The Potential of Sialylated Conjugates as Modulators of the Innate Immune System

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List of Abbreviations

A. fumigates	*Aspergillus fumigates*
A. nidulans	*Aspergillus nidulans*
BCR	B-cell receptor
C. neoformans	*Cryptococcus neoformans*
CCR-7	Chemokine Receptor Type-7
CD22	Siglec-2
CD33r-Siglecs	CD33-related Siglecs
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CGD	Chronic Granulomatous Disease
CPS	Capsular Polysaccharide
DAP10	DNAX activating protein 10
DAP12	DNAX activating protein 12
DCs	Dendritic Cells
DE	Defensin
DHR	Dihydrorhodamine 123
DiBAC	Bis(1,3-dibutylbarbituric acid) trimethine oxonol
Duox	Dual oxidase 1
endoN	Endoneuraminidase
fMLP	Formylmethionine-leucyl-phenylalanine
FSH	Follicle Stimulating Hormone
FYN	Tyrosine-protein kinase FYN
Gal	Galactose
GAS	*Group A streptococcus*
GBS	*Group B streptococcus*
GlcNAc	N-Acetylglucosamine
GnRH	Gonadotropin-Releasing Hormone
HIT	Heparin-induced-thrombocytopenia
HIV	Human Immunodeficiency Virus
HOCl	Hypochlorous acid
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immune receptor tyrosine-based activation motif
ITIM	Immune receptor tyrosine-based inhibition motif
KDN	Deaminated Neuraminic Acid
LF	Lactoferrin
LH	Luteinizing Hormone
LPG	Lipophosphoglycan
LPS	Lipopolysaccharides
MAG	Myelin-associated glycoprotein
ManNAc	N-Acetylmannosamine
Monosia	Monomers of sialic acid
MPO	Myeloperoxidase
MUC	Mucin

MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamidadenindinucelotidphosphate
NE	Neutrophil Elastase
NET	Neutrophil Extracellular Traps
NETosis	The release of NETs
Neu5Ac	N-Acetylneuraminic acid
Neu5Gc	N-Glycolylneuraminic acid
NRP-2	Neuropilin-2
NK-cells	Natural Killer-Cells
NLRP6	NOD-like receptor family pyrin domain containing 6
Nox	NADPH oxidase
P. aeruginosa	*Pseudomonas aeruginosa*
PAD4	Peptidylarginine deimindase 4
PHOX	Phagocytic oxidase
PKC	Proteinkinase C
PMA	Phorbol myristate acetate
PMSF	Phenylmethlysulfonylfluorid
PolySia	Polysialic Acid
ROS	Reactive Oxygen Species
RT-qPCR	Real-time quantitative Polymerase Chain Reaction
S. pneumoniae	*Staphylococcus pneumoniae*
SAMPs	Self-Assiociated Molecular Pattern
SH2	Src homology 2
SHP1	Src homology 2 phosphatase 1
SHP2	Src homology 2 phosphatase 2
Sia	Sialic acid
Siglec	Sialic acid binding immunoglobulin-like lectin
Siglec-1	Sialoadhesin
SLE	Systemic lupus erythematosus
ST3Gal	ST3 beta-galactoside alpha-2,3-sialyltransferase
ST6Gal	ST6 beta-galactoside alpha-2,6-sialyltransferase
ST8Sia	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1
SVV	Small Vessel Vasculitis
Syk-kinase	Spleen tyrosine kinase
TFPI	Tissue Factor Pathway Inhibitor
TGF-β	Transforming Growth Factor Beta
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TR	Tandem Repeats
vWF	von Willebrand factor
ZAP70	Tyrosine-protein kinase ZAP 70

1. Introduction

1.1 Mucins

In order to protect underlying cells the intestinal, the respiratory as well as the reproductive tract are coated with mucus (Hansson 2019), mainly consisting of water, ions and macromolecules (Ma, Rubin and Voynow 2018). The predominant macromolecules are highly glycosylated proteins, called mucins, which can be separated into membrane-bound and secreted proteins (Bansil and Turner 2006, Tsiligianni et al. 2003, Corfield 2015, Pluta et al. 2012, Perez-Vilar and Hill 1999). Produced by epithelial cells, mucins have a protective function against infection, dehydration and physical and chemical injury and play a predominant role in reproduction (Kufe 2009, Perez-Vilar and Hill 1999). As highly glycosylated glycoproteins they are mainly characterized by a large molecular weight (0.5 MDa up to 20 MDa (Pluta et al. 2012, Bansil and Turner 2006)) and a high *O*-glycan content, which accounts for up to 80% of the molecular weight (Bansil and Turner 2006, Tsiligianni et al. 2003, Pluta et al. 2012, Rose and Voynow 2006). Furthermore, secreted mucins manifest an extensive and variable number of tandem repeats (TR) in the protein backbone, which makes up around 20% of the molecular weight (Pluta et al. 2012, Bansil and Turner 2006, Rose and Voynow 2006). Those TR are rich in serine, threonine and proline and account for around 60% of the amino acids (Bansil and Turner 2006). As serine and threonine are the sites of *O*-glycosylation the amount and length of TR determine a mucins degree of glycosylation and therefore its chemical properties (Rose and Voynow 2006). Additionally, the N-terminus of secreted mucins is characterized by a cysteine rich area followed by a sequence similar to von Willebrand factor (vWF) D and the C-terminus hosts a sequences similar to vWF D followed by a sequence similar to vWF C, which ends in a cysteine rich region (Bansil and Turner 2006). Those cysteine rich areas are responsible for the linkage of monomers through disulfide bonds (**Figure 1**), contributing to the formation of mucus gel (Ambort et al. 2011, Desseyn 2009). In contrast, membrane-associated mucins are monomeric with characteristic membrane peptide domains, which facilitate a broad range of biological functions as a part of the glycocalyx (Corfield 2015).

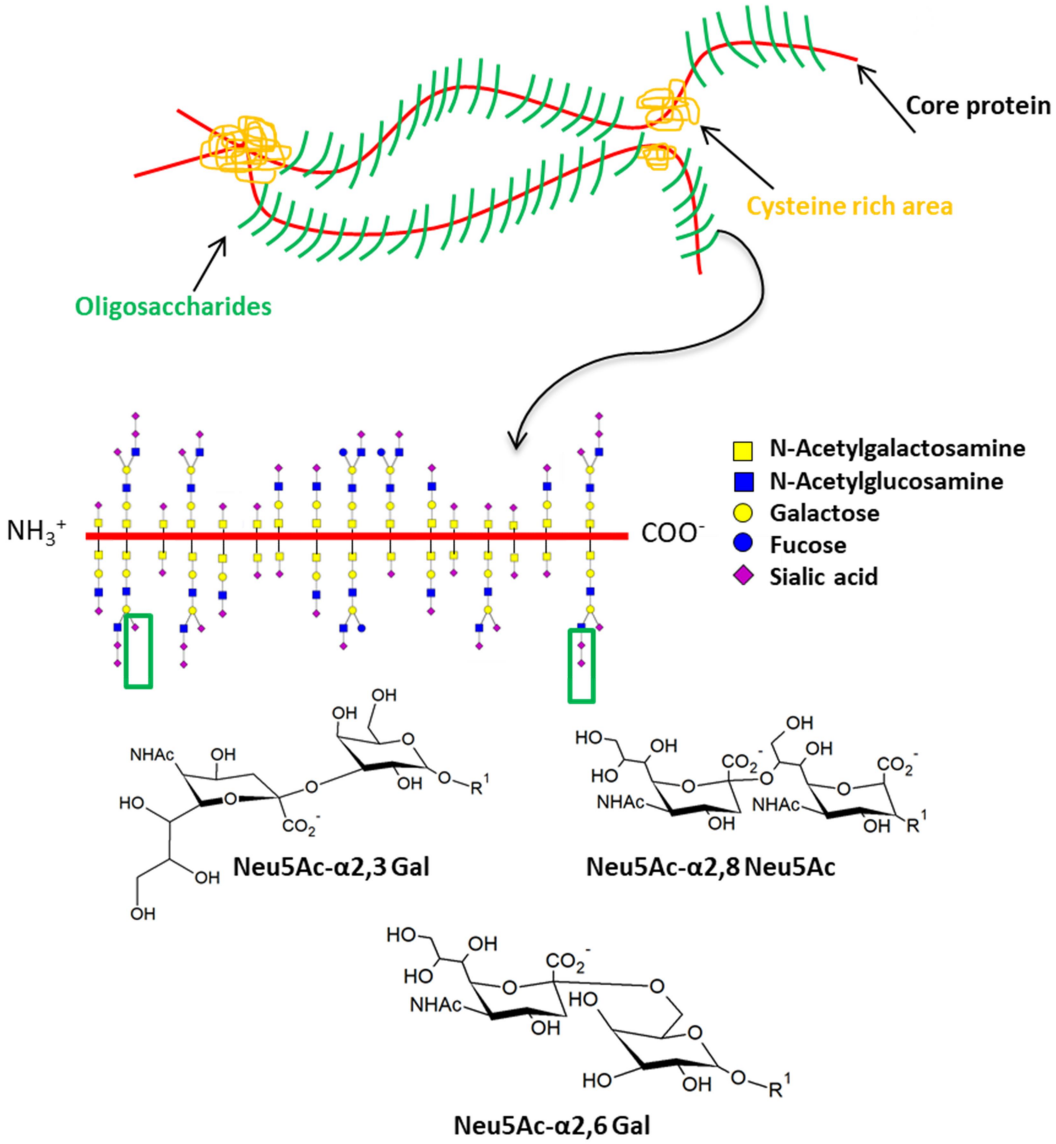

Figure 1: Schematic illustration of secreted mucins (inspired by (Yang and Nauwynck 2015)). Mucin dimer. Mucins are highly glycosylated proteins. At the N-terminus as well as at the C-terminus secreted mucins are characterized by a cysteine rich area, responsible for the linkage of monomers through disulfide bonds (yellow). The glycan structures (green) consist of different amounts of glycans, each beginning with N-Acetylgalactosamine. Depending on the distribution of glycans, the properties of mucins change. The terminating glycan is often a sialic acid (Neu5Ac) residue. These acidic α-keto acids can be linked to galactose (Gal) with α2,3-;2,6-linkages or to each other by α2,8-linkages (Yang and Nauwynck 2015).

1.1.1 Mucins in health and disease

Nowadays two distinct functions of mucins are defined: the cleaning of the surface by washing away bacteria as well as cell debris and the protection of the surface by coating epithelial cells (Hansson 2019). Since the function depends on the localization

of mucins, their role in health and/or disease in different mucin coated organs is focused in the following.

In the small intestine: In healthy intestines the mucus layer effectively protects the epithelial cells (Johansson et al. 2008). Rich in antimicrobial peptides and proteins, produced by Paneth cells at the crypt bottom, the mucus does not exclude bacteria by size but does effect bacterial survival (Clevers and Bevins 2013, Hansson 2019). The most prominent mucin within the small intestine is mucin 2 (MUC2). MUC2 is linked to goblet cells after secretion and detachment is initiated by bicarbonate-mediated unfolding of the N-termini of MUC2, offering the cleavage site for meprin-beta (Hansson 2019, Schütte et al. 2014). Patients suffering from cystic fibrosis (CF) show a mucus accumulation within the distal ileum due to a mutation of the cyclic adenosine monophosphate regulated chloride channel (CFTR channel), causing a decreased bicarbonate release, preventing MUC2 detachment (Hansson 2019, Gustafsson et al. 2012).

In the colon: Within the colon two mucus layers exist, which are separated into the outer and inner mucus layer. The outer mucus layer hosts bacteria (Hansson 2019), whereas the inner mucus layer is linked to goblet cells, excluding pathogens by size (Ambort et al. 2012). Each crypt opening is protected by one sentinel goblet cell (Birchenough et al. 2016, Hansson 2019), which exerts their function by endocytosis of toll like receptor (TLR)-2;-4;-5 ligands. This endocytosis in turn leads to an activation of Myeloid differentiation primary response 88 (MyD88), NADPH oxidase (Nox), Dual oxidase 1 (Duox) and finally the NLRP6 inflammasome and caspase 1 and 11, causing a rise of the calcium level, initiating goblet cells and sentinel goblet cells to exocytose compounds. Goblet and sentinel cells form a mucus plume, able to trap and move away bacteria. Unfortunately, patients suffering from ulcerative colitis lack sentinel goblet cells, contributing to the absence of the bacterial barrier (Hansson 2019).

In the respiratory tract: Within the respiratory tract mucins play a further essential role. Although, we are inhaling particles and pathogens, our lungs are clean, due to mucus-associated pathogen entrapment, phagocytosis of pathogens by airway macrophages and the subsequent removal by mucociliary clearance (Ermund et al. 2017, Hoegger et al. 2014, Dagenais and Keller 2009). Interestingly, patients suffering from CF show a mutation on the gene encoding for the CFTR channel expressed on the apical surface of lung cells and epithelial tissue. The resulting dysfunction causes dehydration and affects the pH as well as the electrolyte and mucus concentration of

the airway surface liquid, contributing to an altered mucociliary transport (Henderson et al. 2014, Cowley et al. 2017, Button et al. 2012, Tang et al. 2016, Hoegger et al. 2014). The increased mucus concentration due to the interplay of increased mucin expression (MUC5AC by goblet cells, MUC5B by submucosal glands and goblet cells) and the missing detachment of MUC5B from goblet cells in combination with decreased hydration ends up in a stagnation of the flow, leading to the accumulation of pathogens (Button et al. 2012, Kirkham et al. 2002, Hansson 2019, Ermund et al. 2018, Gustafsson et al. 2012). The missing mucociliary clearance allows e.g. *Pseudomonas aeruginosa, Staphylococcus aureus* or *Aspergillus fumigates* to germinate and to establish chronic infections (Middleton, Chen and Meyer 2013, Lyczak, Cannon and Pier 2002, Cullen and McClean 2015).

In the breast: The most common cancer type in woman worldwide is breast cancer, showing an overexpression of MUC1, which therefore serves as a tumour marker (Duffy, Evoy and McDermott 2010). In addition, rectal, prostate, lung and pancreatic cancer are also associated with mucins (excellently summarized in (Deng et al. 2013, Chauhan et al. 2006)).

In the reproductive tract: In the early stages, the work on mucins within the reproductive tract was mainly focused on MUC1 expression in the uterus. In mice MUC1 is highly expressed during the peri-implantation period, but has to be removed at the site of implantation between day 1 and 4.5 of pregnancy for a successful implantation of the embryo (Braga and Gendler 1993). Nowadays it is well known, that mucins play many further roles within reproduction. For instance, MUC16 can be found in the peripheral blood of patients suffering from ovarian cancer, serving as a tumour marker, known as cancer antigen 125 (Belisle et al. 2010). In addition, around 90% of malignant ovarian tumours are characterized by MUC4 expression, which is absent or almost undetectable in healthy patients (Chauhan et al. 2006). Regarding cervical mucins, the glycosylation pattern changes during menstrual cycle: while cervical mucins represent an antimicrobial barrier during the ongoing pregnancy, they permit sperm transport during estrus (Pluta et al. 2012, Andersch-Björkman et al. 2007).

The oestrous cycle can be separated in two different phases: the luteal and the follicular phase. In between those phases, ovulation occurs. The follicular phase is devoted to the selection of a dominant follicle. In response to the decreasing levels of oestrogen due to the regression corpus luteum from the previous cycle, the concentration of the follicle stimulating hormone (FSH) increases (Bates and Bowling

2013). As FSH levels continue to rise, the dominant follicle emerges and secretes oestrogen (Goodman and Hodgen 1983), which induces a surge of gonadotropin-releasing hormone (GnRH) from the hypothalamus. GnRH itself induces the increased release of luteinizing hormone (LH) and FSH from the hypophysis (Ramírez-González et al. 2016). FSH and LH cause further follicle growth and stimulate ovulation (Raju et al. 2013). During ovulation, the oocyte is released and the luteal phase is initiated. When fertilization occurred, the corpus luteum is formed from the collapsed ovulated follicle and progesterone levels increase (Vande Wiele et al. 1970, Ramírez-González et al. 2016). The embryo is implanted in the endometrium and produces human chorionic gonadotropin, contributing to the retention of the corpus luteum by supporting its production of progesterone (Catt, Dufau and Vaitukaitis 1975). Human endocervical epithelium express MUC1, MUC4, MUC5AC, MUC5B and MUC6 (Gipson et al. 1997), while bovine endocervical epithelium express MUC1, MUC4, MUC5AC, MUC5B, MUC16 and MUC20 (Pluta et al. 2012). According to Katz *et al.* the release of oestrogen during the follicular phase contributes to the secretion of highly hydrated mucus (up to a water content of 96%), enabling sperm to pass the cervix, whereby through the rising progesterone levels during the luteal phase, cervical mucus gets thick and viscous, serving as a competent antimicrobial barrier during the ongoing pregnancy (Pluta et al. 2012, Katz, Mills and Pritchett 1978, Morales, Roco and Vigil 1993). Thus, it is not surprising, that humoral dysfunctions may cause infertility, by rendering cervical mucus inadequate for sperm transport (Jequier 2006). This is in line with the detection of Bigelow *et al.,* that the mucus hydration is more influential on the incidence of pregnancy than coitus at the time of ovulation (Bigelow et al. 2004).

However, when sperm enter the female reproductive tract, they immediately have to handle further challenges like the low pH of the vaginal fluid (Miller 2018) and the initiated immune response. According to Tyler *et al*. and Pandya *et al*. in rabbit's and humans, vaginal insemination contributes to the migration of neutrophils and macrophages to the vagina as well as the cervix, combatting excess of sperm, seminal debris and invading pathogens mainly by phagocytosis, the release of reactive oxygen species and the liberation of antimicrobial peptides (Tyler 1977, Brinkmann et al. 2004, Pandya and Cohen 1985). Additionally, in 2004 Brinkmann *et al*. detected a further mechanism of neutrophils to combat invading pathogens, the release of neutrophil extracellular traps (NETs) - called NETosis: a trapping and killing instrument of neutrophils, which might not just serve as a pitfall for pathogens within the ejaculate, but also for the sperm themselves (Brinkmann et al. 2004, Hahn et al. 2012).

1.2 Neutrophils

Neutrophil granulocytes, also called polymorphonuclear leukocytes, are the most frequent leukocytes of the innate immune system (Borregaard 2010). They are produced in large numbers in the bone marrow (~1-2*10^{11}cells per day in an adult human) (Mayadas, Cullere and Lowell 2014, Breedveld et al. 2017) and morphologically they are characterized by cytoplasmic granules and their lobed nucleus (Figure **2**) (Lothar Rink 2015). In case of an inflammation, neutrophils are the first mechanism of defence (Kessenbrock et al. 2008, Lekstrom-Himes and Gallin 2000, Nathan 2006) and interestingly, neutrophil production within the bone marrow is upregulated in case of infections (Murphy 2018). They exit circulation selectin-derived via trans endothelial migration (Nel et al. 2016) and arrived at the side of inflammation, they perform their function by phagocytosis as well as by the release of antimicrobial peptides and reactive oxygen species (ROS) (Brinkmann et al. 2004, Nauseef 2007, Papayannopoulos and Zychlinsky 2009, Urban et al. 2009). Moreover, neutrophils are able to perform a 'beneficial suicide', called NETosis-characterized by the release of neutrophil extracellular traps (NETs) consisting of antimicrobial molecules (myeloperoxidases (MPO), neutrophil elastases (NE), lactoferrin (LF), defensins (DE)…), decondensed chromatin fibres and cytotoxic histones (Brinkmann et al. 2004, Nauseef 2007, Papayannopoulos and Zychlinsky 2009, Urban et al. 2009). As for example histones are also toxic for endogenous cells (Hariton-Gazal et al. 2003, Rosenbluh et al. 2004), subsequent clearance of dying neutrophils is not only essential for the controlling of the infection, but also for the resolution of the inflammatory response and the maintenance of homeostasis (Allen and Criss 2019).

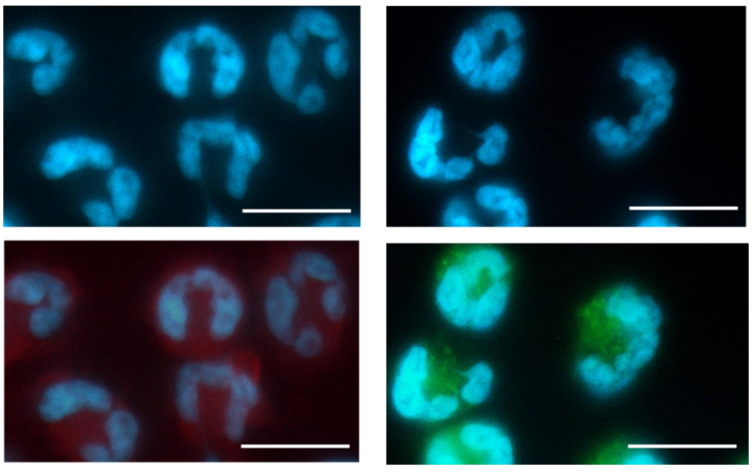

Figure 2: **Characteristics of neutrophils.** Isolated bovine neutrophils show the characteristic segmented nuclei and cytoplasmic granules. Cells were stained with DAPI (blue) and anti-bovine lactoferrin antibody (red) or anti-neutrophil elastase antibody (green). Scale bars 10 µm.

1.2.1 The mechanism of NET release

NETosis can be induced by physiological agonists, such as interleukin-8 (IL-8) or microbe-associated surface proteins like lipopolysaccharides (LPS) or N-formylmethionine-leucyl-phenylalanine (fMLP), gram-positive and gram-negative bacteria, viruses, fungi and platelets as well as non-physiological stimuli, like calcium ionophores and phorbol myristate acetate (PMA) (Brinkmann et al. 2004, Niedźwiedzka-Rystwej et al. 2019). In addition, very recently, Zwarotniak *et al*. described UVB and UVA induced formation of NETs, assumed to be involved in photo aging of the skin (Zawrotniak, Bartnicka and Rapala-Kozik 2019) and Mohanty *et al*. detected sialyl lewis X-L-selectin-interaction dependent NET formation in human saliva (Mohanty et al. 2015).

In general, NETosis can be initiated by ligand binding to TLRs, receptors for IgG-Fc, complement or cytokines (Garcia-Romo et al. 2011, Papayannopoulos et al. 2010, Brinkmann et al. 2010, Brinkmann et al. 2004, Niedźwiedzka-Rystwej et al. 2019). Upon PMA recognition, which resembles diacylglycerol and therefore mimics NET induction by bacteria and fungi (Petretto et al. 2019), a calcium influx from the endoplasmic reticulum is triggered, activating the protein kinase C (PKC), necessary for the activation of NADPH oxidase enzyme complex, also called phagocytic oxidase (PHOX) (Brinkmann and Zychlinsky 2012, Nel et al. 2016, Desai et al. 2016). PKC is presumed to regulate the activation of the Raf-MEK-ERK pathway, whereby ERK1/2 is assumed to activate the NADPH oxidase by phosphorylation (Brinkmann and Zychlinsky 2012, Nel et al. 2016, Desai et al. 2016, Dwivedi and Radic 2014, Brinkmann 2018, Hakkim et al. 2011). In the following, the enzyme complex produces ROS, which serve as substrate for MPO. MPO generates hypochlorous acid (HOCl), which destroys the complex of MPO and NE, releasing NE (stored in the granular), via a not yet fully understood pathway, without rupture of the granular membrane in the cytoplasm (Brinkmann and Zychlinsky 2012, Nel et al. 2016). NE further enters the nuclei. There it degrades histone H4. Furthermore, MPO is detected in the nuclear fraction in later stages of NET formation, assumed to contribute to DNA decondensation independent of its enzymatic activity (Papayannopoulos et al. 2010). Simultaneously, the peptidylarginine deimindase 4 (PAD4) migrates from the cytoplasm to the nucleus (de Bont et al. 2018) and mediates deamination of histones (Hemmers et al. 2011), causing histone hypercitrunillation (conversion of arginine to citrulline) (Wang et al. 2009, Odqvist et al. 2019), promoting NET formation by inducing DNA

decondensation and simplifying the expulsion of DNA coated with antimicrobial peptides (Rohrbach et al. 2012) (**Figure 4**).

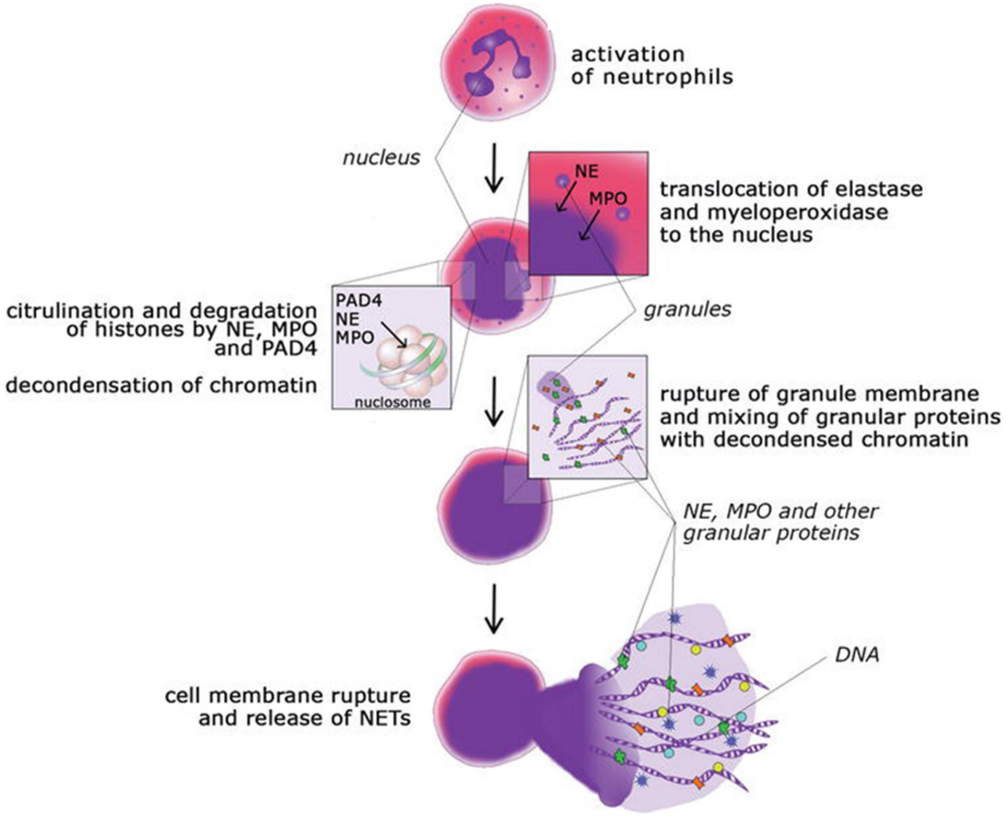

Figure 3: The mechanism of NET release. After the activation of the NADPH oxidase, which causes the production of ROS, NE and MPO translocate to the nuclei, contributing to the decondensation of the DNA by degrading histones. As a further protein, PAD4 translocates to the nuclei, responsible for citrunillation of histones. As with decondensation of the DNA the volume increases, nuclear membrane ruptures, leading to a mixture of decondensed DNA fibers and antimicrobial peptides within the cytoplasm. As a final step, the plasma membrane ruptures, releasing NETs in the extracellular area [modified (Zawrotniak et al. June 7th 2017) CC-BY 3.0.]

However, just a few cells stained positive for citrunillated histone H3 after stimulation with PMA. Interestingly, experiments with phenylmethlysulfonylfluorid (PMSF), a serine protease inhibitor allowed the detection of citrunillated histones on NETs induced by PMA in high quantities, indicating that citrunillated histone H3 is quickly degraded under physiological conditions (de Bont et al. 2018). DNA decondensation results in the swelling of the nuclei, contributing to the rupture of the core membrane and under the influence of ROS the granular membrane ruptures as well, releasing antimicrobial molecules in the cytoplasm. As a final step the plasma membrane ruptures, releasing the mixture, consisting of decondensed chromatin fibres, antimicrobial molecules and

cytotoxic histones in the extracellular area (Papayannopoulos et al. 2010, Brinkmann and Zychlinsky 2012, Yang et al. 2016) (**Figure 4**).

