

THE JANUS-LIKE ROLE OF SIALIC ACIDS IN THE REGULATION OF THE IMMUNE SYSTEM

Towards targeted therapies for hemophilia A patients with inhibitors and house dust mite allergy

ELEONORA NARDINI

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The Janus-like role of sialic acids in the regulation of the immune system

Towards targeted therapies for hemophilia A patients with inhibitors and house dust mite allergy

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1

INTRODUCTION

Part I: The tissue glycome as regulator of immune activation and tolerance mediated by C-type lectins and Siglecs.

Seminars in immunology, 2024

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ABSTRACT

The immune system is a complex network of highly specialized microenvironments, denominated niches, which arise from dynamic interactions between immune and parenchymal cells as well as acellular components such as structural elements and local molecular signals. A critical, yet underexplored, layer shaping these niches is the glycome, the complete repertoire of glycans and glycoconjugates produced by cells. The glycome is prevalent in the outer membrane of cells and their secreted components, and can be sensed by glycan binding receptors on immune cells. These receptors detect changes in glycosylation and consequently modulate immune cell activity, trafficking, and signalling, altering homeostasis. Tissues like the brain and the placenta are prone to accommodate tolerance, while the gut and the thymus are sensitive to inflammation. We provide here an overview of current literature that shows the impact of altered glycosylation of tissues on host immune cells and how interference in this process may lead to new diagnostics and immune therapeutics, aiming to restore the immune balance in autoimmunity and cancer.

1. INTRODUCTION

The immune system is a complex network, composed of many cell types that patrol our body through blood and lymphatics and penetrates peripherally into tissues. This network is organized into niches, which arise from tight interactions between stromal, a-cellular structural and immune cells. Traditionally recognized immune niches are secondary lymphoid organs(1). Their architecture is well known, as well as their embryonically determined cell composition and its changes during development. However, single cell resolution technologies such as spatial transcriptomic and imaging mass cytometry made evident that the concept of niche is much more multifaceted than previously thought, coming to indicate a group as small as three cells which cluster in response to a specifically confined alteration of the local environment (2). Taking this into consideration, immune niches can be defined as microenvironments composed of resident, infiltrating and trafficking immune cells, parenchymal and stromal cells, structural components of the tissue, and local molecular signals, such as chemokines and cytokines. Homeostasis, *i.e.* the dynamic balance between immune activation and suppression, depends on the nature of the niche. Some niches need to maintain tolerance, such as the brain and the placenta, while others accommodate and initiate inflammatory programs, such as the gut and the thymus. To meet these different needs, a tissue-specific regulation of the interplay between the immune system and the environment is needed. Despite the existence of separate microenvironments, they are interconnected via leukocytes that circulate and exchange information in the form of molecular mediators, namely chemokines, cytokines and exosomes, but also via physical interactions and neural control (3, 4).

The glycome is one of the layers that shape the microenvironment which still remains largely unexplored. The glycome is defined as the “complete repertoire of glycans and glycoconjugates that cells produce under specified conditions of time, space, and environment” (5). Often overlooked because of its complex regulation and non-templated nature, it covers the cellular membranes, making it the first to be involved in communications between immune cells and to guide their trafficking in and out niches(6). Glycans have several functions, such as protein folding and stability, cell adhesion, development, differentiation and pathogen recognition(6). They also contribute to the regulation of the immune responses because immune cells are equipped with glycan-binding proteins (GBPs), primarily lectins, which sense changes in glycosylation and consequently alter immune cell activity, trafficking, and signalling(7). GBPs were initially discovered as pathogen recognition receptors, based on the fact that viruses and bacteria have specific glycans expressed on viral/bacterial proteins and or lipids(7). In recent years, it has

become clear that GBPs play a crucial role in tissue sensing of the endogenous glycome(8). Since both innate and adaptive immune cells express GBPs, glycan/lectins interactions control both innate and adaptive immune responses(9). These include not only the modulation of antibodies' function, and leukocytes' signalling and migration but also discrimination between self, non-self and altered-self (9-11). Changes of the glycome have been reported in virtually all immune diseases, from malignant transformation to chronic inflammation and autoimmunity(9). It is therefore crucial to understand the homeostatic function of glycan-lectin interactions, as well as to know what drives the aberrant glycosylation in different diseases, and how this has impact on the glycan sensing by GBPs expressed on immune subsets. This knowledge will be pivotal for the development of targeted therapies to finely tune the immune response in inflammation, autoimmunity and cancer.

2. GLYCANS

Glycosylation is the metabolic pathway that creates the glycome. It is a posttranslational modification that concerns almost all mammalian proteins and lipids and even some forms of small RNAs(6, 12, 13). It consists on a series of biochemical reactions carried out by glycosyltransferases and glycosidases, located mainly in the endoplasmic reticulum and Golgi apparatus(14). These enzymes are organized within the organelles in a way that they sequentially add monosaccharides to form a growing polysaccharide chain(15). In humans, seven monosaccharides form the vast majority of glycans that play a role in the regulation of the immune system: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), sialic acid (Sia) and mannose (Man)(16). These monosaccharides can be assembled in various ways. For example, they can be linked to each other in their α or β anomeric state, at different carbons (1 – 3, 2 – 6, etc.), and attached to the protein or lipid scaffold at various sites(16). Therefore, complex macromolecules that differ in length, branching and composition can be built, generating a plethora of different structures. Monosaccharides that enter the glycosylation pathway come from different sources: from the diet, from the lysosomal degradation of other glycoconjugates or they can be converted from a different sugar substrate(17).

As for proteins, there can be distinguished two main types of glycosylation: N-linked, if the glycan is added to Asn; or O–linked, if it is bound through the hydroxyl group of a Ser or Thr. N-linked oligosaccharides share a common core structure of $GlcNAc_2Man_3$, that can be subsequently extended in diverse grades of complexity, thus generating hybrid and complex structures **(Figure 1)**(18).

The main type of O-linked glycosylation is denominated mucin type, in which glycans begin with a GalNAc that forms the base for the subsequent extension to 4 different core structures(19). Both membrane-bound and secreted proteins, such as the mucins, are glycosylated. Therefore, cells are covered by glycans, which makes them the first players to be involved in the communication of immune cells between each other, in their trafficking and infiltrating into tissues. Another key role of glycans is to contribute to the recognition of self, and sialic acid, which often caps mammalian glycans, plays a prominent role as self-associated molecular pattern(20, 21). The glycome can also be modified after-synthesis by secreted/circulating enzymes(22, 23). Of importance, terminal sialic acids can be cleaved off by a set of enzymes belonging to the neuraminidase (also called sialidase) family. In humans, NEU1 to 4 exist. NEU1 and NEU3 are of particular relevance because they can be recruited to the cell surface, modify the nature of the GBPs' ligands and thus alter their signalling(24, 25).

In homeostatic conditions, tissues display a balanced repertoire of glycans: the majority of N-glycans are complex, highly branched and terminally sialylated **(Figure 1)**(26). O–glycans are variously elongated and branched, to form high molecular weight structures(27). Despite the existence of these common features, every niche presents itself with a peculiar set of glycans, shaped by microenvironmental cues and regulated by the tissue–specific expression of glycosyltransferases. Overall, during an inflammatory response such as chronic lung inflammation, the balance in the glycome is shifted towards N-glycans of lower complexity, high mannose and poly-LacNAc structures, higher incidence of sialyl–Lewis X (a sialylated tetrasaccharide involved in leukocyte recruitment) **(Figure 1)**(28). On the opposite side of the spectrum, niches that need to achieve immune tolerance, such as the gut/microbiome interface, the brain and the placenta, present high levels of sialylation, fucosylation, truncated O-glycans and branching N-glycans **(Figure 1)**(29-32). Glycosylation patterns similar to those described here in inflammation and tolerance have been reported in pathological conditions that arise either from aberrant immune activation (*i.e.*, autoimmunity) or immune suppression, such as in cancer(33, 34).

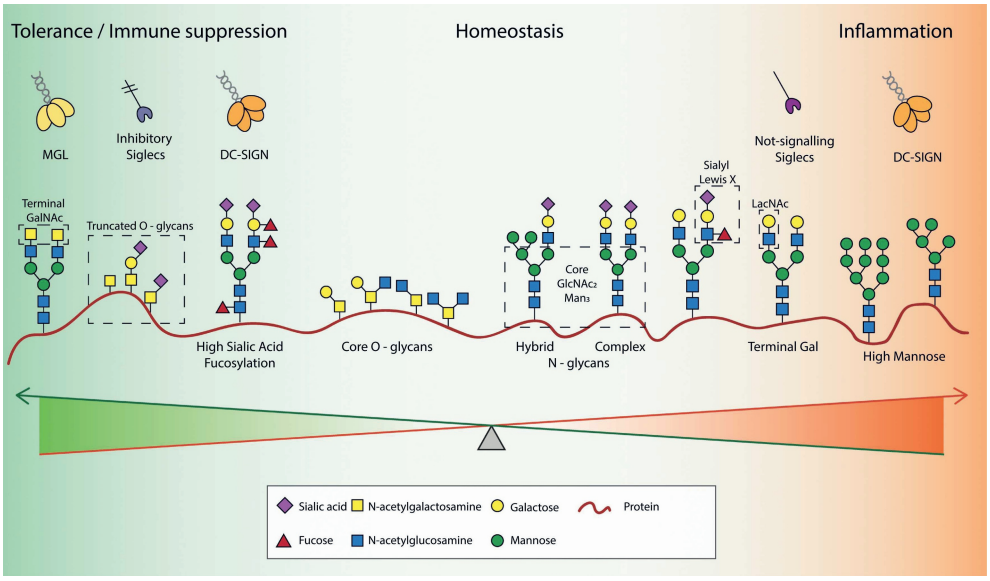


Figure 1 Glycans regulate Tolerance/Inflammation via glycan binding receptors such as MGL, DC-SIGN and Siglecs. *In homeostasis, the glycome is primarily composed of complex and hybrid N-glycans and variously extended forms of all four core O-glycans. During an inflammatory response (on the right), the balance is shifted towards high mannose glycans, as well as terminal galactose and sialyl-LewisX structures. These changes are sensed respectively by DC-SIGN and not-signalling Siglecs. On the opposite side of the spectrum, tolerant niches (on the left) are hypersialylated and truncated O-glycans are present. Moreover, fucosylated glycans are abundant and structures terminating with a GalNAc are also over-represented. These are ligands for inhibitory Siglecs, MGL and DC-SIGN.*

3. GLYCAN BINDING PROTEINS

GBPs on immune cells are responsible to translate changes in the glycosylation of the microenvironment into an immune response. Despite the existence of a great variation of glycans, GBPs have evolved to only recognize specific patterns. Most often, only the terminal bi- or trisaccharide of the glycan is accommodated into the binding pocket. The capping sugar is the most influential determinant for the binding, while the rest of the polysaccharide chain contributes to stabilize the interaction(7). Generally, the affinity of most GBPs for single glycans is low. However, the binding is often strengthened by avidity. This is achieved either by multimerization of the GBPs and/or by a multivalent presentation of the ligand, which induces clustering of the receptor's subunits(35). For example, mucins carry hundreds of glycans, whose high density is responsible to the strong binding of the lectins(36). The lipid or protein scaffold also plays a role in the specificity of binding. Some GBPs evolved to only recognize glycans carried by lipids instead of proteins and vice versa. Among the big family of GBPs, those that play a role in the regulation of the immune response are Siglecs, C-type lectins and galectins. The role of galectins in this context has been recently extensively reviewed (37). We will focus here on the function of the other two families.

3.1 Siglecs

Siglecs (sialic acid–binding immunoglobulin-like lectins) belong to the family of I (Ig)-type lectins due to the homology of their domains to the immunoglobulin superfamily (IgSF) of proteins. Structurally, their extracellular region is composed of an N-terminal V(variable)-set domain and varying numbers of C(constant)1- and C2- domains, all defined based on similarities with the antibody domains(38). As most lectins, the affinity of Siglecs for their ligands is low. Therefore, it is now believed that multivalent presentation of sialic acids is necessary to increase the avidity and initiate signalling(39). However, there is not yet clear evidence of the formation of clusters, though more and more studies on Siglecs ligands indicate that ligand-multivalence is required(40-43). As the name of the family suggests, Siglecs bind through their V-set domain to sialylated glycans. Mammalian cells, as mentioned in paragraph 2, are covered with sialylated glycans in homeostatic conditions(21). Given the high concentration of sialoglycans on the cell surface, Siglecs are typically involved in *cis* interactions (44). Since the variability of sialylated glycans is great, different members of the family have evolved to fine tune pattern specificity recognition and the type of response induced. Fifteen

Siglecs have been identified in humans so far, whose binding preference for sialic acid depends on the glycan scaffold, extension, N- or O-glycosylation, and linkage with the underlining monosaccharide (45). Siglecs also differ structurally in the number of C-set domains, and the type of C-terminal tail, which determines their function. Based on the latter, they can be further classified into three subgroups: inhibitory, activating and not-signalling.

Inhibitory Siglecs are the majority (Siglec-2, 3, 5, 6, 7, 8, 9, 10, 11, 12) and carry in their C-intracellular tail one or more intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) or ITIM-like or associate with adaptor proteins that provide them with one. Once these lectins are engaged, phosphorylation of their intracellular domains recruits SHP-1 or SHP-2 which downstream dephosphorylate activating protein, inhibiting in the end cell activation (46).

Activating Siglecs (Siglec-14, -15, -16) instead lack an intracellular tail but present with a positively charged amino acid in the transmembrane domain. This residue act as a recruiting hook for ITAM-carrying adaptors, mostly DAP10 or 12, which initiate the activation cascade. Not-signalling Siglecs, namely Siglec-1 and -4, lack a functional signalling region and are therefore mainly involved in adhesion and internalization events.

Despite virtually all immune subsets (both adaptive and innate) express Siglecs, not all members of the family are ubiquitous. Rather, distinct cell subtypes express a definite set of Siglecs **(Figure 2)**, which endow them with the ability of recognizing only certain sialoglycans and initiate determined immune responses. Some Siglecs show highly restricted pattern of expression, namely Siglec-2 on B cells, Siglec-4 on myelin forming cells, Siglec-15 on osteoclasts and DC/macrophages, Siglec-16 and Siglec-11 on microglia and macrophages. Moreover, the immuno-microenvironment (characterized for example by the presence of cytokines and chemokines) has a great impact on regulation of Siglec-expression. For instance, it has been recently discovered that T cells start expressing Siglec-9 upon entering the tumour microenvironment, allowing them to sense the immune inhibitory sialic acid signals of cancer cells (47-49). Another example is provided by monocyte-derived DCs, which upregulate Siglec-1 in response to IFNα (50). This upregulation can be interpreted as a strategy to enhance their scavenging abilities in order to address the inflammatory threat associated with IFNα and favor cross presentation of DCs (50). Targeting Siglec-1 has shown promise in cancer vaccines, however Siglec-1 can also be expressed on splenic macrophages and transfer antigens to DC (51).

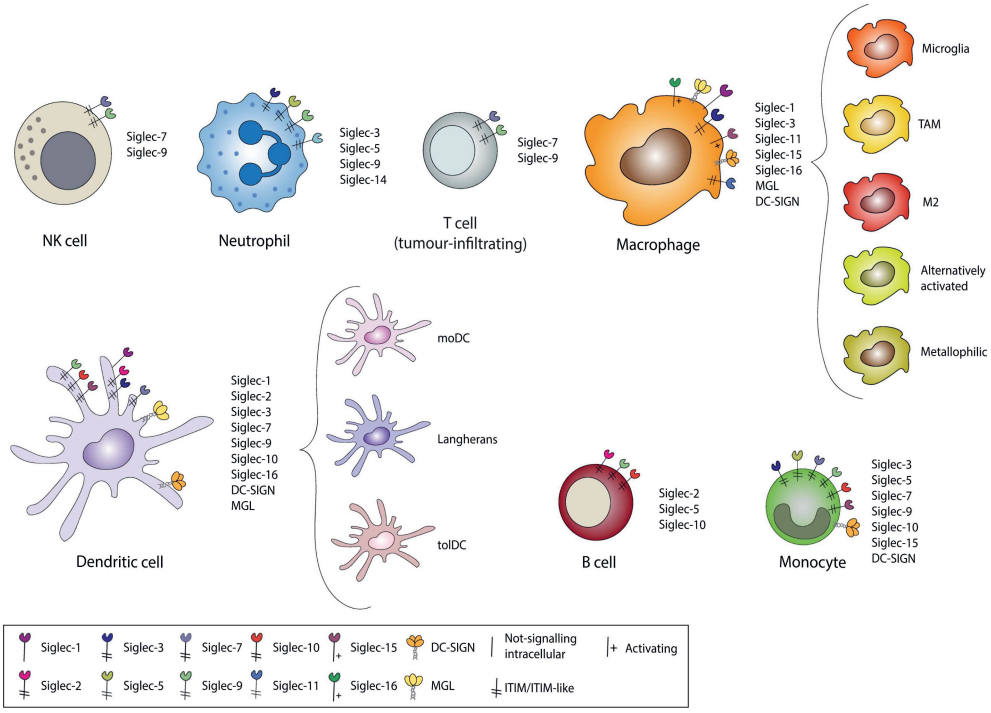


Figure 2 Siglecs, DC-SIGN and MGL expression across immune subtypes. *In curly brackets, different subsets of macrophages and dendritic cells that are described in the review are represented.*

3.2 C-type lectins

C-type lectins (CLRs) are a diverse family of carbohydrate-binding proteins that can recognize exogenous and endogenous structures present in cells and pathogens, playing a crucial role in the immune responses and cell-cell interactions (52). They are characterized by their calcium-dependent binding to their ligands and they are involved in various biological processes including pathogen recognition, cell signalling, and regulation of inflammation (52). Based on their protein structure and domain organization, CLRs can be classified into several different sub-families. In this section, we will discuss the role of some CLRs that have been associated to the modulation of immune responses.

3.2.1 DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), also called CD209, is present as a tetramer on the surface of immune cells and each monomer has one carbohydrate recognition domain (CRD) which can bind both high-mannose and fucosylated glycans (Lewis antigens) (53). These ligands are commonly found on pathogens. Indeed, DC SIGN was first described as the entry receptor for HIV to DCs (54). Since then, it has been implicated in a wide number of viral and bacterial infections (55). Among others, DC-SIGN functions comprise cell-cell interactions, presentation of MHC class I and II and signalling. DC-SIGN signalling is complex, as the composition and presentation of its ligands can significantly alter the binding affinity and subsequently the type of response elicited. Engagement of DC-SIGN by highly mannosylated glycans initiates a pro-inflammatory cascade via phosphorylation of RAF1 that culminates in NFkB translocation in the nucleus and activation of an inflammatory response (56). Fucosylated glycans, on the contrary, activate an immune suppressive programme that is independent of RAF1 (56). The dual nature of DC-SIGN's immune modulation emphasizes the importance of the microenvironment in shaping glycan-mediated immune responses. DC-SIGN expression is associated to cells with antigen-presenting capacities, namely DCs and macrophages subsets **(Figure 2)**(54). In the tumour associated microenvironment (TME), it is present on tumour associated macrophages (TAMs), being associated with their suppressive capacity (57, 58). To date, there is not a complete list of functions in vivo because of technical challenges, since mice have a different orthologue (59).

3.2.2 The macrophage galactose lectin (MGL) is a trimeric C-type lectin whose expression is restricted to myeloid cells, specifically to immature and tolerogenic DCs and activated macrophages **(Figure 2)**(60, 61). It binds to terminal GalNAc, present in O-linked glycans such as LacdiNAc **(Figure 1)**(62, 63). The Tn antigen, a single O-GalNAc attached to a protein, is not commonly found in normal tissue but it is upregulated during malignant transformation in most epithelial cancer (64). Engagement of MGL by GalNAc-containing glycans induces oligomerization and internalization. However, as DC-SIGN, MGL plays a dual role in regulating the immune system, either activating or suppressing a response depending on the ligand and the immune cell. One of the most studied MGL-ligands is MUC1, a glycoprotein of the class of mucins overexpressed in various cancer (65). Tumours express an aberrant glycoform of MUC1, where Tn antigens are overrepresented (66). Binding of MGL to a small Tn-MUC1 peptide promotes cross presentation on MHC-I and initiates a signalling cascade via ERK1,2

phosphorylation which culminates in NFκB translocation into the nucleus and immune activation (67). On the other hand, large glycoforms of Tn-MUC1 similar to those present on the membrane of cancer cells are presented via MHC-II and initiate an MGL–mediated suppressive program that promotes IL10 secretion and downregulation of maturation markers on DCs (67). MGL also directly controls CD4+ and CD8+ T cell activity by binding to the Tn antigen present on CD45 of activated lymphocytes (68). This interaction dampens activation of effector T cells, decreasing proliferation and pro-inflammatory cytokine secretion (68).

3.2.3 Langerin is a specific type of CLR that is primarily expressed on the surface of Langerhans cells, which are specialized myeloid cells found in the epidermis(69). It plays a vital role in the immune system by recognizing and binding to pathogens, facilitating their capture and presentation to T cells, thereby initiating an immune response. Additionally, Langerin is involved in the formation of Birbeck granules, which are unique organelles within Langerhans cells thought to participate in the processing of antigens (69).

3.2.4 Mannose Receptor (CD206), encoded by the gene MRC1, is a multifunctional C-type lectin receptor that binds to mannose-rich carbohydrates, that can be present in the surface of different pathogens, as fungi (70). By binding to these carbohydrates, MR facilitates the internalization and degradation of pathogens through phagocytosis, playing a direct role in the body's defence against infections. The expression of CD206 has been widely used for the identification of alternative activated macrophages with a modulatory phenotype (52).

4. TISSUE AND NICHE REGULATION OF THE IMMUNE RESPONSE BY GLYCANS: HEALTH AND AUTOIMMUNITY

Local alterations of glycosylation driven by environmental or genetic stimuli can result in chronic inflammation associated with autoimmune diseases, such as rheumatoid arthritis (RA) (71) and multiple sclerosis (MS) (72), or immune suppression that favour cancer insurgence and progression (10). Changes in the glyco-microenvironment are sensed by GBPs expressed by the resident population of immune cells. It is therefore relevant to look at how immune responses are determined by glycans/GBPs interactions from a niche-specific perspective.

4.1 Tissue-regulation of the immune response by glycans/ GBP_s

4.1.1 Thymus. The thymus is a primary lymphoid organ devoted to T cell selection and maturation. It is the elected site of T cell development, where naïve single positive (SP) CD4+ and CD8+ T cells are selected and licensed to carry out their function in the periphery. The population of the thymus is composed of epithelial and immune cells. The majority of them are T cells, which are called thymocytes, but also a small population of B cells and innate immune cells such as DCs and macrophages is present (73).

In the thymus, it is known for a decade that glycans play key roles in controlling every step T cell development (74, 75). The repertoire of glycans is modified throughout T cell maturation: double negative and double positive T cells have low levels of sialylation which are restored in the SP that leave the thymus (76). Specifically, mature naïve SP that are ready to go into the periphery start expressing high levels of α2.3 sialylated glycans and localize in the medulla (77). These changes have also been reported in mice, where resting mature thymocytes in medulla also present with higher level of sialylation(78). On the contrary, the outer part of the thymus, the cortex, is highly mannosylated (79). Another interesting observation is that blood and lymphatic vessels in the thymus show different glycan signatures, which is one of the only features that allows discrimination in tissue stainings (79). It is therefore tempting to ascribe a role to GBPs in directing T cell recirculation by sensing differences in the glycome of the vessels. Altered glycosylation of thymocytes gives rise to major complications related to aberrant T cell functionality. This scenario is vastly studied in mice KO of Mgat5, a gene encoding for β1,6 N-acetylglucosaminyltransferase V. This enzyme

is crucial for the synthesis of complex N-glycans, which are indispensable to set the threshold for T cell activation (80). Incomplete TCR glycosylation lowers the threshold of T cell activation by enhancing TCR clustering and signalling (80). Consequently, these mice show higher basal T cell activation and are more prone to develop autoimmune diseases such as psoriasis and EAE or show exacerbated phenotype in already established mouse models of the disease Niemann/Pick Disease (81-83). In humans, M*gat5* expression has been also associated with immune diseases related to T cell hyper activation. Patients with chronic intestinal inflammation (such as inflammatory bowel disease, IBD) have been reported to have lower M*gat5* expression compared to healthy controls (84, 85). They present with fewer branched N-glycans in the lamina propria of the intestine and on the TCR of colon-resident T cells, which is suggested to be the cause of the sterile inflammation that give rise to the disease (85). Recently, another interesting mechanism for M*gat5* deficiency-disease in mice has been proposed. The lack of complex N-glycans results in a relative increase in truncated, mannose-capped glycans in tissues (86). These structures resemble those of lower-complexity organisms (pathogens), which are recognized as DAMPs by a population of γδ T cells expressing DC-SIGN (86). They infiltrate the peripheral tissues and give rise to a sterile inflammation, initiated by secretion of IL17 (86). These data were translated from mice to SLE patients, where stainings of the kidney revealed an overexpression of mannosylated structures and infiltration of γδ T cells comparable to those observed in mice (86).

4.1.2 Brain. The brain is an immune privileged organ, where infiltration of immune cells and antigens from the bloodstream is tightly controlled by the blood brain barrier. A brain-resident population of macrophages called microglia is the primary immune subset found in homeostatic conditions (87). Limited immune surveillance and a number of mechanisms to keep the microglia quiescent make the brain a tolerogenic organ(88). Glycans contribute to the formation of this immunosuppressive milieu. For example, glycopeptidomic analyses both in humans and mice revealed a higher incidence of N- (and to a lesser extent O-) sialylated glycans in the brain compared to other organs (89-91). There are no acetylated sialic acids and more fucosylated high mannose structures (92, 93). Of note, the majority of sialylated glycans is found on lipids (94). All these structures have been associated with tolerance, which supports the hypothesis that glycans contribute to maintenance of brain immune privilege.

Alterations of the brain's glycome have been reported in virtually all immune diseases associated with loss of tolerance in the brain, such as MS and

Alzheimer's disease (AD) (95). Activated microglia contributes to further shift the glycan repertoire towards de-sialylated, lower complexity glycans by secreting βζ–galactosidase (in a study conducted in mice) and Neu1 (96, 97). This feature of the microglia is associated both with neuroinflammation and neurodegeneration (96, 97). Siglecs (specifically -2, -3, -8 and -11) expressed by microglia are the main actors involved in sensing changes in sialylation in the brain (98). In homeostasis, they jointly contribute to the suppression of microglia's neurotoxic activity (namely phagocytosis and secretion of pro inflammatory cytokines) by binding sialylated ligands in cis and on neurons' membranes (*in trans*) (98). However, microglia also plays a protective role by removing protein aggregates (99), suggesting that the inhibition of phagocytosis promoted by Siglecs' engagement might also have a detrimental effect. Removal of Siglecs' ligands by extracellular enzymes release the inhibition on the microglia, promoting the activated phenotype observed in pathological conditions.

In MS, deficiency of GlcNAc has been proposed as an early disease marker, as its serum level negatively correlates with disease severity (72). Microglia (100) and perivascular macrophages (101) both express DC-SIGN, which can bind to fucosylated ligands on Myelin Oligodendrocyte Glycoprotein (MOG), an important myelin-derived autoantigen in MS (100). In healthy individuals, this interaction triggers DC-SIGN-mediated IL-10 secretion from APCs and consequent induction of Tregs (100). However, in the active lesions of MS, the fucosylated ligands of DC-SIGN are downregulated; consequently, DC-SIGN's maintenance of a tolerogenic milieu is lost, giving rise to local inflammation that is typical of the disease (102). In the active lesions of MS patients, it was also reported that anti-inflammatory M2 macrophages upregulate MGL, suggesting an involvement of MGL in the resolution phase of the disease (103).

MGL KO mice shows exacerbated EAE symptoms, hinting at a key role played by this lectin in controlling neuroinflammation (103). Brains from AD patients also show aberrant glycosylation compared with healthy controls. Specifically, less sialylation and N-glycan branching, while increased incidence of bisecting GlcNAc, undecorated complex/hybrid N-glycans and mannosylation have been reported (95). Moreover, spatial glycomics analyses revealed high levels of α2.6 sialylation in amyloid plaques (104). Interestingly, glycosylation of specific proteins is also affected in AD. A high-mannosylated isoform of Tau, a protein known to be implicated in AD pathophysiology, was found in AD patients and absent in not-demented controls (95).

Siglec-3 is a risk factor for AD (105), suggesting its involvement in sensing changes in the glycome of AD patients. Its ligand in the brain has been recently

identified. It is a sialoprotein named RPTPζ, which also binds to Siglec-8 and it is significantly overexpressed in AD patients' brains (106). Engagement of Siglec-3 and Siglec-8 on microglia inhibits phagocytosis, slowing down the removal of the protein aggregates and accelerating the progression of the disease.

4.1.3 Gut. In the gut, the most studied role of glycans is that of the glycan carrier mucins. Here, glycans are key regulators of the balance between tolerance and inflammation, which must be tightly controlled to establish and maintain symbiotic relationships with the microbiota while still being reactive to prevent infections. In IBD, multiple alterations of the gut glycome have been reported, namely the presence in the endothelium of truncated O-glycans and aberrant terminal glycosylation (31, 107). In the lamina propria of IBD patients, decreased N-glycan branching has been reported (85). All of these features of the gut glycome contribute to the insurgence of chronic inflammation via altering the immune response controlled by lectin-glycan interactions. Of note, research revealed decreased Siglec-ligands despite higher basal sialylation, suggesting a role of Siglecs in maintaining the tolerogenic milieu (108). Specifically, α2.6 sialylation of immunoglobulins is increased in the plasma of IBD patients, correlating with higher expression of ST6GAL1 (109, 110). ST6GAL1 KO in rats ameliorates the outcome of the disease by re-balancing the Th17/Treg ratio (111). ST6GAL1 enzymatic activity masks Gal1 ligands, implicating a role for this lectin in controlling intestinal inflammation (110). A role for DC-SIGN in IBD progression has also been suggested, given that its expression correlates with the inflammatory status in the distal colon (112).

4.2 Niche – regulation of the immune response by glycans - GBPs

Apart from anatomically defined tissues, other immune niches exist. These loci arise in determined physiological conditions, such as pregnancy and malignant transformation. Here, specific rules apply for the regulation of the immune response, which deviates from the homeostatic conditions.

4.2.1 Placenta. An elected environment that must be tightly controlled by the immune system is the foetal-maternal interface, which can be considered as an immune privileged site. During pregnancy, tolerance to the not-self-antigens of the foetus must be established in order to prevent rejection of the embryo.

Trophoblasts form the outermost layer of the blastocyst and are therefore responsible for mediating contact with the mother, as well as the exchange of nutrients and information. One of the mechanisms through which trophoblasts directly communicate to the mother's immune system that the antigens they are presenting are not harmful is by decorating them with α2.6 and α2.3 sialic acids (113). Sialylated antigens engage Siglec-2 in humans and Siglec-G in mouse on follicular B cells, which directly reduce foetus-specific CD4+ T cell proliferation (113). Sialylation is also crucial in the very first phases of pregnancy, where α2.8 polysialylation expressed by trophoblasts is needed for placentation (114). Moreover, the placenta starts to show high levels of α2.3 sialylation and fucosylation at week 9 of gestation (115). Genetic depletion of the sialylation pathway by CMAS KO is lethal in mice, and the embryos during gestation show infiltration of neutrophils, macrophages and NK cells from the mother (116). A conditional CMAS KO mouse model allowed to discover that sialylation prevents complement attack, even though the mechanisms behind it still remain to be elucidated (30). Interestingly, mutations that impair other terminal glycosylations are not embryonic-lethal (117).These data highlight the prominent role played by sialic acids in shaping the tolerant immunological environment required for foetal development.

In pregnancy, alterations of the glycan-GBPs axis lead to aberrant immune responses. Recent data showed a correlation between the levels of α2.3 sialylation of the foetal endothelium and trophoblast and the local levels of inflammation in pre-eclampsia biopsies (118). The decrease in sialylation is accompanied by an increase in mannosylated glycans (119). High-mannose glycoforms are also overexpressed in the decidua (the endometrium of the uterus during pregnancy) of patients suffering from miscarriage (120). Premature delivery is another complication of pregnancy where glycans have been shown to play a role. Here, the cervicovaginal fluid presented with lower levels of sialylation and higher levels of non-fucosylated glycans. The altered glycome of pre-term mothers, compared with women delivered at due time, correlates with a pro-inflammatory milieu (121). These data suggest that the maintenance of a *healthy* glycome (high levels of fucosylation and terminal sialylation, low mannose) is key to prevent the insurgence of complications during pregnancy.

A number of studies reported a clear involvement of the immune system in translating these alterations into pathological conditions. Expression of DC-SIGN has been reported on macrophages in the decidua at mid- term (122). Decidual macrophages have features of the homeostatic M2 phenotype: they secrete the immune suppressive cytokines IL10, IL35, the monocyte- and T regs chemokines

CCL2 and CCL18, and have the capacity to induce T regs *in vitro* and reduce NK cytotoxicity (123). It is the placental tissue that modulates and induce these macrophages, via trophoblast – secretion of CXCL16 (123). Therefore, the presence of DC-SIGN expressing M2 macrophages in the decidua in close proximity to the trophoblast might suggest an involvement of this lectin in the maintenance of the tolerogenic milieu indispensable for the survival of the foetus. Human placental trophoblast express Siglec-6(124), and its overexpression was found in the placenta of pre-eclampsia pregnancies (125).

Of note, immune cells also alter the placental glycome. Depletion of NK cells lowers α2.3 sialylation, O-glycosylation but increases complex N-glycans, while expansion of the DCs compartment leads to downregulation of Tn antigen, α2.6 sialylation and increased N-branching in the vasculature (126).

Sialylation also plays an important role in regulating the activity of IgGs that are transferred from the mother to the foetus (127).

During pregnancy, a post-translational modification, specifically the deacetylation of sialic acid residues on the Fab region of antibodies, enhances their ability to protect the newborn from intracellular pathogens. As these antibodies cross the placenta, the removal of the acetyl group exposes the sialic acid, enabling the antibodies to bind to Siglec-2 on pathogen-specific, IL-10-secreting regulatory B cells in the foetus. This interaction inhibits the immunosuppressive function of these regulatory B cells, thereby enhancing the immune response against intracellular pathogens (127).

4.2.2 Cancer. It is now very well described in literature that altered glycosylation is a hallmark of cancer(128). Even though every tumour (and even every subtype) shows its own peculiarities, the TME can be overall considered as a unique niche. Common changes in the glycome reported across multiple cancers are the presence of truncated O-glycans, increased N-glycan branching (33), hyper-sialylation and hyper-fucosylation (129). Glycans that are not present in healthy normal tissue or whose expression is altered in tumour cells are called tumor-associated carbohydrate antigens (TACAs). As expected, Siglecs expressed by a variety of immune cells play major roles in translating alterations of the self-sialoglycans into the immunesuppression that drives tumour survival and progression. Inhibitory Siglecs (specifically Siglec-7 and -9 in humans and –F in mice) are upregulated on tumour-infiltrating CD4+ and CD8+ T cells of various cancers, correlating with their exhausted phenotype (47-49, 130). Sialylated tumour-associated glycans also modulate the innate arm of immunity. In different cancer cell lines, sialylation

inhibited NK killing capacity by engaging Siglec-7 (131). Similarly, removal of sialic acid increased NK killing and cytokine secretion in a Siglec-7 and -9 dependent manner (132). In Pancreatic ductal adenocarcinoma (PDAC), sialic acids polarize monocytes to TAMs and induce their secretion of the anti-inflammatory cytokine IL-10 via Siglec-7 and -9 (133). Further, sialylated tumour ligands also modulate neutrophils' activity, inhibiting their cytotoxic activity through Siglec-7 and -9 engagement (134).

Another feature of the tumour glycome is the presence of Tn and sialylated Tn antigens (135). These are truncated O-glycans which result from incomplete O-glycosylation pathways. It has been reported that MGL-expressing APCs (with a DC-like phenotype) are present in carcinoma patients at the same location of Tn antigen expression (65). The interaction of MGL with Tn on MUC1 of various cancer cell lines has been further validated *in vitro*. Here it was shown to induce a tolerogenic phenotype on MGL-positive DCs and macrophages, which contribute to the formation of the suppressive TME (65, 136).

Tumour cells also secrete soluble glycans which contribute to the formation of the suppressive TME. One of these factors is the tumoral carbohydrate A10 (Ca10h), an heparan-like carbohydrate enriched in the sera of various cancer patients. This glycan is able to drive differentiation of monocytes to tolDCs, which then promotes naïve to Tregs (functional) differentiation (137). To date, the GBPs behind the modulation of APCs activity via Ca10h have not been uniquely identified yet. Interestingly, glycans secreted by cancer cells also modify, modulate and affect distant niches. In prostatic cancer it was found that secretion of aberrantly glycosylated factors (due to altered expression of ST6GAL1) play a crucial role in shaping the pre-metastatic bone niche (138). These glycosylated factors contribute to bone resorption and to the development of an immunosuppressive environment by promoting the formation of TAMs. The modification of the bone niche facilitates the colonization and growth of metastatic tumour cells from the prostatic primary niche (138).

5. THERAPEUTIC OPPORTUNITIES

Alterations of the glycome and its GBP-sensing in immune diseases represent an opportunity for therapeutic interventions, with approaches ranging from reverting the glycome to its homeostatic state, to directly targeting GBPs that regulate the immune response **(Figure 3)**. As discussed so far, specific glycosylation patterns identify every niche. Locally, alterations of the glycome are finely sensed by GBPs. While the most common challenges that new targeted therapies face, namely off-target effects and toxicity do not apply to glycan-based strategies due to their specificity, the real challenge lies in combining them with existing immunotherapies, such as anti-PD1. The modulation of the immune system governed by glycan-GBPs interactions is subtle and should be rather implemented in the current regimens to achieve a stronger, more controlled response.

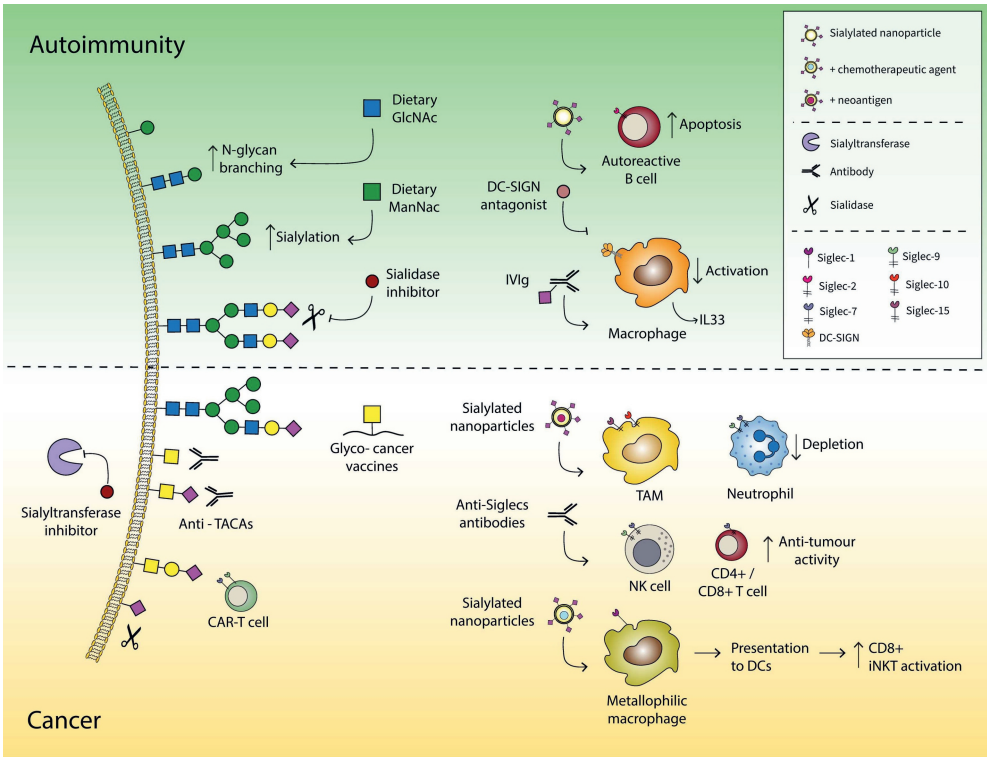


Figure 3 Therapeutic opportunities: targeting altered glycosylation and/or GBPs in autoimmunity and cancer. *At the centre of the figure, various therapeutic strategies are represented. These directly target the glycosylation changes observed in cancer and autoimmune diseases (on the left), with the goal of restoring the homeostatic glycome. Alternatively, they can be targeted to the GBPs on the immune subsets responsible for sensing those changes in glycosylation (on the right), aiming at reverting the detrimental immune response.*

5.1 Autoimmunity

One of the features of the glycome of chronically inflamed tissues discussed in paragraph 2 is the lack of branching N-glycans. This has led to the hypothesis that supplementing the diet with a critical metabolic precursor of N-glycosylation and branching, i.e. GlcNAc, would improve the clinical outcome. This strategy is particularly interesting the way it is delivered, *i.e.* by oral administration, which will likely increase the compliance of the patients. Several studies have already tested it in experimental models of various autoimmune diseases, namely IBD (139), SLE (86), diabetes and EAE (140, 141). The beneficial effects are mediated by a reduction in Th1/Th17 skewing, which are the drivers of inflammation, and by controlling T cell activation and proliferation. General improvement of the disease scores and even inhibition of the insurgence of the disease were reported. These studies are currently being translated to humans. A clinical trial with GlcNAc in MS patients has shown incredibly promising results, by reducing serum levels of Th1/Th17 pro inflammatory cytokines and improving the neurological disease scores with only 4 weeks of treatment (142).

The same metabolic-reprogramming approach has been tested for sialic acid. As mentioned in paragraph 2, a glyco-signature of inflammation and autoimmunity is reduced sialylation. On the other hand, tolerogenic niches show hypersialylation. Therefore, diet supplementation with a precursor of sialic acid, ManNAc, has been tested in an experimental model of autoimmune myopathy, to re-establish the tolerogenic milieu (143). Another approach to restore normal levels of sialylation is inhibiting the sialidases, which has proved useful in a mouse model of pulmonary fibrosis (144). Recently, a study from Mashangva *et al.* suggested the use of small effector molecules targeting downstream players in the sialylation pathway, to restore normal sialic acid levels which are impaired in GNE-deficiency myopathy (145). Oral supplementation of the sialylation pathway is also investigated as a therapy in a mouse model of high fatty liver disease (146). Though not properly considered autoimmunity, a clear dysregulation of inflammation makes it worth discussing it here. Interestingly, feeding the mice a high glucose diet increases Siglec-E ligands on erythrocytes, suggesting an involvement of Siglec-E expressing cells in controlling the systemic inflammation (146).

These findings highlight the potential of using sialic acid to control aberrant immune activation, and lead to the exploration of its role in established immunotherapies. One example of such is IVIg, an immunotherapy already widely used in the clinic for which a peculiar role of sialic acid has been described. Initially used for immune deficiencies, it now also proved useful in autoimmune diseases, both in experimental models as well as in patients, with significant amelioration of the

clinical symptoms. It consists of intravenous infusions of IgGs which systemically induce expansion of the T reg compartment, and suppression of Th1 and Th17 effector cells. The mechanisms responsible for the beneficial effects depend on the presence of an α2.6 sialylated glycan in the Fc portion of the infused antibodies, since mutations in the Fc domain that reproduce the conformational changes induced by sialylation also recapitulate the anti-inflammatory effects (147). The specific molecular modes of actions however seem to be dependent on the microenvironment, the immune cells which inhabit it and the receptors they express. One mechanism of action is via binding of the sialoglycans on IgGs to DC-SIGN on dendritic cells (148). This binding initiates a pathway leading to the suppression of macrophage activation and inflammation through the upregulation of inhibitory Fc receptors (148). In a mouse model of RA, IVIg was shown to induce IL33 production, which increases IL-4 production by basophils, which then upregulates suppressive FcR on monocytes and macrophages (149). In a different mouse model of aberrant hyperactivation of the immune system, airway allergy, the effects of sialylated IVIg were instead mediated by a subset of pulmonary CD11c+ DCs via DCIR, an ITIM-carrying C-type lectin not discussed in this review (150).

Targeted therapies towards lectins are also being investigated. For example, a synthetic antagonist of DC-SIGN in combination with a sialidase inhibitor was tested in an experimental model of chronic liver disease (151). Liposomes displaying Siglec-2 ligands are now widely investigated in pre-clinical studies to induce apoptosis of autoreactive B cells in RA (152) and inhibitors-bearing hemophilic mice (153). Targeting Siglec-3 in the brain of AD mouse models has also proven useful (154), given its involvement in the control of microglia removal of the protein aggregates.

These innovative approaches highlight the great potential of using glycan-based strategies to finely modulate immune responses and treat autoimmune diseases.

