IgG2b	$1x10^5$	$2x10^6$	$6x10^5$	**$2x10^7$**
IgG3	(?)	-	-	-
Mo/Mφ	+/-	+	+	+
Neutrophils	-	+	+	+
Basophils	-	+	+	-

IgG antibodies in the context of anaphylaxis

Recently, the ability of mouse antigen-specific IgG1, IgG2a and IgG2b antibodies to induce anaphylaxis has been demonstrated. It has also been shown that mouse IgG3 antibodies cannot induce anaphylaxis, which is consistent with observations that IgG3 antibodies do not bind any activating FcγR (Beutier *et al.*, 2016; Reber *et al.*, 2017). Furthermore, it has been recognized that murine IgG1 and IgG2 antibodies differ in their anaphylactic potential. In comparison with IgG1 antibodies, passive sensitization with IgG2 antibodies induced a much stronger anaphylactic reaction (Beutier *et al.*, 2016; Epp *et al.*, 2018). One possible reason for this observation is the higher affinity of the inhibitory FcγRIIB for IgG1 antibodies than for IgG2 antibodies. In addition, IgG1 antibodies bind only one activating FcγR, FcγRIII. Consequently, IgG1-mediated anaphylaxis has been abrogated in FcγRIII-deficient mice. By contrast, IgG2a and IgG2b antibodies not only engage FcγRIII with higher affinity than IgG1 antibodies but additionally bind to FcγRIV. Here, a deficiency or depletion of either FcγRIII or FcγRIV led to a reduction but not suppression of the symptoms, suggesting that both receptors are involved in the context of IgG2-mediated anaphylaxis. The involvement of activating FcγRI in the induction of IgG2-mediated anaphylaxis remains under debate (Miyajima *et al.*, 1997; Nimmerjahn *et al.*, 2005; Strait *et al.*, 2006; Jönsson *et al.*, 2011; Beutier *et al.*, 2016; Finkelman *et al.*, 2016; Gillis *et al.*, 2016; Epp *et al.*, 2018).

Furthermore, the inhibitory potential of FcγRIIB has been demonstrated in IgG-mediated anaphylaxis. In IgG1- and IgG2b-mediated anaphylaxis, FcγRIIB-dependent reduction of anaphylaxis could be observed, but the severity of IgG2a-mediated anaphylaxis was

unaltered in FcγRIIB-deficient mice. This might be due to the lower affinity of this receptor for IgG2a antibodies in addition to the relatively high affinity of activating FcγRs for this subclass (Nimmerjahn *et al.*, 2005; Beutier *et al.*, 2016; Gillis *et al.*, 2017; Epp *et al.*, 2018).

Also, many attempts have been made to identify the immune cells involved in the induction of IgG-mediated anaphylaxis and transfer observations to the human situation by using mice transgenic for human FcγRs.

Mouse neutrophils express FcγRIII and FcγRIV, while human neutrophils express FcγRIIA and FcγRIIIB. Both can be activated within the bloodstream by circulating immune complexes. In an attempt to find markers to define IgG-mediated anaphylaxis, Khodoun and colleagues reported a down-regulation of mouse FcγRIII on activated neutrophils upon anaphylaxis induction, suggesting that IgG immune complexes interact with these cells and become internalized (Khodoun *et al.*, 2011). Moreover, in mice transgenic for human FcγRIIA depletion of neutrophils attenuated the anaphylactic reaction. In models of active systemic anaphylaxis the contribution of neutrophils has been further demonstrated by antibody-mediated depletion (αGr-1 or αLy6G Ab) of these cells, leading to reduced IgG-mediated anaphylactic reactions, whereas the transfer of FcγR-expressing neutrophils in FcγR knock-out mice restored anaphylaxis (Jönsson *et al.*, 2011, 2012, 2013; Khodoun *et al.*, 2011, 2013; Bruhns and Jönsson, 2015).

Another cell type discussed as being involved in the induction of IgG-mediated anaphylactic responses are monocytes/ macrophages. In mice and humans, monocytes/ macrophages express all activating and inhibitory FcγRs (except for human FcγRIIIB, which is only expressed by neutrophils). Furthermore, murine FcγRIII-dependent activation of macrophages has been shown in passive IgG-mediated anaphylaxis. However, in models of active systemic anaphylaxis, the depletion of macrophages by clodronate liposomes only reduced the symptoms, suggesting that they are not the only cell type involved. Furthermore, these cells contributed to anaphylaxis severity in mice transgenic for human FcγRIIA, but not in mice transgenic for human FcγRI (Strait *et al.*, 2002; Nimmerjahn and Ravetch, 2008; Arias *et al.*, 2011; Smit *et al.*, 2011; Jönsson *et al.*, 2012; Mancardi *et al.*, 2013; Bruhns and Jönsson, 2015; Balbino *et al.*, 2016).

Even though basophils account for less than 1% of blood leukocytes, they have been demonstrated to contribute to anaphylaxis induction and severity in certain models. Both

human and mouse basophils express activating FcRs (FcεRI, hFcγRIIA/ mFcγRIII), a prerequisite for being involved in the induction of either IgE- or IgG-mediated anaphylaxis, respectively. It has been shown that antibody-dependent depletion of basophils ameliorated IgG-mediated anaphylaxis, although, in another model using basophil-deficient mice these cells were not required for the induction of IgG1-induced anaphylaxis (Tsujimura *et al.*, 2008; Ohnmacht *et al.*, 2010; Beutier *et al.*, 2016). In human subjects, it has been shown that the degree of basophil activation correlated with the severity of the allergic reaction in peanut-induced reactions (Santos, *et al.*, 2015a). However, Cassard and colleagues suggested that human as well as mouse basophils could not be activated in response to IgG immune complexes *ex vivo*, probably due to a high expression and dominant inhibition by FcγRIIB over activating signals (Cassard *et al.*, 2012). Taken together, the contribution of basophils in IgG-mediated anaphylaxis is under debate. Data from mouse and human studies may suggest that basophils contribute to the severity of the anaphylactic response but are not required for its induction (Karasuyama *et al.*, 2010; Bruhns and Jönsson, 2015; Beutier *et al.*, 2016).

In summary, the constant region of IgG antibodies influences their effector functions. Thereby, the elicited signal and the resulting immune response are influenced by the affinities of activating and inhibitory FcγRs for the individual IgG antibody subclasses as well as the expression pattern of these receptors on different immune cells. Accordingly, it could be shown that the different mouse IgG antibody subclasses differ in their potential to induce IgG-mediated anaphylaxis. Another variable influencing IgG antibody effector functions, and thus, their potential to influence anaphylactic responses (e.g. inhibition of IgE-mediated allergic reactions or to induce anaphylaxis themselves) is the type of Fc N-glycosylation.

1.4 IgG antibody Fc glycosylation

Nowadays, it is widely accepted that effector functions of IgG antibodies are not only determined by their subclass but are also influenced by their Fc glycosylation pattern. IgG antibodies have a conserved glycosylation site at asparagine (Asn) 297 located in both C_H2 domains of the antibody Fc part (Figure 6A). The attached glycan comprises 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

and GlcNAc as well as by the terminal extension of galactose and sialic acid at only one or both arms of the biantennary core structure leading to more than 30 different glycosylation patterns (Figure 6B; Kaneko *et al.*, 2006; Nimmerjahn and Ravetch, 2008; Pincetic *et al.*, 2014).

Accordingly, it has been demonstrated that the IgG antibody Fc glycosylation is essential for the induction of antibody-dependent effector functions. The enzymatic removal of the glycan structure resulted in impaired interaction of IgG antibodies with FcγRs and C1q, the initiator of the classical complement pathway (Nandakumar *et al.*, 2007; Allhorn *et al.*, 2008; Dekkers *et al.*, 2018).

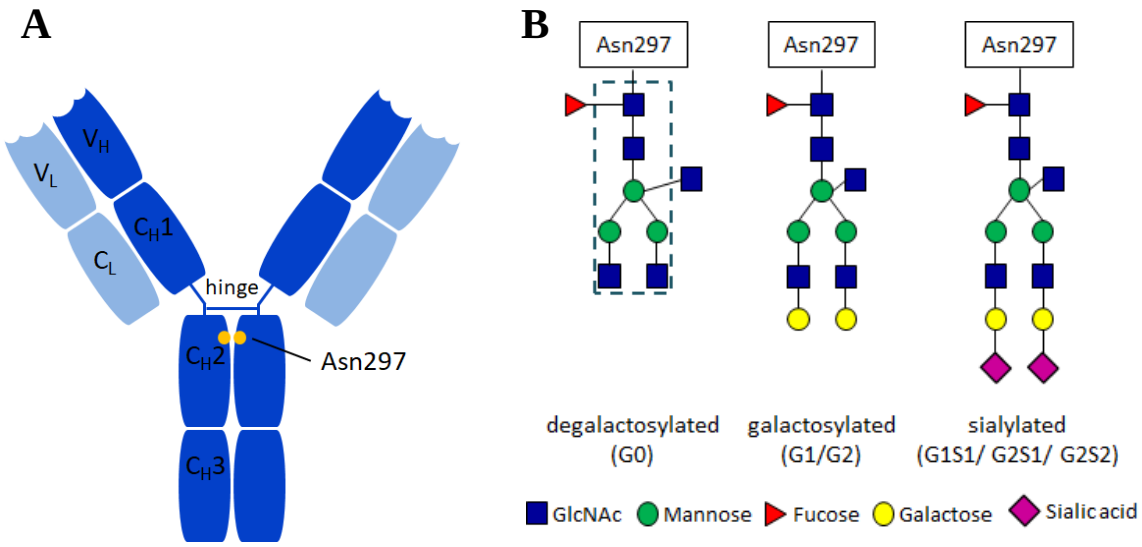

Figure 6: IgG antibody Fc glycosylation. ***(A)*** *IgG antibodies have a conserved glycosylation site at Asn297 of the C_H2 domain.* ***(B)*** *The core structure (dashed line) can be modified by the addition of fucose (red), bisecting N-acetylglucosamine (GlcNAc, blue) as well as by galactose (G, yellow) and sialic acid (S, pink).*

The glycosylation profile of IgG antibodies considerably varies among healthy individuals. Age- and gender-dependent variation as well as modifications upon vaccination have been shown (Yamada *et al.*, 1997; Oefner *et al.*, 2012; Selman *et al.*, 2012; Dall'Olio *et al.*, 2013; Hess *et al.*, 2013; Alter *et al.*, 2018). Moreover, a connection between the IgG antibody Fc glycosylation and some diseases has been observed. A number of human studies have demonstrated that patients with inflammatory autoimmune diseases show an altered IgG antibody glycosylation profile. An enrichment of non-galactosylated (agalactosylated; G0) IgG antibodies has been observed in patients suffering from rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus and

multiple sclerosis and has been correlated with disease progression and severity. Conversely, an increase of galactosylation and sialylation has been linked with disease remission. These observations led to the hypothesis that agalactosylated IgG antibodies contribute to pro-inflammatory effector mechanisms, while galactosylated and sialylated IgG antibodies confer rather anti-inflammatory immune responses (Nimmerjahn *et al.*, 2007; Scherer *et al.*, 2010; Collin and Ehlers, 2013; Trbojević Akmačić *et al.*, 2015; Vučkovïc *et al.*, 2015; Decker *et al.*, 2016; Ohmi *et al.*, 2016; Dekkers *et al.*, 2018).

This notion is also supported by studying the underlying mechanism of IVIG therapy. Intravenous immunoglobulin (IVIG; pooled serum IgG antibodies from thousands of healthy donors) was initially used as substitution therapy for the treatment of immunodeficient individuals but was shown to have broader therapeutic effects. Soon its anti-inflammatory properties in patients with different inflammatory autoimmune diseases, such as immunothrombocytopenia or Kawasaki's disease, was appreciated and attributed to the IgG antibody Fc glycosylation. Subsequently, murine studies have shown that the enrichment of the sialylated fraction of IVIG could enhance the anti-inflammatory properties, whereas the removal of sialic acid by neuraminidase treatment abrogated this protective effect in different animal models (Samuelsson *et al.*, 2001; Kaneko *et al.*, 2006; Schwab and Nimmerjahn, 2013; Fiebiger *et al.*, 2015; Brückner *et al.*, 2017). Furthermore, it has been suggested that the sialylated fraction of IVIG re-establish the endogenous amount of sialylted IgG antibodies in ordere to mediate anti-inflammatory properties via an up-regulation of FcγRIIB on immune effector cells (Anthony *et al.*, 2008a, 2011).

Also, more subtle modifications of the Fc glycan have been analyzed. A lack of fucosylation - which also correlates with a higher content of bisecting GlcNAc - has been shown to enhance antibody-dependent cellular cytotoxicity (ADCC) due to an increased affinity of these IgG antibodies for human FcγRIII (Shields *et al.*, 2002; Kurimoto *et al.*, 2014). Furthermore, terminal galactosylation and sialylation have been demonstrated to influence the antibody effector function due to the introduction of a conformational change of the antibody Fc part influencing the binding with either type I FcRs or type II FcRs, containing members of the C-type lectin receptor family. In the absence of terminal galactose or sialic acid, the IgG antibody Fc part maintains an open conformation which is essential for engagement with type I FcRs. On the other hand, terminal galactosylated and sialylated glycans introduce a closed conformation of the antibody Fc part exposing binding sites and enhancing the affinity for type II FcRs (e.g. 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), dendritic cell immunoreceptor (DCIR), CD23 or Dectin-1). The different type II Fc receptors have been demonstrated to modulate immune responses by impeding signals from e.g. activating type I FcRs (Krapp *et al.*, 2003; Karsten *et al.*, 2012; Sondermann *et al.*, 2013; Ahmed *et al.*, 2014; Massoud *et al.*, 2014; Pincetic *et al.*, 2014; Fiebiger *et al.*, 2015; Wang *et al.*, 2015). The relevance of galactose (without sialic acid) on the antibody effector function remains unclear. Both anti- and pro-inflammatory properties have been described (Karsten *et al.*, 2012; Yamada *et al.*, 2013).
In addition to antigen-unspecific effects of the sialylated fraction of IVIG also effects of sialylated antigen-specific IgG antibodies, particular in the context of autoimmune diseases, have been described (Ohmi *et al.*, 2016; Bartsch *et al.*, 2018).

Taken together, it is well established that changes in IgG antibody glycosylation influence the effector functions of IgG antibodies. Changes in the glycosylation pattern have been observed in health and disease, and thus, function as biomarkers and allow evaluating disease progression/ remission. Moreover, for therapeutic purposes, IgG antibodies, their Fc N-glycosylation and their impact on FcR interaction are investigated in-depth to modulate antibody effector functions. Thereby, most data on IgG antibody glycosylation are reported in the context of autoimmune diseases. However, only few studies provide knowledge about the impact of IgG antibody glycosylation in the context of allergic reactions.

1.5 Aim of the thesis

Allergen-specific IgE antibodies can mediate anaphylaxis by cross-linking the high-affinity FcεRI on mast cells and basophils, and thus, leading to cell activation and the release of inflammatory mediators. By contrast, IgG antibodies, e.g. induced by AIT, have been demonstrated to provide protection in IgE-mediated allergic reactions by allergen masking as well as cross-linking FcεRI with the IgG inhibitory receptor, FcγRIIB. However, at high antigen doses IgG antibodies themselves can cause anaphylaxis in mice by driving FcγR-induced activation of myeloid cells.

There is broad consensus that effector functions of IgG antibodies are not only determined by their subclass but are also influenced by their Fc N-glycosylation pattern. In this regard, the type of IgG antibody Fc glycosylation has been demonstrated to influence the IgG antibody binding affinity towards activating/ inhibitory classical FcγRs (type I FcRs) and sugar-binding receptors, in particular from the C-type lectin receptor family (type II FcRs). However, the role of IgG antibody Fc glycosylation in anaphylactic reactions is unclear.

The main aim of this study was to analyze the role and immunomodulatory mechanisms of total serum IgG subclass glycosylation in comparison to allergen-specific IgG subclass glycosylation in murine passive IgG-mediated as well as IgG-controlled IgE-mediated anaphylaxis.

First, the contribution of complement activation versus classical type I FcγR activation should be analyzed in a passive IgG-mediated anaphylaxis mouse model.

Second, differently glycosylated IgG subclass antibodies should be generated *in vitro*. Here, sialylated and degalactosylated IgG antibodies should be compared concerning their anaphylactic potential regarding their serum half-life, the requirement for antigen-specificity and antibody subclass-dependency in IgG-mediated anaphylaxis. Furthermore, the gained results should be verified in an IgG-controlled IgE-mediated anaphylaxis model.

Third, the contribution of the inhibitory FcγRIIB and type II FcRs SignR1 should be analyzed concerning the impact of differently glycosylated IgG antibodies on antibody-mediated anaphylaxis.

Finally, in an attempt to analyze to what extent the identified immunomodulatory mechanisms could also apply in humans, healthy volunteer blood donors should be recruited and analyzed concerning their total IgG glycosylation pattern in correlation with individual expression levels of FcγRs on different immune cells.

As a side project, the effects of differently glycosylated IgE and IgA antibodies in the context of allergic reactions should be investigated.

Altogether, the gathered results concerning the impact of antibody Fc glycosylation in the context of allergic reactions might bring forward new ideas for therapeutic approaches concerning IgE- as well as IgG-mediated anaphylactic reactions.

2 Materials and Methods

2.1 Materials

2.1.1 Human samples

Thirtysix healthy volunteer blood donors (12 male, 24 female) were recruited. Ethical approval was obtained from the local committee on research in human subjects at the University of Lübeck (approved protocol 18-302, November 2018). All donors provided written informed consent. Venous blood was taken using EDTA as anti-coagulant for cell analysis by flow cytometry. In addition, serum was collected for IgG antibody purification and IgG glycan analysis. Blood and serum samples were analyzed anonymously where only information on the age and gender of the donors were revealed.

2.1.2 Mice

C57BL/6 wild type mice were originally purchased from Jackson Laboratory (Bar Harbor, ME; C57BL/6J) and used for our own breeding colonies. Mice deficient for the FcR γ-chain (Fcrg$^{-/-}$; Takai *et al.*, 1994) or FcγRIIB (*Fcgr2b*$^{-/-}$; Takai *et al.*, 1996) on the C57BL/6 genetic background were obtained from our own breeding colonies. All strains were backcrossed at least 10 generations to the C57BL/6 background. Mice were bred and maintained in a pathogen-free facility at the University of Lübeck. For all experiments 8-12 week old female mice were analyzed. The animal test proposals (V242-45889/2016 (91-7/16), V241-22176/2017 (63-5/17), V242-44603/2018 (79-8/18)) were approved by local authorities of the Animal Care and Use Committee (Ministerium für Landwirtschaft, Umwelt und ländliche Räume, Kiel, Germany).

2.1.3 Cell lines

Isotype switch variant IgG2a anti-2,4,6-trinitrophenol (TNP; clone HA2a) and IgG2b anti-TNP (clone HA2b) hybridoma cell lines (a gift from Lucian Aarden in The Netherlands; Strait *et al.*, 2015; Epp *et al.*, 2018).

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

Human Embryonic Kidney (HEK)293T/17 cell line (ATCC-CRL-11268; Pear *et al.*, 1993).

Human LAD2 mast cell line (Kirshenbaum *et al.*, 2003).

2.1.4 Bacteria

Escherichia coli (*E. coli*; NovaBlue competent cells) were already available in the laboratory.

2.1.5 Expression vectors

For the production of murine IgA anti-TNP antibodies two expression vectors containing either the κ light chain or the Igα heavy chain were co-transfected into HEK293T/17 cells. Both expression vectors have been recently described (Tiller *et al.*, 2009; Hess *et al.*, 2013).

The expression vector with the anti-TNP κ light chain cDNA was already available in the laboratory (Hess *et al.*, 2013). The expression vector for the Igα heavy chain already included the anti-TNP variable heavy chain region (Hess *et al.*, 2013). The Igα constant heavy chain region with additional restriction sites (*SalI* and *BsiWI;* see supplements Table B.1) for cloning into the expression vector was synthesized and purchased from Thermo Fisher Scientific (Regensburg, Germany).

2.1.6 Primers

The following primers were ordered from Eurofins Genomics (Ebersberg, Germany) and used for sequencing of the murine IgA anti-TNP expression vectors:

Name	Orientation	Sequence
muIgA_hc	forward	5´ TTCCGGCACGATGAATGTGACCT
muIgA_hc	reverse	5´ GCTGCTCATGGTGTACCCTC
TNP_kappa	forward	5´ TGTACATTCAGACATTGTGATGTCACAGTCT
TNP_kappa	reverse	5´ TTATTCGGAAGCTTTCAACACTCATTCCTGTTGAA

2.1.7 Antibodies

***In vivo* application**

Antibody	Clone	Company
anti-mouse SignR1	22D1	BioXCell (Lebanon, NH, USA)
mouse IgG1 isotype control	MOPC21	BioXCell (Lebanon, NH, USA)
mouse IgG2a isotype control	C1.18.4	BioXCell (Lebanon, NH, USA)

ELISA antibodies

Epitope	Reactivity	Clone	Conjugate	Company
C3	mouse	polyclonal	-	GeneTex (Irvine, CA, USA)
IgA	mouse	polyclonal	HRP	Bethyl Labs (Montgomery, TX, USA)
IgE	mouse	polyclonal	HRP	Bethyl Labs (Montgomery, TX, USA)
IgM	mouse	polyclonal	HRP	Bethyl Labs (Montgomery, TX, USA)
IgG	goat	polyclonal	HRP	Bethyl Labs (Montgomery, TX, USA)
IgG h+l	mouse	polyclonal	HRP	Bethyl Labs (Montgomery, TX, USA)

FACS antibodies

Epitope	Reactivity	Clone	Conjugate	Company
CCR3	human	5E8	PerCP/5.5	BioLegend (San Diego, CA, USA)
CD14	human	M5E2	AF700	BioLegend (San Diego, CA, USA)
FcγRIII	human	3G8	PerCP/5.5	BioLegend (San Diego, CA, USA)
CD19	human	HIB19	APC	BioLegend (San Diego, CA, USA)
CD3	human	SK7	APC	BioLegend (San Diego, CA, USA)
CD56	human	HCD56	APC	BioLegend (San Diego, CA, USA)
FcγRIIA	human	IV.3	-	BioXCell (Lebanon, NH, USA)
FcγRIIB	human	2B6	-	Creative Biolabs (Shirley, NY, USA)
FcεRI	human	AER-37	AF700	BioLegend (San Diego, CA, USA)
CD11b	mouse	M1/70	BV421	BioLegend (San Diego, CA, USA)
CD49b	mouse	HMα2	FITC	BioLegend (San Diego, CA, USA)
F4-80	mouse	BM8	AF700	BioLegend (San Diego, CA, USA)
FcεRI	mouse	MAR-1	AF700	BioLegend (San Diego, CA, USA)
FcγRIIB	mouse	AT130-2	APC	Fisher Scientific (Schwerte, Germany)

Epitope	Reactivity	Clone	Conjugate	Company
FcγRIII	mouse	275003	PE	R&D Systems (Minneapolis, MN, USA)
Ly6G	mouse	1A8	PerCP/5.5	BioLegend (San Diego, CA, USA)
SignR1	mouse	22D1	-	BioXCell (Lebanon, NH, USA)

2.1.8 Enzymes

α2,6-sialyltransferase (human)	Roche (Basel, Switzerland)
BsiWI + CutSmart buffer	New England BioLabs (Ipswich, MA, USA)
β1,4-galactosidase + 5x reaction buffer B	Prozyme (Hayward, CA, USA)
β1,4-galactosyltransferase	provided by Kelley Moremen, University of Georgia (Athens, GA, USA)
Endoglycosidase S (EndoS)	provided by Mattias Collin, University Lund (Sweden)
SalI + CutSmart buffer	New England BioLabs (Ipswich, MA, USA)
Sialidase A + 5x reaction buffer B	Prozyme (Hayward, CA, USA)
T4 DNA Ligase + 10x reaction buffer	New England BioLabs (Ipswich, MA, USA)

2.1.9 Media and supplements

Cell culture

Phosphate Buffered Saline (PBS)	Life Technologies (Darmstadt, Germany)
DMEM	Life Technologies (Darmstadt, Germany)
Fetal calf serum (FCS)	Life Technologies (Darmstadt, Germany)
Penicillin (10,000 IU mL-^{1})/ Streptomycin (10 mg mL^{-1})	Life Technologies (Darmstadt, Germany)
Primatone	Sigma-Aldrich (St. Louis, MO, USA)
RPMI1640 (L-Glutamine)	Life Technologies (Darmstadt, Germany)
StemPro-34 + Nutrient Supplement	Fisher Scientific (Schwerte, Germany)
L-Glutamine	Life Technologies (Darmstadt, Germany)
Recombinant human stem cell factor (rhSCF)	PeproTech (Hamburg, Germany)

Media composition

Hybridoma culture medium	RPMI 1640 medium (L-Glutamine) + 1% Penicillin-Streptomycin + 10% FCS
Hybridoma production medium	RPMI 1640 medium (L-Glutamine) + 1% Penicillin-Streptomycin + 0.03% Primatone
HEK293T/17 culture medium	DMEM + 1% Penicillin-Streptomycin + 10% FCS
HEK293T/17 production medium	DMEM + 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

Molecular biology

Ampicillin	Sigma-Aldrich (St. Louis, MO, USA)
Bacto Agar	VWR (Radnor, PA, USA)
LB medium	Carl Roth (Karlsruhe, Germany)

Media composition

Bacterial growth medium	12.5 g LB medium in 500 mL ddH_20 + 50 µg/ mL Ampicillin
LB agar plates	12.5 g LB-medium in 500 mL ddH_20 + 7.5 g Bacto Agar + 50 µg/ mL Ampicillin

2.1.10 Chemicals

2-(N-morpholino)ethanesulfonic acid (MES)	Merck (Darmstadt, Germany)
6x DNA loading dye	Thermo Scientific (Waltham, MA, USA)
Acetic acid	Carl Roth (Karlsruhe, Germany)
Acetonitrile Rotisolv HPLC Gradient	Carl Roth (Karlsruhe, Germany)
Agarose peqGOLD	VWR (Radnor, PA, USA)
Aminobenzamide (2-AB)	Sigma-Aldrich (St, Louis, MO, USA)
Ammonia (25%)	Carl Roth (Karlsruhe, Germany)
Bovine Serum Albumin (BSA)	GE Healthcare (Little Chalfont, GB)
Bromphenol blue	AppliChem (Darmstadt, Germany)
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)
Cytofix/ Cytoperm	BD Bioscience (San Diego, CA, USA)
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)
Gelatin from cold water fish	Sigma-Aldrich (St. Louis, MA, USA)
GeneRuler DNA ladder, 100 bp	Thermo Scientific (Waltham, MA, USA)
Glycine	Merck (Darmstadt, Germany)
Graphite (Carbograph)	Grace (Columbia, MD, USA)
Hank's balanced salt solution ($HBSS^{+/+}$)	Life Technologies (Darmstadt, Germany)
Hydrogen chloride (HCl)	Merck (Darmstadt, Germany)
Intratect® (IVIG)	Biotest AG (Langen, Germany)
Iso-propanol	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	Thermo Scientific (Waltham, MA, USA)
p-nitrophenyl N-acetyl-β-D-glucosamide	Sigma-Aldrich (St. Louis, MA, USA)

Polyethylenimin (PEI)	Sigma-Aldrich (St. Louis, MO, 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)
Protein L Sepharose	Biozol (Eching, Germany)
Reaction buffer B, 5x	Prozyme (Hayward, CA, USA)
Rompun 2% (Xylazine)	Bayer (Lerverkusen, Germany)
Sodium acetate (NaOAc)	Sigma-Aldrich (St, Louis, MO, USA)
Sodium azide (NaN_3)	Sigma-Aldrich (St, Louis, MO, USA)
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich (St, Louis, MO, USA)
Sodium carbonate (Na_2CO_3)	Carl Roth (Karlsruhe, Germany)
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)
Sodium phosphate, monobasic (NaH_2PO_4)	Sigma-Aldrich (St. Louis, MA, USA)
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)
Trypsin/ EDTA	Pan Biotech (Aidenbach, Germany)
Tween 20	Sigma-Aldrich (St. Louis, MA, USA)
UDP-galactose	Millipore (Billerica, MA, USA)