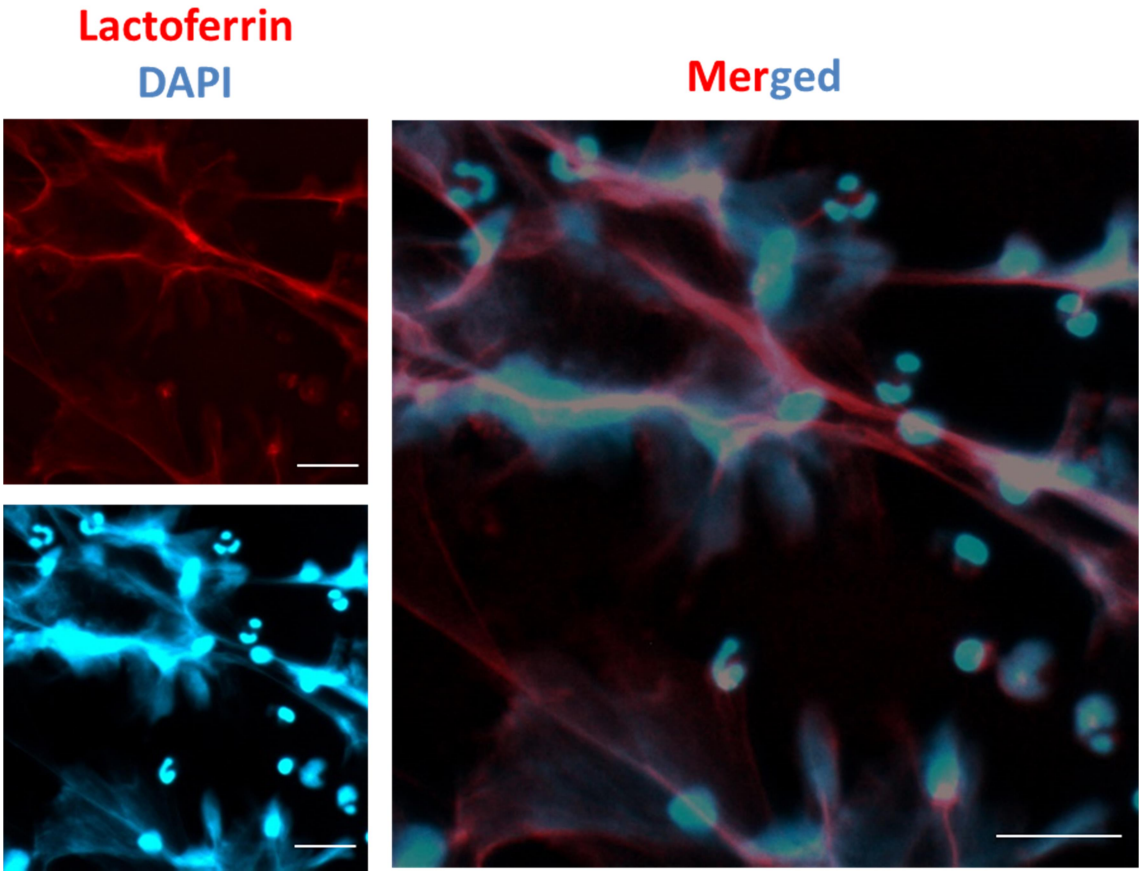

Figure 4: NET release by bovine neutrophils. LPS stimulation (20 µg/mL) leads to the release of DNA fibres (DAPI, blue) decorated with neutrophil specific antimicrobial proteins like lactoferrin (Red). Scale bars: 20 µm. The term "merged" indicates the overlay of both stainings.

1.2.2 NET-associated pathologies

As already mentioned, neutrophils are recruited in the female reproductive tract after insemination and according to Hahn *et al*. an excessive release of NETs would not only eliminate pathogens, induced during mating, but also serve as a trap for sperm, assumed to contribute to infertility (**Figure 5**) (Alghamdi and Foster 2005, Hahn et al. 2012).

Interestingly, independent of the presence of further pathogens, according to Zambrano *et al*., sperm themselves induce NET release dependent on time and dosage, resulting in decreased sperm motility (Zambrano et al. 2016).

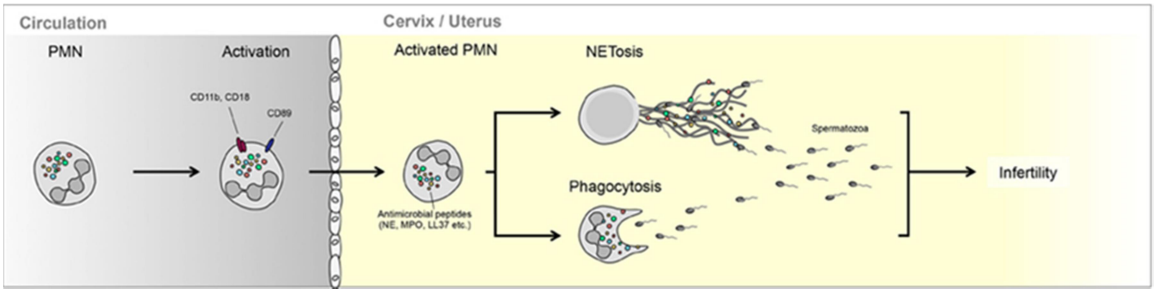

Figure 5: NET release within the reproductive tract. Neutrophils can either phagocytize less motile sperm or can trap those in NETs, contributing to infertility (Hahn et al. 2012) CC-BY.

In addition to infertility, an excessive NET release and a missing clearance of NETs are further associated with life-threatening diseases, like small vessel vasculitis (SVV), preeclampsia, rheumatoid arthritis, ulcerative colitis, thrombosis, atherosclerosis, the formation of gall stones and CF, just to mention a few (Brill et al. 2012, Hakkim et al. 2010, Fuchs et al. 2010, Brinkmann and Zychlinsky 2012, Kaplan 2013, Kessenbrock et al. 2009, von Brühl et al. 2012, Muñoz et al. 2019). For instance, NETs accumulate in case of a reduced blood flow over a certain period of time (venous thrombi) or damage of endothelium (arterial blood clots), acting as a scaffold for the formation of a thrombus. Accessorily, NE cleaves the tissue factor pathway inhibitor (TFPI), thus indirectly activates the coagulation cascade, whereby extracellular histones enhance the thrombin generation triggering platelet activation and coagulation, also contributing to the formation of a thrombus (Fuchs et al. 2010, Clark et al. 2007, Caudrillier et al. 2012, von Brühl et al. 2012). Moreover, healthy lungs are frequently cleaned by sweeping of the surface by the mucus bundles formed by MUC5B. However, in patients suffering from CF, CFTR is mutated, contributing to low levels of bicarbonate, necessary for the detachment of MUC5B from the goblet cells, causing a stagnated mucin layer (Hansson 2019, Ermund et al. 2018, Gustafsson et al. 2012). This attached, tenacious mucus in turn facilitates the accumulation of pathogens like *Staphylococcus aureus*, *Haemophilus influenza* and *Pseudomonas aeruginosa*, leading to a neutrophil-rich inflammation. According to Brinkmann *et al*. due to NET release high amounts of extracellular DNA are present in patients suffering from CF, probably enhancing the viscosity of CF sputum, decreasing lung function (Brinkmann and Zychlinsky 2012, Manzenreiter et al. 2012). With the aim to decrease sputum viscosity, patients are nowadays treated with recombinant DNase (Rahman and Gadjeva 2014).

Thus, it is not surprising, that it is a medical concern to prevent an exaggerated NET release and that several control mechanisms must work efficiently to prevent an excessive release of NETs, as described in the next chapter.

1.2.3 Control mechanisms of NET

Physiological control mechanisms exist to inhibit an exaggerated release of neutrophil extracellular traps. For instance, very recently Lizcano *et al*. published that glycophorin expressed on erythrocytes inhibits NET release within circulation by the binding of its sialic acids to sialic acid binding immunoglobulin-like lectin-9 (Siglec-9) on the surface of neutrophils (Lizcano et al. 2017). Moreover, the biological component heparin is not just able to inhibit histone-mediated cytotoxicity, but is also able to release histones from NET, causing destabilization of NETs (Fuchs et al. 2010). In addition to heparin, polysialic acid (polySia) represents an antagonist of the cytotoxic effects of histones, too (Ulm et al. 2013, Saffarzadeh et al. 2012, Zlatina, Lütteke and Galuska 2017). PolySia, which occurs in the plasma of different species, affects histone-mediated cytotoxicity dependent on the concentration as well as chain-length (Galuska et al. 2017b, Galuska et al. 2017a, Zlatina et al. 2018) (**Figure 6**).

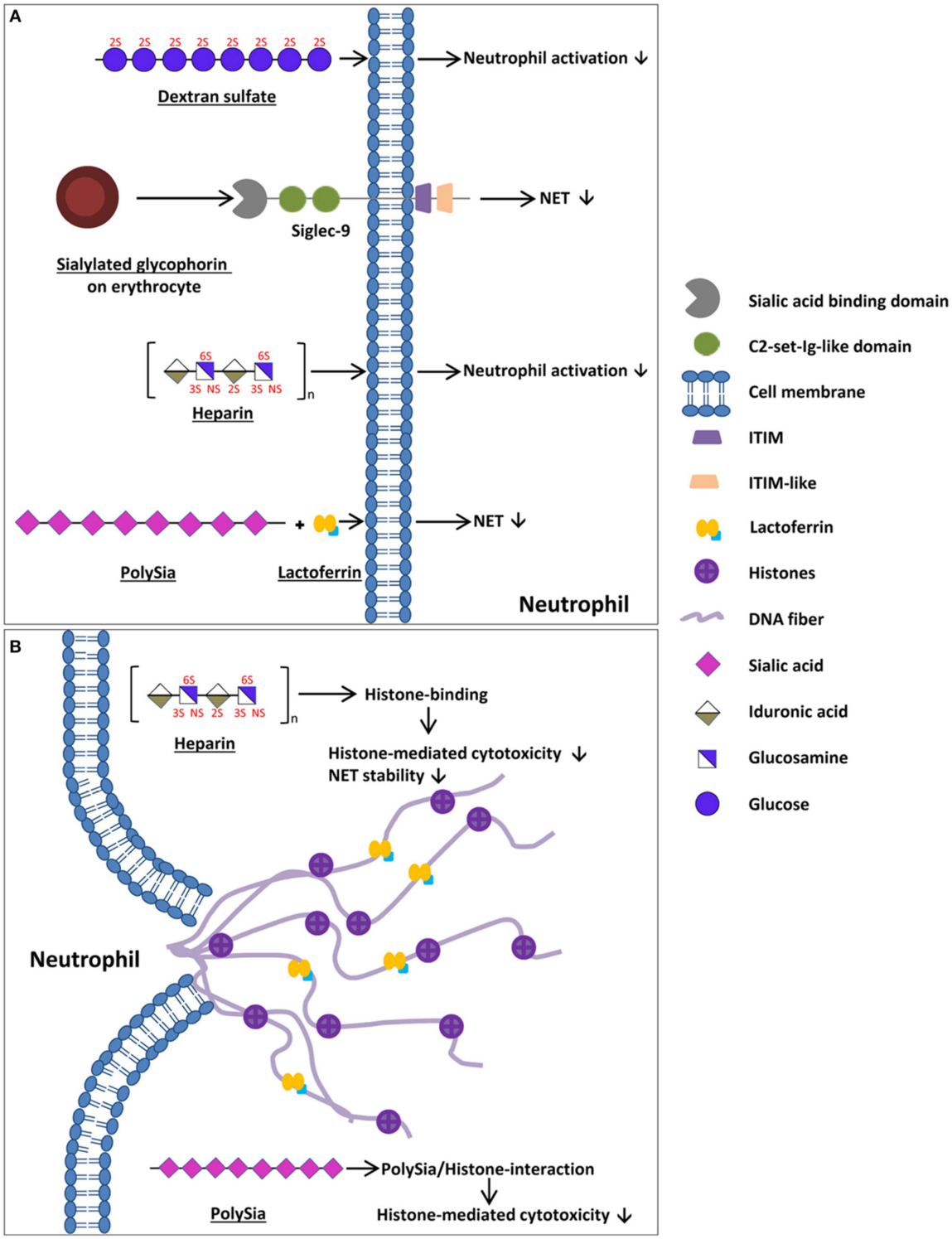

Figure 6: How glycans modulate the formation and outcome of neutrophil extracellular traps (Bornhofft and Galuska 2019) CC-BY. **A)** Naturally occurring components which inhibit NET release. **B)** Naturally occurring components which decrease the negative outcomes of NETs.

In addition, very recently Kuehnle *et al*. detected a further influence of polySia on NET formation. While lactoferrin itself causes a 'lactoferrin shell' (Okubo et al. 2016), preventing NET release, polySia enhances this effect (Kühnle et al. 2018).

Interestingly, polySia is also present on the surface of sperm, neutralizing the cytotoxic characteristics of histones, without affecting the antimicrobial capacity of district histones *in vitro* (Simon et al. 2013, Zlatina and Galuska 2019, Zlatina et al. 2017). Besides polySia, DNase I seems to play a fundamental role within the reproductive tract. Already in 1985 DNase I activity was detected in human seminal plasma (Singer et al. 1985). Since, according to Zambrano *et al*. DNase I treatment of sperm-induced NETs decreased sperm entrapment, the DNase I present in human seminal plasma might decrease the amount of sperm entrapped by NETs (Zambrano et al. 2016). Moreover, according to Tecle *et al*. *in vitro* experiments showed that the interaction of sialic acids on the surface of sperm with Sigle-9 on neutrophils as well as Siglec-10 within the endometrium, might contribute to sperm survival (Tecle et al. 2019).

As this suggests a fundamental role of Siglecs in the modulation of neutrophil activation Siglecs are focused on within the next chapter.

1.3 Sialic acid binding immunoglobulin-like lectins (Siglecs)

Siglecs are immune-regulatory transmembrane receptors mainly found on cells of the haematopoietic system (Varki and Angata 2006, Crocker, Paulson and Varki 2007), which maintain a balance between pathogen defence and pathophysiological manifestations through the recognition of sialic acid (Crocker et al. 2007, Ravetch and Lanier 2000, Macauley, Crocker and Paulson 2014). Nowadays 15 human Siglecs and 9 murine Siglecs are known, while only two Siglecs have been identified in lower vertebrates, like fish (Pillai et al. 2012, O'Reilly and Paulson 2009, Angata et al. 2007, Lehmann et al. 2004). Siglecs are subdivided in two groups: the group of CD33-related (CD33r) Siglecs, marked by a high degree of sequence identity among each other but a low gene conservation across orthologues and the evolutionary conserved group containing Siglec-1 (Sialoadhesin), Siglec-2 (CD22), Siglec-4 (Myelin-associated glycoprotein (MAG)) and Siglec-15, mainly characterized by a high gene conservation across orthologues (**Figure 7**) (Crocker et al. 2007, Angata et al. 2004).

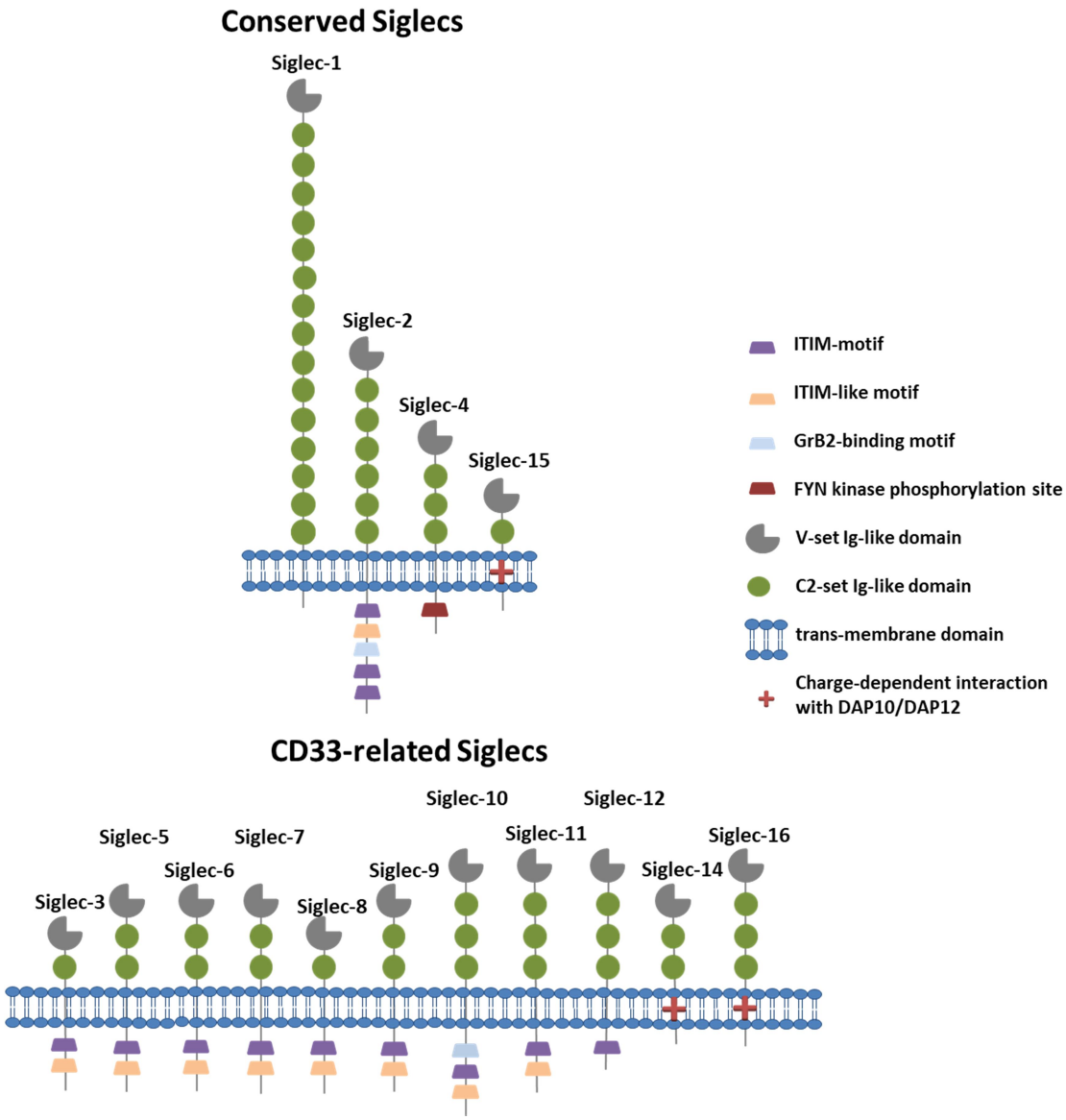

Figure 7: Schematic illustration of CD33-related and highly conserved Siglecs expressed in human-beings (modified (Bornhöfft et al. 2018) CC-BY).

According to Angata and Varki, the high similarity within the group of CD33r-Siglecs might be caused by multifaceted selection pressures caused by pathogens utilizing host sialic acid as ligands during invasion and mimicking host to circumvent the host's immune system, known as 'Red Queen Effect' (**Figure 8**) (Varki and Angata 2006, Varki 2006).

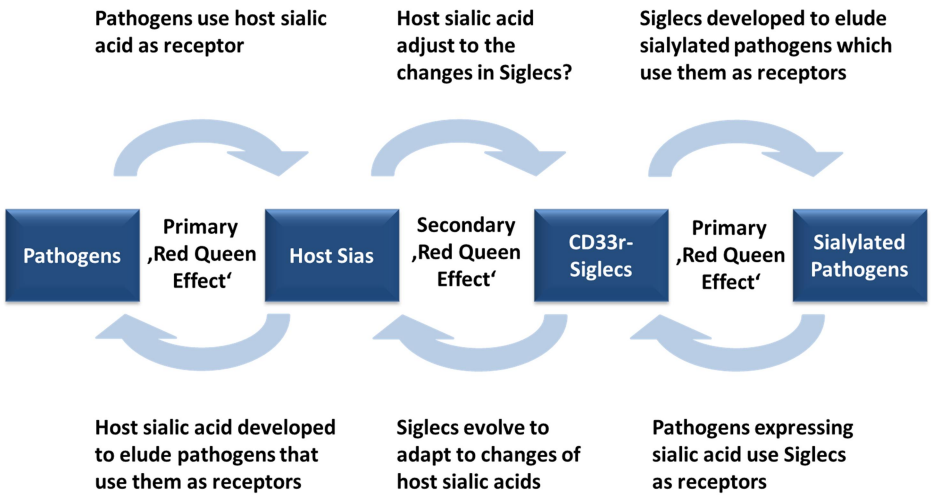

Figure 8: The Red Queen Effect. The assumed cause of the rapid development of CD33r-Siglecs, responsible for the high sequence identity among CD33r-Siglecs (inspired by (Padler-Karavani et al. 2014)).

1.3.1 Structure of Siglecs

As cell-surface-receptors Siglecs comprise of protein domains belonging to the immunoglobulin (Ig) superfamily. Their extracellular area is characterized by a variable number of C2-set Ig-like domains, terminated by one V-set Ig-like domain, responsible for sialic acid binding (Crocker et al. 2007, O'Reilly and Paulson 2009, Angata et al. 2004). Depending on the number of C2-set Ig-like domains, Siglecs prefer binding of sialic acid in cis-or in trans-direction (**Figure 7**) (Crocker et al. 2007). By sialic acid binding, and subsequent phosphorylation of the immune receptor tyrosine-based inhibition motif (ITIM), coming along with the recruitment of phosphatases, inhibitory-acting Siglecs can antagonize the initiation of an immune response, mediated via an immune receptor tyrosine-based activation motif (ITAM) (Crocker et al. 2007, Ravetch and Lanier 2000). In contrast, the activatory-acting Siglecs are associated with DNAX activating protein 10/12 (DAP10/12), bearing an ITAM (Crocker et al. 2007). In general, a highly conserved arginine residue is particularly important for the interaction of Siglecs with sialic acid (Zhuravleva, Trandem and Sun 2008). Remarkably, human Siglec-1 and MAG lack activating/ inhibiting motifs. Nevertheless, Siglec-1, which is mainly expressed on splenic and lymph node macrophages, seems to play an indispensable role in innate and humoral immunity (Chang et al. 2014). Highly expressed during pathogen invasion, Siglec-1 on macrophages contributes to pathogen phagocytosis (Crocker et al. 2007, Chang and Nizet 2014). In addition, Siglec-1 seems to influence humoral immunity, as Siglec-1 null mice showed decreased levels of IgM and decreased serum levels of IgG compared to the wild type

(May et al. 1998). In contrast, MAG is predominantly expressed on Schwann cells, as well as oligodendrocytes and does contain a FYN kinase phosphorylation site instead of ITIMs/ITAMs (Cagnoni et al. 2016, Quarles 2007, Sun et al. 2004). It prefers to bind to the sialylated gangliosides GD1a and GT1b, present in the brain and MAG binding to those, seem to be necessary for axon-myelin-stability, as ganglioside null mice omit among axon degeneration, dysfunction and motor deficiencies (Sun et al. 2004).

1.3.2 Molecular and biological function of DAP10/12 associated Siglecs

Siglec-14,-15,-16 are transmembrane receptors of humans, associated with the activation of the immune system via the interaction with DAP10/DAP12, containing ITAMs (Angata et al. 2007, Ishida-Kitagawa et al. 2012, Angata et al. 2006). The aspartic acid residue present in the transmembrane area of DAP12, can interact charge-dependently with the lysine in the transmembrane domain of Siglec-15 (Angata et al. 2007). Once the V-set Ig-like domain of Siglec-15 recognizes and interacts with sialic acid, the tyrosine residues of the ITAM gets phosphorylated by the kinases of the Src family. Subsequently, the phosphorylated ITAM domains of DAP12 serve as docking station for the Src homology 2 (SH2) domains of the Tyrosine-protein kinase ZAP70 (ZAP70) and the Spleen tyrosine kinase (Syk), associated with the onset of the immune response (Angata et al. 2007, Lanier and Bakker 2000).

Surprisingly, Siglec-15 seems to be a candidate for normalization in cancer immunotherapy. Very recently, Wang *et al*. described that Siglec-15 which is only minimally expressed by health tissues at steady-state but highly expressed in a broad spectrum of human cancers and tumour associated myeloid cells, inhibits antigen specific T-cell responses via an unknown receptor interaction (Wang et al. 2019, Cao, Xiao and Yin 2019). Beside this immunological role Siglec-15 plays an indispensable role during osteoclast differentiation (Teitelbaum 2000). Researcher detected that mice lacking Siglec-15 show evidence of mild osteopetrosis, which is characterized by a high bone density (Hiruma et al. 2013). Intriguingly, *in vitro* studies have demonstrated that the absence of the interaction of Siglec-15 with sialic acid results in the lack of osteoclasts differentiation to its multinuclear state, responsible for bone resorption (Macauley et al. 2014). Thus, an anti-Siglec-15 treatment might be a welcomed and effective possibility to overcome osteoporosis (Sato et al. 2018).

1.3.3 Molecular and biological function of ITIM-bearing Siglecs

Inhibitory Siglecs possess ITIMs with the following signature: (I/V/L/S)-X-Y-X-X-(L/V) (with X representing any amino acid) (Crocker et al. 2007), which enable counteraction

to the activation of the immune system through the inhibition of ITAM-mediated signalling (Crocker et al. 2007, Ravetch and Lanier 2000). Once sialic acid is recognized, the intracellular tyrosine residues of both ITIM and ITIM-like motifs (if available) are phosphorylated and subsequently two Scr homology 2 domain containing protein tyrosine phosphatases SHP1 and SHP2, are recruited, initiating the inhibition of kinase-dependent activation of the immune system (Crocker et al. 2007, Avril et al. 2005). The most prominent example is the Siglec-dependent inhibition of B-cell activation. When the B-cell receptor (BCR) recognizes an antigen, the B-cell differentiates to an antibody producing plasma cell. However, when the recognized antigen is in close proximity of sialylated structures on endogenous cells, these sialylated glycans are bound by CD22 present on B-cells, contributing to the clustering of BCR, together with the CD22 molecules. Subsequently, the intracellular tyrosine residues of the ITIM/ITIM-like motif are phosphorylated and SHP1 and SHP2 are recruited, inhibiting the kinase-dependent signalling pathway and thus contributes to reduced antibody production against the autoantigen (Nitschke 2005, Tedder, Poe and Haas 2005).

In addition, inhibitory Siglecs play a crucial role in tumour cell survival (Daly, Carlsten and O'Dwyer 2019). While sialic acids are indeed also present on the surface of healthy cells, hypersialylation is a characteristic feature of tumour cells (Büll, den Brok and Adema 2014, Fuster and Esko 2005, Jandus et al. 2014). For instance, sialic acid present on the surface of primary chronic lymphocytic leukemia and acute myeloid leukemia cells serve as ligands for Siglec-7 and Siglec-9, expressed by natural killer cells (NK-cells) and contribute to increased tumour cell survival due to the inhibition of NK cell activation (Daly et al. 2019, Barrow and Trowsdale 2006).

Moreover, ovarian cancer cells express MUC16, which serves as a ligand for Siglec-9 (Belisle et al. 2010). Interestingly within the female reproductive tract sialic acid-Siglec-interactions may also play a critical role. They are assumed to contribute to sperm survival, as those interactions inhibit neutrophil activation *in vitro* (Tecle et al. 2019). Therefore, a closer look on the functions of sialic acid is presented in the following chapter.

1.4 Sialic acid

Sialic acids are typically located at the terminating branches of *N*-glycans and *O*-glycans. Thus, as they are easily accessible, it is not surprising, that sialic acids play further roles in several cellular processes besides serving as a ligand for Siglecs

(Schauer 2004, Angata and Varki 2002, Schauer 1996). These sugars, characterized by a 9-carbon backbone, are involved in the stabilization of molecules, protecting them from proteolytic cleavage, elongating their life time, are able to bind and transport ions, due to its negative charge and modulate mechanisms involved in fertility, differentiation, growth and cellular signalling (Varki A 2009). From the chemical point of view, sialic acids contain a carboxyl group at C1 and a keto group at C2 (Schauer 2004, Angata and Varki 2002, Schauer 1996) and the term sialic acid comprises nowadays more than 60 derivatives, whereby the most common derivatives are N-Acetylneuraminic acid (Neu5Ac), N-Glycolylneuraminic acid (Neu5Gc) and deaminated neuraminic acid (KDN). The high diversity of sialic acid is caused by the substitution of one or more hydroxyl groups of Neu5Ac, Neu5Gc or KDN with, e.g., acetyl, methyl or sulfate residues (Angata and Varki 2002, Schauer 2004, Schauer 2009). Sialic acids are mainly linked via the C2 to the C3 or C6-position of galactose, but sialic acid residues are attached to each other via α2,8 linkages (**Figure 9**) (Varki A 2009, Sato et al. 1998, Sato 2004).

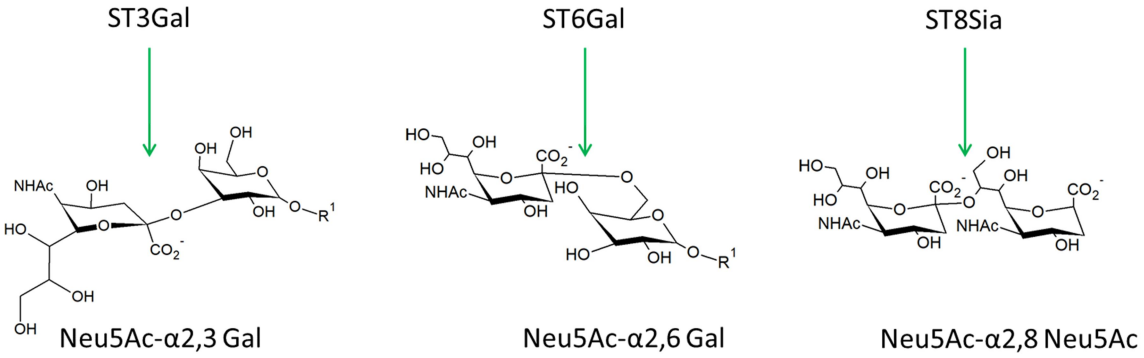

Figure 9: Linkages of sialic acid. Sialic acid can be linked via the C2 to the C3 of galactose mediated through ST3 beta-galactoside alpha-2,3-sialyltransferase (ST3Gal), or to the C6-position of galactose done by ST6 beta-galactoside alpha-2,6-sialyltransferase (St6Gal), whereby sialic acid residues are attached to each other via α2,8 linkages performed by ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1 (ST8Sia) (Varki A 2009).