5.2 Cancer

Malignant transformation is significantly associated with changes in the glycome. Unsurprisingly, recent advancements in cancer immunotherapy have focused on glycan-based strategies, including targeting tumor-associated carbohydrate antigens (TACAs) for drug delivery, modifying the glycome with enzymes or inhibitors, and targeting lectins as immune checkpoints **(Figure 3)**. TACAs are inherently immunogenic due to their absence in healthy tissues (64). Both circulating anti-TACAs IgM and newly identified IgG antibodies have been correlated with improved prognostic outcomes. This knowledge has made TACAs attractive

targets for cancer immunotherapy. Multiple strategies are being investigated, ranging from anti-TACAs antibodies (either unlabelled or conjugated to drugs), to the use of TACAs as cancer vaccines. Tn and sialyl-Tn mucins were investigated as vaccines for colorectal cancer three decades ago (155). Other approaches aim to directly influence the glyco-TME to revert the aberrant suppressive phenotype. A growing body of literature is focusing on targeting sialidases to the TME, to remove sialic acids thereby relieving the inhibition on the immune system (156, 157).

Similarly, inhibitors of sialyltransferases are also being tested in the clinic with the same goal of counteracting immune suppression. In fact, a major challenge in cancer immunotherapy is the presence of an immune population within the TME that is incapable of recognizing and killing tumour cells. This population is composed of exhausted CD4+ and CD8+ T cells, NK cells with impaired cytotoxic capacity, TAMs and tolerogenic DCs. Inhibitory Siglecs have been described in all these phenotypes, and are now broadly acknowledged as immune checkpoints (158). More and more studies are investigating ways of targeting Siglecs, also in combination with classical immune checkpoint inhibition (anti-PD1 and anti-CTLA4). To date, therapies targeting Siglec-7, -9, -10, -15 are in pre-clinical or clinical studies for the treatment of different malignancies (159-162). On the other hand, Siglecs lacking an ITIM have great internalization properties and restricted expression. Targeting Siglec-1 on metallophilic macrophages with sialylated nanoparticles encapsulating tumour neoantigens activates potent CD8+ T (163) and iNKT cells(164) responses that inhibits tumour growth. On a different approach, sialylated nanoparticles encapsulating chemotherapeutic agents were efficiently used in mouse models to deplete TAMs and neutrophils, thus restoring immune killing (165). Another interesting approach is the use of Siglec-7 and -9 extracellular domains in CAR-T cells, to direct them towards the hypersialylated tumour cells (166). *In vitro* and *in vivo* experiments showed efficient anti-tumour activity, underscoring the feasibility of targeting cancer-associated glycosylation patterns with CARs (166).

6. FUTURE DIRECTIONS AND CONCLUSIONS

The glycome plays important roles in the immune system. It can alter local niches to direct immune inflammation or tolerance and immune cell migration via interactions with glycan binding proteins on immune cells. How these glycans have impact on immune cell signalling still remains largely unexplored. One of the primary challenges is understanding how changes in glycans, driven by the non-templated nature of glycosylation, are sensitive to microenvironmental changes, and impact immune modulation. The tissue glycome is shaped by the expression of niche-specific glycosyltransferases, which are differentially regulated. It still needs to be shown whether single cell technologies or spatial analysis can reveal cell specific glycosylation patterns related to their location in the tissues. Moreover, the glycosylation status of immune cells may also be altered during differentiation, inflammation or tolerance, oppositely to its environment. The environment and leucocytes mutually influence and transform each other, and these confined interactions are key to either mounting a proper immune response or maintaining tolerance. Also, in this cell-interactive environment both cis and trans interactions of lectins with their glycan-ligands can modulate the immune state. Further, the same lectin can have different roles in modulating the immune response depending on the immune subset that it is expressed on or the type of glycan to which it binds. Tools to study these interactions are already developed. However, the local tissue-specific interactions between the glycome and the glycan binding proteins remain spatially to be explored, using single cell spatial technologies. Spatial transcriptomics should be employed to study the expression of glycosyltransferases in order to identify niche-specific glycosignatures, This, combined with the new techniques in imaging mass-cytometry will identify the GBPs and immune subset that are responsible for sensing changes in the local glycome by spatially resolving the local interactions. Understanding the glyco-niche dynamics may lead to improved biomarker discovery and targeted therapies for all immune-related diseases, from cancer to autoimmunity and allergy. Overall, employing multi-omics technologies to analyse the glycome and GBPs will uncover the great potential of these interactions to fine-tune the immune response.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (OpenAI's GPT-4 language model) in order to revise and refine the content of the work. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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1

INTRODUCTION

Part II: Translation into the clinic: hemophilia A and house dust mite allergy.

Seminars in immunology, 2024

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As previously mentioned, alterations of the glycome and its GBP-sensing represent an opportunity for therapeutic interventions in virtually all immune diseases. This thesis mainly focuses on the development of anti-FVIII neutralizing antibodies in hemophilia A and house dust mite allergy.

HEMOPHILIA A

Hemophilia A (HA) patients present with an inherited deficiency or dysfunction of factor VIII (FVIII) of coagulation, resulting in prolonged or excessive bleeding, both spontaneous or following an injury. Based on the residual activity of FVIII, measured in international units (IU) / dL or percentage, HA is classified as mild (6 – 40 IU/dL, 6 – 40%), moderate (1 – 5 IU/dL, 1 – 5%) or severe (< 1 IU/dL, < 1%) (1). Clinically, HA is treated with FVIII prophylaxis, administered by biweekly intravenous infusions of plasma derived concentrates or recombinant proteins.

FVIII is a glycoprotein synthetized as a single polypeptide chain primarily by liver sinusoidal endothelial cells (LSEC) (2). Prior to secretion into the blood stream, it is cleaved into a heavy chain of 200 kDa (composed of A1-A2-B domains) and a light chain of 80 kDa (A3-C1-C2) (3). Short peptide sequences rich in negatively charged amino acids designated *a1, a2, a3* are located adjacent to the A1, A2 and A3 domains. The a1, a2 and a3 region contain sulfated tyrosine; sulfation of Tyr1680 in the *a3* region is crucial for binding of FVIII to its molecular chaperone von Willebrand factor (VWF) (4). FVIII bears 25 potential N-glycosylation sites (NXT/S where X can be any aminoacid but proline), of which 19 are located in the B domain ***(Figure 1A)***(5).

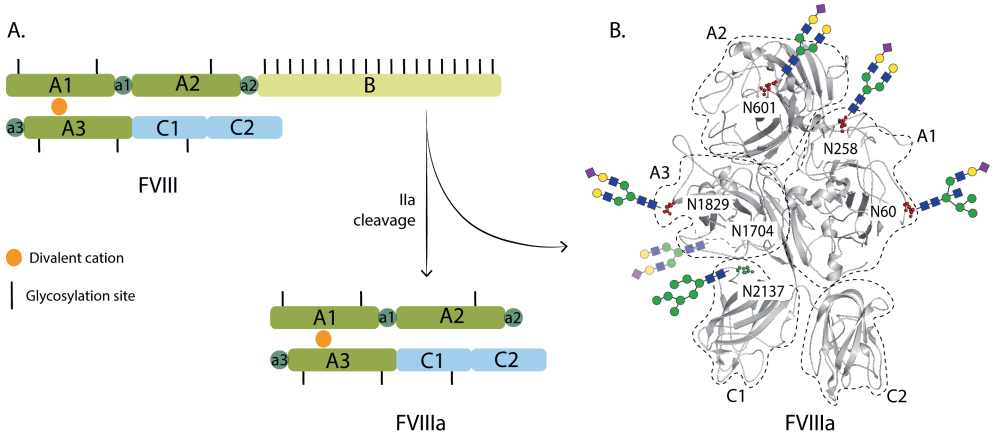


Figure 4 Schematic and structural representation of FVIII N-glycosylation sites. ***A.*** *Schematic representation of FVIII domains with the N-glycosylation sited highlighted by straight black bars. After activation by thrombin (Xa), the B domain is deleted.* ***B.*** *The crystal structure of B domain-deleted FVIII was taken from PDB (3CDZ) and shown as cartoon, while the domains are evidenced by dotted lines. N-glycosylated asparagine are shown as red spheres, except for N2137, which is highlighted in green. Glycans are indicated according to the SNFG nomenclature and were manually added in Adobe Illustrator. N2137 bears a mannose-rich glycan, while all the other are sialic-acid capped. N1704 lies within a flexible region, not resolved in the available crystal structure. It is therefore depicted schematically. The N1704-linked glycan is oriented towards the back of the structure and is therefore displayed as opaque.*

N-glycans are located within the A1 (N60,N258); A2 (N601); A3 (N1829) and C1 domain (N2137) **(Figure 1B)**(5). Of note, glycosylation at N1704 within the A3 domain occurs with approximately 10% site occupancy, suggesting that this modification is present on a limited fraction of the protein population (6). In addition, multiple N-glycans are present in the heavily glycosylated B domain of FVIII (5). The N-glycome of FVIII comprises complex glycans, with a poly-mannosylated core that can be variously extended into sialylated structures. The most common N-glycan identified at these sites is complex antennae, mostly terminally sialylated, both α2.3 and α2.6 **(Figure 1B)** (5). The N-glycosylation site in the C1 domain, N2137, is the only one consistently described as bearing a high mannose structure (5, 6). This site is largely exposed on the surface, and it has been shown to be essential for DC uptake and presentation to T cells (7). While it has been described to be a binding site for CLEC4M, the meaning of this interaction for the clearance of FVIII has not been fully elucidated yet, due to lack of a mouse homologue of the receptor (8). FVIII is also heavily O-glycosylated, with 23 glycoforms identified as of today (6).

Differently than N-glycosylation, which occurs at specific aminoacidic sequences, O-glycosylation happens after protein folding on either serine or threonine residues. O-glycosylation is mostly initiated by the addition N-acetylgalactosamine (GalNAc), defined as Thomsen-nouveau (Tn) antigen (9). Then, it can be variously extended by the sequential action of a number of glycosyltransferases, generating the so-called core structures (9). These are themselves also variously extend, creating an enormous variability of glycans. For FVIII, the core-2 O-glycans are the most commonly described O-linked forms, and, like the N-glycosylation sites, they are mostly located within the B domain (6). While it is true that the B domain is the most heavily glycosylated, it is dispensable for the function of FVIII, and most of the currently marketed recombinant FVIII products are based on B-domain-deleted or B-domain-truncated constructs (10). Therefore, it has been suggested

that the function of N-glycans on the B-domain promotes intracellular trafficking and efficient secretion (11), via binding for example of high mannose structures to the ER-resident mannose receptor LMAN1 (12).

After secretion, FVIII circulates in a noncovalent complex with VWF. The ratio FVIII : VWF is 1 : 50, assuring that the largest part of FVIII is bound, while only up to 5% is free (13). This interaction protects FVIII from its spontaneous inactivation by circulating proteases and extends its half-life up to 4 times by protecting it from clearance, making it essential to stabilize FVIII *in vivo* (14). VWF is a large glycoprotein, composed of several monomers, each of which contains a FVIII-binding site. On FVIII, the a3 acidic region, which contains a sulfated tyrosine, as well as the C1 domain are required for complex formation with VWF, while the A3 and C2 domains contribute to stabilize the binding (15).

FVIII activity in hemostasis

Hemostasis is the process that safeguards vascular integrity, readily arresting bleeding. It can be broadly divided into two phases: primary hemostasis, where a temporary platelet plug forms, and secondary hemostasis, also called coagulation, which stabilizes the plug. At the site of vascular injury, increased local shear stress and exposure of the sub-endothelial matrix causes VWF to bind to collagen, triggering a conformational change in VWF that exposes the binding site for glycoprotein Ib (GPIb) on platelets (16). Consequently, platelets tether on VWF and the formation of the platelet plug initiates; this process comprises primary hemostasis. Secondary hemostasis follows the formation of the platelet plug and leads to its stabilization by the formation of a fibrin mesh.

Coagulation has traditionally been viewed as a series of sequential proteolytic reactions, involving the conversions of zymogens to their activated form in a “cascade” model. Two complementary pathways, the intrinsic and extrinsic, converge in the activation of large amounts of thrombin, which converts fibrinogen to fibrin to form a polymerized network. In the last two decades, this model has been revised to highlight the central role played by activated membrane surfaces of various cell types (17). According with this cell-based model, coagulation is initiated by binding of plasma FVII to tissue factor (TF) bearing cells, where different proteases activate it to FVIIa **(Figure 2)**. TF is a transmembrane protein expressed by a variety of cell types, including fibroblasts, activated monocytes and inflamed endothelial cells (18). FVIIa, FX and TF form the extrinsic Xase complex, which in the cascade model initiate the extrinsic pathway, responsible for producing initial small amounts of FXa and thrombin (FIIa).

During the following amplification phase, newly generated FIIa contributes to platelet activation and the activation of FV and FVIII **(Figure 2)** (19, 20). Proteolytic cleavage of FVIII light chain results in its dissociation from VWF, thereby exposing the phospholipid binding site in the C1 and C2 domain (20). Activated FVIII binds to the anionic phospholipid surface of activated platelets, where it associates with FIXa and FX to form the intrinsic FXase complex. By bridging FIXa and FX, FVIII serves as a cofactor for FIXa and it increases the catalyzing activity of FIXa by several orders of magnitude (20). This initiates the propagation phase described by the cell based model of hemostasis, which produces robust amounts of fibrin crosslinked by FXIIIa to generate a stable clot (17).

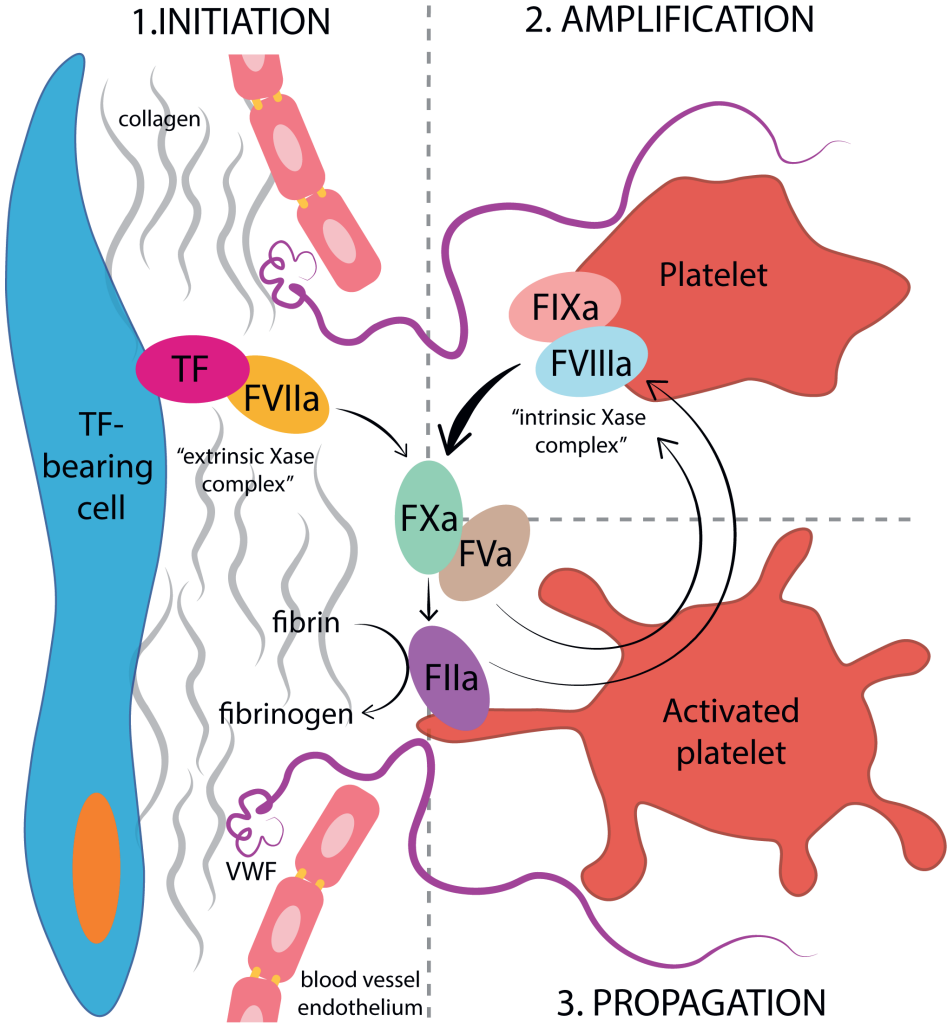


Figure 5 Cellular-based model of the coagulation cascade. *In the initial phase, exposure of the extracellular collagen serves as binding site for the circulating VWF, while TF-bearing cells for FVII. The so-formed extrinsic Xase complex is responsible for the generation of priming concentrations of FXa and FIIa. In the amplification phase, FIX and FVIII are activated by the small amounts of FIIa. This exposes the phospholipid binding sites which allows the formation of the intrinsic Xase complex on the surface of platelets, tethered on VWF. In the propagation phase, robust activation of FIIa is achieved, massively converting fibrin to fibrinogen to start the formation of a stable clot. Abbreviations: TF, tissue factor; FVIIa, activated factor VII; FVIIIa, activated FVIII; FIXa, activated factor IX; FXa, activated FX; FIIa, activated FII; VWF, von Willebrand Factor.*

Role of sialic acid in hemostasis

Terminal sialylation of glycans in circulating coagulation factors is crucial to avoid premature clearance. Removal of capping sialic acid, which in mammals is catalyzed by 4 families of sialidases called Neu1-Neu4 (21), exposes the underlying Gal and GalNAc residues. These monosaccharides are ligands for the hepatic C-type lectin asialoglycoprotein receptor (ASGPR or Ashwell receptor), which is involved in FVIII clearance (22). Another role of sialylation has been proven specifically for FX. Removal of terminal sialic acids from FX slows down its activation by both intrinsic and extrinsic FXase complexes, decreasing the rate of formation of the FXase complex (23).

Clearance and receptors that bind FVIII

In circulation, FVIII fate is closely bound to that of VWF. However, several receptors can still bind FVIII even when it is complexed with VWF. The first receptor described to be involved in the clearance of FVIII was low-density lipoprotein receptor-related protein-1 (LRP1) on hepatic cells (24, 25). Additional receptors expressed on liver sinusoidal endothelial cells (LSECs) include CLEC4M, stabilin-2, and SCARA5 (26). Interestingly, Siglec-5 was also described as a receptor for FVIII, although the binding seems to be independent of the presence of sialic acids (27). Overall, many N- and O-linked glycans on FVIII have been shown to interact with multiple carbohydrate receptors, namely the ASGPR (28), Galectins-1 and -3 (Gal-1, Gal-3) (29), and macrophage galactose lectin (MGL) (30), thereby influencing the half-life of FVIII in circulation and its uptake by antigen presenting cells.

FVIII immunogenicity

Although HA is not per se an immune-mediated disease, an immune-dependent complication of its therapy is the development of neutralizing anti-FVIII antibodies, called inhibitors. Approximately 30% of severe (31) and 13% (32) of non-severe HA patients develop inhibitors, which are polyclonal IgGs mainly of the IgG1 and IgG4 subclasses (33). Interestingly, low levels of IgG against FVIII are also found in healthy individuals and HA patients without inhibitors with a prevalence as high as 19% and 34% respectively (33). Moreover, FVIII specific CD4+ T cells have been identified in healthy donors (34). Of note, these CD4+ T cells recognize the same epitopes of HLA-matched HA CD4+ T cells (35). These data indicate that even though FVIII tolerance should be established at the thymic level, for reasons that are not fully elucidated yet, it is incompletely enforced in a significant proportion of individuals. Nonetheless, substantial differences distinguish HA patients with inhibitors from healthy individuals and inhibitor-free HA patients. First, healthy subjects do not present anti-FVIII antibodies belonging to the IgG4 subclass (33), nor FVIII- specific memory B cells (36). Second, FVIII-specific CD4+ T cells identified in healthy donors include a distinct population of thymus-derived Tregs (37). Collectively, these observations support the existence of distinct immunological pathways underlying inhibitor development in hemophilia A patients. Moreover, several evidence hint at an intrinsic immunostimulatory property of FVIII: inhibitors to FIX are extremely rare in hemophilia B, despite a ~ 17 fold higher dose of FIX than FVIII in HA. However, hemophilia B patients carrying large deletions in the *F9* gene remain at risk of inhibitor development (38); in mice, FIX fail to induce an antibody response at the same dosage as FVIII when injected i.v. (39); and FVIII is more immunogenic than OVA when injected i.p. in the absence of adjuvants (40). Further, genetic background (41, 42), the type of *F8* mutation and the presence of residual FVIII protein (43), intensity of treatment (44), only partially explain the development of inhibitors. Conversely, the presence of concomitant danger signals during treatment such as vaccination (45) or infections are not related to the risk of developing inhibitors. These observations highlight that immunogenicity to FVIII is a complex phenomenon that remains incompletely understood.

FVIII immunogenicity has been extensively investigated in animal models. Experiments in FVIII-KO mice demonstrated that FVIII rapidly clears from the blood to accumulate in the liver and the spleen (46). The spleen appears to be the most important secondary lymphoid organ for mounting an immune response to exogenous FVIII (46). Recently, an elegant study demonstrated that the coordinated presence of marginal zone macrophages, marginal zone metallophilic macrophages and conventional DCs, especially cDC1, is required to achieve

CD4+ T cell priming *in vivo* (39). Among other DC subsets, plasmacytoid DCs and moDCs are not involved in FVIII presentation but likely provide activation signals, since their frequencies rise following FVIII injection (39). Depletion of marginal zone B cells also abolishes Th proliferation (39). Given that they do not travel to the white pulp, it has been suggested that these different APCs subsets act in concert to transfer FVIII to the T cell zone, involving mechanisms such as trogocytosis. Of note, no uptake of FVIII was detected in red pulp macrophages (39), which are the subset responsible for cleaning the blood and induce tolerance to blood-borne antigens. After uptake and processing of FVIII, APCs present it to naïve T cells, which activate and differentiate into follicular Th cells (TFH) **(Figure 3)**(47). TFH play a key role in initiating and maintaining the germinal center response, where FVIII-specific B cells undergo class switch and affinity maturation. More recently, it was found that among TFHs, specifically the regulatory subset plays a key role in providing the help needed to B cells to inhibitor development (48). Germinal center B cells can then migrate to the bone marrow, where they mature into long lived plasma cells (36).

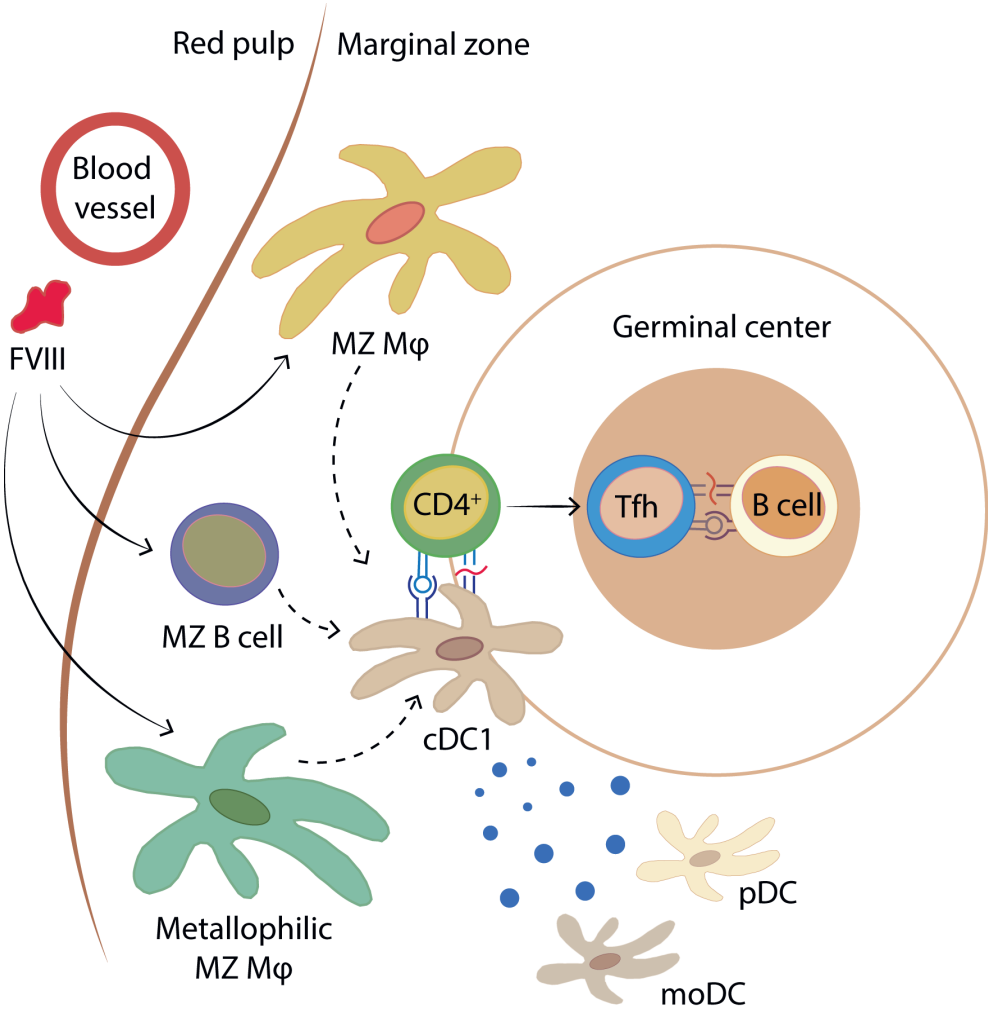


Figure 6 Schematic representation of FVIII trafficking to the white pulp and presentation to germinal center B cells. *Different immune subsets are responsible for taking up FVIII and delivering it to cDC1 in the marginal zone. cDC1 then present it to FVIII-specific CD4+ T cells, which activate B cells in the germinal center. Abbreviations: MZ, marginal zone; cDC1, conventional dendritic cell type 1; moDC, monocyte-derived dendritic cell; pDC, plasmatocytoid dendritic cell; Tfh, follicular helper cell.*

In healthy individuals, peripheral tolerance might compensate for the incomplete central tolerance to FVIII, as supported by the discovery of FVIII-specific T regs (37). However, other mechanisms might also play a role. For example, it is known that VWF protects FVIII from circulating inhibitors. It has been shown that plasma-derived FVIII that is already complexed with VWF is significantly better protected from neutralization by pre-existing FVIII inhibitors than recombinant or isolated FVIII, both *in vivo* and *ex vivo* (49). Pre-complexing rFVIII products with VWF significantly reduced their immunogenicity in FVIII-KO mice (50). Moreover, VWF also plays a role in inhibiting FVIII endocytosis by dendritic cells, consequently reducing antigen processing and presentation to FVIII-specific CD4+ T cells (51). Stabilin-2 expressed on LSEC also modulates FVIII immunogenicity, by mechanisms not fully elucidated yet. In STAB-2 KO mice challenged with pdFVIII, the titer of anti-FVIII IgGs was lower as compared with WT mice, indicating a role of stabilin-2 in the development of inhibitors (52).

Management, therapeutics

The current standard of care for HA is prophylactic replacement therapy with FVIII. The first FVIII concentrates became available in the 1960s, transforming hemophilia A from a fatal to a manageable disease (53). However, complications also arose, like in the 1980s when 60-80% of all HA patients got infected with HIV (53). The cDNA of FVIII was eventually cloned in 1982, which rapidly enabled the production of safer recombinant products (rFVIII) produced in mammalian cells (54). A correlation between the use of rFVIII and the development of inhibitors has been identified in the large SIPPET study of 2016 (55), even though it did not result in a paradigm shift in treatments. Mechanistically, fewer peptides of pdFVIII are presented by moDCs as compared with rFVIII (56). Another factor that might contribute to the lower immunogenicity of pdFVIII is the presence of VWF in the formulation, which has a protective role as already mentioned earlier. More interesting for the purpose of this thesis is the role played by glycans in the immunogenicity of recombinant FVIII products. Differences in glycosylation profiles between pdFVIII and rFVIII were already documented in 1992 (57). These data have been confirmed in HA patients (44, 58) and animal studies (50, 59). Recombinant FVIII products are industrially produced in Baby Hamster Kidney (BHK) and Chinese Hamster Ovary (CHO) cell lines. Although mammalians, they still diverge in the glycosylation machinery from that of humans. Specifically, it has been observed that rFVIII products present incomplete N-glycosylation at various sites, thus exposing the underlying galactose residues, and incomplete occupancy. Surprisingly, desialylation of rFVIII produced in BHK cells did not affect maturation marker expression of murine splenocytes (60). Nonetheless,

increased levels of IgM and IgG were measured in healthy individuals to BHK- and CHO-produced rFVIII, suggesting that N-linked glycans shield epitopes in native FVIII protein, preventing the formation of immune complexes (60). Since the discovery of the FVIII cDNA, various rFVIII products have entered the market, including extended half-life FVIIIs such as the fusion rFVIIIFc-VWF-XTEN, which covalently links FVIII to a recombinant D'D3 fragment of VWF and presents the advantage of requiring only one infusion per week (61).

In recent years, a bispecific antibody has entered the market under the commercial name emicizumab (and subsequently followed by Mim8) (62). One Fab arm binds FIXa and the other FX, thereby mimicking the cofactor activity of FVIII in the coagulation cascade. These bispecific antibodies are administered subcutaneously every two weeks, which has markedly improved treatment adherence and revolutionized HA management by significantly enhancing patients' quality of life and reducing annual bleeding rates (63, 64). Other alternatives to FVIII replacement products are also available to the patients, such as inhibitors of physiological anticoagulants like fitusiran (antithrombin knock down agent), Serpin PC (which inactivates activated Protein C) and inhibitors of Protein S (61).

Excitingly, gene therapy is also fast advancing as a possible solution to achieve endogenous, life-long production of FVIII. Currently, FDA and EMA have approved valoctocogene roxaparvovec (Roctavian) for adults with severe HA without pre-existing anti-AAV5 antibodies (65, 66). Despite this major milestone, several challenges remain, including inter-individual variability in long-term FVIII expression, liver toxicity and potential genotoxicity due to AAV5 integration, and the high cost of the treatment. Moreover, Roctavian is currently not accessible to patients with inhibitors (61), but there are studies investigating platelet-targeted gene therapy that might become available to inhibitors-positive patients (67).

Despite all these advancements in HA management, replacement therapies with FVIII are still needed to achieve 100% FVIII activity during surgeries. In this context, the development of transient inhibitory antibodies still poses a threat to the management of post operative care (68).

HOUSE DUST MITE ALLERGY

Allergies are caused by aberrant and excessive activation of the immune system towards non-harmful foreign molecules such as grass pollen, dietary products and dust, which are called allergens. In the majority of the population, allergens are tolerated and do not trigger immune activation. Mechanistically, the initial encounter between the immune system and an allergen is referred to as sensitization, which leads to Th2 skewing and IgE class switch. Given that this phase happens at very early stages in life, the precise immunological mechanisms involved remain incompletely understood, due to difficulties in accessing infant data. Dendritic cells and other APCs located at the site of contact with the antigen, often the epithelia and mucosa, are involved in antigen processing and presentation to naïve T cells. Studies in mice have shown that maintenance of an IgE memory is guided by follicular DCs (69), which can retain the antigens for long periods of time in their native conformation. This hypothesis is also supported by the evidence that the epitopes recognized by IgG are different from the conformational ones binding to IgE (70, 71). After sensitization, IgE are present in circulation and bind to their high affinity receptors FcεRI. This subclass of Fc receptors is mainly expressed by APCs, mast cells and basophils (72), while low affinity FcεRII receptors are mainly expressed by B cells (73). Second encounter with the allergen triggers a robust immune response guided by allergen-specific IgE. Allergen cross-linking of the IgE bound to FcεRI on mast cells induces a massive release of proinflammatory molecules, such as histamine and leukotriens, proteases but also chemokines and cytokines to recruit other immune subsets (74). The cells recruited include eosinophils, basophils, neutrophils and macrophages. In a delayed phase, IgE-facilitated phagocytosis and antigen presentation by APCs activates memory Th2 cells, thus initiating a complex network of cellular and molecular communication mechanisms that lead to the exacerbation of the allergic symptoms. (review (75)). Interindividual differences in allergic susceptibility are influenced by multiple factors, among which the ability to present allergen-derived peptides to T cells plays a central role. This capacity is largely determined by polymorphisms within human leukocyte antigen (HLA) genes (76, 77). Other factors include environmental cues, such as cigarette smoke and inflammation.

A major class of environmental allergens associated with allergic sensitization is derived from house dust mites. House dust mites belong to the class Arachnida, and different species exhibit distinct geographic ranges. The most common species in Central Europe is Dermatophagoides pteronyssinus (Der p) (78). Clinical manifestations of house dust allergy include allergic rhinitis, asthma and atopic dermatitis. Molecularly, one of the major allergens found in the gut and

fecal pellets of Der p is the Der p 2 protein, with IgE reactivity detected in ~74% of non-hospitalized patients (79). The physiological role of Der p 2 in house dust mites is not completely understood, but the presence of a cholesterol-binding site suggests a role in lipid transport (80).

Role of glycans in house dust mite allergy

The contribution of glycans and GBPs to allergic sensitization to Der p 2 remains poorly explored, despite evidence that Der p 2 is glycosylated and carries both galactose-terminating and sialic acid-capped glycan structures (81, 82). Moreover, it is also well described in literature that both the mannose receptor (MR) and DC-SIGN play a role in the uptake of Der p 2 by dendritic cells (81-83). Higher expression of DC-SIGN is measured in cD11c+ dendritic cells in the dermis of atopic dermatitis (AD) patients compared to non-atopic controls (83). Moreover, DC-SIGN is also involved in immune modulation. Monocyte-derived DCs from AD patients respond to Der p 2 by increasing the secretion of IL-6 and TNF-α, which can be blocked by anti-DC-SIGN antibodies (83). Further, co-cultures with Der p 2-stimulated moDCs with T cells induce a Th2/Th22 phenotype (83). These data demonstrate how Der p 2 can cause Th2 skewing via modulating moDC phenotype in a DC-SIGN dependent way.

Other GBPs are involved in modulating the immune response to HDM. For example, CLEC10a is a C-type lectin that binds to GalNAc-terminating glycans, which appears to be involved in dampening the excessive immune response to HDM in AD mice models (84). On the other hand, HDM extracts can also trigger the C-type lectin Dectin-2 on cD11c+ DC in the lung, initiating a pro-inflammatory response (85).

TREATMENT OPTIONS TO RESTORE TOLERANCE IN HA AND ALLERGY

Both house dust mite allergy and FVIII-inhibitors are managed with tolerance-inducing protocols based on high-dose allergen/antigen exposure. HA patients who develop inhibitors receive immune tolerance induction (ITI) strategy. ITI was first proposed as a treatment for inhibitors in 1977, and it entails large amounts of FVIII infusions. To date, it is still the only accepted protocol to treat HA patients with inhibitors. However, rate of success is ~ 70% (86). ITI also poses other threats, such as increased risk of thrombosis and infections (87). Moreover, it represents a heavy burden for the patients, let alone the healthcare system. House dust mite allergy is treated with allergen immunotherapy (AIT), which consists of gradually increasing doses of allergens given subcutaneously or by oral delivery over a prolonged period of time – usually up to 3 years.

Both ITI and AIT are based upon the same hypothesis: repeated and long-term exposure of the immune system to a not-harmful trigger eventually downregulates the immune response, favoring the induction of tolerogenic circuits. Although successful to a certain extent, it is clear that both ITI and AIT share the same limitations and could benefit from safer, cheaper, patient compliance centered options.

During the past few decades, a growing body of research has therefore focused on developing alternatives to ITI and AIT. As for FVIII, less immunogenic recombinant products have been studied in mice, such as tolerogenic nanoparticles (88, 89), elimination of MHC-II epitopes (90) and Fc fusion chimeras to facilitate transplacental delivery (91). Another approach already in the clinic, is to extend FVIII half-life, thus limiting the number of injections needed for replacement therapies and decreasing the risk of developing inhibitors (92). Recent advances in the field of gene therapy also hold promises for the treatment of HA (93, 94). Similarly, a number of strategies have been proposed for Der p 2, such as modified allergens with reduced IgE-binding capacity and T-cell epitopes (95).

In this thesis, I joined this vibrant field of research to investigate our own tolerogenic solutions for FVIII and Der p 2, based on the coupling of the antigens with sialylated glycans.

SCOPE OF THE THESIS AND OUTLINE

This thesis aims at investigating how to employ sialylation to achieve efficient Siglecs targeting in order to modulate the immune system towards tolerance in hemophilia A and house dust mite allergy.

In **Chapter 1**, we propose to adopt a tissue- and niche- specific point of view to study the role of glycosylation in informing the immune system. Then, the available literature on hemophilia A and house dust mite allergy is reviewed with a focus on the role played by glycans and glycan binding proteins as therapeutical opportunities of intervention in these immune-related conditions.

In **Chapter 2**, we generated a Siglec-7 reporter cell line to demonstrate the importance of using the right tools when studying sialic acid - Siglecs interactions. By comparing data from the reporter cell line with those from Siglec-Fc chimeras, we conclude that the two tools are complementary, and that information on Siglec-Fc binding cannot immediately be translated into functionality. With the goal to eventually develop efficient therapies targeting Siglecs, it is of fundamental importance to start from a solid foundation, where not only binding but also activation of Siglecs is studied which can have impact on the immunological outcome of the interaction.

In **Chapter 3**, we re-iterate the importance of rationally design pharmaceutical formulations to efficiently target Siglecs, in this context Siglec-3 and Siglec-9. I use FVIII as model antigen to study the induction of tolerance via Siglecs *in vitro*. I demonstrate that by conjugating FVIII immunodominant peptides to a sialylated glycan, IL-10 secretion can be achieved from monocyte derived dendritic cells in a Siglec-9 dependent way.

In **Chapter 4**, I translate the findings of chapter 3 to a diseased in vivo setting. I test the sialylated peptide formulation described in chapter 3 in a FVIII-deficient mouse model. I observe that the T reg population is maintained in the spleens of the mice treated with sialylated peptides compared with those treated with the control peptides, while suppressing the IFN-γ and TNF-α secreting CD4+ T cells, suggesting the initiation of a tolerogenic immune program.

Chapter 5 moves to allergy. It studies the immune response in peripheral blood mononuclear cells induced by house dust mite allergen chemically conjugated with the same sialylated glycans used in the previous FVIII studies. An expansion of the LAG3+ T regs subset is observed as well as a reduction in Th2 cytokines in the PBMCs stimulated with the sialylated allergen as compared with the not-sialylated control.

Chapter 6 discusses the results outlined in this thesis and puts them in relation with the current body of knowledge on glycan targeting therapies for the development of inhibitors in hemophilia A and house dust mite allergy.

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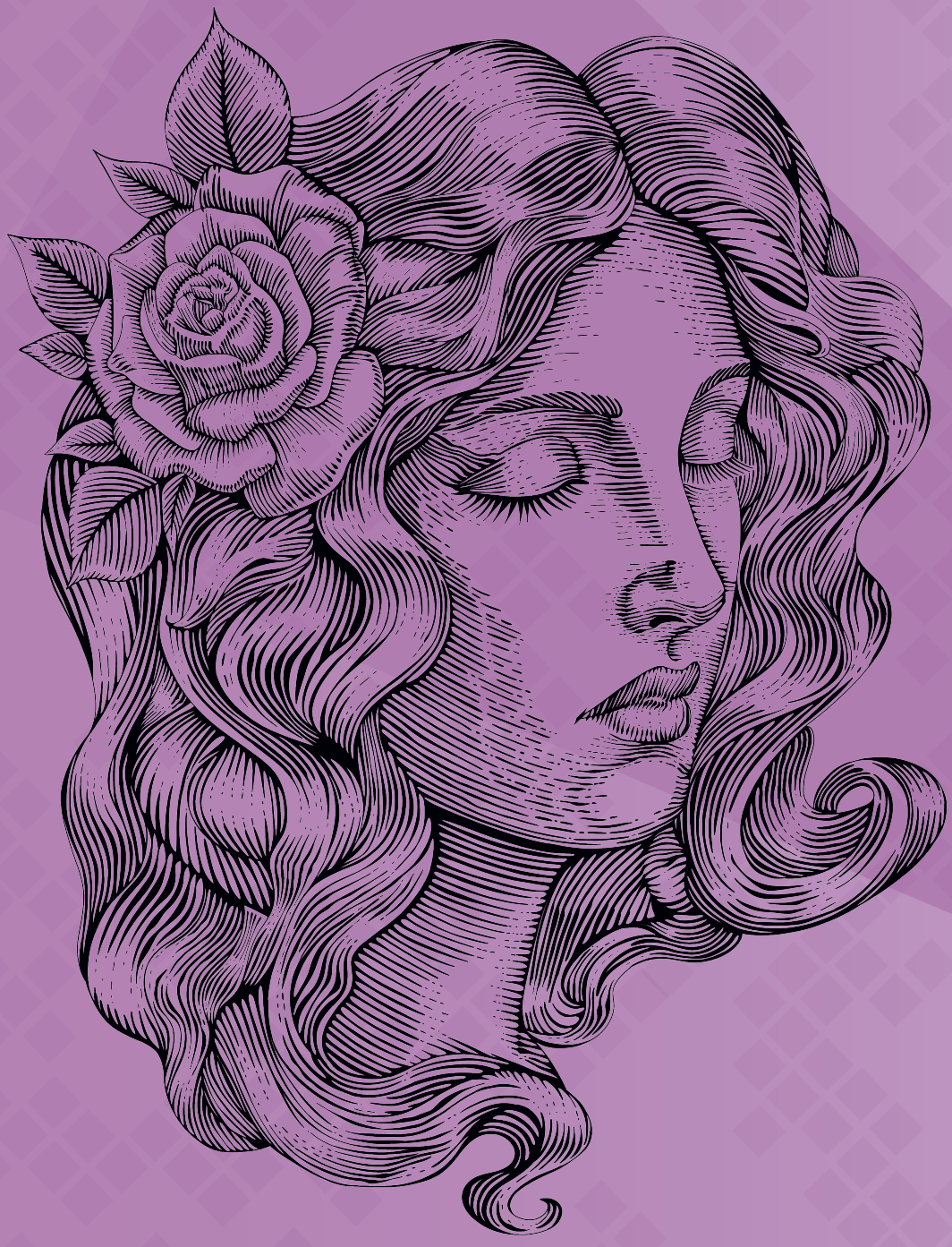
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2

BRIDGING THE GAP BETWEEN LIGAND BINDING AND SIGLEC-7 SIGNALLING WITH REPORTER CELL LINES

Manuscript submitted

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ABSTRACT

Sialylated glycans are emerging as important modulators of the immune response in malignancies, favouring immune evasion by engaging ITIM-carrying Siglec receptors on myeloid cells. However, given the complexity of the glycosylation process, studying Siglecs/ligands interactions is technically challenging. By using Siglec-7 as a model receptor, we provide evidence of how binding to sialylated glycans cannot immediately be translated into functional changes. Here, we developed a Jurkat reporter cell line to measure Siglec-7 activation, we validated it by using known Siglec-7 ligands and proved its sensitivity by testing it in co-culture with K562 cells lacking the expression of the CD43 glycoprotein that carries sialylated glycans. Our study demonstrates that widely used approaches such as Siglec-Fc chimeras may fail to accurately identify functionally relevant Siglec ligands, leading to both false negatives and false positives, as exemplified by CD43. We believe that by complementing Siglec-Fc binding studies with the functional read-out provided by our reporter system might accelerate and validate the discovery of sialylated proteins that contribute to immune resistance in the tumour microenvironment.

1. INTRODUCTION

Glycosylation is a post-translational modification in which glycans are attached to proteins, lipids, and even RNAs, thereby altering their physical properties, signaling capacity, and biological functions. Unlike protein or nucleic acid synthesis, glycosylation is a non-templated process, generating an enormous diversity of glycan structures. This complexity gives rise to the so-called “glycocode,” which is decoded by glycan-binding proteins (GBPs) (1). GBPs recognize defined glycan patterns and mediate a wide range of biological processes, including cell-cell adhesion, self / non-self-recognition, and immune regulation. Understanding the interplay between glycans and GBPs in immunity is of particular importance, as tumors frequently exploit glycosylation to evade immune surveillance (2). Among GBPs, galectins, C-type lectins, and sialic acid-binding immunoglobulin-like lectins (Siglecs) are major modulators of the immune response (3). Specifically Siglecs have emerged as potential immune checkpoints because of their structural and functional parallels with PD-1(4). Most Siglecs, similar to PD-1, carry intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which, upon engagement with sialic acid–terminated glycans, suppress immune activation (5). This has prompted significant interest in targeting Siglecs for immunotherapy (6). However, which sialoglycan carried by glycoproteins serves as immune inhibitory ligand for Siglecs in the tumor microenvironment is not fully explored. Moreover, it is now clear that low-avidity receptors such as Siglecs require multivalency of the ligand or clustering of the receptors themselves to be strongly engaged and the specific requirements of such multivalency are incompletely understood.

To identify the specificity of Siglecs for their sialoglycan and protein carrier, a number of tools have been developed, such as arrays and glycoengineered cell lines (7, 8). Since antibodies against glycans are difficult to generate due to their low immunogenicity, chimeric proteins that fuse the extracellular carbohydrate binding domain of Siglecs to the Fc portion of immunoglobulins (Siglec-Fc) are commonly employed as probes. However, Siglec-Fc chimeras have several limitations: they are unstable in solution (9), making them difficult tools to work with, exist as dimers rather than in their native receptor configuration, and, most importantly, cannot directly link ligand binding to the intracellular signaling events of Siglecs. Conventional methods for studying Siglec signaling, such as monitoring receptor phosphorylation (10), or recruitment of SHP1 (11), are informative but labor-intensive and technically demanding. Therefore, there is a clear need for a system capable of simultaneously characterizing Siglec-ligand binding while directly confirming that such interactions lead to receptor activation.