2.1.11 Buffers and solutions

SDS-PAGE

Coomassie destain solution	40% Methanol 10% Acetic acid in ddH_20
SDS loading buffer	0.25 M Tris (pH 6.8) 0.5 M DTT 25% Bromphenol blue 50% Glycerol 10% SDS in ddH_20
SDS running buffer	25 mM Tris 192 mM Glycin 0.1% SDS in ddH_20, adjusted to pH 8.3

Flow cytometry

Erylysis buffer	150 mM NH4Cl 100 µM EDTA 1 mM NaHCO3 in ddH_20, adjusted to pH 7.2
FACS buffer	0.5% BSA 0.05% sodium azide 10 mM EDTA in 1xPBS

ELISA

Coating buffer	50 mM Carbonate/ bicarbonate buffer in ddH_20
Blocking buffer	30 g BSA 1 g gelatin 7.9 mL 0.38 M EDTA in 1L 1x PBS
PBST	0.5 mL L-1 Tween 20 in 1x PBS

Antibody purification

Blocking buffer	0,1 M Tris-HCl in ddH_20, adjusted to pH 8
Coupling buffer	0,1 M NaHCO3, adjusted to pH 8,3 0,5 M NaCl in ddH_20

Elution buffer	0.1 M Glycin in ddH_20, adjusted to pH 2,8
High pH wash buffer	0.1 M Tris-Hcl 0.5 M NaCl in ddH_20, adjusted to pH 8
Low pH wash buffer	0.1 M Acetic acid 0.5 M NaCl in ddH_20, adjusted to pH 4

2.1.12 Kits

AlexaFluor 488 Abs labeling kit	Thermo Fisher (Waltham, MA, USA)
Nucleo Bond Xtra Maxi Plus Kit	Macherey-Nagel (Düren, Germany)
Nucleo Spin Gel and PCR Clean UP	Macherey-Nagel (Düren, Germany)
PE-conjugation Kit	Abcam (Cambridge, MA, USA)

2.1.13 Consumables

Amicon Ultra Centrifugal Filters, 10K	Merck, (Tullagreen, Ireland)
Blood lancet	Braun (Melsungen, Germany)
Cell Culture Flasks for adherent cells	Greiner Bio-one (Kremsmünster, Austria)
Cell Culture Flasks for suspension cells	Greiner Bio-one (Kremsmünster, Austria)
Cell scraper	Sarstedt (Nümbrecht, Germany)
Cell Strainer 70 µm	BD (Franklin Lakes, NJ, USA)
Centrifugation filter, 10 kDa	Merck (Darmstadt, Germany)
Costar Assay plates, 96 well	Corning (Kennebunk, ME, USA)
EDTA-Monovette/ Microvette	Sarstedt (Nümbrecht, Germany)
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)
Multiscreen filter plate (0.45µm)	Merck (Darmstadt, Germany)
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)
Safety-Multifly (21G)	Sarstedt (Nümbrecht, Germany)
Serological pipettes (5, 10, 25, 50 mL)	Sarstedt (Nümbrecht, Germany)
Serum MiniCollect	Greiner Bio-one (Kremsmünster, Austria)
Serum Monovette	Sarstedt (Nümbrecht, Germany)
Single-use syringes (1 mL)	Braun (Melsungen, Germany)
Suspension culture plate (24-well)	Greiner Bio-one (Kremsmünster, Austria)
Tissue culture dish (100/ 145 mm)	BD (Franklin Lakes, NJ, USA)
Vial lid ND11 PTFE Septum	Carl Roth (Karlsruhe, Germany)
XBridge BEH Glycan 1.7µm column	Waters Corporation (Milford, MA, USA)

2.1.14 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)
Gen Pure Pro Ultrapure water	Thermo Fisher (Waltham, MA, 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-2000C	peqlab Biotechnologie (Erlangen, Germany)
pH-meter FiveEasy F20	Mettler-Toledo (Columbus, OH, USA)
Pipetboy Accu 2	Integra Bioscience (Zizers, Switzerland)
Pipette (single-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	

2.1.15 Software

Serial Cloner v2.6.1	Serial Basics, Freeware
FlowJo v10.0.7	Treestar (Ashland, OR, USA)
GraphPad Prism v. 6.04	GraphPad Software (San Diego, CA, USA)
Chromeleon Software V6.9	Thermo Fisher (Waltham, MA, USA)

2.2 Methods

2.2.1 Cell culturing

Media and growth conditions

HEK293T/17 cells were maintained in DMEM supplemented with 10% FCS/ 1% Penicillin-Streptomycin (100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin).

Hybridoma cell lines HA2a, HA2b and IGEL a2 were maintained in RPMI 1640 medium supplemented with 10% FCS/ 1% Penicillin-Streptomycin (100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin).

The mast cell line LAD2 was maintained in StemPro-34 medium supplemented with 2.5% StemPro-34 Nutrient Supplement, 2 mM L-Glutamine, 1% Penicillin-Streptomycin (100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin) and 100 ng mL^{-1} rhSCF.

For cultivation, cells were placed in a CO_2-incubator with 5% CO_2, at 37 °C and 100% relative air humidity. Cells were regularly examined for their growth behavior, appearance and possible contaminations.

Subculturing of cell lines

All work was performed under sterile conditions. Cells were splitted every 2-3 days when they were 70-80% confluent. In case of the adherent HEK293T/17 cells, first the medium was aspirated and cells were washed with 1x PBS. For detachment, cells were treated with Trypsin-EDTA and incubated for 3-5 minutes at room temperature. Fresh culture medium was added to stop the dissociation reaction. After centrifugation (200x g at room temperature for 5 minutes), cells were resuspended in fresh culture medium. Cells were then usually splitted in a ratio of 1:20 and transferred into a new culture dish.

Hybridoma cells, which grow in suspension, were splitted by withdrawing a portion of the cell suspension from the culture flask and diluting the rest of the cells with new cell culture medium.

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

Freezing of cells

For cryo-preservation, cells were harvested and counted. Cells were frozen in portions of $5x10^7$ cells mL^{-1}. After centrifugation (200x g at room temperature for 5 minutes), the supernatant was discarded and the cell pellet was resuspended in freezing medium (DMEM for HEK293T/17 cells, RPMI 1640 medium for hybridoma cells; each supplemented with 10% of the cryoprotective agent DMSO). 1 mL aliquots of the cell suspension were transferred into cryo-tubes and stored at -80 °C.

Thawing of cells

The frozen cells were taken from the -80 °C storage and placed into a 37 °C water bath. As soon as only a small amount of ice was left, cells were taken up with cell culture medium. In order to remove DMSO from the freezing medium, cells were immediately centrifuged at 200x g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in an appropriate volume of culture medium and transferred into a culture dish. Cells were incubated overnight and the medium was exchanged after 24 hours.

2.2.2 Cloning of murine IgA anti-TNP expression vectors

For the production of murine IgA anti-TNP antibodies, two expression vectors containing either the κ light chain or the Igα heavy chain were used. The expression vector with the κ light chain was already available in the laboratory (Hess *et al.*, 2013). The purchased Igα constant heavy chain cDNA (Supplements Table B1) was cloned into an expression vector that already included the variable anti-TNP VDL heavy chain region (Figure 7). In the following section, the experimental procedure is explained.

Preparative digestion

The former heavy chain expression vector was subjected to preparative digestion with *SalI* and *BsiWI*. For the restriction digest, 4 µg of plasmid DNA were incubated with 1 µL of each restriction enzyme and 3 µL 10x CutSmart reaction buffer in a total volume of 30 µL at 37 °C for 1.5 hours. Next, the digested plasmid DNA was loaded on a 1% agarose gel

and the linearized vector was recovered and gel purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) as described in the manufacturer's manual.

Ligation and transformation

Ligation was performed with the linearized plasmid DNA and the purchased Igα constant heavy chain region construct in a molar ratio of 1:6 and 1:9. Plasmid DNA and insert were incubated with 1 µL T4 DNA ligase (Fermentas) and 2 µL 10x reaction buffer in a total volume of 20 µL at 16 °C overnight. As a control, linearized plasmid was carried along. The next day, the T4 DNA ligase was heat inactivated at 65 °C for 10 minutes.

Next, 5 µL of each ligation product were used for the transformation of 100 µL *E.coli* NovaBlue competent cells. Transformation was performed for one hour on ice. Bacteria were plated on antibiotic-selective LB agar plates and incubated at 37 °C overnight. The next day, single clones were picked and grown in 300 mL selective LB medium at 37 °C and 350 rpm overnight. Plasmid isolation and purification was done using the Nucleo Bond Xtra Maxi Plus Kit (Macherey-Nagel) as described in the manufacturer's manual. A successful integration of the Igα constant heavy chain region was confirmed by sequencing. Samples were sent to Eurofins Genomics (Ebersberg, Germany) for sequencing using the *muIgA_hc forward* and *muIgA_hc reverse* primer.

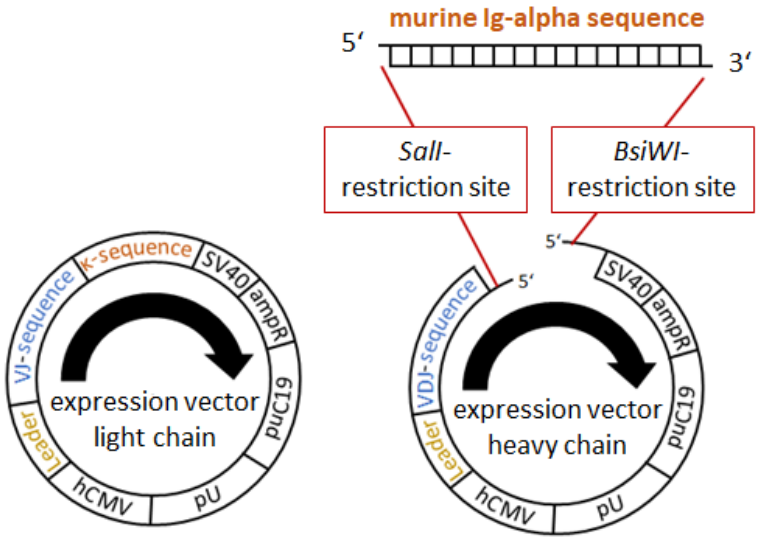

Figure 7: Schematic presentation of κ light chain and Igα heavy chain expression vectors for the production of murine IgA anti-TNP antibodies. *The expression vectors have recently been described by Tiller et al (2009) and Hess et al. (2013). Here, a newly synthesized Igα constant heavy chain region was cloned into an expression vector containing the variable heavy chain region by restriction digest with SalI and BsiWI.*

2.2.3 Antibody production

Hybridoma cell lines

For the production of IgG2a anti-TNP, IgG2b anti-TNP and IgE anti-TNP antibodies, the respective hybridoma cell lines were expanded as described before (2.2.1). Eventually cells were harvested and washed with 1x PBS by centrifugation at 200x g for 5 minutes. The cell pellet was resuspended in FCS-free RPMI medium supplemented with 0.03% Primatone/ 1% Penicillin-Streptomycin (production medium). Cells were incubated for up to 14 days before antibodies were harvested. Therefore, cells were pelleted at 2000x g for 5 minutes and the sterile filtered (0.22 µm filter) supernatant was stored at 4 °C until further use.

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

Transfection of HEK293T/17 cells

For the production of IgA anti-TNP antibodies, HEK293T/17 cells were plated in 145 mm cell culture dishes. When cells reached 80% confluency, they were washed with 1x PBS and received serum-free production medium (DMEM supplemented with 0.03% Primatone/ 1% Penicillin-Streptomycin). Next, cells were co-transfected with expression vectors containing the κ light chain and the Igα heavy chain using the transfection agent polyethylenimine (PEI). For a 145 mm cell culture dish, the following quantities were required: 17.5 µg κ light chain expression vector, 17.5 µg Igα heavy chain expression vector and 70 µg PEI diluted in 3 mL 1x PBS. The transfection mixture was vortexed for 10 seconds and incubated at room temperature for 10 minutes. Next, the transfection mixture was distributed dropwise onto the cells. Cell culture dishes were gently rocked to distribute the mixture and were returned to the incubator. Cells were incubated for up to 7 days before antibodies were harvested. Therefore, the cell culture supernatant was transferred into 50 mL tubes and centrifuged at 2000x g for 5 minutes. The sterile filtered (0.22 µm filter) supernatant was stored at 4 °C until further use.

2.2.4 Antibody purification

Purification of antibodies from cell culture supernatant was achieved by affinity chromatography. Mouse and human IgG antibodies were purified using protein G sepharose (GE Healthcare). Protein G is a *Streptococcal* cell wall protein binding to the Fc part of IgG antibodies (Stone *et al.*, 1989). The murine IgA anti-TNP antibodies were purified using protein L sepharose (Biozol). Unlike protein G, protein L from *Peptostreptococcus magnus* is not specific for a certain antibody class; instead, it binds to the antibody kappa light chain of many species (Wikström *et al.*, 1995). For the purification of IgE anti-TNP antibodies, TNP-sepharose was prepared.

Generation of TNP-Sepharose for antibody purification

The coupling of TNP-BSA to cyanogen bromide (CNBr)-activated sepharose was performed according to Kavran and Leahy (2014). In brief, 6 mg TNP-BSA were dialyzed against coupling buffer using a 10 kDa centrifugal filter. Furthermore, 0.75 mg sepharose were resuspended in 15 mL of 1 mM HCl and transferred into a column. Next, the TNP-BSA solution (2.5 mL) was added to the sepharose and incubated at 4 °C on a tube rotator overnight. TNP-coupled sepharose was washed with coupling buffer and centrifuged (1000x g, 5 minutes) in order to remove unbound TNP-BSA. Uncoupled binding sites of the sepharose were blocked with 0.1 M Tris-HCl (pH 8) for 2 hours at room temperature. Finally, TNP-coupled sepharose was washed six times, alternating between high and low pH wash buffers. The column was stored in 20% ethanol in 1x PBS at 4 °C until further use.

General purification process

First, 2 mL of Protein G-, Protein L- or TNP-coupled-sepharose slurry were transferred into a column and washed with 50 mL 1x PBS (flow by gravity). Next, the cell culture supernatant was loaded onto the column. The flow-through was collected for a second purification step. Afterwards, 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 directly adjusted to pH 7 with 1 M Tris-HCl (pH 8.9). The antibody concentration was determined by Nanodrop and the buffer was exchanged to 1x PBS using 10 kDa centrifugal filters. Finally, antibodies were stored at -80 °C. The columns were washed

with 50 mL 0.1 M glycine followed by 50 mL 1x PBS. Columns were stored in 20% Ethanol in 1x PBS at 4 °C.

2.2.5 ELISA

Anti-TNP antibody ELISA

The TNP-specific ELISA (enzyme-linked immunosorbent assay) was conducted to verify antibody subclasses and reactivity after purification from cell culture supernatants, as well as comparing serum half-lives of anti-TNP antibodies.
Therefore, TNP-Ficoll was diluted to a final concentration of 10 µg/ mL in coating buffer and 100 µL of this solution 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. Next, wells were washed three times with 200 µL PBST. For blocking of residual binding sites, wells were incubated with 200 µL blocking buffer for 1 hour on a plate shaker. In the meantime, samples were diluted as indicated with blocking buffer. Again, wells were washed three times with 200 µL PBST and 100 µL of each sample were added per well. Plates were incubated for 1-2 hours at room temperature on a plate shaker. For detection of the bound antibodies wells were again washed three times with 200 µL PBST prior to incubation with 100 µL of Ig subclass-specific antibodies conjugated to horse-radish peroxidase (HRP) diluted in blocking buffer. Plates were incubated for 1 hour at room temperature on a plate shaker. 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 0.5 M sulfuric acid and absorbance was measured at 450 nm wavelength using an ELISA plate reader.

C3-ELISA

For the detection of complement factor C3 in mouse serum, the C3-ELISA was established by a PhD student of our laboratory, Gina-Maria Lilienthal. First, 96-well plates were coated with serial dilutions of IgG2a antibody allowing subsequent C3 binding. Plates were incubated either for 1 hour at room temperature on a plate shaker or overnight at 4 °C. Next, the wells were washed four times with 200 µL PBST. Mouse serum was diluted 1:20 in HBSS++/ 0.1% Tween20 and 50 µL were transferred to each well. The

plates were incubated for 1 hour on a plate shaker. Afterwards, the plates were washed four times with 200 µL PBST and incubated with a polyclonal goat-anti-mouse C3 antibody (1:10,000 in 1x PBS/ 0.1% Tween20) for 1 hour. Eventually, the plates were washed four times with 200 µL PBST and incubated with an HRP-conjugated rat-anti-goat IgG antibody for 1 hour. For signal detection, the plates were washed four times with 200 µL PBST and 100 µL of freshly prepared TMB substrate per well was added with a multichannel pipette. The reaction was stopped using 50 µL/ well 0.5 M sulfuric acid and absorbance was measured at 450 nm wavelength using an ELISA plate reader.

2.2.6 *In vitro* desialylation and degalactosylation of antibodies

For desialylation, antibodies were incubated in 5x Reaction Buffer B with α2,6-Sialidase A (50 mU/ 1 mg antibody). For further degalactosylation β1,4-galactosidase (40 mU/ 1 mg antibody) was added in addition. Antibodies were incubated at 37 °C on a shaker at 350 rpm for 48 hours. The reaction was stopped by exchanging the buffer to 1x PBS using centrifugal filters.

2.2.7 *In vitro* galactosylation and sialylation of antibodies

For galactosylation, antibodies were incubated with human β1,4-galactosyltransferase (transferase to antibody mass ration = 1:100) and UDP-galactose (UDP-galactose to antibody mass ratio = 1.2:1) in a 100 mM MES buffer supplemented with 20 mM Manganese(II) chloride (pH 7.2) at 37 °C and 350 rounds per minute (rpm) for 24 hours. For further sialylation, human α2,6-sialyltransferase (transferase to antibody ratio = 1:10) and CMP-sialic acid (CMP-sialic acid to antibody mass ratio = 1:2) were added to the galactosylated antibodies for an additional 8 hours. Reactions were stopped by dilution in 100 mM MES followed by a buffer exchange to 1x PBS using centrifugal filters.

2.2.8 Antibody N-glycan analysis by HPLC

For antibody Fc N-glycan release, 50 µg antibodies were incubated with recombinant endoglycosidase S (EndoS) from *Streptococcus pyogenes* (Collin and Olsén, 2001). The

reaction was carried out at 37 °C and 350 rpm for 8 hours. Afterwards, samples were stored at -20 °C until further analysis.

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

Graphite purified glycans were labeled with fluorophore 2-aminobenzamide (2-AB) for detection by a fluorescence detector. Excessive labeling reagent was removed by cellulose chromatography. Here, labeled glycans were eluted by H_2O and again vacuum dried using a vacuum centrifuge. Finally, glycans were reconstituted with 80% acetonitrile. 5 µL of the sample were transferred to the HPLC machine and separated by hydrophilic interaction liquid chromatography. Glycans that were more hydrophobic had a longer retention time.

The resulting chromatograms were analyzed with the software Chromeleon 6. All identified peaks were previously confirmed by MALDI-TOF analysis (Bartsch, 2019).

2.2.9 SDS-PAGE

In order to analyze antibody integrity, 5 µg of each antibody were diluted in 5x sodium dodecyl sulfate (SDS)-loading buffer and heat denatured for 5 minutes at 95 °C. The denatured samples were resolved by SDS-polyacrylamid gel electrophoresis (SDS-PAGE) using pre-casted 10% polyacrylamide gels (Mini-PROTEAN TGX). The gel assembly was placed into a gel tank which was filled up with SDS-running buffer. The comb was removed and 5 µL aliquots of the samples were loaded onto the gel and ran against a protein molecular weight standard (PageRuler Protein ladder). Electrophoresis was performed by voltage 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 the dye Coomassie-Brilliant Blue for 1 hour. Background staining of the gels was reduced by repeated washing steps with fresh destaining solution for 1 hour before the gels were documented photographically.

2.2.10 Passive systemic anaphylaxis (PSA)

IgG-mediated anaphylaxis

Passive systemic anaphylaxis (PSA) can be induced in mice through the intravenous (i.v.) transfer of antigen-specific IgG antibodies, followed by i.v. challenge with the appropriate antigen. Here, mice were sensitized by intravenous transfer of 200 µg TNP-specific IgG antibodies in 200 µL 1x PBS on day 0. Depending on the experimental setup, mice were challenged intravenously with 20 µg TNP-OVA in 200 µL 1x PBS either 30 minutes (30 minutes-model) or 24 hours (24 hour-model) later. The severity of the anaphylactic reaction was assessed by measuring the body core/ rectal temperature.

IgE-mediated anaphylaxis

For studying passive IgE-mediated anaphylaxis, mice were sensitized by the intravenous transfer of 10 µg TNP-specific IgE antibodies in 200 µL 1x PBS on day 0. The next day, mice were challenged intravenously with 1 µg TNP-OVA in a total volume of 200 µL 1x PBS. The severity of the anaphylactic reaction was assessed by measuring the body core/ rectal temperature.

Additional treatment regimens in PSA

Complement depletion

Transient depletion of complement was achieved by the intrapertitoneal injection of 50 µg cobra venom factor (CVF; Quidel) on day 0 one hour before sensitization with IgG2 anti-TNP antibodies. Mice were treated once again with 25 µg CVF on the next day one hour before challenging with TNP-OVA. Complement depletion was verified by C3-ELISA.

IVIG administration

As a treatment approach mice received 20 mg IVIG intraperitoneally 24 hours prior to induction of the anaphylactic response by TNP-specific antibodies and the TNP-OVA challenge.

Anti-SignR1 treatment

For transient depletion of the type II Fc receptor SignR1, mice received intravenously 100 µg anti-SignR1 monoclonal antibody (clone 22D1) in 200 µL 1x PBS. Anti-SignR1 treatment was performed one hour prior to the injection of IVIG or IgG1 anti-TNP antibody on day 0.

2.2.11 Sample collection

Murine samples

For collection of serum, whole blood and spleens mice were anesthetized with Ketamine/ Xylazine (80 mg/kg Ketamine, 10 mg/kg Xylazin; i.p.). Blood was collected by heart puncture using 23G needles. Afterwards, mice were sacrificed by cerebral dislocation before spleens were taken.
Blood was collected in EDTA tubes for whole blood analysis by flow cytometry or in serum gel tubes for antibody analysis by ELISA. Serum tubes were centrifuged at 4,000 g for 5 minutes and the supernatant was transferred into new tubes and stored at -20 °C until further analysis.

Human samples

Thirtysix healthy volunteer blood donors were recruited. Venous blood was taken using EDTA as anti-coagulant for cell analysis by flow cytometry. In addition, serum was collected for IgG antibody purification and IgG antibody glycan analysis.
Upon blood draw, the blood collection tubes were inverted several times. EDTA whole blood samples were analyzed immediately by flow cytometry.
Serum samples were centrifuged at 2000x g for 10 minutes. The supernatant was collected and frozen at -20 °C until further analysis.

2.2.12 Antibody labeling for FACS analysis

Anti-murine SignR1 antibody (clone 22D1) and anti-human FcγRIIB antibody (clone 2B6) were coupled with the Alexa Fluor 488 Abs labeling kit (Thermo Fisher) according to the manufacturer's protocol.

Anti-human FcγRIIA antibody (clone IV.3) was coupled to phycoerythrin (PE) using the PE-conjugation Kit (Abcam) according to the manufacturer's protocol.

2.2.13 FACS analysis

Murine samples

From the collected spleens, a single cell suspension was prepared by meshing the organ through a cell strainer (70 µm). Cells were collected in a 50 mL tube by rinsing the strainer with cold 1x PBS. Next, cells were centrifuged at 300g for 5 minutes at 4 °C. The supernatant was discarded and cells were resuspended with 5 mL Erylysis buffer. Erylysis was allowed for 5 minutes at room temperature before diluting the cells with 20 mL 1x PBS. Cells were again centrifuged and resuspended in 1 mL FACS buffer. Cell concentration was determined by dye exclusion test using trypan blue. Cells were counted using a hemocytometer and light microscopy. Finally, $10x10^6$ splenocytes were transferred into a 96-well plate.
For the analysis of mouse blood, 40 µL EDTA blood were incubated with 500 µL Erylysis buffer for 2 minutes in 1.5 mL reaction tubes. Afterwards, 500 µL FACS buffer were added and cells were centrifuged at 300g for 5 minutes at 4 °C. Cells were resuspended in 200 µL FACS buffer and transferred into a 96-well plate.

Cells were pelleted by centrifugation of the 96-well plate at 300g 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 with 100 µL of a master mix. Two staining protocols were applied: cells were either stained for anti-mouse CD11b (1:200), anti-mouse F4-80 (1:100) and anti-mouse Ly6G (1:200) for the differentiation of monocytes and neutrophils or for anti-mouse CD49b (1:200) and anti-mouse FcεRIα (1:200) for the identification of basophils. In addition, all cells were stained with fixable-viability dye (1:1000), anti-mouse FcγRIIB (1:200), anti-mouse FcγRIII (1:50) and anti-mouse SignR1 (1:100). Cells were stained for 20 minutes on ice in the dark. Next, cells were washed twice by adding 200 µL

FACS buffer per well and centrifuged at 300g for 5 minutes at 4 °C. The supernatant was discarded by inverting the plate and cells were resuspended with 200 µL Cytofix/ Cytoperm fixation buffer. Cells were incubated for 30 minutes at room temperature in the dark. Finally, cells were washed twice and resuspended in 200 µL FACS buffer and measured with the flow cytometer Attune NxT. Evaluation was performed with the software FlowJo.

Human samples

For the analysis of human blood samples, 100 µL EDTA whole blood were incubated with 500 µL Erylysis buffer for 2 minutes in 1.5 mL reaction tubes. Afterwards, 500 µL FACS buffer were added and cells were centrifuged at 300g for 5 minutes at 4 °C. The supernatant was discarded and cells were resuspended in 200 µL FACS buffer. For staining, cells were transferred into a 96-well plate. An additional washing step with 200 µL FACS buffer and centrifugation at 300 g for 5 minutes at 4 °C cells was performed. In a first staining step, cells were resuspended with 100 µL of a master mix containing anti-human FcγRIIB and the viability dye diluted 1:200 and 1:1000 in FACS buffer, respectively. Cells were stained for 25 minutes on ice in the dark. Cells were washed by adding 200 µL FACS buffer per well and centrifuged at 300g for 5 minutes at 4 °C. The supernatant was discarded by inverting the plate. Next, two staining protocols were applied: cells were either stained for anti-human CD14 (1:100), anti-human CD16/ FcγRIII (1:750) and anti-human CD19 (1:100) for the differentiation of monocytes and neutrophils or for anti-human FcεRI (1:100), anti-human CCR3 (1:50), anti-human CD3 (1:100), anti-human CD19 (1:100) and anti-human CD56 (1:100) for the exclusion of T-, B- and NK cells and the identification of basophils. In addition, all cells were analyzed for their surface expression of anti-human FcγRIIA (1:750). Therefore, a master mix for each staining protocol was prepared by diluting the respective amounts of antibodies in FACS buffer. Cells were resuspended with 100 µL of the master mix and incubated for 25 minutes on ice in the dark. Cells were washed twice by adding 200 µL FACS buffer per well and centrifuged at 300g for 5 minutes at 4 °C. The supernatant was discarded by inverting the plate and cells were resuspended with 200 µL Cytofix-Cytoperm fixation buffer. Cells were incubation for 30 minutes at room temperature in the dark. Afterwards, cells were washed twice by adding 200 µL FACS buffer per well and centrifuged at 300g for 5 minutes at 4 °C. Finally, cells were resuspended in 200 µL FACS buffer and

measured with the flow cytometer Attune NxT. Evaluation was performed with the software FlowJo.