1.4.1 The roles of sialic acids in the immune system

Within the immune system sialic acids are indispensable. For instance, the rolling of leukocytes is regulated through a sialic acid-mediated process (Sperandio et al. 2006). Furthermore, sialic acid polymers present on the surface of dendritic cells (DCs) contribute to DCs migration, enabling the presentation of antigens to T-cells (Rey-Gallardo et al. 2010, Yoshie, Imai and Nomiyama 2001). On their surface the polysialylated proteins neuropilin-2 (NRP-2) and the chemokine receptor type-7 (CCR-

7) are expressed and ligand binding to those causes DCs migration from the peripheral sites to the lymph nodes (Rey-Gallardo et al. 2010, Yoshie et al. 2001, Steinman and Banchereau 2007). In addition, as previously mentioned, sialic acids belong to the group of “self-associated molecular pattern” (SAMPs). Once recognized by inhibitory receptors, like Siglecs, SAMPs dampen the immune response (Varki 2017a, Varki 2017b, Varki 2011), inhibiting immune reactions against self (Pillai et al. 2012, Crocker et al. 2007, Varki and Angata 2006, Crocker 2005, Varki and Gagneux 2012).

However, several pathogens mimic hosts sialic acids in order to circumvent hosts immune response, assumed to contribute to the fast development of CD33r-Siglecs as well as activating Siglecs (Lübbers, Rodríguez and van Kooyk 2018, Varki 2006).

For instance, *Pseudomonas aeruginosa (P. aeruginosa)* decorate their own glycoconjugates by adsorbing hosts sialic acids, allowing *P. aeruginosa* to inhibit the activation of host’s immune system by binding to Siglec-9. According to, Khatua *et al*. *P. aeruginosa* directly influences cytokine IL-10 and TGF-β production (Khatua, Roy and Mandal 2013). Regarding neutrophil activation, ROS production was prevented and decreased levels of NE release were detected (Hahn et al. 2013, Fuchs et al. 2007). Similar effects were determined by investigating group B streptococcus (GBS). GBS carry sialic acid on their polysaccharide capsule, which interacts with Siglec-5,-9,-14 on the surface of neutrophils causing the inhibition of neutrophil activation (Carlin et al. 2009). Furthermore, *Campylobacter jejuni, Escherichia coli K1* as well as *Neisseria meningitides* known to synthesize sialylated glycans are assumed to use comparable strategies to evade the host’s immune system (Bhattacharjee et al. 1975, Orskov et al. 1979, Khatua et al. 2013, Vimr and Lichtensteiger 2002). In addition, fungi use sialic acid to evade entrapment by NETs released by neutrophils. For instance, *Aspergillus fumigatus* is resistant against NET-induced injury due to its production of cell-wall-associated galactosaminogalactan and a liberated form of galactosaminogalactan (Fontaine et al. 2011, Gravelat et al. 2013, Gravelat et al. 2010, Lee et al. 2015, Geiser et al. 2007). Furthermore, viruses like Human Immunodeficiency Virus (HIV), counteract NET-mediated killing via its envelope glycoprotein containing more high mannose than complex N-glycans. Viruses like HIV engage the C-type lectin DC-SIGN on dendritic cells with high-mannose glycans, causing the production of IL-10, inhibiting ROS-dependent NET release (Lin et al. 2003, Saitoh et al. 2012). Since also the Ebola virus, the Japanese encephalitis virus and the hepatitis C virus, are decorated by an envelope glycoprotein, a similar DC-SIGN- mannose interaction, to circumvent NET release, is likely (Lin et al. 2003, Lozach et al. 2003, Wang et al. 2016). Interestingly,

although extensive literature research was performed, only one parasite- *Leishmania donovani*- seems to evade NET entrapment, by lipopeptidoglycan on its surface (**Figure 10**) (Roy and Mandal 2016).

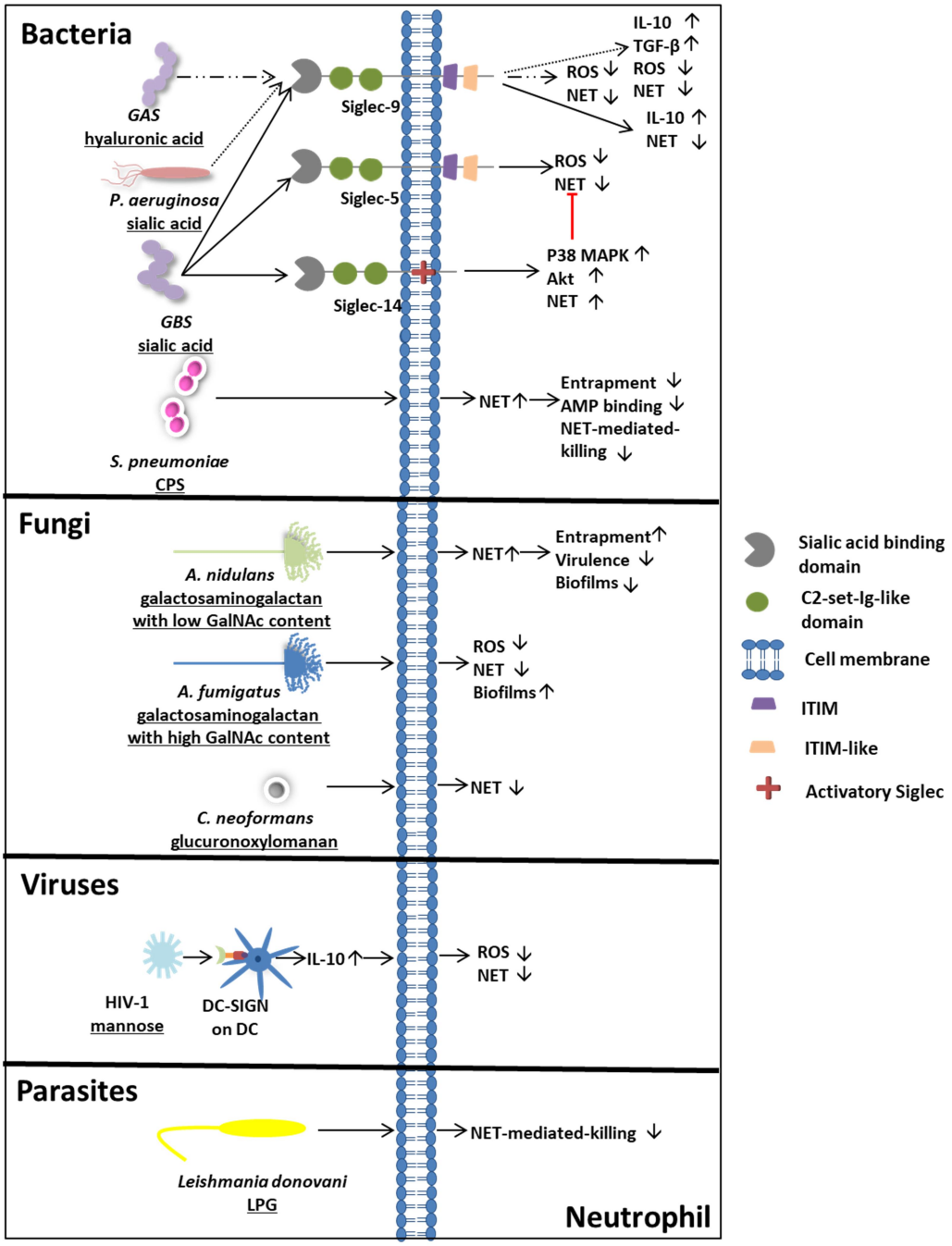

Figure 10: How pathogens use glycans to evade neutrophils immune response (Bornhofft and Galuska 2019) CC-BY.

2. Objective

As previously mentioned an exaggerated NET release is nowadays associated with diseases like preeclampsia, cystic fibrosis, small vessel vasculitis, rheumatoid arthritis, ulcerative colitis as well as infertility (Hahn et al. 2012, Brill et al. 2012, Hakkim et al. 2010, Fuchs et al. 2010, Brinkmann and Zychlinsky 2012, Kaplan 2013, Kessenbrock et al. 2009, von Brühl et al. 2012, Muñoz et al. 2019). Thus, it is obvious that an excessive release of NET needs to be prevented. To this end, several physiological mechanisms exist (Braster et al. 2016, Meier et al. 2019, Lizcano et al. 2017, Spence et al. 2015). For instance, Lizcano and colleagues described that within the blood stream, Siglec-9 on neutrophils inhibits an exaggerated release of NET by the binding of sialylated glycoproteins on erythrocytes (Lizcano et al. 2017) and according to Tecle *et al*. the interaction of Siglec-9 with sialic acids on the surface of sperm promote sperm survival by inhibiting an immune response *in vitro* (Tecle et al. 2019).

As those results show an indispensable role of Siglecs in maintaining a balance between pathogen defense and pathophysiological manifestations, we were first interested in the origin of those receptors.

In the next step, we wanted to investigate the role of those highly conserved receptors within the mammalian female reproductive tract. We assumed that mucins, as highly sialylated conjugates, which line the female reproductive tract, prevent sperm from entrapment by NETs by inhibiting NET release via sialic acid-Siglec interaction, similar to the process described for circulation.

Since the immune system is regulated by sialylated glycoconjugates of the cellular glycocalyx, we further investigated the potential of artificially designed nanoparticles conjugated with α2,8-linked sialic acid residues to inhibit the release of NET.

In sum this work was focused on replying the following questions:

1. Did the evolution of immunological mechanisms come along with the development of distinct Siglecs and does stress, which is linked with increased susceptibility to infections, influence the expression pattern of Siglecs?
2. Could cervical mucins protect sperm from entrapment by NETs by inhibiting the release of NETs within the cervix through sialic acid on its surface and if so, is the involvement of Siglecs likely?

3. Could nanoparticles conjugated with α2,8-linked sialic acid residues trigger similar inhibitory effects on NET release and if so, is also here the involvement of Siglecs likely?

3. Overview of the manuscripts

Developmental and Comparative Immunology 86 (2018) 219–231

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Siglecs: A journey through the evolution of sialic acid-binding immunoglobulin-type lectins

Kim F. Bornhöfft [a], Tom Goldammer [b], Alexander Rebl [b, **], Sebastian P. Galuska [a, *]

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This review deals with the presence and interpretation of Siglecs in different species. Our analysis reveals, that in agreement with previous studies, Siglec-1, CD22, MAG and Siglec-15 were present before the separation of tetrapod's and teleost fishes around 400 million years ago coming along with the development of physiological key elements, such as the adaptive immune system. In addition, the evolution of CD33-related Siglecs occurred just during mammalians evolution. An important incident seems to be the development of lactation, as this initiates the progress of mammalian CD33-related Siglecs (**Figure 11**).

Sequence alignments were performed by Kim Bornhöfft. Phylogenetic trees were designed by Kim Bornhöfft. NCBI protein-protein blasts were performed by Kim Bornhöfft. Figures and tables were designed by Kim Bornhöfft. The first version was written by Kim Bornhöfft and edited by Tom Goldammer, Alexander Rebl and Sebastian P. Galuska.

cells

MDPI

Article

Characterization of Sialic Acid-Binding Immunoglobulin-Type Lectins in Fish Reveals Teleost-Specific Structures and Expression Patterns

Kim F. Bornhöfft [1,†], Joan Martorell Ribera [2,3,†], Torsten Viergutz [1], Marzia T. Venuto [1], Ulrike Gimsa [3], Sebastian P. Galuska [1,*] and Alexander Rebl [2,*]

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† These authors contributed equally to this work.

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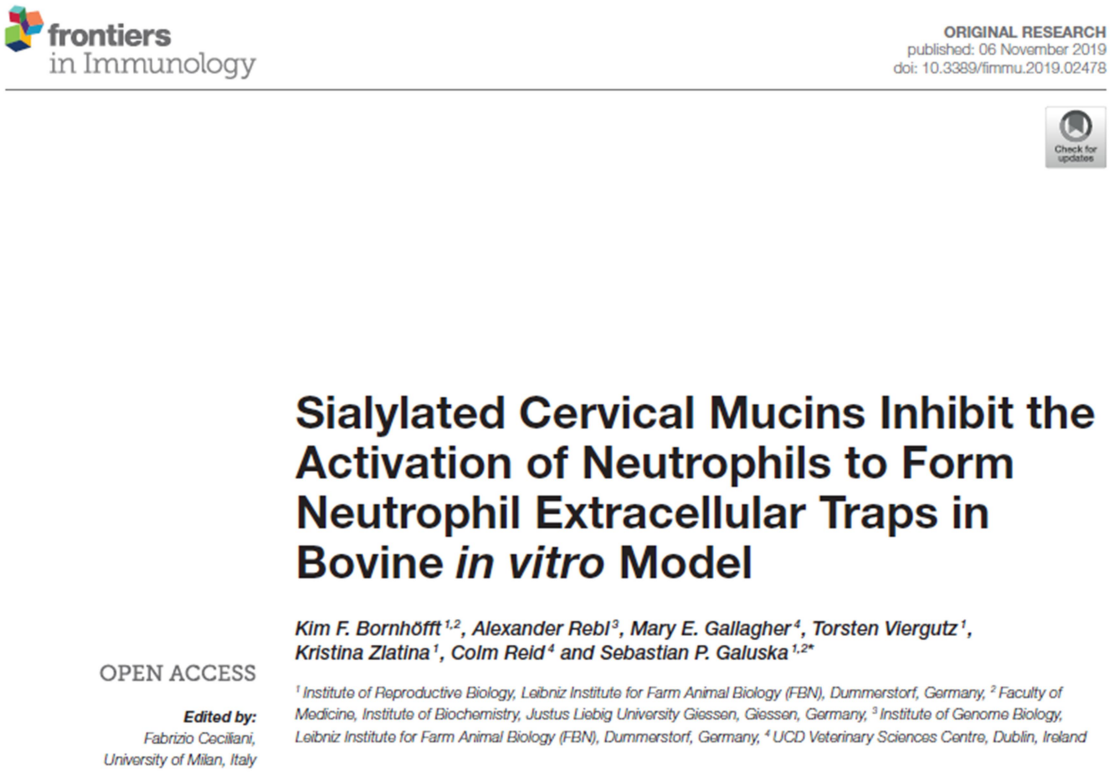
To understand the evolution of the Siglec-functions this manuscript deals with the expression of Siglec-1, CD22, MAG and Siglec-15 in fish. Remarkably, unlike in mammals, the expression of CD22 is not restricted to B-cells. Although MAG is highest expressed in the brain, as it is known for human, MAG is also expressed in several immune cell populations, including erythrocytes. 3D modelling and sequence comparison indicated the presence of an ITIM motif and a conservation of the sialic acid binding domain, suggesting that MAG exert both, immunological functions as well as the maintenance of axon-myelin-spacing. As the genome of fish lacks any CD33-related Siglecs, we assume that MAG and CD22 are mainly responsible for the regulation of sialic acid-dependent mechanisms in fish, whereas in humans Siglecs are more restricted to a specific cell type and function.

Joan Martorell Ribera performed the stress experiments and Marzia Tindara Venuto was significantly involved in the sampling. In addition, Joan Martorell Ribera, Marzia Tindara Venuto and Alexander Rebl performed the qPCR measurements in fish. Kim Bornhöfft, Alexander Rebl and Joan Martorell Ribera analyzed the received raw data. Kim Bornhöfft and Alexander Rebl analyzed the sequencing results. Kim Bornhöfft did the 3D modelling using YASARA, the sequence analysis and sequence alignments and

interpreted the results. Torsten Viergutz did the cell sorting using flow cytometry. Kim Bornhöfft wrote the manuscript; Sebastian P. Galuska, Alexander Rebl and Joan Martorell Ribera edited the manuscript.



frontiers in Immunology

ORIGINAL RESEARCH
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Sialylated Cervical Mucins Inhibit the Activation of Neutrophils to Form Neutrophil Extracellular Traps in Bovine *in vitro* Model

*Kim F. Bornhöfft[1,2], Alexander Rebl[3], Mary E. Gallagher[4], Torsten Viergutz[1], Kristina Zlatina[1], Colm Reid[4] and Sebastian P. Galuska[1,2]**

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Fabrizio Ceciliani, University of Milan, Italy

Front. Immunol. 2019 November, doi: 10.3389/fimmu.2019.02478.

This study shows the potential of cervical mucins of different stages of oestrous cycle to inhibit PMA/Ionomycin as well as LPS induced release of NETs by sialic acids on their surface. When neutrophils were stimulated and co-incubated with mucins, neutrophils nuclei retain their segmented structure, plasma membrane stays intact and granularity was still detectable. However, when sialic acids were chemically or enzymatically modified/ removed, the inhibitory effects of mucins diminished-NET release occurred, indicating a sialic acid-dependent mechanism.

Torsten Viergutz performed the flow cytometric measurements. Mary E. Gallagher taught me the technique of mucin purification. Kristina Zlatina and Kim Bornhöfft purified mucins. Alexander Rebl did the RT-qPCR measurements. Colm Reid provided cervix samples. All further experiments shown in this publication were designed and performed by Kim Bornhöfft. Figures were designed by Kim Bornhöfft.

nanomaterials

MDPI

Article

Nanoparticles Equipped with α2,8-Linked Sialic Acid Chains Inhibit the Release of Neutrophil Extracellular Traps

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Nanomaterials (Basel). 2019 April;9(4). pii: E610. doi: 10.3390/nano9040610.

This study demonstrates that oligosialylated nanoparticles have the potential to inhibit the release of NET, although the nuclei of neutrophils are swollen and NE translocate to the nuclei.

Torsten Viergutz performed the flow cytometric measurements and analysed together with Kim Bornhöfft the results. Andrea Kühnle created the supplemental video and performed the experiments resulting in Figure 7 of the manuscript. All further experiments shown in this publication were designed and performed by Kim Bornhöfft. The first version was written by Kim Bornhöfft.

frontiers in Immunology

MINI REVIEW
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Glycans as Modulators for the Formation and Functional Properties of Neutrophil Extracellular Traps: Used by the Forces of Good and Evil

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Front. Immunol. 2019 May, doi: 10.3389/fimmu.2019.00959.

This review deals with the influence of glycans on the formation of NETs, focusing on the one hand on glycan-dependent mechanism of endogenous cells to prevent an exaggerated release of NETs or to decrease the negative outcomes of NETs and on the other hand on glycan-dependent mechanisms of pathogens to escape entrapment by NETs as well as killing by NETs.

The manuscript was written by Kim Bornhöfft and Sebastian Galuska. Figures were designed by Kim Bornhöfft.

4. Manuscripts

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Siglecs: A journey through the evolution of sialic acid-binding immunoglobulin-type lectins

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ABSTRACT

Siglecs (sialic acid-binding immunoglobulin-type lectins) are a family of immune regulatory receptors predominantly found on the cells of the hematopoietic system. A V-set Ig-like domain mediates the recognition of different sialylated glycoconjugates, which can lead to the activation or inhibition of the immune response, depending on the involved Siglecs. Siglecs are categorized into two subgroups: one including all CD33-related Siglecs and the other consisting of Siglec-1 (Sialoadhesin), Siglec-2 (CD22), Siglec-4 (myelin-associated glycoprotein, MAG) and Siglec-15. In contrast to the members of the CD33-related Siglecs, which share ~50–99% sequence identity, Siglecs of the other subgroup show quite low homology (approximately 25–30% sequence identity). Based on the published sequences and functions of Siglecs, we performed phylogenetic analyses and sequence alignments to reveal the conservation of Siglecs throughout evolution. Therefore, we focused on the presence of Siglecs in different classes of vertebrates (fishes, amphibians, birds, reptiles and mammals), offering a bridge between the presence of different Siglecs and the biological situations of the selected animals.

Contents

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1. Introduction

All eukaryotic cells are surrounded with proteoglycans, glycosphingolipids and glycoproteins that form an essential functional unit: the glycocalyx (Varki, 2017b). Indeed, no living cell can survive without its "sugar jacket" (Gagneux et al., 2015). These sugar residues are directly involved in the modulation of numerous crucial cellular processes, like cell-cell interaction and communication (Crocker et al., 2007; Green et al., 1995; Kelm and Schauer, 1997; Ohtsubo and Marth, 2006; Sato, 2004; Schauer, 2000; Varki, 2007, 2017b; Varki and Angata, 2006; Varki and Varki, 2007). In vertebrates, most of these glycans have sialic acid residues on their outermost positions.

Sialic acids consist of a 9-carbon backbone; the most common forms of these acidic sugars are *N*-acetylneuraminic acid (Neu5Ac), followed by *N*-glycolylneuraminic acid (Neu5Gc) (Angata and Varki, 2002; Schauer, 1996, 2004). The substitution of one or more hydroxyl groups of Neu5Ac, Neu5Gc or deaminated neuraminic acid (KDN) with, e.g., acetyl, methyl or sulfate residues results in high diversity and more than 50 derivatives were described until now (Angata and Varki, 2002; Schauer, 2004, 2009). Since sialic acids are located at the distal part of the non-reducing end of a glycan, these α-keto acids play a special role in physiological processes. Intriguingly, sialic acids belong to the self-associated-molecular patterns (SAMPs), which are molecules that are recognized by inhibitory receptors in order to dampen immune reactions (Varki, 2011b; Varki, 2017a; b). Such modulations can be mediated via the interaction of sialic acid-binding immunoglobulin-like lectins (Siglecs) with distinct sialylated glycoconjugates (Crocker, 2005; Crocker et al., 2007; Pillai et al., 2012; Varki and Angata, 2006; Varki and Gagneux, 2012). Already in 2009, 14 different human Siglecs and 9 murine Siglecs have been identified (O'Reilly and Paulson, 2009; Pillai et al., 2012); each type of Siglec prevalently recognizes a particular set of sialylated structures (Crocker et al., 2007; Macauley et al., 2014; Pillai et al., 2012), carrying different α2,3-, α2,6- or α2,8-linked sialic acid residues (Bakker et al., 2002; Blixt et al., 2003).

Siglecs are mostly located on the cell surface of hematopoietic cells (Crocker et al., 2007; Varki and Angata, 2006), but cells outside the immune system can also express Siglecs. For instance, Siglec-4 (myelin-associated glycoprotein, MAG) is predominantly expressed in Schwann cells, as well as oligodendrocytes (Quarles, 2007).

Based on sequence homologies of human Siglecs, members of the Siglec family can be subdivided in two groups: one group comprises CD33 (Siglec-3)-related Siglecs, showing low gene conservation across orthologues but a high degree of sequence identity throughout the subfamily members; the other group includes Siglec-1 (Sialoadhesin), Siglec-2 (CD22), Siglec-4 and Siglec-15, which show low sequence identity between each other (Angata et al., 2004; Crocker et al., 2007). Both groups include both activating and inhibitory receptors. The inhibitory Siglecs contain the so-called 'immune receptor tyrosine-based inhibition motifs' (ITIM) antagonizing the initiation of an immune reaction, which is mediated via the 'immune receptor tyrosine-based activation motif' (ITAM) (Crocker et al., 2007; Ravetch and Lanier, 2000). Such an ITAM-dependent activation can be mediated by Siglec-14, Siglec-15 and Siglec-16. They support activation via the interaction partner DAP10/12 (DNAX-activation protein of 10/12 kDa), which includes an ITAM domain required for the production of proinflammatory cytokines (Crocker et al., 2007).

In addition, there are two Siglecs in humans without a direct connection to the ITIM and ITAM systems. Both Siglec-1 and Siglec-4 lack activating and inhibiting motifs. In the case of Siglec-1, it has been suggested that it is mainly responsible for cell-cell interactions and/or the phagocytosis of sialylated pathogens (Chang and Nizet, 2014; Crocker et al., 2007), whereas Siglec-4 is prevalently involved in myelination processes and the stabilization of cell-cell contacts (Owens and Bunge, 1989; Quarles, 2007).

Sialylated glycoconjugates have been present on the cell surfaces and/or been released to modulate physiological processes for more than 500 million years (Gagneux et al., 2015; Varki, 2011a, 2017a; b). Since not only eukaryotic cells but also distinct prokaryotic cells are able to build differently sialylated glycans, immune-competent cells use sialylated glycans as recognition molecules for endogenous cells and to detect pathogens forming a complex network of several identification, activation and inhibition processes (Varki, 2006, 2009; 2017a; b). As already outlined by Varki and colleagues, the co-presence of distinct sialylated structures on endogenous cells and on pathogens might represent one of the most important triggers for the evolution of both inhibitory and activating Siglec family members (Varki, 2006, 2009; 2017a; b). However, whereas there is already quite lot knowledge about the Siglec expression in mice and primates including humans, the knowledge about Siglec expression in other vertebrates like fishes, amphibians, reptiles and birds is still fragmentary, leading to an insuperable gap within evolutionary explanations. With the aim to contribute to the organization of the ever-growing number of complex immunoregulatory receptor families, we summarized the presence of Siglecs in several species by the performance of Blast searches and discuss the findings in the context of physiological changes throughout evolution (placenta types, lactation, and ecological niche/habitat).

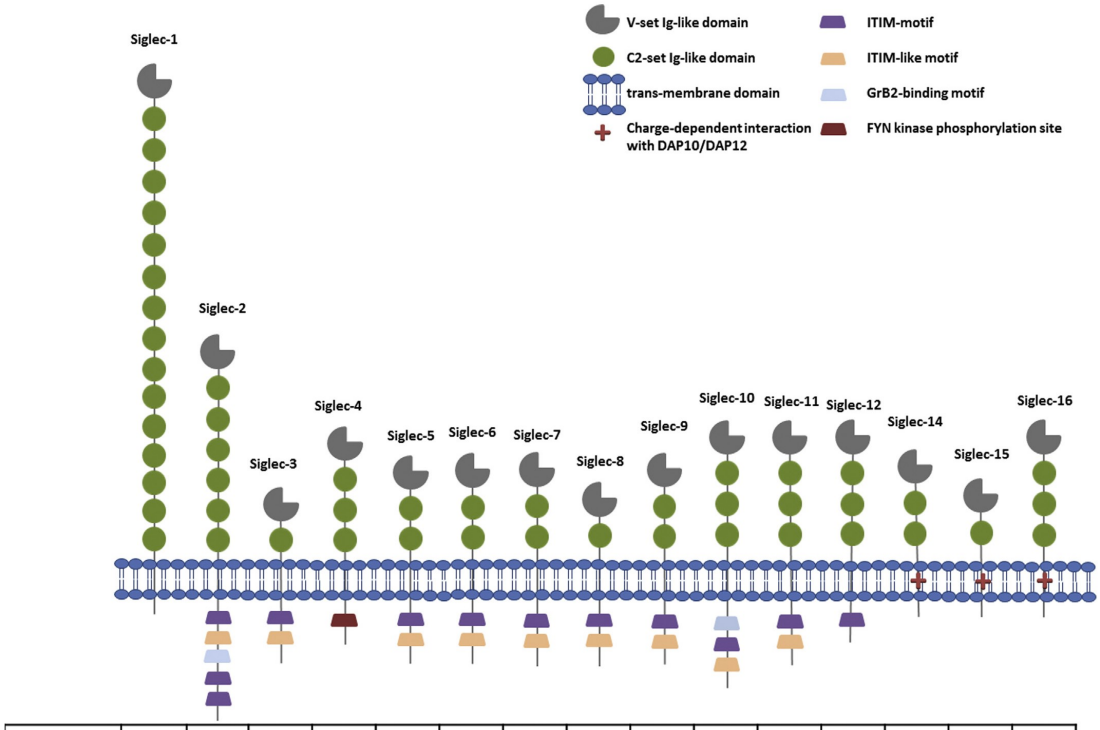
2. Structure of Siglecs

Siglecs are cell-surface receptors containing protein domains belonging to the Immunoglobulin (Ig) superfamily. Their extracellular part consists of a variable number of so-called 'C2-set' Ig-like domains with high sequence and folding similarities to the constant region of immunoglobulins (Fig. 1) (Jandus et al., 2011), and an amino-terminal Ig-like domain with high similarities to the variable domain of antibodies, abbreviated as the V-set domain. This domain contains the sialic acid binding domain (Angata et al., 2004; Crocker et al., 2007; O'Reilly and Paulson, 2009). Depending on the number of C2-set Ig-like domains, Siglecs tend to bind sialic acid residues on the same cell surface in cis-mode or on adjacent cells in trans-mode. For example, Siglec-1 (15 Ig-domains) commonly binds glycans in trans-mode, whereas Siglec-3, Siglec-8 and Siglec-15 (one Ig-domain) are believed to favor the cis binding of sialylated sugar structures (Angata et al., 2004; Crocker et al., 2007; Hartnell et al., 2001).