To address this gap, we set out to establish a reporter cell line that enables functional read-outs of Siglec engagement on cells. Using Siglec-7 as a model, we show that commonly used tools to measure Siglec-7 binding, such as Siglec-7Fc chimeras, fail to predict receptor signaling. Our reporter system provides a more direct and rapid mean to screen Siglec ligands, offering a platform that can accelerate the identification of novel interactions and potentially advance the development of Siglec-targeted cancer immunotherapies.

2. MATERIALS AND METHODS

2.1 Cell culture

Jurkat and K562 cell lines were acquired from ATCC and cultured in Roswell Park Memorial Institute (RPMI) (Gibco, 11875093) complete medium containing 10% fetal calf serum (FCS) (Biowest), 2 mM L-Glutamine (Gibco) and 1000 U/mL Penicillin-Streptomycin (Gibco). Jurkat parental cell lines were maintained in the same RPMI complete medium supplemented with 330 µg/mL of Hygromycin B Gold (Invivogen, ant-hg-1) while Jurkat Siglec-7 reporter cells were cultured in RPMI complete medium supplemented with 330 µg/mL of Hygromycin B Gold and 0.33 µg/mL of puromycin (Invivogen ant-pr-1). HEK293T cells were obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FCS, 2 mM L-Glutamine (Gibco) and 1000 U/mL Penicillin-Streptomycin. All cell lines were incubated in a humidified environment at 37 °C in 5% CO2 and they were routinely tested for mycoplasma contamination by PCR.

2.2 Generation of HEK293T GCNT1 KO and K562 CD43 KO cell lines

To knockout GCNT1, we used the sgRNA 5'TAGTCGTCAGGTGTCCACCG 3', previously validated by Narimastu *et al.* (12). For the knock-out of CD43 in K562 cells, the sgRNA 5'GGCTCGCTAGTAGAGACCAA3' described by Wisnovsky et al. was employed (13). The top and bottom strands of the sgRNAs were synthetized including the sticky ends 5' CACC 3' and 5' CAAA 3' respectively. After annealing and phosphorylation with T4 PNK kinase, the sgRNAs were cloned into PX459 (Addgene 62988) with BbsI-HF (New England Biolabs) and T7 DNA ligase (New England Biolabs). Non ligated and residual DNA was digested with PlasmidSafe exonuclease (Epicentre). The plasmids were then amplified in XL-1 Blue Sublconing-Grade competent bacteria (Stratagene) and purified using

the Nucleobond Xtra Midi kit (Macherey-Nagel). The plasmids' sequences were checked by Sanger sequencing and then stored at –20 °C until further use. PX459 plasmids without any gRNA were used to generate the mock cell lines. To obtain HEK293T mock and GCNT1 KO, 0.1 x 10^6 cells were plated on a 12-well plate and transfected the day after with 1 µg of relevant plasmids pre-complexed with lipofectamine in PLUS reagent (Invitrogen). The following day the medium was changed and the selection with 0.1 µg/mL of puromycin was started. After one week, the alive 5% positive population for Siglec-7Fc (R&D Systems) staining in GCNT1 KO cells was sorted at the BD FACS Aria Fusion sorter and the procedure was repeated a second time to maximize the percentage of cells presenting with Siglec-7 ligands. As for the mock cell line, all the cells were sorted on the alive population. The CD43 KO and mock cell lines were obtained by nucleofecting 1 x 10^6 K562 cells with the relevant plasmids using the 4D- nucleofector and the SF Cell Line Nucleofector Kit (Lonza) according to the manufacturer's instructions. Selection with 1 µg/mL of puromycin was started one day after nucleofection and after one week the negative population for CD43 staining, performed with the APC anti-human CD43 antibody (Biolegend, clone CD43-10G7), was sorted at the BD FACS Aria Fusion sorter. The mock cell line was sorted without any gating applied.

2.3 Generation of parental and Siglec-7 Jurkat reporter cell lines

To generate the parental Jurkat cell line, the cells were electroporated with pGL4.30[luc2P/NFAT-RE/Hygro] (Promega) by using the SE Cell Line 4D-Nucleofector® X Kit S (Lonza, V4XC-1032) and following the manufacturer's instructions. Once recovered, the cells were then put in selection RPMI medium with 330 µg/mL of Hygromycin B Gold and tested for luciferase activity with PMA/ionomycin. The parental cell line was subsequently genetically modified to generate the Siglec-7 reporter cell line. A gene fragment was designed to contain the extracellular and transmembrane domains of Siglec-7 in frame with the ITAMs 1, 2 and 3 of CD3ξ and delivered to the nucleus by nucleofection as described in the previous paragraph. After 1 week of selection with 0.33 µg/mL of puromycin, the cells were bulk sorted at the BD FACS Aria Fusion and the expression of Siglec-7 was routinely checked at LSR FortessaX-20 (BD Bioscience).

2.4 Luciferase assays

To prepare the Jurkat reporter cells for the luciferase assay, they were washed once in PBS and resuspended in phenol red-free, serum free AIM-V medium (Gibco) at a concentration of 1 x 10^6 cells/mL. Flat bottom 96-well plates were previously coated at 4°C overnight with 10 µg/mL of anti-CD3 (Biolegend, clone OKT3), anti-Siglec-7 (Biolegend, clone S7.7), isotype matched mIgG1 controls (Thermofisher, clone eBM2a and Biolegend, clone MG1-45 respectively) or 2 µM of glycodendrimers. The dendrimers were generated as previously reported (2) by conjugating the PAMAM cystamine core to the following glycans: α2.3 = LS-Tetrasaccharide (LSTd, Neu5Acα2-3Galβ1-4GlcNAcβ1-3Gal, Elicityl), α2.8 = Disialylated pentaose type 1 (Neu5Acα2-8Neu5Acα2-3Galβ1-3GlcNAcβ1-3Gal, Elicityl), α2.3x = sialylated tetraose type 1 (Neu5Acα2-3Galβ1-3GlcNAcβ1-3Gal, Elicityl), LeY = Lewis y pentaose (Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal, Elicityl). The plates were washed twice with PBS before addition of 25 µL of serum free AIM-V medium and 25 µL of Jurkat Siglec-7 reporter cells and incubated overnight at 37°C in 5% CO_2. To assess Siglec-7 signalling upon binding of membrane-bound ligands, co-cultures of the Jurkat reporter cells with HEK293T mock, HEK293T GCNT1 KO, K562 mock and K562 CD43 KO cells were established at different Jurkat : cell lines ratios as explicated in each graph. Luciferase activity was assessed using the Bright-Glo™ Luciferase Assay System (Promega, E2620) according to the manufacturer's protocol using a plate reader (Mithras LB 940). Data were analyzed by diving the measurements per the corresponding medium-only background values and plotted as fold change.

2.5 Siglec-7Fc ELISA

MaxiSorp ELISA plates were coated overnight at RT with 5 µM of glycodendrimers diluted in 50 µL of PBS. Then, the plates were washed twice with HBSS (Invivogen) and blocked with carbo-free blocking buffer (Vector, SP5040) 30 minutes at 37°C. Siglec-7Fc was used at a concentration of 3.3 µg/mL and incubated on a shaking rotor for 1h at RT. After washing 3 times with HBSS, the plates were incubated with goat anti-human Fc-PO antibody (Jackson, 109-036-098) and the binding was visualized using TMB followed by measurement at 450 nm.

2.6 Flow cytometry

For flow cytometry staining, 0.1 x 10^6 cells per well were washed twice with PBA (PBS + 0.5% BSA (Roche) + 0.02% sodium azide (Sigma)) and stained for 15 minutes on ice with the fixable viability die eFluor780 (eBioscience 65-0865-18) diluted 1:1000 in PBS. Next, the cells were washed twice with PBA and stained with the antibody mixes prepared in PBA. The following antibodies were used in the study: Siglec-7 PerCP Vio700 (Miltenyi Biotec, clone REA214), CD43-APC (Biolegend, clone CD43-10G7). Alternatively, the cells were stained with 1 µg/mL of Siglec-7Fc (R&D Systems) pre-complexed 1:400 for 30 minutes at RT with anti-human IgG Fc APC (Biolegend, clone M1310G05) in HBSS + 0.5% BSA or with SiaFind™ Pan-Specific Lectenz® Biotinylated Kit (LectENZ Bio) pre-complexed with streptavidin-AF647. After staining, the cells were washed twice in PBA, fixed in 1% PFA diluted in PBS for 15 minutes on ice, washed to remove the excess PFA and acquired at the LSR Fortessa X-20 (BD Biosciences). Where indicated, the cells were incubated for 30 mins at 37^{0}C with 1:200 of Neuraminidase (Sialidase) from Arthrobacter ureafaciens in PBS or with 200 mM sialylainhibitor for 3 days in the cell culture, to remove sialic acids on the cellular surface and confirm sialic-acid dependency of Siglec-7Fc binding.

3. RESULTS

3.1 Development of a Siglec-7 reporter cell line

To study Siglec-7 activation, we developed a Siglec-7 reporter Jurkat cell line. The intracellular tail of the wild type Siglec-7 protein, including the ITIM and ITIM/like motifs, was exchanged for that of CD3ξ, which carries three ITAMs. The construct was stably expressed into Jurkat cells previously engineered to carry the luciferase gene under the control of an NFAT response element (thereafter called parental cell line) **(Figure 1a-c)**. The functionality of the Siglec-7 and parental reporter cell lines was controlled by stimulating the cells overnight with plate-bound anti-CD3 antibodies. Triggering of CD3 induced the translocation of NFAT into the nucleus and consequent transcription of Luc, whose activity was measured by luminescence **(Figure 1a-c)**. No differences between the Siglec-7 reporter and parental cell lines could be detected in response to anti-CD3, confirming comparable activation of both the cell lines in response to ITAM-triggering signals independent of chimeric-Siglec-7 signalling **(Figure 1d)**. Chimeric Siglec-7 activation was confirmed by stimulating the cells overnight with plate-bound anti-Siglec-7 antibodies. As shown in Figure 1e, luminescence is only measured in the Siglec-7 reporter cell lines, indicating selective triggering of the chimeric Siglec-7 protein.

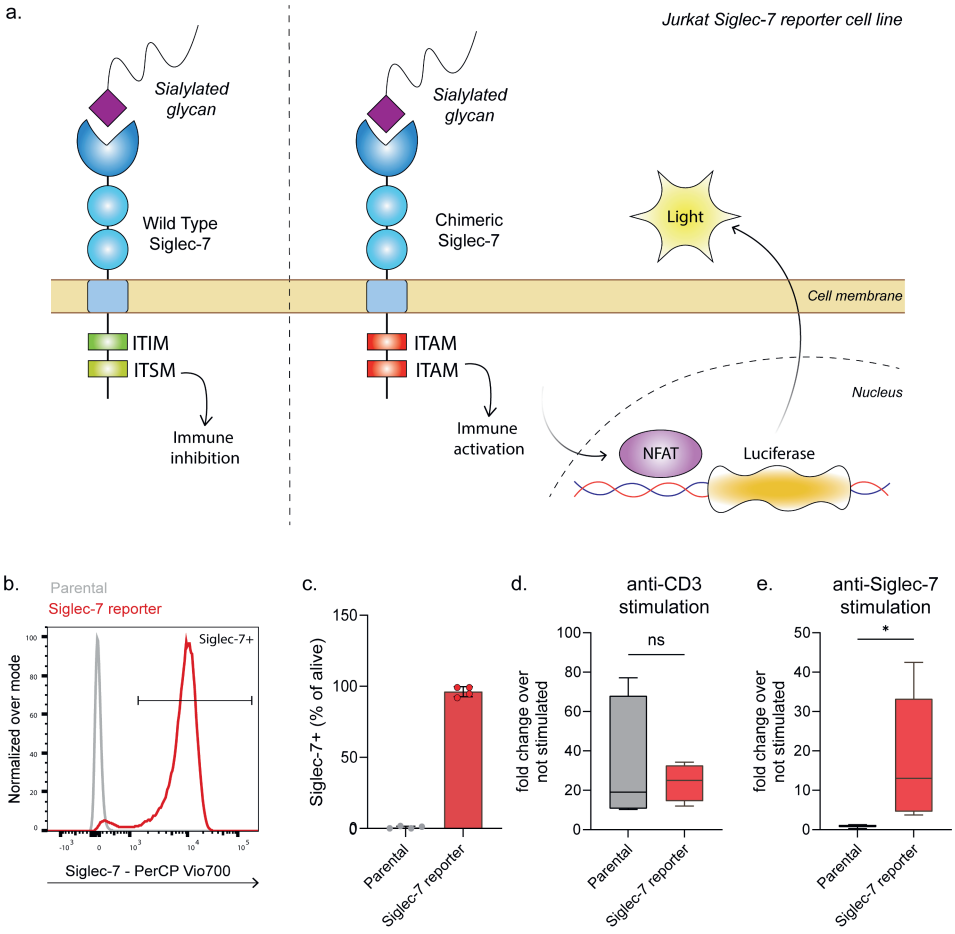


Figure 1: Development of a Siglec-7 reporter cell line. A. *To generate the Siglec-7 reporter cell line, the intracellular signalling ITIM and ITIM-like motifs of the native protein were substituted for the ITAMs of CD3. The chimeric receptor was then cloned into a Jurkat cell line previously engineered to bear the luciferase gene under the control of a NFAT response element (parental cell line). Siglec-7 engagement can be measured by luminescence.* ***B.*** *Flow cytometry staining of Siglec-7 on the surface of Siglec-7 reporter and parental cell lines. Representative histogram of 4 independent experiments.* ***C.*** *Quantification of the percentages of Siglec-7 positive cells, the gating is shown in b. Mean ± SD of 4 independent experiments performed in duplicate.* ***D.*** *and* ***E.*** *Siglec-7 reporter or parental cells were stimulated overnight with 10 µg/mL of anti-CD3 (d.) or anti-Siglec-7 (e.) antibodies previously coated in a 96-well plate. Luminescence was read the day after and the data are plotted as fold change over the not-stimulated control. Mean ± SD of 4 independent experiments performed in duplicate. Statistics: unpaired t test, ns = not significant, * = $p < 0.05$.*

3.2 Siglec-7 reporter cell line identifies α2.3 and α2.8 sialylated dendrimers as ligands for Siglec-7, which are missed by Siglec-7Fc ELISA

We next employed our Siglec-7 reporter cell line to screen for novel Siglec-7 ligands. For this purpose, we made use of PAMAM dendrimers chemically conjugated in-house to various sialylated glycans **(Figure 2a)**. We included the non-sialylated Lewis Y (LeY) glycans as a negative control. By coating the dendrimers on an ELISA plate and detecting them with the Siglec-7Fc chimeras, only the α2.3x dendrimer showed binding **(Figure 2b)**. On the contrary, all the sialylated dendrimers induced luminescence in the Siglec-7 reporter cell line **(Figure 2c)**, whereas the non-sialylated LeY, as expected, did not. Of note, none of the dendrimers induced signalling when added in soluble form to the reporter cell line **(Supp. Fig. 1)**. This indicates that both surface expression of Siglec-7 and plate-bound presentation of the ligands are required to influence binding specificity and signalling.

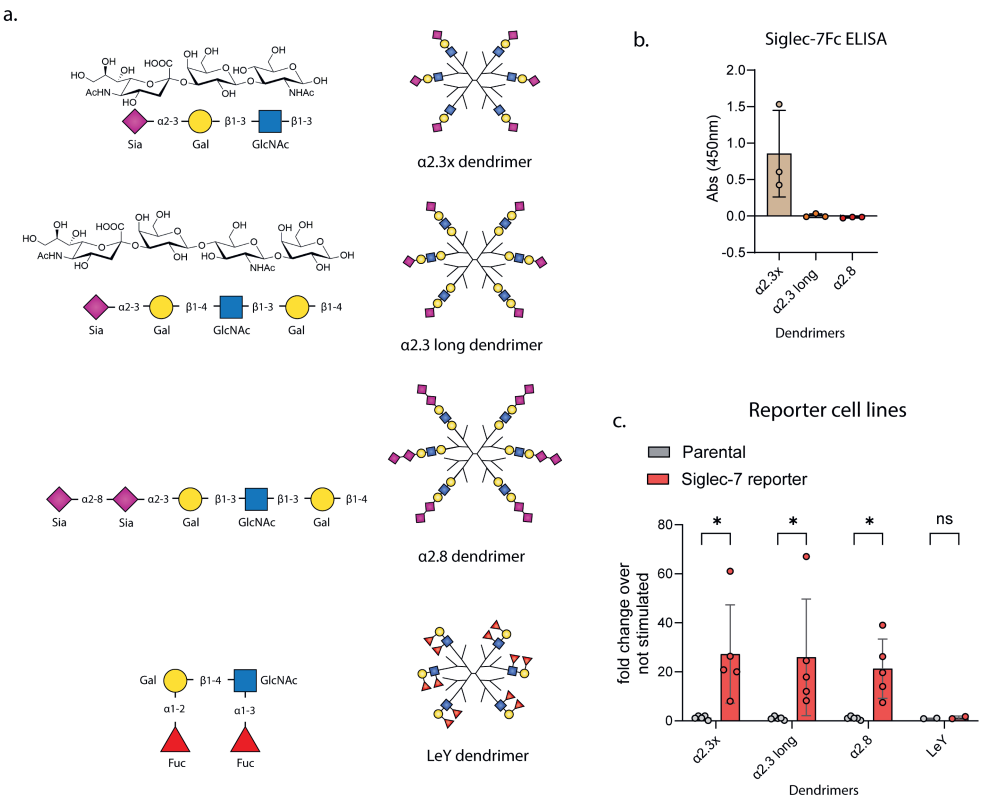


Figure 2: Siglec-7 reporter cell line identifies α2.3 and α2.8 terminating glycans as a ligands for Siglec-7, which are missed by Siglec-7Fc ELISA. A. *Schematic of the dendrimers used in the study. Structures are drawn using ChemDraw, using the chair representation of monosaccharides. The glycans are also shown in SFNG nomenclature.* ***B.*** *Siglec-7Fc binding to the dendrimers detected by ELISA with anti-IgG-PO. Each dot represents an independent experiment performed in duplicate. Data are presented as mean ± SD.* ***C.*** *Parental and Siglec-7 reporter cells were stimulated overnight with 2 μM of coated dendrimers. Luminescence was read the day after and plotted as fold change over the not stimulated controls. Each dot represents the mean of an independent experiment performed in duplicate. Data are presented as mean ± SD of 2 to 5 independent experiments. Statistics: multiple unpaired t tests. * = $p < 0.05$; ns = not significant.*

3.3 Siglec-7 ligands on the cell surface of HEK293T cell lines fail to induce signaling in the Siglec-7 reporter cell line, despite positive binding to Siglec-7Fc

Moving onto a more physiologically relevant context, we investigated Siglec-7 binding and triggering induced by ligands presented on the cellular membrane, which mimics the situation in vivo where immune cells encounter target cells. As a model, we generated HEK293T cell lines lacking glucosaminyl (N-acetyl) transferase 1 (GCNT1), since it has been previously reported that knock-out of this enzyme increases the concentration of Siglec-7 ligands on the cellular surface (8). In the ER, GCNT1 competes with ST3Gal-I for core 1 O-glycans (T antigen) as a substrate. In the absence of GCNT1, T antigens within the cell feed the pathway initiated by ST3Gal-I, which catalyzes the addition of an α2,3-linked sialic acid to the β1,3-linked galactose **(Figure 3a)**. Ultimately, increased levels of disialyl T antigen are generated, which is a known ligand of Siglec-7 (14). When stained with Siglec-7Fc, GCNT1 KO cells doubled the presence of Siglec-7 ligands compared to the mock cell line **(Figure 3b-c)**. The dependency of the binding to sialic acids was confirmed by complete abrogation of the signal when mock and GCNT1 KO cells were pre-treated with neuraminidase **(Figure 3c)**. However, when the Siglec-7 reporter cell line was tested in co-culture with the HEK293T cell lines, no signal was detected in all the cell ratios tested **(Figure 2d)**. This indicates that despite ~ 10% of the cells display Siglec-7 ligands on the cell membrane, as detected by Siglec-7Fc, it might be too low to induce signaling, or HEK293T cells are missing the correct scaffold to present multivalent sialoglycan ligands to Siglec-7 to allow signaling.

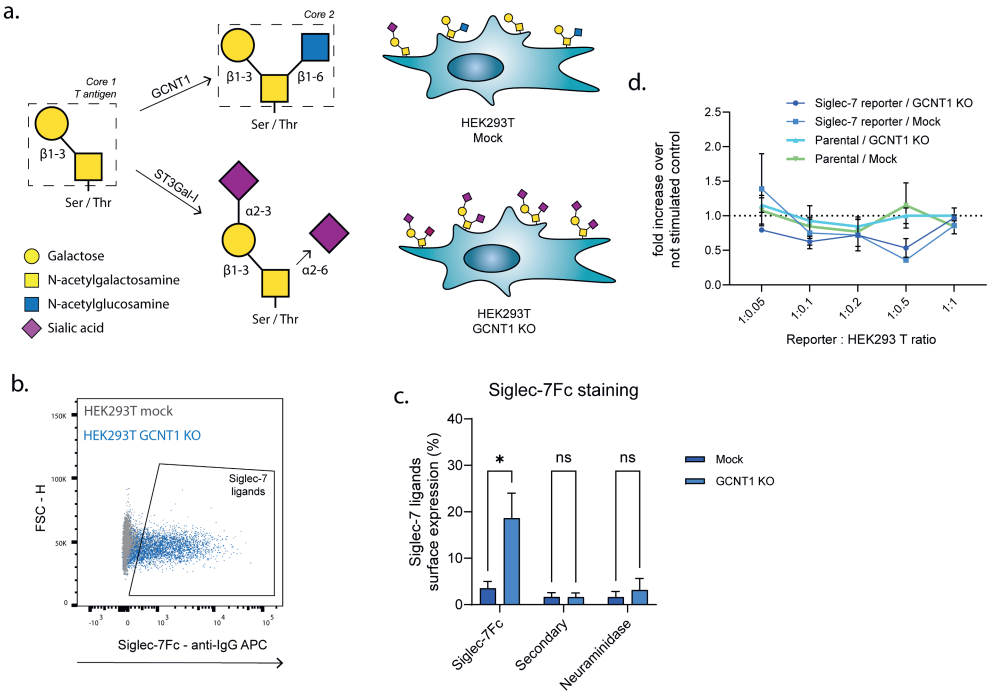


Figure 3: Siglec-7 ligands presented on the cell surface of HEK293T cell lines fail to induce signaling in the Siglec-7 reporter cell line, despite positive binding to Siglec-7Fc. ***A.*** *Enzymatic reaction catalyzed by GCNT1. C2GlcNAcT-I and ST3Gal-I compete for the core 1 (T antigen) as a substrate. ST3Gal-I activity catalyzes the addition of an α2,3-linked sialic acid to the β1,3-linked galactose.* ***B.*** *Representative dot plot showing the final gate used to identify Siglec-7 ligands positive cells in the mock or GCNT1 KO cell lines. The cells were stained for flow cytometry analysis with Siglec-7Fc pre-complexed with anti-IgG-APC.* ***C.*** *Percentage of mock or GCNT1 KO cells positive for Siglec-7 sialoglycan ligands, quantification of b. Data are shown as mean ± SD of 4 independent experiments performed in duplicate. Statistics: multiple unpaired t tests. * = $p < 0.05$; ns = not significant.* ***D.*** *Jurkat parental or Siglec-7 reporter cells were co-cultured overnight with HEK293T mock or GCNT1 KO cells in different ratios, indicated in the x axis. The next morning, luminescence was measured and plotted as fold change over the not stimulated control. Representative of 2 independent experiments. Data are shown as mean ± SD of 2 duplicates.*

3.4 K562 cells carry high concentrations of Siglec-7 ligands and are able to trigger its signaling, partially independently of CD43.

Given that HEK293T is an epithelial, non-transformed cell line and that strong engagers of Siglec-7 are cancer cells, we investigated whether this specificity could be reproduced in vitro by using the lymphoblastoid, tumor-derived K562 cell line. As shown in Figure 4a-b, K562 cells express high concentrations of Siglec-7 ligands, close to 100%, as identified by Siglec-7Fc stainings of the cellular surface. This high concentration of Siglec-7 ligands is also able to strongly induce signaling in the Siglec-7 reporter cell line in a cell-number dependent manner **(Figure 4b)** and sialic acid-dependent manner, as shown by the abrogation of the signalling in the reporter cell line when the K562 cells are cultured in the presence of sialylinhibitor **(Supp. Figure 2a,c)**. It is known that the carrier of glycans matters, providing multivalency of sialoglycans for efficient binding and signaling Siglecs (15). While some lectins show specificity for glycolipids, others are selectively engaged by glycans carried by a specific protein. It was recently described that Siglec-7 interacted with disialyl-T antigen on CD43, with evidence showing strong engagement of Siglec-7 (13, 16). We therefore knocked-out CD43 on K562 cells and observed a reduction in Siglec-7Fc staining **(Figure 4d)**, as previously reported (13). However, we observed no difference in signalling when the Siglec-7 reporter cell line are cultured with the mock or the GCNT1 KO K562 cells **(Figure 4e)**, indicating that CD43 might not be crucial for Siglec-7 signaling, with the presence of other carriers (proteins or lipidic) that play more important roles.

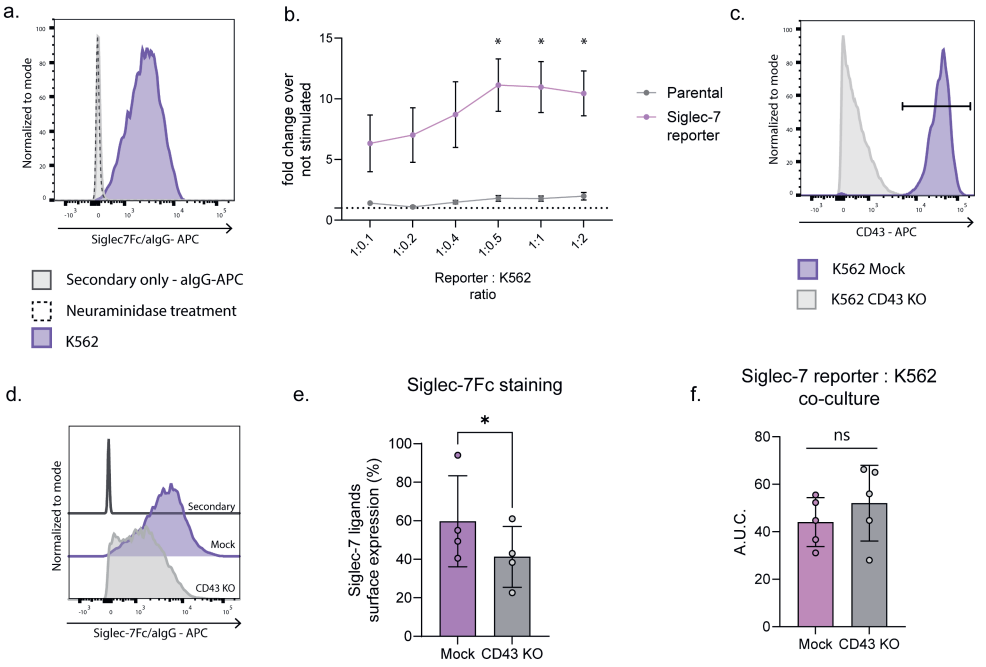


Figure 4. K562 cells carry high concentrations of Siglec-7 ligands, which are not limited to CD43. ***A.*** *Flow cytometry staining of K562 cells with Siglec7Fc pre-complexed with anti-IgG-APC. As control for sialic-acid dependency of Siglec-7Fc binding, part of the sample was pre-treated with neuraminidase. Representative histogram of 3 independent experiments.* ***B.*** *Jurkat parental or Siglec-7 reporter cells were co-cultured overnight with K562 cells in different ratios, indicated in the x axis. The next morning, luminescence was measured and plotted as fold change over the not stimulated control. Mean ± SEM of 4 independent experiments each performed in duplicate. Statistics: Unpaired t tests with Holm-Šídák correction method for multiple comparisons. * = p < 0.005. C. K562 cells were genetically engineered to knock-out CD43 with CRISPR/Cas9 technology. Flow cytometry analysis of CD43 in mock and CD43 KO cells. Representative histograms of 3 independent experiments. D. Flow cytometry staining of K562 mock and CD43 KO cells with Siglec7Fc pre-complexed with anti-IgG-APC. Representative histogram of 3 independent experiment. E. Percentage of K562 mock or CD43 KO positive to Siglec-7Fc staining. Mean ± SD of 4 independent experiments performed in duplicates. Statistics: paired t tests. * = p < 0.05; F. Jurkat parental or Siglec-7 reporter cells were co-cultured overnight with K562 mock cells of CD43 KO in different ratios, as in B. The next morning, luminescence was measured and plotted as area under the curve (A.U.C.). Mean ± SD of 5 independent experiments performed in duplicates. Statistics: paired t test, ns = not significant.*

4. DISCUSSION

Despite the existence of a number of tools to investigate the ligand specificities of GBPs, the field is still missing a way to confidently connect binding to activation. In this study, we addressed this gap by generating a reporter cell line for Siglec-7. Building up on a previously developed reporter cell line (17), we improved this by conserving the transmembrane domain of Siglec-7. Although no studies have ultimately proved for Siglec-7 that the transmembrane domain is crucial for their suppressive function, based on the functional similarities with PD-1 it could be postulated that the transmembrane domain is crucial for their suppressive function (18). Moreover, our system is developed to be easily engineered to remove the extracellular part of Siglec-7 and substitute it for any other Type I or Type II GBP, making it a highly versatile tool to study lectins triggering.

We first validated the functionality of the reporter system using anti-CD3 stimulation **(Figure 1d)**. Despite considerable variability across experiments, no statistically significant difference in activation was observed between the parental and Siglec-7 reporter cell lines, supporting the use of the parental line as an appropriate negative control. The high standard deviation likely reflects heterogeneity within the reporter population, which resulted from bulk rather than clonal sorting, and could be minimized by generating single-cell–derived clones. In contrast, stimulation with an anti-Siglec-7 antibody induced a strong reporter response, with a fold increase exceeding 10, indicating a robust dynamic range and suggesting high assay sensitivity **(Figure 1e)**.

By employing our Siglec-7 reporter cell line, we found that α2,3- and α2,8-sialic acid–terminated glycans displayed on PAMAM dendrimer scaffolds can activate the receptor when coated on a plate, whereas no binding was detected using the Siglec-7Fc probe **(Figure 2b-c)**. Our results are confirmed by a previous study in our group, which measured Siglec-7 phosphorylation following stimulation of monocytes with the α2,3 dendrimer (2). These findings reveal the ability of the reporter system to detect functional Siglec-7 engagement that may be missed by conventional binding assays. Siglec-7 was originally identified as a novel member of the family of Siglecs binding to α2.3- and α2.6-sialylated glycans carried by a polyacrylamide polymer (PAA) (19), and later also shown to recognize α2,8-linked sialoglycans (20, 21). ST3Gal1, the enzyme responsible for synthesizing α2,3-linked sialic acid termini, has been implicated in generating Siglec-7 ligands (22). Our observation that α2,3- and α2,8-sialylated dendrimers both bind and activate Siglec-7 aligns with these studies. On the other hand, our data show that α2.3x-dendrimers both bind and trigger Siglec-7. Out of the structures tested, this

sialoglycan resembles the most that of the putative endogenous ligand of Siglec-7, the disialylted Core 1 O-glycan, differing only by the absence of a second Neu5Ac residue on the GlcNAc. Although most studies report that Siglec-7 preferentially recognizes disialylated O-glycans, our data indicate that even monosialylated glycans can trigger receptor activation when presented in a multivalent context. This suggests that the spatial organization or density of sialic acids, rather than strict disialylation, may suffice for receptor engagement. Our work further demonstrates the potential of using a reporter system to identify novel Siglec ligands. This approach could be used to screen a broader repertoire of sialylated structures with potential immune modulatory properties. In particular, sulfated sialoglycans represent an intriguing class of potential ligands. Previous work employing glycan microarrays of chemically synthesized sulfated sialoglycans revealed that sulfation enhances Siglecs binding, with the combination of α2,8-linked sialylation and 6-O-sulfation of galactose exerting a synergistic effect on Siglec-7 recognition (23). Extending our analysis to include such sulfated sialylated glycans would help to determine whether sulfation not only promotes Siglec-7 binding but also translates into functional receptor activation as detected by the reporter cell line.

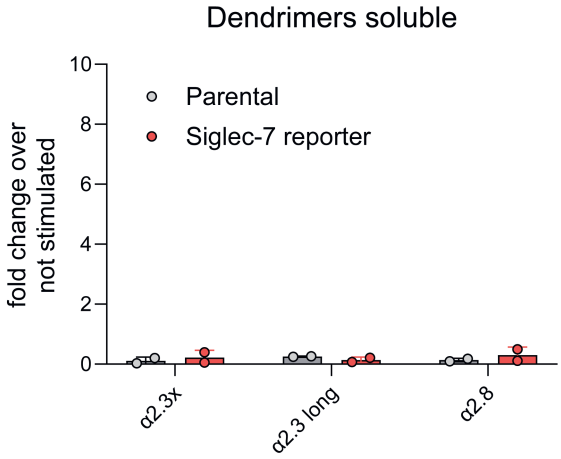
We next assessed whether the Siglec-7 reporter cell line could detect receptor engagement when ligands are presented in their native context of the cell surface **(Figure 3a)**. HEK293 cells have previously been used to dissect the glycosyltransferases required for the generation of Siglec-7 ligands. Consistent with the findings of Bull *et al.* (8), we observed an increased Siglec-7Fc binding signal in GCNT1 KO cells **(Figure 3b-c)**. GCNT1 has been identified as a master negative regulator of the synthesis of Siglec-7 ligands, both in immune subsets (15), and in leukemia (24). However, despite this enhanced Siglec-7Fc binding, the glycoengineered HEK293T cells failed to activate the Siglec-7 reporter cell line **(Figure 3d)**. This observation is in agreement with van Houtum *et al.* (17), who reported a similar lack of Siglec-7 activation with HEK293 cells with their reporter system. This discrepancy between binding and signaling is perhaps not unexpected. Increasing evidence indicates that Siglecs recognition depends not only the linkage between the sialic acid and the underlying glycan, but also on the higher-order presentation of sialylated motifs, such as their density, multivalency, and organization on the carrier molecule. The absence of activation in HEK293T cells may therefore reflect the lack of appropriate glycoprotein scaffolds rather than the absence of suitable glycans. Indeed, HEK293 cells do not express the mucin-like proteins that have been identified as major carriers of Siglec-7 ligands, including PDPN (25), CD45 (24), and CD162/PSGL-1 (26).

Unlike HEK293T cells, K562 cells display nearly complete surface staining with Siglec-7Fc chimeras **(Figure 4a, d-e)**. While this strong binding correlates with activation of the Siglec-7 reporter, our data demonstrate that this functional engagement is independent of CD43 **(Figure 4b, f)**. Although CD43 knockout reduces Siglec-7Fc binding **(Figure 4e)**, it does not impair receptor activation, indicating that CD43-associated sialoglycans are insufficient to trigger Siglec-7 signaling. These findings highlight a critical distinction between ligand binding and receptor activation, and challenge the prevailing assumption that CD43 functions as a *bona fide* Siglec-7 ligand. Instead, our results suggest that other sialoglycan structures expressed by K562 cells are responsible for productive Siglec-7 engagement. This interpretation is supported by recent work showing that disruption of the CD43-Siglec-7 axis does not alter NK cell cytotoxicity, arguing against a functional role for this interaction despite detectable biochemical binding (27). Collectively, our data provide direct functional evidence that CD43 is dispensable for Siglec-7 signaling and emphasize the need to reassess previously identified Siglec ligands in a functional context.

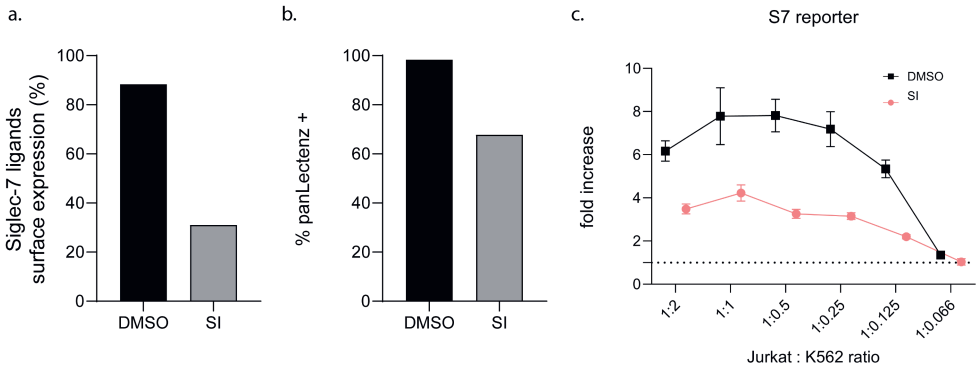
Discrepancies between Siglec-7 binding and its functional outcomes have been noted previously. More than a decade ago, Hudak *et al.* (28) reported that incorporation of GD3 into target cell membranes did not further inhibit NK cell cytotoxicity compared with other sialylated polymers, despite exhibiting higher Siglec-7Fc binding. Similarly, inconsistencies between Siglec-9 binding and downstream signaling have also been observed (29), underscoring the importance of studying receptor activation directly rather than inferring functionality from binding alone.

Given that glycans are emerging as key mediators of immune suppression during malignant transformation (2, 30), it is crucial to develop technologies capable of distinguishing between binding and functionally relevant interactions. The reporter system described in this study offers a complementary approach to conventional binding assays, enabling the identification of physiologically active Siglec-7 ligands with potential relevance for immunotherapy development.

Supplementary figures



Supplementary Figure 1. α2.3, α2.3x and α2.8 terminating glycans fail to trigger the Siglec-7 reporter cell line when presented as soluble ligands. *Parental and Siglec-7 reporter cells were stimulated overnight with 2 μM of soluble dendrimers. Luminescence was read the day after and plotted as fold change over the not stimulated controls. Each dot represents the mean of an independent experiment performed in duplicate. Data are presented as mean ± SD of two independent experiments.*



Supplementary Figure 2. A reduction in surface sialic acids abrogates the signalling in the Siglec-7 reporter cell line. A. *and* ***B.*** *K562 cells were cultured for 3 days in the presence of a sialylinhibitor (SI) or a corresponding concentration of DMSO. Flow cytometry staining with Siglec-7Fc precomplexed with anti-IgG-APC (a.) or PanLectENZ (b). C. K562 cells cultured with SI or control DMSO were co-cultured overnight at different ratios, indicated on the x axis, with the Jurkat Siglec-7 reporter cell lines. The next morning, luminescence was measured and plotted as fold change over the not stimulated control. Representative of 3 independent experiments. Mean ± SEM of technical duplicates.*

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3

RATIONAL DESIGN OF FVIII SIALYLATED PEPTIDES TO TARGET SIGLEC-3 AND SIGLEC-9 AND IMPROVE PEPTIDE FORMULATIONS FOR REVERSE VACCINES

Frontiers in Bioengineering and Biotechnology, 2025

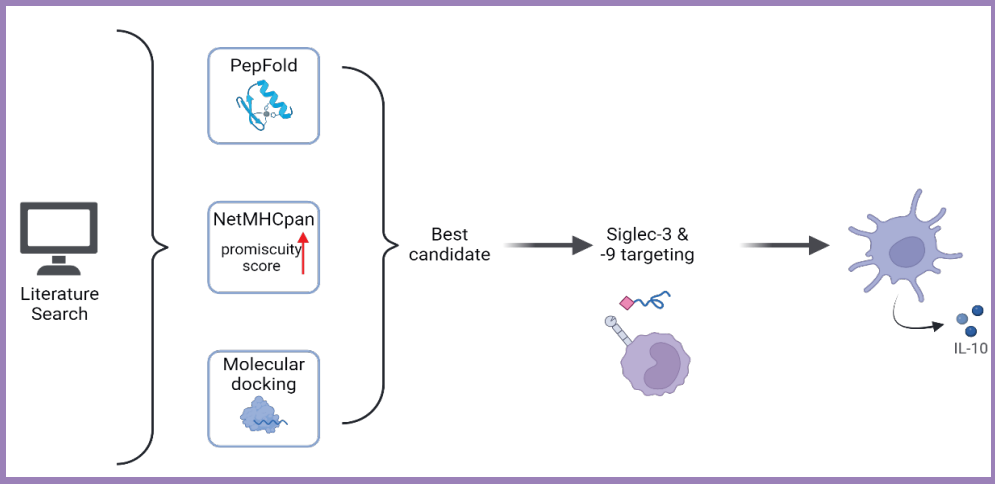
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ABSTRACT

Reverse vaccine formulations have shown their potential for the treatment of allergies and other autoimmune diseases by the design of antigens that modify dendritic cell (DC) function towards tolerogenic responses. We here demonstrate that modification of an immunodominant peptide from factor VIII (FVIII) with a tolerizing molecule, sialic acid, improves existing peptide formulations towards the induction of tolerogenic cytokine secretion by DCs. Sialic acids are the end-standing moiety of mammalian N- and O- glycans, which are naturally recognized as self-associated molecular pattern. In this paper we show that sialic acid modified FVIII peptides target Siglec-3 and -9 on DCs and increase IL-10 secretion. Our work proposes a method to select, synthetize and test sialylated immunodominant peptides with the aim of ameliorating the efficacy of peptide immunotherapy. Based on our results, we propose that the sialylated FVIII peptide designed in this study may be useful for re-establishing tolerance to FVIII in hemophilia A patients who developed neutralizing antibodies following treatment.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Peptides are used in reverse vaccination as an alternative to whole-antigen formulations to achieve tolerance in allergies and autoimmune diseases, as demonstrated by the ongoing clinical trials in type 1 diabetes (1), Graves' (2) and MS (3). The use of immunodominant sequences allows for the reduction of costs and risks associated with infusing the full antigens, while still maintaining control over the amounts injected. Recently, peptide immunotherapy has been tested in preclinical models of hemophilia A (HA) (4), which is an X-linked genetic disorder caused by mutations of coagulation factor VIII (FVIII) (5). Although the quality of life of these patients significantly improved in the last decades, up to 30% of them still develop neutralizing antibodies to FVIII, called inhibitors, hampering the success of the treatment. Currently, the only available option to eradicate the inhibitors is Immune Tolerance Induction (ITI), which consists of repeated infusions of FVIII at high doses for a prolonged period of time. ITI poses a high burden on the patients and the healthcare system, and success rate varies between 60% and 90% (6). Better alternatives to promote tolerance to FVIII are therefore needed, aiming at 100% eradication of the inhibitors while using limited amount of antigen and fewer injections. A recently published approach which employs FVIII peptides has proved promising in an experimental model of HA (4). The strategy from Pletinckx *et al.* is based on the use of antigen processing-independent T cell epitopes (apitopes) (4). Strict chemical requirements allow apitopes to directly bind MHC-II on steady state APCs in lymphoid organs. In the absence of concomitant danger signals, these immature APCs presents antigens to T cells in a tolerogenic way, skewing them to type 1 regulatory T cells (Tr1s). Tr1s express inhibitory receptors (CTLA4, TIM3, LAG3, TIGIT) and mostly secrete IL-10, creating a microenvironment that suppresses the expression of co-stimulatory molecules on neighboring APCs and promotes the generation of IL-10-secreting B_{regs} and myeloid-derived suppressor cells (4). However, complete eradication of the inhibitors was not achieved with apitopes in the preclinical models and the protocol required frequent injections, which will likely affect patients' compliance. To improve the efficacy of such peptide formulations, we propose to modify FVIII peptides by chemically conjugating them to a tolerogenic signal, sialic acid (sialylation). Sialic acids are monosaccharides capping the majority of mammalian glycans (7). Sialylated glycans are the ligands for immunosuppressive receptors called Sialic-acid-binding immunoglobulin-like lectins (Siglecs), broadly expressed by immune cells (8). The majority of Siglecs signals through an Immunoreceptor tyrosine-based inhibitory motif (ITIM) (9). Upon binding to sialylated ligands, phosphorylation of the ITIM by adaptor proteins, mainly SHP1 and 2, initiates a cascade that culminates in inhibition of

NFkB translocation in the nucleus, resulting in immune suppression (9). The family of Siglecs comprise fifteen lectins, which differ in their preference of binding to specific sialoglycans and their pattern of expression in immune subsets (9). Targeting Siglec-9 with sialylated dendrimers induced a tolerogenic profile in monocyte derived dendritic cells (moDCs) *in vitro* (10). Moreover, in the context of allergies, sialylation of the house dust mite allergen reduced the detrimental secretion of Th2 cytokines from PBMCs (11) and treatment with sialylated peptides ameliorated the symptoms of grass pollen allergy *in vivo* (12). In this study, we present an improvement of sialylated peptide formulations by rational design of the peptide sequence, in order to target the largest portion of population, and of the sialylated glycan to improve targeting to Siglec-9. We demonstrate that our implemented peptide formulation binds both to Siglec-3 and -9. We further show that stimulation of monocyte-derived DCs (moDCs) with sialylated peptides induces IL-10 secretion in a Siglec-3 and Siglec-9 dependent manner. IL-10 was shown to be a key signal in the response to FVIII to restore and maintain tolerance (13), suggesting that our FVIII peptide formulation might prove useful in the treatment of HA patients with inhibitors.