2.2.14 Mast cell activation assay

Determination of the activation and degranulation of the human mast cell line LAD2 was performed according to Kuehn *et al.* (2010). Thereby, the release of the granule component β-hexosaminidase was measured. In brief, using a 24-well plate, $2.5x10^5$ cells per 0.5 mL culture medium were sensitized with 20 ng IgE anti-TNP antibodies for 5 hours at 37 °C. Following sensitization, cells were washed three times with 10 mL 1x PBS/ 0.1% BSA in order to remove excess IgE. Next, $5x10^4$ cells were stimulated wit 0.2 µg TNP-OVA in a total volume of 100 µL for 30 minutes on a plate shaker at 37 °C. Cells were centrifuged at 400x g for 10 minutes at 4 °C. Subsequently, 100 µL of cell free supernatant was transferred into a 96-well ELISA plate and incubated with 50 µL p-nitrophenyl N-acetyl-β-D-glucosamide (PNAG; 1.36 mg mL^{-1}) in citrate buffer for 90 minutes at 37 °C. In addition, for total β-hexosaminidase release cells were incubated with 0.05% TritonX-100 solution. 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.

2.2.15 Statistical analysis

Statistical analysis was conducted using GraphPad Prism, Version 6. Data are presented 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 Tukey's post-test correction for comparison of more than two groups. For analysis of the expression levels of the different cell surface receptors, the median fluorescent intensity (MFI) of each cell population was calculated by FlowJo and is presented as the mean (+/- standard deviation). One out of at least two independent experiments is shown. Linear regression analysis was carried out to correlate human total IgG sialylation with the expression level of distinct FcγRs on different immune cell populations. 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 demonstrated that antigen-specific IgG antibodies have the potential to induce anaphylaxis in mice when antigen doses are high. Thereby, murine IgG2 antibodies provoked a more severe anaphylactic response compared with murine IgG1 antibodies in different passive IgG-mediated anaphylaxis mouse models (Jönsson *et al.*, 2011; Beutier *et al.*, 2016; Epp *et al.*, 2018).

Here, I used a passive IgG-mediated systemic anaphylaxis mouse model:

- to clarify the contribution of complement activation versus classical type I FcγR activation
- to compare the anaphylactic potential of sialylated versus degalactosylated IgG antibodies regarding their serum half-life, the requirement for antigen-specificity and antibody subclass-dependency
- to analyze the contribution of the inhibitory FcγRIIB and type II FcR SignR1 concerning the impact of differently glycosylated IgG antibodies on antibody-mediated anaphylaxis.

3.1.1 Complement activation plays a minor role in the severity of IgG2-mediated anaphylaxis

IgG2a and IgG2b antibodies have been demonstrated to induce a more severe anaphylactic response compared with IgG1 antibodies (Beutier *et al.*, 2016; Epp *et al.*, 2018). Thereby, the strong interaction of IgG2 antibodies with activating FcγRIII and FcγRIV as well as the lower affinity of inhibitory FcγRIIB for IgG2 antibodies than for IgG1 antibodies may contribute to the amplified immune response. Accordingly, it has been demonstrated that FcγRs are mandatory for IgG-mediated anaphylaxis and that FcγRIIB attenuates IgG1- and IgG2b-mediated anaphylaxis, but not IgG2a-mediated anaphylaxis (Table 1; Beutier et al., 2016; Epp *et al.*, 2018).

However, besides the differential engagement of FcγRs, additional activation of the complement system might increase the severity of anaphylaxis mediated by IgG2 antibodies.

The complement system comprises a group of self-activating proteases that mediate inflammatory responses, contributing to innate defense mechanisms. It is initiated by different pathways that converge at the activation of the complement factor C3 and can result in the membrane attack complex (MAC) as well as the release of inflammatory mediators/ anaphylatoxins. Thereby, IgG2 but not IgG1 immune complexes can interact with complement factor C1q leading to the activation of the classical pathway of complement (Ricklin *et al.*, 2010; Murphy *et al.*, 2012; Lilienthal *et al.*, 2018).

Here, I analyzed whether complement activation contributes to the severity of IgG2-mediated anaphylaxis. Therefore, TNP-specific IgG2 subclass antibodies were produced and their anaphylactic potential was studied in complement depleted mice.

For use in passive IgG-mediated anaphylaxis, both monoclonal IgG2a anti-TNP antibodies (clone HA2a) and monoclonal IgG2b anti-TNP antibodies (clone HA2b) were produced by culturing the corresponding hybridoma cell lines. IgG2 anti-TNP antibodies were purified from cell culture supernatants by Protein G sepharose columns. Finally, purified IgG2 subclass antibodies were analyzed concerning their TNP-reactivity by ELISA. As expected, the TNP-specific IgG2a and IgG2b antibodies sharing the identical VDJ-region showed the same affinity for TNP (Figure 8).

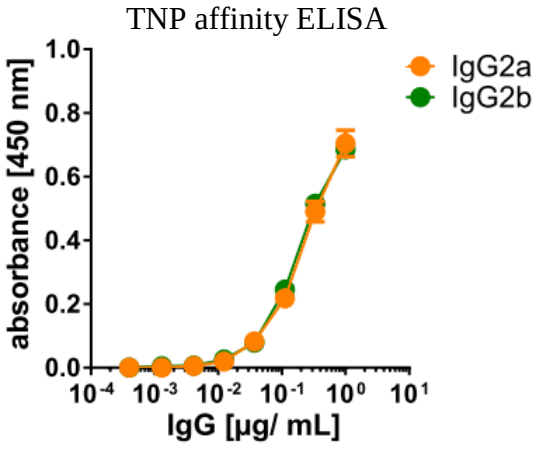

Figure 8: Generation of IgG2a and IgG2b anti-TNP subclass antibodies. *TNP-affinity of purified IgG2 antibodies was analyzed by ELISA. ELISA plates coated with 1 µg TNP-Ficoll per well were incubated with serial dilutions of IgG2a anti-TNP (clone HA2a) or IgG2b anti-TNP (clone HA2b) antibodies. Detection was performed using an HRP-conjugated goat-anti-mouse IgG antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are shown as mean +/- SEM.*

Next, passive systemic anaphylaxis (PSA) using either IgG2a anti-TNP antibodies or IgG2b anti-TNP antibodies was conducted. PSA can be induced in mice through the intravenous (i.v.) transfer of antigen-specific IgG antibodies, followed by challenging the mice i.v. with the appropriate antigen. Here, for the induction of IgG2-mediated PSA, mice were sensitized with 150 µg IgG2 anti-TNP antibodies followed by a challenge with 20 µg TNP-OVA 24 hours later. The severity of the anaphylactic response was assessed by measuring the drop in body core/ rectal temperature (Figure 9A). In addition, to test the contribution of complement in IgG2-mediated anaphylaxis, mice were treated intraperitoneally (i.p.) with cobra venom factor (CVF) one hour before the transfer of IgG2 antibodies and one hour before the antigen challenge as indicated in Figure 9A. CVF functionally resembles complement factor C3, leading to the activation and transient exhaustion of complement (Kock *et al.*, 2004). A successful depletion of complement by CVF was verified. Therefore, serum of CVF-treated and untreated mice was analyzed for the presence of C3 by ELISA (Figure 9B).

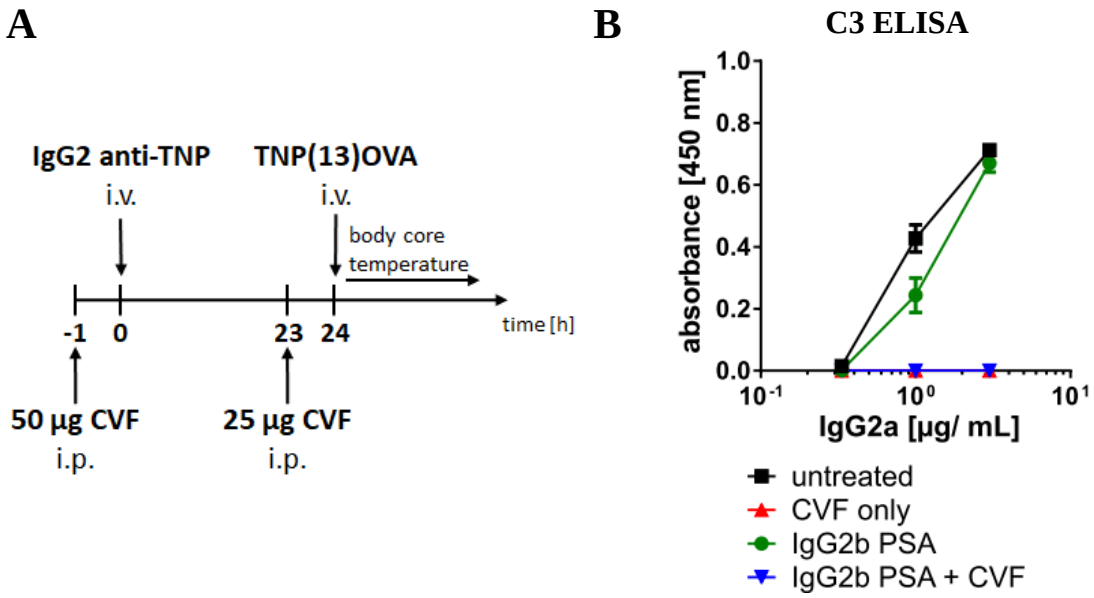

Figure 9: Complement depletion by cobra venom factor in IgG2-mediated anaphylaxis. (A) *Experimental setup of IgG2-mediated anaphylaxis. Mice were sensitized i.v. with 150 µg IgG2 anti-TNP antibodies and were challenged i.v. with 20 µg TNP-OVA the next day. To assess the severity of the anaphylactic reaction the body core/ rectal temperature of individual mice was monitored. Complement depletion was achieved by two i.p. injections of cobra venom factor (CVF).* ***(B)*** *Complement depletion by CVF was verified by ELISA. ELISA plates coated with serial dilutions of IgG2a antibodies (clone HA2a) were incubated with serum diluted 1:20 in HBSS++/ 0.1% Tween20. Detection was performed by subsequent incubation with a goat-anti-mouse C3 antibody followed by an HRP-conjugated rabbit-anti-goat IgG antibody. Absorption at 450 nm was measured using an ELISA plate reader. All data are shown as mean +/- SEM, n=4.*

Successful complement depletion in mice could be verified upon treatment with CVF. Furthermore, serum of untreated mice was compared with mice experiencing IgG2b-

mediated PSA (Figure 9B). Here, no significant decrease in C3 could be observed, suggesting that complement was hardly activated in the course of the anaphylactic response. This was also shown by the anaphylactic response itself. No significant difference could be observed between CVF-treated and CVF-untreated mice in IgG2a- (Figure 10A) or IgG2b-mediated anaphylaxis (Figure 10B). The results indicate that FcγR interaction but complement activation hardly, if at all, influences the severity of IgG2-mediated anaphylaxis in our model.

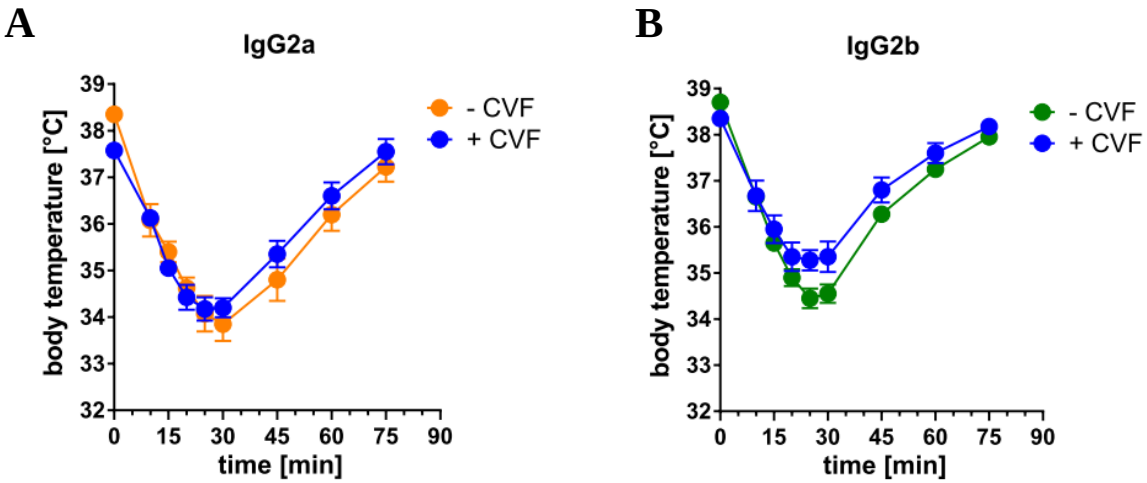

Figure 10: Complement activation hardly, if at all, contributes to the severity of IgG2-mediated anaphylaxis. *Mice were sensitized i.v. with 150 µg* ***(A)*** *IgG2a anti-TNP (clone HA2a) or* ***(B)*** *IgG2b anti-TNP (clone HA2b) antibodies and challenged i.v. with 20 µg TNP-OVA 24 hours later. The drop in body temperature of CVF-treated (blue) and CVF-untreated mice was compared. Data from one experiment are presented as mean +/- SEM, n = 4.*

3.1.2 Potential of differently glycosylated IgG antibodies in IgG-mediated PSA

As mentioned before, it has been shown that the effector functions of IgG antibodies not only depend on their subclass but also on their Fc glycosylation. At the time when I started this project the finding in our laboratory has been that *in vitro* sialylated IgG1 anti-TNP antibodies display a reduced anaphylactic potential compared with degalactosylated IgG1 anti-TNP antibodies in the 24 hour-model (Figure 11B; Epp *et al.*, 2018).

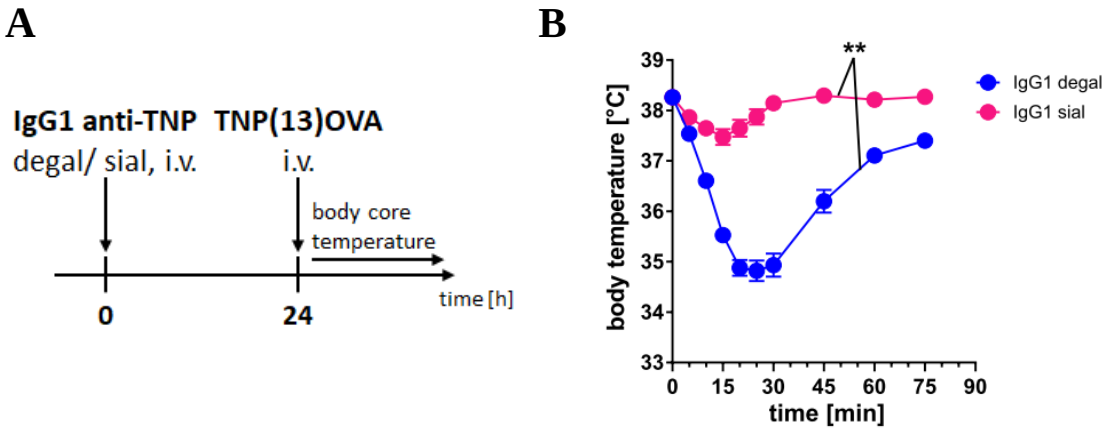

Figure 11: Anaphylactic potential of IgG1 anti-TNP antibodies depends on their Fc glycosylation. (A) *Experimental setup of IgG1-mediated anaphylaxis.* ***(B)*** *From Epp et al., 2018: mice were sensitized i.v. with 200 µg of degalactosylated (blue, "degal") or sialylated (pink, "sial") IgG1 anti-TNP antibodies. After challenging mice i.v. with 20 µg TNP-OVA, 24 hours later, the body temperature of individual mice was monitored. Data are presented as mean +/- SEM, n = 5. **P < 0.01 by paired t-test in (B).*

However, the mechanisms modulating the efficiency of differently glycosylated IgG1 antibodies to induce anaphylaxis have not yet been investigated. The following section will present data on the production of differently glycosylated IgG antibodies. Furthermore, immunomodulatory mechanisms in IgG-mediated anaphylaxis are analyzed.

3.1.2.1 Generation of differently glycosylated IgG1 anti-TNP antibodies

In order to investigate the potential of differently glycosylated IgG1 anti-TNP antibodies in murine IgG-mediated PSA *in vitro* glycan modification was performed.

The IgG1 anti-TNP antibodies (clone H5) were produced and purchased from InVivo (Henningsdorf, Germany). Next, *in vitro* sialylated as well as degalactosylated IgG1 antibody versions were generated to study their impact on anaphylaxis. Therefore, native IgG1 anti-TNP antibodies were galactosylated and subsequently sialylated ("sialylated") using beta 1,4-galctosyltransferase and alpha-2,6-sialyltransferase, respectively. Another portion of native IgG1 anti-TNP antibodies was desialylated and additionally degalactosylated ("degalactoslyated") using Sialidase A and beta-1,4-galactosidase, respectively. The native state of the IgG1 anti-TNP antibodies as well as the successful *in vitro* sialylation and degalactosylation was analyzed by high-performance liquid chromatography (HPLC). Therefore, Fc N-glycans were cleaved by recombinant endoglycosidase S (EndoS) from *Streptococcus pyogenes* (a gift from Mattias Collin (Lund, Sweden); Collin and Olsén, 2001) and purified as described in section 2.2.8.

Figure 12 shows the glycan distribution of representative IgG1 anti-TNP antibody batches. Native IgG1 anti-TNP antibodies from the hybridoma cell line H5 contained 42.0% of non-galactosylated plus non-sialylated ("agalactoslyated", G0) glycans. Furthermore, 32.8% and 7.8% of all glycans comprised one (G1) or two (G2) terminal galactose, respectively. Terminal sialylation (G1S1, G1S2 and G2S2) made up 17.4% of Fc glycans from native IgG1 anti-TNP antibodies.

In vitro galactosylation and additional sialylation enhanced the proportion of terminal sialylated Fc glycans to 95.5%. Only a small fraction of Fc glycans was still terminal galactosylated (4.5%) and no glycans remained agalactosylated. On the other hand, *in vitro* desialylation and additional degalactosylation increased the proportion of non-galactosylated IgG1 Fc-glycans from 42.0% to 94.2%, whereas terminal galactosylation was reduced from 38.2% to 5.2% and terminal sialylation was completely absent.

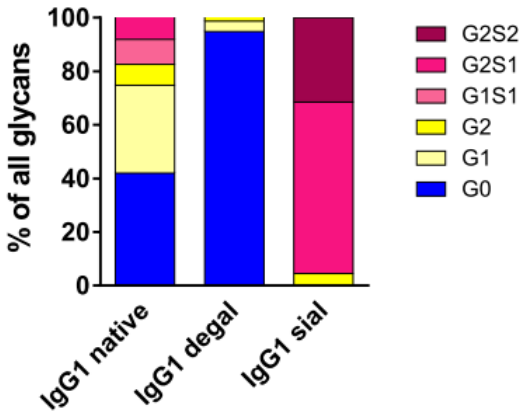

Figure 12: Fc glycosylation of IgG1 anti-TNP antibodies. *Glycan distribution of native IgG1 anti-TNP antibodies from the hybridoma cell line H5 and after in vitro desialylation plus degalactosylation ("degal") or galactosylation and subsequent sialylation ("sial"). G0 = agalactosylated, G1 = mono-galactosylated, G2 = bi-galactosylated, S1 = mono-sialylated, S2 = bi-sialylated. The analysis of the IgG1 anti-TNP antibody glycosylation was performed by Yannic Bartsch in our laboratory.*

Next, the differently glycosylated IgG1 anti-TNP antibodies were examined concerning their affinity to TNP by ELISA. Comparison of degalactosylated and sialylated IgG1 anti-TNP antibodies by TNP-ELISA revealed unaltered antigen-binding activity upon *in vitro* modification of the antibody Fc glycosylation (Figure 13), as already shown in previous studies (Hess *et al.*, 2013; Epp *et al.*, 2018).

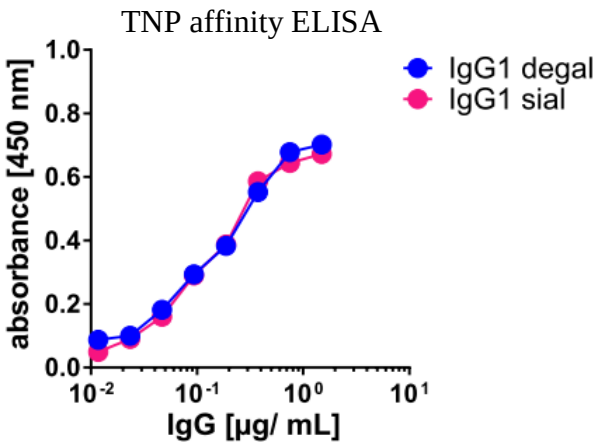

Figure 13: TNP-affinity of differently glycosylated IgG1 anti-TNP antibodies. *ELISA plates coated with 1µg TNP-Ficoll per well were incubated with serial dilutions of degalactosylated ("degal"; blue) or sialylated ("sial"; pink) IgG1 anti-TNP antibodies. Detection was performed using an HRP-conjugated goat-anti-mouse IgG antibody and absorption at 450 nm was measured using an ELISA plate reader. All data are shown as mean +/- SEM.*

In the following part, the two differently glycosylated IgG1 anti-TNP antibodies were used to investigate underlying mechanisms that may contribute to the increased or decreased anaphylactic potential upon Fc degalactosylation or sialylation, respectively, in the 24 hour-model of IgG-mediated PSA.

3.1.2.2 Sialic acid-induced attenuation of IgG1-mediated PSA depends on the type II Fc receptor SignR1

For the model of passive IgG1-mediated anaphylaxis, mice were sensitized with 200 µg sialylated or degalactosylated IgG1 anti-TNP antibodies (clone H5) followed by a challenge with 20 µg TNP-OVA 24 hours later (Figure 14A). The severity of the anaphylactic response was assessed by measuring the drop in body core temperature. The newly generated sialylated IgG1 anti-TNP antibodies also showed a reduced anaphylactic potential compared with degalactosylated IgG1 anti-TNP antibodies (Figure 14B, Figure 11B from Epp *et al.*, 2018).

However, it has not been analyzed so far whether the different anaphylactic potential of sialylated and degalactosylated IgG1 anti-TNP antibodies was due to a different half-life. Therefore, serum from IgG1 anti-TNP antibody sensitized mice was collected just before challenging the mice with TNP-OVA and again 75 minutes after the challenge when mice had recovered. The relative serum concentration of sialylated and degalactosylated IgG1 anti-TNP antibodies 24 hours after sensitization was analyzed by TNP-ELISA (Figure 14C). Here, no difference concerning the half-life of the differently glycosylated IgG1 anti-

TNP antibodies could be observed. Thus, different half-lives could not explain the different anaphylactic potential of the two IgG1 antibody glyco-versions.

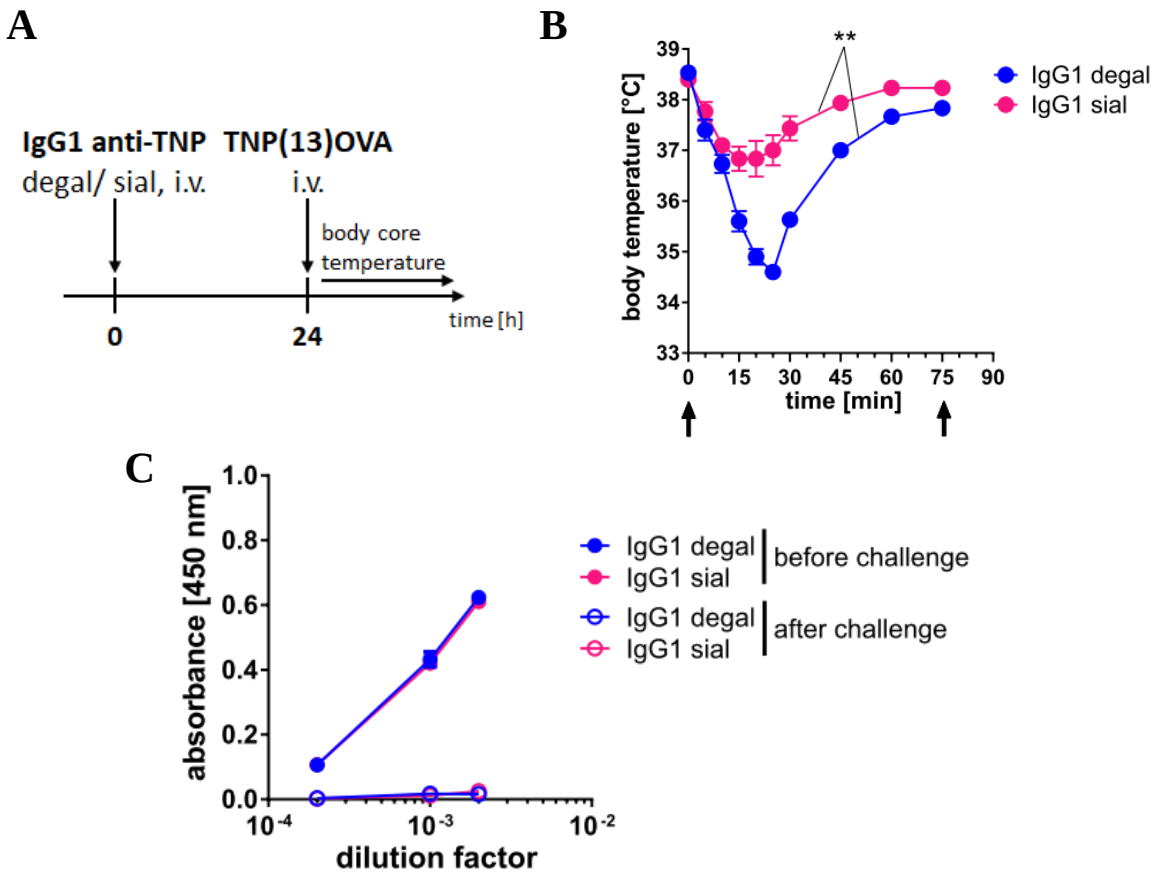

Figure 14: The glycosylation pattern of IgG1 antibodies, but not their half-life, influences IgG-mediated PSA. (A) *Experimental setup of IgG1-mediated anaphylaxis.* ***(B)*** *Mice were sensitized i.v. with 200 µg of degalactosylated (blue, "degal") or sialylated (pink, "sial") IgG1 anti-TNP antibodies. After challenging mice i.v. with 20 µg TNP-OVA, 24 hours later, the body temperature of individual mice was monitored. Arrows indicate the time points of serum collection.* ***(C)*** *ELISA plates coated with 1 µg TNP-Ficoll per well were incubated with serial dilutions of serum collected from IgG1-sensitized mice before and after the challenge with TNP-OVA. Detection of IgG anti-TNP antibodies was performed using an HRP-conjugated goat-anti-mouse IgG antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are presented as mean +/- SEM, n = 3. **P < 0.01 by paired t-test in (B).*

In comparison with the serum concentration of IgG1 anti-TNP antibodies before the TNP-OVA challenge, serum concentrations of IgG1 anti-TNP antibodies were drastically reduced 75 minutes after the challenge, irrespective of their Fc glycosylation pattern (Figure 14C). The depletion of IgG1 anti-TNP antibodies upon antigen challenge suggests that antibody immune complexes were formed and internalized by effector cells upon binding to FcγRs (Bonnerot *et al.*, 1998; Khodoun *et al.*, 2011; Bruhns and Jönsson, 2015).