2.1. Sialic acid binding by Siglecs

In general, it seems that one salt bridge, mediated by an arginine residue, is particularly important for the interactions (Supplement 1A). Regarding human Siglec-1 (hSiglec-1), it is known that the sialic acid binding domain is located at the N-terminus of the protein (May et al., 1998). The guanidine group of R116 of hSiglec-1 forms a salt bridge with the carboxyl group of Neu5Ac. The acetyl group of Neu5Ac is in van der Waals contact with the indole ring of W21 and the C9 of the glycerol residue of sialic acid interact with the aromatic chain of W125.

The interaction of hSiglec-5 and the sialylated structures involves a salt bridge between arginine (R124) and the carboxyl group of Neu5Ac (Zhuravleva et al., 2008), similar to Siglec-1 (Supplement 1B). In addition, K132 and S134 interact via hydrogen bonds with the secondary amine and the hydroxyl group of C8 of sialic acid, respectively. Moreover, van der Waals



hSiglec	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16
ITIM	-	+++	+	-	+	+	+	+	+	+	+	+	-	-	-
ITIM-like	-	+	+	-	+	+	+	+	+	+	+	-	-	-	-
ITAM	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+

Fig. 1. Structure of the 15 Siglecs known to be expressed in humans. The illustrations were plotted using the SMART (Simple Molecular Architecture Research Tool, Heidelberg) tool. For ITIM and ITIM-like motifs positive signs indicate the availability of signaling motifs in the cytoplasmic area, whereas for ITAM motifs positive signs indicate the charge-dependent association with DAP10/12, ITAM bearing proteins. Negative signs show the absence of ITIMs, ITIM-likes and ITAMs. Based on Jandus et al. (2011).

interactions take place between the aromatic part of Y133 and position C9 of sialic acid (Zhuravleva et al., 2008). Thus, distinct structural changes affect the character of individual Siglec binding pockets and modulate their affinity to a particular panel of ligands.

2.2. The signaling motifs of Siglecs

As mentioned above, some members of the Siglec family—Siglec-14, Siglec-15 and Siglec-16—are able to promote immune responses by interacting with DAP10/12 (Fig. 1) (Angata et al., 2007; Arase and Lanier, 2004). DAP10/12 contains ITAM domains (Angata et al., 2006, 2007; Ishida-Kitagawa et al., 2012) with the signature sequence Y-X-X-L/I-X_{6-8}Y-X-X-X-L/I (with X representing any amino acid) (Lanier and Bakker, 2000). This interaction with DAP10/12 is initiated by positive residues in the transmembrane region of these lectins (Ishida-Kitagawa et al., 2012; Pillai et al., 2012).

However, Fig. 1 illustrates that most Siglecs contain ITIMs in the intracellular domain (Ando et al., 2008). ITIMs share the signature (I/V/L/S)-X-Y-X-X-(L/V) (Crocker et al., 2007), which allows counteracting the activation of the immune system via the inhibition of ITAM-mediated signaling (Crocker et al., 2007; Ravetch and Lanier, 2000). Additionally, most of the known Siglecs possess not only an ITIM, but also ITIM-like motifs. Mutagenesis experiments with CD33-related Siglecs showed that ITIM functionally dominates over the ITIM-like motif, although both are relevant for recruiting the Scr-homology-2 domain containing protein tyrosine phosphatases SHP1 and SHP2 (Avril et al., 2005; Paul et al., 2000; Taylor et al., 1999; Ulyanova et al., 2001; Yu et al., 2001). The binding of SHP1 and SHP2 requires the phosphorylation of the tyrosine residues of both the ITIM and the ITIM-like motif (Avril et al., 2005; Crocker et al., 2007). One exception is Siglec-5, which can weakly bind SHP1, although it cannot be phosphorylated since the required tyrosine residue is replaced by alanine (Avril et al., 2005).

3. Functions of Siglecs

3.1. DAP-associated Siglecs

Siglec-14, Siglec-15 and Siglec-16 have been reported to interact with DNAX-activation proteins. However, Siglec-15 is the most investigated DAP12- and DAP10-associated Siglec. Due to the aspartic acid residue in its transmembrane area, DAP12 can interact in a charge-dependent mechanism with lysine located in the transmembrane domain of Siglec-15 (Angata et al., 2007). When Siglec-15 interacts with a sialylated binding partner, the tyrosine residues of ITAM are phosphorylated by the kinases of the Src family. The phosphorylated ITAM domains of DAP12 serve as docking sites for the SH2 domains of the ZAP70 and the Syk kinases, which in turn activates the immune system (Angata et al., 2007; Lanier and Bakker, 2000).

3.1.1. Biological role of DAP-associated Siglecs

Besides a possible immunological role, Hiruma et al. described the critical role of Siglec-15 in the differentiation of osteoclasts (hematopoietic-origin) (Hiruma et al., 2011), which is important for bone resorption (Teitelbaum, 2000). Mice lacking Siglec-15 show mild osteopetrosis characterized by dense bones (Hiruma et al., 2013). *In vitro* studies have demonstrated that the interaction of Siglec-15 with DAP12 after sialic acid binding leads to a signal-activating osteoclast differentiation into their multinucleated states, which is responsible for bone resorption (Hiruma et al., 2011). This differentiation step can be inhibited using anti-Siglec-15 antibodies, causing dimerization and the subsequent internalization and degradation of Siglec-15 dimers (Stuible et al., 2014). Thus, Tremblay and co-workers recommended an administration of antibodies against Siglec-15 to counteract bone loss (Stuible et al., 2014).

3.2. ITIM-bearing Siglecs

In contrast to DAP-associated Siglecs, ITIM-containing Siglecs are able to silence ITAM-dependent immune responses. The binding of different sialylated glycans induces the phosphorylation of the intracellular tyrosine residues of the receptor by Src-family members. Phosphorylation initiates the recruitment of SHP1 and SHP2, which bind to the receptor and are able to inhibit kinase-dependent pathways (Avril et al., 2005; Crocker et al., 2007).

One prominent example of a Siglec-dependent inhibition-mechanism is the modulation of antibody production. When its B-cell receptor (BCR) recognizes a specific antigen, the B-cells differentiate into antibody-producing plasma cells. In the case that the recognized antigen is co-localized with sialylated structures on endogenous cells, these sialylated glycans are bound by the Siglec-2 present on B-cells (Nitschke, 2005; Tedder et al., 2005). The resulting clustering of BCR, together with the Siglec-2 molecules, recruits SHP1 and SHP2, followed by the inhibition of the kinase-dependent signaling pathway. Thus, reduced antibody production against the autoantigen is initiated (Nitschke, 2005; Tedder et al., 2005). However, Siglec-2 is also known to recruit GRB2 (growth-factor-receptor-bound protein 2), SHC (SH2-domain-containing transforming protein C), PI3K (phosphoinositide 3-kinase) and PLCγ2 (phospholipase Cγ2), which are effectors of cellular activation, suggesting that Siglec-2 related tasks depend on the respective activated B-cells (Tedder et al., 2005).

3.2.1. Biological role of ITIM-bearing Siglecs

Another example of Siglec-mediated suppression of immune cell function was recently described by Varki and colleagues (Lizcano et al., 2017). They demonstrated that Siglec-9 inhibits neutrophil activation via binding to sialylated glycoproteins on erythrocytes. The authors suggested that this might explain the phenomenon that isolated neutrophils can be very easily stimulated to form neutrophil extracellular traps (NETs), for example. Thus, a sialylated structure may act as a SAMP on erythrocytes to counteract the activation of neutrophils in the blood stream.

Interestingly, cancer cells also seem to exploit Siglec-mediated mechanisms. Leukemic cells from patients suffering from acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) produce, for instance, a much higher amount of ligands for Siglec-7 and Siglec-9. These Siglecs are present on the cell surfaces of NK cells and the detection of their ligands on tumor cells inhibits their activation, thus promoting tumor growth (Hudak et al., 2014; Jandus et al., 2014; Macauley et al., 2014).

Furthermore, an imbalance in the Siglec-mediated system can provoke pathophysiological situations and might be associated with neurodegenerative diseases (Angata et al., 2002; Bradshaw et al., 2013; Cao et al., 2008; Griciuc et al., 2013; Linnartz-Gerlach et al., 2014; Malik et al., 2013). Siglec-3, for instance, is known as a high potential risk factor for Alzheimer, since an increased expression of Siglec-3 on microglia, tissue macrophages of the neuronal system, leads to an insufficient uptake of toxic plaques of amyloid β (Bertram et al., 2008; Hollingworth et al., 2011; Naj et al., 2011).

In addition, polymorphisms of the gene encoding Siglec-8 are associated with the outbreak of allergic asthma (Gao et al., 2010). Siglec-8 is mainly present on human eosinophils. Current research has revealed that ligand binding to Siglec-8 on eosinophils regulates the levels of eosinophils in the tissue by promoting the apoptosis of eosinophils. In mouse models of ovalbumin-induced airway inflammation, epithelia cells and mucins showed an increased amount of ligands for Siglec-F (homologue of hSiglec-8), followed by a decreased number of invading eosinophils (Hudson et al., 2009; Kiwamoto et al., 2013; Macauley et al., 2014).

These five examples (of many) demonstrate from a medical point of view that ITIM-bearing Siglecs play an essential role in controlling the immune response and might represent a powerful therapeutic target during pathophysiological conditions. However, pathogens also seem to make use of the Siglec functions. Pathogens like *Neisseria meningitides, Haemophilus influenza, Campylobacter jejuni, Pseudomonas aeruginosa* and *group B Streptococcus* generate sialylated binding partners of Siglecs to influence the immune response (Chang and Nizet, 2014). *Group B Streptococcus*, for example, produces Siaα2,3-Galβ1,4-GlcNAc, a ligand recognized by Siglec-9, on its capsule. The interaction decreases neutrophil oxidative burst, thus reducing NET formation and promoting the survival of pathogens (Carlin et al., 2009).

4. Vertebrates and highly-conserved Siglecs

The development of the first vertebrates dates back to the Paleozoic era more than 500 million years ago. Distinct Siglecs—Siglec-1 (Cao et al., 2009), Siglec-2 (Cao et al., 2009), Siglec-4 (Lehmann et al., 2004) and Siglec-15 (Angata et al., 2007)—are conserved in several branches of vertebrates including fish. In order to prove and summarize the presence of Siglecs in invertebrates as well as vertebrates, we performed BLAST analyses (NCBI Blastn (Altschul et al., 1990)) based on published human and mice sequences. Most likely, the first vertebrates possessed Siglec-2 and Siglec-15 and, accordingly, one pro- and one anti-inflammatory receptor, both of which are still present in all extant species of the five vertebrate classes: fishes, amphibians, reptiles, birds and mammals (Supplements 2, 3 and Fig. 2). It is also likely that Siglec-1 and Siglec-4 are ancient receptors, having been expressed together with Siglec-2 and Siglec-15 in the common ancestor of modern vertebrates.

4.1. Siglec-4

Jawless fishes are obviously an exception, as this most primitive form of vertebrates seems to be Siglec-negative (Supplement 2). It might be reasonable to assume that striking events took place, including the emergence of the first Siglec molecules linked to particular cellular immune function, after the separation of bony fishes from jawless fishes. Jawless fishes are the only vertebrates without myelin and these fishes seem to lack Siglec-4 (Knowles, 2017; Zalc, 2006), which is mainly responsible for the stabilization of the myelin sheath in vertebrates. The same applies to invertebrates. In invertebrates, as well as jawless fishes, glial cells surround the axons, while in higher animals Schwann cells or oligodendrocytes are myelin-forming cells (Knowles, 2017; Zalc, 2006). Our alignment of the amino acid sequences of the sialic acid binding domains exhibited a low degree of structural variance between humans, amphibians, reptiles and birds (Supplement 4A) and likewise, hSiglec-4 was similar to its counterpart in different myelin positive fishes (Supplement 4B). Besides sharks, belonging to the cartilaginous fishes and thus representing the "oldest" fish after jawless fishes, only fugu showed a higher degree of sequence differences. Nevertheless, manifest key nucleotide positions were preserved across different vertebrate species. Consequently, our comparison strongly suggests that the development of myelination evolved together with the emergence of Siglec-4. However, when we examined the published intracellular amino acid sequences of Siglec-4 for immunomodulatory elements, we observed that in three of four analyzed bony fishes, ITIM and ITIM-like sequences were present (Fig. 3). This was already observed by Dietz and colleagues and an involvement of Siglec-4 in signal transduction was suggested in fish by the authors (Lehmann et al., 2004). In contrast, in nearly all examined terrestrial vertebrates, these two motifs were absent, except for anole, gecko and koala. This pattern indicates that Siglec-4 might modulate more physiological functions in fish than in terrestrial vertebrates (Lehmann et al., 2004). This interesting aspect is now under investigation in our laboratories. Nonetheless, it is also possible that these sequences are adventitiously present. In this context, reference should be made to a very recent work showing that rock breams challenged with the relevant pathogens *E. tarda*/ *S. iniae*/ red seabream iridovirus (RSIV) respectively, showed significant changes in the expression levels of a Siglec in several organs that has been assigned to Siglec-3 (Jeswin et al., 2018). We hypothesize, however, that the described Siglec was Siglec-4. The sialic acid binding domain has characteristic motifs that are highly conserved in Siglec-4 orthologs (Supplement 5). For instance, the amino acids of the sequence motif **RAIW** in rock bream have chemical characteristics shared with the Siglec-4 sequences of medaka, zebrafish and coelacanth (Fig. 4). This is also the case in higher vertebrates (Supplement 4A). In Siglec-4, a nonpolar amino acid is always attached to the highly conserved tryptophan. In contrast, in Siglec-3, amino acid residues containing a hydroxyl group occupy the same position. Another interesting sequence motif of Siglec-4 is **GRT** (Fig. 4). This motif is highly conserved in medaka, zebrafish, coelacanth, rock beam (Fig. 4) and other vertebrates. In Siglec-3, however, the arginine of this motif is followed by the aromatic and highly hydrophobic amino acid phenylalanine (F), instead of the hydrophilic amino acids serine or threonine (S or T). In sum, the distinctive parallels in the sequences between the Siglec-4 of the analyzed fishes suggest that the described Siglec-3 in rock bream (Jeswin et al., 2018) belongs to Siglec-4 and that Siglec-4 has also immunomodulatory functions in fish. However, it has to be noted here that Cao and colleagues found evidence that the first ancestral regions for CD33-related Siglecs might have already emerged in at least some fish species (Cao et al., 2009).

4.2. Siglec-2

Looking at Siglec-2 during evolution, there is another Siglec-dependent system that differs between jawless fishes and higher vertebrates and directly affects the adaptive arm of the immune system. In contrast to higher vertebrates, jawless vertebrates and

	Mammalians														
	Ornitho-rhynchidae	Diproto-dontia	Afrotheria	Laurasiatheria								Glires			Human
Siglec	Platypus	Koala	Elephant	European shrew	Cat	Horse	Pig	Killer whale	Cow	Sheep	Goat	Guinea pig	Beaver	Mouse	
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+
6	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
8	-	-	+	-	-	+	-	-	+	-	+	+	-	+	+
9	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
10	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+
12	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
14	-	+	+	-	+	+	+	-	+	+	+	+	+	-	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Fig. 2. Presence of Siglec family members in selected mammalian species. Based on NCBI protein-protein BLASTs against the respective human and mouse sequences, the presence of Siglecs were investigated. Siglec-1, Siglec-2, Siglec-4 and Siglec-15 are labeled in green. Dark blue indicates CD33-related Siglecs, which are presumably expressed by more than 73% of the analyzed species, whereas fields labelled in light blue denote Siglecs expressed only by certain mammalian species. The positive sign indicates the availability of the referring gene sequence; the negative sign marks its absence. For the accession number of a respective sequence see Supplement 17. The sources of all photos are listed in Supplement 18. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Species	ITIM	ITIM-like
Shark	-	-
Medaka	+	+
Zebrafish	+	+
Fugu	-	-
Coelacanth	+	+
Xenopus	-	-
Tibetan frog	-	-
Phyton	-	-
Alligator	-	-
Turtle	-	-
Anole	-	+
Gekko	+	-
Chicken	-	-
Goose	-	-
Platypus	-	-
Koala	+	-
Mouse	-	-
European Shrew	-	-
Guinea Pig	-	-
Beaver	-	-
Pig	-	-
Cow	-	-
Sheep	-	-
Goat	-	-
Horse	-	-
Killer whale	-	-
Dog	-	-
Cat	-	-
Elephant	-	-
Human	-	-

Fig. 3. The presence of ITIM and ITIM-like motifs in Siglec-4 during evolution. Positive signs indicate the presence of ITIM and ITIM-like motifs in different species based on published cDNA sequences in the NCBI library, negative signs the absence. For the accession number of a respective sequence see Supplement 17.

invertebrates do not express BCR. Invertebrates have no corresponding antigen receptors and jawless vertebrates use variable lymphocyte receptors (VLRBs). As described for Siglec-4, the common ancestors of sharks and bony fishes were apparently the first organisms on earth to express Siglec-2, a regulatory partner of BCR. Thus, in a timely manner a mechanism to counteract the production of autoimmune antibodies had possibly developed. In contrast to the amino acid sequence of Siglec-4, the sequence of Siglec-2 seems to be more variable (Supplement 4A: Siglec-4: identity: 33.34%; Supplement 6B: Siglec-2: identity: 4.9%). Whereas mammalian Siglec-2 sequences are comparably well conserved (Supplement 6A), a large majority of amino acids were exchanged in amphibians, reptiles and birds (Supplement 6B). At first glance, hSiglec-2 seems to share no homology with its orthologs in fish (Supplement 7A), but a pairwise comparison reveals that certain key residues of the sialic acid binding domain of Siglec-2 in fishes and humans are conserved (Supplement 7B). We observed the same when the orthologous sequences from amphibians, reptiles and birds were separately aligned (Supplement 8).

4.3. Siglec 15

Siglec-15 appeared during the evolution from cartilaginous fishes to bony fishes (Arctic lamprey: Siglec-15 negative and shark: Siglec-15 positive). The amino acid sequence of the sialic acid binding domain is highly conserved in Siglec-15 positive vertebrates (Supplement 9). Since the deletion of Siglec-15 in mice leads to osteopetrosis (Hiruma et al., 2013), it seems likely that Siglec-15 of osteoclasts plays an important role during bone development. It seems that chondroclasts are mature osteoclasts (Knowles et al., 2012). Thus, in sharks, belonging to the cartilaginous fishes, Siglec-15 might be involved in the regulation of mechanical strength of cartilages. However, until now it is unknown, if Siglec-15 can be also expressed by chondroclasts.

4.4. Siglec-1

Siglec-1 is another highly conserved Siglec. This Siglec receptor lacks tyrosine-based motifs in its cytoplasmic tail (Crocker et al., 1995). Instead of implementing inhibitory or activating functions, Siglec-1 is tasked with phagocytic functions (Chang and Nizet, 2014; Chang et al., 2014; Crocker et al., 2007). Its V-set domain is highly conserved in all analyzed species (Supplement 10). Like the above Siglec-2 alignments, evolutionarily conserved residues became obvious in pairwise sequence comparisons (Supplement 11).

Taken together, the genomes of the analyzed bony fishes contain a comparably low number of Siglec types: Siglec-1, Siglec-2, Siglec-4 and Siglec-15. Remarkably, no dramatic changes have occurred during the evolution of the first terrestrial vertebrates, amphibians, reptiles and birds—still encoding species-specific variants of Siglec-1, Siglec-2, Siglec-4 and Siglec-15— although a complete change of the environment took place (Supplements 2, 3 and Fig. 2). However, we do not exclude that further possibly Siglec types may be present, which might also be species specific, besides the confidently identified Siglec-1, Siglec-2, Siglec-4 and Siglec-15 sequences, in the analyzed vertebrates.

Interestingly, in turtles as well as in anoles Siglec-variants with a high sequence homology with hSiglec-14 were detected (Supplement 3). When we compared the V-set domain of hSiglec-14 with these sequences, the obtained alignments showed a high similarity (Supplement 12). As mentioned above, Siglec-14 is an activating receptor. Angata et al. suggested that an arginine residue (R) in the transmembrane domain of hSiglec-14 interacts with DNAX-activation proteins (Angata et al., 2006). Consequently, this domain also was compared in detail (Fig. 5A). The transmembrane domains in turtle and in anole exhibit lysine residue (K) instead of arginine residue (R), which can also mediate the binding of DNAX-activation proteins. The similarity of the compared regions is approximately 50% and 70% in anole and turtle, respectively. Consequently, these two Siglecs may indeed represent orthologues of Siglec-14. However, since these orthologues could only be detected in two reptiles by database search, no evidence had been provided that a common ancestor of mammals and reptiles already expressed a variant of Siglec-14. In addition, in mammals, the Siglec-14 transmembrane domains are almost identical in all analyzed species (Fig. 5B), indicating that the structure of the transmembrane domain is very important for the functionality of Siglec-14. The very strong similarity of the transmembrane domain

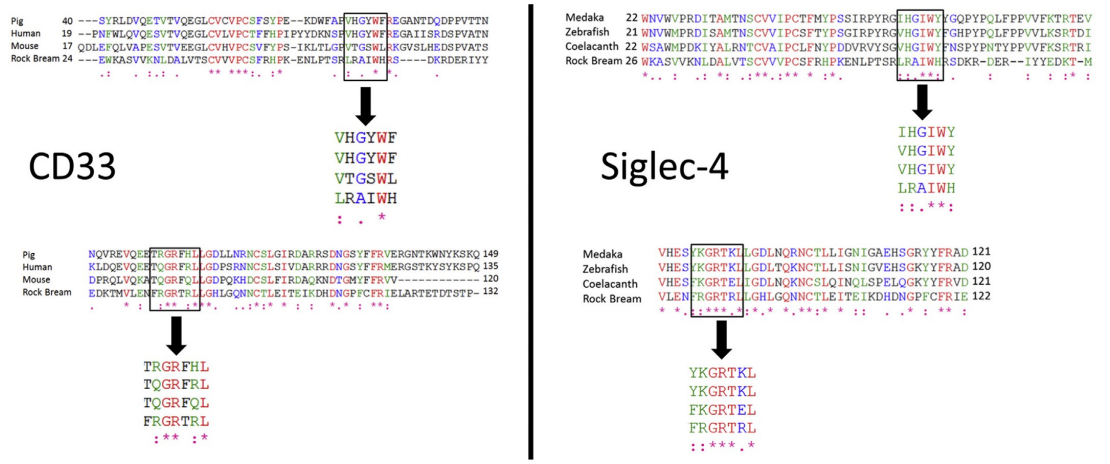


Fig. 4. Multiple alignments of the V-set domain of CD33 and Siglec-4 sequences from selected species. Multiple sequence alignments were performed using the Clustal Omega tool of EMBL-EBI using the currently published CD33 sequence in rock bream (GenBank accession number MF377634), as well as the CD33 sequences and Siglec-4 of several fishes. For the accession number of a respective sequence see Supplement 17. (*) equal amino acids; (:) highly similar amino acids; (.) similar amino acids.

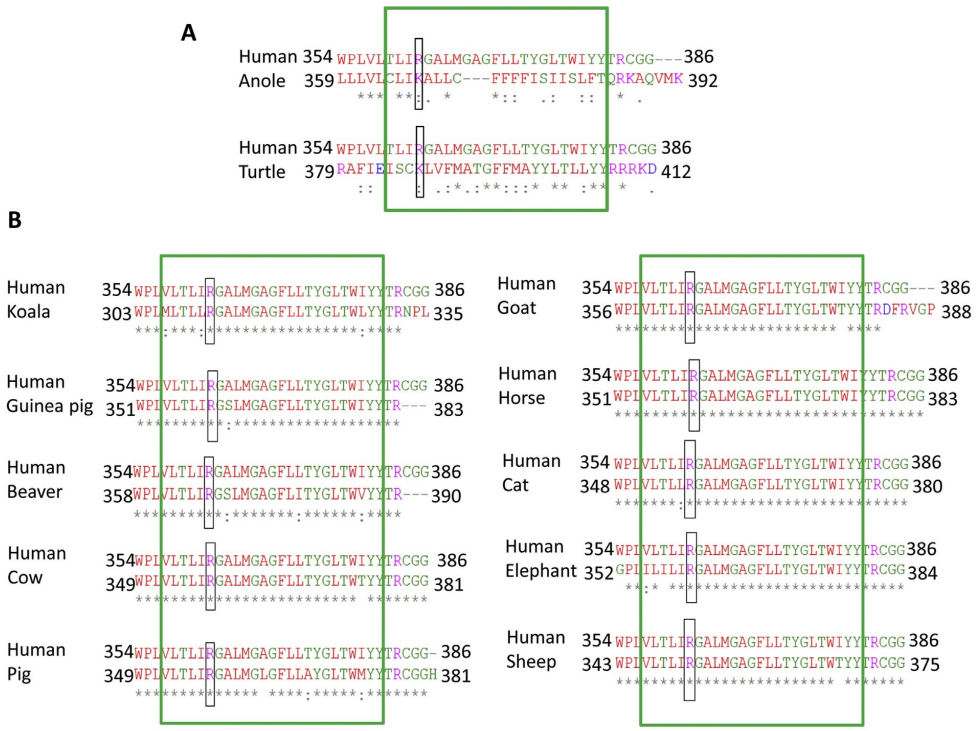


Fig. 5. Multiple alignments of the transmembrane domain of Siglec-14 from selected species. A) The transmembrane domain of human Siglec-14 was compared with potential membrane domains of the assigned Siglec-14 variants in turtle and anole. B) In addition alignments of the transmembrane domains of all human Siglecs were performed. Using the Clustal Omega tool of EMBL-EBI Multiple, sequence alignments were performed. For the accession number of a respective sequence see Supplement 17. (*) equal amino acids; (:) highly similar amino acids; (.) similar amino acids.

in mammals places the similarities between mammals and these two reptiles (turtles and anoles) in perspective. Actually, the similarity of the transmembrane domain of all different human Siglecs is comparable with the similarities between human and anole Siglec-14 (Supplement 13). Taken together, although noticeable similarities exist, at the moment one can only speculate whether these two proteins in anoles and turtles are really Siglec-14 variants and, if so, whether a common ancestor of reptiles and mammals already generated a Siglec-14-variant or whether parallel and independent developments took place. In addition, it should be noted in this context that this brief evolutionary outline refers basically to GenBank and Ensembl database entries, which might still be incomplete or incorrectly annotated, leading ultimately to inaccurate estimates.

Nevertheless, the outlined million-year lasting expansion of the Siglec family apparently mirrors well, to a degree, upward evolutionary development across vertebrate classes: humans contain at least 15 different Siglec types, whereas only up to four Siglecs are present in bony fishes. This particularity is somehow remarkable inasmuch as bony fish underwent several rounds of additional whole-genome duplications and simultaneous independent gene duplications (Hurley et al., 2007; Robinson-Rechavi et al., 2001), leading to the "more genes in fish" theory (Wittbrodt et al., 1998). The teleostean innate immune system has a much broader collection of complement proteins, including up to eight isoforms of the central complement component C3, in all fish species investigated so far (Forn-Cuni et al., 2014; Kobis et al., 2015; Lovoll et al., 2007; Nakao et al., 2000; Sunyer et al., 1997; Zarkadis et al., 2001). The family of Toll-like receptors (TLRs) represents a similarly well-investigated example of immune gene expansion in the teleostean lineage. TLRs recognize structurally-conserved components of microorganisms (Kawasaki and Kawai, 2014) and have moreover been demonstrated to interact with Siglecs to fine-tune the transduction of danger signals (Chen et al., 2014). While up to a dozen TLRs have been identified in mammals, fishes possess a significantly-expanded repertoire of TLRs (Ahn et al., 2014; Altmann et al., 2016; Gong et al., 2017; Quiniou et al., 2013; Solbakken et al., 2016), including counterparts to all human TLRs (except TLR6 and TLR10) plus 'non-mammalian' or even 'fish-specific' TLRs (up to TLR28 (Wang et al., 2016), as well as multiple isoforms of certain TLR types (Lee et al., 2013; Sundaram et al., 2012). Several downstream molecules of the TLR signaling cascade have also multiplied in the teleostean lineage (Brietzke et al., 2014; Rebl et al., 2008). Compared with the mammalian repertoire of TLRs, amphibians are equipped with additional TLRs, too (Babik et al., 2015). These examples illustrate once more that gene expansion does not necessarily indicate an upward development during evolution.