2. METHODS

2.1 Peptide synthesis and conjugation to the sialylated glycan and to TAMRA

FVIII derived peptide (KHNIFNPPIIARYIRLHPTHYSIRST; residues 2155-2180) was synthesized by solid-phase using Fmoc chemistry on a CEM Liberty Blue peptide synthesizer and conjugated to Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc (LSTd) and 5(6)-Carboxytetramethylrhodamine (TAMRA, Sigma). The detailed method is described in the supplementary materials.

2.2 In silico analyses

Predictions of the binding affinities to various HLA-DRB alleles were computed using the publicly available software NetPanMHCII4.0 (14), while PEP-FOLD$_3$ was used to predict the secondary structures (15).

2.2.1 Ligand preparation for computational analysis. The 3D coordinates for Sia were generated using the GLYCAM database. Subsequently, the ligand geometries underwent optimization through molecular dynamics (MD) simulations. Siglec-9 model was built using AlphaFold (16). An AlphaFold homology model

was used to run MD simulations. MD simulations were carried out with AMBER18 package implemented with the ff14SB for proteins. As for natural glycan moieties, the GLYCAM06j-1 forcefield was used.

2.2.2 Docking calculations. Docking calculations of the glycan were performed using AutoDock 4.2 (17). The docking protocol was validated by using the AlphaFold modelled structure from Siglec-9. A total of 200 runs using Lamarckian Genetic algorithm was performed, with a population size of 100. After docking, the 100 solutions were clustered in groups with root-mean-square deviation less than 1.0 Å. The clusters were ranked to the lowest energy representative of each one.

2.2.3 MD simulations. MD simulations were conducted using AMBER 18 with explicit water and specific forcefields (Glycam06j-1 for glycans, FF14SB for proteins, and Gaff for organic moieties) (18). Proteins were prepared by adding missing hydrogens, setting ionizable group states, and capping termini. Input files were created using the AMBER tleap module, energy minimizations were done with Sander, and MD simulations were performed with PMEMD. Systems were placed in a TIP3P water box, neutralized with counterions, and refined using energy minimization. Simulations included heating (0–300 K), equilibration, and a 100 ns production run with 1 fs timesteps. Trajectories were analyzed for stability and structural properties using Cpptraj, and visualized with PyMOL.

2.3 Validation of sialylation and binding to Siglecs

The presence and functionality of the sialylated glycan was confirmed by ELISA with α2,3-specific Lectenz (Lectenz Bio), Maackia Amurensis Lectin I (MAL-I) (Vector Laboratories), and Sambucus Nigra Lectin (SNA, EBL) (Vector Laboratories), while binding to Siglecs was similarly assessed by ELISA with Siglec-Fc chimeras. The detailed methods are described in the supplementary materials.

2.4 Binding/uptake experiments of TAMRA-(sia) $FVIII_{2155-2180}$

PBMCs were isolated from the buffy coats of healthy donors (Sanquin, reference: S03.0023-XT) by Ficoll density gradient and monocytes were obtained by Percoll density gradient. These were cultured for five days to generate monocyte-derived dendritic cells (moDCs), as previously described (19). At day 5 of differentiation, 0.1x106 moDCs per time point incubated on ice for 30 mins with 10 μM of TAMRA-

$FVIII_{2155-2180}$ or TAMRA-$siaFVIII_{2155-2180}$ diluted in blocking buffer (HBSS (Invitrogen) + 0.5% BSA). Next, $0.1x10^6$ moDC were placed on ice as time point zero, while the rest of the sample was incubated at 37°C for the number of minutes indicated in Fig. 3E. After the last incubation step, cells were washed twice with blocking buffer, stained with the fixable-viability-dye efluor780 (Invitrogen), fixed in 1% PFA and measured at the LSRFortessaX-20 (BD Bioscience). The data were analyzed with FlowJo (v10) by gating on the alive cells and extracting the MFI of TAMRA. Every time point of each peptide ($FVIII_{2155-2180}$ or $siaFVIII_{2155-2180}$) was normalized over time point 0.

2.5 Phosphorylation analysis

The phosphorylation of Siglec-9 induced by sialylated peptides in monocytes was assessed with the Human Phospho-immunoreceptor array kit (R&D systems) according with manufacturer's instructions. For the generation of the cell lysates, $1x10^5$ CD14+ isolated monocytes were first starved in FCS-free RPMI-1640 medium for 4 hours at 4°C and then stimulated for approximately 15 minutes with 10 µM FVIII2155-2180 or siaFVIII2155-2180 at 37°C, previously coated overnight at 4°C in a 24-well plate. The cell lysates were then obtained with the kit's buffers, their concentration was determined with the micro-BCA protein assay kit (Thermo Scientific) and 50 µg were used for the array.

2.6 Generation of the knock-outs

One million monocytes were obtained from PBMCs of healthy donors by positive selection using CD14+ MACS beads (Miltenyi) and then nucleofected with 2.5 µL of a 20 µM solution of precomplexed RNPs, as previously described (20). Briefly, stock solutions of 20 µM RNPs were generated by incubating equal volumes of 40 µM Cas9 nuclease protein NLS (Horizon CAS12206) and 80 µM gRNAs at 37°C for 15 minutes. These were then immediately used or stored at -80°C. GuideRNAs targeting Siglec-3 CGGTGCTCATAATCACCCCA and Siglec-9 CCCTCTCCCTCCCCCAGAGC were synthesized by Genscript using their CRISPR gRNA Synthesis Service. Mock monocytes were obtained by precomplexing tracrRNA (Dharmacon, U-002005-0050) with Cas9 and double KO were generated by mixing equimolar ratios of single gRNAs to a final concentration of 80 µM. After nucleofection, the monocytes were cultured for five days to generate moDCs as described in paragraph 2.4.

2.7 Cytokines secretion from moDCs and surface marker expression

At day 5 of differentiation, $0.5x10^5$ wild type, mock, Siglec-9, Siglec-3/-9 double knock-out moDCs were stimulated with 10µM of $FVIII_{2155-2180}$ or $siaFVIII_{2155-2180}$ and matured overnight with 10ng/mL LPS (LPS-EB Ultrapure from E. coli O111:B4, Invivogen). Samples without LPS were taken along as controls. The supernatants were collected and stored at -20°C to be processed with LEGENDplex™ Human Inflammation Panel 1 (13-plex) (BioLegend, 740809), which was then performed according with the manufacturer's instructions. Cytokine concentrations were determined using BioLegend's LEGENDplex™ data analysis software, while the cells were processed for flow cytometry analysis. Briefly, the cells were incubated on ice for 15 minutes with fixable viability dye eFluor450 (Thermo Fisher) and Fc-block (BD Biosciences). Then, moDCs were stained for 30 mins on ice with the antibody mix (Supp. Table 2) diluted in PBS + 0.1% BSA (Roche) + 0.02% sodium azide. Finally, samples were fixed in 1% PFA, measured at the LSRFortessa X-20 and analyzed with FlowJo (v10).

2.8 Statistics

All tests were performed using Graphpad PRISM version 10 or the R package ggstats (Larmarange, 2024). P-values ≤ 0.05 were considered significant.

3. RESULTS

3.1 Selection of the peptide sequence

To choose the peptide sequence, we shortlisted FVIII immunodominant T cell epitopes identified by Steinitz and colleagues (21) **(Fig. 1A, Supp. Table 3)**. For each core peptide, *i.e.* the 21-aminoacid sequence fitting into the MHC-II groove, the prediction of binding to the most common HLA-DRB alleles in the Caucasian population (22, 23) was computed using NetMHCIIpan4.0 (14). The percentile rank score was used to summarize the promiscuity of binding of every sequence. This value represents the predicted affinity of the input sequence compared to a set of 100000 random natural peptides, it is thus not inherently biased. We then calculated the sum of ranks to each HLA-DRB haplotype and plotted it as the inverse figure **(Fig. 1B)**. Core sequence #8 scored as the most promiscuous, being classified among the top 1% binders for 4 alleles and within the top 17% for the others **(Fig. 1C)**. As expected, all the sequences are predicted to be strong binders for DRB1*15:01, since they were identified by Steinitz *et al.* in a mouse model humanized with this haplotype. This confirms the reliability of the percentile rank score as a proxy measure for promiscuousness. Based on these results, we selected core peptide #8 and synthesized a 25-mer peptide spanning residues 2155-2180 of FVIII, which included core sequence #8. This longer peptide was designed to require antigen processing by APCs prior to MHC-II presentation, thereby preventing direct binding of the core peptide to empty MHC-II molecules. In the following sections, this peptide is referred to as "$FVIII_{2155\text{-}2180}$ ". We next interrogated the IEDB software to compute population coverage worldwide for $FVIII_{2155\text{-}2180}$, which resulted to be higher than 75% (24). Overall, these findings underscore the rationale for selecting core peptide #8 and designing the extended $FVIII_{2155\text{-}2180}$ peptide, which demonstrated broad predicted HLA-DRB binding promiscuity and global population coverage.

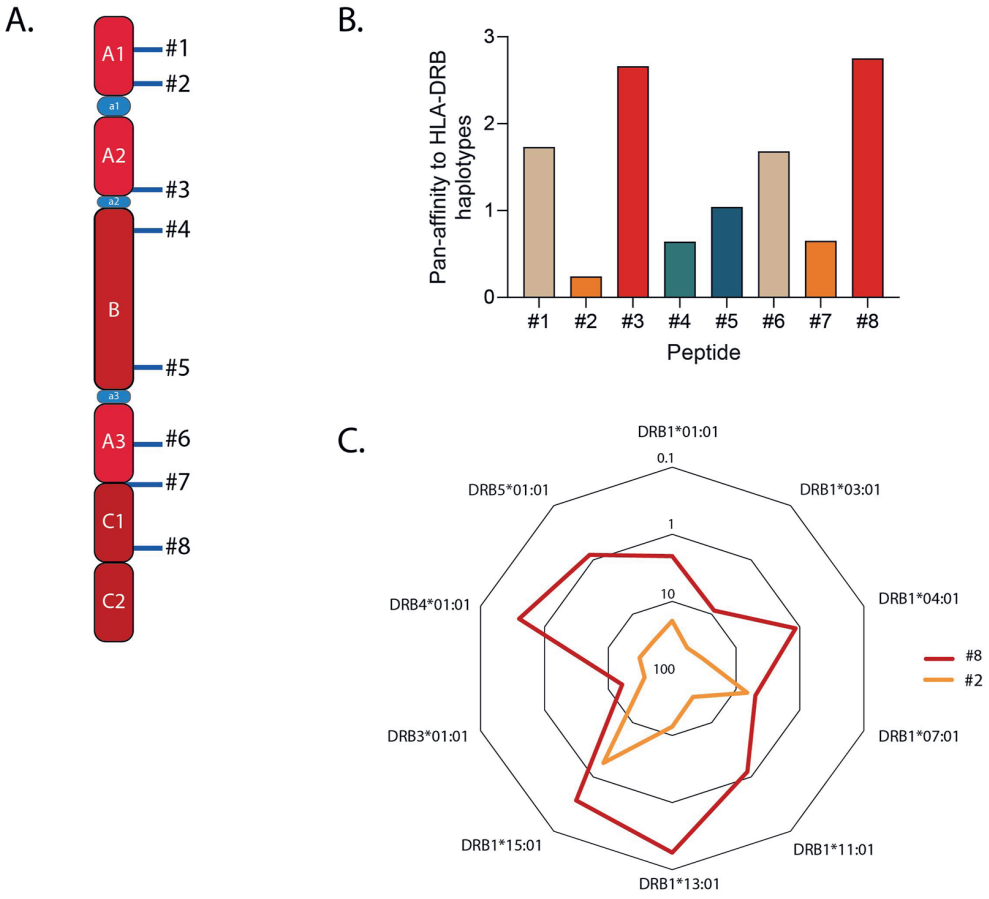


Figure 1 Selection of the FVIII sequence to be conjugated to the Siglec-9 ligand (Sia). ***A.*** *Schematic showing the immunodominant sequences identified in Steinitz et al. 2012.* ***B.*** *Promiscuity of binding to the most common HLA haplotypes in the Caucasian population, plotted as the inverse of the sum of ranks predicted in silico.* ***C.*** *The closed hexagonals shown in the radar charts represent the percentile rank (ranging from 0.1% to 100%) to each HLA-DRB allele for peptides #2 and #8, representative of the most and the least promiscuous peptides respectively.*

3.2 Selection of Siglec-9 ligand and conjugation to $FVIII_{2155-2180}$

We then conjugated $FVIII_{2155-2180}$ to sialic acid (Neu5Ac), to target it to the immune inhibitory receptor on DCs, Siglec-9 (25). Siglec-9 belongs to a family of 15 lectins, the majority of which carries an intracellular inhibitory motif to suppress the immune response upon binding to sialic acid. Siglecs show preferential binding to different sialylated glycans. It is known that Siglec-9 preferentially binds N-glycans with terminal α2.3-Neu5Ac (26-29), so we used the tetrasaccharide Neu5Acα2-3Galβ1-4GlcNAcβ1-3Gal (Sia) to chemically modify $FVIII_{2155-2180}$ (depicted in **Fig. 2A** and addressed to as $siaFVIII_{2155-2180}$ in the following sections). To study the interactions of Sia with Siglec-9, docking to the V-set domain of Siglec-9 was performed. The interactions were mapped to propose a molecular description of the complex **(Fig. 2A-C)**. The 3D models showed a stable salt-bridge interaction between the carboxylate group of Neu5Ac and the guanidinium group of R120 along the simulation **(Fig. 2B)**. Neu5Ac established polar interactions involving the O9 of its glycerol chain, through the formation of H-bonds with the lateral chain of N129. An additional contact from O7 of the glycerol chain with K127 was also observed **(Fig. 2B)**. The results from the MD simulation further showed that D69 from the *CC*-loop and K131 and K127 from the F-β strand were highly implicated on the accommodation of Gal, stabilizing the ligand into the binding pocket **(Fig. 2B-C)**. Specifically, the position O6 of Gal established a hydrogen bond with the lateral chain of N67 and its O4 with D69. The GlcNAc and the reducing Gal established transient interactions within K127 and R134, respectively. This suggests that this is the particular position where the following peptide $FVIII_{2155-2180}$ is coupled **(Fig. 2A)**. We next modelled the secondary structure of $FVIII_{2155-2180}$ with PepFold3, of which one representative conformation is shown in Fig. 2D. Amino acids from P2143 to Y2148 formed an alpha helix. We reasoned that it would be an advantage to guarantee flexibility and availability of binding to Siglec-9 to conjugate Sia to the N-terminus, indicated by the red arrow, given that it is not involved in the helix. We subsequently tested for the functionality of Sia after chemical conjugation to the peptide by ELISA. We observed that $siaFVIII_{2155-2180}$ but not $FVIII_{2155-2180}$ bound to α2.3PanLectENZ (Fig. 2E) and to MAL-1 but not to SNA **(Supp Fig.1A-B)**, known to only recognize α2,6 sialic acids and used as a negative control. In conclusion, sialylation at the N-terminus of $FVIII_{2155-2180}$ with Sia demonstrated strong *in silico* engagement with the V-set domain of Siglec-9 and enhanced the binding of α2.3-specific lectins.

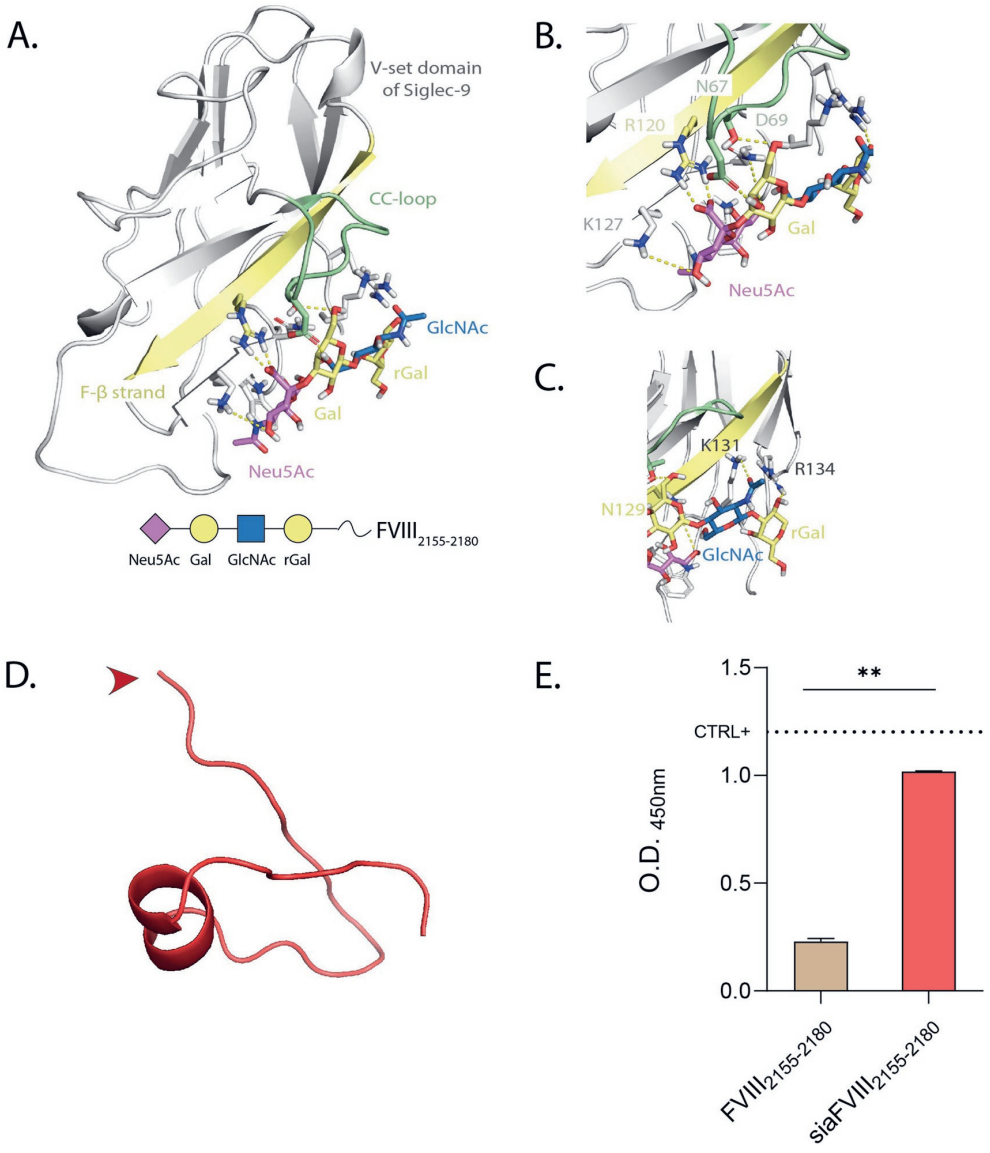


Figure 2. Selection of Siglec-9 ligand (Sia) and conjugation to FVIII. ***A.*** *Different views highlighting H-bonds between the 3D model of Siglec-9 and the Sia, monitored by MD simulation. The strands involved in the recognition are colored by type: F-β strand in yellow and CC-loop in green. The ligand is represented following SNFG color-code.* ***B, C.*** *Close up views of the interactions between Siglec-9 and the first (B) or second half (C) of the sialoglycan.* ***D.*** *PEP-FOLD3 prediction of the secondary structure of* $FVIII_{2155\text{-}2180}$ *in physiological conditions. The red arrow indicates the site of conjugation to Sia.* ***E.*** *α2.3PanLectENZ binding to* $siaFVIII_{2155\text{-}2180}$*. The dotted line marks the mean O.D. value of α2.3 PAA used as a positive control. Representative of 3 independent experiments performed in duplicate. Statistics: unpaired t test.*

3.3 siaFVIII$_{2155\text{-}2180}$ efficiently binds to Siglec-3 and Siglec-9 and it is bound/internalized by moDCs

Dendritic cells are the main immune subtype involved in antigen presentation to T cells and they express several Siglecs, Siglec-1, -3, -7, -9, -10 and -15 (30). We tested these Siglecs by ELISA for binding to siaFVIII$_{2155\text{-}2180}$ observing that siaFVIII$_{2155\text{-}2180}$ bound Siglec-9, and also engaged Siglec-3 **(Fig. 3A)**. A concentration dependent-binding was observed both for Siglec-3 and Siglec-9 to siaFVIII$_{2155\text{-}2180}$ **(Fig. 3B-C)**, further confirming that both the lectins can recognize the sialylated glycan on the peptide backbone. To investigate the capacity of siaFVIII$_{2155\text{-}2180}$ to be internalized by DCs, binding/uptake to monocyte-derived DCs (moDCs) was investigated by flow cytometry with (sia)FVIII$_{2155\text{-}2180}$ fluorescently labelled with TAMRA. Siglec-3 and Siglec-9 expression by moDCs was confirmed by flow-cytometry in unstimulated (naïve) and LPS-matured moDCs **(Fig.3D, Supp. Fig. 2A)**. Naïve cells were incubated for 30 min with TAMRA(sia)FVIII$_{2155\text{-}2180}$ at 4°C and then at 37°C for different time points. Given that the variability between donors was great **(Supp. Fig.2C)**, data were normalized over time point 0, addressed to as "binding" **(Fig. 3E)**. From the resulting curves, it can be inferred that moDCs are equally able to bind/internalize FVIII$_{2155\text{-}2180}$ and siaFVIII$_{2155\text{-}2180}$.

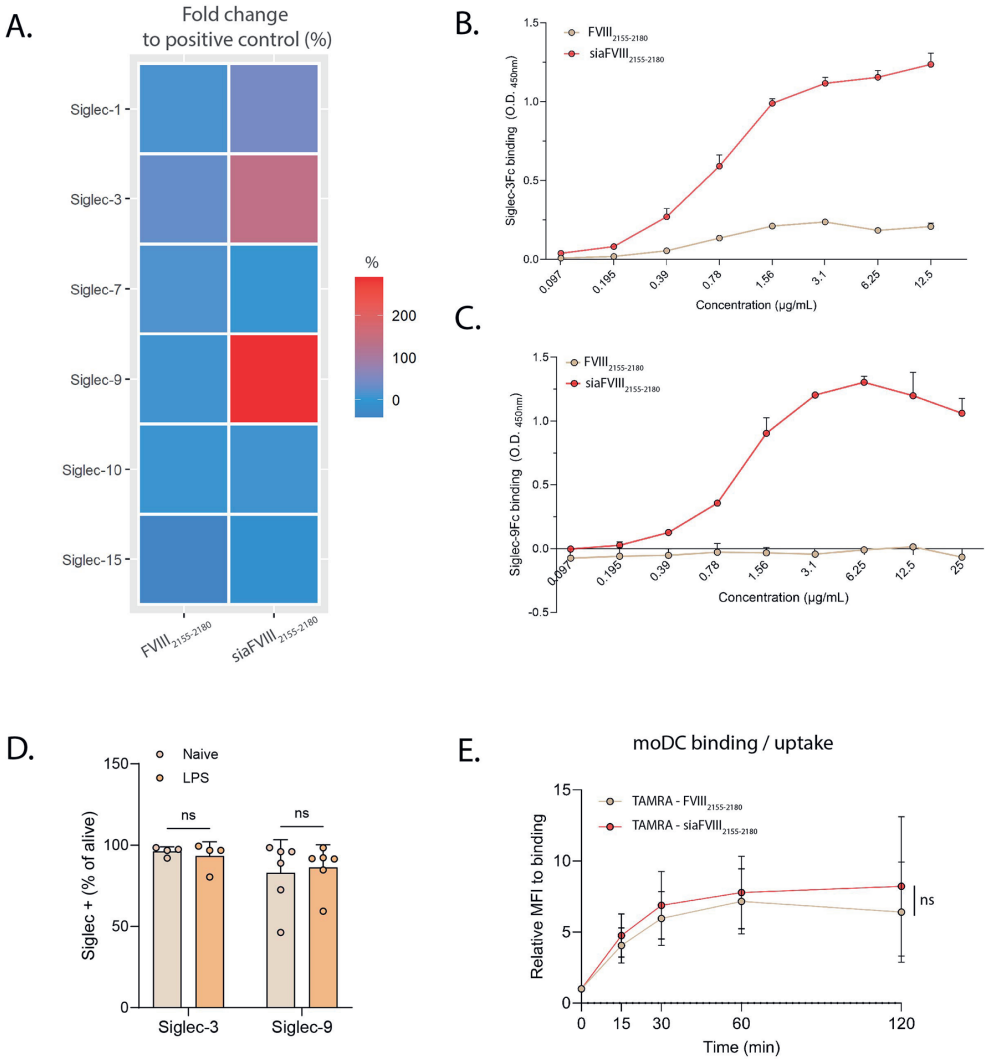


Figure 3. siaFVIII$_{2155-2180}$ efficiently binds to Siglec-3 and Siglec-9. ***A.*** *Heatmap showing binding of siaFVIII$_{2155-2180}$ to various Siglec-Fc chimeras. The O.D. values are plotted as fold change to the positive control, set as 100% of binding. Data are presented as mean of three independent experiments.* ***B, C.*** *Concentration dependent curves of binding to Siglec-3 (C) and Siglec-9 (D) of siaFVIII$_{2155-2180}$. Data are shown as mean ± SD of two technical duplicates of three independent experiments.* ***D.*** *Siglec-3 and Siglec-9 expression in unstimulated moDCs (naïve) and moDCs stimulated overnight with 10 ng/mL LPS assessed by flow cytometry. The data represent the percentages of Siglec-positive cells, expressed as mean of 4 (Siglec-3) or 6 (Siglec-9) donors ± SD. Each dot represents one donor. Statistics: paired t test, ns = not significant.* ***E.*** *Binding/uptake of TAMRA-(sia)FVIII$_{2155-2180}$ in moDCs measured by flow cytometry. Median fluoresce intensities (MFIs) are plotted as relative to time point 0 (binding), as mean of 8 donors ± SD. Statistics: paired t test, ns = not significant.*

3.4 siaFVIII$_{2155\text{-}2180}$ activates Siglec-9 on monocytes and promotes IL-10 secretion from dendritic cells via Siglec-3 and Siglec-9

We then tested whether siaFVIII$_{2155\text{-}2180}$ could activate Siglecs. We used tyrosine phosphorylation as marker for activation of Siglecs on monocytes stimulated with (sia)FVIII$_{2155\text{-}2180}$ tested with an array of immunoreceptors blotted on a membrane and detected with anti-phophoTyr-HRP. Interestingly, siaFVIII$_{2155\text{-}2180}$ selectively induced Siglec-9 phosphorylation **(Fig. 4A-B, Supp.Fig. 3A-B)**, indicating that Siglec-9 is the main receptor targeted by the sialylated peptide. To further explore the functional modulatory properties of the immune response induced by Siglec-targeting with siaFVIII$_{2155\text{-}2180}$, we analysed maturation markers and cytokine secretion in LPS-matured moDCs. Primary monocytes knocked out of Siglec-9, Siglec-3, or a double KO of both Siglec-9 and Siglec-3 were generated by CrisprCas9 **(Fig.4C-F)**, cultured for 5-days with IL-4 and GM-CSF to generate moDCs and then stimulated overnight with FVIII$_{2155\text{-}2180}$ or siaFVIII$_{2155\text{-}2180}$ in the presence of LPS. Binding of TAMRA(sia)FVIII$_{2155\text{-}2180}$ to the mock and KO cells was confirmed by flow cytometry **(Supp. Fig. 3C-D)**. No differences were detected in the expression of maturation (CD80, CD86) or tolerogenic (TIM3, PDL1) markers **(Supp. Fig. E-H)**. A panel of essential cytokines was tested by bead array, of which IL-10 was the only cytokine showing significant differences between the groups **(Supp. Fig. 3I)**. In mock moDCs, siaFVIII$_{2155\text{-}2180}$ induced IL-10 secretion **(Fig.G)**, while in the Siglec-KO cells this effect was not observed **(Fig. 4H-J)**, suggesting that functionally both Siglecs are needed to achieve immune modulation with siaFVIII$_{2155\text{-}2180}$.

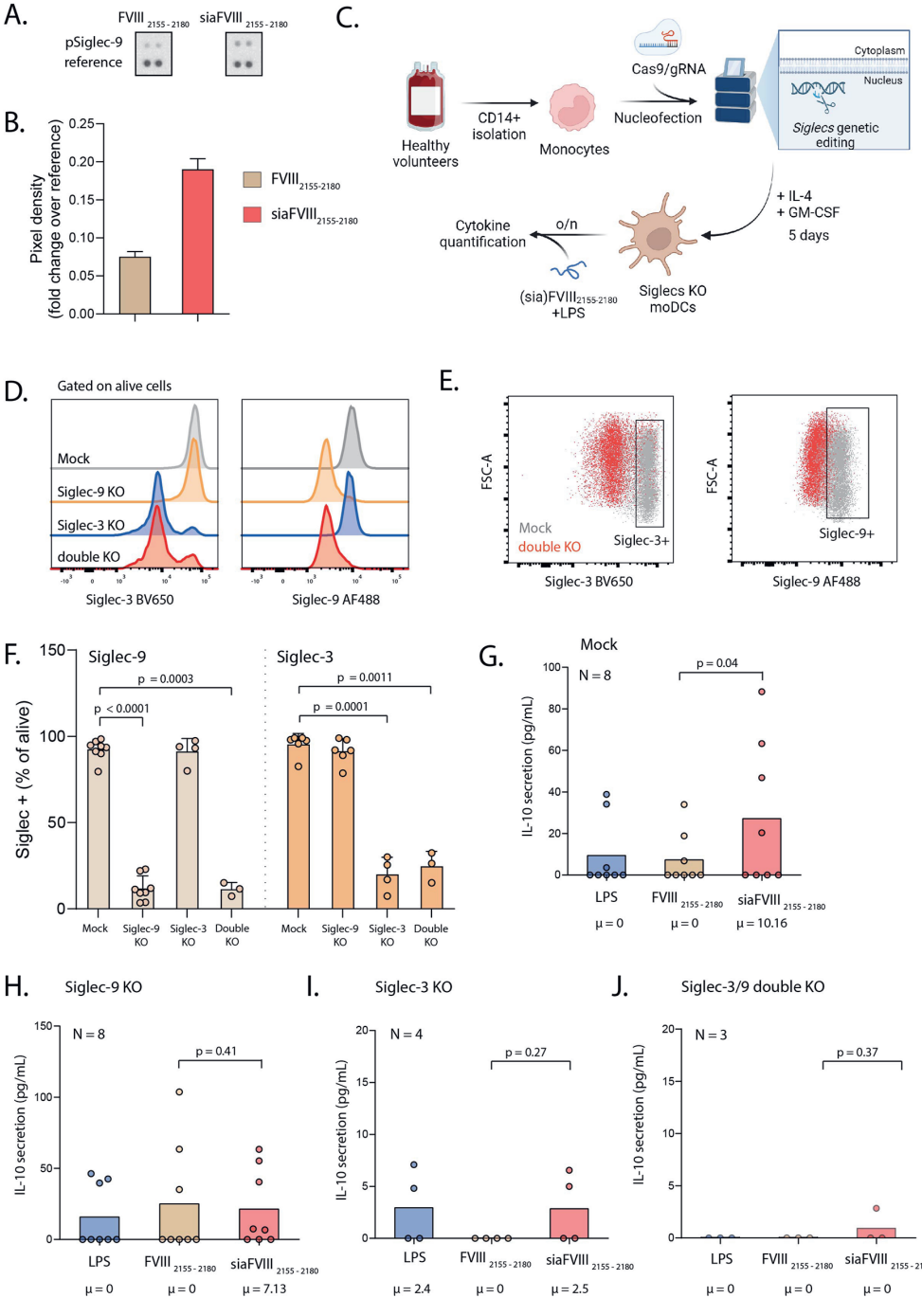

A.
FVIII 2155 - 2180
siaFVIII 2155 - 2180
pSiglec-9 reference
B.
Pixel density (fold change over reference)
0.25
0.20
0.15
0.10
0.05
0.00
FVIII2155-2180
siaFVIII2155-2180
C.
Healthy volunteers
CD14+ isolation
Monocytes
Cas9/gRNA
Nucleofection
Cytoplasm
Nucleus
Siglecs genetic editing
+ IL-4
+ GM-CSF
5 days
Siglecs KO moDCs
o/n
Cytokine quantification
(sia)FVIII2155-2180 +LPS
D.
Gated on alive cells
Mock
Siglec-9 KO
Siglec-3 KO
double KO
Siglec-3 BV650
Siglec-9 AF488
E.
FSC-A
Mock
double KO
Siglec-3+
Siglec-3 BV650
Siglec-9+
Siglec-9 AF488
F.
Siglec-9
Siglec-3
Siglec + (% of alive)
150
100
50
0
p = 0.0003
p < 0.0001
p = 0.0011
p = 0.0001
Mock
Siglec-9 KO
Siglec-3 KO
Double KO
G.
Mock
N = 8
p = 0.04
IL-10 secretion (pg/mL)
100
80
60
40
20
0
LPS
FVIII 2155 - 2180
siaFVIII 2155 - 2180
μ = 0
μ = 0
μ = 10.16
H.
Siglec-9 KO
N = 8
p = 0.41
IL-10 secretion (pg/mL)
150
100
50
0
LPS
FVIII 2155 - 2180
siaFVIII 2155 - 2180
μ = 0
μ = 0
μ = 7.13
I.
Siglec-3 KO
N = 4
p = 0.27
IL-10 secretion (pg/mL)
20
15
10
5
0
LPS
FVIII 2155 - 2180
siaFVIII 2155 - 2180
μ = 2.4
μ = 0
μ = 2.5
J.
Siglec-3/9 double KO
N = 3
p = 0.37
IL-10 secretion (pg/mL)
20
15
10
5
0
LPS
FVIII 2155 - 2180
siaFVIII 2155 - 2180
μ = 0
μ = 0
μ = 0

Figure 4. Stimulation of monocytes with siaFVIII$_{2155-2180}$ activates Siglec-9 and promotes IL-10 secretion via Siglec-3 and Siglec-9. ***A.*** *Phospho-Immunoreceptor array of monocytes from one donor stimulated with siaFVIII$_{2155-2180}$ or FVIII$_{2155-2180}$.* ***B.*** *Quantification of A, plotted as pixel density analyzed with Fiji. Data are presented as* ***C.*** *Schematic of the experimental workflow; monocytes were nucleofected with Cas9 pre-complexed with gRNAs targeting Siglec-9 or a combination of Siglec-9 and Siglec-3. The cells were differentiated to moDCs for 5 days and then stimulated overnight with siaFVIII$_{2155-2180}$ in the presence of LPS. Illustration was generated with biorender.com.* ***D.*** *Histograms showing Siglec-3 (left) and Siglec-9 (right) expression across moDC mock, Siglec-9, Siglec-3 and double KO.* ***E.*** *Gating strategy used to select Siglec-9 and Siglec-3 positive cells.* ***F.*** *Percentages of Siglecs KO in the moDC cultures, analyzed by flow cytometry.* ***G-J.*** *IL-10 secretion in mock, Siglec-9 single KO, Siglec-3 single KO and Siglec-3 and -9 double KO respectively, quantified with cytokine bead assay. Data are presented as box plots, the median is specified in text underneath. Statistics: Durbin-Conover pairwise test with Holm-Bonferroni correction for multiple comparisons. Each dot is the mean of two technical duplicates per donor, number of donors is specified in text in each graph. μ = median.*

4. DISCUSSION

The objective of the present study was to develop a novel FVIII-peptide formulation with improved targeting to two immune inhibitory receptors, Siglec-3 and Siglec-9 on DCs to be employed as reverse vaccines for hemophilia A patients with inhibitory FVIII antibodies.

In silico analyses of FVIII core peptides' binding promiscuity to frequent Caucasian HLA-DRB alleles shortlisted a sequence predicted to cover 75% of the global population **(Fig. 1 A-C)**. This estimate should be confirmed *in vitro* by employing an array of MHC-II molecules. However, independent studies that characterized the immunodominant peptidome of FVIII using mass spectrometry identified peptides within the $FVIII_{2155\text{-}2180}$ region across various donors (31, 32). Moreover, T cells reactive with peptide I2163-T2179, within $FVIII_{2155\text{-}2180}$, were isolated from an HA patient (33). Altogether, this evidence reinforces our results. Population coverage could be increased by synthetizing a pool of epitopes, given that chemical coupling of Sia is potentially feasible for every FVIII epitope, but using one sequence enhances scalability and standardization. Moreover, chemical synthesis is preferable over production of whole antigens in cell-based systems due to cost and ease of standardization.

$PEP\text{-}FOLD_3$ predictions of the secondary structure of $FVIII_{2155\text{-}2180}$ showed an α-helix encompassing P2143 to Y2148, corresponding to P3-P8 of the core sequence fitting into the MHC-II groove. P4 and P6 anchor amino acids are part of the helix, but since $FVIII_{2155\text{-}2180}$ requires processing before presentation, the rigidity of the helix is not expected to interfere with binding. Moreover, the analysis of the secondary structure guided the choice of coupling Sia in a non-structured end-terminal of the peptide, so that binding of Sia to Siglecs would not be impeded by the rigidity of the aminoacidic scaffold.

Although Pletinckx *et al.* demonstrated that peptide immunotherapy reduced inhibitors in HA mouse models, the development of these antibodies was not impeded (4). To enhance efficacy, we proposed to couple the peptides to sialic acids to target Siglecs. It is well established that Siglecs engagement promotes immune tolerance in various contexts, such as controlling excessive inflammation during sepsis (34). In HA, targeting Siglec-2 on B cells with sialylated nanoparticles containing FVIII achieved complete tolerance (35). Given that DCs are the main antigen-presenting cells and that the development of inhibitors requires APCs' licensing, we designed a sialylated saccharide to target Siglec-9, highly expressed on DCs **(Fig. 3D)**. While we selected the α2.3-sialylated tetrasaccharide based on MD simulations to Siglec-9 **(Fig. 2A-C)**, we also observed binding to Siglec-3

(Fig.3A-C). This is not unexpected, as Siglecs are known for binding promiscuity, and the specific ligands are not yet fully elucidated for all the lectins. Interestingly, targeting Siglec-3 was explored for Alzheimer's disease, where its suppression of microglial activity was found to be detrimental (36). However, in the present study Siglec-3 engagement could be advantageous, since it is an inhibitory Siglec and its pattern of expression is mainly restricted to APCs. Despite plate-bound siaFVIII$_{2155-2180}$ was shown to bind Siglec-3 and Siglec-9 **(Fig.3A-C)**, no differences in peptide binding/uptake were observed, regardless of sialylation, on moDCs **(Fig. 3D, Supp. Fig. 1B-C)**. While Siglec-1 and Siglec-2 are described to be endocytic receptors (37, 38), to our knowledge there are no studies linking Siglec-3 and Siglec-9 to improved internalization, their main role being immunosuppression via ITIMs. It is therefore expected that α2.3-sialylation would not improve endocytosis of FVIII-peptides by moDCs. Given the phagocytic function of moDCs, a non-specific uptake of the unsialylated peptide is also expected. However, our detection system with TAMRA as fluorochrome may have lacked sensitivity, and results should be confirmed by a different tracking system.

Interestingly, when we measured Siglecs activation, we could only detect phosphorylated Siglec-9 **(Fig.4A-B)** and not Siglec-3 **(Supp. Fig. 3A-B)**. This confirms that Siglec-9 will likely be the main target of siaFVIII$_{2155-2180}$, while binding to Siglec-3 might not be strong enough to also induce signalling. Low Siglec-9 phosphorylation (pSiglec-9) was detected in samples treated with FVIII$_{2155-2180}$. Recent research also reported pSiglec-9 in control-treated samples (10, 39). These and our data suggest that pSiglec-9 observed with FVIII$_{2155-2180}$ is not induced by the treatment with the peptide but represents a physiological condition of unstimulated monocytes.

Lectins bind glycans with low affinity, with avidity enhancing the interaction (40). A multivalent presentation of the ligand is generally advantageous (41). Since a single sialylated moiety on an aminoacidic sequence may not achieve optimal multivalency, we coated the peptides on a plate for binding and monocyte-stimulation assays, to enhance the sensitivity required for assessing Siglec-triggering *in vitro*.

In the present study, we demonstrated that IL-10 secretion from moDCs can be induced by triggering Siglecs with siaFVIII$_{2155-2180}$ **(Fig. 4G)**. While previous research from our group showed that α2.3-sialylated dendrimers can achieve the same effect (10), the key advantage of our approach is its antigen specificity. By conjugating Sia to an immunodominant-FVIII-derived peptide, we ensured that the tolerogenic signal was directly linked to the antigen towards which tolerance is to be induced. However, further research is needed to confirm this hypothesis.

We also demonstrated that IL-10 secretion is dependent on Siglec-3 and Siglec-9, as confirmed by knock-out experiments **(Fig. 4H-J)**. We detected low IL-10 secretion in Siglec-3 and double KOs, suggesting that Siglec-3 might play an essential function in moDC biology which cannot be functionally tested with a knockout. We did not observe any differences in cell viability between the nucleofected cells, and we could detect most of the cytokines tested due to the high sensitivity provided by the bead array. Even though IL-10 was the only cytokine significantly altered in the mock moDCs by siaFVIII$_{2155\text{-}2180}$ treatment, other cytokines were upregulated that are considered to be pro-inflammatory (IP-10, IL-4, TNF-α, and IFN-γ). However, the maturation and tolerogenic markers were not affected regardless of peptide-treatment or Siglec-expression **(Supp. Fig. 3E-H)**, which is an observation in line with previous research on immune-modulation induced by sialylated glycans (10). Overall, despite the presence of pro-inflammatory cytokines, the activation status of moDCs was not altered by siaFVIII$_{2155\text{-}2180}$, and we measured significant induction of IL-10 secretion. IL-10 is a key cytokine in immune homeostasis, and dysregulations of its pathway have been linked to a number of autoimmune and inflammatory diseases, such as IBD, RA and SLE (42). The development of inhibitors is an immune-related complication with an unclear cause, but it shares mechanisms with tolerance breakdown tolerance observed in autoimmunity (43). Therapies aiming at restoring IL-10 levels are already widely being investigated for autoimmune diseases (42). Moreover, it was shown that IL-10 is a key cytokine to restore tolerance to FVIII in hemophilic mice (44). Concluding, we suggest that promoting IL-10 secretion from moDCs via Siglec-3 and Siglec-9 with sialylated FVIII-derived peptides as shown in this study could also could help restore a tolerogenic milieu in HA patients that developed neutralizing antibodies to FVIII.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization, EN and YvK; investigation, EN and TGA; writing—original draft preparation, EN; writing—review and editing, BCK, JV, FC; proof-reading, all the others; supervision, YvK. All authors have read and agreed to the published version of the manuscript.

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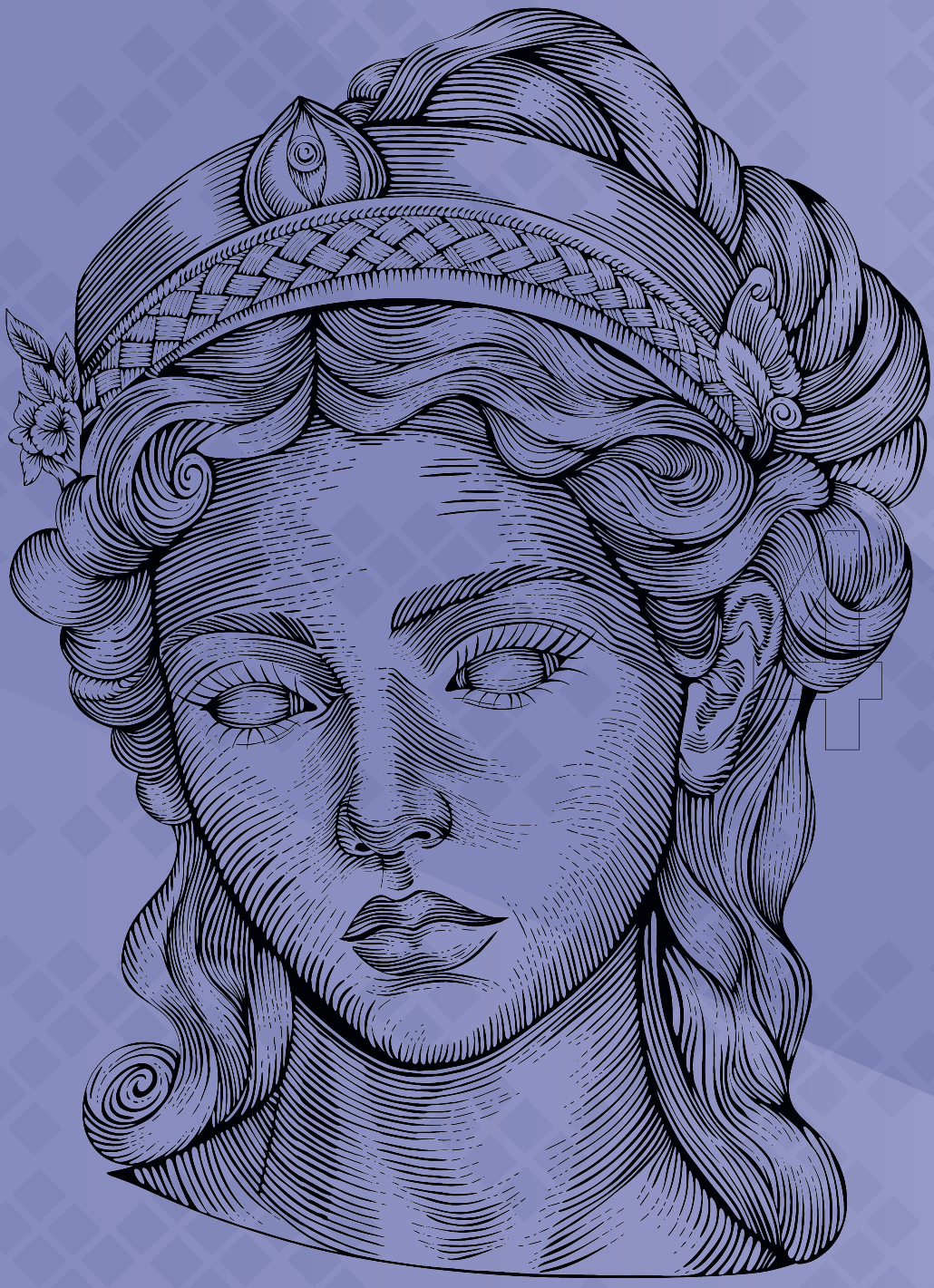
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4

SHAPING T CELL RESPONSES TO FACTOR VIII IN HEMOPHILIA A WITH SIALYLATED IMMUNODOMINANT PEPTIDES

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ABSTRACT

Hemophilia A (HA) patients present a deficiency of coagulation factor VIIII (FVIII). One of the complications of replacement therapy using exogenous FVIII is the development of FVIII neutralizing antibodies (inhibitors). Despite the fact that various tolerogenic solutions have been investigated, the immune response towards FVIII remains complex, with CD4+ T cells being key players in promoting inhibitor-secreting plasma cells. A strategy to foster tolerance is to engage immune-inhibitory receptors such as Siglecs, a family of sialic-acid binding receptors which carry an immune-inhibitory intracellular motive. In this study, we employed sialylated FVIII-derived (sia-FVIII) peptides to investigate whether we could skew the anti-FVIII immune response towards tolerance. We showed that DCs loaded with sia-FVIII-peptides dampened allogenic T cell proliferation and activation of a HA patient-derived CD4+ T cell clone. Moreover, we translated these results to a preclinical model of development of inhibitors, where prophylactic treatment with sia-FVIII-peptides restrained Teff cells differentiation while preserving the Tregs compartment. Our study proposes a novel approach to fine tune the immune system towards FVIII tolerance.