Accordingly, FcRγ-deficiency protected mice from passive systemic anaphylaxis induced by either degalactosylated or sialylated IgG1 anti-TNP antibodies (Figure 15).

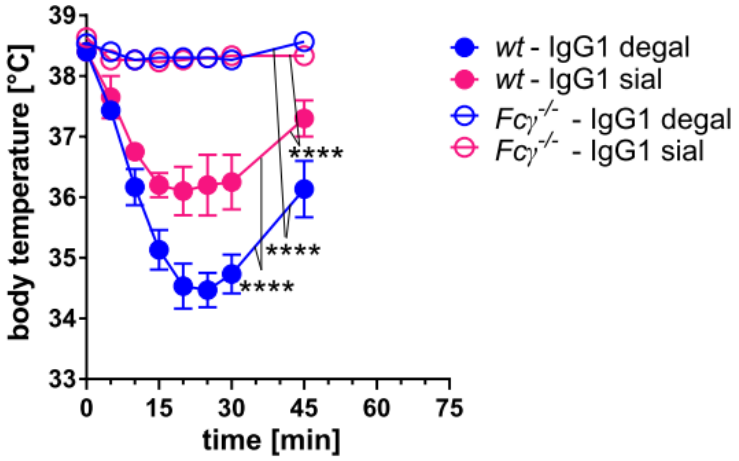

Figure 15: Induction of IgG1-mediated anaphylaxis depends on the presence of the FcRγ-chain. *C57BL/6 wild type mice (wt, filled symbols) or Fcγ-deficient mice (empty symbols) were sensitized i.v. with 200 µg of degalactosylated (blue, "degal") or sialylated (pink, "sial") IgG1 anti-TNP antibodies. Mice were challenged i.v. with 20 µg TNP-OVA, 24 hours later, and the body temperature of individual mice was monitored. Data from one experiment are presented as mean +/- SEM, n = 3. ****P < 0.0001 by two-way ANOVA.*

Murine IgG2 antibodies bind all activating FcγRs, whereas IgG1 antibodies bind only activating FcγRIII. Thus, in the context of IgG1-mediated anaphylaxis, FcγRIII expressed by monocytes/ macrophages, neutrophils and/ or basophils is a prerequisite for the induction of the anaphylactic response (Beutier et al., 2016). Accordingly, IgG1 antibody effector functions are regulated by their affinity for activating FcγRIII in relation to the inhibitory FcγRIIB as well as by the relative expression of these receptors by effector cells.

Next, I compared the anaphylactic potential of differently glycosylated IgG1 anti-TNP antibodies in wild type and FcγRIIB-deficient mice in the 24 hour-PSA model (Figure 16). As presumed, IgG1 anti-TNP antibodies induced a more severe anaphylactic response in FcγRIIB-deficient mice compared with wild type mice.

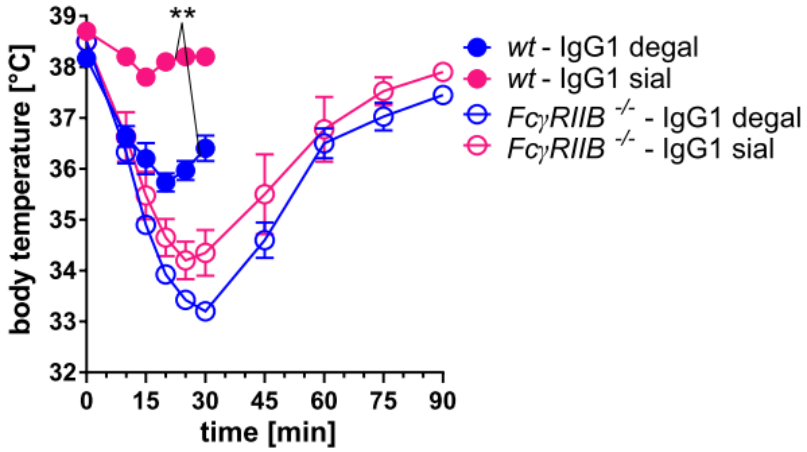

Figure 16: FcγRIIB attenuates IgG1-mediated anaphylaxis. *C57BL/6 wild type mice (wt, filled symbols) or FcγRIIB-deficient mice (empty symbols) were sensitized i.v. with 200 µg of degalactosylated (blue, "degal") or sialylated (pink, "sial") IgG1 anti-TNP antibodies. Mice were challenged i.v. with 20 µg TNP-OVA, 24 hours later, and the body temperature of individual mice was monitored. Data from one experiment are presented as mean +/- SEM, n = 4-5. **P < 0.01 by unpaired t-test.*

In summary, the experiments demonstrated a sialic acid-induced attenuation of IgG1-mediated anaphylaxis. Terminal IgG Fc sialylation of the conserved glycosylation site at Asn297 has been shown to promote structural changes of the IgG antibody Fc part, thus, exposing binding sites for type II FcRs while reducing the affinity for type I FcRs (Kaneko *et al.*, 2006; Sondermann *et al.*, 2013).

In the context of IgG-mediated anaphylaxis, sialylated IgG1 antibodies probably engage the classical activating FcγRIII with lower affinity. However, it has not been shown whether the increased affinity of sialylated IgG1 antibodies for type II FcRs introduces additional inhibitory signals that are responsible for its reduced anaphylactic potential. Type II FcRs (particular from the C-type lectin receptor family) have been demonstrated to induce modulatory or even inhibitory signals (Kanazawa *et al.*, 2002; Anthony *et al.*, 2008; Osorio and Reis e Sousa, 2011; Karsten *et al.*, 2012; Massoud *et al.*, 2014; Pincetic *et al.*, 2014).

Here, the contribution of the type II FcR SignR1 (specific ICAM-3-grabbing non-integrin-related 1) in IgG1-mediated anaphylaxis was analyzed. SignR1 is expressed particularly on monocytes/ macrophages. Furthermore, Kang and colleagues demonstrated that the i.v. application of the anti-SignR1 antibody (clone 22D1) induced a down-regulation of SignR1 on monocytes/ macrophages in the spleen, but not in the lymph nodes. Here, as demonstrated by flow cytometry 24 hours later (Figure 17), the i.v. application of 100 µg anti-SignR1 antibody selectively down-regulated the receptor on monocytes/ macrophages

in the spleen (Figure 18A), but not in the blood (Figure 18B; Kang *et al.*, 2003; Anthony *et al.*, 2008).

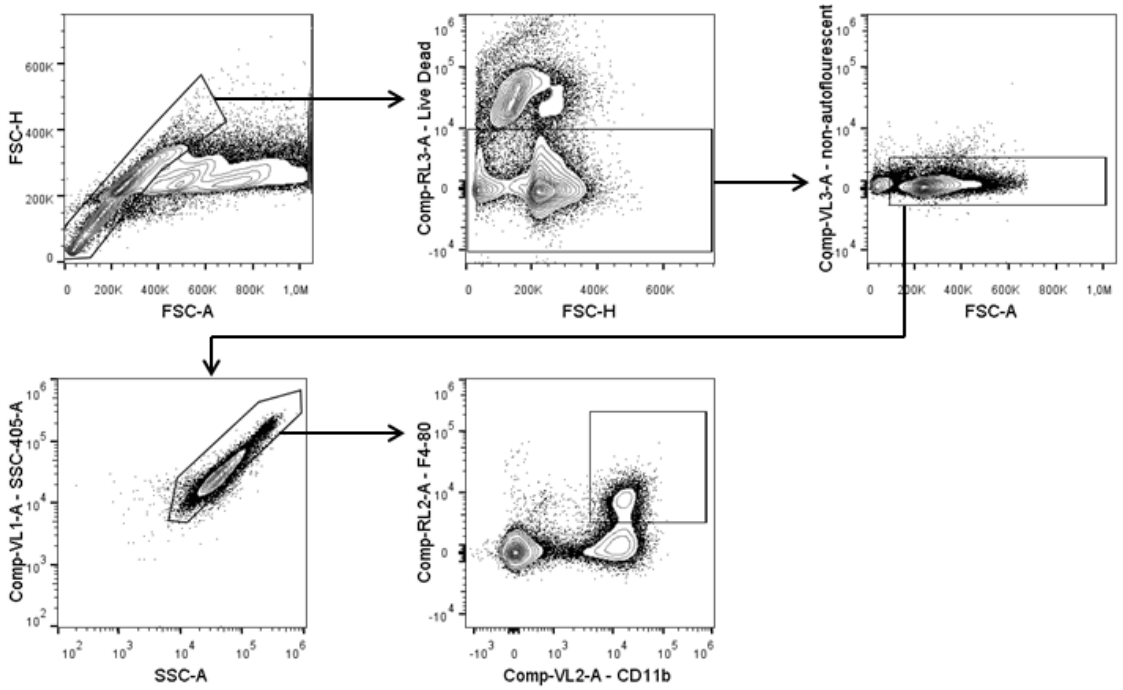

Figure 17: Gating strategy for murine splenic and blood monocytes/ macrophages. *Mice were treated i.v. with 100 µg anti-SignR1 antibody (clone 22D1) and were analyzed by flow cytometry 24 hours later. Splenocytes and whole blood cells were pre-gated on single, viable, non-autoflourescent leukocytes. Next, CD11b$^+$F4-80$^+$ monocytes/ macrophages were identified.*

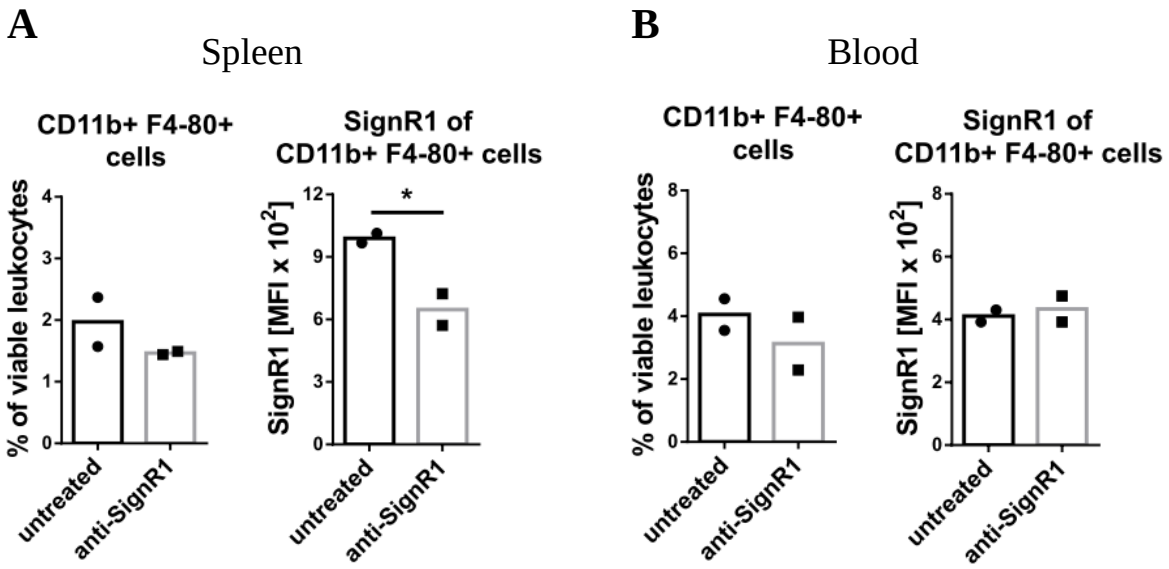

Figure 18: Treatment with an anti-SignR1 antibody down-regulates type II Fc receptor SignR1 on splenic monocytes/ macrophages. *Mice were treated i.v. with 100 µg anti-SignR1 antibody (clone 22D1) and were analyzed by flow cytometry 24 hours later.* ***(A)*** *Splenocytes and* ***(B)*** *whole blood cells were pre-gated on single, viable, non-autoflourescent leukocytes. Next, murine CD11b$^+$F4-80$^+$ monocytes/ macrophages were analyzed for their cell surface expression of the type II Fc receptor SignR1. Frequencies of monocytes/ macrophages of untreated or anti-SignR1 treated mice as well as the Mean fluorescent intensity (MFI) of*

*SignR1$^+$ monocytes/ macrophages are shown. Individual symbols represent individual mice. *P < 0.05 by unpaired t-test.*

In order to analyze the potential role of SignR1 in IgG-mediated anaphylaxis mice were treated intravenously with 100 µg anti-SignR1 one hour prior to sensitization with differently glycosylated IgG1 anti-TNP antibodies. Challenging with TNP-OVA 24 hours later revealed that sialylated IgG1 anti-TNP antibodies were no longer protective upon the transient down-regulation of SignR1 (Figure 19). Hence, it could be shown that the SignR1 expression affected by the pre-treatment with the anti-SignR1 antibody 22D1 contributes to the reduced anaphylactic potential of sialylated IgG1 antibodies.

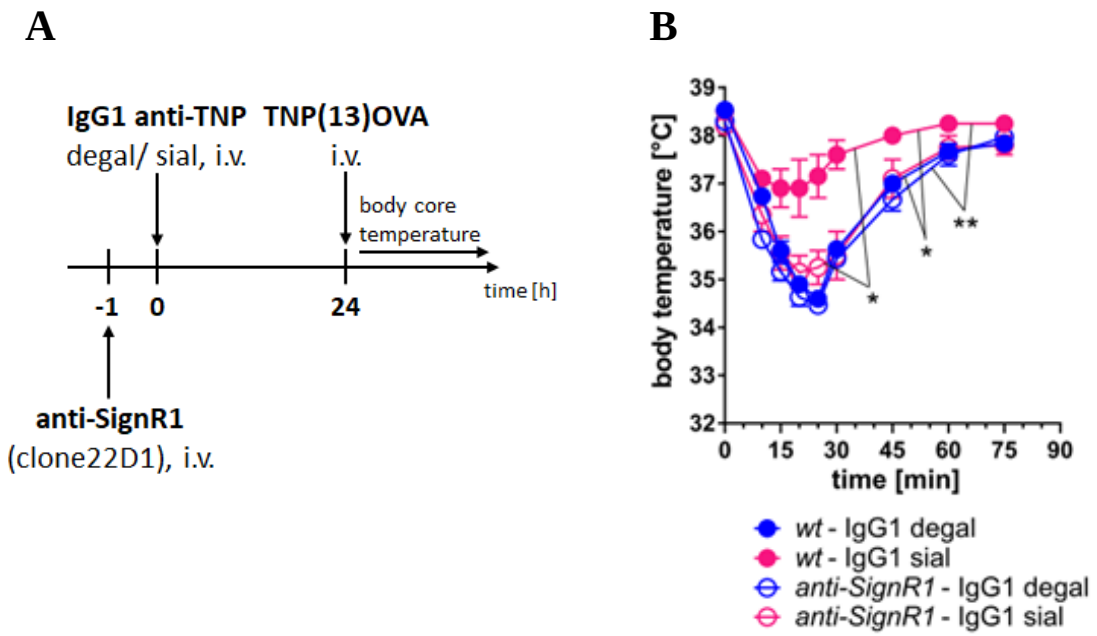

***Figure 19: Type II Fc receptor SignR1 is required for the reduced anaphylactic potential of sialylated IgG1 anti-TNP antibodies**. **(A)** Experimental setup of IgG1-mediated anaphylaxis. **(B)** Mice were sensitized i.v. with 200 µg of degalactosylated (blue, "degal") or sialylated (pink, "sial") IgG1 anti-TNP antibodies. 24 hours later, mice were challenged i.v. with 20 µg TNP-OVA. The drop in body temperature in C57BL/6 wild type mice (wt, filled symbols) was compared with mice treated with 100 µg anti-SignR1 antibody (clone 22D1; empty symbols). One of two independent experiments is shown. Data are presented as mean +/- SEM, n = 3-4. *P < 0.05 and **P < 0.01 by two-way ANOVA.*

Taken together, IgG1 antibodies showed different anaphylactic potentials upon *in vitro* degalactosylation or sialylation with the above-described 24 hour-model of IgG-mediated PSA.

It could be demonstrated that the reduced anaphylactic potential of sialylated IgG1 antibodies in the 24 hour-model was not due to a shorter half-life of these antibodies.

Furthermore, the anaphylactic potential of both IgG1 anti-TNP glyco-versions was dependent on the FcR γ-chain and was attenuated by FcγRIIB. Eventually, it could be demonstrated that the sialylation-dependent reduction of the IgG1-mediated anaphylactic reaction required the expression of the type II Fc receptor SignR1.

Parts of the presented experiments were published in Epp A*, Hobusch J*, Bartsch YC*, Petry J*, … Ehlers M. Sialylation of IgG antibodies inhibits IgG-mediated allergic reactions. *J Allergy Clinic Immunol 2018* (*these authors contributed equally).

3.1.2.3 Sialylated IgG1 antibodies attenuate IgG-mediated anaphylaxis in an allergen-unspecific but time-dependent manner

With the above-described 24 hour-model of PSA, IgG1 antibodies showed different anaphylactic potentials upon *in vitro* degalactosylation or sialylation (Figure 14B). Furthermore, the type II Fc receptor SignR1 was demonstrated to contribute to the reduced anaphylactic potential of sialylated IgG1 anti-TNP antibodies.

As mentioned above, SignR1-mediated anti-inflammatory effects have been described before for the antigen-unspecific effects of IVIG (Kaneko *et al.,* 2006; Anthony *et al.,* 2008; Anthony *et al.,* 2011; Galeotti *et al.,* 2017).
Here, I continued my investigation to figure out whether the SignR1-dependent inhibitory effect of sialylated IgG1 antibodies was antigen-specific or antigen-unspecific in the before applied 24-hour model of passive IgG1-mediated anaphylaxis compared to a 30 minutes-model.

First, I analyzed the anaphylactic potential of sialylated and degalactosylated IgG1 anti-TNP antibodies in a 30 minutes-model of PSA (Figure 20). Here, mice were challenged i.v. with TNP-OVA already 30 minutes after sensitization with the differently glycosylated IgG1 anti-TNP antibodies (clone H5). Surprisingly, the reduced anaphylactic potential of sialylated IgG1 antibodies could not be observed in the 30 minutes-PSA model. Degalactosylated and sialylated IgG1 anti-TNP antibodies induced comparable temperature drops.
FcγRIIB-deficiency enhanced the temperature drop for both glyco-versions. This was also observed before, when FcγRIIB-deficient mice were challenged after 24 hours (Figure 16).

Thus, attenuation of IgG1-mediated PSA by FcγRIIB is observed irrespective of the time point of the antigen challenge. On the other hand, sialylation-dependent inhibition is abrogated and seems to be time-dependent in wild type mice.
The anti-SignR1 treatment showed no effect in the 30 minutes-PSA model (Figure 20C).

Thus, the results are indicative of an antigen-unspecific, but time-dependent inhibitory mechanism of sialylated IgG1 antibodies.

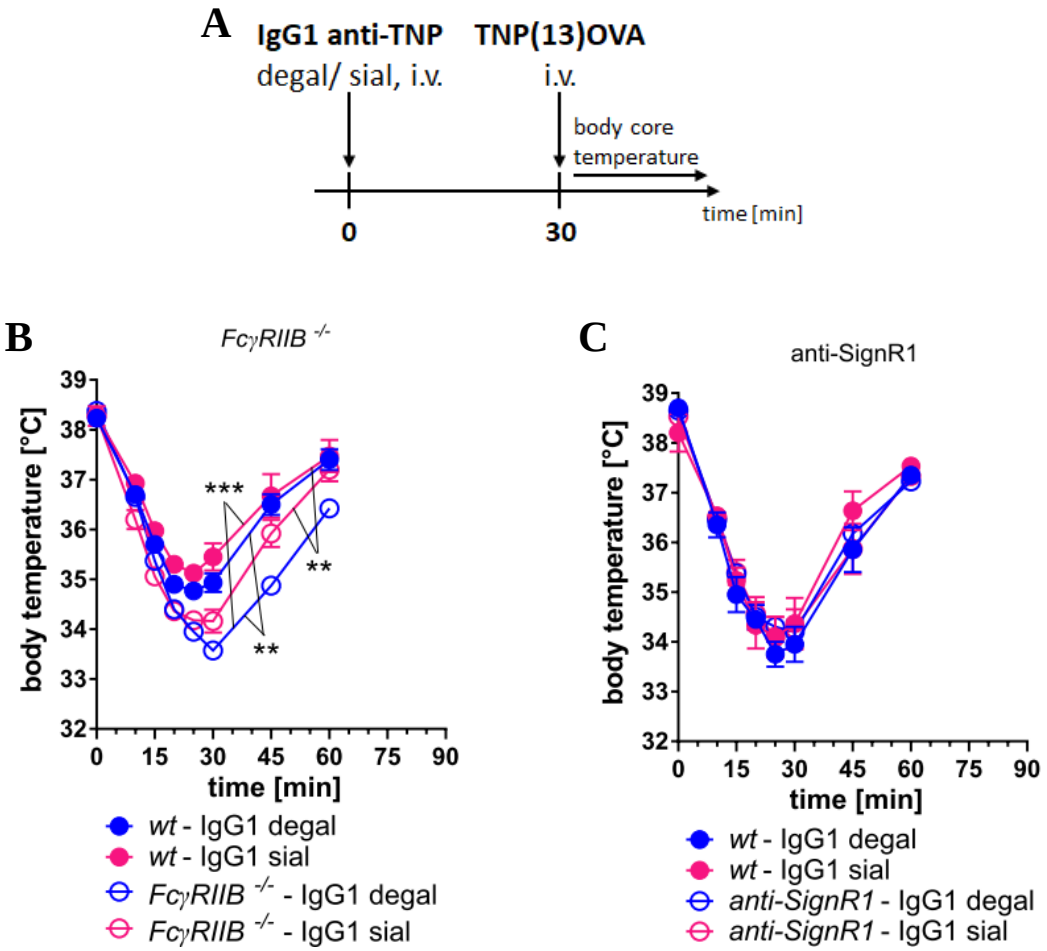

Figure 20: Sialylation-induced inhibition of IgG1-mediated anaphylaxis is abrogated with reduced sensitization time. (A) *Shortened experimental setup of passive IgG1-mediated anaphylaxis ("30 minutes model"). Mice were sensitized i.v. with 200 µg of degalactosylated (blue, "degal") or sialylated (pink, "sial") IgG1 anti-TNP antibodies before challenged i.v. with 20 µg TNP-OVA, only 30 minutes later. The drop in body temperature in C57BL/6 wild type mice (wt, filled symbols) was compared with* ***(B)*** *FcγRIIB-deficient mice or* ***(C)*** *mice treated with 100 µg anti-SignR1 antibody (clone 22D1; empty symbols). One of two independent experiments is shown. Data are presented as mean +/- SEM, n = 3-5. *P < 0.05, **P < 0.01 and ***P < 0.001 by two-way ANOVA.*

Next, an immunomodulatory potential of IVIG was tested under preventive treatment conditions in the 30 minutes-model of IgG-mediated anaphylaxis. Therefore, mice received i.p. 20 mg (1g/ kg) IVIG 24 hours before sensitization with sialylated or degalactosylated

IgG1 anti-TNP antibodies and challenging with TNP-OVA 30 minutes later (Figure 21A). As shown in Figure 21B, high amounts of IVIG attenuated the anaphylactic potential of both IgG1 antibody glyco-versions in wild type mice. In comparison with wild type mice, SignR1-depleted mice were not protected by IVIG treatment (Figure 21C).

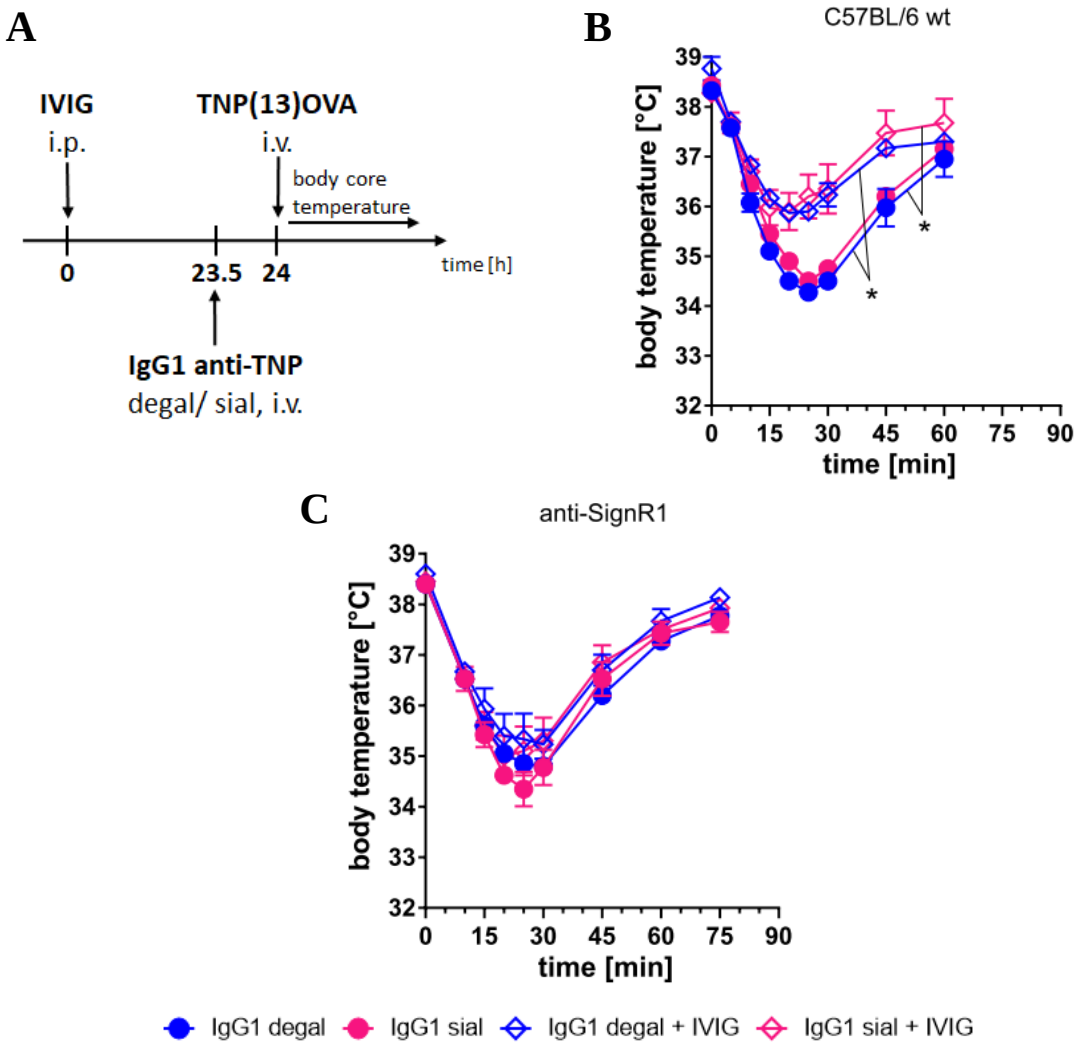

Figure 21: Treatment with IVIG attenuates IgG1-mediated anaphylaxis in wild type mice in a SignR1-dependent manner. ***(A)*** *Experimental setup. Mice were treated i.p. with 20 mg IVIG (empty symbols) or not (filled symbols). The next day, mice were sensitized i.v. with 200 µg of degalactosylated (blue, "degal") or sialylated (pink, "sial") IgG1 anti-TNP antibodies before challenging i.v. with 20 µg TNP-OVA, 30 minutes later. The drop in body temperature in* ***(B)*** *C57BL/6 wild type (wt) mice or* ***(C)*** *in mice treated with 100 µg anti-SignR1 antibody (clone 22D1; one hour before the IVIG-treatment) 24 hours before the TNP-OVA challenge was monitored. Data are presented as mean +/- SEM, n = 3-5. *P < 0.05 by two-way ANOVA.*

The potential of high amounts of IVIG to suppress the subsequent induction of IgG1-mediated anaphylaxis in the 30 minutes-model (Figure 21B) suggested that also the reduced anaphylactic potential of sialylated IgG1 anti-TNP antibodies in the 24 hour-model (Figure 14B) was mediated in an antigen-independent manner. In order to explore

this assumption, antigen-unspecific IgG1 antibodies (clone MOPC21, Figure 22A) were *in vitro* degalactosylated and sialylated (Figure 22B) for their application in the model of passive IgG1-mediated anaphylaxis.