5. Siglecs in mammals

Presumably in conjunction with the appearance of the earliest mammals, the family of CD33-related Siglecs has been growing for over 200 million years (Capuco and Akers, 2009). Our phylogenetic tree of mammalian Siglec sequences clearly clusters the sequences of Siglec-1, Siglec-2, Siglec-4 and Siglec-15 (Supplement 14), while the CD33-related Siglecs are less clearly separated (Fig. 6), especially with regard to Siglec-7, Siglec-8 and Siglec-9. The high degree of sequence similarity among CD33-related Siglecs might be driven by diverse selection pressures caused by pathogens using sialic acid-based molecular mimicry mechanisms, as well as pathogens utilizing host sialic acids as ligands during invasion ("Red Queen Effect"), already mentioned by Angata and Varki (Varki, 2006; Varki and Angata, 2006). With regard to Siglec-3, Siglec-5 and Siglec-6, it may be assumed that the high sequence identity of CD33-related Siglecs may lead to incorrect annotations (Supplement 15). Moreover, the amino acid sequences of Siglec-5 and Siglec-14 seem to be relatively similar, possibly complicating unambiguous separation (Supplement 15) (Angata et al., 2006). These two receptors interact as antagonists—one inhibits the immune response and the other one activates the immune system—suggesting that these two receptors evolved from each other (Angata et al., 2006). Interestingly, the activating Siglec-14 is absent in some humans and this circumstance allows group B *Streptococcus* to suppress the activation of neutrophils (Ali et al., 2014). Varki and colleagues suggested that the loss of Siglec-14 may increase the risk of prematurity (Ali et al., 2014), since amniotic epithelium—representing a part of the placenta system—expresses Siglec-14 and is a known target of invading group B *Streptococcus* (Vanderhoeven et al., 2014).

Fig. 2 gives a fair overview of the Siglec system in mammals. As expected, the highly conserved Siglec-1, Siglec-2, Siglec-4 as well as Siglec-15 are present in all analyzed species. Furthermore, in nearly all analyzed mammals hits for Siglec-3, Siglec-5, Siglec-10 and Siglec-14 were found. No ortholog sequences of hSiglec-7 have been published so far, while for all other Siglecs putative orthologue sequences were identified in one or more of the screened mammals. However, the reason for the presence of distinct CD33-related Siglecs in different mammals remains a mystery.

We wanted to investigate whether the different types of placentas in mammals could represent triggers for the development of different ITIM-containing CD33-related Siglecs. During evolution, different types of placentas developed. Human beings, primates and some rodents possess a hemochorial placenta that is characterized by direct exchange between the maternal part and the fetus, responsible for gas, metabolite, nutrition, hormone and antibody transfer between the embryo and the mother (Newman, 1960). Therefore, we expected that species with a hemochorial placenta would possess a bench of Siglecs in order to prevent immune reactions against the fetus. Human beings seem to be an exception within the species with hemochorial placentas (Supplement 16), as they possess the highest number of Siglec types. Animals like dogs, cats and elephants have an endotheliochorial placenta (missing blood contact between the fetus and the maternal blood vessels (Furukawa et al., 2014)) characterized by the maintenance of the blood vessel endothelia; these species bear comparable numbers of inhibitory Siglecs, like animals with a hemochorial placenta. In addition, mainly farm animals have epitheliochorial placentas (Furukawa et al., 2014), which are characterized by the maintenance of the maternal uterus epithelia, the maternal connective tissue and the maternal blood vessels (Furukawa et al., 2014). Consequently, no direct contact between the maternal blood—and thus the maternal immune system—and the fetus exists, leading to the prospect that these species might handle pregnancy without a high amount of transmembrane immune regulatory receptors like Siglecs. Altogether, we did not find any correlation between the number of Siglecs and the placenta type. However, we note that only the general presence of Siglec-encoding genes in an organism is retrievable from databases and information on the tissue-specific expression of Siglecs during pregnancy is limited.

Intriguingly, lactation apparently developed alongside with the growing of the mammalian CD33-related Siglec family and their modulation possibilities of the immune system (Cao and Crocker, 2011; Cao et al., 2009). Actually, egg-laying mammals—evolving for ~200 million years—seem to express at least one variant of inhibitory the mammalian CD33-related Siglec, since the platypus was positive for Siglec-5 (Figs. 2 and 7). Data from studies analyzing the milk oligosaccharides of platypus suggest that the milk of monotremes contains different α2,3- and α2,6-sialylated milk oligosaccharides (Urashima et al., 2015). Interestingly, koalas already

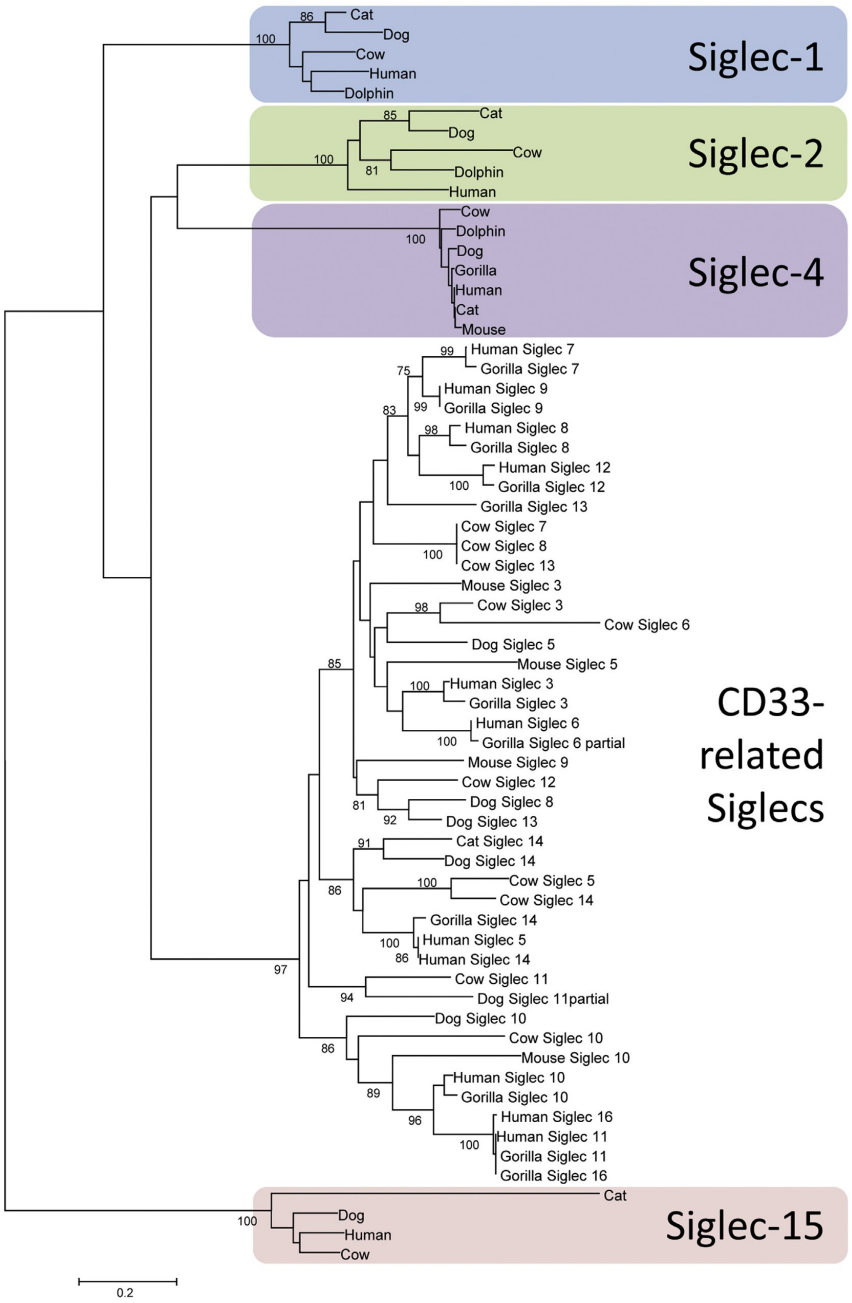


Fig. 6. Phylogenetic relationship of Siglec proteins from representatives of selected mammalians. This dendrogram was constructed using the Neighbor-Joining Method applying the Poisson correction distance model included in the Molecular Evolutionary Genetics Analysis package (MEGA, version 6.0) as conducted in our previous report (Rebl et al., 2011). Bootstrap confidence values represent the percent frequency of appearances of each clade in 1000 replicas. Labeling includes the species name. For the accession number of the respective sequence, see Supplement 17. Due to high sequence identities, the CD33-related Siglecs could not be separated clearly (Supplement 15). Used animals: Cat, Dog, Cow, Dolphin, Human, Mouse and Gorilla.

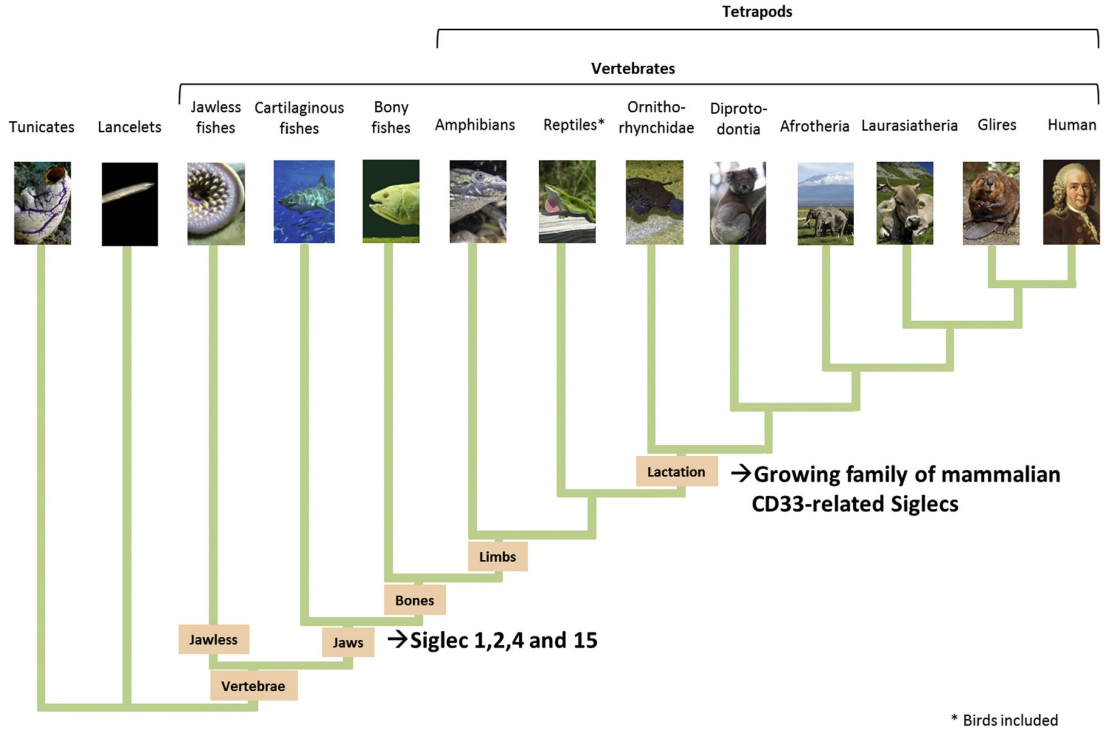


Fig. 7. Milestones of Siglec evolution. With the emergence of jaws, the highly conserved Siglec-1, -2, -4 and -15 occurred. Around 200 MY ago, with the emergence of lactation (Capuco and Akers, 2009), the family of CD33-related siglecs expanded. The sources of all photos are listed in Supplement 18.

express three different CD33-related Siglec variants, which bear strong sequence homology with Siglec-3, Siglec-10 and Siglec-14. Also, koala milk oligosaccharides contain α2,3- as well as α2,6-linked sialic acid residues.

Since the microbiome of milk directly influences the composition of the gut microbiome in offspring (Pannaraj et al., 2017), the interplay during lactation between sialylated milk oligosaccharides and the sialome of the microbiome of the mammary gland, as well as in the intestine of the offspring, might represent important triggers for the evolution of CD33-related Siglec mechanisms. This might explain the parallel gene expansion of the mammalian CD33-related Siglec family and the development of lactation.

6. Conclusion

Our analyses of Siglecs from numerous species from evolutionary distinct clades suggest, in agreement with previous studies, that Siglec-1, Siglec-2, Siglec-4 and Siglec-15 were present in the ancestral vertebrate before the separation of tetrapod's (e.g. humans) and teleost fishes (e.g. salmon) more than 400 million years ago (Angata et al., 2007; Cao and Crocker, 2011; Cao et al., 2009; Lehmann et al., 2004). Thus, an ecological niche/habitat-independent high evolutionary pressure on these four highly conserved receptors exists, whereas a significant expansion of the CD33-related Siglec genes took place during the evolution of mammals (Betancur et al., 2015). Siglec-2 might prevent autoimmunity (Poe and Tedder, 2012). Siglec-4, which is expressed on the innermost myelin wrap, may stabilize the myelin-axon interaction (Sun et al., 2004). The conservation of Siglec-15 might reflect its important function for bone resorption (Macauley et al., 2014). Thus, it seems very likely that during the evolution of first vertebrates, the emergence of Siglec-2, Siglec-4 and Siglec-15 is closely linked to the development of the BCR system, myelination and bones, respectively (Fig. 7). A further important key step concerning the evolution of Siglecs might be the development of lactation, which may initiate the progress of the mammalian CD33-related Siglec family (Fig. 7). Thus, sialic acid and Siglec machinery underwent remarkable changes during essential stages of vertebrate evolution.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2018.05.008.

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Characterization of Sialic Acid-Binding Immunoglobulin-Type Lectins in Fish Reveals Teleost-Specific Structures and Expression Patterns

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Abstract: The cellular glycocalyx of vertebrates is frequently decorated with sialic acid residues. These sialylated structures are recognized by sialic acid-binding immunoglobulin-type lectins (Siglecs) of immune cells, which modulate their responsiveness. Fifteen Siglecs are known to be expressed in humans, but only four Siglecs are regularly present in fish: Siglec1, CD22, myelin-associated glycoprotein (MAG), and Siglec15. While several studies have dealt with the physiological roles of these four Siglecs in mammals, little is known about Siglecs in fish. In the present manuscript, the expression landscapes of these Siglecs were determined in the two salmonid species *Oncorhynchus mykiss* and *Coregonus maraena* and in the percid fish *Sander lucioperca*. This gene-expression profiling revealed that the expression of *MAG* is not restricted to neuronal cells but is detectable in all analyzed blood cells, including erythrocytes. The teleostean MAG contains the inhibitory motif ITIM; therefore, an additional immunomodulatory function of MAG is likely to be present in fish. Besides *MAG*, *Siglec1*, *CD22*, and *Siglec15* were also expressed in all analyzed blood cell populations. Interestingly, the expression profiles of genes encoding Siglecs and particular associated enzymes changed in a gene- and tissue-specific manner when *Coregonus maraena* was exposed to handling stress. Thus, the obtained data indicate once more that stress directly affects immune-associated processes.

Keywords: acute stress; ITIM; Salmonidae; siglecs; sialic acids; vertebrate evolution

1. Introduction

Innate immunity is of even more paramount importance for fish health than adaptive immune mechanisms [1]. More than 50 regulators of innate immunity are known in fish today [2]; these regulators maintain the balance between pathogen defense and pathophysiological manifestations. Some of these innate-immune regulators recognize self-associated molecular patterns (SAMPs), a heterogeneous group of molecules that stimulate inhibitory receptors to dampen immune responses [3–5]. The family of sialic acids consists of more than 50 members [6] and belongs to the group of SAMPs [7]. Sialic acids are frequently located at the terminal ends of glycans on glycoproteins and the glycolipids that coat all mammalian cells in a glycocalyx. Immune cells abundantly express sialic acid-binding

immunoglobulin-type lectins (Siglecs), which interact with these sialylated glycoconjugates and thus prevent an excessive immunological response [8,9].

The Siglecs are divided into two subgroups: the CD33-related Siglecs and the highly conserved Siglecs, including Siglec1 (sialoadhesin), CD22 (Siglec2), MAG (Siglec4), and Siglec15. Both subgroups comprise Siglecs that suppress the immune response or activate the immune system [9,10]. The inhibitory function of Siglecs is mediated by an intracellular immune-receptor tyrosine-based inhibition motif (ITIM; signature: [I/V/L/S]-X-Y-X-X-[L/V] [9]), which is absent in immune-activating Siglecs (Siglec14, Siglec15, and Siglec16 in humans). These Siglec receptors interact with DNAX proteins (DAP10/DAP12), which carry immune-receptor tyrosine-based activation motifs (ITAMs) [9,11]. To date, fifteen and nine Siglecs have been identified in human and mouse, respectively, each expressed by defined cell types and responding to a particular set of ligands [12–14]. Remarkably, Siglec1, CD22, MAG, and Siglec15 were already present in the animal kingdom before the separation of tetrapods and teleost fishes more than 400 million years ago [15]. However, little is known about the physiological role of Siglecs in teleosts [16,17] including their tissue-specific basal expression profiles. For this reason, the present study aimed at the analysis of the expression landscapes of Siglec1, CD22, MAG, and Siglec15 in teleost fish. The expression profiling was complemented by in-depth analyses of the structures of the aforementioned Siglec receptors. These investigations focused on three aquaculture fish species of economic importance, (i) pikeperch (*Sander lucioperca*) as a representative of diploid percid fish, (ii) rainbow trout (*Oncorhynchus mykiss*) and (iii) maraena whitefish (*Coregonus maraena*) as representatives of the pseudotetraploid salmonid fishes that are currently undergoing species-specific reploidization processes [18]. Notably, the rainbow trout has been adapted to intensive farming for decades, whereas maraena whitefish is a novel aquaculture species and a useful model for dissecting the response of a pseudo-wild fish to anthropogenic environments. Our previous transcriptomic analyses on the stress physiology of the maraena whitefish revealed that the level of MAG transcripts is modulated by a factor of approximately two in different organs after exposure to temperature and stocking-density stress [19,20]. These findings suggested that Siglecs could potentially be biomarkers for immune and stress responses in teleost fish. To address whether stress affects the expression of Siglec-encoding genes, maraena whitefish were exposed to handling procedures [19,20]. The presently outlined study provides novel insights into the physiological roles of Siglecs in teleost fish.

2. Materials and Methods

2.1. Fish Husbandry and Experimental Treatment

The animals used for this study were provided by the Institute for Fisheries of the State Research Centre for Agriculture and Fishery Mecklenburg–Western Pomerania and BiMES Binnenfischerei GmbH (Friedrichsruhe, Germany). Fish were held in freshwater recirculation systems under a 12:12 day-and-night cycle at 18 °C. Water quality was maintained by automated purification and disinfection (bio-filter and UV light). In addition, the concentrations of NH_4^+, NO_2^-, NO_3^-, and NH_3 in the water, pH value, temperature, and oxygen saturation were constantly recorded. The feeding material consisted of commercial dry pellets (4.5 mm, INICIO Plus; BioMar, Brande, Denmark), which were distributed by automatic feeders at a daily rate of 0.8–4.0%, depending on the biomass of the fish.

Maraena whitefish were acclimatized in the experimental tanks connected to a recirculation system for a period of at least three weeks. Acute handling stress was induced by chasing, netting and the transfer of fish to another tank for a period of one minute. Eight stressed and eight control fish were euthanized with an overdose of 2-phenoxyethanol (0.7 mL/L; Sigma-Aldrich/Merck, Munich, Germany) and then underwent spine sectioning at the skull level. These procedures followed the standards described in the German Animal Welfare Act (§ 4(3) TierSchG) and were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (Mecklenburg–Vorpommern, Germany; approval ID: LALLF M-V/TSD/7221.3-1-069/18; date of approval: January 16, 2019). The brain of each maraena whitefish was dissected into the hypothalamus, telencephalon, and hindbrain and sampled together

with the spleen, liver, white muscle, gills, head kidney (HK), and heart. Samples were snap-frozen in liquid nitrogen and stored at −80 °C. Husbandry and sampling of pikeperch and rainbow trout have been described elsewhere [21,22].

2.2. Isolation and Microscopic Characterization of Head Kidney Cells from Maraena Whitefish

For HK cell isolation and cell sorting, HKs from freshly slaughtered maraena whitefish were dissected and put into a Dulbecco's Modified Eagle Medium (DMEM; Gibco/Thermo Fisher Scientific, Bremen, Germany). The tissue was homogenized using a steel sieve (500 µm, Carl Roth, Karlsruhe, Germany) and then filtered through two cell strainers (200 µm, pluriSelect Life Science, Leipzig, Germany; 100 µm, Falcon/Fisher Scientific, Schwerte, Germany). After centrifugation (524× *g*, 5 min) and resuspension in 3 mL DMEM, the cell suspension was added onto an isotonic Percoll (Sigma-Aldrich/Merck) gradient (3 mL, ρ = 1.084 g/mL) and centrifuged at 800× *g* for 30 min at 6 °C with minimum deceleration. The erythrocyte pellet was stored at −80 °C for further RNA extraction, while the cell band at the interface was collected in the DMEM and the volume was adjusted for cell counting. Cell number and cell viability were determined using the Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA, USA). In addition, a portion of the cells separated by the Percoll gradient were placed on glass slides, stained with a May-Grünwald-Giemsa solution (Brand, Wertheim, Germany; Carl Roth), and then microscopically observed using a Nikon TMS-F microscope and a Nikon Coolpix E5000 camera with an MDC Lens (Nikon, Tokyo, Japan).

2.3. Flow Cytometry

Flow cytometry was performed using a MoFlo XDP high-speed cell sorter (Beckman Coulter, Krefeld, Germany) with an incorporated, air-cooled sapphire laser (488 nm, 100 mW). A total of ~20 million HK cells were sorted through a 70-µm nozzle at 60 psi on purify mode into two fractions, low side-scattering intensity (fraction I) and high side-scattering intensity (fraction II). Fractions I and II were collected in phosphate-buffered saline (PBS), centrifuged at 500× *g* for 5 min, and used for RNA extraction. Subsequently, cell type-specific gene expressions were profiled, as described in detail in [23].

2.4. RNA Isolation

Approximately 50 µg of each of the individual tissue samples were placed in separate reaction tubes containing 1 mL of TRIzol Reagent (Life Technologies/Thermo Fisher Scientific) and homogenized using the Precellys24 Homogeniser (6000 rpm, 30 s). After the addition of chloroform and a centrifugation step (12,000× g, 15 min, 4 °C), the RNA contained in the resulting aqueous phase was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was isolated from cells and purified using the Isolate 2 RNA Micro Kit (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions and without a previous treatment with TRIzol Reagent. The quality of the purified RNA was checked using horizontal agarose-gel electrophoresis. RNA concentration was determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

2.5. Primer Design and Quantitative PCR

Species-specific quantitative PCR (qPCR) primers were designed for the target genes using PSQ Assay Design 1.0.6 software (Biotage AB, Uppsala, Sweden). Amplicon length ranged from 140 to 180 bp (Table S1). Coding sequences for rainbow trout were retrieved from the NCBI public database. To identify Siglec sequences from pikeperch or maraena whitefish, we aligned the orthologous sequences from yellow perch (*Perca flavescens*) or rainbow trout, Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*), respectively, with the recently published pikeperch genome [24] or our RNA-seq read collection from maraena whitefish [25] using Bowtie 2 software (v. 2.2.4; http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). These alignments were then indexed and sorted with the Samtools software package (v. 16; http://www.htslib.org/), and final consensus

sequences were obtained with Ugene software (v. 1.29; http://ugene.net/). The amplicon sequences selected from pikeperch were highly identical to the counterpart sequences selected from yellow perch (*SIGLEC1*: 92%; *CD22*: 98%; *MAG*: 91%), but they shared only moderate levels of identity with the respective sequences of other fish species. Moreover, we did not find any *SIGLEC15*/*CD33L3* sequences from percid fish species in the public databases, so we used a *CD33* sequence from barred knifejaw (*Oplegnathus fasciatus*) instead. To take the uncertain assignment of the aforementioned sequences into account, we extended the gene names of the pikeperch sequences by '-like' (abbreviated as 'L').

The integrity and specificity of the PCR products were assessed via standard PCRs (HotStarTaq Plus DNA Polymerase, Qiagen) and single qPCR analyses (LightCycler 480 System, Roche, Mannheim, Germany; SensiFAST SYBR No-ROX Kit, Bioline). A multiplex qPCR analysis was performed with the 48.48 Dynamic Array IFC chip (Fluidigm, South San Francisco, CA, USA) and the BioMark HD-System (Fluidigm) using the nucleotide-binding EvaGreen fluorescence dye (Bio-Rad, Feldkirchen, Germany). In detail, 1 µL of the extracted RNA was reverse-transcribed with the Reverse Transcription Master Mix (Fluidigm). Then, the designed primer pairs and the PreAmp Master Mix (Fluidigm; 100 µM of mix per pair) were used to perform 11 pre-amplification cycles with the individual cDNA samples adjusted at 10 ng/5 µL. The pre-amplified products were treated with exonuclease I (ExoI; New England BioLabs, Frankfurt am Main, Germany) and subsequently diluted in a pre-mixed solution of SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) and 20 × DNA binding dye sample loading reagent. The sample and primer mixes were transferred to the respective inlets of the 48.48 Dynamic Array IFC chip, which was thereafter primed in the BioMark IFC Controller MX (Fluidigm), running the Load Mix 48.48 GE script. The loaded array chip was then placed in the BioMark HD-System (Fluidigm) to proceed with the qPCR according to the GE 48 × 48 Fast PCR+Melt v2.pcl cycling program. Fluidigm RealTime PCR Analysis Software (v. 3.0.2, https://www.fluidigm.com/software) was used to analyze the qPCR results. To obtain the relative copy number of each transcript, a serial dilution-based standard curve (10^2–10^6 copies) was used and the copy number was normalized with the geometric mean of three suitable reference genes (*EEF1A1b*, *RPL9*, and *RLP32* for maraena whitefish; *EEF1A1*, *ACTB*, and *RPS5* for rainbow trout; and *EEF1A1*, *RPS5*, and *RLP32* for pikeperch) [21,26–28].

2.6. Cloning

Since we retrieved only gene fragments of *CD22* and *MAG* from our transcriptome of maraena whitefish, we derived primers from the 5′ and 3′ ends of the respective open reading frames. First, a SuperScript II Reverse Transcriptase Kit (Invitrogen/Thermo Fisher Scientific) was used to transcribe a total of 1 µg of RNA into cDNA. This reverse transcription was carried out at 42 °C for 50 min, followed by an inactivation step at 70 °C for 15 min. Purification of the cDNA was performed using a High Pure PCR Product Purification Kit (Roche), and the resulting cDNA was diluted in 100 µL of distilled water. Subsequently, we used the HotStarTaq Plus DNA Polymerase (Qiagen) to generate the PCR products of the full-length open reading frames. The purified (High Pure PCR Product Purification Kit; Roche) amplicons were inserted into a pGEM-T-Easy vector (Promega, Walldorf, Germany). The obtained plasmids were sequenced using the universal SP6/T7 primers and a MegaBACE capillary sequencer (GE Healthcare, Freiburg im Breisgau, Germany). Twelve clones were picked and analyzed per amplified sequence fragment.

2.7. In Silico Analyses

Sequence alignments were performed using the Clustal Omega tool of EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/). The sequences of CD22, MAG and Siglec15 were derived by blasting the orthologous sequences of rainbow trout (*O. mykiss*; Om), coho salmon (*O. kisutch;* Ok), Atlantic salmon (*S. salar*; Ss), yellow perch (*P. flavescens*; Pf), and barred knifejaw (*O. fasciatus*; Of) against the sequence assemblies of maraena whitefish and pikeperch [24,29]. In addition, the CD22 and MAG sequences of maraena whitefish were completed by sequencing the results.

We retrieved the following sequences from the NCBI database: *Danio rerio* (zebrafish) MAG: XP_021337068; *Takifugu rubripes* (pufferfish) MAG: XP_011616490; *Mus musculus* (mouse) Siglec15: NP_001094508, MAG: XP_030098048; CD22: NP_033975; and *Homo sapiens* (human) Siglec15: NP_998767, MAG: AAB58805, CD22: NP_001762. The positions of the transmembrane domains of murine and human Siglec15 were retrieved from Uniprot; the transmembrane areas of Siglec15 from maraena whitefish and rainbow trout were predicted using SMART (http://smart.embl-heidelberg.de/). The V-set, Ig-like domains of murine and human Siglec15 were determined using the SMART program; the V-set, Ig-like domains of MAG and Siglec15 from fishes were estimated based on sequence alignments; and the V-set, Ig-like domains of human MAG and murine MAG (pdb sequence: 5LF5) were determined using the SMART program. In addition, the V-set, Ig-like domain of human and mouse CD22 was determined using the SMART program, while the V-set, Ig-like domains of CD22 from maraena whitefish and rainbow trout were defined by sequence alignment.

The 3D modelling of the sialic acid-binding domain (V-set, Ig-like domain) of CD22 from human and maraena whitefish was performed using YASARA 19.9.17. The structure of human CD22 was given by the pdb sequence: 5VKM, published by Ereño-Orbea et al. [30]. Based on Uniprot, the sequence was shortened to the first Ig-domain, responsible for sialic acid recognition. The sequence of the V-set domain of CD22 from maraena whitefish was obtained by sequence alignment with the sequence of the V-set, Ig-like domain of human CD22.The 3D-modelling of the sialic acid-binding domain (V-set, Ig-like domain) of MAG from mouse and maraena whitefish was also performed using YASARA 19.9.17. The structure of the murine MAG was determined by Pronker et al. [31]. Using the generated alignments, the sequence of murine MAG was shortened to the first Ig domain, which is responsible for sialic acid-binding. The sequence of the V-set, Ig-like domain of MAG from maraena whitefish was based on our cloned sequences.