MAIN TEXT

Hemophilia A (HA) is a genetic disorder originating from a deficiency of coagulation factor VIII (FVIII). Up to 30% of severe HA patients develop neutralizing antibodies (inhibitors), which pose a serious threat to the success of replacement FVIII therapies. Immunologically, the development of inhibitors is a CD4-driven complication where T helper cells are key actors in inducing FVIII-specific memory B cells and plasma cells which secrete high levels of pathogenic IgGs[1]. Promising studies in HA preclinical models aiming at transiently depleting T cells showed an expansion of T_{regs} [2], which are the counter-actors involved in maintaining tolerance towards FVIII in healthy individuals and likely restoring/promoting it in HA patients. A vast landscape of other tolerogenic solutions has been proposed, however, the immune response to FVIII in HA patients remains complex, warranting further studies to develop new tolerance-inducing strategies. A largely unexplored layer of complexity in the regulation of the immune response is glycosylation, the post-translational modification that adds glycans to proteins, lipids and RNAs to modify their function or signaling. In mammals, glycans are often capped by one specific monosaccharide, sialic acid. Sialylated glycans are recognized by immune cells via Siglecs, a family mainly comprising immune-inhibitory receptors. Engagement of Siglec-9 has already been explored as a treatment for allergies, showing induction of T_{regs} *in vitro* and *in vivo* via modulation of dendritic cell (DC) function[3,4]. However, the way sialylation would modify the immune response to FVIII remains unclear as yet. In this study, we employed sialylated FVIII-derived (siaFVIII) peptides to investigate whether we could skew the anti-FVIII immune response towards tolerance. We demonstrate here with *in vitro* and *in vivo* experiments that siaFVIII-peptides can redirect CD4+ T cell responses by reducing activation, pro-inflammatory cytokine secretion and promoting T_{reg} expansion.

FVIII has 4 glycosylation sites outside of the B domain, 3 of which are reported to be mostly sialylated[5]. Previous studies have linked a loss of sialic acids to increased inhibitor development[6,7], suggesting a role for sialylation in regulating the immune response to FVIII. To examine how sialylation affects the immune response to FVIII, we chemically conjugated the C-terminal of various immunodominant peptides derived from FVIII to a sialylated glycan (siaFVIII-peptides, **Figure 1A, Table 1**).

ID	SEQUENCE	USE
FVIII1	**K_{2155}HNIFNPPIIARYIR**LHPTHYSIRST$_{2180}$	In vitro moDC-T cell co cultures. In bold is the mouse epitope, the human epitope is underlined.
FVIII2	A_{2108}RQKF**SSLYISQFIIMYSLDGKKWQ**TYRGN-STG$_{2140}$	In vitro stimulation of the T cell clone. In bold the epitope recognized by the T cells.
FVIII1.1	S_{2152}GI**KHNIFNPPIIARYIR**LHPTHYSIRSTLRM$_{2183}$	In vivo prophylactic treatment of HA mice. In bold is the mouse epitope, underlined the human epitope.
FVIII1.3	K_{2155}HNIFNPPIIARYIR$_{2169}$	Ex vivo restimulation of the splenocytes.

Table 1. List of peptides used in the study. The full-length numbering for FVIII residues is used.

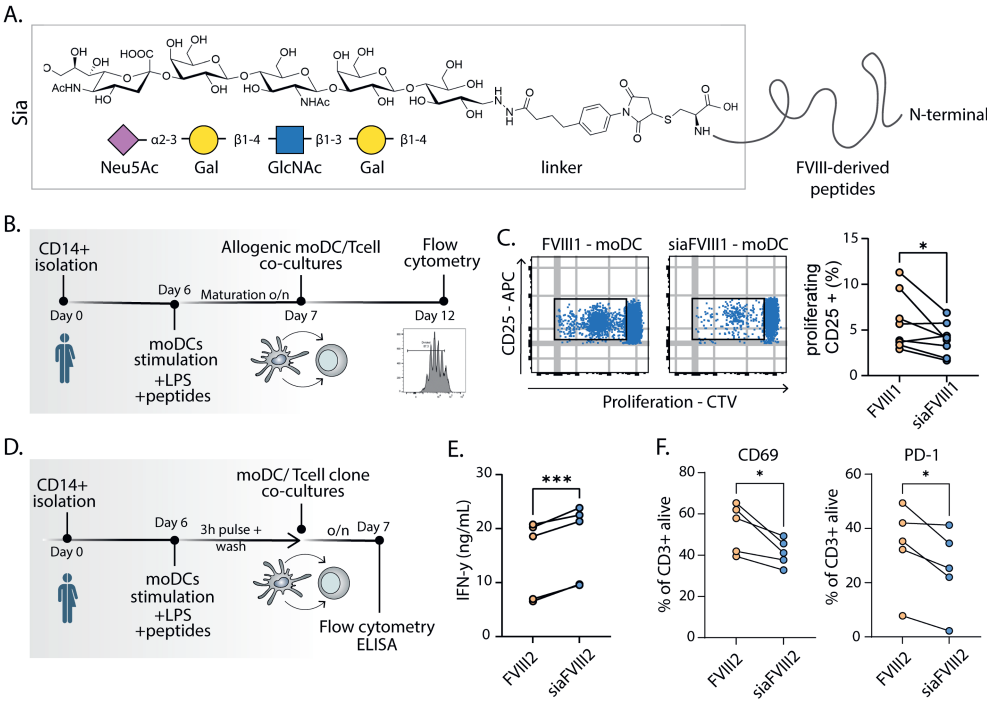


Figure 1. moDCs loaded with siaFVIII-peptides dampen CD4+ T cell responses, both allogenic and HA-specific. ***A.*** *Schematic representation of the chemical structure of the tetrasaccharide and the linker coupled to the FVIII-derived peptides, obtained with ChemDraw. The tetrasaccharide is also shown following SNFG color-code.* ***B.*** *Schematic of the experimental workflow: moDCs obtained from CD14+ isolated monocytes from healthy donors were stimulated with FVIII1 or siaFVIII1 and matured overnight with 10 ng/mL of LPS. At day 7, co-cultures of moDCs : allogenic naïve CD4+ T cells (ratio 1 : 5) were established and kept in culture for 5 days to study T cell activation and proliferation.* ***C.*** *left, Representative dot plots showing the final gate of the gating strategy to select CD4+CD25+CTV- (proliferated) T cells. (not shown time, singlets, alive, CD4+); right, Quantification of proliferated T cells stimulated by FVIII1 (orange dots) or siaFVIII1 (blue dots)- loaded moDCs, shown as percentage of CD4+ alive. Each dot represents one moDC donor. N = 8.* ***D.*** *Schematic of the experimental workflow: moDCs obtained from CD14+ isolated monocytes from HLA-DRB1*01:01/DRB1*15:01 genotyped donors were stimulated with 0.5 μM FVIII2 or siaFVIII2 for 3h in the presence of 10 ng/mL of LPS. Then, moDC : T cells (ratio 1 : 5) co-cultures with an HLA-matched CD4+ T cell clone isolated from an HA patient were established. The day after, the supernatants were collected to measure secreted IFN-γ by ELISA and the cells were stained for flow cytometry analysis.* ***E.*** *Concentration of IFN-γ in the supernatants of the T cell clone co-cultured with moDCs pulsed with 0.5 μM of FVIII2 (orange dots) or siaFVIII2 (blue dots). Each dot represents the mean of 3 technical duplicates of one donor, N = 5. F. Percentages of CD69+ (left) and PD1+ (right) T cells from the T cell clone stimulated by FVIII2 (orange dots) or siaFVIII2 (blue dots)- loaded moDCs, measured by flow cytometry. N = 5 moDCs donors. Statistics: the data distribution was tested for normality by Kolmogorov-Smirnov test and parametric, paired t tests were computed accordingly. * = p < 0.05, *** = p < 0.001.*

To meet experimental needs, different peptides were used throughout the study. Given that we both investigated human and mouse responses, we first used a 26 amino acid (FVIII1) peptide spanning a mouse and a human epitope. *In vivo*, a longer version, (FVIII1.1) of this peptide was employed, to account for correct antigen processing of the mouse epitope. The selection of this peptide sequence was guided by literature and by our previous study[8], where we investigated in silico the promiscuity of binding to the most common HLA-DRB haplotypes and predicted > 75% worldwide population coverage. Moreover, to investigate the FVIII specific responses in vitro, a sequence specific for a CD4+ T cell clone was synthesized (FVIII2). All the sequences are within the C1 domain and the sialylated ones bind to Siglec-9 and its mouse homologue Siglec-E (Figure S1A, B).

We first investigated the ability of monocyte-derived DCs (moDCs) loaded with siaFVIII1 (sia-loaded moDCs) to instruct T cell responses **(Figure 1B)**. Allogeneic co-cultures of $CD4^+$ T cells with moDCs loaded with siaFVIII1 showed reduced proliferation within the activated CD25+ subset compared to controls **(Figure 1C)**, and this effect was partially dependent on Siglec-9, as suggested by knockout experiments **(Figure S1C, D)**. This indicates that sia-loaded moDCs are less capable of initiating responses to non-FVIII allogeneic antigens, pointing to a broad,

antigen-independent immunosuppressive effect mediated by siaFVIII1. Next, to investigate FVIII specificity, we employed a CD4+ T cell clone isolated from a HA patient **(Figure 1D)**[9]. Since this clone is specific for the epitope spanning amino acids 2113-2132, we used a longer peptide (FVIII2, **Table 1**) that includes this region to ensure proper antigen processing. HLA-DRB1*01:01 / DRB1*15:01 healthy donors were selected as source of moDCs, to match the genetic background of the T cell clone. Interestingly, we measured higher IFN-γ concentrations in the supernatants from the T cell clone co-cultured with sia-loaded moDCs than control moDCs **(Figure 1E)**. While IFN-γ secretion from CD4+ T cells often signals a pathogenic Th1 response, in the context of a T cell clone it mainly reflects antigen presentation through MHC-II. Our results indicate that sialylation does not hinder, but rather enhances, MHC-II loading. By making use of a biotinylated version of FVIII2 and siaFVIII2, we measured binding / uptake in moDCs, showing that at 4°C siaFVIII2 binds better than FVIII2, partially dependently on Siglec-9 **(Figure S1E)**. When the cells are put at 37°C and endocytosis is activated, siaFVIII2 and FVIII2 are equally taken up by moDCs **(Figure S1F)**. Moreover, in the native FVIII protein, residue N2137 carries a mannosylated glycan, whose removal has been shown to reduce uptake by moDCs[10], but synthetic peptides FVIII2 and siaFVIII2 lack this glycan. For these reasons, we postulate that the observed differences in antigen presentation are due to a receptor-mediated effect of sialylation on antigen processing, rather than altered peptide uptake by moDCs. We further observed that the T cell clone co-cultured with sia-loaded-moDCs expressed lower levels of CD69 and PD-1 than the control **(Figure 1F, S1G,H)**. At such an early stimulation time point, this is indicative of reduced activation. Other cell surface markers were unaffected **(Figure S1I, J)**. Our results suggest that moDCs process and present siaFVIII2 more efficiently than the unsialylated peptide to HA CD4+ T cell clones, while inducing a qualitatively different activation profile.

Then, we studied the immune response induced by siaFVIII-peptides in the preclinical model of HA **(Figure 2A)**. The mice were treated with siaFVIII1.1 or the unsialylated peptide for 2 weeks prior of FVIII reconstitution, following a previously published dose-escalation protocol[11], which is also an established method for immunotherapy in allergies. The development of anti-FVIII IgG was not affected by prophylactic treatment with siaFVIII1.1, but was increased in the unsialylated FVIII1.1-treated group, along with the inhibitor titers **(Figure 2B)**. Accordingly, FVIII-specific splenic B cell populations were not altered upon pretreatment with siaFVIII1.1 **(Figure S2A, B)**. Since FVIII1.1 is largely exposed on the surface of the C1 domain **(Figure 2C)**, we measured anti-C1-peptide IgG, which correlated in our cohort with the inhibitor titers **(Figure S2C)**. IgG towards FVII1.1 and siaFVIII1.1 could be equally detected in both groups, but not in the PBS treated

mice **(Figure 2D)**, suggesting that the antibody response is directed against the peptide sequence, irrespective of the presence of the sialylated moiety. These results indicate that pre-treatment with a single FVIII epitope selectively primed the immune response to that sequence. Therefore, the PBS group was excluded from further analysis. Based on our *in vitro* results, we expected the T cell compartment to be modulated by siaFVIII pre-treatment. Remarkably, the analysis of the splenic T cell populations revealed higher percentages of T_{regs} in the siaFVIII1.1-treated mice than in the unsialylated FVIII1.1-treated group **(Figure 2E)**. Previous research already showed T_{regs} induction with a model sialylated antigen12. Here, we show that a single sialylated FVIII immunodominant peptide can similarly modulate the immune response in the context of HA, maintaining the T_{regs} compartment. T_{regs} and conventional T cells (T_{convs}) displayed distinct memory/effector phenotypes **(Figure S2D)**, consistent with previous reports showing that naïve cells are more abundant among Tconvs, while T_{regs} are enriched in central memory and effector subsets[13]. In our study, T_{regs} phenotypes did not differ between the groups, whereas the siaFVIII1.1-treated group showed reduced frequencies of naïve and central memory T_{convs} and increased effector cells **(Figure S2E, F)**. We further tested *ex vivo* for the presence of peptide-specific T cells secreting IFN-γ and TNF-α. Strikingly, the frequency of these cells in the siaFVIII1.1 group was reduced by half as compared to the unsialylated FVIII1.1-treated mice **(Figure 2F)**. Previous research has shown that splenocytes from successfully tolerated mice restimulated with FVIII fail to secrete IFN-γ[14]. Our findings *in vivo* imply that even though no differences in the humoral response were observed, the immune profile in the siaFVIII1.1 pre-treated mice may reflect a state of tolerance, characterized by suppression of T cells secreting IFN-γ and TNF-α, while maintaining the T_{regs} compartment. Concluding, this study demonstrates that sialylation of immunodominant peptides promotes a reduced-proinflammatory response to FVIII in vitro and modulates the $CD4^+$ T cell response *in vivo* by restraining T_{eff} cells differentiation while preserving the T_{regs} compartment. The shift in T cell fate towards T_{regs}, together with previous studies assessing later time points in FVIII-tolerance[6,10,11], suggest that a decline in inhibitor levels might occur over a longer follow-up period. Overall, these results suggest a novel approach to fine tune the immune system towards FVIII tolerance, laying the groundwork for future studies aimed at translating these immunological changes into clinical benefits.

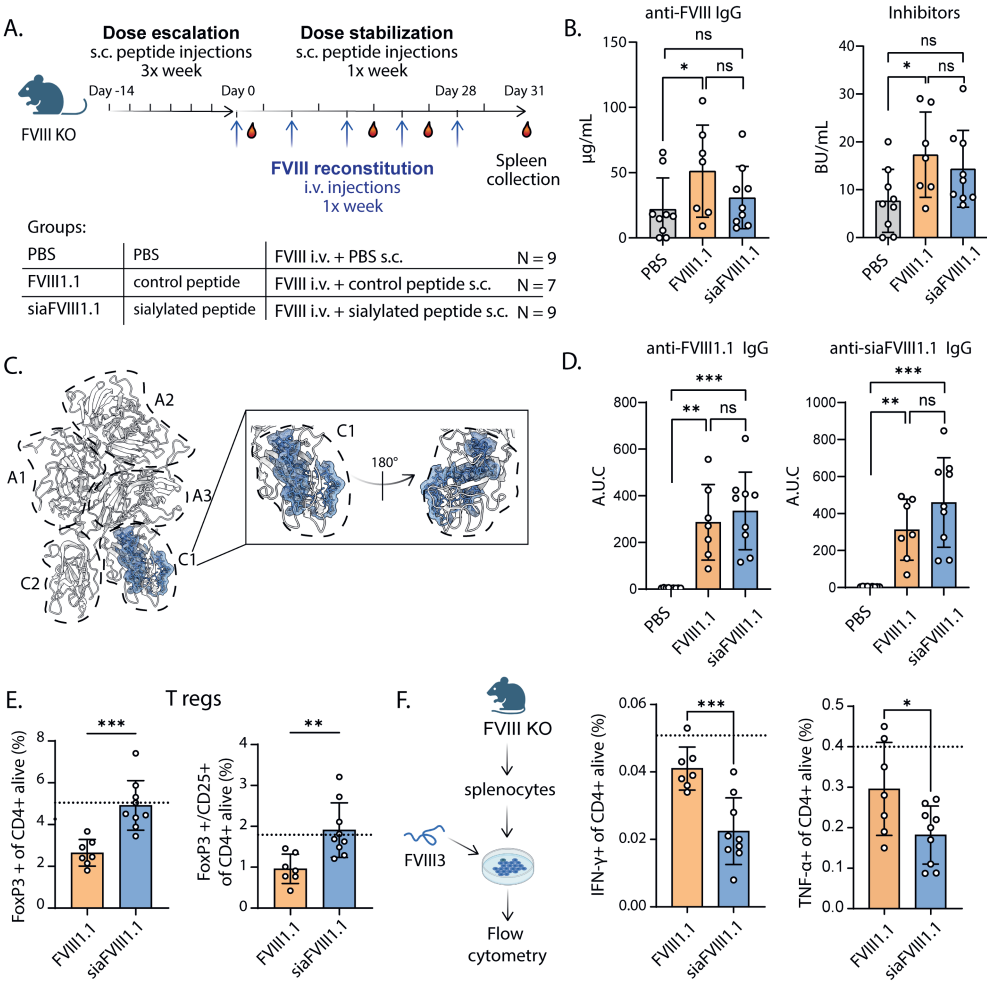


Figure 2. Prophylactic treatment with siaFVIII-peptides significantly expands T_{regs} and suppresses the activation of peptide-specific T cells. A. *Schematic of the immunization protocol. Groups of 7 or 9 FVIII-KO mice were treated 3 times per week for 2 weeks with s.c. injections in the neck of FVIII1.1 or siaFVIII1.1, or PBS according to a dose-escalation regimen (0.3 – 3 – 30 – 300 – 300 – 300 µM of each peptide). After the peptide pretreatment, mice were immunized once per week for 5 weeks, via i.v. injections with 1 IU of full-length human FVIII. Subcutaneous treatment with FVIII1.1 or siaFVIII1.1, or PBS was continued s.c. once per week 3 days after every FVIII intravenous injection, following a dose stabilization regimen (300 µM of each peptide). Blood samples were collected when indicated (red droplet).* ***B.*** *Titrations of anti-FVIII IgGs (left) and neutralizing anti-FVIII IgGs (right) in the sera samples collected at day 31 from mice pre-treated with PBS, FVIII1.1 or siaFVIII1.1, measured by ELISA and Bethesda Assay, respectively. Data are plotted as mean ± standard deviation (SD). Each dot represents 1 mouse. Statistics: Kruskal-Wallis test with Dunn's post hoc correction for multiple comparisons. ns = non-significant, * = $p < 0.05$.* ***C.*** *Three-dimensional structure of FVIII, the domains are*

indicated by a dotted line and FVIII1.1 is shown in blue in stick representation and dotted surface. The right panel shows a close-up view of the C1 domain with FVIII1.1. The model was obtained with Chimera X software based on PDB file of FVIII 3CDZ. ***D.*** *Quantification of anti- FVIII1.1 (left) and anti- siaFVIII1.1 (right) IgG in the sera samples collected at day 31 from mice pre-treated with PBS, FVIII1.1 or siaFVIII1.1. Maxi Sorp NUNC ELISA plates were coated with 2 µM of FVIII1.1 (left) or siaFVIII1.1 (right) and incubated with serial dilutions of the sera. Anti-peptide IgG were detected with an anti-mouse-IgG-HRP and TMB substrate. The O.D. was plotted against the sera titration and the area under the curve (A.U.C.) was calculated. Data are plotted as mean ± SD. Each dot represents 1 mouse. Statistics: Kruskal-Wallis test with Dunn's post hoc correction for multiple comparisons. ns = non-significant, ** = $p < 0.01$, *** = $p < 0.001$.* ***E.*** *Quantification of the percentages of FoxP3+ and FoxP3+/CD25+ Tregs in the spleens. The dotted line represents the average of the PBS control mice.* ***F.*** *Schematic of the workflow for the ex vivo restimulation of the splenocytes and quantification of the percentages of IFN-γ (left) and TNF-α (right) positive CD4+ T cells obtained from single cell suspensions of the spleens of mice pre-treated with FVIII1.1 or siaFVIII1.1. The cultures were restimulated ex vivo with the short peptide FVIII3, representing the CD4-restricted epitope included into the peptide FVIII1.1 used to immunize the mice. Cytokines are measured intracellularly by flow-cytometry. The dotted line represents the average of the PBS control mice. Data are plotted as mean ± SD. Each dot represents 1 mouse. Statistics: the data distribution was tested for normality by Kolmogorov-Smirnov test and parametric or not-parametric unpaired t tests were computed accordingly. ns = non-significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.*

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of Sanquin Blood Supply. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Animals were handled in agreement with local ethical authorities (APAFIS #44404-202308101204894 v5).

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Authors' disclosures

The authors declare no competing financial or non-financial interests.

Authors' contributions

Y.v.K., E.N. and S.D.L designed the research; E.N, S.D., K.O., B.C.K.D. performed research; E.P., H.K., M.J. and R.L. contributed vital reagents; E.N. analyzed data and made the figures; E.N. wrote the paper; all the others contributed to critically edit and review the paper.

Data sharing statement

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

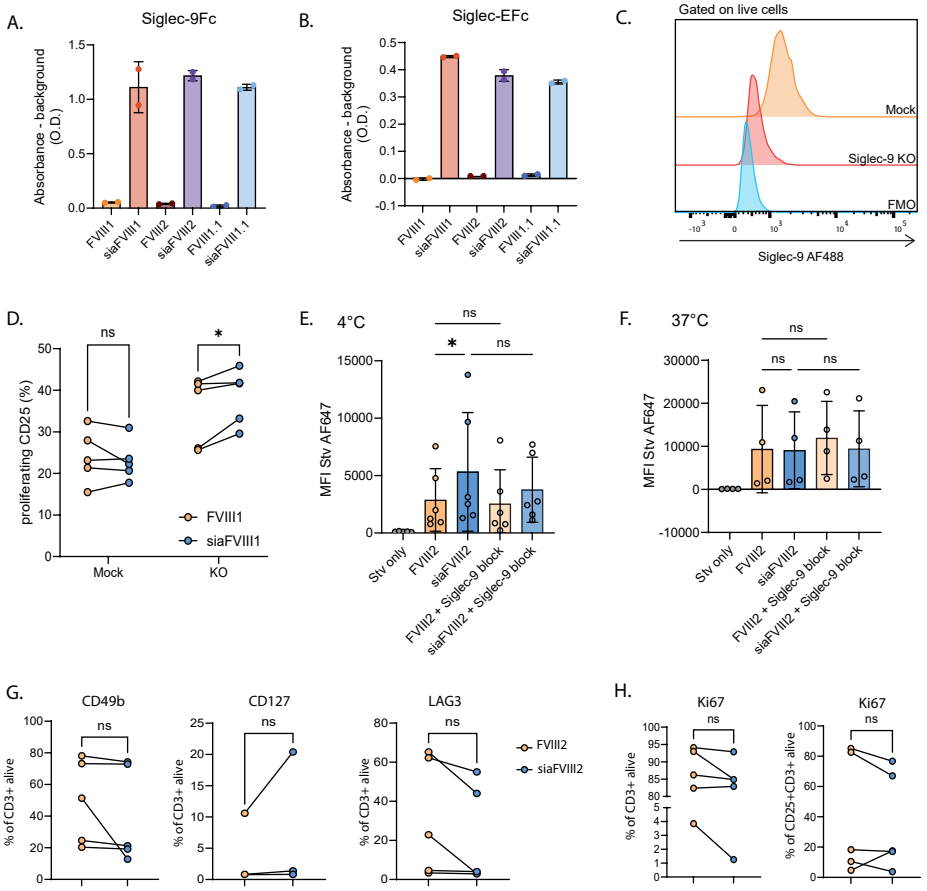
Use of generative AI disclosure

The authors declare that they used ChatGPT (OpenAI, GPT-4) to assist in language revision and refinement of the manuscript. All content was subsequently reviewed, verified, and edited by the authors.

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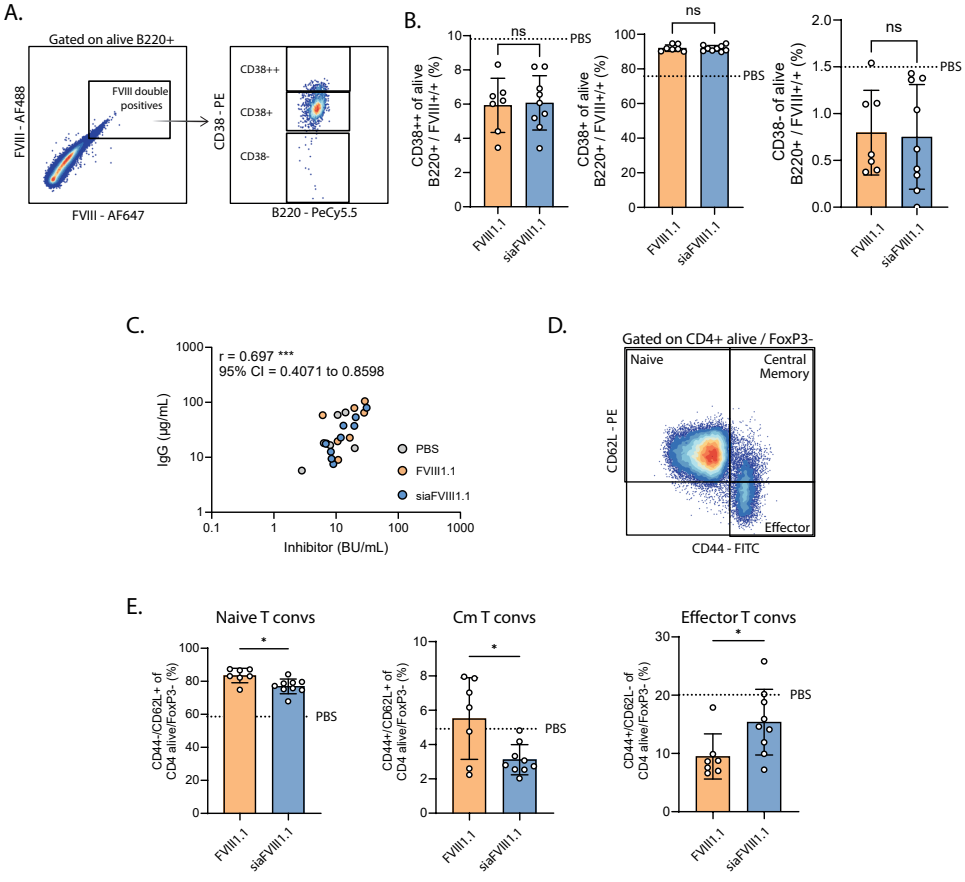
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Supplementary information



Supplementary Figure 1: A and B. *All the sialylated peptides used in the study bind to Siglec-9 and Siglec-E. The peptides were coated on a MaxiSorp ELISA plate at a concentration of 10 µM and detected with 0.2 µg/mL (Siglec-9Fc, A) or 2 µg/mL (Siglec-EFc, B), and 0.2 µg/mL of anti-IgG-HRP, visualized with TMB substrate. Data are presented as mean ± SD. Each dot represents a technical duplicate.* ***C. and D.*** *Allogeneic co-cultures of CD4+ T cells with moDCs nucleofected with Cas-9 (mock) and loaded with siaFVIII1 showed reduced proliferation within the activated CD25+ subset compared to controls. On the other hand, moDCs nucleofected with Cas-9 pre-complexed with a gRNA targeting Siglec-9 (Siglec-9 KO) and loaded with siaFVIII1 induce higher proliferation of allogenic CD4+ T cells.* ***C.*** *Representative histogram showing Siglec-9 expression across moDCs mock and Siglec-9 KO. FMO is the fluorescence minus one control.* ***D.*** *Quantification of proliferated T cells stimulated by FVIII1 (orange dots) or siaFVIII1 (blue dots)- loaded moDCs, etiher mock or Siglec-9 KO. Each dot represents one moDC donor. N = 6. Statistics: paired t test. ns = not significant, * = $p < 0.05$.* ***E. and F.***

*Biotinylated FVIII2 and siaFVIII2 are bound/internalized by moDCs. The binding/uptake was measured by pre-complexing the peptides with streptavidin-AF647 and incubating 0.05 x 106 moDCs/well with 0.5 µM of the peptides/streptavidin complexes for 1h at 4°C (E) or 37°C (F). Where indicated, the cells were pre-incubated with 10 µg/mL of anti-Siglec-9 antibody. Data are presented as mean ± SD. Each dot represents one donor and it is the average of two technical duplicates. Statistics: the data distribution was tested for normality by Kolmogorov-Smirnov test and Friedman test with Dunn's multiple comparisons correction was computed. * = p < 0.05, ns = not significant. stv = streptavidin.* ***G and H.*** *Representative dot plots showing the final gate of the gating strategy to select live CD3+CD69+ (G.) and CD3+PD-1+ (H.) cells. FMO = fluorescence minus one control.* ***I. and J.*** *Exhaustion markers and proliferation of the CD4+ T clone are not modified upon stimulation with moDCs loaded with FVIII2 or siaFVIII2. G. Percentages of CD49b, CD127, LAG3 positive T cell clones stimulated by FVIII2 (yellow dots) or siaFVIII2 (blue dots), measured by flow cytometry. N = 5 moDCs donors.* ***H.*** *Percentages of Ki67+ (left) and Ki67+/CD25+ (right) T cell clones stimulated by FVIII2 (yellow dots) or siaFVIII2 (blue dots), measured by flow cytometry. N = 5 moDCs donors. Statistics: the data distribution was tested for normality by Kolmogorov-Smirnov test and parametric or not-parametric paired t tests were computed accordingly. ns = not significant.*



Supplementary Figure 2. Prophylactic treatment with siaFVIII1.1 does not prevent the insurgence of FVIII-specific B cells. A. *Representative dot plots of the gating strategy used to identify FVIII double positive B cells by flow cytometry in the spleens of mice pre-treated with FVIII1.1 or siaFVIII1.1 or PBS.* ***B.*** *Percentages of CD38++ , CD38+ and CD38- B cells in the spleens of mice pre-treated with FVIII1.1 or siaFVIII1.1, analyzed by flow cytometry. The mean of PBS group mice is indicated as a dotted line in each graph.* ***C.*** *Correlation between anti-FVIII IgG and inhibitors in sera collected at day 31 from animals treated with PBS (grey dots), FVIII1.1 (orange dots) or siaFVIII1.1 (blue dots). Statistics: Spearman correlation (r); 95% confidence intervals (C.I.) are also shown.* ***D.*** *Stacked plots showing the distribution of naïve, central, effector and double negative T cells in the T conventional (convs, left) and T reg (right) subsets measured by flow cytometry based on CD44/CD62L expression.* ***E.*** *Gating strategy to identify different conventional T cell subsets by flow cytometry in the spleens of mice pre-treated with FVIII1 or siaFVIII1.1 or PBS. F. Quantification of the percentages of naïve, central memory (Cm) and effector T conventional (T convs) cells in the spleens of mice pre-treated with FVIII1.1 or siaFVIII1.1 or PBS analyzed by flow cytometry. The dotted line represents the means of mice pre-treated with PBS. Data are plotted as mean ± standard deviation (SD). Each dot represents 1 mouse. Statistics: the data distribution was tested for normality by Kolmogorov-Smirnov test and parametric or not-parametric paired t tests were computed accordingly. ns = not significant, * = $p < 0.05$.*

SUPPLEMENTAL METHODS

Peptides-synthesis and conjugation to the sialylated glycan

Peptides (KHNIFNPPIIARYIRLHPTHYSIRST, residues 2155-2180;

SGIKHNIFNPPIIARYIRLHPTHYSIRSTLRM, 2152-2183;

ARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTG, 2108-2140;

KHNIFNPPIIARYIR, residues 2155-2169) were synthetized by solid Fmoc chemistry and coupled to the sialylated glycan as previously described (1).

moDC stimulation and co-culture with T cells

Monocytes were obtained by positive selection with CD14+ MACS beads (Miltenyi Biotec, CA) from PBMCs isolated from the buffy coats of healthy donors (Sanquin, reference: S03.0023-XT) by Ficoll density gradient. Once purified, 1.25 x 10^6 monocytes per mL were kept in culture at 37°C, 5% CO2 in medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine) supplemented with 500 U/ml IL-4 and 800 U/ml GM-CSF to generate monocyte-derived dendritic cells (moDCs), as previously described (2). At day 5 of differentiation, 0.1 x 10^6 cells per condition were seeded in a U-bottom

96-well plate and incubated for 1 to 3h with 10 µM FVIII1, siaFVIII1 or medium before maturing them overnight with 10 ng/mL LPS (LPS-EB Ultrapure from E. coli O111:B4, Invivogen). The next morning the supernatants were collected and kept at -20°C until further analysis while the cells were prepared for flow-cytometric analyses of maturation markers. Alternatively, at day 6 of differentiation, the moDCs were harvested, counted and 0.2 x 10^6 cells per condition were plated in a 24-well culture plates (Greiner Bio-One) to be stimulated overnight at 37°C, 5% CO_2 with 10 µM $FVIII_{2155-2183}$, $siaFVIII_{2155-2183}$ or medium in the presence of 10 ng/mL LPS (LPS-EB Ultrapure from E. coli O111:B4, Invivogen). The next morning, the cells were harvested and counted and used to perform 1:5 (20000 moDCs : 100000 T cells) co-cultures in triplicate with allogenic naïve CD4+ T cells isolated from PBMCs of healthy donors by negative selection with a kit (Miltenyi Biotec), as recommended by the manufacturer. To track proliferation, the T cells were stained with 5 µM of CellTraceViolet in PBS prior of seeding them. The co-cultures were plated in 96 flat-bottom culture plates (Greiner Bio-One) in T cell medium (IMDM, Gibco, supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine) and kept for 6 days at 37°C, 5% CO2. Then, the supernatants were collected and kept at -20°C until further analysis, while the cells were stained for flow cytometry.

Generation of moDCs Siglec-9 KO and co-culture with allogenic CD4 T cells

Monocytes were obtained by positive selection with CD14+ MACS beads (Miltenyi Biotec) from PBMCs isolated from the buffy coats of healthy donors (Sanquin, reference: S03.0023-XT) by Ficoll density gradient. Once purified, the cells were divided among three conditions: wild type, mock and Siglec-9 KO. The wild type cells were left untouched. One million of the mock and the Siglec-9 KO conditions underwent nucleofection (Lonza) with 2.5 µL of a 20 µM solution of precomplexed RNPs, as previously described (Hiatt et al., 2021). Briefly, stock solutions of 20 µM RNPs were generated by incubating equal volumes of 40 µM Cas9 nuclease protein NLS (Horizon CAS12206) and 80 µM gRNAs at 37°C for 15 min. The guideRNA targeting Siglec-9 CCCTCTCCCTCCCCCAGAGC was synthesized by Genscript using their CRISPR gRNA Synthesis Service. Mock monocytes were obtained by precomplexing tracrRNA (Dharmacon, U-002005–0,050) with Cas9. Next, all cell suspensions were plated in medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine) supplemented with 500 U/ml IL-4 and 800 U/ml GM-CSF to generate monocyte-derived dendritic cells (moDCs) at a concentration of 1.25 x 10^6 monocytes/mL in a 24-well plate. At

day 6 of differentiation, the moDCs were harvested, counted and 0.02 x 10^6 cells per condition were plated in 96-well U bottom plates (Greiner Bio-One) previously coated with 10 µM of FVIII1, siaFVIII1 or medium in the presence of 10 ng/mL LPS (LPS-EB Ultrapure from E. coli O111:B4, Invivogen) to be stimulated overnight at 37°C, 5% CO_2 . The next morning, co-cultures with T cells were performed in triplicate. The allogenic naïve CD4+ T cells were isolated from PBMCs of healthy donors by negative selection with a kit (Miltenyi Biotec), as recommended by the manufacturer. To track proliferation, the T cells were stained with 5 µM of CellTraceViolet in PBS prior of seeding them. The T cells were added at 0.1 x 10^6 cells per condition in T cell medium (IMDM, Gibco, supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine) to the moDCs and the co-cultures were kept for 6 days at 37°C, 5% CO2. Then, the supernatants were collected and kept at -20°C until further analysis, while the cells were stained for flow cytometry.

Antigen presentation experiments

The CD4 T cell clone used in the experiments was kindly provided by Dr. Marc Jacquemin (3). HLA-DR matched (DRB1 01:01:01/15:01:01) moDCs were generated from healthy donors PBMCs by Percoll gradient as previously described (2). At day 6 of differentiation, moDCs were harvested, counted and pulsed for 3h with 10 µM FVIII2, siaFVIII2 or medium in the presence of 10 ng/mL of LPS (LPS-EB Ultrapure from E. coli O111:B4, Invivogen). After washing, an overnight 1:5 co-culture of 2000 moDCs and 10 000 T cells was performed. The next morning, the supernatants were collected and stored at -20°C until further analyses, while the cells were collected and prepared for flow cytometric analyses.

Flow cytometry

To prepare the samples for flow cytometry, the cells were first incubated for 15 mins on ice with the fixable/viability dye eFluor780 (Thermo Fisher Scientific) or LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Thermo Fisher Scientific) and Fc-block (BD Biosciences). Then, cells were incubated for 30 mins on ice with the antibody mixed **(Table 1)** diluted in PBS + 0.1% BSA (Roche) + 0.02% sodium azide and brilliant stain buffer (BD Biosciences). After staining cell surface markers, cells were fixed-permeabilized using FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher, Waltham, Mass) following the manufacturer's instructions and stained intracellularly. Alternatively, a solution of 0.5% saponin in PBS was used

to permeabilize the cells, previously fixed in 1% PFA diluted in PBS. The samples were measured at the LSR Fortessa X-20 (BD Bioscience) and analyzed with FlowJo (v10). Alternatively, samples obtained from *in vivo* and *ex vivo* experiments were acquired at the ID7000™ Spectral Cell Analyzer (Sony), the co-cultures with the CD4+ T cell clone were acquired at the Aurora 4L (Cytek Biosciences) and analyzed with using the OMIQ software from Dotmatics (www.omiq.ai,). The data were first cleaned by using PeacoQC, then the files were gated for lymphocytes (based on FSC-A/SSC-A), single cells (based on FSC-A/FSC-H), CD3+/LiveDead Blue-, and subsampled to the maximum number of cells available in the final gate, so as to input the same number of events in the downstream analysis. In all the stainings, fluorescence-minus-one control samples were generated and used to manually set the gates.

Antigen	Fluorochrome	Clone	Manufacturer
CD86	FITC	2331 (FUN-1)	BD Biosciences
CD40	PE/Cy7	5C3	Biolegend
HLA-DR	BV510	G46-6	BD Biosciences
CD80	BV605	L307.4	BD Biosciences
CD3	BV510	UCHT1	Biolegend
CD25	APC	BC96	Biolegend
CD69	FITC	FN50	Biolegend
CD49b	BV605	AK-7	BD Biosciences
CD127	BUV395	HIL-7R-M21	BD Biosciences
LAG-3 (CD223)	APC/Cy7	7H2C65	Biolegend
CD279 (PD-1)	AF700	EH12.2H7	Biolegend
Ki67	APC	Ki-67	Biolegend
CD4	BV510	GK1.5	Biolegend
CD25	BV421	PC61	Biolegend
CD44	FITC	IM7	Biolegend
CD62L	PE	MEL-14	eBioscience
FoxP3	PE/Cy5	FJK-16s	Thermo Fisher Scientific
B220	PE/Cy5.5	RA3-6B2	eBioscience
CD38	PE	90/CD38	BD Biosciences
IFN-γ	APC	XMG1.2	Biolegend
TNF-α	PE/Cy7	MP6-XT22	Invitrogen

Table 1 List of antibodies used for flow cytometry experiments.

Cytokine ELISAs

The supernatants from the in vitro human cultures were tested for the presence of cytokines by ELISA using the following antibody pairs: IL-6 (Invitrogen AHC0562 and eBioscience 13-7068-85), IL-12p70 (eBioscience 14-7128-82 and 13-7129-85), IFN-γ (eBioscience; 14-7318-85 and 13-7319-85). As a standard curve, different dilutions of commercial cytokines were used (IL-6 from Immunotools; IL-12p70 from BD; IFN-γ from Invitrogen). Briefly, the capture antibodies were coated in NUNC MaxiSorp plates (Greiner Bio-One) overnight at 4°C in 0.05 mol Na2CO3 of PBS (pH 9.7). The plates were blocked with 1% BSA for 30 mins at 37°C and then incubated with the supernatants and the biotinylated detection antibodies for 2 h at room temperature. Detection was performed with HRP-conjugated anti-human IgG Fc (Jackson ImmunoResearch) and revealed using TMB. Absorbance was read at 450 nm with iMarkTM Microplate Absorbance Reader (Bio-Rad).

Mice

Six- to 10-week-old male and female FVIII exon 16 knock-out (FVIII-KO) mice on the C57BL/6 background were used. Animals were handled in agreement with local ethical authorities (APAFIS #44404-202308101204894 v5).

In vivo and ex vivo studies:

Mice were treated with subcutaneous injections in the neck 3 times per week for 2 weeks with FVIII1.1 or siaFVIII1.1 dissolved in 100 µL of PBS following a dose escalation regimen (0.3-3-30-300-300-300 µM). Control mice received PBS. All groups were then immunized weekly by retro-orbital injections of 1 IU of full-length FVIII (Helixate Nexgen,CSL Behring) in 100 µL of PBS for 5 weeks. Two days after every FVIII injection, mice received subcutaneously 100 µl of FVIII1.1 or siaFVIII1.1 at 300µM in PBS, while control mice received 100 µL of PBS. Blood was collected from the retro-orbital sinus at different time points, as indicated in Figure 3A, in citrated tubes (Sigma Aldrich). Serum was obtained by 15-minute centrifugation step and stored at - 20°C until further analyses. At day 31, the mice were sacrificed and the spleens were collected to generate single-cell suspensions. Three million splenocytes per mouse were stained for flow cytometry, while 3 million splenocytes per mouse were seeded in 96-well U-bottom plates (Greiner Bio-One) to be restimulated overnight with 100 µg/mL of the FVIII-derived peptide FVIII1.3 (KHNIFNPPIIARYIR), residues 2155-2169. Next, the cells were prepared for flow cytometric intracellular detection of cytokines by blocking protein transport with GolgiPlug (BD Biosciences) for 5 hours.