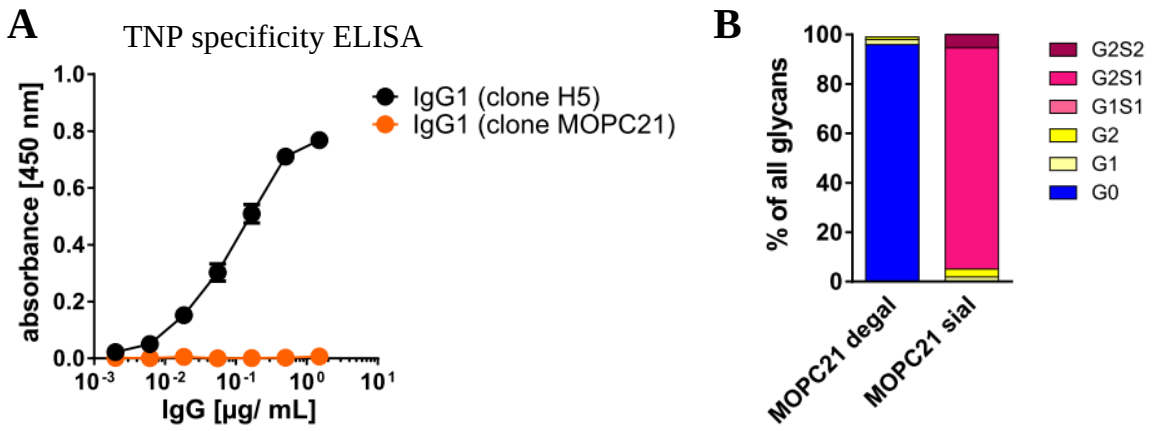

Figure 22: Analysis of antigen-unspecific IgG1 antibodies. (A) *ELISA plates coated with 1µg TNP-OVA per well were incubated with serial dilutions of IgG1 anti-TNP antibodies (clone H5, black) or antigen-unspecific IgG1 antibodies (clone MOPC21, orange). Detection was performed using an HRP-conjugated goat-anti-mouse IgG antibody and absorption at 450 nm was measured using an ELISA plate reader. All data are shown as mean +/- SEM.* ***(B)*** *Glycan distribution of in vitro degalactosylated ("degal") or sialylated ("sial") IgG1 antibodies from the hybridoma cell line MOPC21. G0 = degalactosylated, G1 = mono-galactosylated, G2 = bi-galactosylated, S1 = mono-sialylated, S2 = bi-sialylated.*

The immunomodulatory potential of degalactosylated and sialylated antigen-unspecific IgG1 antibodies (clone MOPC21) was tested in IgG1-mediated anaphylaxis. Therefore, mice received 1 mg of degalactosylated or sialylated antigen-unspecific IgG1 antibodies 24 hours before sensitization with IgG1 anti-TNP antibodies and challenging with TNP-OVA 30 minutes later. Notably, sialylated, but not degalactosylated, antigen-unspecific IgG1 antibodies were able to attenuate the anaphylactic response mediated by IgG1 anti-TNP antibodies on the next day (Figure 23A; these results were also observed with another antigen-unspecific IgG1 antibody clone (data not shown)). Furthermore, the pre-treatment with degalactosylated antigen-unspecific IgG1 antibodies did not further exacerbate the severity of anaphylaxis (Figure 23A). By contrast, sialylated antigen-unspecific IgG1 antibodies were no longer protective upon the transient down-regulation of SignR1 (Figure 23A) and failed to induce immunoinhibitory effects in FcγRIIB-deficient mice (Figure 23B).

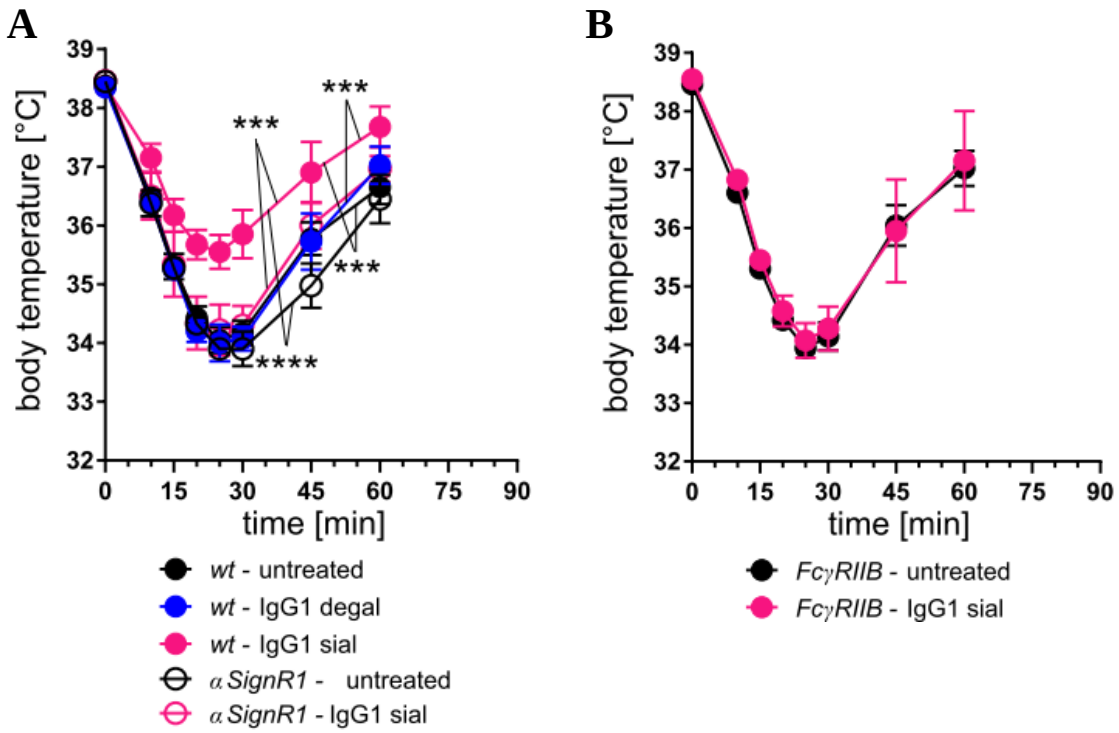

Figure 23: Antigen-unspecific sialylated IgG1 antibodies inhibit IgG1-mediated anaphylaxis in a SignR1 and FcγRIIB-dependent manner. *Mice were untreated (black) or treated with 1 mg of a degalactosylated (blue, "degal") or sialylated (pink, "sial") antigen-unspecific IgG1 antibodies (clone MOPC21). The next day, mice were sensitized i.v. with 200 µg of degalactosylated IgG1 anti-TNP antibody and challenged i.v. with 20 µg TNP-OVA. The drop in body temperature in* ***(A)*** *wild type mice (filled symbols) or in mice treated with 100 µg anti-SignR1 antibody (clone 22D1; empty symbols) on day 0 or* ***(B)*** *in FcγRIIB-deficient mice was monitored. Data are presented as mean +/- SEM, n = 4-5. ***P < 0.001 and ****P < 0.0001 by two-way ANOVA.*

Taken together, in the model of passive IgG1-mediated anaphylaxis suppressive effects could be directed to the Fc sialylation of IgG1 antibodies and were observed in an antigen-independent manner over time. Pre-treatment with high amounts (20 mg) of IVIG as well as lower amounts (1 mg) of sialylated antigen-unspecific IgG1 antibodies but not degalactosylated antigen-unspecific IgG1 antibodies attenuated IgG1-mediated anaphylaxis. Thereby, the inhibition by IVIG as well as sialylated antigen-unspecific IgG1 antibodies was dependent on the type II FcR SignR1. The inhibition by sialylated antigen-unspecific IgG1 antibodies was further dependent on FcγRIIB.

3.1.2.4 IgG subclass-dependent attenuation of IgG-mediated anaphylaxis

Next, the ability of sialylated antigen-unspecific IgG2a antibodies to attenuate IgG1 anti-TNP-mediated anaphylaxis was analyzed. Therefore, antigen-unspecific IgG2a antibodies (clone C1.18.4; Figure 24A) were purchased and *in vitro* degalactosylated and sialylated (Figure 24B).

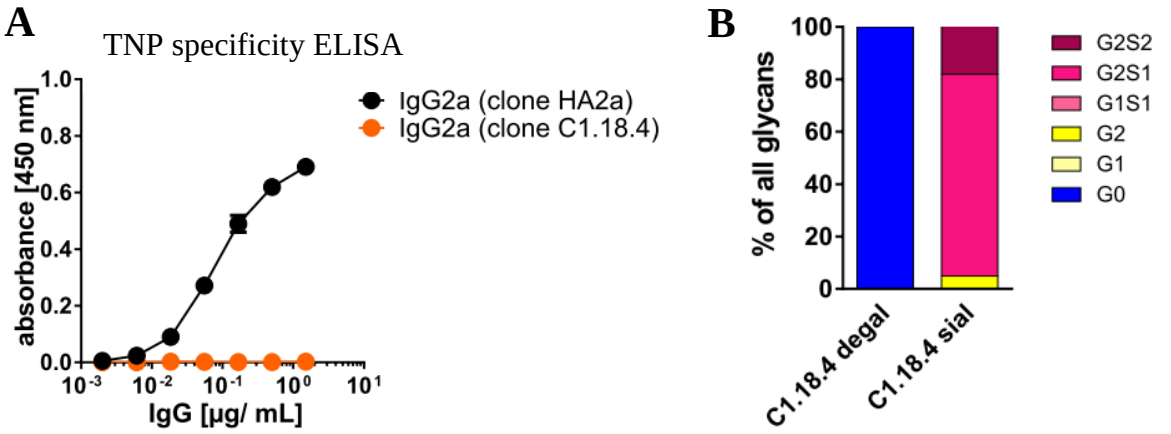

Figure 24: Analysis of antigen-unspecific IgG2a antibodies. (A) *ELISA plates coated with 1µg TNP-OVA per well were incubated with serial dilutions of IgG2a anti-TNP antibodies (clone HA2a, black) or antigen-unspecific IgG2a antibodies (clone C1.18.4, orange). Detection was performed using an HRP-conjugated goat-anti-mouse IgG antibody and absorption at 450 nm was measured using an ELISA plate reader. All data are shown as mean +/- SEM.* ***(B)*** *Glycan distribution of in vitro degalactosylated ("degal") or sialylated ("sial") IgG2a antibodies from the hybridoma cell line C1.18.4. G0 = degalactosylated, G1 = mono-galactosylated, G2 = bi-galactosylated, S1 = mono-sialylated, S2 = bi-sialylated. The analysis of the IgG2a antibody glycosylation was performed by Yannic Bartsch in our laboratory.*

The immunomodulatory potential of degalactosylated and sialylated antigen-unspecific IgG2a antibodies (clone C1.18.4) was tested in the 30 minutes IgG1-mediated PSA model. Therefore, mice received 1 mg of degalactosylated or sialylated antigen-unspecific IgG2a antibodies 24 hours prior to sensitization with IgG1 anti-TNP antibodies and challenging with TNP-OVA 30 minutes later. In comparison with sialylated antigen-unspecific IgG1 antibodies (Figure 23A), sialylated antigen-unspecific IgG2a antibodies failed to attenuate IgG1-mediated anaphylaxis (Figure 25).

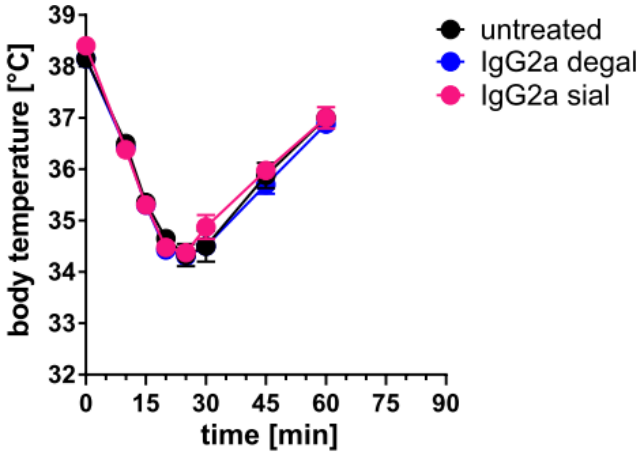

Figure 25: Sialylated IgG2a subclass antibodies are unable to reduce the severity of IgG1-mediated anaphylaxis. *Mice were untreated (black) or treated with 1 mg of degalactosylated (blue, "degal") or sialylated (pink, "sial") antigen-unspecific IgG2a antibodies (clone C1.18.4). The next day, mice were sensitized i.v. with 200 µg of degalactosylated IgG1 anti-TNP antibodies and challenged i.v. with 20 µg TNP-OVA. The drop in body temperature of individual mice was monitored. Data are presented as mean +/- SEM, n = 4.*

Comparable results have been found with sialylated IgG2a anti-TNP antibodies in the 24 hour-model of IgG-mediated PSA (Epp *et al.*, 2018). Thus, sialylated IgG2a antibodies might fail to interact with SignR1.

It would be of great interest to investigate whether other highly sialylated antibody isotypes could also facilitate immunosuppressive effects like observed for sialylated IgG1 antibodies. In the course of AIT, allergen-specific IgA antibodies are also induced (Jutel *et al.*, 2003; Scadding *et al.*, 2010). Furthermore, IgA antibodies are highly sialylated and have been demonstrated to interact with the type II Fc receptor SignR1 (Arnold *et al.*, 2007; Diana *et al.*, 2013).

In order to start to analyze the inhibitory potential of native/ highly sialylated IgA antibodies, I generated murine IgA anti-TNP antibodies (see section 2.2.2 and Supplements Table B1, Figure B1). As a first attempt, it could be demonstrated that IgA anti-TNP antibodies do not induce anaphylaxis in mice, however, they were able to inhibit IgG2-mediated anaphylaxis (Supplements Figure B2). Future studies will investigate the underlying antigen-unspecific or -specific mechanisms.

3.1.3 Sialylation-dependent attenuation of IgG1-mediated anaphylaxis is associated with FcγRIIB up-regulation

It has been shown in mice before that the sialylated IgG antibody fraction of IVIG interacts with the type II FcR SignR1 expressed by splenic macrophages, and thus, leads to an up-regulation of the inhibitory FcγRIIB and a down-regulation of FcγRIV on effector cells (Anthony *et al.*, 2008; Anthony *et al.*, 2011; Hirose *et al.*, 2015). Thus, in the following section I tested the hypothesis that IgG antibody Fc sialylation influences the FcγR expression on effector cells involved in IgG1-mediated anaphylactic immune responses. More precisely, I analyzed the capacity of sialylated IgG1 antibodies to modify the expression level of inhibitory FcγRIIB or activating FcγRIII on monocytes/ macrophages, neutrophils and basophils.

Mice were treated with degalactosylated or sialylated IgG antibodies. The next day, blood monocytes/ macrophages and neutrophils were analyzed by flow cytometry. Unfortunately, an insufficient number of blood basophils prevented the analysis of FcγR expression on these cells.

Blood cells were pre-gated on single, viable, non-autoflourescent leukocytes as shown before (Figure 17). Next, $CD11b^+$ $F4\text{-}80^+$ monocytes/ macrophages (Figure 26) and $CD11b^+$ $Ly6G^+$ neutrophils (Figure 27) were identified and analyzed for their expression of FcγRIIB and FcγRIII.

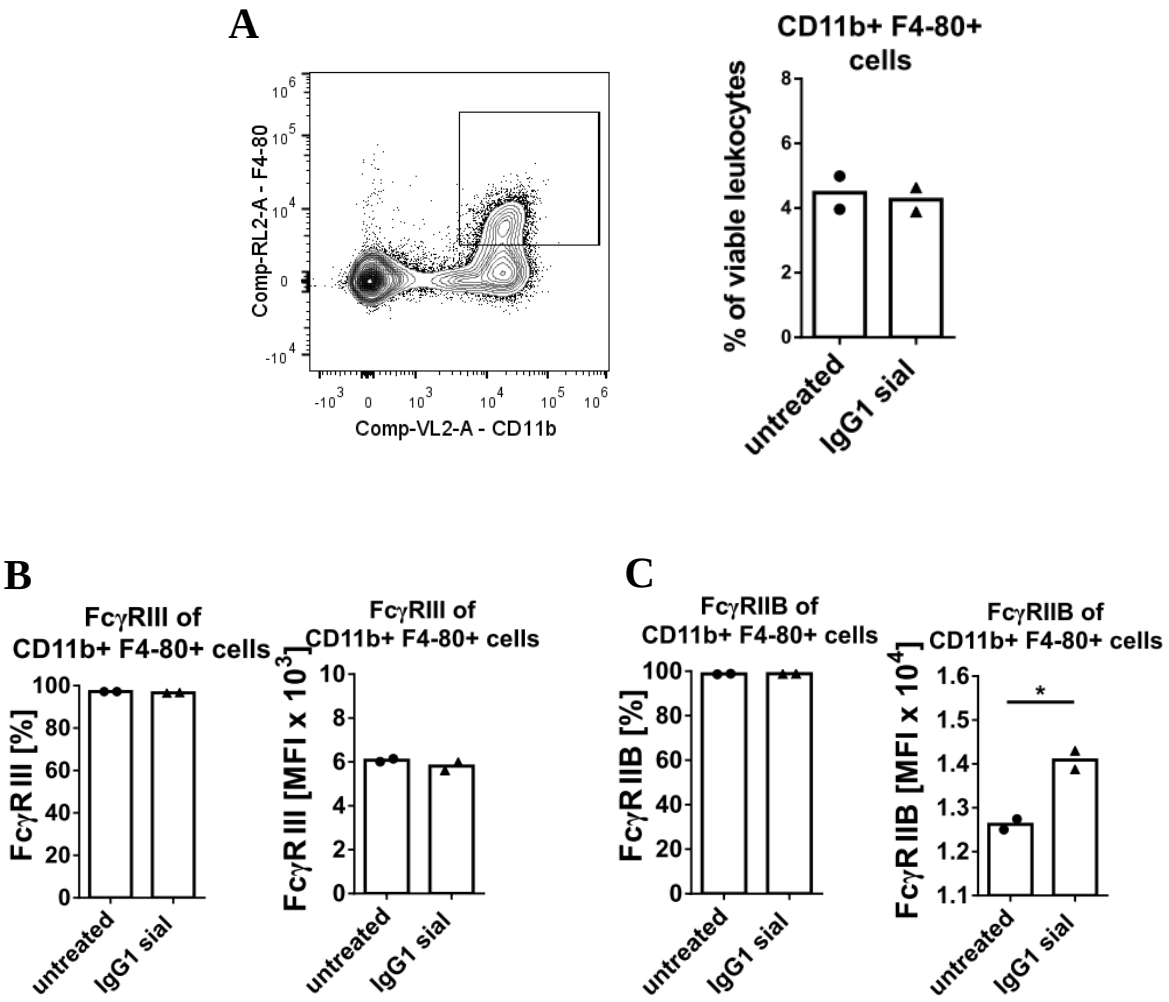

Figure 26: Expression of FcγRIIB and FcγRIII on monocytes/ macrophages upon treatment with sialylated IgG1 antibody. *Mice were treated i.v. with 200 µg sialylated ("sial") IgG1 anti-TNP antibodies and were analyzed by flow cytometry 24 hours later without antigen challenge. Whole blood cells were pre-gated on single, viable, non-autoflourescent leukocytes and* ***(A)*** *$CD11b^+F4\text{-}80^+$ monocytes/ macrophages were identified. Frequencies of monocytes/ macrophages of untreated or IgG1 anti-TNP antibody treated mice are shown. Next, monocytes/ macrophages were analyzed for their cell surface expression of* ***(B)*** *FcγRIII and* ***(C)*** *FcγRIIB. The frequency of FcγRIII and FcγRIIB expressing monocytes/ macrophages and the Mean fluorescent intensity (MFI) of FcγRIII and FcγRIIB are shown. Individual symbols represent individual mice. *P < 0.05 by unpaired t-test.*

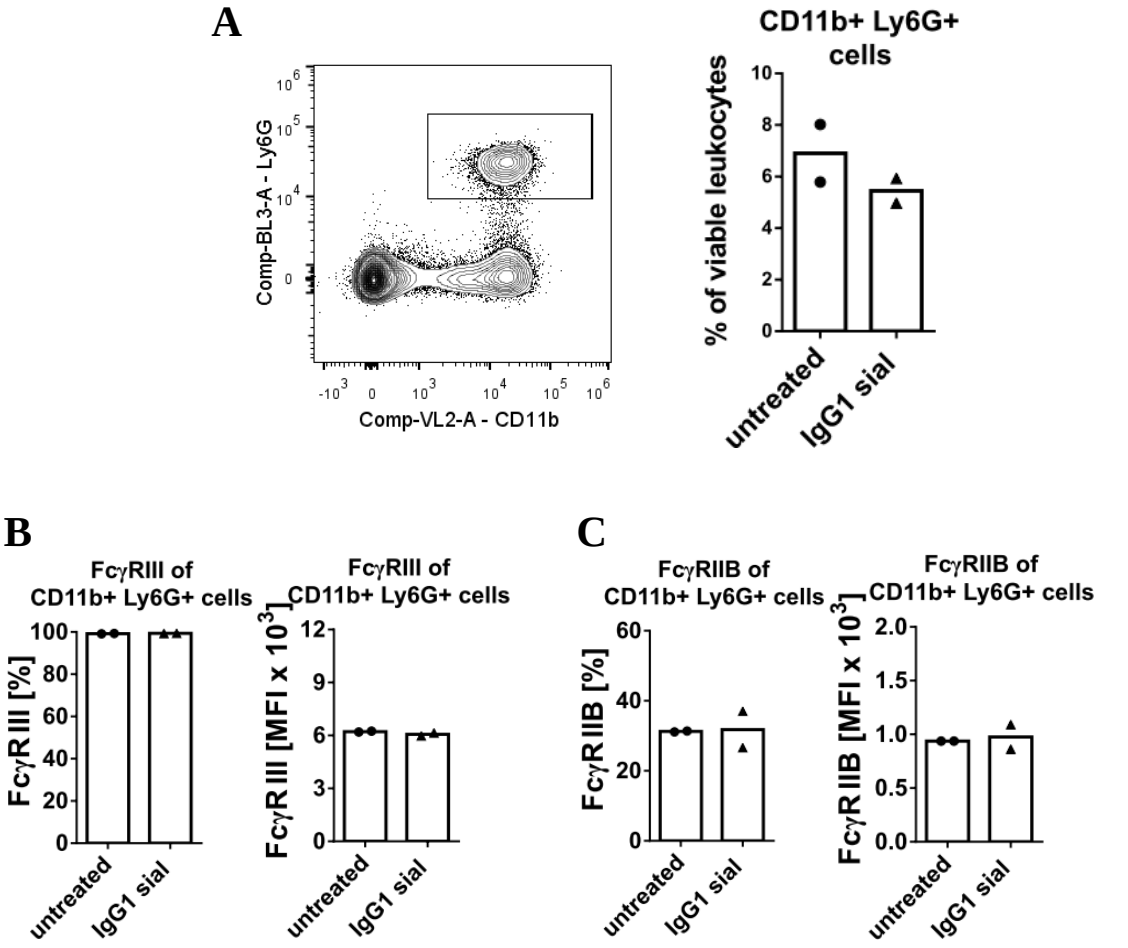

Figure 27: Expression of FcγRIIB and FcγRIII on neutrophils upon treatment with sialylated IgG1 antibody. *Mice were treated i.v. with 200 µg sialylated ("sial") IgG1 anti-TNP antibodies and were analyzed by flow cytometry 24 hours later without antigen challenge. Whole blood cells were pre-gated on single, viable, non-autoflourescent leukocytes and* ***(A)*** *$CD11b^+Ly6G^+$ neutrophils were identified. Frequencies of neutrophils of untreated or IgG1 anti-TNP antibody treated mice are shown. Next, neutrophils were analyzed for their cell surface expression of* ***(B)*** *FcγRIII and* ***(C)*** *FcγRIIB. The frequency of FcγRIII and FcγRIIB expressing neutrophils and the Mean fluorescent intensity (MFI) are shown. Individual symbols represent individual mice.*

According to the literature, both murine blood monocytes/ macrophages (Figure 26B) and neutrophils (Figure 27B) presented high levels of FcγRIII. By contrast, FcγRIIB was detected at high levels on monocytes/ macrophages (Figure 26C), whereas FcγRIIB was detected at lower levels on neutrophils (Figure 27C). Upon treatment with sialylated IgG1 antibodies, an up-regulation of inhibitory FcγRIIB on monocytes/ macrophages but not on neutrophils could be observed 24 hours later (Bruhns and Jönsson, 2015). No changes in the FcγRIII-expression could be observed.

Further results showed that in comparison to sialylated IgG1 antibodies the treatment with sialylated IgG2a antibodies does not affect the expression level of FcγRIIB on monocytes/ macrophages (data not shown).

In summary, inhibition of IgG-mediated anaphylaxis by IgG1 antibody Fc sialylation was demonstrated to occur in an antigen-independent albeit time-dependent manner. Furthermore, protection mediated by sialylated IgG1 antibodies required the presence of the type II Fc receptor SignR1 and was associated with an up-regulation of the inhibitory FcγRIIB on blood monocytes/ macrophages. By contrast, sialylated IgG2a antibodies failed to modify the anaphylactic potential of IgG1-mediated anaphylaxis.

3.2 IVIG as a treatment approach in IgG-controlled IgE-mediated anaphylaxis

In the context of IgE-mediated allergic reactions, the blocking property of allergen-specific IgG antibodies has been demonstrated. Antigen interception as well as FcγRIIB-dependent inhibition contributed to the ability of IgG antibodies to inhibit murine IgE-mediated anaphylaxis (Strait *et al.*, 2006; Möbs *et al.*, 2012; Epp *et al.*, 2018).

As demonstrated above, IVIG as well as sialylated (antigen-unspecific) IgG1 antibodies convey protection in IgG1-mediated anaphylaxis due to SignR1-dependent up-regulation of FcγRIIB. The same mechanism might enhance the blocking property of existing allergen-specific IgG antibodies. Therefore, the therapeutic potential of IVIG to attenuate IgG-controlled IgE-mediated anaphylaxis was investigated next.

IgE anti-TNP antibodies were produced by culturing the IGEL a2 hybridoma cell line. Purification of IgE anti-TNP antibodies from cell culture supernatants was achieved using self-made TNP-coupled sepharose columns. Ig isotype and TNP-specificity were frequently analyzed by TNP-ELISA (Figure 28).

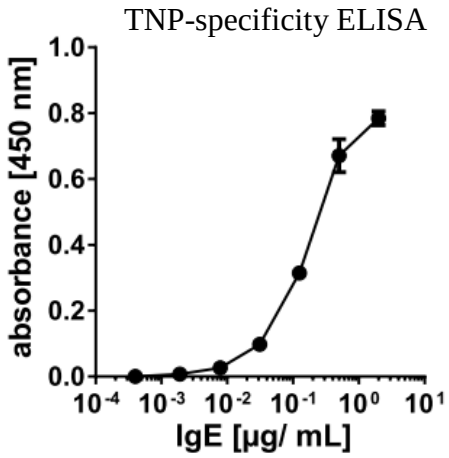

Figure 28: Generation of IgE anti-TNP antibodies. *Ig isotype and TNP-specificity of IgE anti-TNP antibodies produced by the hybridoma cell line IGEL a2 was analyzed by ELISA. ELISA plates coated with 1 µg TNP-Ficoll per well were incubated with serial dilutions of IgE anti-TNP antibodies. Detection was performed using an HRP-conjugated goat-anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are shown as mean +/- SEM.*

For the induction of passive IgE-mediated anaphylaxis, mice were sensitized i.v. with 10 µg IgE anti-TNP antibodies and challenged i.v. with 1 µg TNP-OVA the next day. The therapeutic potential of IVIG in passive IgE-mediated anaphylaxis was tested under preventive treatment conditions. Therefore, mice received 20 mg IVIG i.p. one hour prior to the i.v. transfer of IgE anti-TNP antibodies (Figure 29A).