The mechanistic interaction of Siglecs and associated factors was illustrated using the Ingenuity Pathways Analysis software (IPA; Qiagen Bioinformatics software solutions) based on the Ingenuity Knowledge Base. The constructed informal diagram was manually edited using the Path Designer tool (IPA).

3. Results and Discussion

3.1. The Expression of Siglec-Encoding Genes in Different Tissues of Salmonid and Percid Fishes

The expression of Siglec-encoding genes in different lymphoid and non-lymphoid tissues of the economically important farm-fish families *Salmonidae* and *Percidae* has, to our knowledge, not yet been described in detail. To pave the road for future immunological studies of the sialic acid-dependent regulation of immune processes in bony fish, we performed structural analyses and multiplex qPCR measurements of the piscine Siglecs *Siglec1*, *CD22*, *MAG*, and *Siglec15*.

Siglec1 plays an indispensable role in innate and humoral immunity, even though it contains no immunomodulatory motifs [32]. The expression analyses (Figure 1) demonstrated that *Siglec1* mRNA was most abundant in the spleens and HKs of pikeperch (>2 × 10^7 copies/µg RNA) and in the spleens and gills of maraena whitefish (>2 × 10^3 copies/µg RNA) and rainbow trout (>3 × 10^3 copies/µg RNA). The teleostean HK is considered the functional counterpart of the mammalian bone marrow [1]. It contains considerable amounts of lymphocytes and macrophages [33], similar to the spleen and gills. Probably, *Siglec1* is mainly expressed on those immune cells, since it has been reported that mammalian *Siglec1* is highly expressed on splenic and lymph-node macrophages [9,13].

CD22 was assigned as an activation marker for mature B cells in mammals, and the interaction of CD22 with the B-cell receptor (BCR) has been well established in mammals. The highest levels of *CD22* were found in the HKs of the salmonids maraena whitefish and rainbow trout (>2 × 10^5 copies/µg RNA) as well as in the HKs of pikeperch (>3 × 10^6 copies/µg RNA). The high *CD22* copy number might indicate an analogous interaction between the CD22 and B cells of the teleostean HK.

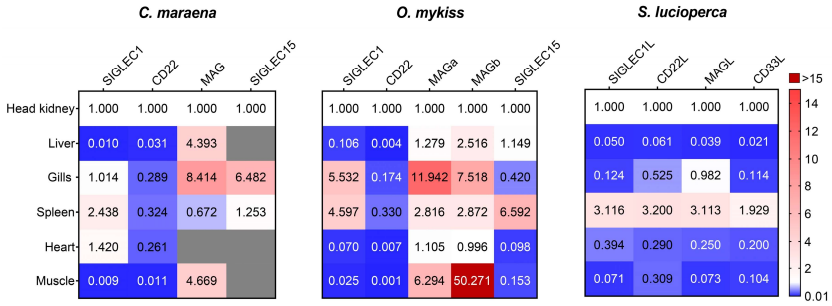


Figure 1. Tissue-specific expression of *Siglec1*, *CD22*, *MAG*, and *Siglec15*/*CD33L* in maraena whitefish (*C. maraena*), rainbow trout (*O. mykiss*), and pikeperch (*S. lucioperca*). The qPCR data were normalized by three reference genes. The resulting transcript numbers from the head kidney (HK) were set at 1.0, and the transcript numbers of the same gene in all other tissues were expressed as fractions. Lower and higher transcript values than those of the HK are highlighted in blue and red, respectively, according to the given color code. Non-detectable transcript numbers are indicated by gray fields.

Siglec15 is an immune-activating Siglec interacting with DNAX proteins [9,11]. The salmonid fish species shared high expression levels of *Siglec15* in the spleen (>15 × 10^3 copies/µg RNA). This observation was in line with the fact that mammalian macrophages are the dominant expression site of *Siglec15* [13,34].

MAG is mainly involved in the stabilization of axon-myelin interactions, the inhibition of neurite growth, and the inhibition of axon regeneration in mammals [35]. The copy numbers of *MAG* were present in a range of tissues across the three analyzed fishes, with the highest *MAG* levels in the gills (>8 × 10^3 copies/µg RNA). Notably, two *MAG* ohnologs were expressed in rainbow trout: *MAGa* and *MAGb*, which are located on chromosomes 2 and 3, respectively. *MAGa* showed at least twice as many transcript numbers as *MAGb* in the analyzed tissues, with muscle containing the highest number of *MAGb* transcripts (>2 × 10^3 copies/µg RNA).

Since in mammals the highest amounts of MAG can be found in the central nervous system [35], we also inspected the expression of *MAG* together with that of *SIGLEC1*, *CD22*, and *SIGLEC15* in different regions of the brains (hypothalami, telencephalons, and hindbrains) of maraena whitefish (Figure 2). While the transcripts of *Siglec1*, *CD22*, and *Siglec15* were detected at low or moderate levels (<2.5 × 10^3 copies/µg RNA), we detected extremely high levels (~2.6 × 10^7 copies/µg RNA) of *MAG* in the brains of maraena whitefish, especially in the hindbrains (>5 × 10^7 copies/µg RNA). The *MAG* levels in the hindbrains exceeded even the relatively high *MAG* copy numbers in the gills, muscles, and livers of maraena whitefish by 300- to 4000-fold (Figures 1 and 2).

The expression of *Siglec15* was absent in telencephalon, but comparably high in the hypothalamus of maraena whitefish (~2.5 × 10^3 copies/µg RNA). The murine SiglecH has previously been described as activating an immune response in microglia cells [36]. SiglecH is known to interact with DAP12 and enhance the phagocytotic activity of glioma cells in mice [36,37]. It is conceivable that Siglec15 may play a similar immune-regulatory function in fish brains. This also applies to CD22, which is expressed in mammalian microglia cells to decrease inflammatory effects [38].

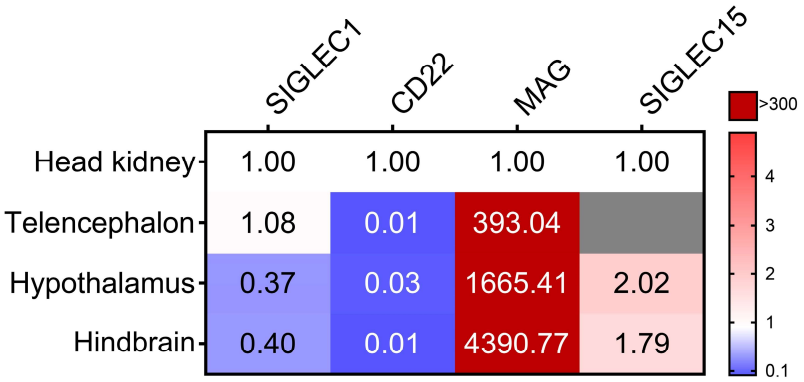


Figure 2. Expression of *Siglec1*, *CD22*, *MAG*, and *Siglec15* in the different brain regions of maraena whitefish. The qPCR data were normalized by the reference genes *RPL9*, *EEF1A1b*, and *RPL32*. The resulting transcript numbers of the same genes in different brain regions are shown relative to the respective transcript levels in the HKs, which were set at 1.0. Transcript values compared with those found in HKs are colored according to the code on the right. Non-detectable transcript numbers are indicated by a gray field.

3.2. The Expression Patterns of Siglec-Encoding Genes in Cell Populations of Maraena Whitefish

Since in mammals Siglecs are heterogeneously expressed in immune cells [39], we conducted a subsequent qPCR analysis to determine which immune-cell populations express Siglecs in maraena whitefish. To this end, we isolated the HKs (Figure 3A1) and extracted cells from these tissues (Figure 3A2). The resulting cell suspension was then separated into leukocyte (Figure 3A3) and erythrocyte (Figure 3A4) suspensions via Percoll treatment. Eventually, the leucocyte suspension was further separated into a fraction I, enriched with less granular and smaller cells (presumably lymphocytes and monocytes/macrophages), and a fraction II with more granular and larger cells (presumably granulocytes) (Figure 3B). Profiling the copy numbers of *Siglec1*, *CD22*, *MAG*, and *Siglec15* via multiplex qPCR revealed that the erythrocytes were the main cell population expressing all four Siglecs (Figure 3C1–6).

The main function of mammalian erythrocytes is the transportation of oxygen, whereas teleostean erythrocytes also exert immunological functions [40–43]. This may be due to the fact that teleostean erythrocytes contain nuclei and are capable of regulating their gene expression if necessary [44], in contrast to their mammalian counterparts [45,46]. In particular, fish erythrocytes are considered as antigen-presenting cells, recognize pathogen-associated molecular patterns (PAMPs), phagocytose, and influence the activity of other immune cells [40–43]. It is likely that Siglecs are also involved in these erythrocyte-pathogen interactions. A closer look at the Siglec expression in erythrocytes from maraena whitefish revealed that *Siglec15* and *CD22* were most highly expressed. This also applied to all other immune cell fractions indicating that a high expression of *CD22* in fish is not restricted to B cells [47]. The expression of mammalian *CD22* has been described as being predominately located in B cells [47]. As the genomes of fish obviously lack any inhibitory CD33-related Siglecs (containing the ITIM motif), it might be possible that CD22 may take over this role in the immune cells of fish. In addition, the expression of *MAG* and *Siglec1* was detectable in the analyzed immune cell populations of maraena whitefish.

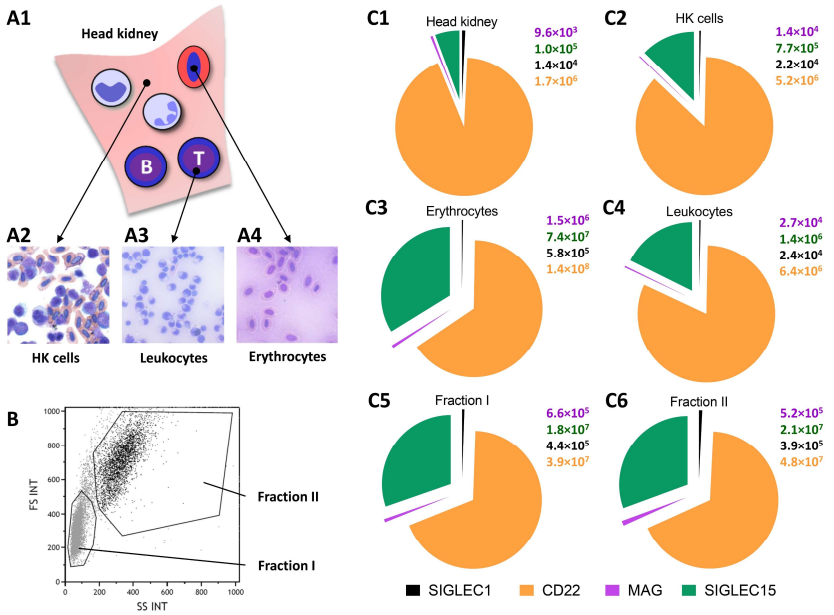


Figure 3. Copy numbers (per µg RNA) of *Siglec1*, *CD22*, *MAG*, and *Siglec15* in (**A1**) whole head kidneys (HKs), (**A2**) extracted HK cells, (**A3**) a heterogeneous leukocyte suspension, (**A4**) erythrocytes, and (**B**) one sorted cell fraction (I), enriched with lymphocytes and monocytes/macrophages, and one fraction (II) enriched with granulocytes (n = 5). (**C**) Siglec expression in the isolated cell fractions (**C1**) HK, (**C2**) HK cells, (**C3**) erythrocytes, (**C4**) leukocytes, (**C5**) fraction I, and (C6) fraction II. QPCR expression data for *Siglec1* (black), *CD22* (orange), *MAG* (purple), and *Siglec15* (green) was normalized against the GeoMean of the reference genes *RPL9*, *EEF1A1b*, and *RPL32*.

3.3. Sequence Comparison of Siglecs Expressed by Salmonid and Percid Fishes

We analyzed the nucleotide and amino-acid sequences of the evolutionarily conserved Siglecs in fish and mammals in more detail because their expression profiles exhibited remarkable differences. Siglec1 contains no regulatory domains; for this reason, only the sequences of CD22, MAG, and Siglec15 are presented here. Since the Siglec sequences of the pikeperch (SIGLEC1L, CD22L, MAGL, and CD33L) revealed poor homology with its piscine orthologs (see Section 2.5), we disregarded these sequences in the more detailed sequence analysis.

3.3.1. Sequence Comparison of Siglec2 (CD22)

In mammals, CD22 can counteract the BCR-triggered activation of B cells, when defined sialylated structures are detected simultaneously with an antigen [48,49]. The clustering of a BCR with CD22 molecules causes the recruitment of SHP1 and SHP2 (encoded by the genes *PTPN6* and *PTPN11*), which leads to the inhibition of the kinase-dependent signaling pathway, along with the reduced production of antibodies against the autoantigen [48,49]. This mechanism inhibits thus the synthesis of autoantibodies. Teleosts produce three main types of immunoglobulins (IgM, IgD, and IgT/IgZ) that act as BCRs [50,51]. The interplay between these immunoglobulins and CD22 might be regulated in a mammalian-analogous way on teleostean B cells.

The *N*-glycosylation status of mammalian CD22 seems to be important for its activity. Twelve *N*-glycosylation sites are known in mammalian CD22, six of which are close to the sialic acid-binding domain (N_{67}; N_{101}; N_{112}; N_{135}; N_{164}; N_{231}). According to Orbea et al., N_{67}, N_{112}, N_{135}, N_{164}, N_{231} can be exchanged by an alanine without functional loss, while the mutation of N_{101} disrupted protein expression [30]. N-glycans at N_{101} are probably involved in the correct folding of the receptor. Regarding the sequence comparison of the V-set Ig-like domain, which is responsible for sialic acid-binding (Figure 4A), N_{101} seems to be conserved from mammals to lower vertebrates (N_{105} in fishes), while N_{67} and N_{135} seem to be absent. Recently, Wasim et al. determined that mutations of N_{67}, N_{112}, N_{135}, N_{164} and N_{231} resulted in a higher density of CD22 nanoclusters, along with a decreased CD22-phosphorylation rate and an increased B-cell signaling, culminating in a reduced functionality of CD22 [52]. Therefore, we took a closer look at the N-glycosylation sites in the CD22 orthologs from maraena whitefish and rainbow trout. The alignment of the CD22 sequences from maraena whitefish and rainbow trout suggested (Figure 4A and Figure S1) that the majority of N-glycosylation sites are also present in CD22 of fish, although they are at slightly different positions compared to their human orthologs. The N-glycosylation at N_{67}, N_{101}, N_{112}, N_{164} and N_{231} in human corresponds to N_{59}, N_{105}, N_{112}, N_{167} and N_{221} in fish. Moreover, we searched for ITIM domains in the CD22 orthologs of maraena whitefish and rainbow trout, since these domains characterize inhibitory receptors in mammals [53]. An ITIM is present in CD22 of rainbow trout but not in the orthologous sequence of maraena whitefish (Figure S1). However, our CD22 sequence of maraena whitefish was severely truncated, and we cannot exclude the possibility that an ITIM is present there.

Furthermore, we analyzed the sialic acid-binding domain in more detail. Human CD22 preferentially binds α2,6-linked sialic acid. The binding is mediated by the amino-acid residues R_{120}, R_{131}, E_{126} and W_{128} in addition to Y_{64}, which is responsible for the preference for α2,6-linked sialic acid [30]. The sequence comparison (Figure 4A) demonstrated that R_{120} is conserved from mammals to fish, while almost all other amino acids, necessary for sialic acid-binding in humans, are missing in the investigated fish. In mice, Y_{64} is replaced by F, indicating the conservation of the aromatic properties, and also in fish, W resides close to Y_{64} in human [30].

In addition, we simulated 3D models of the V-set Ig-like domain of CD22 from maraena whitefish based on the known 3D structure of the human counterpart [30]. This 3D model exhibited remarkable structural differences between the CD22 orthologs from human and maraena whitefish (Figure 4B–D). Hence, based on the modelling of CD22 from maraena whitefish (Figure 4B–D) combined with the sequence alignment (Figure 4A), we suggest that the binding properties of CD22 have changed during evolution. However, experimental data is needed to define the glycan-binding properties of CD22 in fishes.

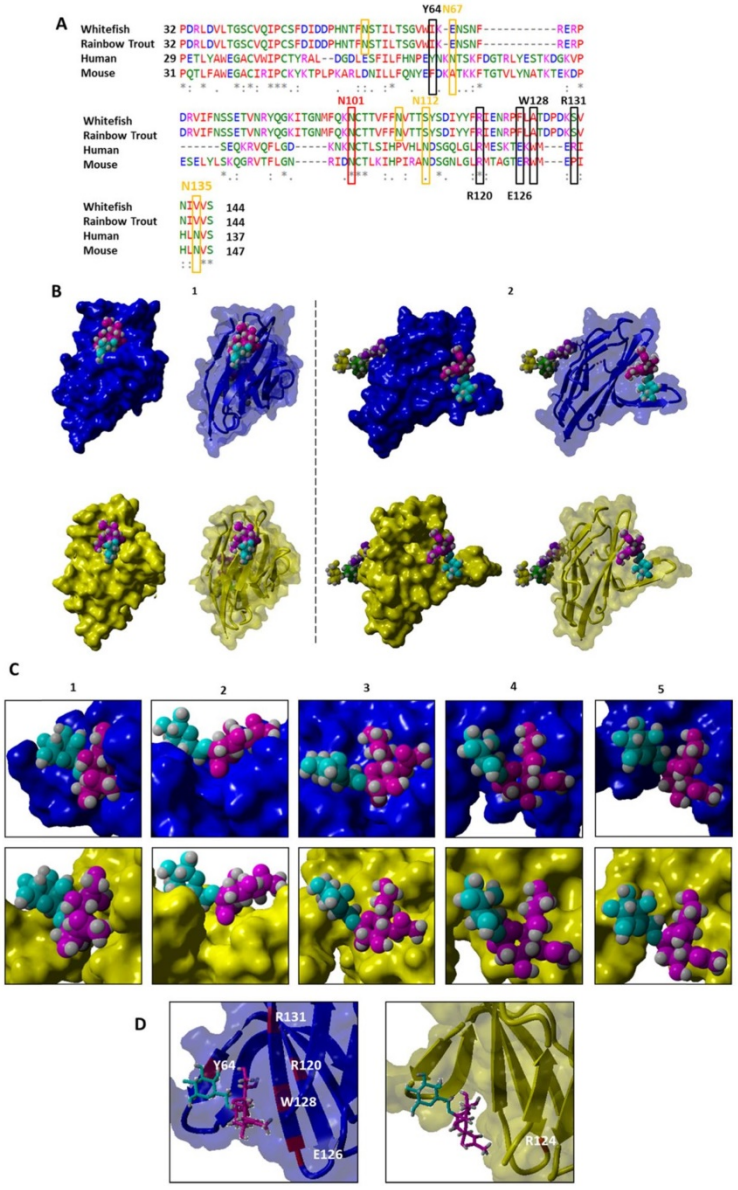


Figure 4. Sequence and structural comparison of CD22 from human and maraena whitefish. (**A**) The first Ig domain of maraena whitefish and rainbow trout was determined by aligning sequences. The different colors indicate the chemical properties of the amino acids as follows: red, small hydrophobic/aromatic

amino acids; blue, acidic amino acids; magenta, basic amino acids; green, hydrophilic, polar, and small amino acids. Black boxes show the amino acids that are essential for sialic acid-binding; red boxes show the conserved N-glycosylation site N_{101}. In addition, the orange boxes indicate further amino acids known to be a target for N-glycosylation. Numbering is based on the human CD22 sequence. (**B**) YASARA was used to model the 3D structure of the sialic-acid-binding domain (V-set, Ig-like domain) of CD22 from human (pdb: 5VKM, including 5 point mutations $N_{67}A$, $N_{112}A$, $N_{135}A$, $N_{164}A$, $N_{231}A$ [30]) and maraena whitefish. The sequence of the human MAG was shortened to the first Ig domain. The sequence of the V-set, Ig-like domain of CD22 from maraena whitefish was based on the alignments (see (**A**)). CD22 models from human and whitefish are labelled in blue and yellow, respectively. Two different perspectives of the surface with a transparency of 30% are shown along with the corresponding secondary structures. (**C**) Enlargement of the sialic-acid-binding domain of CD22 from maraena whitefish in five different perspectives. (**D**) Amino acids responsible for sialic acid-binding by CD22 from human and maraena whitefish. Surface and secondary structures are shown. Bound glycans are specifically labeled as follows: galactose, cyan; *N*-acetyl-D-galactosamine, purple; sialic acid, pink; α-D-mannose, yellow; β-D-mannose, green.

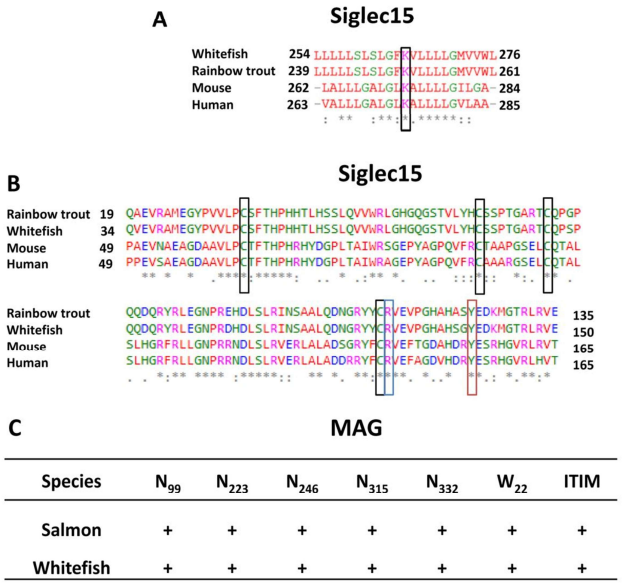
3.3.2. Sequence Comparison of Siglec15

Siglec15 belongs to the activating receptors, interacting with DAP10/12 via a lysine residue in the transmembrane domain [17]. This residue is well-conserved from fish to mammals (Figure 5A). The exchange of lysine by alanine has been demonstrated to abrogate the interaction of Siglec15 with DAP10 or DAP12; this confirms the functional importance of the lysine residue. However, this exchange might not impact the minimal FcRγ interaction with Siglec15 [17].

Furthermore, sequence alignments of the first Ig domain were performed. Angata and colleagues showed that an exchange of R_{143} to alanine results in the loss of sialic acid-binding and thus, in a loss of the immune regulatory function of Siglec15 [17]. This amino acid is conserved from mammals to lower vertebrates (Figure 5B). In addition, cysteine residues in the V-set, Ig-like domain are highly conserved across vertebrates. One likely reason for this remarkable conservation is pathogen-driven selection pressure. Our sequence alignments of the first Ig domain of the Siglec15 orthologs suggested that the well-conserved cysteine residues that contribute to the tertiary structure by forming disulfide bonds are present in the Siglec15 of the investigated salmonid species (Figure 5B). Nevertheless, the cloned ortholog from zebrafish did not show strong binding to the tested glycans ex vivo, irrespective of the presence or absence of cysteine residues [17].

3.3.3. Sequence Comparison of Siglec4 (MAG)

In mammals, MAG is involved in myelination processes through interactions with gangliosides. The expression of *MAG* is restricted to Schwann cells and oligodendrocytes. The dimerization of MAG is essential for specific axon-myelin spacing (9–12 nm) and strongly depends on the glycosylation pattern of MAG [15,35,54,55]. Therefore, we also inspected potential N-glycosylation sites of the MAG sequences from maraena whitefish and Atlantic salmon. Eight sites of N-glycosylation are known in human MAG (N_{99}, N_{223}, N_{246}, N_{315}, N_{332}, N_{406}, N_{450}, and N_{454}). The residue W_{22} is targeted by *C*-mannosylation and conserved from fish to humans (W_{21} in fish). Therefore, it is likely that this residue contributes to the functionality of MAG [31]. Our sequence alignment showed that the N-glycosylation sites of the three Ig-domains of the MAG were conserved together with residue W_{22} of the murine MAG sequences (Figure 5C and Figure S2).



Species	N_{99}	N_{223}	N_{246}	N_{315}	N_{332}	W_{22}	ITIM
Salmon	+	+	+	+	+	+	+
Whitefish	+	+	+	+	+	+	+

Figure 5. Analysis of specific properties of Siglec15 and MAG. (**A**) Sequence alignment of the transmembrane domain of Siglec15. The positions of the transmembrane domains of murine and human Siglec15 were retrieved from Uniprot, while the transmembrane area of Siglec15 from maraena whitefish and rainbow trout were predicted using SMART. The black box highlights the transmembrane lysine residue, which interacts with DAP10/12. (**B**) Sequence alignments of the first Ig domain of Siglec15. The alignment illustrates the conserved cysteine residues (black boxes) of Siglec15 as well as the conserved arginine residue (blue box) and a conserved hydrophobic amino acid (Y, red box), which are involved in sialic acid-binding [17]. Sequence alignments were performed using the Clustal Omega tool of EMBL-EBI. Different colors label the chemical properties of the amino acids: red, small hydrophobic/aromatic amino acids; blue, acidic amino acids; magenta, basic amino acids; green, hydrophilic, polar, and small amino acids. (**C**) The presence of specific N-glycosylation sites and IIM motifs of MAG from salmon and whitefish are shown. The position of the amino acid residue refers to the human sequence. For accession numbers, see Section 2.7.

In addition, these MAG sequences harbor immune-regulatory ITIMs in their intracellular sections, whereas their mammalian counterparts lack these motifs. In 2004, Lehmann et al. detected a proximal ITIM motif in the MAG orthologs of zebrafish and pufferfish [16]. Lehmann et al. suggested that these motifs are involved in signal transduction and contribute to biological functions different from those described for mammalian MAG [16]. Furthermore, our previous sequence analysis [15] confirmed the presence of ITIMs in coelacanth, while almost all investigated higher vertebrates were ITIM-negative. Consequently, the ITIM motif seems to be an ancestral feature that has persisted in most Siglecs but was lost during the evolution of higher vertebrates [15,16]. The presence of ITIM motifs in the intracellular part of MAG in salmonid fish might contribute to inhibiting immunological responses, while mammalian MAGs merely stabilize axon-myelin interactions by binding to GD1a and GT1b, two gangliosides present in the brain. Unlike the MAG in mammals, the MAG in fish seems to be involved in immunomodulatory processes. To analyze its carbohydrate-binding pocket in more detail, the 3D structure of MAG from maraena whitefish was simulated and compared with its murine ortholog (Figure 6A–C).