Anti-FVIII or anti-FVIII peptide IgG ELISA and FVIII inhibitor assays

Nunc MaxiSorp plates (Greiner Bio-One) were coated overnight at 4°C with 2 µg/mL of full-length FVIII (Advate, Takeda) or 2 µM or FVIII1.1 or siaFVIII1.1. After blocking buffer with PBS with 3% BSA (Sigma Aldrich) for 1 hour at 37°C, plates were washed and incubated with serial dilutions of the mouse sera for 1 hour at room temperature. Peptide or FVIII-bound IgGs were detected with goat anti-Mouse IgG Fc-HRP (SouthernBiotech) and 3,3′,5,5′-tetramethylbenzidine (TMB) or o-phenylenediamine dihydrochloride (OPD) substrate (Sigma Aldrich). Absorbance was read at 492 nm with a TECAN Infinite 200 for the anti-FVIII IgGs or at 450 nm with iMarkTM Microplate Absorbance Reader (Bio-Rad). As standard curve for the anti-FVIII IgG ELISAs, a pool of anti-FVIII mouse IgGs (clones GMA8015, ESH4, GMA 8026) was used, whereas for the anti-FVIII peptide ELISA the area under the curve was used to quantify the amount of anti-peptide IgGs, computed with GraphPad PRISM (v10). To determine the inhibitor titers, the sera samples were first decomplemented and then incubated at different dilutions with a standard human plasma (Siemens Healthineers) for 2 hours at 37°C. Next, FVIII residual activity was measured by chromogenic assay (Siemens Helthineers) following the manufacturer's instructions. FVIII-inhibitor titers were calculated as previously described (4) and expressed in Bethesda units (BU)/mL.

Statistics

Statistical tests were run with Graphpad PRISM (v10). Specific tests performed are indicated in the legend of each figure.

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SIALIC ACID-MODIFIED DER P 2 ALLERGEN EXERTS IMMUNOMODULATORY EFFECTS ON HUMAN PBMCS

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ABSTRACT

Background: House dust mite (HDM) extract based allergen immunotherapy (AIT) to treat HDM allergy is substantially effective but still presents some safety and efficacy concerns that warrant improvement. Several major allergen-based approaches to increase safety and efficacy of AIT have been proposed. One of them is the use of the group 2 allergen, Der p 2.

Objective: In this study, we investigated the immunomodulatory effects of sialic acid-modified major allergen recombinant Der p 2 (sia-rDer p 2) on PBMCs from healthy volunteers.

Methods: We activated PBMCs with anti-CD3/CD28 antibodies and incubated them at 37°C for 6 days in the presence or absence of either native rDer p 2 or α2-3 sialic acid-modified rDer p 2 (sia-rDer p 2). We assessed the changes in CD4+T cell activation and proliferation by flow cytometry and changes in T lymphocyte cytokine production in cell culture supernatant by ELISA.

Results: We observed that, PBMCs treated with sia-rDer p 2 presented with a markedly decreased expression of CD69 and an increased abundance of LAG-3+ lymphocytes compared to cells treated with rDer p 2. Moreover, PBMCs treated with sia-rDer p 2 showed a reduced production of IL-4, IL-13 and IL-5 and displayed a higher IL-10/IL-5 ratio compared to rDer p 2-treated PBMCs.

Conclusion: We demonstrate that α2-3 sialic acids-modified rDer p 2 might be a safer option than native rDerp2 for Der p 2-specific AIT. This is most relevant in the early phase of AIT that is often characterized by heightened Th2 responses, as sia-rDer p 2 does not enhance the production of Th2 cytokines.

INTRODUCTION

The standard therapeutic strategies for managing immune dysregulation, such as the excessive immune response in allergic reactions, mostly involve the use of immunosuppressive drugs, like corticosteroids, Janus kinase and Calcineurin inhibitors, which lead to generalized immunosuppression (1). Because of the multiple side effects associated with immunosuppressive drugs, preventing or attenuating immune dysregulation in a specific manner is highly desirable. Most strategies under investigation for inducing targeted immune tolerance often utilize (nano- or lipid-) particles encapsulating allergens and/or immunosuppressive compounds (2-4). These strategies generally aim to alter antigen presenting cells (APC) to suppress inflammatory T cells and induce regulatory T cells (5).

Directly targeting APC including dendritic cells (DC), monocytes and B cells is an attractive strategy to modulate T cell function and to induce antigen-specific tolerance (5). In particular, inhibitory receptors expressed by APC, such as the Siglec (sialic acid-binding immunoglobulin-like lectin) family of carbohydrate-binding proteins are a good target. Siglecs recognize sialic acid-containing glycoproteins and -lipids and can be targeted to induce immune suppressive responses (5,6). The majority of the Siglecs, including Siglec-9 and -10, discussed in this study, possess immunoreceptor tyrosine-based inhibitory motif (ITIMs), which transmit immune deactivating signals upon recognition of their ligand (sialic acids) and can thus be therapeutically exploited for the management of inflammatory diseases (6).

We previously demonstrated that *ex-vivo* and *in-vivo* DC-targeting of α2-3 sialic acid-modified antigen to the mouse homologue of human Siglec-9, Siglec-E, drove naive CD4+T cell differentiation into antigen-specific regulatory T cells (7). Moreover, DCs treated with sialic acid-modified antigen dampened T cell differentiation to effector T cells even in the presence of native antigen-loaded DCs (7). Also, exposing LPS-stimulated human monocyte-derived DCs to Siglec-9 ligands, α2-3 sialic acids or anti-Siglec-9 antibodies, suppressed the production of IL-6 and IL-12 (8, 9). Together, these reports show that targeting Siglec-9 inhibits Th1 responses. However, very little is known about whether Siglec-9-mediated interferences can also alter allergen-associated Th2 responses, characterized by the secretion of IL-4, IL-5 and IL-13. We therefore evaluated the immunomodulatory effects of α2-3 sialic acid-modified antigen on the Th2 arm of T helper cells in human PBMCs using Der p 2 as a model antigen. Der p 2 is one of the established major allergens from house dust mite (HDM) and is an important risk factor for the development of allergic rhinitis and asthma (10,

11). Consequently, research into the use of Der p 2 for Allergen immunotherapy (AIT) against Der p 2-HDM allergy instead of HDM extracts has grown in the past decades since AIT with HDM still presents some safety and efficacy concerns that warrant improvement (4). Exploiting the sialic acid-Siglec axis by modifying Der p 2 via its 12 free Lysine residues (14) with sialic acids, could provide Siglec-mediated crosslinking and signaling to attenuate allergen-specific Th2 mediated immune responses (12).

METHODS

α2-3 sialic acid-rDer p 2 binding to Siglec-human Fc chimeras

NUNC maxisorb plates (Greiner Bio-One, Denmark) were coated with 10 µg/ml of rDer p 2 or sia-rDer p 2 overnight at room temperature (RT). After washing with Hanks' Balanced Salt Solution (HBSS) (Gibco, USA), wells were blocked with Carbo-free Blocking Buffer (CFBB) – glycoprotein-free blocking agent (Vector Laboratories, USA) diluted 1:10 in HBSS for 1 hour at RT and subsequently incubated with Siglec-Fc chimeras and goat anti-human Fc-PO antibody (Jackson Laboratory, USA). The following Siglec-Fc chimeras (R&D Systems, USA) were used: Siglec-2-hFc (1968-SL-050), Siglec-3-hFc (1137-SL-050), Siglec-7-hFc (1138-SL-050), Siglec-9-hFc (1139-SL-050), and Siglec-10-hFc (2130-SL-050).

For all colorimetric assays, plates were developed using 3,3′,5,5′-tetramethylbenzidine (TMB) (Merck, Germany) as a substrate and H2SO4 as stop solution. The iMarkTM Microplate absorbance reader (Bio-RAD, USA) was used to measure absorbance at 450nm.

Cell isolation and culture

Blood from healthy volunteers for the isolation of peripheral blood mononuclear cells (PBMCs) was supplied by the Sanquin Blood Bank (Sanquin, The Netherlands). Healthy donors (non-allergic) gave their written consent for the use of blood donation for research purposes. We performed an ImmunoCAP assay to confirm the allergic status of donors. PBMCs were isolated from whole blood by density gradient centrifugation (2000 rpm, 30 min) using Lymphoprep (Serumwerk, Germany). After isolation, PBMCs were frozen and stored at -80°C and/or liquid Nitrogen until use. PBMCs were thawed and plated in 96-well round-bottomed plates (Greiner Bio-One, The Netherlands) at a density of 10×10^6 cells/

ml, in RPMI 1640 (Thermo Fisher) supplemented with 10% inactivated fetal bovine serum (FBS) (Biowhittaker, Switzerland), 1% glutamine (Thermo Fisher), and 1% penicillin-streptomycin (Lonza, Switzerland). The PBMCs were left inactivated or activated with plate-bound anti-CD3 (10 µg/ml) (clone SPV-T3b, Monoclonal Antibody Facility MCBI, The Netherlands) and soluble anti-CD28 (2 µg/ml) (Sanquin). The activated PBMCs were subsequently either left untreated (control) or treated with rDer p 2 (10 µg/ml) or sia-rDerp2 (10 µg/ml), for 6 days, at 37 °C, 5% CO2 in humidified air. Supernatants were harvested and stored at -20°C for cytokine measurements and cells were harvested for flow cytometry.

Prevention or reversion of PBMC responses

To investigate whether sia-rDer p 2 can revert an existing inflammation involving rDer p 2, anti-CD3/CD28-activated PBMCs (10×10^6 cells/ml) were first treated with rDer p 2 (10 µg/ml) for 2 days, after which cells were washed with RPMI medium and sia-rDer p 2 (10 µg/ml) was added to the cells and cultured for another 4 days. To investigate whether sia-rDer p 2 can prevent inflammation involving rDer p 2, anti-CD3/CD28-activated PBMCs were first treated with sia-rDer p 2 (10 µg/ml) for 2 days, cells were washed with RPMI medium and rDer p 2 (10 µg/ml) was added to the cells and cultured for another 4 days. This setup was adapted from that previously described (13).

Flow cytometry

For the characterization of the different cell populations within PBMCs and the evaluation of Siglec expression, cells (1×106/well) were stained with monoclonal antibodies (Table 1) for 30 minutes on ice in a 96-well V-bottomed plate (Greiner Bio-One). Antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA) (Roche, Switzerland), 0.02% sodium azide, Fc block (in-house) and True-Stain monocyte blocker (Biolegend, The Netherlands). Fixable Viability stain, Zombie NIR (Biolegend) or LIVE DEAD Blue (Thermo Fisher) were used to stain dead cells for 15 minutes prior to staining surface markers. For intracellular Ki67, IL-10 and FoxP3 detection, cells were incubated with 0.5 µg/ml of PMA (Sigma-Aldrich, Bulgaria) and 1 µg/ml of ionomycin (Sigma-Aldrich) in the presence of GolgiStop and Golgiplug (BD Biosciences, Belgium) for 4 hours. After staining cell surface markers, cells were fixed-permeabilized using FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher) as directed by the manufacturer and stained with intracellular antibodies **(Table 1)**. Labelled cells were fixed with 1%

paraformaldehyde (PFA) (Electron microscopy sciences, USA) and stored at 4°C until acquisition. Fluorescence minus 1 controls were prepared from for each cell marker and used for gating. Stained samples were acquired with the 4 or 5 laser Aurora flow cytometer (Cytek Biosciences, The Netherlands). FCS files were analyzed using FlowJo software v10.8 (BD Biosciences).

Fluorochrome	Antigen	Clone	Manufacturer	Catalog no.
PE-Cy5	CD33	WM53	BioLegend	303406
Atto647	CD56	NBL-1	Monoclonal Antibody Facility MCBI	NA
PE-CF-594	CD56	NCAM16.2	BD Horizon	564849
BV510	CD3	UCHT1	BioLegend	300448
APC-Cy7	CD3	UCHT1	BioLegend	300425
BV605	CD3	UCHT1	BioLegend	300460
PerCP	CD4	OKT4	BioLegend	317432
BV711	CD4	RPA-T4	BioLegend	300558
BV421	CD8	RPA-T8	BioLegend	301036
BV750	CD19	HIB19	BioLegend	302262
BUV496	CD19	SJ25C1	BD Biosciences	612938
FITC	CD69	FN50	BD Biosciences	555530
BUV737	CD25	2A3	BD Biosciences	612806
PE-Dazzle594	CRTH2	BM16	BioLegend	350126
CXCR3	PE-Fire810	G025H7	BioLegend	353760
BV605	CD49b	AK-7	BD Biosciences	742646
APC-Cy7	LAG-3	7H2C65	BioLegend	369219
Alexa Fluor 700	PD-1	EH12.2H7	BioLegend	329952
V450	FoxP3	259D/C7	BD Biosciences	560459
APC	Ki67	Ki-67	BioLegend	350513
PE-Cy7	IL-10	JES-9D7	BioLegend	501420
eFluor450	CD14	61D3	Invitrogen	48-0149-42
BV605	CD14	M5E2	BioLegend	301834
BV480	HLA-DR	G46-6	BD Biosciences	566113
BUV563	HLA-DR	L203.rMAb	BD Biosciences	752494
BV510	CD16	3G8	BioLegend	302048
PE-Cy5	CD16	3G8	BioLegend	302010
BV785	CD123	6H6	BioLegend	306032
BV650	CD11c	3.9	BioLegend	301638

FITC	CD1c	L161	SONY Biotechnologies	2257585
BV711	CD141	1A4	BD Biosciences	563155
PE-Cy7	Siglec-1	7-239	BioLegend	346014
PE	Siglec-2	HIB22	BioLegend	302506
BV650	Siglec-3	P67.6	BioLegend	366612
BB515	Siglec-5	194128	BD Biosciences	565198
Alexa Fluor 700	Siglec-6	767389	R&D Systems	FAB2859N
PerCP vio700	Siglec-7	REA214	Miltenyi Biotec	130-100-979
Alexa Fluor 488	Siglec-9	191240	R&D Systems	FAB1139G
APC	Siglec-10	5G6	BioLegend	347606

FITC, Fluorescein isothiocyanate; MCBI, Molecular Cell Biology and Immunology; NA, not applicable; PE, phycoerythrin.

Table 1. *mAbs used for flow cytometry, including clone, manufacturer, and catalog number*

Binding of sia-rDer p 2 to PBMC

1x10^6 PBMCs/well were washed with 0.5% BSA in HBSS (ice-cold) in a 96 well V-bottomed plate. 10 µg/mL rDer p 2 or sia-rDer p 2 or 10 µg/mL Polyacrylamide (PAA)-α2-3-biotin (positive control, PC) was added to the cells and incubated for 1 hour at 37°C. Subsequently, 10 µg/mL of biotinylated anti-Der p 2 antibody (Absolute antibody, The Netherlands) was added and incubated for 45 min at 4°C. Cells were then stained with monoclonal antibodies to characterize PBMC subsets **(Table 1)** and Streptavidin-PE to detect binding (Jackson Laboratories, USA) for 30 min at 4°C. Stained cells were fixed in 1% PFA and acquired with the 5 laser Aurora flow cytometer (Cytek Biosciences).

Cytokine quantification

The concentrations of IL-4, IL-5, IL-10, IL-13, IL-17, and IFN-γ in supernatants were quantified by sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) using the following anti-cytokine antibody pairs: IL-4 (Biosource, USA, AHC0642 and AHC0749), IL-5 (Biolegend, 500902 and 501002), IL-10 (eBioscience, 14-7108-85 and 13-7109-85), IL-13 (Invitrogen, P130E and M130B), IL-17 (eBioscience, 14-7178-85 and 13-7179-85), and IFN-γ (eBioscience, 14-7318-85 and 13-7319-85). The capture antibody was coated in NUNC MaxiSorp plates (Greiner Bio One) overnight at 4 °C in 0.05M Na2CO3 buffer, pH 9.7 (Merck). The plates were blocked with 1% BSA for 1 hour at 37 °C. Supernatants and biotinylated antibodies were added and plates were incubated for 2 hours at RT.

Statistical analysis

Data represent the mean ± SD as indicated in figure legends. P-values were determined by Wilcoxon paired test using GraphPad Prism v9 (Graphpad, USA) as indicated in figure legends. Differences in values were considered significant at a p value <0.05.

RESULTS

Sia-rDer p 2 binds to APCs

α2-3 sialic acids were chemically conjugated to rDer p 2 through a maleimide-thiol reaction to produce glyco-allergen conjugates (sia-rDer p 2) (see the Methods section, supplementary materials) **(Fig 1, A)**. We performed a lectin-binding ELISA to confirm the presence of α2-3 sialic acids on sia-Der p 2 (see the Methods section and **Fig E1**, supplementary materials). We then assessed whether sia-rDer p 2 would bind to selected Siglec-Fc chimeras and found that sia-rDer p 2 but not rDer p 2 interacted primarily with Siglec-9–Fc **(Fig E1)**. To investigate whether sia-rDer p 2 would bind to Siglecs expressed on immune cells, we first measured the expression of Siglec-9 and Siglec-10 on the different immune cells within PBMCs (gating strategy; **Fig E1**). Monocytes and DCs but not B cells expressed high levels of Siglec-9, whereas CD14−CD16+ nonclassical monocytes, DCs, and B cells expressed Siglec-10 **(Fig E1)**. CD4+ T cells did not express Siglec-9 or Siglec-10 **(Fig E1)**. We demonstrate that sia-rDer p 2 bound to monocytes, DCs, and B cells **(Fig 1, B and C)**, but not to CD4+ T cells **(Fig E1)**. Binding of sia-rDer p 2 to these cells partially reduced the Siglec-9 expression on these cells on preincubation with sia-rDer p 2 **(Fig 1, D)**, which may indicate partial receptor occupancy. Overall, we illustrate that sia-rDer p 2 bound to APCs and that this binding may be Siglec-mediated.

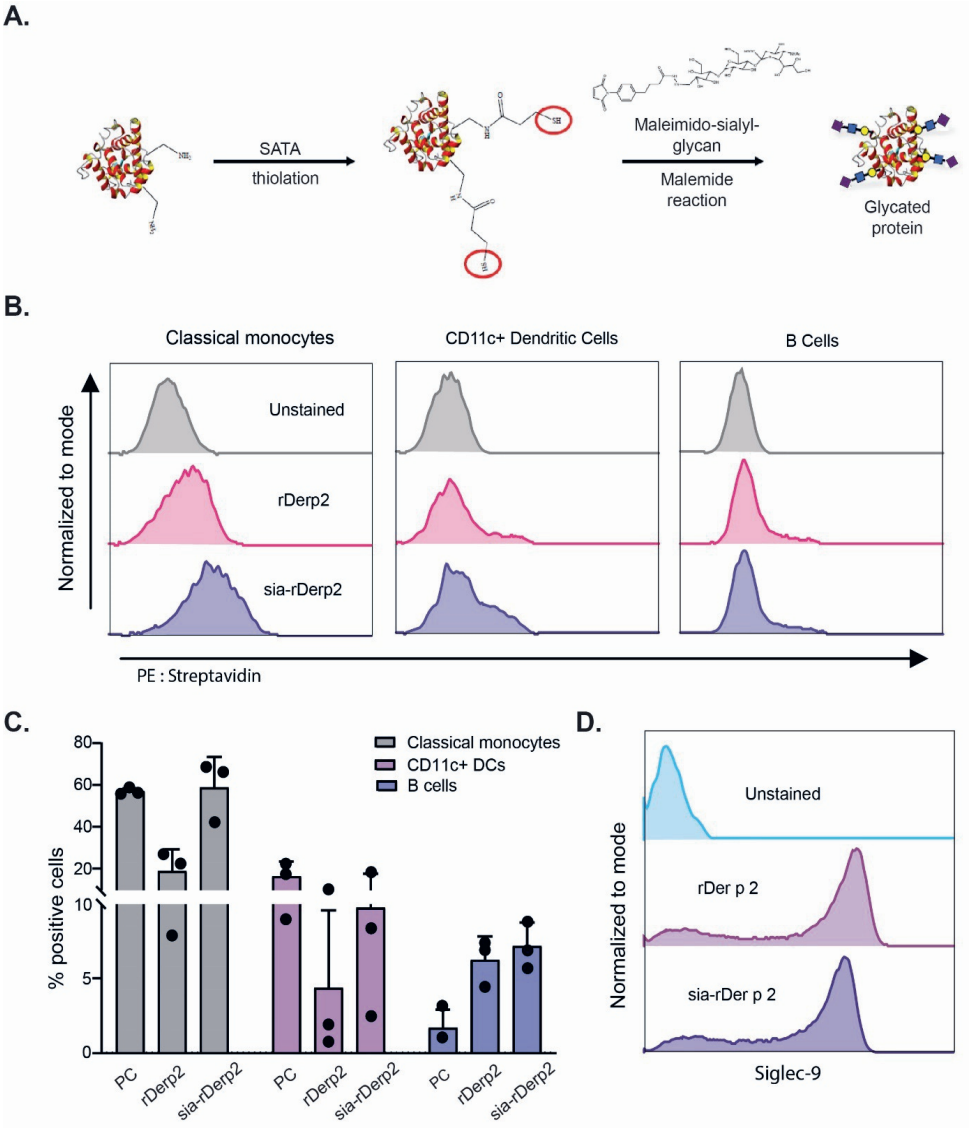


Figure 1. sia-rDer p 2 binds to Siglec-9 and/or Siglec-10 expressed on APCs. ***A****, Schematic representation of the thiol-malemide reaction for the conjugation of α2-3 sialic acids to rDer p 2.* ***B****, Representative histograms showing the binding of rDer p 2 and sia-rDer p 2 on classical monocytes (CD14+CD16+), CD11c+ DCs, and B cells.* ***C****, The percentage of cells among monocytes, CD11c+ DCs, and B cells that bound polyacrylamide-α2-3 (positive control [PC]), rDer p 2, and sia-rDer p 2 (n = 3). Data are shown as mean ± SD.* ***D****, Representative histograms showing the expression of Siglec-9 on classical monocytes after incubation of cells with either rDer p 2 or sia-rDer p 2.*

TH1- and TH2-cell activation is equally suppressed by sia-rDer p 2

To assess whether coincubation of anti–CD3-/CD28-activated PBMCs with sia-rDer p 2 altered the proliferation (expression of intracellular Ki67) and activation (surface expression of CD69 and CD25) of CD4+ T cells, PBMCs were cultured for 6 days with rDer p 2 or sia-rDer p 2 and assessed by flow cytometry (**Fig 2**; see also gating strategy in **Fig E2**). Treatment of PBMCs with rDer p 2 significantly enhanced CD4+ T-cell proliferation and the expression of CD25 and CD69 compared with the control (baseline) **(Fig 2, A-D)**, whereas sia-rDer p 2 did not. Of note, sia-rDer p 2 reduced the percentage of CD69+CD4+ T cells **(Fig 2, C, and Fig E2, B)** and the level of expression of CD69 **(Fig E2)** to lower than baseline levels. This reduction was similar in both T_H1 lymphocytes (on the basis of expression of CXCR3) and T_H2 lymphocytes (on the basis of CRTH2 expression) **(Fig 2, F)**. The difference in proliferation and CD25 expression between the control, rDer p 2, and sia-rDer p 2 within T_H1 and T_H2 lymphocytes did not reach statistical significance **(Fig 2, E and G)**. In conclusion, these data demonstrate that rDer p 2 enhances CD4+ T-cell activation and proliferation, whereas sia-rDer p 2 appears to suppress both T_H1- and T_H2-cell activation.

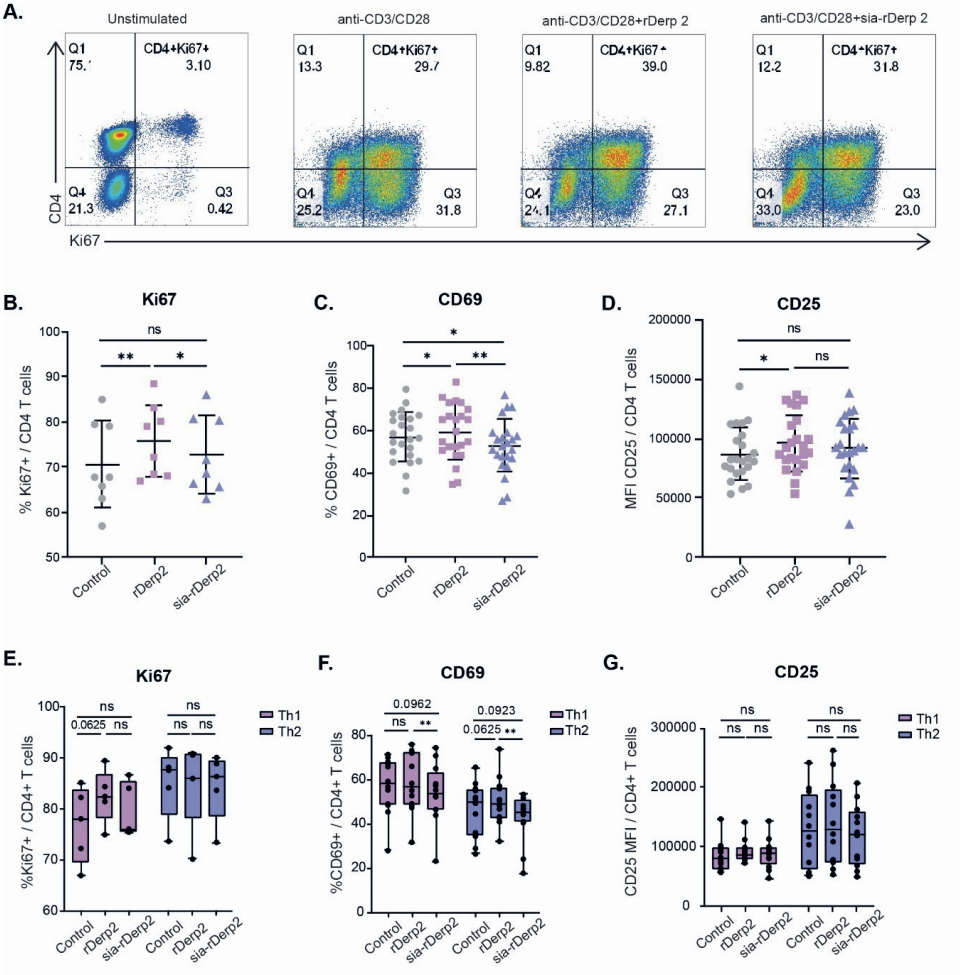


Figure 2. CD4+ T-cell activation is moderately suppressed by sia-rDer p 2. *A, Representative dot plot showing the percentage of Ki67+CD4+ T cells, indicative of the percentage of proliferating CD4+ T cells.* ***B****, The percentage of Ki67+CD4+ T cells (n = 8).* ***C****, The percentage of CD69+CD4+ T cells (n = 23).* ***D****, The MFI of CD25 on CD4+ T cells (n = 23).* ***E****, The percentage of Ki67+ cells among CD4+CXCR3+ T_H1 cells (pink) and CD4+CRTH2+ TH2 cells (purple) (n = 5).* ***F****, The percentage of CD69+ cells among CD4+CXCR3+ T_H1 cells (pink) and CD4+CRTH2+ TH2 cells (purple) (n = 12).* ***G****, The MFI of CD25 on CD4+CXCR3+, TH1 cells (pink) and CD4+CRTH2+ TH2 cells (purple) (n = 12). All results are shown as mean ± SD. MFI, Mean fluorescence intensity; NS, not significant. *P < .05; **P < .01. Wilcoxon matched-pairs test.*

sia-rDer p 2 modulates TH2 cytokine production but not TH1 or TH17 cytokines

Given that sia-rDer p 2 equally modulated both T_H1- and T_H2-cell activation, we investigated whether a similar effect would occur in their cytokine profiles. When compared with baseline, rDer p 2 upregulated the production of IL-4 **(Fig 3, A)** and IL-13 **(Fig 3, B)** but not IL-5 **(Fig 3, C)**, IFN-γ **(Fig 3, D)**, or IL-17 **(Fig 3, E)**. However, sia-rDer p 2 downregulated the production of IL-5 **(Fig 3, C)** but did not affect IL-13, IL-4, IL-7, and IFN-γ **(Fig 3, A, B, D, and E)**. Moreover, compared with rDer p 2, sia-rDer p 2 induced lower production of IL-13 and IL-4 **(Fig 3, A and B)**. We then investigated whether the effects of sia-rDer p 2 on IL-5 were a result of direct binding to CD4+ T cells or mediated by APCs present in PBMCs by co-incubating rDer p 2 or sia-rDer p 2 with pure CD4+ T cells activated with anti-CD3/CD28. The production of IL-5 was not affected **(Fig 3, F)**. We can therefore conclude that sia-rDer p 2downregulates IL-5 secretion and that this may be via APC–T-cell interactions.

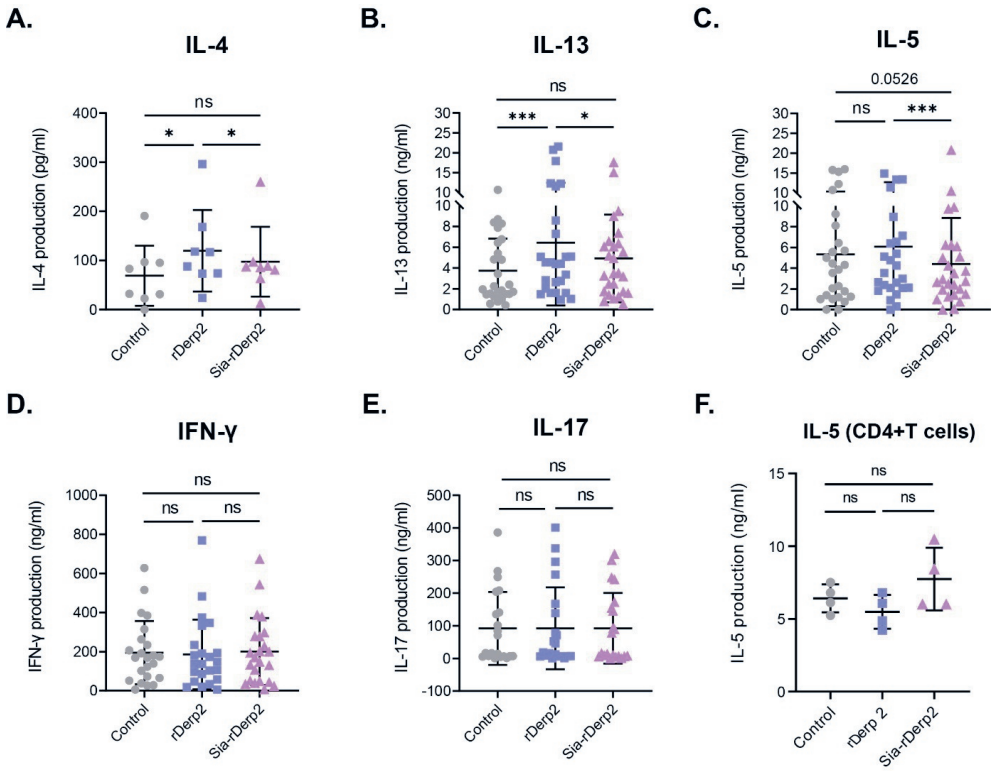


Figure 3. A-E, sia-rDer p 2 modulates the secretion of TH2 cytokines, but not TH1 or TH17 cytokines. *Levels of IL-4 (n = 8)* ***(Fig 3, A)****, IL-13 (n = 25)* ***(Fig 3, B)****, IL-5 (n = 25)* ***(Fig 3, C)****, IFN-γ (n = 12)* ***(Fig 3, D)****, and IL-17 (n = 12)* ***(Fig 3, E)*** *in day 2 (IL-4) or day 6 cell culture supernatants were determined by ELISA.* ***F****, IL-5 was measured in day 6 cell culture supernatants after culturing anti–CD3-/CD28-activated pure CD4+ T cells with rDer p 2 or sia-rDer p 2. All results are shown as mean ± SD. NS, Not significant (Wilcoxon matched-pairs test). *P < .05; ***P < .001.*

The Treg/TH2 cytokine ratio is higher with sia-rDer p 2 than with native rDer p 2

Successful AIT can be characterized by a high IL-10/IL-5 ratio (12). We therefore analyzed culture supernatants of anti–CD3-/CD28-activated PBMCs to determine the impact of rDer p 2 or sia-rDer p 2 on IL-10 production. IL-10 secretion was lower in PBMCs treated with rDer p 2 compared with the control. Sia-rDer p 2 also attenuated IL-10 secretion but to a lesser extent than rDer p 2 **(Fig 4, A)**. We then determined the T_{reg}/T_H2 cytokine balance by calculating the ratio of IL-10 to IL-4, IL-13, and IL-5, respectively. We observed that the IL-10/IL-4, IL-10/IL-13, and IL-10/IL-5 ratios were higher in sia-rDer p 2–treated PBMCs than in rDer p 2–treated PBMCs **(Fig 4, B-D)**. To further determine the role of IL-10 in the reduced expression of TH2-cell cytokines, we stimulated PBMCs in the presence of an IL-10–blocking antibody and analyzed the IL-5 and IL-13 production. In the presence of an IL-10–blocking antibody, Sia-rDer p 2–treated PBMCs produced more IL-5 and IL-13 **(Fig 4, E and F)**. These data suggest that sia-rDer p 2, unlike rDer p 2, prevents the disruption of the T_{reg}/T_H2 cytokine balance and that IL-10 plays a role in the downregulation of IL-5.

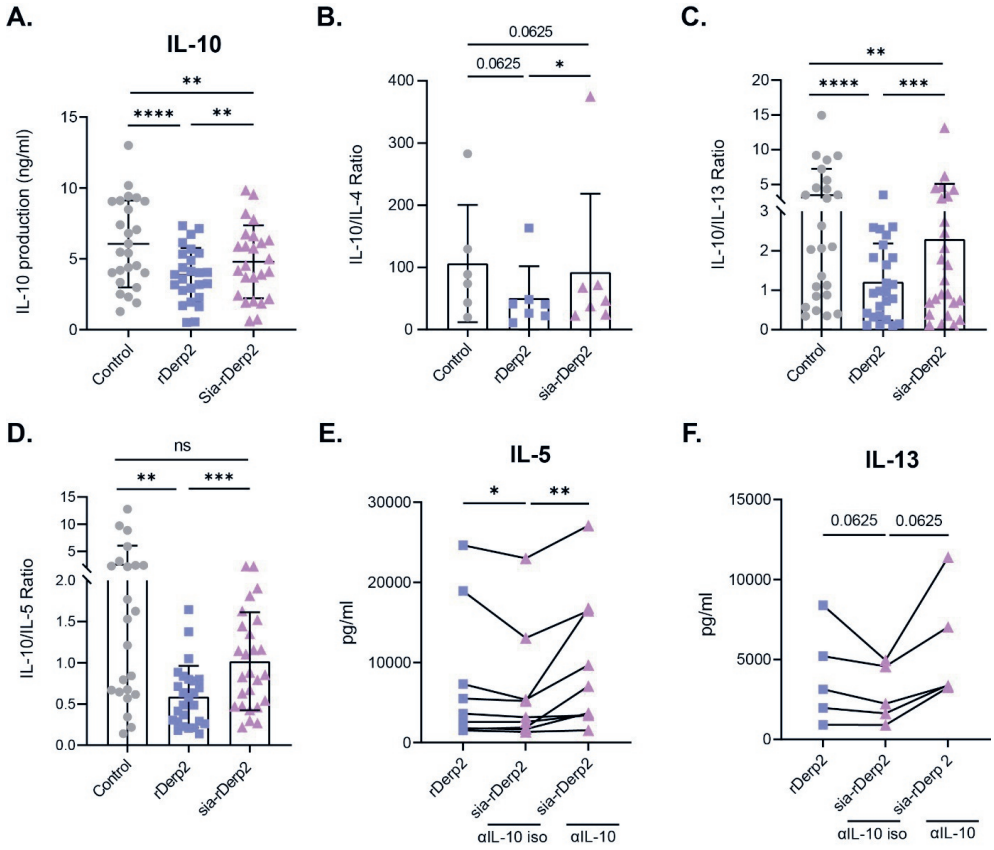


***Fig 4. A-D**, The Treg/TH2 ratio is higher with sia-rDer p 2 than with rDer p 2. The levels of IL-10 (n = 25) **(Fig 4, A)** were determined by ELISA in day 6 PBMC culture supernatants. The ratios of IL-10 to IL-4 (n = 8) (Fig 4, B), IL-10 to IL-13 (n = 25) **(Fig 4, C)**, and IL-10 to IL-5 (n = 25) **(Fig 4, D)** were determined by dividing the values of IL-10 by the values of IL-4, IL-13, and IL-5, respectively, for each condition (control, rDer p 2 and sia-rDer p 2). PBMCs were activated with anti-CD3/CD28 antibodies and coincubated with either rDer p 2 or sia-rDer p 2 in the presence of anti–IL-10 (αIL-10) or an isotype antibody (αIL-10 iso) and cultured for 6 days. **E**, The levels of IL-5 produced when PBMCs were coincubated with rDer p 2 without anti–IL-10 or with sia-rDer p 2 and αIL-10 iso or anti–IL-1 αIL-10 (n = 9). F, The levels of IL-13 produced when PBMCs were coincubated with rDer p 2 without anti–IL-10 or with sia-rDer p 2 and αIL-10 iso or anti–IL-1 αIL-10 (n = 5). Results are shown as mean ± SD. NS, Not significant (Wilcoxon matched-pairs test). **P < .01; ***P < .001; ****P < .0001.*

The frequency of LAG-3+ lymphocytes is higher in sia-rDer p 2–treated PBMCs than in rDer p 2–treated PBMCs

T_{reg} cells, especially the FoxP3+ and CD49b+LAG-3+ (Tr1) subsets, are known to play a role in the development of tolerance in AIT (12, 15, 16, 17). We therefore assessed whether sia-rDer p 2 could expand Treg cells. Given the vast phenotypic heterogeneity of human Treg cells, we analyzed only the following subsets: CD4+CD25+FoxP3+, CD25+LAG-3+, Tr1, PD-1+, and PD-1+LAG-3+ T_{reg} cells. For the gating strategy, see Fig E3. sia-rDer p 2 did not affect the frequencies of CD25+FoxP3+ T_{reg} cells **(Fig E3)** nor the levels of PD-1 expression **(Fig E3)**. However, compared with rDer p 2, sia-rDer p 2 expanded the frequencies of CD25+LAG-3+ **(Fig 5, A)**, Tr1 **(Fig 5, C)**, and PD-1+LAG-3+ **(Fig 5, D)** T_{reg} cells. Interestingly, among the CD25+LAG-3+ lymphocytes, only the FoxP3– subset was expanded in sia-rDer p 2–treated PBMCs but not the FoxP3+ subset **(Fig 5, B)**. Collectively, these data indicate that sia-rDer p 2 but not native rDer p 2 increases the frequency of LAG-3+ CD4+ T cells.

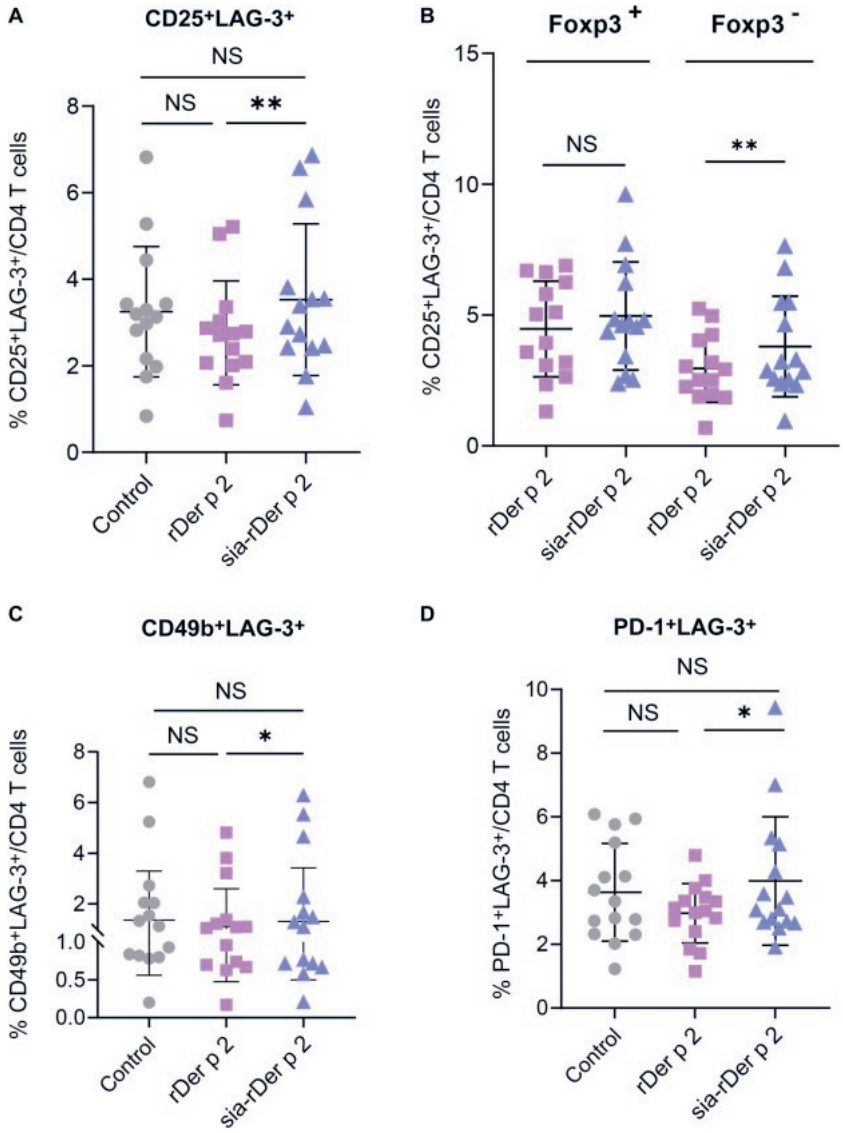
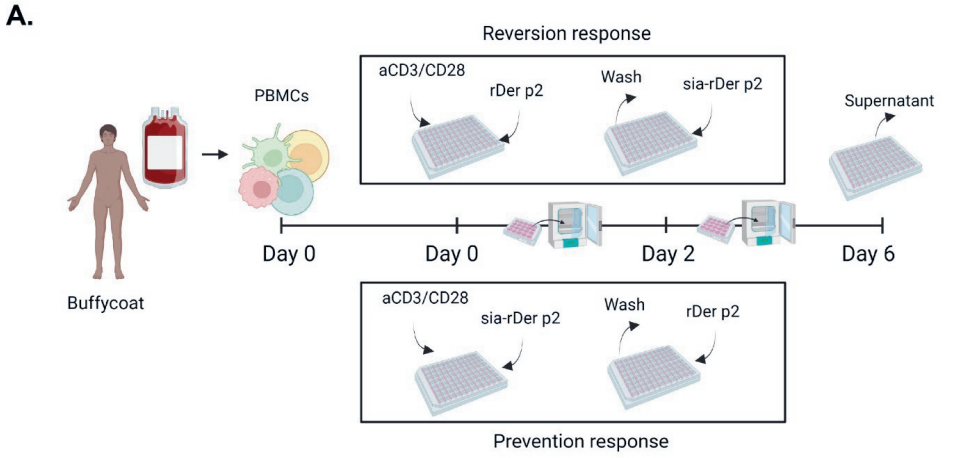


Fig 5. *The frequency of LAG-3+ lymphocytes is higher in sia-rDer p 2–treated PBMCs.* ***A****, The percentage of CD25+LAG-3+CD4+ T cells in control, rDer p 2, or sia-rDer p 2–treated PBMCs.* ***B****, The proportion of FoxP3+ and FoxP3– subsets within the CD25+LAG-3+CD4+ T cells.* ***C****, The percentage of CD49b+LAG-3+CD4+ T cells (Tr1).* ***D****, The percentage of PD-1+LAG-3+CD4+ T cells (n = 15). All results are shown as mean ± SD. NS, Not significant (Wilcoxon matched-pairs test). *P < .05; **P < .01.*

Treatment of PBMCs with sia-rDer p 2 before treatment with rDer p 2 downregulates T_H2 cytokine production and CD4+ T-cell activation

Following our observation that sia-rDer p 2 suppresses the expression of CD69 **(Fig 2, C)** and the production of IL-5 **(Fig 2, C, and Fig 3, C)**, we investigated its capacity to either prevent or revert CD4+ T-cell activation in rDer p 2–treated PBMCs. To do this, anti–CD3-/CD28-activated PBMCs were exposed to sia-rDer p 2 either 48 hours before (prevention of response) or after (reversal of response) coincubation with rDer p 2 **(Fig 6, A)**. We observed that treating PBMCs with sia-rDer p 2 before treating with rDer p 2 resulted in the downregulation of both IL-5 and IL-13 **(Fig 6, B)**. However, the percentages of both CD69+CD4+ and Ki67+CD4+ T cells **(Fig 6, C)** were unaltered. In contrast, treating PBMCs with sia-rDer p 2 after pretreating them with rDer p 2 resulted in an increase in IL-5 and IL-13 **(Fig 6, D)**. Nonetheless, the proportions of CD69+CD4+ and Ki67+CD4+ T cells appeared to be reduced **(Fig 6, E)**. Together, these data demonstrate that sia-rDer p 2 can prevent the activation of T_H2 cytokine responses on challenge with rDer p 2.