As shown in Figure 29B, IVIG alone did not attenuate the anaphylactic potential of IgE antibodies. However, the transfer of 1.5 µg antigen-specific IgG1 antibodies (clone H5, IgG1 anti-TNP) 30 minutes prior to the TNP-OVA challenge attenuated IgE-mediated anaphylaxis and demonstrated the blocking property of these antibodies, as previously shown (Strait *et al.*, 2006; Epp *et al.*, 2018). Furthermore, IVIG-treatment (assumed to up-regulate FcγRIIB expression on effector cells) enhanced the inhibitory potential of antigen-specific IgG1 antibodies in IgE-mediated anaphylaxis (Figure 29C).

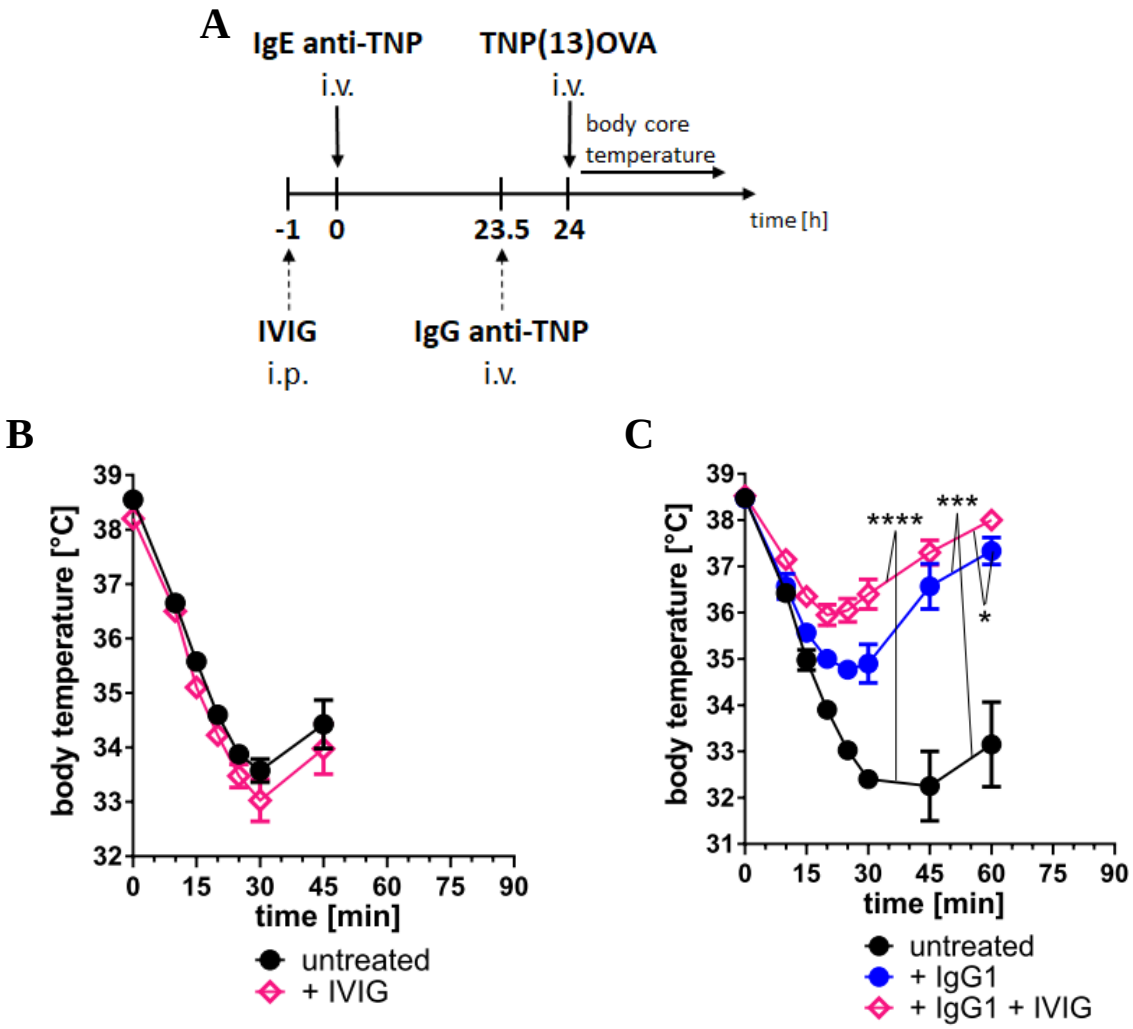

Figure 29: IVIG-treatment enhances IgG1-dependent inhibition of IgE-mediated anaphylaxis. (A) *Experimental setup of IgE-mediated anaphylaxis. Mice were sensitized i.v. with 10 µg IgE anti-TNP antibodies and challenged with 1 µg TNP-OVA the next day. In addition, mice were treated (dashed arrows) with* ***(B, C)*** *20 mg IVIG (empty symbols)* ***(C)*** *and/ or with 1.5 µg IgG1 anti-TNP antibodies. The drop in body temperature upon the TNP-OVA challenge was measured in order to assess the severity of the anaphylactic reaction. Data are presented as mean +/- SEM, n = 4. *P < 0.05, ***P < 0.001 and ****P < 0.0001 by two-way ANOVA in (C).*

Thus, IVIG-treatment enhanced the protective effect of allergen-specific IgG1 antibodies in IgE-mediated anaphylaxis probably due to an up-regulation of FcγRIIB on corresponding immune effector cells.

3.3 Total serum IgG antibody Fc sialylation correlates with FcγRIIB levels of human immune effector cells

In this study, the suppressive effects of high amounts of IVIG as well as lower amounts of highly sialylated IgG1 antibodies in murine IgG1-mediated anaphylaxis were demonstrated. Thereby, the suppressive effects were dependent on the type II FcR SignR1 and could be associated with an up-regulation of the inhibitory FcγRIIB on blood monocytes/ macrophages. The results suggest that a shift in total serum IgG antibody glycosylation patterns might influence the receptor expression on immune effector cells and ultimately contribute to altered IgG-mediated or IgG-controlled IgE-mediated allergic immune responses.

As a first approach and to test the hypothesis whether differences in the total IgG glycosylation pattern reflect individual expression levels of activating and/ or inhibitory FcγRs, I conducted a pilot study. Thirtysix healthy volunteer blood donors (12 male, 24 female; 23-58 years old) were recruited for the collection of EDTA-blood and serum. Blood cell analysis by flow cytometry as well as purification and analysis of total serum IgG antibody Fc glycosylation was performed.

Human blood cells were characterized for their expression of FcγRIIB and FcγRIIA by flow cytometry. Therefore, cells were pre-gated on single, viable, non-autoflourescent leukocytes. Next, three different leukocyte populations were characterized (Figure 30): neutrophils, monocytes/ macrophages and basophils. Neutrophils were identified by subsequent gating on side scatter $(SSC)^{high}$ granulocytes. Within the granulocyte-gate, neutrophils were defined as $CD16^{+}$ (FcγRIII) for the discrimination from eosinophils (neutrophils 52.27% +/- 13.60% of leukocytes; mean +/- SD). Classical monocytes were gated as SSC^{int}, forward scatter $(FSC)^{high}$ and delineated as $CD14^{+}$ $CD16^{-}$ leukocytes (monocytes/ macrophages 4.68% +/- 1.56% of leukocytes; mean +/- SD). Basophils were selected as SSC^{low} $CCR3^{+}$ $FcεRI^{+}$ leukocytes (basophils 0.53% +/- 0.29% of leukocytes; mean +/- SD).

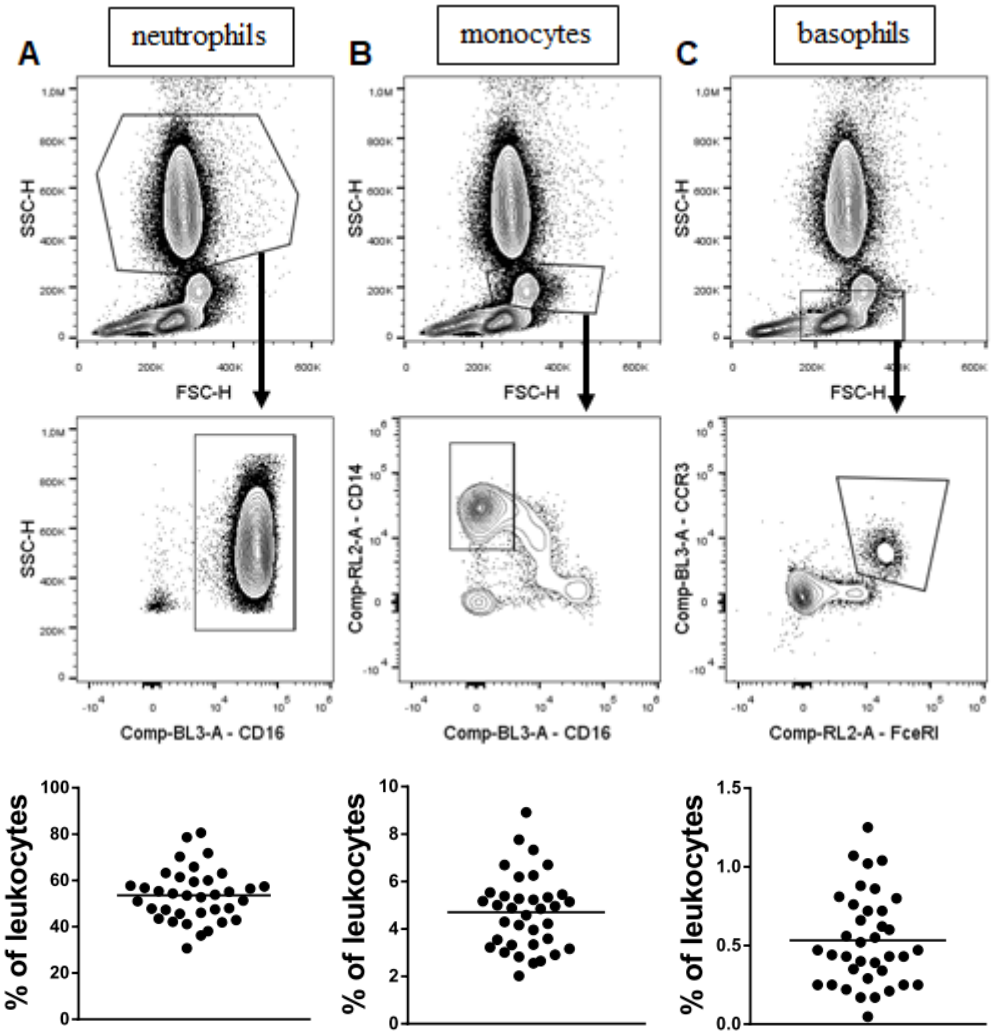

Figure 30: Gating strategy for human blood neutrophils, monocytes/ macrophages and basophils. *Cells were pre-gated on single, viable, non-autoflourescent leukocytes. The following leukocyte populations have been characterized:* ***(A)*** *$SSC^{hi}CD16^{+}$ neutrophils,* ***(B)*** *$SSC^{int}FSC^{hi}CD14^{+}CD16^{-}$ monocytes and* ***(C)*** *SSC^{low} $CCR3^{+}Fc\varepsilon RI^{+}$ basophils. Individual symbols represent individual blood donors.*

The blood donors presented high FcγRIIB levels (Figure 31) on basophils (97.85% +/- 0.25%; mean +/- SEM), whereas for monocytes/ macrophages and neutrophils lower FcγRIIB levels were detected (17.97% +/- 3.22% and 10.79% +/- 1.70%, respectively; mean +/- SEM). By contrast, FcγRIIA (Figure 32) was detected at high levels on monocytes/ macrophages and neutrophils (99.30% +/- 0.08% and 99.96% +/- 0.01%, respectively; mean +/- SEM) and at lower levels varying on basophils (47.74% +/- 3.67%; mean +/- SEM).

Furthermore, total serum IgG antibodies from the 36 healthy donors was purified. IgG Fc glycans were released by EndoS treatment and analyzed by HPLC. As expected, the analysis of the IgG antibody Fc glycan distribution revealed major differences among healthy individuals. The degree of degalactosylated (G0) and sialylated (S) IgG Fc glycans

varied between 17.6% – 44.1% and 4.2% – 14.2%, respectively. Supplement Tables B2 and B3 provide an overview of the individual donors.

Next, the individual IgG antibody Fc sialylation level was correlated with the individual expression levels of inhibitory FcγRIIB (Figure 31) and activating FcγRIIA (Figure 32) on blood neutrophils, monocytes/ macrophages and basophils. Interestingly, a correlation between IgG antibody Fc sialylation and FcγRIIB expression on female blood neutrophils ($p = 0.0027$) and male basophils ($p = 0.0355$) has been revealed. No correlation was found for FcγRIIA expression.

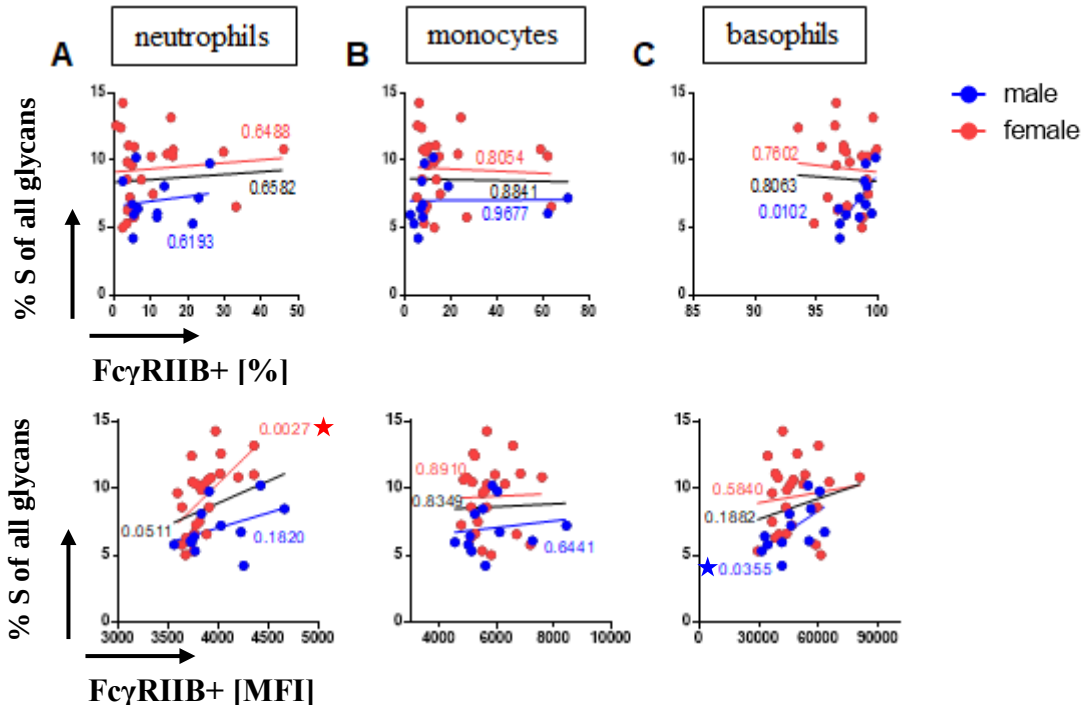

Figure 31: IgG Fc sialylation in correlation with inhibitory FcγRIIB expression on human blood neutrophils, monocytes/ macrophages and basophils. *The total IgG Fc sialylation (S) analyzed by HPLC is shown in correlation to the FcγRIIB expression (given as percentage or mean fluorescent intensity (MFI) of FcγRIIB-positive cells) on* ***(A)*** *neutrophils,* ***(B)*** *monocytes/ macrophages and* ***(C)*** *basophils analyzed by flow cytometry. Individual symbols represent individual blood donors and lines represent the median of each group (black = all donors, blue = male, red = female).*

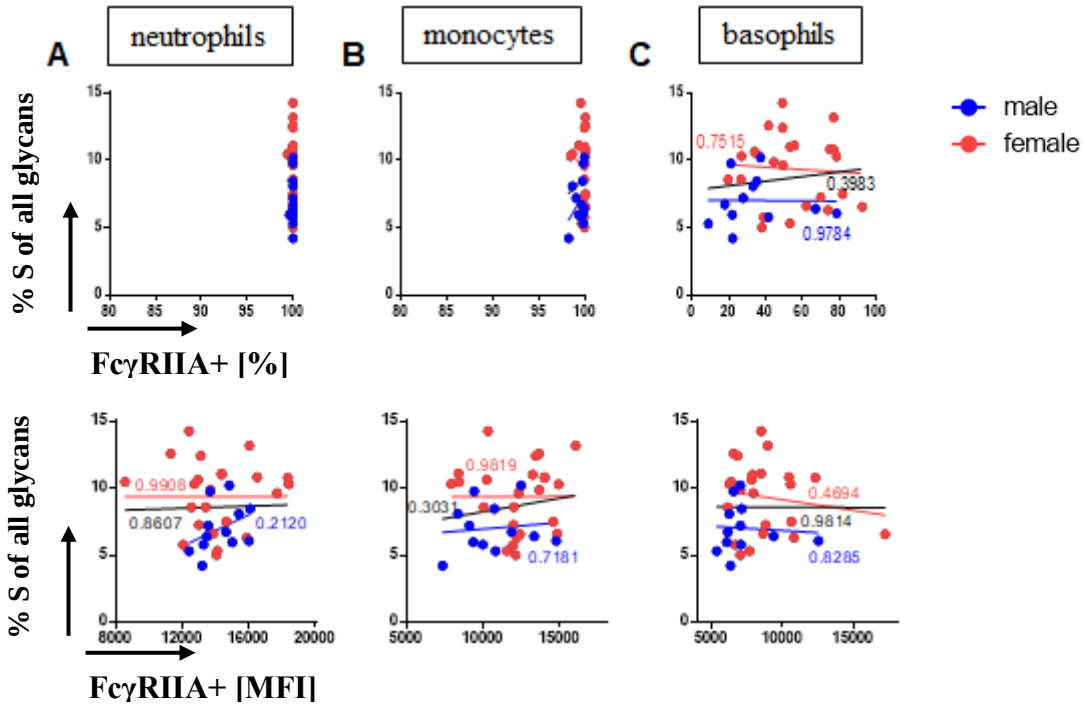

Figure 32: IgG Fc sialylation in correlation with activating FcγRIIA expression on human blood neutrophils, monocytes/ macrophages and basophils. *The total IgG Fc sialylation (S) analyzed by HPLC is shown in correlation to the FcγRIIB expression (given as percentage or mean fluorescent intensity (MFI) of FcγRIIA-positive cells) on* ***(A)*** *neutrophils,* ***(B)*** *monocytes/ macrophages and* ***(C)*** *basophils analyzed by flow cytometry. Individual symbols represent individual blood donors and lines represent the median of each group (black = all donors, blue = male, red = female).*

Taken together, it could be demonstrated that not only sialylated murine IgG1 antibodies could modify the expression levels of inhibitory FcγRIIB on immune effector cells in mice, but also physiological levels of human total IgG antibody sialylation correlated with the individual expression level of this receptor on human blood neutrophils (in females) and basophils (in males). This observation might be used in the future to evaluate e.g. AIT success. Furthermore, in search of new therapeutic approaches, restoring tolerogenic conditions in patients suffering from allergic reactions by IVIG or sialylated IgG antibodies might improve AIT efficacy.

3.4 *In vitro* activation of mast cells by differently glycosylated IgE antibodies

The importance of IgG antibody glycosylation for its interaction with different Fc receptors, and thus, for its effector function has been acknowledged. However, the contribution of glycan modifications to the IgE antibody-receptor interaction has hardly been investigated. Here, I intended in a side project to provide the basis for future studies analyzing the impact of IgE antibody glycosylation on its effector functions.

IgE antibodies are heavily glycosylated. Human and mouse IgE antibodies are decorated with seven and nine glycosylation sites, respectively (Figure 33A). Thereby, the conserved Fc glycan at Asn384 of murine IgE antibodies (corresponding to human IgE antibody Asn394) has been reported to be orthologous to the single Fc glycosylation site of IgG antibodies and to be obligatory for binding to FcεRI. Asn384 is occupied by oligomannose glycans, whereas the other glycosylation sites are of the complex type like existent at IgG antibodies (Figure 6B). As opposed to IgG antibodies, complex glycans of native IgE antibodies have been demonstrated to be mainly terminal sialylated (Arnold *et al.*, 2004, 2007; Plomp *et al.*, 2013; Shade *et al.*, 2015, 2019).

Here, I intended to provide the basis for future studies analyzing the impact of glycosylation on IgE antibody effector functions. Therefore, IgE anti-TNP antibodies were produced by culturing the IGEL a2 hybridoma cell line and purified via TNP-coupled sepharose columns (Figure 28). Next, differently glycosylated IgE anti-TNP antibodies were generated *in vitro*. Therefore, IgE anti-TNP antibodies were galactosylated and subsequently sialylated ("sialylated") using beta 1,4-galctosyltransferase and alpha-2,6-sialyltransferase, respectively. Another portion of IgE anti-TNP antibodies was desialylated ("desialylated") or desialylated and additionally degalactosylated ("degalactoslyated") using Sialidase A and beta-1,4-galactosidase, respectively.
IgE antibody glycosylation contributes to up to 12% of the antibody molecular weight (Plomp *et al.*, 2013). Thus, successful *in vitro* glycan modification could be demonstrated by SDS-PAGE (Figure 33B).

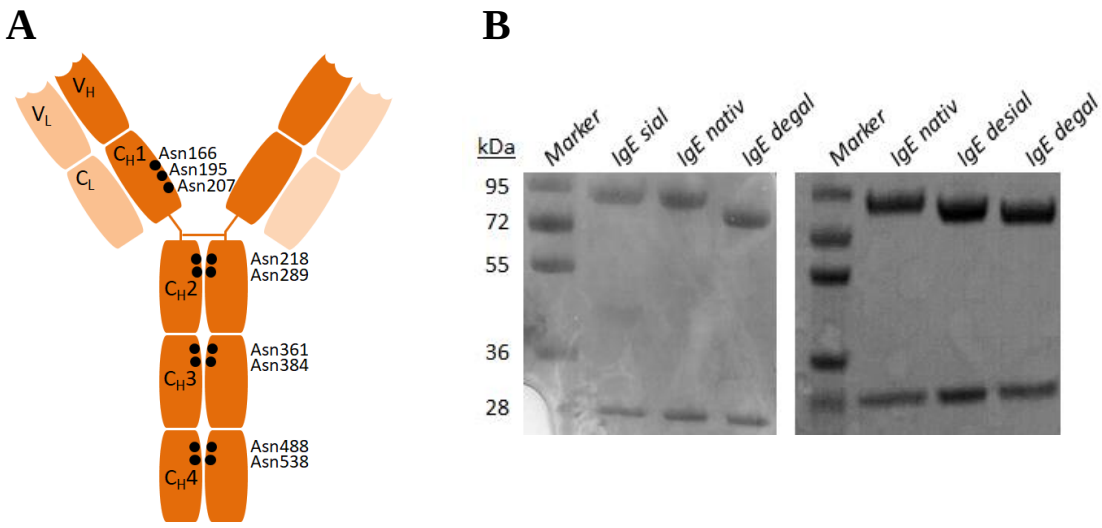

Figure 33: Murine IgE antibody glycosylation. (A) *IgE antibodies consist of two identical heavy (H, dark orange) and two identical light chains (L, light orange). The light chains comprise a variable (V_L) and constant (C_L) domain. The heavy chains comprise a variable (V_H) and several constant (C_H1- C_H4) domains. Furthermore, murine IgE antibodies have nine N-glycosylation sites (black circles;* Shade *et al.*, 2015). ***(B)*** *SDS-PAGE analysis of 5 µg IgE anti-TNP antibodies under reducing conditions showed two bands corresponding to the antibody heavy and light chains. Following in vitro galactosylation and additional sialylation ("sial"), desialylation ("desial") or desialylation and additional degalactosylation ("degal"), the IgE antibody heavy chains migrated at a different molecular weight. The experiment was conducted together with Marie Kubiak, a bachelor student in our laboratory.*

Next, differently glycosylated IgE anti-TNP antibodies were analyzed for their ability to activate the human mast cell line LAD2 (Figure 34). Mast cell activation by differently glycosylated IgE anti-TNP antibodies was assessed by the release of the preformed granule component β-hexosaminidase following cross-linking of FcεRI (Kuehn *et al.*, 2010). Therefore, LAD2 cells were sensitized with 20 ng of differently glycosylated IgE anti-TNP antibodies for 5 hours before cells were activated by 0.2 µg TNP-OVA for 30 minutes. The activation assay showed increased mast cell activation upon IgE antibody desialylation and degalactosylation. Furthermore, galactosylation and additional sialylation could further suppress the activation potential of native IgE anti-TNP antibodies. Future studies will investigate the mechanisms contributing to the disparate effects mediated by differently glycosylated IgE antibodies *in vitro* and have to verify the results *in vivo*.

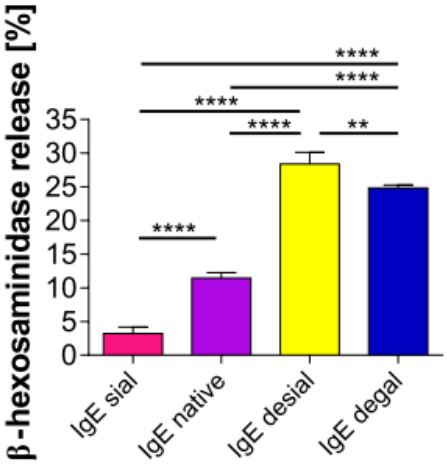

Figure 34: Mast cell activation by differently glycosylated IgE antibodies. *Mast cell activation was analyzed by β-hexosaminidase release. Therefore, 2.5x10^5 LAD2 cells were sensitized with 20 ng of differently glycosylated IgE anti-TNP antibodies for 5 hours. Next, cells were activated with 0.2 µg TNP-OVA for 30 minutes. The released β-hexosaminidase was calculated as percentage of total release by 0.05% TritonX-100. Data are presented as mean +/- SEM, n = 4. **P < 0.01 and ****P < 0.0001 by one-way ANOVA. The experiment was conducted together with Gina-Maria Lilienthal, a PhD student in our laboratory, and Marie Kubiak, a bachelor student in our laboratory.*

4 Discussion

Anaphylaxis is a severe and systemic allergic reaction that results from the rapid release of inflammatory mediators and cytokines by myeloid cells. Thereby, two different pathways have been described in mice. Classically, anaphylaxis is attributed to IgE-mediated immune responses. Thereby, cross-linking of the high-affinity receptor FcεRI by allergen-specific IgE antibodies leads to an activation of mast cells and basophils. By contrast, IgE-mediated allergic reactions can be inhibited by allergen-specific IgG antibodies via allergen masking and particular by cross-linking of the inhibitory IgG receptor FcγRIIB with FcεRI. However, when allergen doses are high, allergen-specific IgG antibodies themselves can cause anaphylaxis in mice by driving FcγR-induced activation of neutrophils, monocytes/ macrophages and/ or basophils (Simons *et al.*, 2014; Finkelman *et al.*, 2016; Beutier *et al.*, 2016).