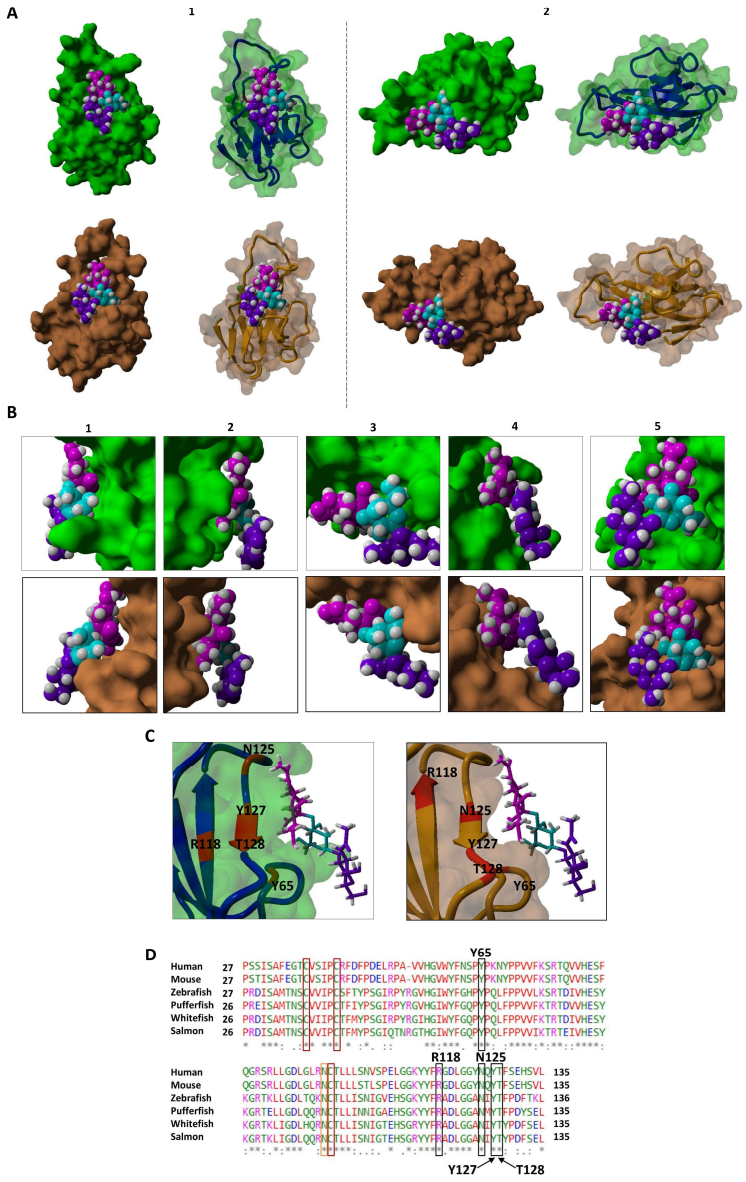


Figure 6. Sequence and structural comparison of MAG from mouse and maraena whitefish. (**A**) YASARA was used to model the 3D structure of the sialic-acid-binding domain (V-set, Ig-like domain) of MAG from mouse and maraena whitefish. Based on the sequence alignments performed, the sequence of the murine MAG was shortened to the first Ig domain. The sequence of the V-set Ig-like domain of MAG

from maraena whitefish was based on the performed alignments (see (**D**)). MAG models from mouse and maraena whitefish are labelled in green and brown, respectively. Two different perspectives of the surface with a transparency of 30% are shown along with the corresponding secondary structures. (**B**) Enlargement of the sialic-acid-binding domain of MAG from maraena whitefish in five different perspectives. (**C**) Amino acids responsible for sialic acid-binding by MAG from mouse and maraena whitefish. Surface and secondary structures are shown. Bound glycans are specifically labeled as follows: galactose, cyan; *N*-acetyl-D-galactosamine, purple; sialic acid, pink. (**D**) Sequences of MAG from zebrafish, pufferfish, human, and mouse were available in the NCBI database: zebrafish MAG: XP_021337068; pufferfish MAG: XP_011616490; murine MAG: XP_030098048; human MAG: AAB58805. The V-set, Ig-like domain of the mouse MAG was determined by analyzing the pdb sequence (pdb: 5LF5) with SMART. The V-set Ig-like domain of the human MAG was assessed using SMART. For zebrafish, pufferfish, maraena whitefish, and rainbow trout, V-set, Ig-like domains were detected using sequence alignment. The different colors indicate the chemical properties of the amino acids as follows: red, small hydrophobic/aromatic amino acids; blue, acidic amino acids; magenta, basic amino acids; green: hydrophilic, polar, and small amino acids. Black boxes show the amino acids that are essential for sialic acid-binding, red boxes show conserved cysteine residues [16] and the orange box indicates a glycosylation site.

Based on the crystal structure of murine MAG, the program determined that the secondary structure of MAG from maraena whitefish contains 37.6% beta sheets, 18.3% alpha-turn-helices, and 44.0% coiled coils, whereas that of murine MAG contains only 34.9% beta sheets, 22.0% alpha-turn-helices, and 43.1% coiled coils (Figure 6A). Although several amino acid residues differed between the MAG sequences from mouse and maraena whitefish, no significant changes were visible with regard to the sialic-acid-binding domain (Figure 6B,C). The binding pockets of MAG from mouse and maraena whitefish for Neu5Ac-α2,3-Gal-β1,3-GalNAc are highly comparable. The data suggest analogous functions of MAG in maraena whitefish and mammals. In addition, we aligned the sequences of the selected MAG orthologs to compare the presence of essential amino acids that mediate the protein-carbohydrate interaction. In human MAG, amino acids R_{118}, Y_{65}, N_{125}, T_{128}, and Y_{127} are responsible for sialic acid-binding [31]. These amino acids were conserved throughout evolution (Figure 6D), indicating once more [16] the preserved potential of MAG to bind to sialic acid across a range of vertebrate classes.

3.4. The Influence of Handling Stress on the Expression of Siglecs in Maraena Whitefish

Stress is known to affect immune processes [2]. To investigate the impact of stress on a panel of nine selected target genes related to the Siglec signaling, we exposed maraena whitefish to one-minute handling procedures (including chasing and exposure to air) and sampled the fish three hours after this treatment. Subsequently, we recorded the expression of the genes encoding the four Siglecs present in fish (*Siglec1*, *CD22*, *MAG*, and *Siglec15*), the associated non-receptor tyrosine kinases *LYN*, *SYK*, and *ZAP70* in addition to the non-receptor tyrosine phosphatase *PTPN6* (alias SHP1) and *PTPN11* (alias SHP2) (Figure 7A). Gene profiling revealed that the transcript levels of the four Siglecs were modulated in a tissue-specific fashion after exposure to stress (Figure 7B). *Siglec1* and *CD22* were 2.0- to 4.3-fold upregulated in telencephalon and hindbrain but substantially downregulated in the heart as well as the spleen (*Siglec1*) and muscle (*CD22*). All other tissues exhibited comparable values in untreated and stressed fish.

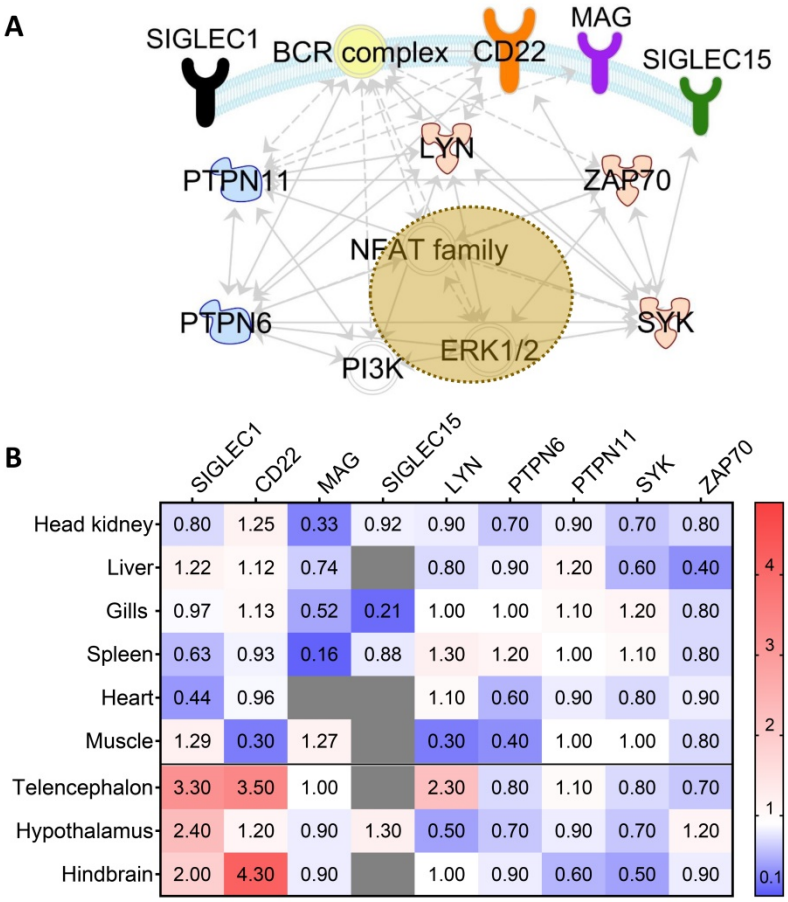


Figure 7. (**A**) Functional relationship between Siglec receptors and associated kinases (red symbols) and phosphatases (blue symbols) in a schematic B cell. Interactions are displayed by broken (indirect influence) or full (direct influence) lines. Cellular and nuclear membranes are colored in light blue and brown, respectively. Please note that these relationships are supported by at least one reference, which are based exclusively on investigations in mammalian species (accessible in the Ingenuity Knowledge Base). (**B**) Tissue-specific expression of genes encoding Siglecs (*Siglec1*, *CD22*, *MAG*, *Siglec15*) and downstream factors (*LYN*, *PTPN6*, *PTPN11*, *SYK*, *ZAP70*) in maraena whitefish exposed to three hours of handling stress. QPCR data were normalized by the reference genes *RPL9*, *EEF1A1b*, and *RPL32*. The heat map shows the averaged fold-change values in the respective tissue relative to the same tissue from unstressed fish, colored according to the code on the right. Non-detectable transcript numbers are indicated by gray fields.

Surprisingly, *MAG* expression was not influenced in the brain and was downregulated in nearly all other tissues. This effect was especially pronounced in both lymphoid organs spleen and HK. Since

MAG from maraena whitefish contains an ITIM, this data might indicate the immunomodulatory capacity of MAG in salmonid fish. Furthermore, few *MAG* transcripts were detected in gills of stressed maraena whitefish, and these fish also exhibited reduced *Siglec15*-transcript level in their gills. As the respiratory organs of fish, gills are directly exposed to significant environmental changes, including exposure to air, and are thus expected to induce fast responses.

The genes encoding the three Siglec-associated kinases (*LYN, SYK,* and *ZAP70*) and two phosphatases (*PTPN6* and *PTPN11*) were expressed at high levels (between ~2200 and ~71,500 copies/µg RNA) in HK, gills and spleen, but at low levels (<450 copies/µg RNA) in muscle, telencephalon, hypothalamus, and hindbrain. Handling stress did not affect the expression of the aforementioned enzyme genes, except for *LYN* in muscle (~4-fold downregulated), hypothalamus (~2-fold downregulated), and telencephalon (~2-fold upregulated), as well as *PTPN6* in muscle (~3-fold downregulated) (Figure 7B). Since we recorded these alterations in those tissues that had only relatively low basal concentrations of the respective transcripts, the observed expression data should not be overestimated. We rather assume that the stress-dependent regulation of the activity of Siglec-associated enzymes in fish does not occur at the transcript level. In contrast, the expression patterns of Siglec-encoding genes showed characteristic factor-specific alterations, both under homeostatic conditions and in response to handling stress. Although the biological significance of these changes remains unknown, the data obtained in the present study strongly suggests that the function of individual Siglecs has partially changed during the evolution of vertebrates.

4. Conclusions

The present study draws three main conclusions:

- Our qPCR analyses suggested that the basal gene-expression patterns of *Siglec1*, *CD22*, *MAG*, and *Siglec15* are largely conserved across salmonid and percid fishes. In contrast to mammals, *CD22* is highly expressed in several blood-cell populations. Similarly, the expression of *MAG* in fish is not restricted to the cells of the nervous system but is detectable in a range of blood cells.
- Stress modulates the expression of Siglecs (but not of the associated enzymes) in a tissue-dependent fashion and most likely influences the cellular reactivity against PAMPs and DAMPs.
- The genomes of fish lack CD33-related Siglecs, which exert inhibitory functions. Our structural analyses indicated that CD22 and MAG contain inhibitory motifs (ITIM) in salmonid fish. We speculate that these ITIM-containing Siglecs may compensate the deficiency of the canonical inhibitory Siglecs. This first assumption might be the starting point for subsequent studies to clarify whether CD22 and MAG have an immunosuppressive effect in fish.

Supplementary Materials: The following materials are available online at http://www.mdpi.com/2073-4409/9/4/836/s1: Figure S1: Sequence alignment of CD22 of rainbow trout (XM_021620093) and the obtained sequence of CD22 of maraena whitefish, Figure S2: Sequence alignment of MAG from salmon and the obtained sequence of MAG of maraena whitefish, Table S1: Primer sequences and accession codes.

Author Contributions: S.P.G., A.R., and U.G. designed the study. K.F.B. analyzed the sequences and generated the 3D models. J.M.R. and M.T.V. performed the qPCR analyses and calculated the data. J.M.R. and M.T.V. conducted stress experiments. T.V. performed flow cytometric cell sorting. K.F.B. wrote and S.P.G., A.R., and J.M.R. edited the manuscript. All authors have read and approved the final article. All authors have read and agreed to the published version of the manuscript.

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Sialylated Cervical Mucins Inhibit the Activation of Neutrophils to Form Neutrophil Extracellular Traps in Bovine *in vitro* Model

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In order to combat invading pathogens neutrophils can release neutrophil extracellular traps (NETs). However, since NETs can also damage endogenous cells, several control mechanisms for the formation of NETs must work effectively. For instance, neutrophil activation is silenced within blood circulation by the binding of sialylated glycoconjugates to sialic acid binding immunoglobulin-like lectins (Siglecs) on neutrophils. As neutrophils are recruited within the female reproductive tract, after mating, a comparable mechanism may also take place within the bovine cervix to prevent an exaggerated NET formation and thus, infertility. We examined, if the highly glycosylated mucins, which are the major functional fraction of biomolecules in mucus, represent a potential regulator of NET formation. The qPCR data revealed that in polymorphonuclear neutrophils (PMNs) inhibitory Siglecs are the most frequently expressed Siglecs and might be a potential target of sialylated glycans to modulate the activation of PMNs. Remarkably, the addition of bovine cervical mucins significantly inhibited the formation of NET, which had been induced in response to lipopolysaccharides (LPS) or a combination of phorbol myristate acetate (PMA) and ionomycin. The inhibitory effects were independent of the stage of estrous cycle (estrus, luteal, and follicular mucins). PMNs retained their segmented nuclei and membrane perforation was prevented. However, the inhibitory effects were diminished, when sialic acids were released under acidic conditions. Comparable results were achieved, when sialic acids were targeted by neuraminidase digestion, indicating a sialic acid dependent inhibition of NET release. Thus, bovine cervical mucins have an anti-inflammatory capability to modulate NET formation and might be further immunomodulatory biomolecules that support fertility.

Keywords: neutrophil extracellular traps (NET), mucins, sialic acids, reproduction, sialic acid binding immunoglobulin-like lectins (siglecs), bovine neutrophils

INTRODUCTION

When sperm enter the female reproductive tract an immune response is initiated and polymorphonuclear neutrophils (PMNs) are recruited (1, 2). There, several defense mechanisms are initiated such as phagocytosis (3). Furthermore, the formation of neutrophil extracellular traps (NETs) can be activated (1, 2). These molecular traps are mainly targeted against invading pathogens and their mechanism of action includes trapping and killing instruments (4–6). The rapidly released chromatin meshwork traps viruses, fungi, and bacteria. Since the DNA is loaded with high amounts of antimicrobial biomolecules, such as histones, lactoferrin, neutrophil elastases, and antimicrobial peptides, the trapped pathogens can be efficiently eliminated. However, several of these antimicrobial molecules are also toxic for endogenous cells. Extracellular histones play a predominant role in this context (7). Interestingly, an exaggerated formation of NET and its adverse effects were reported during several diseases (5, 8–11). Besides life-threatening diseases such as sepsis, thrombosis and acute lung failure, it is likely that NET formation can affect the reproductive system influencing infertility, preeclampsia, and fetal loss (2). Thus, throughout the body a tight control of neutrophil activation is required to regulate the NET response.

For instance, in circulation, sialylated glycans on erythrocytes seem to silence neutrophils in humans. The activation of neutrophils is suppressed immediately subsequent to the detection of these sialylated glycoconjugates by sialic acid binding immunoglobulin-like lectin-9 (Siglec-9) (12). Thus, in blood, sialic acid dependent mechanisms exist to prevent an excessive NET formation.

Possibly, similar to the circulatory system, the female reproductive tract also produces glycoconjugates to modulate the activation of neutrophils. The surface of the female reproductive tract is coated with large amounts of mucus that mainly consist of mucins (13–18). These are highly glycosylated glycoproteins and are essential to counteract infections, prevent dehydration, and both physical and chemical injury (13, 19). Besides secretory mucins, which are the main functional components in mucus, mucins can be anchored in cell membranes. Remarkably, the covalently linked *O*-glycans can typically comprise over 70% of the molecular mass. In addition to *O*-linked glycans, *N*-glycans are present on mucins. Both, *N*- and *O*-glycans are frequently terminated by sialic acid residues (20, 21) and may trigger the activation of inhibitory Siglecs on neutrophils. An inhibition of activation via Siglecs is possible, since inhibitory Siglecs contain intracellular tyrosine based inhibition motifs (ITIM), which mediate the inhibitory action. These immunomodulatory lectins are transmembrane receptors which are expressed in different immune cells, including PMNs, of vertebrates (22).

Similar to sialylated glyconjugates in circulation, sialylated cervical mucins may exert an inhibitory effect on the activation of PMNs in the female reproductive tract. To address this hypothesis, we isolated bovine cervical mucins and tested their capability to inhibit the activation of NET formation. Since the glycosylation of cervical mucins can change during estrous cycle (14, 16), mucins were collected from estrus, luteal, and follicular stage samples. In addition to the native form, mucins decorated with chemically modified sialic acid residues and de-sialylated mucins were employed allowing an assessment of the biological impact of these sugar residues on the activation of PMNs.

Abbreviations: DAPI, 4′,6-Diamidin-2-phenylindol; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; HRP, Horse-rabbit-peroxidase; ITIM, Immunoreceptor tyrosine-based inhibition motif; ITAM, Immunoreceptor tyrosine-based activation motif; Gal-6′-*S*′, 6-*S*′Galactose; 6′-S-sialyl Le^x, 6′sulfo-sialyl-lewis X; Gdn HCl, Guadinium hydrochloride; LPS, Lipopolysaccharides; GlcNAc, N-acetylgalactosamine; MAG, Myelin-associated glycoprotein; MAL II, Maackia Amurensis II; NETs, Neutrophil extracellular traps; PFA, Paraformaldehyde; PCR, Polymerase chain reaction; PenStrep, Penicillin/streptavidin; PMA, Phorbol myristate acetate; PBS, Phosphate buffered saline; PMNs, Polymorphonuclear neutrophils; RT-qPCR, Real-Time quantitative Polymerase Chain Reaction; PVDF, Polyvinylidine fluoride; Rt, Room temperature; ROS, Reactive oxygen species; SDS, Sodium dodecyl sulfate; Siglec, Sialic acid-binding immunoglobulin-like lectins; SNA, Sambucus Nigra Lectin; SSC, saline sodium citrate; TFA, Trifluoroacetic acid; TAE buffer, TRIS-acetate buffer.

MATERIALS AND METHODS

All reagents used were of analytical grade.

Isolation of Bovine PMNs

The isolation procedure for bovine PMNs was based on the protocol of Schuberth et al. (23). Minor changes, as described in the following, were implemented. For the isolation of bovine neutrophils, fresh ethylenediaminetetraacetic acid (EDTA) - blood samples of normally cycling Holstein cows (7 months up to 7 years of age, female animals) were diluted 1:1 with 1 × sterile phosphate buffered saline (PBS) and the diluted samples were transferred to a layer of histopaque 1,077 (Sigma Aldrich, St. Louis, Missouri, USA) with a volume equal to the amount of fresh blood used. Following this, the samples were centrifuged at 1,280 × g for 30 min at 10°C without break. Based on the density of the cells, lymphocytes and monocytes as well as some thrombocytes are localized in the interphase between the plasma and the histopaque, whereas the PMNs are co-localized with the erythrocytes. Therefore, in the next step, the supernatant was removed. Subsequently, a hypotonic lysis was performed. Ten milliliter of sterile water was added to 5 mL of blood sample for 10 s then 10 mL of 2 × PBS was added to re-generate isotonic conditions. After a further centrifugation step (500 × g, 10 min, 4°C), hypotonic lysis was repeated followed by centrifugation at 220 × g for 10 min at 4°C. Finally, the pellet was washed twice with 1 × PBS and the cells were resuspended and counted in RPMI 1640 (Thermo Fisher Scientific, Waltham, USA) with 1% penicillin/streptomycin (PenStrep, Thermo Fisher Scientific) without the addition of fetal bovine serum. PMNs were isolated from blood received from the slaughterhouse or from the remaining blood samples of regulatory blood collections from other projects (7221.3-1-010/16-1). Both the blood sampling and the slaughter processes were performed in accordance with the guidelines, applicable laws and provisions for ethical regulations.

Quantification of the Siglec-Expression

Real- Time quantitative Polymerase Chain Reaction (RT-qPCR) analyses were performed with the LightCycler 96 System (Roche, Basel, Switzerland) using the Sensi-FAST SYBR No-ROX Kit (Bioline, Luckenwalde, Germany). RNA was isolated by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) including digestion with RNase-free DNase I for 15 min. The concentration and the purity of the isolated RNA were then checked with the NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific). Agarose gel electrophoresis validated the presence of intact 18S and 28S rRNA bands without genomic DNA contamination. For cDNA synthesis, 100 ng of RNA was individually reverse-transcribed in a total volume of 100 μL (final concentration 1 ng/μL) using the Super Script II kit (Thermo Fisher Scientific). A total cDNA equivalent of 5 ng RNA was used for copy number analysis. The qPCR program included an initial denaturation (95°C, 5 min.), followed by 40 cycles of denaturation (95°C, 5 min.), annealing (60°C, 15 s) and elongation (72°C, 15 s) steps and the fluorescence measurement (72°C, 10 s). No-template controls and positive controls (containing PCR-generated fragments of the target genes) were included to monitor contaminations and the accuracy of the detection, respectively. The used oligonucleotide primers amplified gene-specific products with lengths between 74 and 194 bp (**Table 1**). The performance of the chosen primers was checked before qPCR measurements via standard PCR and the resulting PCR products were sequenced. In addition, a standard curve was applied ($R^2 > 0.9999$) and efficiency of the primers was verified. All primers utilized here had an efficiency score between 91.5 and 103.0%. The quality of the PCR products was assessed based on gel electrophoresis and melting-curve analysis. Light-Cycler data was analyzed using the LightCycler 96 analysis software v. 1.1; C_q values < 35 were considered detectable. GAPDH (24), YWHAH, and RPL19 (25) were used as reference genes. For Siglec-8, two different primer pairs were used. In addition, PCR products were partly sequenced in order to confirm Siglec-8 expression. Sample values were normalized by calculating the GeoMean of the reference genes. The numbers of the individual Siglec transcripts were calculated as a percentage relative to the summed transcript numbers of all measured Siglec transcripts. The outlined method has been previously described (26).

Flow Cytometry

In order to determine the purity of the isolated bovine PMNs, flow cytometry was performed. Therefore, an aliquot of the isolated cells was taken, diluted in sterile PBS and placed in the Gallios, Beckman Coulter instrument. Granularity was examined by measuring the side scattered light and the forward scattered light as described previously in detail (27, 28).

Isolation and Purification of Cervical Mucins

Bovine cervices were collected from animals sent to the abattoir for meat production. The stage of estrous cycle was determined by observation of gross ovary morphology (assessment of corpus luteum and follicle development), as well as cervix conformation and cervical mucus characteristics. Estrus samples were collected from reproductive tracts with clear mucus, a well-developed follicle as well as a regressing corpus luteum. Luteal samples were collected from tracts with ovaries showing a large and well-developed corpus luteum [stage 3 of luteal phase according to the classification of estrous stages by Ireland et al. (29)]. Follicular samples were collected from tracts showing no viscous mucus secretion and a well-developed follicle. Samples were stored on ice prior to mucus isolation. The first step of mucin purification was the repeated treatment of the tissue with 8 M guadinium hydrochloride (GdnCl) (Sigma Aldrich) in order to release the mucus over ~30 min. Then, after overnight rolling in at least an equal volume of GdnCl, Dithiothreitol (DTT) (final concentration 10 mM) was added and incubated for 5 h. Iodacetamide (final concentration 25 mM) was added and the samples were incubated overnight in the dark at room temperature (Rt). The isopycnic density gradient centrifugation was carried out in CsCl/GdnCl. The density of the samples was brought to 1.4 g/mL using solid CsCl and samples were placed in Beckman Ultra-clear tubes. Ultracentrifugation (Beckmann Coulter, Optima L-100 XP) took place at 65,000 rpm for 18 h at 10°C, using the 70 Ti rotor without break. Samples were then unpacked sequentially as 1 mL fractions and mucin containing samples were identified by taking 5 μL for blotting onto a polyvinylidine fluoride (PVDF) membrane using a Whatmann manifold Slotblot apparatus. The membrane was incubated in 1% periodic acid in 3% acetic acid for 30 min, washed twice with 0.1% Na metabisulphite in 1 mM HCl, then stained with Schiff's Reagent (VWR, Radnor, Pennsylvania, USA). 0.5 mL of each fraction was also weighed in order to calculate the density in g/ml. Samples rich in carbohydrate and above 1.35 g/mL were further pooled and loaded on a Sepharose CL-4B column (Sigma Aldrich) and eluted with 50 mM Tris/100 mM KCl, pH 7.5. Fractions of 4 mL were collected and those rich in carbohydrate were identified using Schiff's Reagent as before. Carbohydrate rich factions were pooled and freeze dried. In order to get rid of remaining buffer salts, the samples were dissolved in a small volume of water and loaded on a Biogel p6 column (Bio-Rad, Hercules, California, USA) for desalting. Fraction collection and mucin identification took place as described previously for the Sepharose CL4B column. As a final step samples were freeze dried and the quantity of purified mucins was determined by weighing. Purified mucins have a cobweb like appearance and are highly electrostatic. The purified samples of different cows were separately stored dry at −20°C in sealed cryotubes until required.

NET Induction

After counting, 19,500 cells/well were seeded in a 12-well silicone chamber slide (Ibidi, Gräfelfing, Germany) in a total volume of 130 μL RPMI 1640 (containing 1% PenStrep) and incubated for 1 h at 37°C and 5% CO_2 prior to NET induction. NET induction was performed by adding 1.5 μM phorbol myristate acetate (PMA) (Sigma Aldrich) in combination with 3 μM ionomycin (Cell Signaling, Danvers, Massachusetts, USA) for 4 h at 37°C and 5% CO_2. In order to investigate the potential of bovine cervical mucins to inhibit the release of NET, NET was induced and

TABLE 1 | The primer pairs used for qPCR.

Primer name (F: sense, R: anti-sense)	Sequence 5′→ 3′	NCBI accession code (nt. position)	Amplicon length (bp)
cattle_siglec_1_F	GTATGAAGGGGTCCTGCTTCGT	XM_025001079 (354-376)	194
cattle_siglec_1_R	AGCTCAGGATTCCCCACCAT	XM_025001079 (547-527)	
cattle_siglec_2_F	TGAGGTTGGAGCCTGTGAAG	XM_010814857 (4028-4048)	74
cattle_siglec_2_R	CTCTGGCCCAGACGGTTG	XM_010814857 (4101-4083)	
cattle_siglec_3_F	CATCTTCTCCTGGACGTCAGC	XM_015458139 (488-508)	183
cattle_siglec_3_R	CGTGAAGCATAGGTGACATTGAG	XM_015458139 (670-648)	
cattle_siglec_4_F	CGCTTCAGCTTCCCTGATGAG	NM_001040570 (224-245)	140
cattle_siglec_4_R	TGCGTCCCTGGAAGCTCTC	NM_001040570 (363-344)	
cattle_siglec_5_F	GGGACCCCAGCAACAATGACT	XM_005219563 (460-481)	154
cattle_siglec_5 R	CGGGTTTCTCTCCTGTCACCT	XM_005219563 (613-592)	
cattle_siglec_8(I)_F	ACGTGCCCTGCTCCTTCT	XM_024978778 (543-560)	172
cattle_siglec_8(I)_R	TCCCCGAGGAGATGGAATC	XM_024978778 (714-696)	
cattle_siglec_8(II)_F	GCGGTCCTGGTAGCCATCTG	GGVB01041119 (1038-1057)	87
cattle_siglec_8(II)_R	TGTGCAGCACCTCACTAGGAG	GGVB01041119 (1124-1104)	
cattle_siglec_10_F	AGAAGGCCTGTGCATGGTCGT	NM_001206277 (286-307)	186
cattle_siglec_10_R	CGCCAAGGAGCTGGAATCGG	NM_001206277 (471-451)	
cattle_siglec_14_F	TGGATCTACTACACCAGGTGTG	XM_019978755 (1242-1263)	114
cattle_siglec_14_R	TCTGTCCTCTGGGCTCTGCAT	XM_019978755 (1355-1335)	
cattle_siglec_15_F	ATCGCCTAGAGCACCCAGTCA	NM_001192567 (869-889)	93
cattle_siglec_15_R	CCCGAGGGCTCATCTGGTTC	NM_001192567 (961-942)	

different final concentrations of cervical mucins were applied: 1, 5, 10, 15 μg/μL as well as 20 μg/μL.

Further experiments were performed using hydrolyzed mucins, C7 modified mucins and neuraminidase digested mucins (final concentration each 20 μg/μL).

In addition to the combination of PMA and ionomycin, lipopolysaccharides (LPS) was used to induce NET formation. After counting 19,500 cells/well were seeded in a 12-well silicone chamber slide (Ibidi) in a total volume of 130 μL of RPMI 1640 medium supplemented with PenStrep and incubated for 1 h at 37°C and 5% CO_2. The formation of NET was induced with LPS from *Pseudomonas aeruginosa* (Sigma Aldrich) using a final concentration of 20 μg/mL and again incubation took place for 4 h at 37°C with 5% CO_2. In order to determine the potential of bovine cervical mucins to inhibit NET released induced by LPS, different final concentrations of cervical mucins were applied: 1, 5, 10, 15 μg/μL as well as 20 μg/μL.

To determine the pH of the medium, pH strips were used to monitor the pH throughout the experiments. No significant change of the pH was detectable over the duration, independent of the added substance (PMA, ionomycin, LPS, mucins).