A.
Reversion response
aCD3/CD28
rDer p2
Wash
sia-rDer p2
Supernatant
PBMCs
Buffycoat
Day 0
Day 0
Day 2
Day 6
aCD3/CD28
sia-rDer p2
Wash
rDer p2
Prevention response

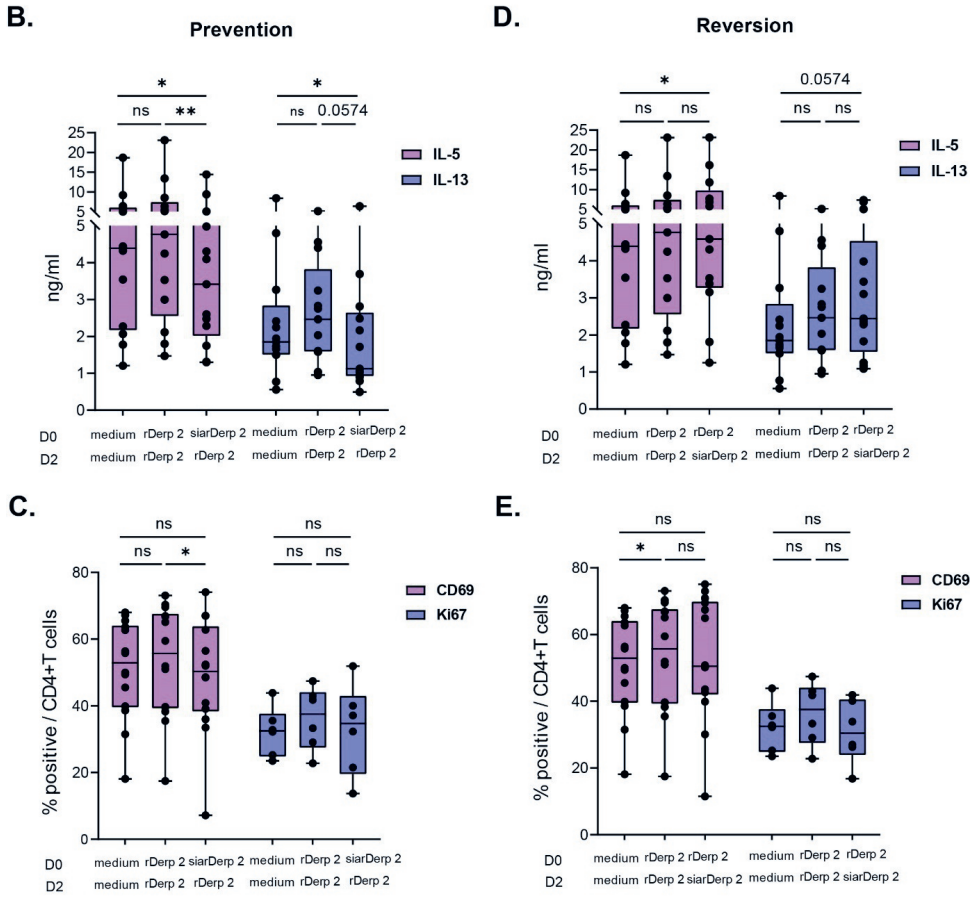

B.
Prevention
D.
Reversion
IL-5
IL-13
ng/ml
C.
E.
CD69
Ki67
% positive / CD4+T cells
D0
D2
medium
rDerp 2
siarDerp 2
rDerp 2

Fig 6. *Prophylactic treatment of PBMCs with sia-rDer p 2 before challenge with rDer p 2 downregulates IL-5 and IL-13 production.* ***A****, A schematic representation of the in vitro experimental setup to investigate the effect of sia-rDer p 2 in a prophylactic (prevention response) and therapeutic (reversion response) setting.* ***B-E****, IL-5 (pink boxes) and IL-13 (purple boxes) levels (n = 11) were measured in day 6 PBMC supernatant by ELISA in the prevention response* ***(Fig 6, B)*** *and the reversion response* ***(Fig 6, D)****. The percentage of CD69+ (pink boxes) (n = 11) and Ki67+ (purple boxes) (n = 5), CD4+ T cells in day 6 PBMCs in the prevention response* ***(Fig 6, C)*** *and the reversion response* ***(Fig 6, E)****. All results are shown as mean ± SD. NS, Not significant (Wilcoxon matched-pairs test). *P < .05; **P < .01.*

DISCUSSION

This study aimed to investigate the immunomodulatory capacity of sialic acid–modified recombinant Der p 2 on the T_H2 arm of CD4+ T cells in human PBMCs from nonallergic individuals *ex vivo*. We also evaluated whether sia-rDer p 2 could induce the expansion of T_{reg} cells. We noted that sia-rDer p 2 moderately suppressed the activation of CD4+ T cells, evidenced by a reduction in the expression of CD69 in both T_H1 and T_H2 cells and downregulated the production of T_H2 cytokines IL-5 and IL-13 but not the T_H1 cytokine IFN-γ or the T_H17 cytokine IL-17. We also showed that sia-rDer p 2 binds to Siglec-9 present on monocytes, DCs, and B cells, and that these cells may be involved in the suppression of CD4+ T-cell activation and in the diminished production of T_H2 cytokines.

The Der p 2 protein has gained attention as an alternative option to prevent and treat HDM allergy besides crude HDM extracts (3,18, 19, 20). This is because there is still ongoing debates about properly standardizing the components of the crude HDM extracts that are currently used in clinics (21), and the risk of unwanted side effects is still quite high (22). Moreover, 79.2% of people with asthma have high IgE titers specific for Der p 2 (23), and Der p 2 itself is a strong risk factor for the development of asthma (10). Moreover, Der p 2 is a good model antigen for investigating the effects of sialic acid–protein modification on immune responses because it contains 12 free lysine residues on which sialic acid molecules could be conjugated. In sialic acid–Siglec interactions, the use of multivalent ligands is necessary to achieve sufficient avidity to stably bind Siglecs and subsequently transduce strong immunomodulatory signals (24). We hypothesized using Der p 2 decorated with sialic acids instead of native Der p 2 would prevent the initial spike in T_H2 responses (25), observed during HDM AIT and potentially decrease the time needed to develop full tolerance because of the induction of immunomodulatory responses mediated by the sialic acid–Siglec axis.

We observed that when PBMCs from nonallergic individuals were treated with rDer p 2, they produced more IL-4 and IL-13, features that are typical of an allergic phenotype. Indeed, nonatopic individuals have been reported to possess allergen-specific cells (26,27), although the response to the allergen is of a lower intensity compared with those of atopic individuals (28). This could explain the observed increase in IL-4 and IL-13. Notably, however, the production of IL-4 and IL-13 from sia-rDer p 2–treated PBMCs from the same nonallergic individuals was unaltered, whereas the production of IL-5 was downregulated. This indicates that sia-rDer p 2 may be safer than native Der p 2 because it does not enhance T_H2 cytokine production. In addition, sia-rDer p 2 did not affect the production of both IL-17 and IFN-γ, which can enhance the severity of allergic diseases and asthma (29,30). In an *in vitro* prophylactic setting, where we treated PBMCs with sia-rDer p 2 before treating them with rDer p 2, sia-rDer p 2 downregulated the production of IL-5 and IL-13. Collectively, these data show that sia-rDer p 2 inhibits T_H2 cytokine production, especially IL-5, and may therefore be a good candidate for Der p 2–specific AIT or be used for prophylactic vaccination against Der p 2–HDM allergy.

When tested in an *ex vivo* therapeutic setting (PBMCs were first treated with rDer p 2 and then with sia-rDer p 2), sia-rDer p 2 did not produce the suppressive effects described earlier. We hypothesize that the activation signal produced by a combination of anti-CD3/CD28 and rDer p 2 was too strong to be overcome by sia-rDer p 2. The fact that lowering the amounts of anti-CD3/CD28 did not result in a different outcome (data not shown) might indicate that the changes provoked in the PBMCs after activation with anti-CD3/CD28 cannot be reversed by the immunosuppressive signals generated by sialic acids. Indeed, anti-CD3–mediated T-cell activation can be overridden only when the suppressive compound is administered simultaneously with anti-CD3 (31). Notably, the fact that sia-Der p 2 modulated only T_H2 cytokines, and not IFN-γ or IL-17, as opposed to previous reports with ovalbumin as antigen,7 indicates that the activity of sia-Der p 2 may be driven by APCs. Moreover, the involvement of APCs is also substantiated by our finding that sia-rDer p 2 does not affect T_H2 cytokine secretion when incubated with pure CD4+ T cells.

Beyond the T_H2 cytokine response, we measured the effect of sia-rDer p 2 on the activation of CD4+ T cells by the expression of CD25 and CD69. CD25 is widely recognized as the primary marker for cellular activation (32), whereas a prolonged increase in the expression of CD69 is associated with allergic inflammation (33). Our findings show that sia-Der p 2 markedly suppressed the expression of CD69. The effects of sia-rDer p 2 on CD69 were similar in both T_H1 and T_H2 cells. Shinoda *et al.* (34) reported that CD69-deficient CD4+ T cells cannot help B cells

to produce high-affinity antibodies and fail to mediate the generation of long-lived plasma cells. Therefore, immunotherapy with sia-rDer p 2 would indirectly inhibit the generation of long-lived allergic plasma cells.

Another important mechanism that has been proposed to be involved in successful AIT is the induction of T_{reg} cells (12,22). The 2 most commonly described subsets of Treg cells that play a key role in allergen tolerance are FoxP3+ T_{reg} cells (35,36), and IL-10+ Tr1 cells (16,37). We showed that, compared with rDer p 2, treatment of PBMCs with sia-rDer p 2 did not affect the frequency of FoxP3+ Treg cells but expanded the frequency of Tr1 cells. Of note, Tr1 cells, having strong suppressive activity, are expanded after AIT, and they correlate with a decrease in clinical scores (16). Because LAG-3 is increasingly implicated in the downregulation of T-cell responses (38,39), we measured the frequency of LAG-3+ Treg-cell subsets. We observed that the frequencies of CD25+LAG-3+ Treg cells, notably the FoxP3– subset and PD-1+LAG-3+, were higher in the sia-rDer p 2 than in the rDer p 2 condition. Dawicki et al. have reported that CD25+LAG-3+FoxP3– Treg cells can contribute to tolerance induction (40). These data suggest that AIT with sia-rDer p 2 instead of Der p 2 might maintain the abundance of necessary T_{reg}-cell populations, which may accelerate the development of tolerance to Der p 2.

Overall, we have demonstrated that α2-3 sialic acid–modified rDer p 2 is able to suppress the activation of both T_H1 and T_H2 cells and downregulate the production of T_H2 cytokines IL-5 and IL-13 in PBMCs from nonallergic individuals. Moreover, sia-rDer p 2, unlike rDer p 2, does not enhance the activation and proliferation of CD4+ T cells, does not alter the T_{reg}/T_H2 cytokine balance, and does not alter the frequency of Treg cells. All these findings illustrate that the use of sia-rDer p 2 instead of rDer p 2 for Der p 2–specific AIT would potentially be more beneficial especially in the early phase of AIT, which is often characterized by a heightened T_H2 response and corresponding allergic side effects, although this needs to be confirmed using cells from subjects with established HDM allergy. Further studies, especially in cells from HDM-allergic donors, are needed to broaden the knowledge of the present findings and to confirm their possible use in routine clinical practice.

Clinical implications

Our findings provide evidence that can support research into the development of a safe prophylactic compound against HDM allergy, which may accelerate the development of necessary regulatory responses.

Disclosure statement

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Disclosure of potential conflict of interest: E. R. J. Li and Y. van Kooyk are involved in DC4U Technologies, which develops glycan-based technologies that enable steering the human immune response. R. van Ree receives consultancy fees from HAL Allergy BV, Citeq BV, Angany, Inc, Reacta Healthcare Ltd, AB Enzymes, Mission MightyMe, and The Protein Brewery; receives speaker fees from HAL Allergy BV, ALK, and Thermo Fisher Scientific; and possesses stock options at Angany, Inc. The rest of the authors declare that they have no relevant conflicts of interest.

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SUPPLEMENTARY MATERIAL

Methods

Modification of recombinant Der p 2 with Sialic acids

To obtain sialic-acid conjugated rDer p 2 (Sia-rDer p 2), maleimide-activated 3′- sialyl-N-acetyllactosamine (SLN302; Neu5Acα2,3Galβ1,4Glc, DEXTRA Labs) were conjugated to thio-activated rDer p 2 (Amsterdam University Medical Center (AmsUMC)) and through a thiol-ene reaction. The glycans were activated with the bifunctional cross linker 4-N-maleimidophenyl butyric acid hydrazide (MPBH) (Pierce) and rDer p 2 was activated with the linker N-succinimidyl S-acetylthioacetate (SATA) (Pierce). The hydrazide moiety of MPBH was covalently linked to the reducing end of the carbohydrate via reductive amination at a 3:1 molar ratio, rDer p 2 was reacted with SATA via the amino groups on its surface at a 6:1 molar ratio, and the final reaction of rDer p 2-SATA with the derivatized carbohydrate was performed at a molar ratio of 1:10. Briefly, SATA, dissolved in DMSO, was added to a filtered solution of rDer p 2 in phosphate buffer (pH 8.2; filtered to 200 nm). After vigorous stirring, the resulting rDer p 2-SATAAc solution was purified using a PD10 column (GE Healthcare) and diluted with 10% vol/vol of 0.5 M NH2OH·HCl solution (pH 7.2). After another 40 min of vigorous stirring and a second purification using a disposable size exclusion cartridge (PD10 column; GE Healthcare Life Sciences), rDer p 2-SATASH was ready for coupling with the MPBH-glycans. A mixture of MPBH (3 eq), 3′-sialyl-N-acetyllactosamine (or 6′-sialyl-N-acetyllactosamine) (1 eq), and picoline borane complex (10 eq; Sigma-Aldrich) dissolved in DMSO/AcOH (8:2) was incubated for 2 h at 65 °C, cooled to room temperature (RT), 1.4 mL of ice-cold isopropanol (anhydrous; Sigma-Aldrich) was added, and then incubated at –20 °C for 1 h. Subsequently, the precipitated MPBH– carbohydrates were pelleted, washed twice with cold isopropanol, and dissolved in 50 µL of PBS. The derivatization was confirmed by electrospray ionization mass spectrometry. The obtained MPBH–3′ sialyl-N-acetyllactosamine was used immediately for coupling to rDer p 2-SATASH. Conjugation of rDer p 2-SATASH to the activated glycans was performed o/n at 4 °C, and the neo-glycoconjugates were purified by size exclusion chromatography. The concentration of rDer p 2 was determined using the bicinchoninic acid assay (Pierce). The presence of α2-3-linked sialic acids on rDer p 2 was confirmed by ELISA (Fig. S1). Briefly, NUNC maxisorb plates (Thermo Fisher Scientific, USA) were coated overnight at room temperature (RT) with 10 µg/ml of either sia-rDer p 2 or non-conjugated rDer p 2 (rDer p 2). A polyacrylamide polymer containing α2-3 sialic acid (PAA-α2-3) (GlycoNZ, New Zealand) was used a positive control.

After overnight incubation, the wells were blocked with carbo-free blocking buffer (CFBB) (Vector Laboratories, USA) for 1 hour at RT. Plates were then incubated with biotinylated MAL-I and peroxidase-labeled streptavidin (Sigma-Aldrich) was use to quantify binding.

Amino Acid Sequence of Der p 2

MMYKILCLSLLVAAVARDQVDVKDCANHEIKKVLVPGCHGSEPCIIHRGKPFQLE-
AVFEANQNTKTAKIEIKASIDGLEVDVPGIDPNACHYMKCPLVKGQQYDIKYTWN-
VPKIAPKSENVVVTVKVMGDDGVLACAIATHAKIRD

Supplementary Figures

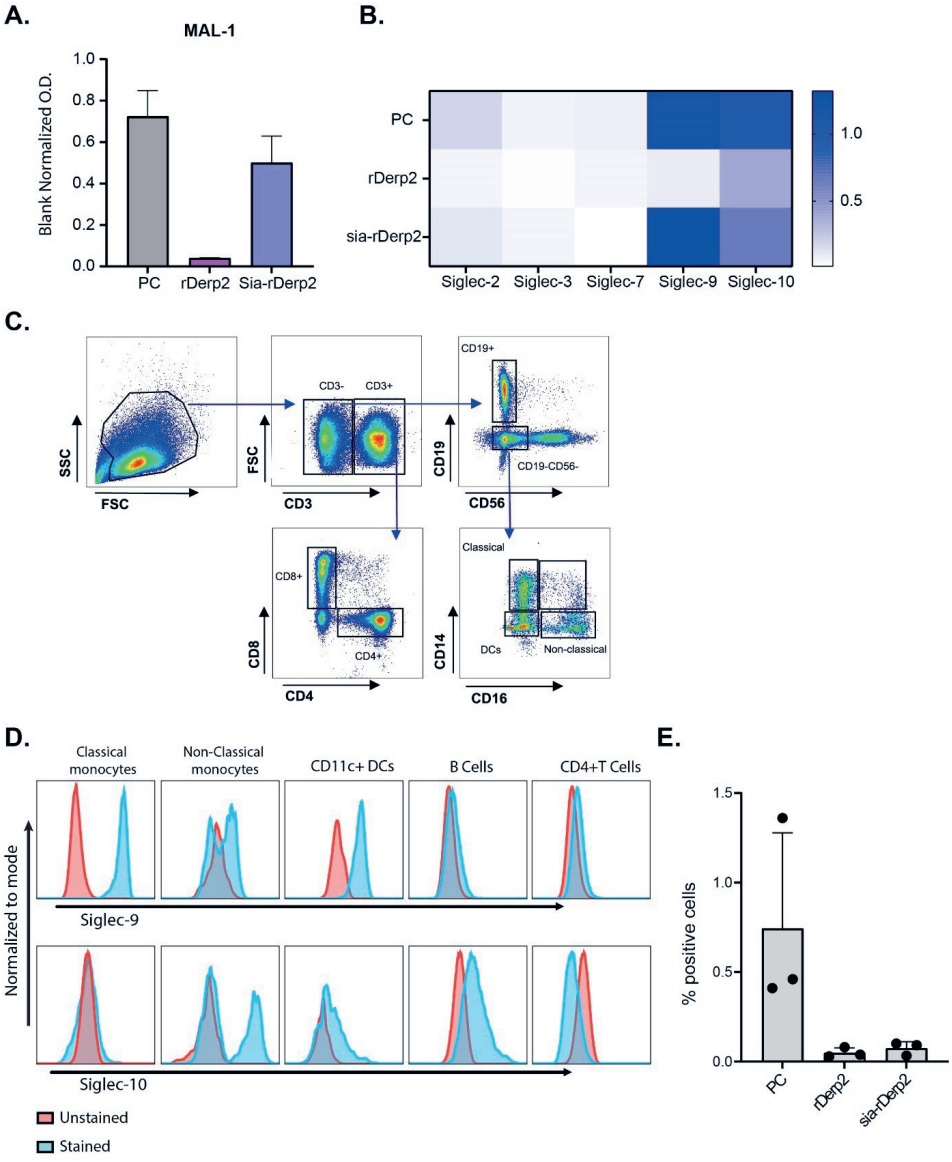


Figure E1. *Binding of α2-3 sialic acid-rDer p 2 to Siglec-9 and -10.* ***A****, Binding of polyacrylamide (PAA)-α2-3 sialic acid (PC), native rDer p 2 (rDer p 2), and α2-3 sialic acid-conjugated rDer p 2 (sia-rDer p 2) to Maackia amurensis lectin I (MAL-1).* ***B****, Binding of PC, rDer p 2, and sia-rDer p 2 to different Siglec-Fc chimeras.* ***C****, Gating strategy used to identify monocytes, dendritic cells (DCs), B cells and CD4+T cells in PBMCs.* ***D****, Representative histograms showing the expression of Siglec-9 and -10 on different immune cell populations within PBMCs.* ***E****, Binding of PC, rDer p 2, and sia-rDer p 2 to CD4+T cells.*

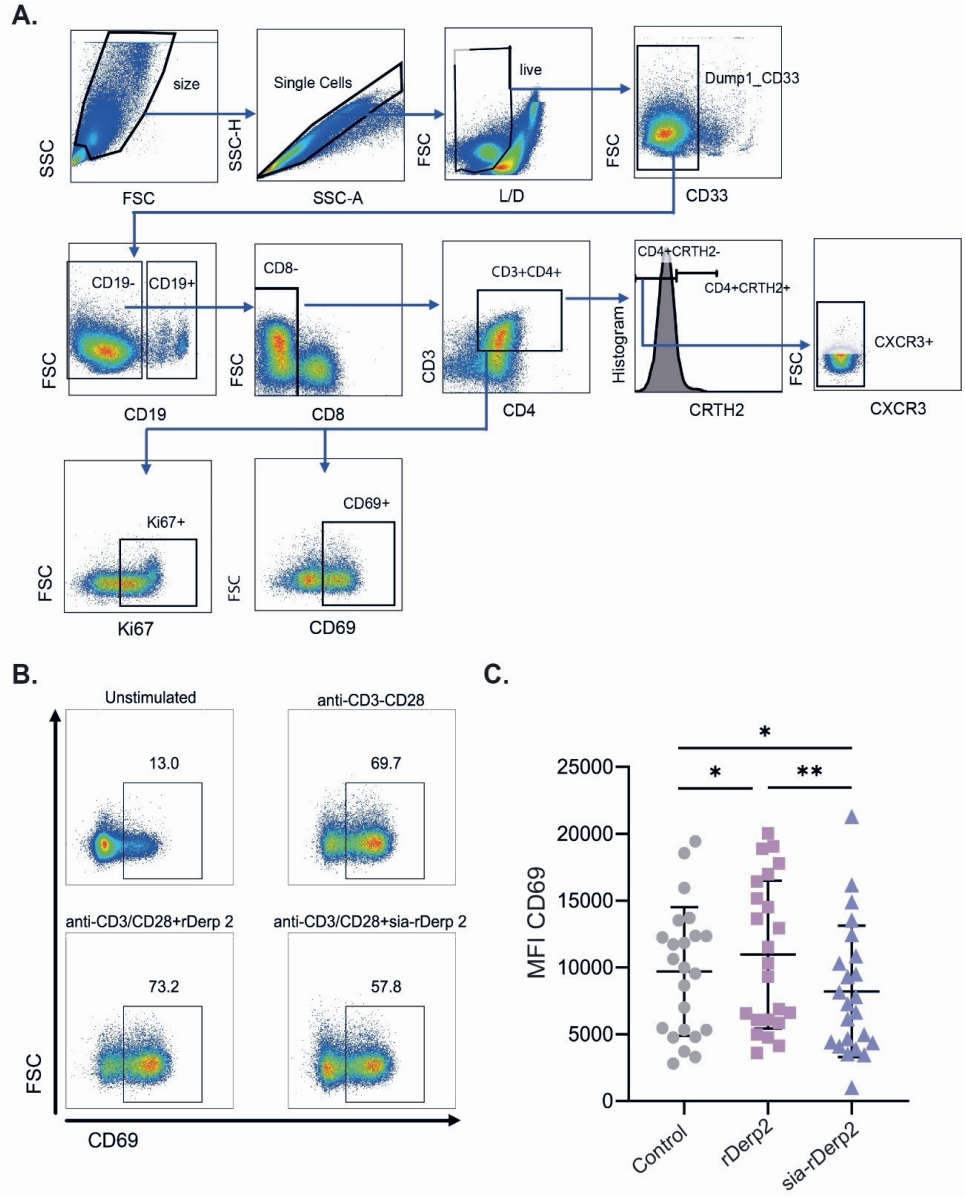


Figure E2. *Activation and proliferation of α2-3 sialic acid-rDer p 2 and rDer p 2-treated CD4+T cells.* ***A****, Gating strategy used to identify CRTH2+ (Th2), CXCR3+ (Th1), CD69+ and Ki67+ CD4+T cells in PBMCs left unactivated or activated with anti-CD3/CD28 antibodies and co-incubated with either RPMI medium (control), or rDer p 2, or sia-rDer p 2 and cultured for 6 days.* ***B****, Representative dot plot showing the percentage of CD69+CD4+T cells in PBMCs treated with medium (control), rDer p 2 or sia-rDer p 2.* ***C****, The mean fluorescent intensity (MFI) of CD69 on CD4+T cells. All results are shown as mean ± SD. * p <0.05; ** p< 0.01; Wilcoxon matched-pairs test.*

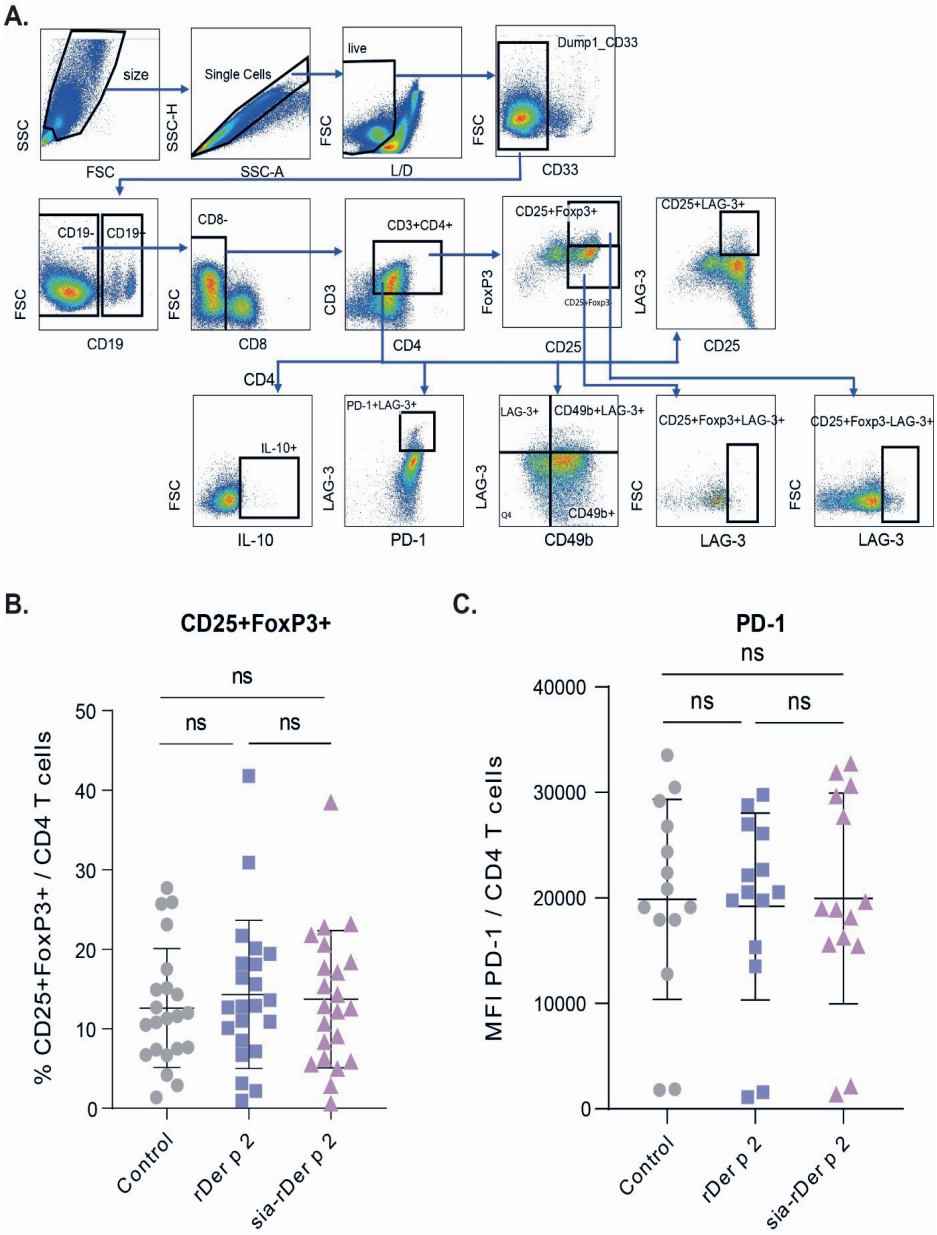


Figure E3. Different Treg populations in PBMCs treated with rDer p 2 or sia-rDer p 2. Non-allergic PBMCs were left unactivated or activated with anti-CD3/CD28 antibodies, co-incubated with either RPMI medium (control), or rDer p 2, or sia-rDer p 2 and cultured for 6 days. A, Gating strategy used to quantify CD19+IL-10+, CD4+IL-10+, PD-1+LAG-3+, CD49b+LAG-3+, FoxP3-/FoxP3+ CD25+LAG-3+ lymphocytes. B, The percentage of CD25+Foxp3+ CD4+T cells. C, The MFI of PD-1 on CD4+T cells. Results are shown as mean ± SD. ns, not significant, Wilcoxon matched-pairs test.



6

GENERAL DISCUSSION

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DISCUSSION

The immune system as a regulated threat: setting the framework

The immune system can be viewed as a powerful defensive apparatus embedded within the body. Its extraordinary destructive capacity is essential for eliminating pathogens and mutated cells, yet that same capacity poses an inherent danger to the host. For this reason, immune surveillance is not governed by a single central entity, but distributed across multiple cell types that democratically regulate and license one another. Each cell type is further controlled by multiple layers of inhibitory mechanisms. When one or more of these brakes fail, the consequences can be severe, leading to chronic inflammation, or autoimmunity - often difficult to reverse. Thus, immune function is not defined solely by its capacity to respond, but by its ability to maintain equilibrium and **homeostasis**. The immune system operates under a dual imperative: to respond rapidly and potently to danger while remaining unresponsive to self and other unharmful antigens. This controlled restraint is known as immunological tolerance. Cases where the immune system is hyper- or wrongly activated are increasing worldwide, causing autoimmune diseases (1, 2) and allergies (3). The significance of this issue if highlighted by the awarding of the 2025 Nobel Prize in medicine to Mary E. Brunkow, Fred Ramsdell, and Shimon Sakaguchi for their groundbreaking discoveries on peripheral tolerance. These observations indicate that maintaining immune tolerance is becoming a challenge to health. Autoimmune diseases already affect approximately 10% of the population, with their overall burden continuing to rise over time (4). I would therefore speculate that they will soon join cardiovascular diseases and cancer as a major population problem. A deeper understanding of the mechanisms that regulate tolerance and finding novel approaches to reinforce it or restore it are urgent goals for biomedical research.

In this thesis, I tackled the topic of developing a strategy to instruct/re-establish immune tolerance by using two models: FVIII in hemophilia A (HA) and Der p 2 in house dust mite (HDM) allergy. FVIII is a circulating glycoprotein which is part of the coagulation cascade; genetic deficiencies of FVIII cause HA, whose therapeutic treatment still requires infusions of recombinant FVIII. A complication of the therapy is the development of neutralizing antibodies against FVIII, which underlie an aberrant activation of the immune system towards a harmless antigen (5, 6). HDM allergy faces the same problem: the immune system reacts with an inflammatory response towards a non-pathogenic antigen. One of the most common species of house dust mite in Central Europe is *Dermatophagoides*

pteronyssinus (Der p), of which the protein Der p 2 is classified as a major allergen (7, 8). I investigated how chemically modifying these antigens, FVIII and Der p 2, with a ligand known to act as a self-associated molecule pattern (SAMP) could instruct the immune system to inhibit the inflammatory processes against FVIII and Der p 2. SAMPs are molecular patterns expressed on the surface of self-cells which are recognized by patter-recognition receptors with inhibitory roles on the immune system (9). Among SAMPs, we chose sialic acids to target Siglecs, specific sialic acid receptors on immune cells that have been shown to play a dominant role in cancer evasion (10, 11). The hypothesis which founded the basis of this thesis was that targeting FVIII and Der p 2 with sialylated glycans to ITIM-carrying Siglecs would initiate a tolerogenic programme via DCs culminating in the suppression of pathogenic immune responses.

On the other hand, the presence of dysregulated immune tolerance programs can also contribute to disease. That is the case in cancer, where tumor cells can actively inhibit immune responses that foster their growth. In fact, the idea of blocking inhibitory receptors on T cells to unleash their potential revolutionized the field treatment of cancer (12, 13). But myeloid cells are also equipped with a series of inhibitory receptors that can be triggered by tumour cells, for example FcγRIIB, members of the Leukocyte Immunoglobulin-Like Receptors (LILR) family, and ITIM-carrying Siglecs (14). Therefore, in the last few decades, the possibility to block these receptors on myeloid cells was also widely investigated in the field of cancer immunotherapy (15). In this thesis, we inverted this paradigm, and proposed that these tumour-induced tolerogenic programs can be used as potential therapeutic options for autoimmune pathologies. Rather than blocking inhibitory receptors as a strategy to increase cancer immunity, we aimed to reinforce inhibitory signalling by targeting these receptors and thereby promote immune suppression and tolerance **(Figure 1)**.

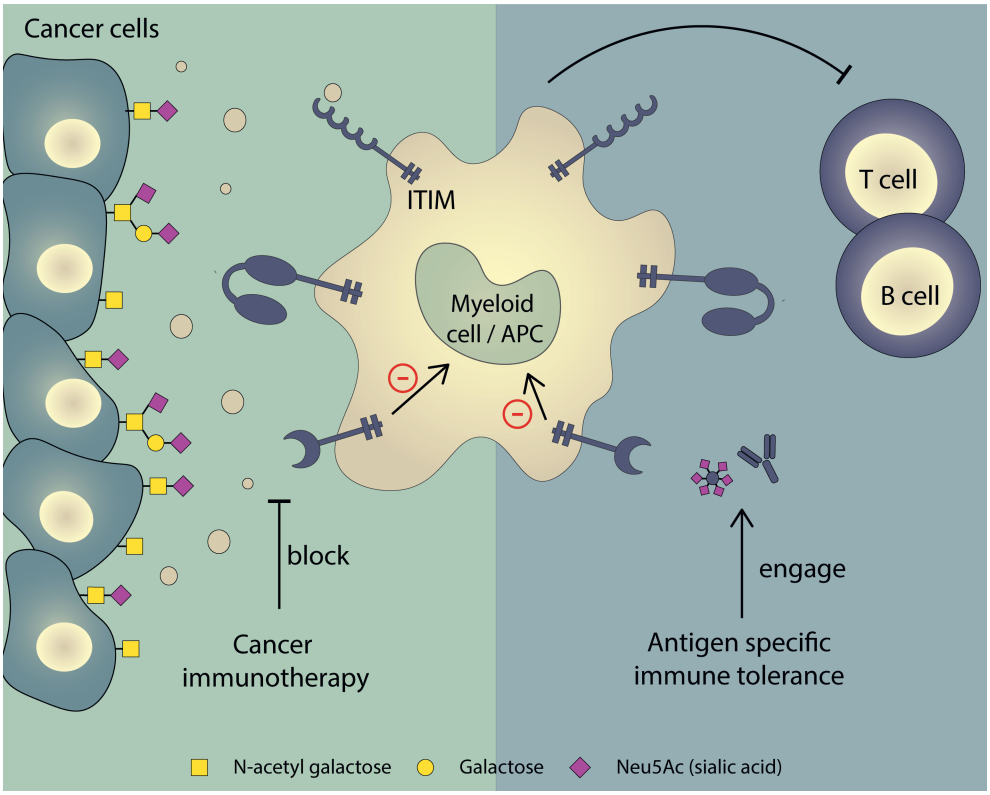


Figure 1. *Strategies for immunotherapy treatment of cancer (in green, left) and autoimmunity/ allergies (in grey, right). In cancer immunotherapy the aim is to block the ITIM carrying receptors which are engaged by membrane bound and secreted components of the tumour cells, thereby releasing the inhibition on the immune system. On the other hand, antigen specific immune tolerance strategies aim at engaging ITIM carrying receptors, thus suppressing the exacerbated immune response.*

In light of the results presented in this thesis, the following points will be addressed **(Figure 2)**:

1. Sialylated peptides and proteins as tolerance-inducing platforms;
2. Benefit of targeting Siglecs when compared to other ITIM-carrying receptors on myeloid cells?
3. Pros and cons of targeting DCs as opposed to other immune cells?
4. T_{reg} responses required for achieving long term tolerance to FVIII and Der p 2?

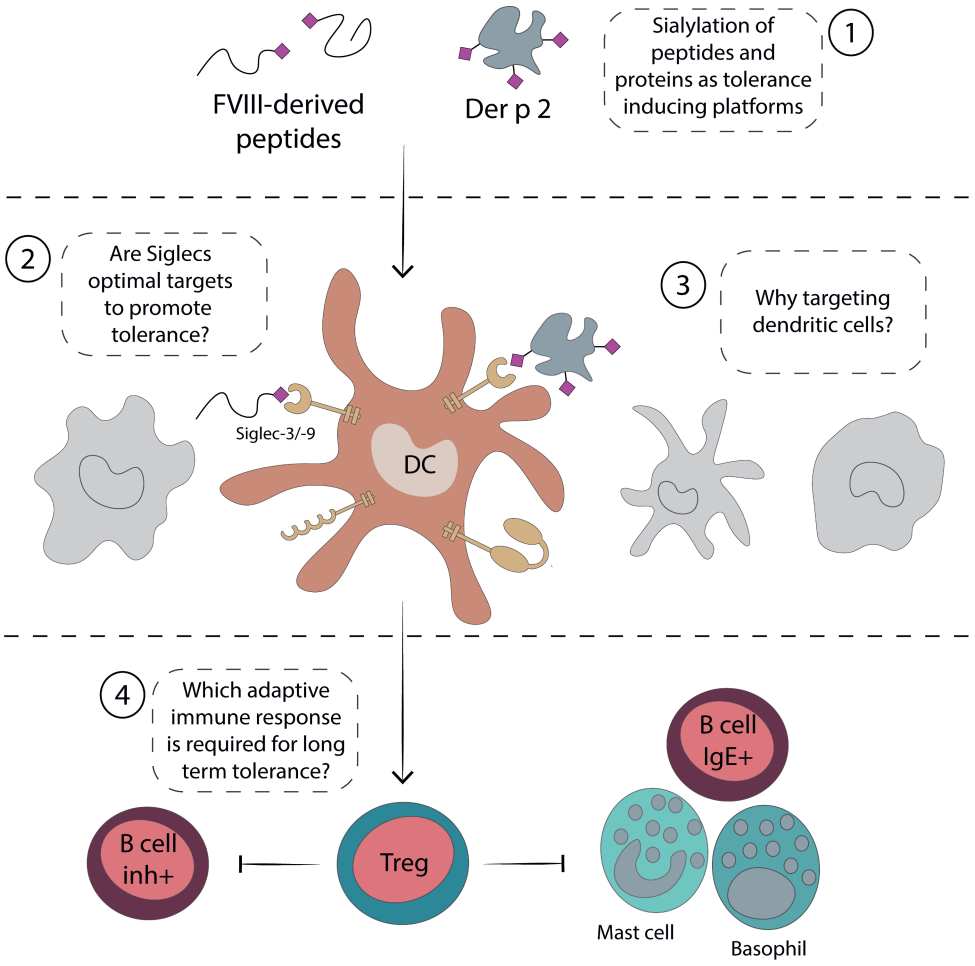


Figure 2. *Overview of the main points addressed in this discussion. Sialylated antigen platforms for dendritic cell–mediated induction of immune tolerance. Sialylated factor VIII (FVIII)-derived peptides and the allergen Der p 2 (sialic acid residues are represented in purple diamonds following the SNFG nomenclature) are taken up by a dendritic cell (DC) that expresses Siglec-3/-9 and other receptors. The conditioned DCs shape the adaptive immune response, promoting regulatory T cell (T_{reg}) induction.*

1. Sialylating peptides and proteins as tolerance-inducing platforms

Successful Siglecs binding and signalling critically depends on the type of sialic acid (N-acetylneuraminic acid and N-glycolylneuraminic acid), its modifications, *e.g.* O-acetylation and sulfation, the linkage to the underlying glycan – α2.3, α2.6 or α2.8 – and the nature of the carrier molecule on which the sialylated glycans are displayed (16). In addition, the spatial organization and repetition pattern of these glycans, for example, their dense, regularly spaced presentation on mucins, also promote Siglecs engagement (17). In **Chapter 2**, we emphasized the importance of choosing an appropriate carrier to present sialylated glycans in the correct conformation for efficient Siglec 7 triggering, and we showed that receptor binding can occur without productive signalling. Moreover, it is now widely accepted that **multivalency** is a *conditio sine qua non* for Siglecs activation (18). In this thesis, we explored the use of single sialylated peptides (**Chapters 3 and 4**) and sialylated proteins (**Chapter 5**) to trigger Siglec signalling, demonstrating that functionally relevant multivalency can be achieved both *in vitro* and *in vivo* using different formats. In the field, targeting Siglecs has been investigated with Siglec-engaging tolerance-inducing antigenic liposomes (STALs), where the targeting moiety is a modified high affinity sialoglycan (19, 20). However, we intriguingly observed that even a single sialylated glycan on one peptide showed indications of tolerance induction *in vitro* and *in vivo*, which is puzzling in light of the current view on the critical role for multivalency. The underlying mechanisms, such as peptide secondary structure, aggregation, or locally increased ligand concentration at the injection site or in draining lymph nodes, remain to be elucidated.

The FVIII peptide sequences used throughout this thesis were selected by performing a bioinformatic analysis focusing on promiscuity of binding to various HLA haplotypes (**Chapter 3**). Immunodominant FVIII epitopes presented by HLA DR, DP, and DQ is well described which allowed for optimal selection of FVIII derived peptides (21, 22). The fact that T cell clones specific for the FVIII peptides used in our studies were identified and isolated from an HA patient with FVIII inhibitors highlights the relevance of these peptides in the immune response to FVIII (23). Ideally, a single peptide, optimized by the bioinformatic analysis performed in **Chapter 3**, would have been used throughout **Chapters 3 and 4**; however, distinct experimental constraints such as the use of a mouse model and a T cell clone specific for other FVIII-derived sequenced required us to employ various peptides. I would argue that this variability ultimately strengthens our conclusions, demonstrating that immune modulation towards tolerance can be achieved across diverse experimental settings using multiple FVIII-derived peptides.

It must also be discussed here that the immunomodulatory effects of sialylated antigens measured ***in vivo* are not readily recapitulated *in vitro***. *In vivo*, the effects are rather dramatic and easily measured, such as the 30% increase in splenic T_{regs} demonstrated by seminal work from our group with α2.3- and α2.6-sialylated OVA (24), the alleviation of allergic symptoms in grass pollen allergy mouse model with α2.3-peptides (25), and the maintenance of the splenic FoxP3+ compartment in our own work in **Chapter 4**. *In vitro* however, the results we measured, are not as striking, which might also reflect the biological inter-species differences between human and mice. In **Chapter 4**, we showed a small decrease in the proliferation of allogenic CD4+CD25+ T cells when co-cultured with moDCs loaded with sialylated peptides from FVIII as compared with the non-sialylated control, but we did not observe induction of T_{regs}. In Chapter 5, we could demonstrate an increase in the CD25+LAG3+, CD49b, +LAG3+ and PD1+LAG3+ frequencies in the PBMC population treated with sialylated Der p2 compared to the natural protein, however this was not significant compared to the not treated control, indicating that sialylation of Der p 2 rather preserves the abundance of these T_{reg} subsets. This discrepancy between *in vivo* and *in vitro* results points at an urgent need in glycobiology for the development of novel assays and improved tools to better model what is happening *in vivo*. The induction of tolerance may require more complex context than the classical in vitro work, it may depend on complex tissue-level interactions that are not easily recapitulated *in vitro*.

In this thesis, we exploited sialylated glycans as SAMPs, but the nature of the immune response elicited highly depends on the molecular and cellular context, which I addressed to as “niche”. We specifically focussed on ITIM-carrying Siglecs, which represents the majority of the family members. We did not study Siglec-1 since it lacks an intracellular signalling motif and its main function is to act as adhesion molecule (18). The sialylated products used in our work did not show binding to Siglec-1Fc in ELISA, even though this does not rule out a possible functional engagement of this lectin when injected *in vivo*. Interestingly, the use of sialylated glycans to target Siglec-1 on splenic macrophages is explored in the context of cancer immunotherapy, with the aim of inducing potent CD8+ T cell responses (26, 27). Given, the choice of the **injection site** becomes crucial to determine the nature of the immune response elicited. Intravenous injections as performed in cancer immunotherapy will rapidly target the spleens, where Siglec-1+ macrophages initiate the adaptive immune response. On the other hand, the subcutaneous injections performed in **Chapter 4** reduce systemic distribution to organs such as the spleen and liver and instead favour delivery to draining lymph nodes through lymphatic drainage and uptake by local antigen-presenting cells, thereby bypassing Siglec-1 uptake by splenic macrophages.

Janus, the Roman god of beginnings and transitions, is traditionally depicted with two faces looking in opposite directions, symbolizing duality. Based on all these observations (the difficulty of modelling sialic acid-induced tolerance in vitro and the potentially activating effects of Siglec-1 targeting) I argue that sialic acids play a **Janus-like** role in immune regulation, with the capacity to either dampen or promote immune activation depending on the molecular and cellular context.

It is well established that the majority of secretory proteins contains N- and O-linked glycans with terminal sialic acid. How do the natural glycans on FVIII and Der p 2 relate to the sialylated tetrasaccharide used in this thesis? As outlined in **Chapter 1**, Der p 2 is not heavily glycosylated, and there are conflicting reports as to whether it naturally carries sialic acid terminated glycans (28-30). On the other hand, FVIII and its recombinant products are well established as glycoproteins, carrying mostly sialic acid capped glycans (31), both α2.3 and α2.6 (32), and blood group antigens (31). However, a defined tetrasaccharide identical to the one used in our studies throughout this thesis - Neu5Acα2-3Galβ1-4GlcNAcβ1-3Gal (modified LSTd) - has not been reported on native FVIII **(Figure 3)**.