Four different IgG antibody subclasses have been identified in mice (IgG1, 2a, 2b and 3), which substantially differ in their FcγR binding (Table 1). FcγRs regulate immune responses by inducing activating or inhibitory signals. In mice, three activating FcγRs have been recognized: FcγRI, FcγRIII and FcγRIV. On the other hand, inhibitory signals are conveyed by FcγRIIB that interferes with activating signals, thus, modulating the activation threshold of effector cells (Vidarsson *et al.*, 2014; Bruhns and Jönsson, 2015). Accordingly, it could be shown that the different mouse IgG antibody subclasses differ in their potential to induce IgG-mediated anaphylaxis. Correlating with their affinity for activating and inhibitory FcγRs, IgG2 antibodies were demonstrated to induce a more severe anaphylactic response compared with IgG1 antibodies. Murine IgG1 antibodies interact with only one activating FcγR, FcγRIII, and have a relatively high affinity for inhibitory FcγRIIB. On the other hand, the high affinity of IgG2 antibodies for activating FcγRs as well as the rather low affinity for inhibitory FcγRIIB are thought to contribute to the amplified anaphylactic response observed for IgG2 antibodies (Table 1; Beutier *et al.*, 2016; Epp *et al.*, 2018).

As demonstrated for antibody-mediated protection against pathogens as well as for autoimmune diseases, the different IgG antibody subclasses have different protective or pathogenic potentials, respectively. Thereby, besides the differential engagement of FcγRs, additional activation of the complement system determines the effector functions of the

different IgG antibody subclasses (Sitaru *et al.*, 2007; Hovenden *et al.*, 2013; Ito *et al.*, 2014; Vidarsson *et al.*, 2014). In the context of anaphylaxis, Jiao and colleagues analyzed passive systemic anaphylaxis mediated by monoclonal IgG1 antibodies in complement factor C3-deficient mice. In comparison with wild type mice, the symptoms remained unchanged in C3-deficient mice and led to the conclusion that IgG-mediated anaphylaxis is independent on complement activation (Jiao *et al.*, 2014). However, unlike IgG2 antibodies, murine IgG1 antibodies were found to be unable to activate the classical complement pathway via C1q (Yamada *et al.*, 2013; Ito *et al.*, 2014; Lilienthal *et al.*, 2018). Therefore, I analyzed the anaphylactic potential of IgG2a and IgG2b antibodies upon transient depletion of complement by CVF (Figure 10). Complement depletion hardly render mice more susceptible to IgG2-mediated anaphylaxis, suggesting that complement activation does not substantially contribute to the severity of the anaphylactic reaction.

Previous studies have confirmed that passive IgG1-mediated anaphylaxis occurs via activating FcγRIII (the only activating FcγR for murine IgG1) expressed by monocytes/ macrophages, neutrophils and/ or basophils (Miyajima *et al.*, 1997; Strait *et al.*, 2002; Beutier *et al.*, 2016). Furthermore, attenuation of the anaphylactic reaction is mediated by inhibitory FcγRIIB. Thereby, FcγRIIB modulates the activation threshold of immune effector cells when co-engaged by IgG immune complexes with activating FcγRs expressed on the same cell (Beutier *et al.*, 2016).
However, nowadays it is widely accepted that effector functions of IgG antibodies are not only determined by their subclass but are also influenced by their Fc N-glycosylation pattern. An enrichment of agalactosylated IgG antibodies has been observed in patients suffering from different inflammatory autoimmune diseases. At the same time, the anti-inflammatory properties of IVIG therapy have been appreciated and attributed to the IgG antibody Fc sialylation in mice. Thus, agalactosylated IgG antibodies have been generally associated with pro-inflammatory immune responses, whereas terminal galactosylation and additional sialylation of IgG antibodies have been demonstrated to suppress inflammation (Kaneko *et al.*, 2006; Collin and Ehlers, 2013; Pincetic *et al.*, 2014).

Most data on IgG antibody glycosylation are reported in the context of inflammatory autoimmune diseases. However, only few studies provide knowledge about the impact of IgG antibody glycosylation on anaphylaxis.

At the time I started my investigations, the laboratory had found that TNP-specific sialylated IgG1 antibodies display a reduced anaphylactic potential compared with degalactosylated IgG1 antibodies in a 24 hour IgG-mediated PSA mouse model (Figure 11B; Epp *et al.,* 2018). However, the mechanisms modulating the efficiency of differently glycosylated IgG1 antibodies to induce anaphylaxis had been unclear.

Initial experiments of my study demonstrated that the reduced anaphylactic activity of sialylated IgG1 antibodies was not the result of changes in the antigen affinity (Figure 13) or a reduced serum half-life of these antibodies (Figure 14C). Furthermore, degalactosylated as well as sialylated IgG1 antibodies were equally impaired in their ability to induce anaphylaxis in FcRγ-deficient mice (Figure 15), thus, demonstrating the fundamental requirement of activating FcγRs also in our model of IgG-mediated anaphylaxis.

IgG antibody Fc glycosylation has been demonstrated to influence the antibody effector function due to the introduction of a conformational change of the antibody Fc part, consequently influencing the binding affinity towards FcRs. In the absence of terminal sialic acid and galactose, the IgG antibody Fc part maintains an open conformation essential for engagement with type I FcRs, the classical FcγRs. On the other hand, terminal galactosylated and sialylated glycans introduce a closed conformation of the IgG antibody Fc part reducing the affinity to classical type I FcRs but enhancing the affinity for type II FcRs, in particular from the C-type lectin receptor family. Accordingly, several studies have shown that sialylated IgG antibodies drive anti-inflammatory immune responses in the context of IVIG by interacting with type II Fc receptors (Krapp *et al.,* 2003; Karsten *et al.,* 2012; Sondermann *et al.*, 2013; Pincetic *et al.*, 2014; Schwab *et al.*, 2014; Fiebiger *et al.*, 2015).
Also, in the context of IgG-mediated anaphylaxis, sialylated IgG1 antibodies might engage type II Fc receptors, and thus, diminish the anaphylactic reaction. Here, the immunomodulatory potential of the type II Fc receptor SignR1 was analyzed.

In this study, amelioration of the anaphylactic reaction by IgG1 antibody Fc sialylation was shown to depend on the type II Fc receptor SignR1. Upon depletion of SignR1 by a monoclonal anti-SignR1 antibody (clone 22D1), IVIG as well as sialylated antigen-specific and sialylated antigen-unspecific IgG1 antibodies were no longer protective in IgG1-mediated anaphylaxis (Figures 21C, 19 and 23A, respectively).

It has been previously reported that the anti-inflammatory properties of IVIG are mediated by a minor, yet highly sialylated IgG antibody fraction. Thereby, sialylated IgG antibodies interact with the type II FcR SignR1 expressed by macrophages of the splenic marginal zone. This interaction resulted in the secretion of IL-33, which in turn provoked an expansion of IL-4 producing basophils inducing an up-regulation of the inhibitory FcγRIIB on effector cells (Anthony *et al.*, 2008; Anthony *et al.*, 2011). Here, intravenous administration of the anti-SignR1 antibody 22D1 allowed depletion of splenic SignR1 on monocytes/ macrophages, whereas blood monocytes/ macrophages showed an unaltered expression of this receptor (Figure 18, Kaneko *et al.*, 2006). The results suggest that in IgG1-mediated anaphylaxis the SignR1 expression on monocytes/ macrophages in the spleen is required to induce the anti-inflammatory setting mediated by sialylated IgG1 antibodies. Furthermore, it could be shown by flow cytometry that mice sensitized with sialylated IgG1 antibodies (or IVIG) showed an enhanced expression of inhibitory FcγRIIB, 24 hours later (Figure 26 and Supplement Figure B3, respectively). On the other hand, the expression of FcγRIII remained unaltered upon sensitization with sialylated IgG1 antibodies (Figure 26).

Furthermore, several studies addressing mechanisms in IgG-mediated anaphylaxis devote strong effort to identifying the immune effector cells involved in the reaction. My findings might also provide further data for the ongoing discussion concerning which cell population contributes to IgG1-mediated anaphylaxis. Here, an altered FcγR expression profile could be observed for monocytes/ macrophages but not for neutrophils (Figures 26/ 27 and B3/ B4), thus, suggesting that the former is the main contributor in IgG1-mediated anaphylaxis in mice.

Altogether, the data suggest that in the context of anaphylaxis sialylated IgG1 antibodies interact with type II FcR SignR1, and thus, mediate a subsequent up-regulation of inhibitory FcγRIIB. Furthermore, in accordance with these results, it could be demonstrated that sialylated IgG1 antibodies propagated their suppressive effect in IgG-mediated anaphylaxis in a time-dependent manner. The reduced anaphylactic potential of sialylated IgG1 antibodies was not observed when mice were challenged with TNP-OVA only 30 minutes after immunization (Figure 20), instead of 24 hours after immunization (Figure 14B). On the other hand, FcγRIIB-deficiency provoked an enhanced anaphylactic response irrespective of the time point of the antigen challenge (30 minutes-model: see figure 20B versus 24 hour-model: see figure 16). Thus, the results further suggest that as

demonstrated for autoimmune inflammation (Anthony *et al.*, 2008; Schwab *et al.*, 2014) also in IgG-mediated anaphylaxis the type II Fc receptor SignR1 was essential for sialylation-dependent suppression of the anaphylactic response only under preventive treatment conditions. On the other hand, inhibitory FcγRIIB also determined disease severity after the onset of the anaphylactic reaction.

In my study, the binding of sialylated IgG1 antibodies to type II Fc receptor SignR1 initiated a yet unknown pathway promoting the up-regulation of FcγRIIB on blood monocytes/ macrophages and ultimately reduced the anaphylactic potential of IgG1 antibodies. The involvement of an IL-33 and/ or IL-4 dependent up-regulation of FcγRIIB as described for the IVIG-induced therapeutic effect in mouse models of rheumatoid arthritis by Anthony and colleagues (2011) could not be demonstrated in the context of anaphylaxis thus far. Altered serum levels of these cytokines were tried to analyze by ELISA, however, the concentrations were too low and could not be detected (data not shown). Future studies could analyze the missing link between the IgG antibody-SignR1 interaction and FcγRIIB up-regulation by using more sensitive methods such as determining gene expression levels of IL-4 and IL-33 by quantitative polymerase chain reaction (PCR). Alternatively, blocking antibodies to the IL-4- and/ or IL-33-receptor could provide another hint at the contribution of these cytokines. However, it is also possible that these cytokines are dispensable for the sialylated-mediated suppression of anaphylactic reactions. As has been described for mouse models of immunothrombocytopenia (ITP), IVIG-induced protection was dependent on sialic acid and SignR1, although, a role for IL-33 or IL-4-producing basophils could be excluded (Schwab *et al.*, 2012).

Different mechanisms are discussed to contribute to the efficacy of IVIG in different disease models, by using varying doses, different application routes or in mice with varying genetic backgrounds. Furthermore, as described above, the sialylated fraction of IVIG was identified to mediate anti-inflammatory properties. However, sialic acid-independent mechanisms have also been attributed to the anti-inflammatory properties of IVIG. Among others, the neutralization of auto-antibodies by anti-idiotype antibodies has been described in the context of autoimmunity. This mechanism may not be true in IgG-mediated anaphylaxis, since similar effects could be achieved by IVIG and sialylated antigen-unspecific IgG1 antibodies in wild type mice. Alternative modes of action by IVIG

also involved the ability of IVIG to block complement components, and thus, prevent complement-dependent inflammation. However, here the contribution of complement in IgG-mediated anaphylaxis was excluded. Furthermore, saturation of the neonatal FcR (FcRn; which is involved in the catabolism of IgG antibodies) by IVIG was demonstrated to reduce the half-life of pathogenic IgG antibodies. However, here sialylated and degalactosylated IgG1 antibodies displayed different anaphylactic potential even though no difference concerning the serum half-life could be observed, thus, arguing against a role for FcRn (Basta and Dalakas, 1994; Anthony *et al.*, 2008; Schwab and Nimmerjahn, 2013; Galeotti *et al.*, 2017).
However, my studies show equal anti-inflammatory effects of high amounts of IVIG as well as of lower amounts of sialylated antigen-unspecific murine IgG1 antibodies confirming the inhibitory effect of the sialylated fraction of IVIG. Furthermore, my studies excluded an antigen-specific effect via SignR1.

Previous studies have demonstrated that Fc sialylation failed to attenuate IgG2a-mediated anaphylaxis (Epp *et al.*, 2018). Moreover, in a murine model of autoimmune hemolytic anemia, disease progression was independent of the extent of IgG2a antibody sialylation (Ito *et al.*, 2014) suggesting that the high-affinity engagement of multiple activating FcγRs by IgG2a antibodies as well as a rather low affinity of IgG2a antibodies for inhibitory FcγRIIB interferes with potential sialic acid-dependent up-regulation of inhibitory FcγRIIB. This presumption does not exclude the idea that sialylated IgG2a antibodies might have the potential to modulate IgG1-mediated anaphylaxis. However, here I have demonstrated that sialylated IgG2a antibodies are also unable to reduce the anaphylactic potential of IgG1 antibodies (Figure 25). Furthermore, preliminary results suggest that despite being highly sialylated (Figure 24B), IgG2a antibodies might not be able to interact with SignR1, and thus, are unable to induce an up-regulation of FcγRIIB (data not shown).

In search of new therapeutic approaches, it would be of great interest to investigate whether other highly sialylated antibody isotypes could also facilitate immunosuppressive effects, like displayed by sialylated IgG1 antibodies. For example, in the course of AIT also allergen-specific IgA antibodies are induced (Jutel *et al.*, 2003; Scadding *et al.*, 2010) and neutralizing serum IgA antibodies have been demonstrated to prevent IgE-mediated anaphylaxis in mice (Strait *et al.*, 2011). Furthermore, IgA antibodies are highly sialylated and have been demonstrated to interact with type II Fc receptor SignR1, thus, dampening

autoimmunity in mice (Arnold *et al.*, 2007; Diana *et al.*, 2013). Here, as a first attempt it could be demonstrated that IgA anti-TNP antibodies do not induce anaphylaxis themselves; however, they are able to inhibit passive IgG2-mediated anaphylaxis (Supplements Figure B2). Future studies will investigate whether inhibition by these IgA antibodies is due to antigen-specific effects alone or if further immunomodulatory mechanisms as described for IgG1 antibodies are involved. Furthermore, the contribution of the human IgA Fc receptor, FcαRI, which is expressed by human myeloid cells and has been demonstrated to induce both activating and inhibitory signals, will be considered (Watanabe *et al.*, 2011; Hansen *et al.*, 2017). Therefore, human TNP-specific IgA1 and IgA2 antibodies were cloned by Lena Höbel, a former bachelor student in our laboratory.

Together, the suppressive effects of high amounts of IVIG as well as of lower amounts of highly sialylated IgG1 antibodies in murine IgG1-mediated anaphylaxis were demonstrated. Thereby, the conducted experiments elucidated that sialylated IgG1 antibodies propagated their suppressive effect in an antigen-unspecific, yet time-dependent manner. The mechanisms contributing to the reduced anaphylactic potential of sialylated IgG1 antibodies involved the splenic expression of the type II Fc receptor SignR1 and a subsequent up-regulation of FcγRIIB. In turn, this contributed to peripheral tolerance due to an enhanced threshold of IgG immune complex-mediated activation of effector cells. Consequently, a reduced anaphylactic potential during IgG1-mediated anaphylaxis could be observed. Importantly, this could be demonstrated for IgG1 antibodies, but not for IgG2a antibodies, thus further highlighting the differences of murine IgG antibody subclasses.

In addition, a similar mechanism is proposed for the enhanced inhibitory potential of IgG1 antibodies in IgE-mediated anaphylaxis upon IVIG treatment (Figure 29C). Here, an up-regulation of inhibitory FcγRIIB on mast cells and/ or basophils is assumed upon IVIG-treatment. The results suggest that modifications in total IgG antibody glycosylation influence the receptor expression on immune effector cells and ultimately contribute to altered immune responses. Thus, modification of the ratio of activating to inhibitory FcγRs might be an efficient tool to induce peripheral tolerance in patients suffering from allergic reactions (Figure 35). Moreover, it is necessary to further elucidate underlying mechanisms in search of new therapeutic approaches.

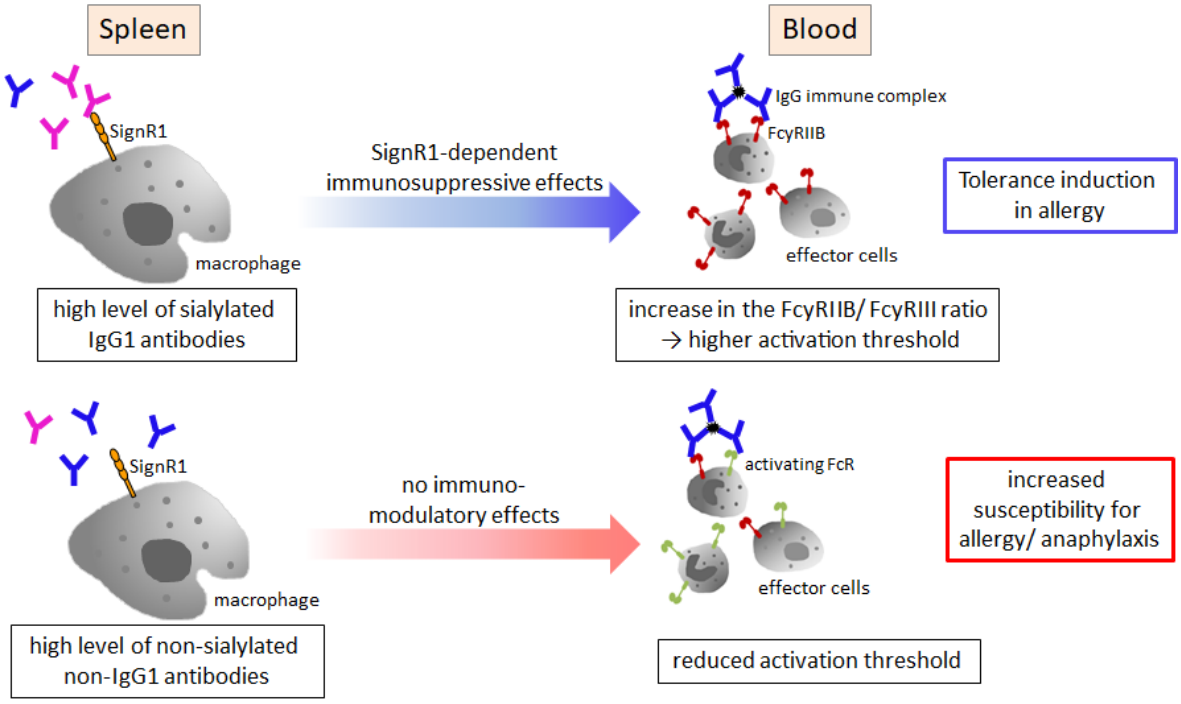

Figure 35: Proposed model for the tolerance induction in IgG- and IgE-mediated allergic reactions. *Increased serum levels of sialylated murine IgG1 antibodies interact with the type II Fc receptor SignR1 on splenic monocytes/ macrophages, and thus, initiate the up-regulation of inhibitory FcγRIIB on blood effector cells. Consequently, the enhanced activation threshold of effector cells contributes to the induction of peripheral tolerance and a reduced anaphylactic potential in both IgE- and IgG-mediated anaphylaxis. On the contrary, reduced levels of sialylated IgG1 antibodies associated with a lower activation threshold of effector cells results in an increased susceptibility for allergic/ anaphylactic responses.*

The existence of human IgG-mediated anaphylaxis is debated. However, human cases of anaphylaxis without detectable allergen-specific IgE antibodies and without apparent mast cell activation have been described. Instead, in these patients the severity of the anaphylactic response correlated with serum levels of allergen-specific IgG antibodies, supporting the notion of an IgE-independent mechanism (Finkelman *et al.*, 2016).

Human effector cells seem to be potential candidates in an alternative IgG-mediated pathway of anaphylaxis. The activation of human monocyte/ macrophages, neutrophils and basophils results in the release of PAF, which has been associated with murine IgG-mediated anaphylaxis. In addition, human basophils have a larger number of granules than their mouse counterpart. Thus, activation and degranulation of human basophils seems to be more effective for the inductions of anaphylaxis. Similarly, human monocytes/ macrophages and their FcγR profile makes them even more potent in the induction of IgG-mediated anaphylaxis as their murine counterpart. FcγRIIB expression is diminished on human monocytes/ macrophages (Figure 31), whereas murine monocytes/ macrophages

show a high FcγRIIB expression (Figure 26C; Karasuyama *et al.*, 2010; Bruhns and Jönsson, 2015; Nimmerjahn *et al.*, 2015; Finkelman *et al.*, 2016).
Consequently, even though the existence of IgG-mediated anaphylaxis in humans has not yet been clarified it is important to further investigate the underlying pathomechanisms in order to avoid adverse allergic reaction and improve therapeutic approaches.

Murine studies have demonstrated that IgG-mediated anaphylaxis requires higher amounts of allergen compared with IgE-mediated anaphylaxis. In humans, exposure to large quantities of antigen may occur during the parenteral administration of drugs. Thus, it has been argued that IgG-mediated anaphylaxis may be of great importance in cases of drug-induced anaphylaxis. Furthermore, during AIT patients receive increasing doses of allergen inducing increasing levels of allergen-specific IgG antibodies, thus, providing yet another setting for human IgG-mediated allergic reactions to occur (Finkelman *et al.*, 2016; Jutel *et al.*, 2016; Epp *et al.*, 2018).
Nevertheless, AIT is the only treatment regimen for patients suffering from severe allergic reactions thus far that has the potential to modulate the course of the disease and induce lasting improvement. Thereby, treatment success has been attributed to the increasing serum levels of allergen-specific IgG antibodies, and especially human IgG4 antibodies. Furthermore, in the context of murine IgE-mediated anaphylaxis, the blocking properties of antigen-specific IgG antibodies have been demonstrated. Thereby, antigen interception as well as FcγRIIB-dependent inhibition contributed to the ability of IgG antibodies to inhibit murine IgE-mediated anaphylaxis. However, prediction of therapy efficiency based on levels of allergen-specific IgG(4) antibodies was not reliable in all patients. One explanation for this disparity put forward included the presence of polyclonal IgG antibodies specific for allergen epitopes different from the one to which allergy-inducing IgE antibodies are directed (Müller, 2005; Strait *et al.*, 2006; Möbs *et al.*, 2012; Jutel *et al.*, 2016). However, based on my results gathered in the context of IgG-mediated anaphylaxis, it appears obvious that the efficiency of AIT is not only based on the amount and the affinity of blocking IgG antibodies but also depends on their glycosylation profile. Here, IVIG as well as sialylated IgG1 antibodies were able to up-regulate FcγRIIB (Figure B3 and 26C, respectively). Unbalanced signaling via activating and inhibitory FcγRs is the basis of inflammatory diseases. Modulation of cell activation by enhanced expression of inhibitory FcγRIIB or reduced expression of activating FcγRs would offer strong potential to overcome the limited therapeutic strategies in antibody-mediated anaphylaxis.

Transferring the generated results to the human system, it would be indicated that during AIT sialylated IgG antibodies should be induced (or even substituted by e.g. IVIG-treatment) in order to dampen potential allergic reactions mediated by either IgE or IgG antibodies.

Thereby, the composition of the allergen extract might be considered. Adjuvants in AIT are used to provide a sustained and potent immune response. In conventional AIT, aluminium hydroxide (alum) is a commonly used adjuvant. However, its use was found to have some limitations including the induction of unwanted Th2 responses as well as an increased IgE response. In search of new therapeutic approaches, the adjuvant Monophosphoryl lipid A (MPL-A) has been approved for the use in AIT. It has been associated with reduced levels of allergen-specific IgE antibodies, a stronger induction of IgG antibodies and improved outcome in a murine model of airway hyperreactivity (Mothes *et al.*, 2003; Larché *et al.*, 2006; Jensen-Jarolim, 2015; De La Torre *et al.*, 2018; Zubeldia *et al.*, 2018).

The selected adjuvants also have a strong influence on the IgG antibody glycosylation. Antigen presentation under tolerogenic conditions was demonstrated to promote the production of galactosylated and sialylated IgG antibodies, whereas pro-inflammatory conditions induced low galactosylated and low sialylated IgG antibodies (Oefner *et al.*, 2012). We previously compared the impact of eCFA (enhanced complete Freund adjuvant) providing a strong inflammatory stimulus with alum and MPL-A on IgG antibody subclass distribution and Fc glycosylation profiles in OVA-immunized mice. Alum and MPL-A induced high levels of total IgG antibody as well as subclass-specific sialylation that conveyed protection in IgG-mediated anaphylaxis (Epp *et al.*, 2018).

Thus, the induction of sialylated IgG antibodies by AIT protocols seems favorable. Moreover, the observation that IVIG-induced up-regulation of FcγRIIB probably contributes to the enhanced blocking property of IgG1 antibodies in murine IgE-mediated anaphylaxis (Figure 29C) might explain observations from earlier studies describing the successful treatment of drug-induced hypersensitivity by IVIG (Scheuerman *et al.*, 2001; Kito *et al.*, 2012).

However, from another perspective, sialylation-induced up-regulation of FcγRIIB may also serve as an explanation why some individuals have a higher propensity to develop allergies. It indicates that individuals with an increased susceptibility to allergy and

anaphylactic reactions probably might be armed with a lowered activation threshold of effector cells. Also in the context of autoimmunity, FcγRIIB expression has been demonstrated to be associated with a predisposition to develop disease (Bolland and Ravetch, 2000; Boross *et al.*, 2011). In my study, with only few volunteer blood donors varying expression levels of activating and inhibitory FcγRs among individuals has been demonstrated. Furthermore, a correlation between the serum level of total IgG antibody sialylation and the expression level of inhibitory FcγRIIB on human blood neutrophils and basophils has been identified.

Contradictory to the above assumptions, in humans, IVIG-mediated up-regulation of inhibitory FcγRIIB has not been observed so far. Instead, treatment might be rather associated with a down-regulation of activating FcγRs (Abe *et al.*, 2005; Tjon *et al.*, 2014). These findings, however, do not diminish the value of the results of this study. Down-regulation of activating FcγRs would also contribute to an improved balance of activating and inhibitory signals and might allow the induction of tolerance. Moreover, the lower affinity of human IgG antibodies than mouse IgG antibodies for FcγRIIB (Bruhns and Jönsson, 2015) would appreciate a down-regulation of activating FcγRs rather than an up-regulation of inhibitory FcγRIIB for treatment success in humans.
Here, as a first attempt the analysis of human activating FcγRIIA was chosen, which is thought to be the functional homolog of murine FcγRIII. Furthermore, induction of active and passive IgG-mediated anaphylaxis in mice transgenic for human high- (FcγRI) and low-affinity IgG receptors (FcγRIIA/ IIB/ IIIA/ IIIB) demonstrated a pre-dominant role of FcγRIIA, which also displays expression on all human myeloid cells (Bruhns and Jönsson, 2015; Gillis *et al.*, 2016, 2017). However, in this study no correlation was found for total IgG antibody glycosylation and the expression level of activating FcγRIIA on healthy volunteer blood donors. Thus, it would be interesting to check similar correlations for other activating FcγR in both healthy and allergic individuals.

Thus, a fundamental question for future studies is whether the immunosuppressive mechanisms providing amelioration in both murine IgE- and IgG mediated anaphylaxis demonstrated here are functional in humans. It is also important to clarify whether human subclass-specific differences, like observed for sialylated murine IgG1 and IgG2a antibodies, exist or if already increased levels of total IgG antibodies sialylation are sufficient to modulate effector cell activation. Furthermore, it is of great interest to

ascertain the degree to which such mechanisms are effective at a time when the disease has already been established.