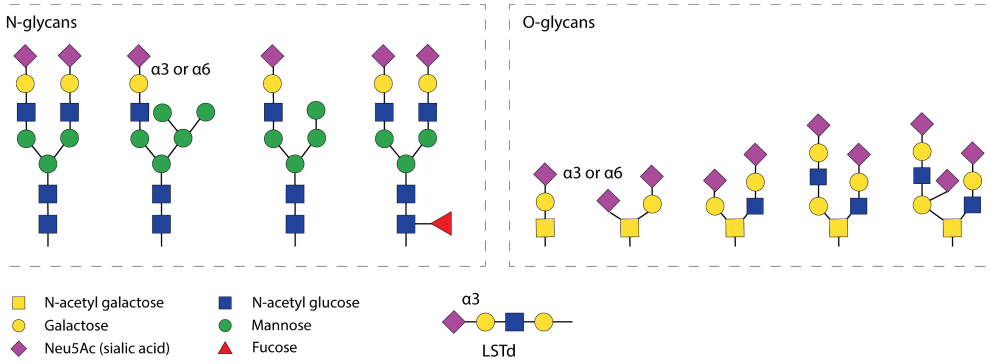


Figure 3. Examples of N- and O-glycans present on FVIII as compared to the LSTd.

Despite the presence of α2.3 and α2.6 ending sialic acid containing N-glycans on FVIII a subset of patients with hemophilia A develop inhibitors. Apparently, the presence of α2.3 and α2.6 ending sialic acid containing N-glycans on FVIII is not sufficient to induce tolerance. Interestingly, Siglec-5 has been identified as a clearance receptor for FVIII and its binding partner VWF (33), suggesting that the native glycosylation of FVIII permits interactions with at least some Siglecs. However, the structural organization and glycan presentation of the intact

protein may not provide the optimal spatial arrangement or multivalency required for effective engagement of immunomodulatory Siglecs. The role of sialic acid-terminating glycans is not limited to marking self-associated molecules, but they can also shield epitopes on the carrier protein or underlying glycan structures, thereby preventing aberrant antibody, complement or lectin binding (34, 35). More broadly, they also contribute to create a negative, anti-adhesive charge on the surface of cells (36). Moreover, in specific cases such as the Fc portion of IgG, the presence of sialic acid on the N-glycan modifies the activity of the protein (37). As for FVIII, there are no specific roles ascribed to sialylated N-glycans, even though some studies linked a higher incidence of inhibitors when children are treated with rFVIII, which presents a different glycosylation profile than pdFVIII (38, 39). Moreover, the sialylated glycans on FVIII might not be accessible to binding so Siglecs, since the majority of FVIII circulates in the blood in tight association with VWF (40). Indeed, it has been shown that presence of VWF prevents FVIII from being endocytosed by DCs *in vitro* and consequently presented to T cells (41). Additional *in vitro* experiments have suggested that plasma-derived (pd) FVIII concentrates containing VWF are less immunogenic, as showed by a lower amount of peptides presented on MHC-II by DCs (42). However, another study found that still less FVIII peptides are presented from pdFVIII than recombinant full length FVIII when the same excess of VWF is provided (43), suggesting that other factors are at play and that VWF does not completely protects FVIII from being seen by the immune system. Lastly, replacement therapies with FVIII might simply not reach Siglecs expressing cells, as pharmacokinetics, route of delivery, biodistribution are all sensitive parameters that strongly influence target cell exposure. We therefore speculate that the introduction of a defined sialylated motif such as that of the LSTd on FVIII-derived peptides, which is not physiologically present on the FVIII molecule itself, enhances or redirects the interactions particularly towards ITIM-carrying Siglecs, which may underlie the tolerogenic effects observed in this thesis.

While other studies have also explored glycosylation as a mean to induce tolerance, such as mannosylated chimeras of hemagglutinin 1 and Der p 2 (44) or Siglec-engaging tolerance-inducing antigenic liposomes (STALs) containing FVIII (45), a key difference of the strategy presented in this thesis is to directly couple the sialylated glycan to the antigen, either to immunodominant FVIII derived peptides (**Chapters 3 and 4**) or to the full-length Der p 2 protein (**Chapter 5**). In doing so, we provided both antigen specificity and the tolerogenic cue within a single formulation. Conceptually, the peptide or protein sequence dictates antigen specificity through presentation on MHC II, while the sialoglycan allows for engagement of inhibitory Siglec receptors, like a “glycan delivery barcode”.

2. Are Siglecs the best targets?

As mentioned in the first paragraph, all immune cells are tightly controlled by a number of checkpoints. Among inhibitory receptors, we specifically focused on Siglecs. In literature, Siglec-2 targeting was investigated in HA with the aim to induce B cell apoptosis, thus eliminating the inhibitor-producing cells (45). Siglec-8, selectively expressed on basophils, eosinophils and mast cells, has been targeted with a monoclonal antibody that induces Siglec 8-mediated eosinophil apoptosis and suppresses mast cell degranulation (46), and it is now undergoing clinical trials for mast cell-driven allergic and inflammatory disorders (47-49). However, whether other ITIM carrying Siglecs can contribute to novel treatment strategies for autoimmune diseases remains largely unexplored.

There is accumulating evidence that Siglecs function as critical inhibitory checkpoints (50). For instance, Siglec-2 knock-out *in vivo* leads to higher BCR-dependent Ca^{2}+ influx, which is a proxy measure for BCR signalling and B cell activation (51). Combined knockout of Siglec 2 and Siglec G (the murine orthologue of Siglec 10) results in the development of spontaneous autoimmune diseases (52), similarly to CTLA-4 KO (53, 54) and PD-1 KO (55). This substantiates the theory that in humans Siglec-2 and Siglec-10 likely function as baseline brakes in B cells, just like PD-1 and CTLA-4 do in T cells. In this thesis, we provided *in vitro* evidence for a similar role of Siglec 9 in myeloid cells. In **Chapter 3**, we observed that monocytes stimulated with the non sialylated FVIII peptide already display Siglec 9 phosphorylation. Consistent with this, in **Chapter 4** we showed that moDCs lacking Siglec 9 drive higher proliferation of allogeneic $CD4^{+}$ T cells. These observations align with previous work from our group: Rodríguez et al. reported basal phosphorylation of Siglec 9 in control treated monocytes (56), and Lübbers *et al.* observed a similar effect in moDCs stimulated with an unsialylated control ligand (57). These findings can be explained by the fact that membrane proteins are highly sialylated, leading to Siglecs being largely engaged in continuous cis interactions. The extent of baseline sialylation of immune cells may also regulate the availability of Siglecs to engage in trans binding to sialylated FVIII peptides and Der p 2. *In vivo*, data from Siglec E knockout mice, the murine orthologue of Siglec 7 and 9, demonstrate increased susceptibility to some tumours (58), suggesting that the tissue specific immunological niche critically shapes the impact of Siglec 7/ 9 on myeloid cell function. Along the same line, in a high fat diet–induced obesity model, Siglec E deficiency remodelled the adipose tissue myeloid compartment towards a pro inflammatory phenotype (59). Collectively, these and our own findings suggest that Siglec 9 is constitutively engaged as a "baseline activated" inhibitory checkpoint in myeloid cells, helping to maintain

the balance between activation and suppression. This, in my view, makes Siglec 9 an extremely attractive candidate to target in order to steer immune responses towards tolerance.

Nevertheless, the interplay and **cooperation between different Siglecs**, and between Siglecs and other receptor classes, remain poorly understood. In **Chapter 3**, we checked binding of sialylated FVIII peptides to Siglecs expressed by moDCs (Siglec-1, Siglec-3, Siglec-7, Siglec-9, Siglec-10 and Siglec-15) by SiglecFc ELISA and showed engagement of Siglec-3 and Siglec-9. Next, by using Siglec 3 and Siglec 9 knockout moDCs, we further demonstrated that IL 10 secretion depends on both lectins, pointing to potential cooperative signalling. As for Der p 2, the sialylated version of the protein binds to Siglec-9Fc and Siglec-10Fc. Although both FVIII peptides and Der p 2 have been chemically conjugated to the same sialylated glycan (Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc, LSTd), differences in the scaffold – peptide vs protein – and multivalency (Der p 2 contains various sialylation conjugation sites while the FVIII peptides only present one glycan attached to its C-terminus) could potentially explain differences in SiglecsFc binding. Whether binding of sialylated Der p 2 to multiple Siglecs effectively translates to signalling was not investigated. It is therefore still to be determined whether strategies that simultaneously engage multiple Siglecs, or Siglecs in combination with other inhibitory receptors, would be more effective at inducing tolerance, and whether this multiple engagement would eventually strengthen the signalling or rather compete with each other.

Moreover, it is important to note that Siglecs are not the only receptors for sialylated glycans. Selectins also recognize sialylated, though often sulphated, ligands, but they instead mediate leukocyte adhesion and rolling at sites of inflammation, thereby facilitating leukocyte recruitment. This further supports the idea of a Janus-like role of sialylation, where context and the immune niche determine either immune suppression via Siglecs or inflammatory recruitment via selectins. In our work, selectin binding was not assessed, as it was considered less relevant.

Advancing our understanding of how cooperative interactions arise between different Siglecs and other inhibitory receptors, is a major challenge. Addressing this question will be essential for the rational design of next generation Siglec directed therapies in allergy, HA, autoimmune diseases, and also cancer.

3. Why targeting DCs?

Which immune cells are the key drivers of the pathogenic immune response in HA and HDM allergy; is targeting dendritic cells (DCs) the optimal strategy? We mainly focused on myeloid cells expressing ITIM-bearing Siglecs, in particular DCs and monocytes. The immune responses to FVIII and Der p 2, although culminating in distinct clinical outcomes, induction of IgG and IgE, respectively, share a common initiation phase. DCs take up antigen and present it to naïve T cells, which subsequently differentiate into effector T cells and initiate the adaptive inflammatory response (60, 61). We made use of the *in vitro* model of monocytes-derived DCs, but the extent to which it is representative of DC function is under debate. In humans, monocyte-derived DCs share a different ontology than other DC subsets and some argue that they are not able to migrate to the secondary lymphoid organs to prime naïve T cells, excluding them from even being referred to as DCs (62). Moreover, they seem to have a pro-inflammatory phenotype (63, 64) which do not make them the perfect model to test tolerance inducing strategies. In light of this, I believe that the results obtained *in vitro* in **Chapters 3 and 4** are of great relevance, despite the evidence for tolerance-induction is limited.

In humans, moDCs represent merely one of the various subsets, *i.e*. cDC1, cDC2, DC3, pDCs, moDCs, Langerhans Cells, each of which is allocated to specific functions (65). In **Chapter 5**, we employed whole PBMC to model this complexity, even though the percentages of conventional DCs, pDCs and DC3 together only make up to 1% of PBMCs (66). An interesting alternative approach could be to directly isolate the subsets relevant for HDM allergy and inhibitors development in HA, which are cDCs (67) and cDC2 (68) respectively, as identified in mouse studies, even though this will be be technically challenging. While waiting for better ways to model DCs *in vitro*, monocyte derived DCs still represent a rapid-to-obtain and valuable model, which allows to reproduce the inter-donor variability.

Given the prominent role of DCs (69), establishing an antigen-specific tolerogenic strategy targeting these cells represents a promising therapeutically relevant model system. Therapies that target only effector cells, such as basophils and mast cells in HDM allergy, or B cells in inhibitor-positive HA patients, are not curative because they fail to address the underlying immune imbalance, while DCs act as central orchestrators of the immune response. Their key role in HA was already demonstrated more than 20 years ago, when anti-CD40L antibodies were shown to prevent inhibitor development in a HA mouse model (70), highlighting DCs as attractive targets for tolerance-inducing therapies. This idea is not novel in the field, as tolerogenic DCs (tolDCs), characterized by low expression of maturation markers and production of IL-10, are widely regarded as a promising

strategy for treating autoimmune diseases. This concept has also already been explored in the context of FVIII: Dao Nyesiga *et al.* generated tolDCs by mimicking the tolerogenic gut environment and showed that these cells can endocytose and present FVIII (71). In **Chapter 3**, we demonstrated that monocyte-derived DCs acquire a tolerogenic feature, namely IL-10 secretion, upon exposure to FVIII-immunodominant peptides coupled to a sialylated glycan. Although we also observed a rather pro-inflammatory cytokine milieu (**Supp. Fig. 3E–H**, **Chapter 3**), no clear changes in maturation or tolerogenic markers were detected **(Supp. Fig.)**. Furthermore, in **Chapter 4**, the same moDCs loaded with sialylated FVIII peptides were shown to dampen the proliferation of activated $CD25^+$ $CD4^+$ T cells, providing additional evidence for the induction of a tolerogenic program in sialylated peptide-loaded moDCs. In **Chapter 5**, we translated these findings to Der p 2 and HDM allergy. In PBMCs treated with sialylated Der p 2, we observed an expansion of $LAG3^+$ regulatory T cells (Tr1), a subset previously shown to be crucial for establishing peripheral tolerance to HDM allergens following allergen immunotherapy (72). However, as depletion experiments were not performed, we cannot definitively conclude that DCs are solely responsible for skewing T cells toward a Tr1 phenotype. PBMCs contain multiple APC subsets, including monocytes (approximately 10-20% of total cells) and B cells, among others.

Apart from DCs and effector cells, additional tolerogenic strategies targeting macrophages have recently been proposed by Miranda *et al*, where the authors generated FVIII-Annexin V fusion proteins which bind phosphatidylserine on apoptotic cells, thereby hijacking the tolerogenic pathway mediated by scavenger macrophages (73). The same group also explored targeting splenic scavenger macrophages by loading red blood cells with FVIII, which are then naturally cleared without eliciting a detrimental immune response (74).

In summary, converging evidence from DC based tolerogenic approaches and our own work underscores the central role of DCs in shaping FVIII and Der p 2 specific immune responses. This provides a strong rationale for DC targeted, glycan based strategies to induce antigen specific tolerance.

4. Which type of T_{regs}/adaptive immune response is required to achieve long term tolerance to FVIII and Der p 2?

Results presented in **Chapter 4** *in vivo* revealed maintenance of the FoxP3+ CD4+ T cell compartment in the spleens of mice treated with subcutaneous injections of sialylated FVIII peptides when compared to the non-sialylated control peptides. More extensive phenotypic analyses would have been helpful to dissect in more detail the contribution of different T_{reg} subsets. In the FVIII KO mouse model, we observed higher titers of inhibitors compared to the control mice which did not receive any peptide pretreatment. We hypothesize that by injecting subcutaneously an immunodominant sequence with a dose escalation regimen we might have primed the immune system towards that sequence, thereby eliciting an additional response that adds to that induced by FVIII alone. Nevertheless, the addition of a sialylated glycan did not show the same effect, keeping the inhibitor titers to the same level as the control group. These observations suggest that the impact of sialylated FVIII peptides on regulatory mechanisms may extend beyond the "classical" FoxP3+ compartment and warrants a more detailed analysis of specialized T_{reg} subsets. While the role of CD4+CD25+CD127low "classical" T_{regs} in the immune response to FVIII is well established (75, 76), recently, it has been discovered in mice that a major role is played by regulatory T follicular helper cells (77). Therefore, it would be essential to understand how and if the sialylated FVIII peptides, as employed in **Chapters 3-4**, would be able to also affect this specific subset. It has to be noted that our knowledge on FVIII immunogenic mechanisms is mainly based on results from mouse studies, including our own study in **Chapter 4**. Can these results be eventually translated to humans? Although broad common features can be identified in FVIII KO mice, such as T cell activation in the spleen and inhibitor formation following FVIII infusions (78), there is evidence showing that a different endocytic pathway is active in mice compared to humans (32). This may reflect important interspecies differences in the mechanisms underlying inhibitor development, being endocytosis by APCs the initiating step of the immunogenic cascade. These considerations highlight the need to directly assess the relevance of our findings in patient samples, which would be following logical step to test our approach. However, access to patient material is limited, given that HA patients with inhibitors only represent the 25-30% of a disease with a low incidence of 1 in 10000 male births (79).

Concerning HDM allergy, our study in **Chapter 5** demonstrated that sialylated Der p2 mostly increased the percentages of the LAG3+ T_{reg} subsets in PBMCs , while the increase in FoxP3+ T_{regs} did not reach significance. Increased levels of

LAG3+ regulatory T cells have been shown to correlate directly with successful outcome of tolerance inducing therapies (72, 80) indicating that our results are of significant interest for achieving long term tolerance to Der p 2, despite the lack of an effect on the level of classical FoxP3+ T_{regs}.

Concluding remarks and future perspective

In summary, we explored a tumour-derived strategy for inducing immune tolerance as a potential therapeutic approach for autoimmune disease and allergy. The work presented in this thesis shows that directly sialylating antigens and targeting ITIM-carrying Siglecs on myeloid cells can bias the immune system towards tolerance in two different clinical contexts, inhibitors in HA and HDM allergy. Siglec-9 and potentially other related Siglecs act as inhibitory checkpoints that can be harnessed to enforce tolerance. We also highlighted how route of delivery and immune niche critically determine whether sialic acids drive suppression or activation, uncovering what I called the **Janus-like** role of sialic acid in immune regulation. Mechanistic studies on the tolerance inducing properties are currently hampered by the lack of suitable in experimental model systems to study sialylation-induced tolerance. Throughout our work, we made use of *in vitro* systems such as monocyte-derived dendritic cells and PBMC cultures which simplify the complexity of the immune system and do not include physiologically relevant human DC subsets. Moreover, it remains to be dissected how different Siglecs and other inhibitory receptors cooperate, and what the relative contribution of distinct T_{reg} subsets is in achieving long term tolerance. In order to translate this research into clinical benefit for the life of a patient the formulation, protein versus peptide for example, could still be modified to improve multivalency and achieve stronger Siglec-engagement. Our data support the idea that glycan editing of therapeutic and allergic proteins provides an highly interesting therapeutic approach to establish antigen-specific tolerance, with potential applications across the ever-growing spectrum of autoimmune diseases and allergies. As science works, there is no end to research, and no limit to curiosity.

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7

APPENDICES

English Summary
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ENGLISH SUMMARY

The immune system is a powerful machinery designed to protect us from external dangers such as virus and bacteria and to keep the order internally by eliminating rogue cells such as cancer. Unfortunately, during the past few decades, cases are on the rise where the immune system itself represents a threat. These range from autoimmunity to allergy, which arise when the balance between immune activation and tolerance is lost. Equilibrium in the immune system is kept by a series of breaks and checkpoints.

One of these breaks, which is gaining more and more attention, is represented by the sialic acid/Siglec axis. Sialic acid is a monosaccharide which caps mammalian glycans. Given its position as outermost glycan, it is used as self-associated molecular pattern, to signal "self" to the immune system. Accordingly, cells of the immune system, specifically belonging to the myeloid compartment, are equipped with receptors called Siglecs to sense sialylated moieties and suppress the response. Cancer cells exploit this axis to evade immune surveillance, by increasing the sialylation pathway and the concentration of sialylated glycans on their surface to be seen as "self".

The research conducted in this thesis was guided by the idea that the natural immunosuppressive properties of the sialic acid / Siglec axis could be harnessed to reprogram the immune response to FVIII in hemophilia A and Der p 2 in house dust mite allergy towards tolerance. As reviewed in **Chapter 1**, FVIII is a coagulation factor that is used in replacement therapies for hemophilia A patients, whom presents with a genetic mutation in the FVIII locus which hampers the coagulation cascade. In up to 30% severe cases the patients develop anti-FVIII neutralizing antibodies which makes the therapy ineffective. On the other hand, Der p 2 is a protein produced by house dust mite. It is an unharmful molecule for humans, but some individuals develop an immune response towards it guided by IgEs which culminates in exacerbation of asthma, sneezing, coughing and itch. We hypothesized that by coupling FVIII and Der p 2 to a sialylated glycan we could engage Siglec-9 on dendritic cells and thus initiate a tolerogenic program.

First, in **Chapter 2** we investigated how to study this system by developing a reporter cell line for one of the Sigecs. By engineering a Jurkat cell line with a NFAT-Luc plasmid and Siglec-7-ITAM chimera, we revealed by ELISA and luminescence that one of the most renowned Siglec-7 ligand, sialylated CD43, might not be capable of inducing signalling. We propose to use this reporter to improve the screening of targets of Siglec-7, to potentially accelerate the discovery of novel points of intervention in the treatment of cancer.

Next, we investigated how to translate this system into a pharmaceutical formulation of FVIII. In **Chapter 3**, we outlined a strategy to select immunodominant peptides from FVIII and chemically conjugate them to a sialic acid-terminating tetrasaccharide. We tested Siglecs binding by ELISA and further demonstrated that IL-10 secretion induced by sialylated FVIII peptides in monocyte-derived dendritic cells was Siglec-9 dependent by making use of knock-outs. In **Chapter 4** we moved onto testing these sialylated FVIIII peptides in a FVIII-KO model, showing that compared with the control group treated with not-sialylated peptides there was an increase in T_{regs} in the spleens and lower percentages of IFNγ- and TNFα-secreting Teff.

Chapter 5 extends the investigation of sialic acid/Siglec interactions to the context of allergy. We conjugated the same sialylated glycan employed in chapters 3 and 4 to Der p 2, and analyzed its effects on peripheral blood mononuclear cells (PBMCs) from non-allergic volunteers. Our data showed that sialylated Der p 2 both suppressed the production of Th2 cells cytokines (IL-4, IL-5, IL-13), and the activation of CD4+ T cells. Moreover, higher frequencies of two subsets of lymphocyte activation gene 3 (LAG-3+) regulatory T cells were observed in PBMCs cultured with sialylated Der p 2.

In conclusion, as discussed in **Chapter 6**, within this thesis we investigated how directly sialylating antigens and targeting ITIM-carrying Siglecs on myeloid cells can bias the immune system towards tolerance in two different clinical contexts, inhibitors in HA and house dust mite allergy. We also highlighted how the route of delivery and the immune niche critically determine whether sialic acids drive suppression or activation, uncovering what I called the Janus-like role of sialic acid in immune regulation.

NEDERLANDSE SAMEVATTING

Het immuunsysteem beschermt ons tegen externe gevaren, zoals virussen en bacteriën. Tevens houdt het intern de orde door afwijkende cellen, zoals kankercellen, te elimineren. De afgelopen decennia zien we echter een toename van situaties waarin het immuunsysteem zelf een bedreiging vormt. Dit varieert van het ontstaan van auto-immuniteit tot allergieën, welke een steeds grotere belasting vormen voor de gezondheidszorg in de toekomst. De balans binnen het immuunsysteem wordt bewaakt door een reeks remmen en controlepunten die voorkomen dat het immuunsysteem zich richt tegen onschadelijke of lichaamseigen structuren.

Een van deze remmen, die steeds meer aandacht krijgt, wordt gevormd door de siaalzuur/Siglec axis. Siaalzuur is een monosacharide dat aan het einde van glycaanstructuren aanwezig is waardoor het een makkelijk herkennings signaal is voor immuuncellen. Siaalzuur fungeert daardoor als een self-associated molecular pattern, waarmee het een signaal van “zelf” aan het immuunsysteem afgeeft. Cellen van het immuunsysteem, zijn uitgerust met receptoren die siaalzuren herkennen en worden Siglecs genoemd. Deze herkenning van gesialyleerde structuren door Siglecs onderdrukken een immuunrespons. Kankercellen maken gebruik van deze as om aan immuunsurveillance te ontsnappen, onder andere door de sialyleringsroute te versterken en de hoeveelheid gesialyleerde glycaanstructuren op hun oppervlak te verhogen waardoor immuun cellen geremd worden in hun functie.

Het onderzoek dat in dit proefschrift wordt beschreven, werd geleid door het idee dat de natuurlijke immunosuppressieve eigenschappen van de siaalzuur/Siglec axis kunnen worden benut om de immuunrespons tegen FVIII bij hemofilie A en tegen Der p 2 bij huisstofmijtallergie te herprogrammeren richting tolerantie. FVIII is een stollingsfactor die wordt gebruikt als substitutietherapie bij patiënten met hemofilie A. Deze patiënten hebben een genetische mutatie in het FVIII-gen, waardoor de bloedstollingscascade wordt verstoord. Bij tot 30% van de patiënten met ernstige hemofilie A ontwikkelen zich neutraliserende anti-FVIII-antistoffen, waardoor de therapie ineffectief wordt. Der p 2 daarentegen is een eiwit dat wordt geproduceerd door de huisstofmijt. Hoewel dit molecuul voor de meeste mensen onschadelijk is, ontwikkelen sommige individuen er een IgE-gemedieerde immuunrespons tegen, die kan leiden tot verergering van astma, niezen, hoesten en jeuk. Onze hypothese was dat we, door FVIII en Der p 2 te koppelen aan een gesialyleerd glycan, Siglec-9 op dendritische cellen konden activeren en zo een tolerogeen programma konden initiëren.

Eerst onderzochten we in **Hoofdstuk 2** hoe we dit systeem konden bestuderen door een reportercellijn voor een van de Siglecs te ontwikkelen. Door een Jurkat-cellijn te modificeren met een NFAT-Luc-plasmide en een Siglec-7-ITAM-chimeer, lieten we met behulp van ELISA en luminescentie zien dat een van de bekendste liganden van Siglec-7, gesialyleerd CD43, mogelijk niet in staat is om signalering te induceren. We stellen voor om deze reportercellijn te gebruiken om de screening van potentiële Siglec-7-targets te verbeteren en daarmee mogelijk de ontdekking van nieuwe aangrijpingspunten voor de behandeling van kanker te versnellen.

Vervolgens onderzochten we hoe we dit systeem konden vertalen naar een farmaceutische formulering van FVIII. In **Hoofdstuk 3** beschreven we een strategie om immunodominante peptiden uit FVIII te selecteren en deze chemisch te conjugeren aan een tetrasacharide dat eindigt in siaalzuur. We onderzochten de binding aan Siglecs met behulp van ELISA en toonden vervolgens aan dat de door gesialyleerde FVIII-peptiden geïnduceerde IL-10-secretie in monocyte-derived dendritic cells afhankelijk was van Siglec-9, door gebruik te maken van knock-outcellen. In **Hoofdstuk 4** gingen we vervolgens verder met het testen van deze gesialyleerde FVIII-peptiden in een FVIII-KO-model. In vergelijking met de controlegroep die behandeld werd met niet-gesialyleerde peptiden, zagen we een toename van Tregs in de milt en lagere percentages IFNγ- en TNFα-producerende effector-T-cellen.

Hoofdstuk 5 breidt het onderzoek naar siaalzuur/Siglec-interacties uit naar de context van allergie. We conjugereerden hetzelfde gesialyleerde glycan dat in hoofdstukken 3 en 4 werd gebruikt aan Der p 2 en onderzochten vervolgens de effecten ervan op perifere mononucleaire bloedcellen, ofwel PBMCs, van niet-allergische vrijwilligers. Onze resultaten lieten zien dat gesialyleerd Der p 2 zowel de productie van Th2-cytokinen, waaronder IL-4, IL-5 en IL-13, als de activatie van CD4+ T-cellen onderdrukte. Daarnaast zagen we hogere frequenties van twee subsets van LAG-3+ regulatoire T-cellen in PBMCs die met gesialyleerd Der p 2 waren gekweekt.

Tot slot hebben we, zoals besproken in **Hoofdstuk 6**, onderzocht hoe het rechtstreeks sialyleren van antigenen en het targeten van ITIM-dragende Siglecs op myeloïde cellen het immuunsysteem richting tolerantie kan sturen in twee verschillende klinische contexten: hemofilie A en huisstofmijtallergie. Daarnaast hebben we laten zien dat zowel de toedieningsroute als de immuunomgeving in belangrijke mate bepalen of siaalzuren immunosuppressie of juist activatie bevorderen. Daarmee hebben we de Janus-achtige rol van siaalzuur in de regulatie van het immuunsysteem blootgelegd.

RIASSUNTO IN ITALIANO

Il sistema immunitario è una potente macchina progettata sia per proteggerci da pericoli esterni, come virus e batteri, sia per mantenere l'ordine eliminando cellule anomale, come le cellule tumorali. Tuttavia, negli ultimi decenni sono aumentati i casi in cui è proprio il sistema immunitario a rappresentare una minaccia. Questi comprendono condizioni che vanno dalle malattie autoimmuni alle allergie e che in futuro rappresenteranno un peso sempre maggiore per il sistema sanitario. L'equilibrio del sistema immunitario è mantenuto da una serie di freni e checkpoint che impediscono al sistema immunitario di attaccare strutture innocue o proprie dell'organismo definite self.

Uno di questi freni, che sta ricevendo sempre più attenzione, è rappresentato dall'asse acido sialico/Siglec. L'acido sialico è un monosaccaride che si trova all'estremità dei glicani dei mammiferi. Grazie alla sua posizione più esterna, viene utilizzato come self-associated molecular pattern, fornendo al sistema immunitario un segnale di "self". Le cellule del sistema immunitario, in particolare quelle appartenenti al compartimento mieloide, sono dotate di recettori chiamati Siglecs, che riconoscono le strutture sialilate e sopprimono in risposta il sistema immunitario. Le cellule tumorali sfruttano questo asse per sfuggire alla sorveglianza immunitaria, aumentando i pathway enzimatici di sialilazione e la concentrazione di glicani sialilati sulla loro superficie.

La ricerca condotta in questa tesi è stata guidata dall'idea che le proprietà naturalmente immunosoppressive dell'asse acido sialico/Siglec potessero essere sfruttate per riprogrammare la risposta immunitaria verso FVIII nell'emofilia A e verso Der p 2 nell'allergia agli acari della polvere domestica, indirizzandola verso la tolleranza. Come descritto nel **Capitolo 1**, il FVIII è un fattore della coagulazione utilizzato nella terapia per i pazienti affetti da emofilia A, una condizione caratterizzata da una mutazione genetica nel locus FVIII che compromette la cascata coagulativa. Fino al 30% dei pazienti con emofilia A grave sviluppa anticorpi neutralizzanti anti-FVIII, rendendo inefficace la terapia. Der p 2, invece, è una proteina prodotta dagli acari della polvere domestica. Sebbene sia una molecola innocua per la maggior parte delle persone, alcuni individui sviluppano contro di essa una risposta immunitaria mediata da IgE, che può culminare nell'aggravamento dell'asma, starnuti, tosse e prurito. Abbiamo ipotizzato che, coniugando FVIII e Der p 2 a un glicano sialilato, avremmo potuto coinvolgere Siglec-9 sulle cellule dendritiche e quindi avviare un programma di tolleranza immunologica.

Innanzitutto, nel **Capitolo 2**, abbiamo studiato come analizzare questo sistema sviluppando una linea cellulare reporter per uno dei Siglecs. Modificando una linea cellulare Jurkat con un plasmide NFAT-Luc e una chimera Siglec-7-ITAM, abbiamo dimostrato, mediante ELISA e luminescenza, che uno dei ligandi più conosciuti di Siglec-7, il CD43 sialilato, potrebbe non essere in grado di indurre signalling. Proponiamo quindi di utilizzare questo sistema reporter per migliorare lo screening di potenziali target di Siglec-7 e, potenzialmente, accelerare la scoperta di nuovi punti di intervento per il trattamento del cancro.

Successivamente, abbiamo studiato come tradurre questo sistema in una formulazione farmaceutica di FVIII. Nel **Capitolo 3**, abbiamo delineato una strategia per selezionare peptidi immunodominanti di FVIII e coniugarli chimicamente a un tetrasaccaride terminante in acido sialico. Abbiamo analizzato il legame ai Siglec mediante ELISA e abbiamo successivamente dimostrato, utilizzando cellule knock-out, che la secrezione di IL-10 indotta dai peptidi di FVIII sialilati nelle cellule dendritiche derivate da monociti era dipendente da Siglec-9. Nel **Capitolo 4**, abbiamo quindi proseguito testando questi peptidi di FVIII sialilati in un modello FVIII-KO. Rispetto al gruppo di controllo trattato con peptidi non sialilati, abbiamo osservato un aumento dei Treg nella milza e una riduzione delle percentuali di cellule T effettrici secernenti IFNγ e TNFα.

Il **Capitolo 5** estende lo studio delle interazioni tra acido sialico e Siglec al contesto dell'allergia. Abbiamo coniugato lo stesso glicano sialilato utilizzato nei capitoli 3 e 4 a Der p 2 e ne abbiamo analizzato gli effetti sulle cellule mononucleate del sangue periferico, dette PBMCs, di volontari non allergici. I nostri dati hanno mostrato che Der p 2 sialilato sopprimeva sia la produzione di citochine Th2, i.e. IL-4, IL-5 e IL-13, sia l'attivazione delle cellule T CD4+. Inoltre, nelle PBMCs coltivate con Der p 2 sialilato, abbiamo osservato una maggiore frequenza di due sottopopolazioni di cellule T regolatorie LAG-3+.

In conclusione, come discusso nel **Capitolo 6**, in questa tesi ho ricercato come la sialilazione diretta degli antigeni e il targeting dei Siglec contenenti domini ITIM sulle cellule mieloidi possano indirizzare il sistema immunitario verso la tolleranza in due diversi contesti clinici: l'emofilia A e l'allergia agli acari della polvere domestica. Abbiamo inoltre evidenziato come la via di somministrazione e la nicchia immunitaria siano determinanti nel definire se l'acido sialico promuova soppressione o attivazione immunitaria, mettendo in luce quello che ho definito il ruolo da Giano bifronte dell'acido sialico nella regolazione della risposta immunitaria.

RIASUNTO IN DIAETO VENESSIANO

El sistema immunitario el xe na machina potente, fata par proteggerne. El ne proteze da pericoli esterni, come virus e bateri, e el tien in ordine quel che succede dentro al corpo, eliminando le cellule che le xe andà a remengo, come le cellule tumorali. Però, negli ultimi decenni, xe aumentà i casi in cui xe proprio el sistema immunitario a diventar na minaccia. Se va da le malatie autoimmuni a le alergie, e queste condizioni le diventerà presto un peso sempre pi grande par la sanità. L'equilibrio del sistema immunitario el xe mantenù da na serie de freni e checkpoint che i ghe impedisse de scatar contro strutture innocue o contro quel che xe nostro.

Un de questi freni, che el sta ricevendo sempre più attenzione, el xe rappresentà da l'asse acido sialico/Siglec. L'acido sialico el xe un monosacaride che el se trova a la fine dei sùcari dei mammiferi. Proprio perché el xe el sùcaro pì esterno, el vien usà come un self-associated molecular pattern, par mandar al sistema immunitario el segnale de "self". Le cellule del sistema immunitario, soprattutto quee del compartimento mieloide, le ga dei recettori ciamà Siglec, che i riconosce le strutture sialilà e i pol quindi sopprimere la risposta immunitaria. El tumor sfruta questo asse par scampàr a la sorveianza immunitaria, aumentando la via de sialilazion e la quantità de glicani sialilà su la so superficie.

La ricerca fata in sta tesi la xe stà guidà da l'idea che le proprietà naturalmente immunosoppressive de l'asse acido sialico/Siglec le podesse esser sfrutà par riprogrammar la risposta immunitaria contro FVIII ne l'emofilia A e contro Der p 2 ne l'alergia a la polvere domestica, portandola verso la tolleranza. Come gavemo spiegà nel **Capitolo 1**, FVIII el xe un fator de la coagulazion che vien usà pa i pazienti con emofilia A, che li gà na mutazion genetica nel locus FVIII, in paroe povare li pérda sangue. Fin al 30% dei casi gravi i pazienti i sviluppa anticorpi contro FVIII, e a quel punto la terapia no la funziona più. Der p 2, invece, el xe una proteina prodota da i acari dea polvare. Par la maggior parte dei omeni la xe una molecola innocua, ma in alcuni individui la se sviluppa na risposta immunitaria guidà da IgE, che la pol culminar in un peggioramento de l'asma, starnui, tosse e prurito. La nostra ipotesi la gera che, coniugando FVIII e Der p 2 a un glicano sialilà, podessimo coinvolger Siglec-9 su le cellule dendritiche e quindi far partir un programma tolerogenico.

Prima de tuto, nel **Capitolo 2**, gavemo studià come analizzar sto sistema, sviluppando una linea cellulare reporter par uno dei Siglec. Modificando una linea cellulare Jurkat con un plasmide NFAT-Luc e una chimera Siglec-7-ITAM, gavemo mostrà, con ELISA e luminescenza, che uno dei ligandi più conosciù de Siglec-7,

el CD43 sialilà, el podarìa no esser capace de indur la segnalazion. Proponemo quindi de usar sto reporter par migliorar lo screening dei possibili target de Siglec-7 e gò caro de accelerar la scoperta de nòvi modi de trattar del cancro.

Dopo, gavemo studià come portar sto sistema verso una formulazion farmaceutica de FVIII. Nel **Capitolo 3**, gavemo sviluppà na strategia par selezionar i peptidi immunodominanti de FVIII e coniugarli chimicamente a un tetrasacaride che finisse con acido sialico. Gavemo studià el legame ai Siglec con ELISA e dopo gavemo dimostrà, usando cellule knock-out, che la secrezion de IL-10 indota dai peptidi de FVIII sialilà ne le cellule dendritiche derivate da monociti la dipende da Siglec-9. Nel **Capitolo 4**, semo quindi andà avanti testando sti peptidi de FVIII sialilà in un modello FVIII-KO. Rispetto al gruppo de controllo tratà co' peptidi non sialilà, gavemo visto un aumento dei Treg ne la milza e una riduzion de le percentuali de cellule T effetrici che secreta IFNγ e TNFα.

El **Capitolo 5** el porta vanti lo studio de le interazioni tra acido sialico e Siglec nel contesto de l'alergia. Gavemo coniugà el stesso sucaro sialilà usà nei capitoli 3 e 4 a Der p 2 e gavemo studià i so effetti su le cellule mononucleari del sangue periferico, le PBMC, de volontari non alergici. I nostri dati i ga mostrà che Der p 2 sialilà el sopprimeva sia la produzion de citochine Th2, cioè IL-4, IL-5 e IL-13, sia l'attivazion de le cellule T CD4+. Inoltre, ne le PBMC coltivate co' Der p 2 sialilà, gavemo osservà una frequenza più alta de due sottopopolazion de cellule T regolatorie LAG-3+.

In conclusione, come discusso nel **Capitolo 6**, in sta tesi gavemo studià come la sialilazion direta degli antigeni e el targeting dei Siglec con domini ITIM su le cellule mieloidi i possa indirizzar el sistema immunitario verso la tolleranza in due diversi contesti clinici: l'emofilia A e l'alergia a la polvare. Gavemo anca mostrà come la via de somministrazion e el microambiente imunitario i sia fondamentali par determinar se l'acido sialico el porta a soppression o ativazion immunitaria. E questo el mette in luce quel che mi go ciamà el ruolo da Giano bifronte de l'acido sialico ne la regolazion imunitaria.

Portfolio of education

Eleonora Nardini

March 2021 – April 2025

Promotors: Yvette van Kooyk, Jan Voorberg

Copromotors: Juan Ernesto Rodriguez, Fabrizio Chiodo

PhD training	Year	ECTS
Courses		
Advanced Cytometry Course	2022	2
Research Integrity Course	2022	2
Basic ImageJ/Fiji Course	2022	0.60
Basic microscopy	2021	1.5
Animal Science Course	2021	3
Advanced Immunology Course	2021	3
Statistics course	2022	1
Hemostasis and immunology	2021	0.36
Clinical training	2022	0.86
Omics and Bioinformatics	2022	1.5
Entrapreneuership and innovation	2023	0.36
Other		
MCBI meetings & seminars	2020-2024	2.00
TRIM meetings	2020-2024	2.00
Tumor Immunology meetings	2020-2024	3.00
Journal Club	2020-2024	1.00
AI&I Seminar series	2021-2024	1.00
Organizing CCA PhD retreat	2022	1
AI&I PhD retreat	2021	0.5
Science Awards Day	2024	0.89
Secondment at CRC in Paris	2024	5
Teaching	**Year**	**ECTS**
Supervising literature thesis		
Jenny Heijkoop: The role of Siglecs in immune tolerance	2023	1.0

Supervising master internship		
Tjalle Dijkstra: Glyco-engineering HEK293T cell lines to produce a tolerogenic recombinant FVIII protein overexpressing the Siglec-7 ligand	2022-2023	3.00

List of publications

1. Hogan R, Flamier, A; Nardini, E; Bernier G., The Role of BMI1 in Late-Onset Sporadic Alzheimer's Disease. Genes 2020
2. Nardini E, Hogan R, Flamier A, Bernier G. Alzheimer's disease: a tale of two diseases? Neural Regen Res. 2021
3. Keumatio Doungtsop BC, Nardini E, Kalay H, Versteeg SA, Lübbers J, van Barneveld G, Li ERJ, van Vliet SJ, van Ree R, de Jong EC, van Kooyk Y. Sialic acid-modified der p 2 allergen exerts immunomodulatory effects on human PBMCs. J Allergy Clin Immunol Glob. 2023
4. Nardini E, Rodriguez E, van Kooyk Y, The tissue glycome as regulator of immune activation and tolerance mediated by C-type lectins and Siglecs, Seminars in Immunology, 2024
5. Nardini E, Keumatio Doungstop BC, Gerpe-Amor T, de Haas AM, de Kok M, Peterse E, Kalay H, Li RE, Chiodo F, Silipo A, Voorberg J, van Kooyk Y. Rational design of FVIII sialylated peptides to target Siglec-3 and Siglec-9 and improve peptide formulations for reverse vaccines. Front Bioeng Biotechnol. 2025
6. Keumatio Doungtsop BC, Nardini E, Peterse EE, Kalay H, Versteeg SA, van Ree R, de Jong EEC, van Kooyk Y. α2-3 Sialic Acids-Decorated Allergens Exert a Tolerogenic Effect on CD4+ T Cells from Der p 2-Allergic Patients. Int Arch Allergy Immunol. 2025
7. Ray GJ, Nardini E, Keys HR, Lin DH, Sabatini DM, Bartel DP. Lysosomal RNA profiling reveals targeting of specific types of RNAs for degradation. bioRxiv [Preprint]. 2025 Nature, 2026
8. van der Haar Àvila I, Lenos K, Lorrain V, Nardini E, Rodríguez E, García-Vallejo JJ, van Kooyk Y, Grootjans J, van Vliet SJ. Sialylation in Colorectal Cancer Rewires Antitumor Immunity at the Peritoneal Metastatic Site. Eur J Immunol. 2026
9. Nardini E, Delignat S, Doungstop BK, Olesek K, Reyes-Ruiz A, Lavend'homme R, Balzarini F, Peterse E, Kalay H, Li RE, Rodriguez E, Van Vliet SJ, Jacquemin M, Lacroix-Desmazes S, Van Kooyk Y. Shaping T cell responses to factor VIII in hemophilia A with sialylated immunodominant peptides. Haematologica. 2026
10. van den Brekel TMEV, Goossens-Kruijssen LJW, Olesek K, Nardini E, Koningstein GM, van Elk J, Jong WSP, Duinkerken S, Luirink S, van Kooyk Y. Soluble multi-epitope protein vaccination leverages antigen availability to drive CD8+ T cell immunity and tumor control. Front Immunol. 2026
11. Coccimiglio M, Ibáñez-Molero S, Springer BO, Goossens-Kruijssen L, Clayton G, Davison R, Zhang T, Wijnen S, Rodriguez E, Olesek K, Veenstra SPGR, van den Brekel T, Nardini E, van Elk J, Keuning E, Garcia-Garcia S, Boelaars K, de Haan N, Labots M, de Gruijl T, Blank C, Chiodo F, van Kooyk Y. CMAS dampens anti-tumor immunity and associates with response to neoadjuvant immunotherapy in melanoma. bioRxiv. 2026

ABOUT THE AUTHOR



Eleonora Nardini was born on the 23rd December 1996 and she grew up in San Donà di Piave (VE), where the principles of scouting - service, loyalty, resilience, optimism, and respect for nature - shaped much of her childhood. When she was 18, she moved to Bologna to study biotechnology during her BSc and pharmaceutical biotechnology for her MSc, from both of which she graduated cum laude. Her bachelor thesis was carried out in Oxford under the supervision of dr. Dianne Newbury, while for her master thesis she moved to Boston at MIT, where she worked on RNA autophagy in the laboratory of dr. David Sabatini. During her master, she was also selected to be part of Collegio Superiore, a degree of honor imprinted to excellence, thanks to which she kept her knowledge broad with courses on various topics such as philosophy, philology and finance. In 2021, she started her PhD in Amsterdam in the laboratory of dr. Yvette van Kooyk. This was carried out thanks to the Marie Curie fellowship EDUC8, which brought together eight European institutions to work on factor VIII immunogenicity. As part of this network, she was visiting researcher in the laboratory of dr. Sebastien Desmanez Lacroix in Paris where she conducted experiments on hemophilic mice. After her PhD contract ended in 2025, she joined AviSia therapeutics, a spin-off company of Yvette van Kooyk, where she could continue pursuing her passion for glycoimmunology by developing sialylated medicines for type 1 diabetes. As from July 2026, she joined MAT BioTech as a scientist, entering the vibrant field of pyrogen testing. The time for constantly running around and moving is over, now it is time to enjoy and rest. Throughout her life she pursed curiosity and the highest goal of understanding the world around her, looking at something that no one has ever looked at before. At MAT BioTech, she has found a new home where she hopes to continue doing just that for many years to come, in her beloved Amsterdam.

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I could call you and (if you hadn't lost your phone) you would be there for me.

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