In addition, as mentioned before, the type II FcR SignR1 expressed by splenic monocytes/ macrophages was indispensable for the reduced anaphylactic potential of sialylated IgG1 antibodies. Aside from monocytes/ macrophages, the human ortholog of SignR1, DC-SIGN, is also expressed by dendritic cells. DC-SIGN has been demonstrated to interact with sialylated IgG antibodies and transgenic expression of this receptor protected SignR1-deficient mice from arthritis and experimental autoimmune encephalomyelitis (EAE; Anthony *et al.*, 2008; Anthony *et al.*, 2011; Fiebiger *et al.*, 2015). However, these results have been questioned by others who could not reproduce an interaction of human DC-SIGN and sialylated IgG or their involvement in IVIG-mediated protection (Bayry *et al.*, 2009; Yu *et al.*, 2013). Thus, preventive treatment with IVIG or sialylated IgG antibodies via DC-SIGN-dependent mechanisms needs to be critically reconsidered in the context of human IgE- and IgG-mediated anaphylaxis.

The importance of IgG antibody glycosylation for its interaction with different Fc receptors, and thus, for its effector function has been acknowledged. However, the contribution of glycan modifications to the IgE antibody-receptor interaction is less clear. Therefore, I intended to provide the basis for future studies analyzing the impact of the glycosylation on IgE antibody effector functions. The initial *in vitro* experiments demonstrating differential activation of mast cells by differently glycosylated IgE antibodies have to be confirmed by future *in vivo* studies. Furthermore, IgE glycan analysis by Liquid Chromatography Mass Spectrometry (LC-MS) will be established by our PhD student, Alexander Wagt, at the Center for Proteomics and Metabolomics (Prof. Manfred Wuhrer) at Leiden University Medical Center (Leiden, the Netherlands) in order to analyze (allergen-specific) IgE antibody glycosylation from healthy and allergic individuals.

In summary, the suppressive effects of high amounts of IVIG as well as lower amounts of highly sialylated antigen-unspecific IgG1 antibodies in murine IgG1-mediated anaphylaxis have been demonstrated. Thereby, the conducted experiments elucidated that the glycosylation of allergen-unspecific IgG antibodies were important in the induction but not in the effector phase of antibody-mediated anaphylaxis. Furthermore, murine sialylated IgG1 antibodies propagated their suppressive effect in a SignR1-dependent up-regulation of FcγRIIB. The immunomodulatory mechanisms contributed to a reduced anaphylactic

potential during IgG1- and IgE-mediated anaphylaxis. These findings suggest that in addition to the presence of allergen-specific IgE and IgG antibodies a profiling of activating and inhibitory FcγRs as well as of total IgG sialylation levels may identify individuals with increased risk of developing allergic reactions and might allow an interpretation about which patient may profit from AIT and which not.

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B Supplements

Table B.1: Sequence of the TNP κ light chain and the TNP Igα heavy chain. *For the synthesis of murine IgA anti-TNP antibodies the complete kappa light chain gene (AgeI (green) - HindIII (purple)) was cloned into an expression vector by Hess et al., 2013. The constant Igα heavy chain region (SalI (red) - BsiWI (blue)) was cloned into an expression vector already including the variable anti-TNP heavy chain region (AgeI (green) - SalI (red)). The leader sequences (underlined) of the recently described expression vectors were used (Tiller et al., 2009; Hess et al., 2013).*

TNP-κ light chain	ATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAACTGCA**ACCGGT**GTACATTCA GACATTGTGATGTCACAGTCTCCATCCTCCCTAGCTGTGTCAGTTGGAGAGAAGGTT ACTATGAGCTGCAAGTCCAGTCAGAGCCTTTTATATAGTAGCAATCAAAAGAACTAC TTGGCCTGGTACCAGCGGAAACCAGGGCAGTCTCCTAAACTGCTGATTTACTGGGCA TCCACTAGGGAATCTGGGGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGAT TTCACTCTCACCATCAGCAGTGTGAAGGCTGAAGACCTGGCAGTTTATTACTGTCAG CATTATTATAGCTCTCCGTACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAACG GGCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATC TGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACATCAATGT CAAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTG ATCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACCAAG GACGAGTATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAAC TTCACCCATTGTCAAGAGCTTCAACAGGAATGAGTGTTGA**AAGCTT**
TNP-Igα heavy chain	ATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAACTGCA**ACCGGT**GTACATTCC GAGGTGCAGCTTCAGGAGTCAGGACCTAGCCTCGTGAAACCTTCTCAGACTCTGTCC CTCACATGTTCTGTCACTGGCGACTCCATCACCAGTGGTTACTGGAACTGGATCCGG CAAGTCCCAGGGAATAAACTTGAGTACATGGGTTTCATAAATTACAGTGGTAACACT TACTACAATCCATCTCTGAGAAGTCGAATCTCCATCACTCGAGACACATCCAAGAAC CAGTACTTCCTGCACTTGAATTCTGTGACTACTGAGGACACAGCCACATATTACTGT GCAAGGGCTAACTGGGACGTCTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTC TCTGCAGC**GTCGAC**GAGAAATCCCACCATCTACCCACTGACACTCCCACGAGCTCTG TCAAGTGACCCAGTGATAATCGGCTGCCTGATTCACGATTACTTCCCTTCCGGCACG ATGAATGTGACCTGGGGAAAGAGTGGGAAGGATATAACCACCGTAAACTTCCCACC TGCCCTGGCCTCTGGGGGAGGGTACACCATGAGCAGCCAGTTGACCCTGCCAGCTGT CGAGTGCCCAGAAGGAGAATCCGTGAAATGTTCCGTGCAACATGACTCTAACGCCGT CCAAGAATTGGATGTGAAGTGCTCTGGTCCTCCTCCTCCTTGTCCTCCTTGTCCTCCT TCCTGCCATCCCAGCCTGTCACTGCAGCGGCCAGCTCTTGAGGACCTGCTCCTGGGTT CAGATGCCAGCCTCACATGTACTCTGAATGGCCTGAGAAATCCTGAGGGAGCTGTCT TCACCTGGGAGCCCTCCACTGGGAAGGATGCAGTGCAGAAAAAAGCTGTGCAGAAT TCCTGCGGCTGCTACAGTGTGTCCAGCGTCCTGCCTGGCTGTGCTGAGCGCTGGAAC AGTGGCGCATCATTCAAGTGCACAGTTACCCATCCTGAGTCTGACACCTTAACTGGC ACAATTGCCAAAATCACAGTGAACACCTTCCCACCCCAGGTCCACCTGCTACCGCCG CCGTCGGAGGAGCTGGCCCTGAATGAGCTCGTGTCCCTGACATGCCTGGTGCGAGCT TTCAACCCTAAAGAAGTGCTGGTGCGATGGCTGCATGGAAATGAGGAGCTGTCCCCA GAAAGCTACCTAGTGTTTGAGCCCCTAAAGGAGCCAGGCGAGGGAGCCACCACCTA CCTGGTGACAAGCGTGTTGCGTGTATCAGCTGAACTCTGGAAACAGGGTGACCAGTA CTCCTGCATGGTGGGCCACGAGGCCTTGCCCATGAACTTCACCCAGAAGACCATCGA CCGTCTGTCGGGTAAACCCACCAATGTCAGCGTGTCTGTGATCATGTCAGAGGgAGA TGGCATCTGCTACTGACG**CGTACG**

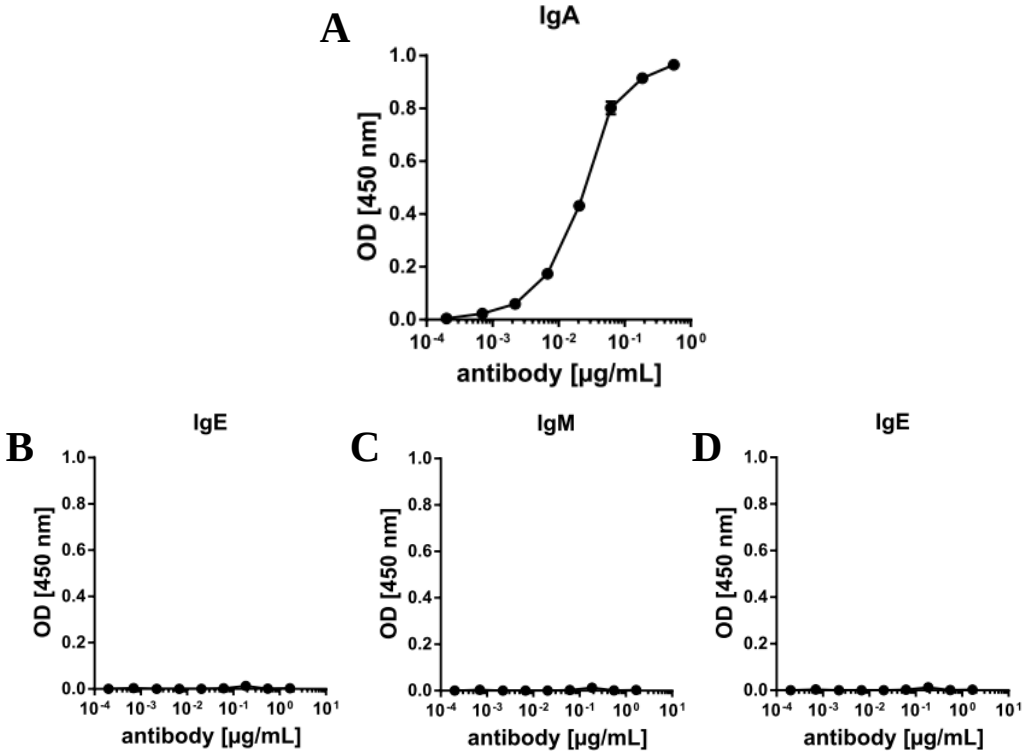

Figure B1: Production of murine IgA anti-TNP antibodies. *Two expression vectors containing either the κ light chain or the Igα heavy chain were co-transfected into HEK293T/17 cells. The IgA anti-TNP antibodies were purified using protein L sepharose that is specific for the antibody kappa light chain. In order to verify the IgA antibody isotype and its TNP-reactivity TNP-ELISA was performed. ELISA plates coated with 1 µg TNP-Ficoll per well were incubated with serial dilutions of protein L purified antibodies. Detection was performed using a HRP-conjugated* ***(A)*** *goat-anti-mouse IgA,* ***(B)*** *goat-anti-mouse IgG,* ***(C)*** *goat-anti-mouse IgM, or* ***(D)*** *goat-anti-mouse IgE antibody and absorption at 450 nm was measured using an ELISA plate reader. Data are shown as mean +/- SEM.*

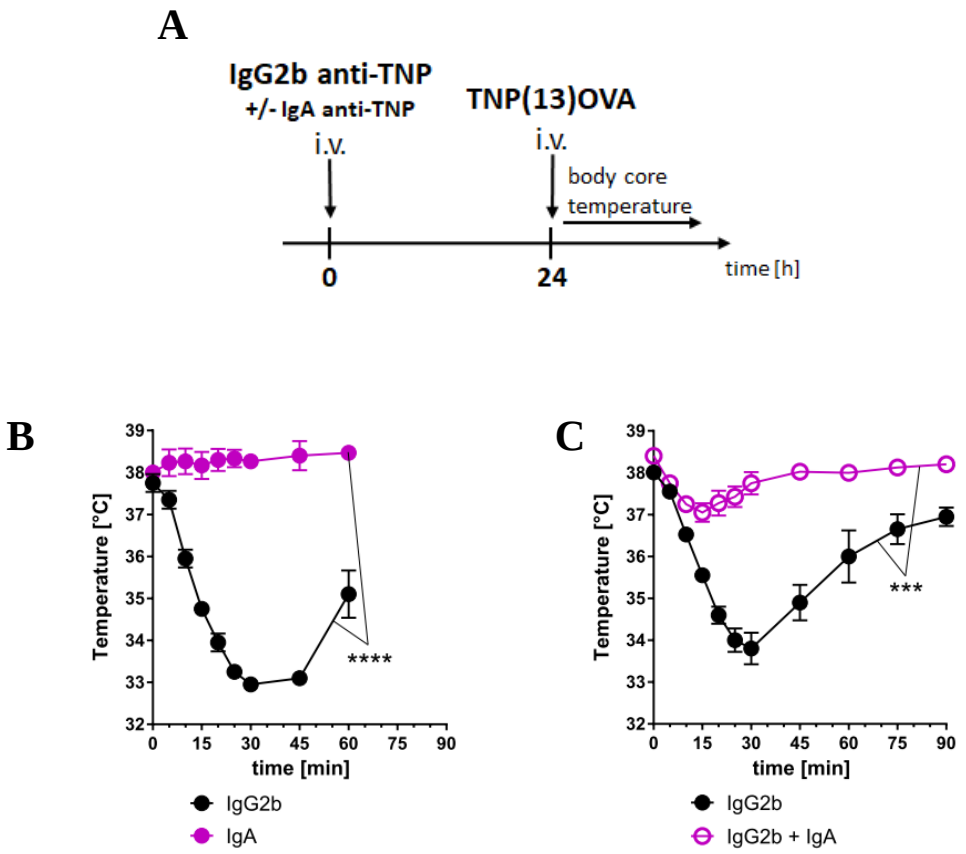

Figure B2: Potential of IgA anti-TNP antibodies in passive systemic anaphylaxis. (A) *Experimental setup.* ***(B)*** *IgA anti-TNP antibodies do not induce anaphylaxis in mice. Mice were sensitized i.v. with 150 µg IgG2b anti-TNP (clone HA2b, black) or 150 µg IgA anti-TNP (pink) antibodies and were challenged i.v. with 20 µg TNP-OVA 24 hours later.* ***(C)*** *IgA anti-TNP antibodies inhibit IgG2b-mediated passive anaphylaxis. Mice were sensitized i.v. with 150 µg IgG2b anti-TNP antibodies (black) or additionally with 250 µg IgA anti-TNP antibodies (pink). Eventually, mice were challenged i.v. with 20 µg TNP-OVA 24 hours later. The body temperature of individual mice was monitored. Data from one experiment are presented as mean +/- SEM, n = 3-4. ***P < 0.001 and ****P < 0.0001 by unpaired t-test.*

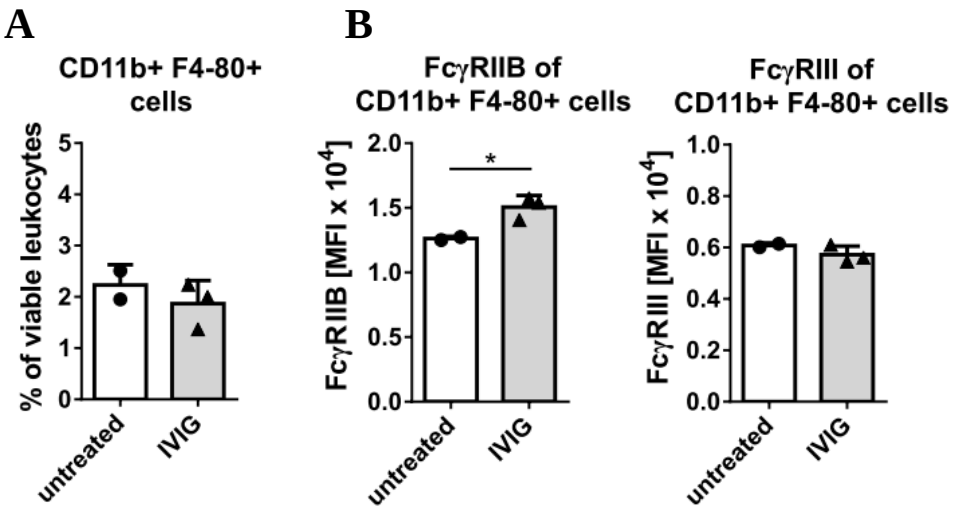

Figure B3: IVIG-induced up-regulation of inhibitory FcγRIIB on blood monocytes/ macrophages. *C57BL/6 wild type mice were treated i.p. with 1 mg IVIG 24 hours before FACS analysis.* ***(A)*** *Frequencies of CD11b+ F4-80+ blood monocytes/ macrophages pre-gated on single, viable, non-autoflourescent leukocytes.* ***(B)*** *Median fluorescent intensities (MFI) of FcγRIIB and FcγRIII of monocytes/ macrophages. Individual symbols represent individual mice. Data from one experiment are presented as mean +/- SD, n = 2-3. *P < 0.05 by unpaired t-test.*

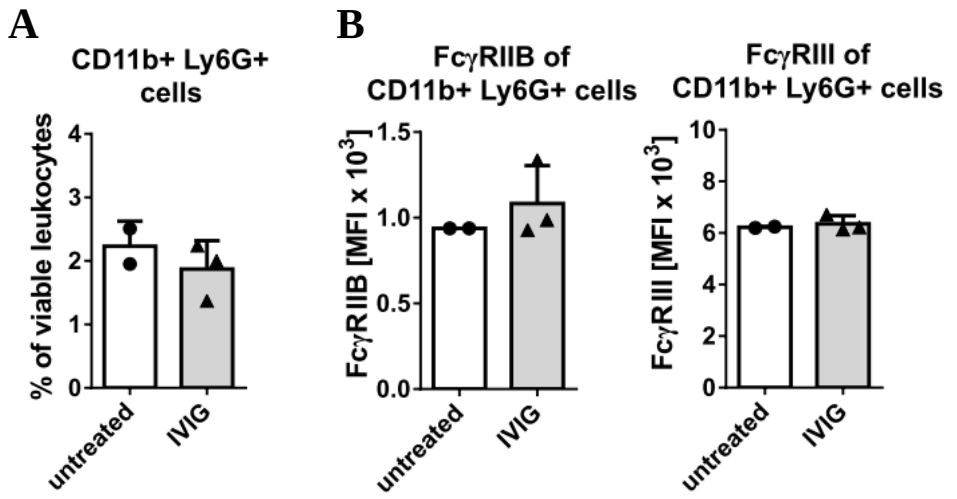

Figure B4: Analysis of murine blood neutrophils upon treatment with IVIG. *C57BL/6 wild type mice were treated i.p. with 1 mg IVIG 24 hours before FACS analysis.* ***(A)*** *Frequencies of CD11b+ Ly6G+ blood neutrophils pre-gated on single, viable, non-autoflourescent leukocytes.* ***(B)*** *Median fluorescent intensities (MFI) of FcγRIIB and FcγRIII of neutrophils. Individual symbols represent individual mice. Data from one experiment are presented as mean +/- SD, n = 2-3.*

Table B.2: Glycan distribution of total serum IgG from healthy blood donors. *Thirtysix healthy volunteer blood donors (12 male (m), 24 female (f)) were recruited. Serum was collected for IgG antibody purification by protein G sepharose columns. Subsequent IgG glycan analysis was performed by HPLC. Presented are the relative proportion of the individual peaks previously identified by MALDI-TOF anaylsis (Epp et al., 2018).*

Donor #	Age	Gender	G0	G0GNAc	G1	G1GNAc	G2	G2GNAc	G1S1	G2S1
14	34	m	31.30	5.85	37.65	5.85	12.51	0.88	1.55	4.42
15	32	f	18.10	3.75	34.41	6.73	22.09	1.72	1.94	11.25
16	30	f	21.99	3.18	36.93	4.17	21.86	1.07	1.67	9.12
17	29	f	23.31	4.19	36.90	6.27	21.32	1.40	0.93	5.68
18	32	f	18.41	2.90	37.74	6.91	21.40	1.81	1.57	9.26
19	28	m	37.92	6.18	35.68	4.87	10.51	0.63	1.17	3.04
20	31	m	33.04	5.07	37.57	4.78	12.72	0.76	1.55	4.51
21	30	m	30.32	5.87	38.69	6.77	11.63	0.94	1.68	4.10
22	36	f	27.65	4.23	37.58	5.99	15.88	1.18	1.35	6.15
23	31	f	27.68	4.32	37.51	5.92	14.84	1.15	2.06	6.51
24	29	f	29.78	6.53	35.65	5.83	14.87	1.04	1.54	4.76
25	31	m	28.84	5.17	36.73	5.81	14.33	1.04	1.90	6.18
26	27	m	28.18	6.36	36.86	7.48	12.76	1.16	1.60	5.60
27	45	f	22.05	3.46	38.28	6.32	17.41	1.38	2.46	8.64
28	29	f	24.10	4.64	37.47	5.27	20.06	1.20	1.17	6.09
29	58	f	28.28	6.59	37.73	7.52	13.45	1.13	1.17	4.15
30	46	f	33.30	4.89	36.93	5.55	11.89	0.88	1.65	4.91
31	27	f	19.50	4.20	37.33	6.48	20.30	1.56	1.68	8.96
32	52	m	32.16	6.30	36.40	6.67	10.81	0.94	1.96	4.76
33	29	f	21.53	3.55	39.38	6.12	19.44	1.39	1.38	7.21
34	35	m	34.37	7.13	35.01	6.95	10.26	0.97	1.43	3.87
35	25	f	29.52	5.01	38.66	6.83	13.76	1.21	1.34	3.67
36	24	m	27.33	4.79	37.33	5.73	15.40	0.95	2.30	6.17
37	23	m	24.96	4.58	37.48	6.14	15.45	1.17	2.37	7.85
39	27	f	19.82	4.17	36.14	7.44	20.54	1.59	1.20	9.10
40	32	f	14.99	2.64	35.88	6.51	25.50	1.90	1.74	10.85
41	41	m	26.67	6.92	33.71	8.29	13.08	1.55	2.33	7.44
42	36	f	20.28	5.02	35.85	8.51	18.02	1.85	1.94	8.53
43	31	m	34.98	5.09	35.44	4.71	12.52	0.87	1.70	4.70
44	45	f	19.36	2.89	37.49	4.76	23.12	1.35	1.85	9.17

Donor #	Age	Gender	G0	G0GNAc	G1	G1GNAc	G2	G2GNAc	G1S1	G2S1
45	26	f	20.77	3.05	39.80	6.02	19.14	1.34	1.77	8.11
46	29	f	28.53	6.98	35.80	7.85	13.83	1.23	1.14	4.63
47	30	f	15.28	3.17	33.11	6.91	24.84	2.41	2.25	12.03
48	31	f	19.90	5.38	32.44	8.06	22.15	1.75	1.84	8.47
49	28	f	20.96	4.07	33.49	6.31	21.13	1.62	1.89	10.54
50	23	f	25.35	3.42	39.55	4.17	16.93	0.93	2.50	7.14

Table B.3: Flow cytometric analysis of human leukocytes. *Frequencies of human SSC^{hi} $CD16^{+}$ neutrophils, SSC^{int} FSC^{hi} $CD14^{+}CD16^{-}$ monocytes and SSC^{low} $CCR3^{+}$ $Fc\varepsilon RI^{+}$ basophils [% of single, viable, non-autoflourescent leukocytes]. Cells were further analyzed for the expression of FcγRIIB and FcγRIIA.*

Donor #	CD16+ granulocytes			CD14+ CD16- monocytes			CCR3+ FcεRI+ basophils		
	total [%]	FcγRIIA [%]	FcγRIIB [%]	total [%]	FcγRIIA [%]	FcγRIIB [%]	total [%]	FcγRIIA [%]	FcγRIIB [%]
14	63.2	99.6	5.4	7.7	99.3	2.3	0.4	22.0	97.4
15	55.1	100.0	15.5	2.7	100.0	24.2	0.9	77.0	99.6
16	51.3	100.0	16.1	5.0	99.9	7.7	0.4	77.0	97.2
17	53.8	100.0	4.7	2.6	99.9	9.5	0.2	62.2	97.5
18	48.0	100.0	46.0	5.0	99.9	58.8	0.6	75.1	99.9
19	51.0	100.0	5.2	6.3	98.2	5.7	0.7	22.1	96.9
20	41.9	100.0	11.8	2.8	99.8	62.0	0.5	78.7	99.5
21	38.1	100.0	11.7	2.0	99.6	7.7	0.5	41.6	98.5
22	52.7	100.0	10.6	4.2	100.0	15.2	0.6	81.9	99.2
23	43.5	100.0	7.5	3.6	99.9	13.5	0.6	19.7	99.1
24	57.4	100.0	3.8	3.0	99.8	8.4	0.3	74.0	96.7
25	56.8	100.0	13.7	5.3	98.6	18.7	0.9	33.2	99.1
26	47.8	100.0	23.0	6.2	99.0	70.7	0.3	27.9	98.5
27	63.0	100.0	4.1	5.5	99.3	13.3	0.2	55.8	97.2
28	65.8	99.9	4.4	3.3	99.9	5.1	0.2	70.0	95.9
29	71.8	100.0	3.5	6.7	99.6	8.3	0.2	53.3	94.8
30	42.1	100.0	33.1	3.5	100.0	63.5	0.4	92.6	97.4
31	60.0	100.0	29.7	5.2	100.0	9.2	0.3	34.1	97.4
32	36.2	100.0	5.0	4.9	99.6	7.7	0.8	17.9	99.0
33	57.7	100.0	3.6	4.9	99.9	7.3	0.7	26.7	99.1

Donor #	CD16+ granulocytes			CD14+ CD16- monocytes			CCR3+ FcεRI+ basophils		
	total [%]	FcγRIIA [%]	FcγRIIB [%]	total [%]	FcγRIIA [%]	FcγRIIB [%]	total [%]	FcγRIIA [%]	FcγRIIB [%]
34	78.6	100.0	21.4	5.5	99.8	3.9	0.4	8.9	96.9
35	60.0	100.0	2.4	7.3	99.9	12.6	0.3	38.1	98.7
36	46.0	100.0	2.5	6.7	99.7	7.2	1.1	35.3	98.9
37	56.4	100.0	6.1	4.0	99.9	12.2	0.6	37.3	99.8
39	47.5	100.0	10.2	5.2	99.9	14.7	1.0	78.6	99.1
40	70.2	100.0	0.6	3.2	100.0	5.3	0.3	41.7	96.5
41	47.3	100.0	26.0	5.3	99.8	8.4	1.3	21.2	99.0
42	80.6	99.4	14.4	4.3	98.6	22.9	0.5	35.6	99.7
43	55.3	100.0	6.6	5.1	99.9	6.8	0.7	67.2	96.8
44	54.3	100.0	5.5	3.2	99.9	10.1	0.1	53.4	95.4
45	61.4	100.0	3.7	4.6	99.9	12.6	0.8	44.6	97.7
46	59.4	100.0	5.4	8.9	99.9	26.7	0.4	39.0	98.8
47	42.9	100.0	2.5	2.9	99.5	6.1	1.0	49.2	96.6
48	45.6	100.0	16.0	4.2	98.4	61.9	0.4	27.0	98.7
49	41.1	100.0	1.9	3.3	99.9	7.2	0.3	49.3	93.5
50	53.5	100.0	4.9	5.4	99.9	9.8	0.8	49.5	96.6

C Abbreviations

ADCC	Antibody-dependent cytotoxicity
AIT	Allergen-specific immunotherapy
ANOVA	Analysis of variance
Asn	Asparagine
BSA	Bovine Serum Albumin
C1q	Complement component 1q
CD	Cluster of differentiation
CMP	Cytidine monophosphate
D	Diversity
DC	Dendritic cell
DCIR	Dendritic cell immunoreceptor
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
E. coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
EndoS	Endoglycosidase S
Fab	Antigen-binding fragment
Fc	Fragment crystallizable
FcR	Fc receptor
FCS	Fetal calf serum
G0	Degalactosylated
GlcNAc	N-acetylglucosamine
HEK	Human Embryonic Kidney
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
i.p.	Intraperitoneal
i.v.	Intravenous
Ig	Immunoglobulin
IL	Interleukine
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IVIG	Intravenous immunoglobulin
J	Joining
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
OD	Optical density
Ova	Ovalbumin
PAF	Platelet-activating factor

PBS	Phosphate Buffered Saline
PNAG	p-nitrophenyl N-acetyl-β-D-glucosamide
rhSCF	recombinant human stem cell factor
S	Sialic acid
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
STAT	Signal transducer and activator of transcription
Th	T helper cell
TNP	2,4,6 Trinitrophenyl
V	Variable
wt	Wild type

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