Nuclei Staining

After 4 h of stimulation, cells were fixed with 4% paraformaldehyde (PFA) for 30 min at 4°C. After washing several times, cells were permeabilized for 1 min with 0.5% Triton-X-100 followed by further washing steps. Subsequently, nuclear staining was performed with 4′,6-Diamidin-2-phenylindol (DAPI) (Carl Roth, Karlsruhe, Germany, 1 μg/mL) before a further fixation step with 2% PFA for 20 min at Rt took place. Samples were then mounted and analyzed using fluorescence microscopy (Zeiss Axio Imager A1, Carl Zeiss). The determination of the percentage of activated cells was based on the release of de-condensed DNA fibers during NETosis (DAPI staining). During NETosis neutrophil elastase and other neutrophilic granule proteins translocate to the nucleus, de-condensation of chromatin is triggered and the DNA is decorated by several granular proteins. The resulting chromatin swelling directly leads to cell rounding and rupture of the cell and release of the meshwork of chromatin, which is associated with granule proteins such as lactoferrin and neutrophil elastase (30). In contrast, apoptotic cells condensate their chromatin, the cell and its nucleus shrink and cell membrane blebs are formed (31). During necrosis cells lose their membrane integrity and the resulting influx of water and ions leads to swelling of the cytoplasm, the nucleus and the cell organelles. Subsequently, cell lysis and an uncontrolled release of cellular content such as proteins, partially degraded DNA and granules takes place. In contrast to NETosis, no initiated decoration of chromatin with granule proteins occurs and thus, no long DNA filaments are commonly visible, which are highly associated with granule proteins. Taken together, all cellular death occurs with a loss of the segmented nucleus structure and can be distinguished by their characteristic morphological changes of chromatin (32).

Total cell number was evaluated and the number of segmented nuclei was determined. Analysis was carried out on multiple (random) pictures of different biological samples.

Immunofluorescence Staining

After 4 h of stimulation, cells were fixed with 4% PFA for 30 min at 4°C. After washing several times cells were permeabilized for 1 min with 0.5% Triton-X-100 followed by further washing

steps. Blocking was performed with 2% IgG-free bovine serum albumin (BSA) for 30 min at 37°C. Subsequently, the antibodies, diluted in the blocking buffer, were added [rabbit pAb to Neutrophil Elastase (1:200; Abcam, Cambridge, Great Britain), goat anti-bovine Lactoferrin (final concentration 5 μg/mL, Biomol, Hamburg, Germany)]. Incubation of anti-Neutrophil Elastase took place overnight at 4°C, whereas the incubation of anti-bovine Lactoferrin took place for 1 h at 37°C. After the incubation of the first antibody, samples were washed and the secondary antibody was added. In order to detect neutrophil elastase Alexa Fluor 488 goat anti-rabbit IgG (final concentration 2 μg/mL, Invitrogen, Carlsbad, California, USA) was used and to detect Lactoferrin Alexa Fluor 568 donkey anti-goat IgG (H+L) was applied (final concentration 2 μg/mL, Molecular Probes, Eugene, Oregon, USA). Incubation took place for 1 h at 37°C (Alexa Fluor 568) or for 1 h at Rt (Alexa Fluor 488). Afterwards a further washing step was performed and subsequently, nuclear staining was performed with DAPI (Carl Roth, 1 μg/mL) before a fixation with 2% PFA for 20 min at Rt took place. Samples were then mounted and analyzed using fluorescence microscopy (Zeiss Axio Imager A1, Carl Zeiss). For each stage of estrus cycle, neutrophils from three independent cell isolations (2 different animals) were used. Total cell number and cells positive for neutrophil elastase/lactoferrin were counted and the percentage of neutrophil elastase/lactoferrin positive cells was calculated.

Video-Fluorescence Microscopy

Neutrophils were isolated as described in section Isolation of bovine PMNs. Again, 19,500 cells were seeded in a 12-well silicone chamber slide (Ibidi) and preincubated for 1 h at 37°C and 5% CO_2. To detect DNA, neutrophils were incubated at 37°C for 20 min with Hoechst 33342 (final concentration 10 μg/mL, Sigma Aldrich) prior to a washing step with RPMI 1640. The medium was then exchanged once more and mucins (20 μg/μL) were added to the cells. Subsequently, cells were analyzed for 4 h using fluorescence microscopy (Carl Zeiss confocal laser scanning microscope LSM 800). Cells were monitored using a shooting interval of 1 picture/min.

Hydrolysis of Purified Bovine Cervical Mucins

Isolated cervical mucins were hydrolyzed with 1 N acetic acid for 30 min at 80°C and 350 rpm in a thermos-shaker. Hydrolyzed samples were dried via lyophilization overnight, resuspended and dialyzed in water with Spectra-Por® Float-A-Lyzer® (G2 blue, 1 mL, MWCO 100 kDa, Sigma Aldrich) in order to remove mono-sialic acid. Successful hydrolysis was verified by lectin staining (please see section Lectin staining).

C7 Modification of Bovine Cervical Mucins

The first step of the conversion of a 9-carbon sugar (sialic acid) to a C7-corpus was the oxidation via sodium-metaperiodate. Therefore, dried samples of isolated cervical mucins were resuspended in 50 μL of sodium acetate buffer (pH 5.5, 40 mM) and 4 μL of 0.25 M sodium-metaperiodate were added as described previously (33–35). Incubation, which took place for 3 h at 0°C without shaking was stopped by the addition of 10 μL of 3% ethylene-glycol (30 min, Rt). Subsequently, 64 μL of 0.2 N borohydride dissolved in 0.2 N sodium borate buffer pH 8.0 was added and incubated at 0°C overnight before samples were dried.

In order to determine the success of the oxidation of sialic acids with sodium-metaperiodate, sample aliquots were hydrolyzed with 0.2 N trifluoroacetic acid (TFA) for 4 h at 80°C. Dried samples as well as appropriate standards were then labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) under the following conditions: 40 μL DMB reagent [9 mM sodium hydrosulfite, 0.5 M β-mercaptoethanol, 20 mM TFA, and 1.35 M DMB (Dojindo, Kumamoto, Japan)] and 40 μL of MilliQ water was added and incubated for 2 h, 55°C, 350 rpm and the labeling was stopped by the addition of 20 μL 0.2 M NaOH (36, 37). The separation was performed with LiCroCart 250-2 Merck and a SuperSpher 100 reverse-phase- C18 column as described previously (38, 39).

Neuraminidase Treatment of Mucins

Samples were treated with an α-2,3;6;8 specific sialidase (New England Biolabs, Ipswich, Massachusetts, USA). The incubation of the α-2,3;6;8 neuraminidase (6 Units) in a final volume of 30 μL (1x Glyco buffer) took place for 5 min at 37°C. The reaction was stopped by heating the sample to 65°C for 10 min, followed by dialysis in water with Spectra-Por® Float-A-Lyzer® (G2 blue, 1 mL, MWCO 100 kDa, Sigma Aldrich) in order to remove mono-sialic acid.

Lectin Staining

The exoglycosidase treatment and the chemical release of sialic acid residues were assessed by Western blotting agarose gels. A 0.8% agarose gel was prepared in 40 mM Tris-Acetate, 1 mM EDTA (TAE, pH 8.0) containing 0.1% sodium dodecyl sulfate (SDS). Samples for electrophoresis were dried and diluted 1 to 10 in sample buffer (1x TAE-buffer, 50% glycerol, 0.25% bromophenol blue, 1% SDS), according to Ramsey et al. (40). The gel was run for 90 min at 80 V. Then the gel was washed briefly with water, prior to incubating the gel in 4 × saline sodium citrate (SSC: 0.6 M sodium chloride, 60 mM trisodium citrate, pH 7.0), containing DTT (final concentration 10 mM) for 20 min at Rt. After a further washing step with DTT free 4 × SSC, the gel was placed onto a VacuGene XL blotting apparatus (GE Healthcare, Dornstadt, Germany) and samples were blotted onto a PVDF membrane under vacuum (60 mbar, 2 h) following the manufacturer's instructions. For lectin staining, membrane was then rinsed with PBS and blocked with 3% milk in PBS for 1 h. Lectins [Biotinylated Maackia Amurensis II (MAL II, final concentration 1 μg/mL, Vector, Burlingame, California, USA); Biotinylated Sambucus Nigra Lectin (SNA, final concentration 1 μg/mL, Vector)] were added and incubated for 30 min at Rt. Then further washing steps were carried out prior to application of HRP-coupled streptavidin (final concentration 50 mU/mL diluted in PBS-T, Roche). Incubation took place for 40 min at Rt, followed by 3 further washing steps. For visualization enhanced chemiluminescence reagent (ECL solution: 1.27 mM Luminol, 0.6 mM p- Coumaric acid, 0.008%

H_2O_2) was used in combination with an imaging system (Bio-Rad). Experiments were performed in triplicate with freshly hydrolyzed or neuraminidase digested mucins.

CellToxGreen Assay

Isolated PMNs were seeded in black 96-well plates at a total cell number of 150,000 cells/well. Cells were stimulated with 1.5 μM PMA and 3 μM ionomycin for 4 h. In addition, estrus mucins were added (final concentration 20 μg/μL) and co-incubated with 1.5 μM PMA and 3 μM ionomycin. Subsequently, CellToxGreen (Promega, Madison, Wisconsin, USA) was added as described in the user's manual. This assay is based on a cyanine dye that is not absorbed by viable cells, but exhibits an enhanced fluorescence signal, when binding to DNA occurs in cells with loss of their membrane integrity. Measurement of increasing fluorescence intensity was performed for 4 h every 15 min at 485 nm for excitation and 520 nm for emission. The received values were corrected by subtracting blank values and $t = 0$ h values, in order to determine the increase of the fluorescence signal.

Sequence Alignments

Sequence alignments were performed using the EMBL EBI Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). Protein sequences used are available in the NCBI data bank (Accession numbers: human Siglec-8: XP_010813599.1; bovine Siglec-8: XP_010813599.1) The V-set Ig-like domain of human Siglec-8 was determined based on PDB 2n7b (https://www.ebi.ac.uk/pdbe/entry/pdb/2N7B). V-set Ig-like domain of bovine Siglec-8 was estimated by sequence alignment. The different colors indicate the properties of the amino acids. Red, small hydrophobic/aromatic amino acids; blue, acidic amino acids; magenta, basic amino acids; green: hydrophilic, polar and small amino acids.

3D Modeling

The modeling of the 3D structure of the binding-model between human as well as bovine Siglec-8 to 6′-S-sialyl-Lex was performed using UCSF Chimera 1.13.1. The structure of human Siglec-8 is based on that reported by Pröpster et al. (41) (PDB 2n7b). The structure of bovine Siglec-8 was generated using the Phyre[2] web portal for protein modeling (42). The sequence of the V-set Ig like domain of bovine Siglec-8 is based on the performed alignment. The components of the glycans are differently labeled: sialic acid (purple), fucose (yellow), galactose-6-P (cyan), N-acetylgalactosamine (magenta), 3-aminopropan-1-ol (green).

Statistical Analysis

Data sets were analyzed with Graph Pad Prism 7.0 software using paired ANOVA and a multiple-comparison Tukey test. The data have been initially evaluated by D'Agostino and Pearson test ($n \geq 8$) or Shapiro-Wilk test ($n < 8$) to calculate the distribution of the values. The calculated differences were considered statistically significant at $p \leq 0.05$. Statistically significant differences are given: ns, not significant; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

RESULTS

Activating and Inhibitory Siglecs Are Expressed in Bovine PMNs

As mucins are very heavily glycosylated glycoproteins, carrying numerous sialylated glycans, an inhibitory effect on NETosis, induced by the interaction of bovine cervical mucins with Siglecs, is likely. To address this hypothesis, we firstly analyzed the range of Siglecs in isolated bovine PMNs by RT-qPCR.

In order to evaluate the isolation strategy, the amount of granular containing cells was calculated by measuring the side scattered light and the forward scattered light using a FACS system. Based on the obtained values, ∼92% of the isolated cells show high internal granularity (**Supplementary Figures 1A,B**). In addition, neutrophil elastase and lactoferrin were visualized. Approximately 96 and 98% of the analyzed cells were lactoferrin and neutrophil elastase positive, respectively (**Supplementary Figures 1B,C**). Thus, it can be assumed that mainly PMNs were isolated. However, low numbers of other blood cells, such as eosinophils and lymphocytes, might be present.

The RT-qPCR analyses demonstrated that mRNA products for Siglec-2, -4, -5, -8, and -14 were detectable in the isolated cell populations (**Figure 1**). Since the highest copy numbers were detectable for Siglec-8 (**Figures 1B,C**) and in humans this inhibitory Siglec is only present on eosinophil/mast cells, but absent from neutrophils, we derived two different sets of primers. The primers pairs are specific for different regions of the bovine Siglec-8 cDNA sequence to verify the qPCR results. In addition, the resulting PCR products were sequenced. All results confirmed Siglec-8, as the Siglec with the highest copy number in the isolated cell populations (**Figure 1B**). The second largest Siglec fraction was Siglec-4, also known as myelin-associated glycoprotein (MAG) (**Figures 1B,C**). Finally, transcripts were detected for Siglec-5 and Siglec-2, which function as inhibitory receptors.

Siglec-8 has not only the highest copy numbers but also the highest circumscribed ligand preference of the detected Siglecs [based on human Siglec data (43, 44)]. In contrast to hSiglec-8, which prevalently recognizes a very special glycan motif, namely 6′-sulfo-sialyl-Lewis X (6′-S-sialyl-Le^{x}), Siglec-2, -4, -5, and -14 can bind several sialylated motifs, which are frequently present on numerous sialylated glycans (44). For this reason, the glycan-binding domain of hSiglec-8 was compared with the presumed binding domain of bovine Siglec-8 (**Figure 2**). For human Siglec-8 the selective binding mechanism for the sulfated galactose residue of the 6′-S-sialyl-Le^{x} motif is well-known (41). The amino acids R56 and Q59 stabilize the binding with the negatively charged sulfate group of the 6′-S-galactose (6′-S-Gal) residue. In addition, Y58 interacts with the galactopyranose ring. The overlay of the human and the calculated bovine 3D structure suggests that the binding area for 6′-S-Gal is missing in bovine Siglec-8 (**Figure 2B**). A sequence alignment of the glycan-binding domain of hSiglec8 (pdb: 2N7B) and the presumed binding domain of bovine Siglec-8 (XP_010813599.1) confirmed the calculated model. The amino acid R56 is replaced by A, Y58 is replaced by S and Q59 is replaced by K. Consequently, all amino

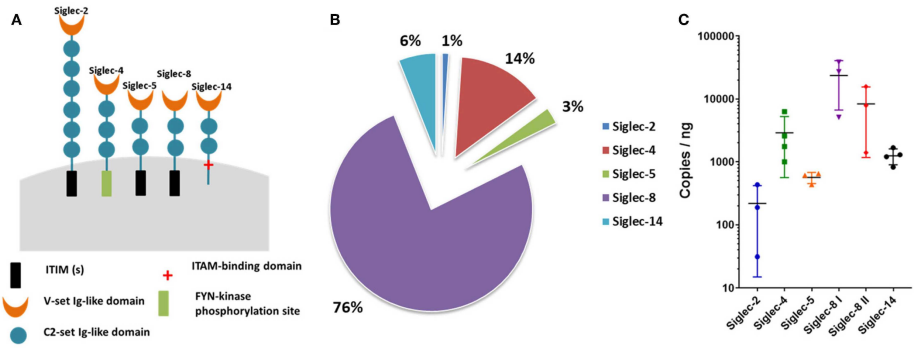

FIGURE 1 | Siglecs expressed in bovine neutrophils. **(A)** Cartoon of the Siglecs expressed by bovine neutrophils and their functional intracellular domains. The number of C2-set Ig-like domains was determined by SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) based on the following NCBI sequences: Siglec-2: XP_024834447.1; Siglec-4: XP_0248341235.1; Siglec-5: XP_005219620.2; Siglec-8: XP_010813599.1; Siglec-14: XP_019834314.1. **(B)** Pie chart of the percentage Siglec expression in bovine neutrophils. qPCR raw data was normalized against the reference genes. Siglec-2, -4, -5, -8, and -14 were detected by RT-qPCR and the total of the detected Siglecs was set to 100%. **(C)** Determined copies of Siglecs per ng RNA used during qPCR. X-axis shows the Siglecs expressed by bovine neutrophils; Y-axis shows the log 10 values. Mean values and standard deviations are displayed in the diagrams (Siglec-2, Siglec-5, Siglec-8 I, Siglec-8 II, $n = 3$ different animals; Siglec-4, Siglec-14, $n = 4$ different animals). For Siglec-8, values generated with two different primer pairs are displayed.

acids, which are essential for the circumscribed ligand preference to 6′-S-sialyl-Le^x, are absent in bovine Siglec-8 (**Figure 3**). In contrast, most amino acids responsible for sialic acid binding are highly conserved in human and bovine Siglec-8. Thus, remarkable structural differences exist mainly in the area that is essential to interact with the circumscribed 6′-S-Gal residue.

In summary, the results demonstrated that prevalently inhibitory Siglecs were expressed in bovine isolated cell population and, as determined by copy numbers, Siglec-8 was the most highly expressed.

Cervical Mucins Inhibit PMA/Ionomycin and LPS Induced NET Formation

Since the qPCR data revealed that mainly inhibitory Siglecs are expressed in bovine neutrophils, we were interested in the potential of sialylated cervical mucins to inhibit NET release. To this end, we stimulated bovine neutrophils in the presence and absence of cervical mucins. The mucins were applied with a final concentration of 20 μg/μL, since mucins are mainly present in concentrations between 10 and 50 μg/μL in mucus (45). As shown in **Figure 4,** unstimulated neutrophils exhibit a characteristic segmented nucleus. When neutrophils are stimulated with a combination of 1.5 μM PMA and 3 μM ionomycin nearly all nuclei lose their segmented structure and NET is released (**Figure 4** and **Supplementary Figure 2**). In addition to DNA, neutrophil elastase and lactoferrin were visualized by immunofluorescence staining. Both proteins are located in selected granules of PMNs and during NETosis the content of these granules are combined with the meshwork of DNA (5, 6, 46–48). The obtained results demonstrate that the released chromatin is decorated with neutrophil elastase and lactoferrin (**Figure 4**). However, when cervical mucins from different phases of the estrous cycle were applied, the activation of neutrophils seems to be inhibited (**Figures 4A,B**). The nuclei still show segmented structure similar to unstimulated neutrophils and the immunostaining of lactoferrin and neutrophil elastase exhibited the maintenance of the internal granularity. In addition to stimulated PMNs, cervical mucins were added to un-stimulated cells and no alterations were detectable (**Supplementary Figure 3**, **Video**).

Since NETosis comes along with membrane rupture, we further analyzed membrane integrity. Samples of estrus mucin were applied to stimulated neutrophils and membrane integrity was determined using CellToxGreen Assay. After 4 h of neutrophil stimulation with PMA/ionomycin, the fluorescence intensity increased, due to the initiated NETosis (**Supplementary Figure 4**). However, when cervical estrus mucins were applied, the fluorescence signal remained unchanged indicating that membrane integrity is maintained. Comparable fluorescence intensities to those of unstimulated neutrophils were achieved. In order to test the efficiency of mucins to inhibit the activation of PMNs by PMA/ionomycin, different concentrations of cervical mucins were applied. Whereas, 1 μg/μL mucin or 5 μg/μL mucin showed no significant effects on NET formation induced by the combination of PMA and ionomycin, the addition of 10 μg/μL mucin reduced the release of NET (**Supplementary Figure 5**) by ∼40%. Thus, with increasing concentrations of mucins, fewer numbers of PMNs were activated demonstrating a concentration dependent mechanism.

In addition to the combination of PMA and ionomycin, 20 μg/mL LPS was used as NET inducer. According to

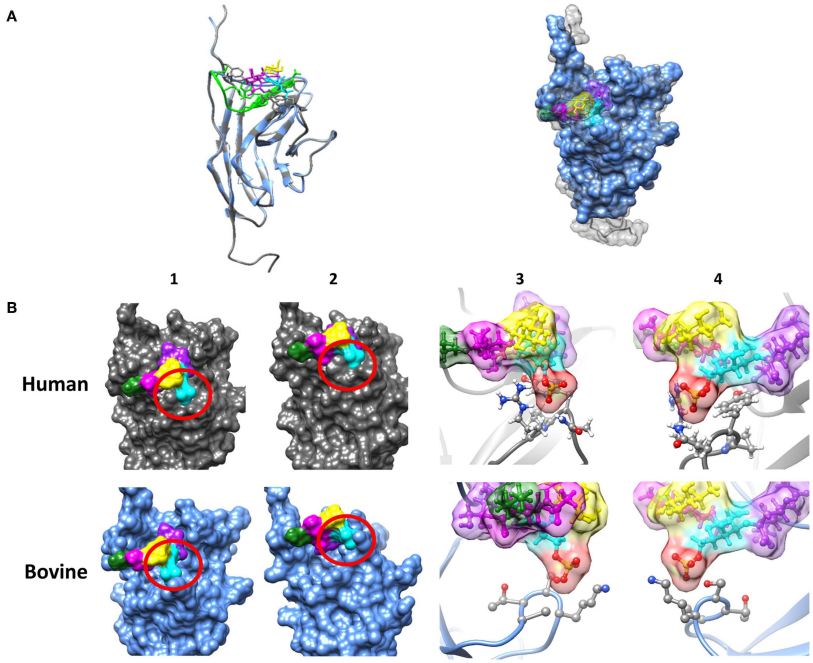

FIGURE 2 | Investigation of the sialic acid binding properties of human and bovine Siglec-8. **(A)** Overlay of the tertiary structure of hSiglec-8 (gray) and bovine Siglec-8 (blue). The components of the glycan are differently labeled: sialic acid (purple), fucose (yellow), galactose-6-P (cyan), N-acetylgalactosamine (magenta), 3-aminopropan-1-ol (green). **(B)** Differences in the tertiary structure of hSiglec-8 (gray) and bovine Siglec-8 (blue). Different views of the glycan-binding domain are shown (1–4). (3,4) 6-P is labeled in red. All figures were created by Chimera 1.13.1. Red circle demonstrates potential differences in glycan-binding properties. The structure of hSiglec-8 is based on Pröpster et al. (41) and the structure of bovine Siglec-8 was designed using Phyre^2.

Pieterse and colleagues LPS from *Pseudomonas aeruginosa* is a potent stimulator for NETosis (49). Comparable to the PMA/ionomycin stimulation, neutrophils undergo NETosis in the presence of LPS (**Figure 5A**). Again, the DNA filaments are highly decorated with the granular proteins neutrophil elastase and lactoferrin. When cervical mucins are present during stimulation, the effects of LPS was abolished (**Figure 5B**). Independent of the stage of estrous cycle NETosis was prevented by mucins and neutrophils retained their segmented nuclei (**Figure 5C**). However, in contrast to PMA/ionomycin, mucins at concentrations of 1 μg/μL completely inhibited LPS induced NETosis (**Supplementary Figure 6**).

The Inhibition of NET Formation Depends on the Sialylation Status of Cervical Mucins

To investigate, whether sialic acids are involved in the observed inhibition of NET formation, we first released sialic acid residues from bovine cervical mucins using acidic conditions. The released sialic acid residues were removed by dialysis. The mild hydrolysis led to a significant decrease of the sialylation status, as verified by lectin staining of agarose gels (**Supplementary Figure 7**). When we applied the acidic treated estrus mucins, no inhibitory activity against the stimulation of neutrophils was observed and NET formation occurred (**Figure 6A** and **Supplementary Figure 8**). Similar results were achieved when desialylated follicular mucins were used (**Figure 6B** and **Supplementary Figure 9**). However, unlike follicular and estrus mucins, where released NET fibers were visible after desialylation, the reduced sialylation of luteal mucins lead to an activation of neutrophils and a loss of the segmented nuclei structure but NET release was prevented (**Figure 6C** and **Supplementary Figure 10**).

Since the hydrolytic conditions may also result in additional alteration of the mucin structure, we mildly oxidized the terminal sialic acid residues. The oxidation of sialic acids with sodium periodate is a well-established method that specifically modifies

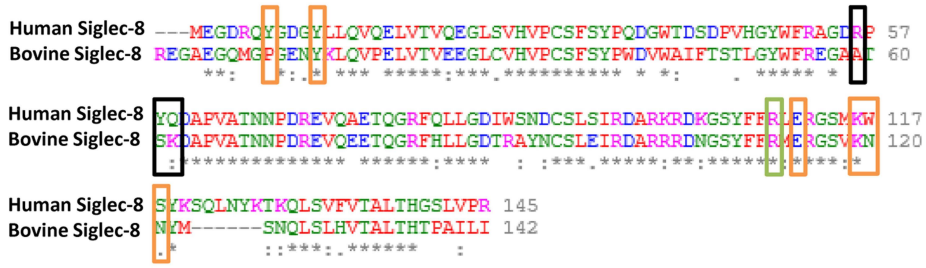

FIGURE 3 | Comparison of the sialic acid binding domain between human and bovine Siglec-8. Sequence alignment of the V-set domain of hSiglec-8 (pdb: 2N7B) and the predicted sialic acid binding domain of bovine Siglec-8 (NCBI: XP_010813599.1). Sequence alignments were performed using the Clustal Omega tool of EMBL-EBI. The different colors indicate the properties of the amino acids. Red, small hydrophobic/aromatic amino acids; blue, acidic amino acids; magenta, basic amino acids; green, hydrophilic, polar and small amino acids. The boxes indicate amino acids, which are mainly involved in binding of the 6′-S-sialyl-Lex motif based on Pröpster et al. (41): black boxes indicate amino acids required for the binding of galactose-6-S. Orange boxes indicate amino acids mainly involved in Neu5Ac binding. In addition, the highly conserved Arginine (R), that is essential for sialic acid binding, is boxed in green.

the exocyclic side chain of unmodified terminal sialic acid residues. During oxidation, C9 and C8 are released resulting in a C7-backbone. Subsequently, the resulting aldehyde group is reduced and a hydroxyl group is formed at C7 of the terminal sialic acid residues (34). This chemical reaction was confirmed by HPLC (**Supplementary Figure 11**). The conversion of the C9-carbon sugar into a C7-carbon sugar, partially inhibited the observed effects of estrus and luteal mucins (**Figure 6** and **Supplementary Figures 8–10**). In contrast to oxidized luteal and estrus mucins, the C9/C7 conversion of follicular mucins has no effect (**Figure 6C** and **Supplementary Figure 9**). Oxidized and native follicular mucins showed comparable effects.

In addition, neuraminidase was employed to enzymatically release sialic acid residues. After digestion, neuraminidase was heat-inactivated and released sialic acids were removed by dialysis. The enzymatic treatment completely eliminated the previously detected inhibitory effects of estrus and luteal mucins (**Figures 6A,C**). However, in the case of follicular mucins ∼50% of the neutrophils were still inactive (**Figure 6B**). This might be the consequences of a poor detachment of release of sialic acid by neuraminidases. The staining with SNA demonstrated that the detachment was not efficient for α2,6-linked sialic acid in the case of follicular mucins (**Supplementary Figure 12**).

In summary, the results demonstrate that cervical mucins from different phases of the estrous cycle have the capability to suppress the activation of neutrophils to form NET in a sialic acid dependent manner.

DISCUSSION

The route for sperm to reach the oviduct is challenging. They have to evade areas with low pH, to pass through highly viscous areas and to navigate or circumvent the female immune system (50, 51). After mating, neutrophils are recruited in cervical, uterine and fallopian tube tissue to protect the female reproductive tract against bacterial contamination (52). In mated Fresian cows, for instance, 6.4×10^5 PMNs were counted in the lumen of the cervix (52). The polymorphonuclear cells do not just target pathogens present in semen but also spermatozoa (3, 50). Most bacteria and spermatozoa are eliminated via phagocytosis and probably degranulation of antimicrobial peptides/proteins in addition to the release of reactive oxygen species (ROS) additionally damage spermatozoa. For example, it is known that ROS can influence the motility and the survival of sperms (53–55). However, besides these mechanisms, the formation of extracellular traps appear to play a role in reproductive immunology and the tight control of PMN response is critical to normal fertility (2). The mechanisms of PMN regulation that control pathogen phagocytosis or NET entrapment are poorly understood (56). The same applies for the possibility of phagocytosis triggered NETosis. Interestingly, ROS is a key player during phagocytosis (57, 58) and NETosis (6). However, during phagocytosis ROS is mainly produced in the phagosomal lumen, whereas during NETosis cytosolic ROS production is induced (56). Remarkably, PMNs, which have phagocytosed apoptotic cells, seems to be unable to form NET (59). However, it is unknown, if phagocytosed sperm induce similar effects inhibiting an exaggerated NET formation to prevent a blockade of the cervical canal by NET and thus, to ensure a transit of spermatozoa through this duct system.

While this would be one possibility to silence the induction of NETosis, we hypothesized that sialylated cervical mucins are further modulators to influence the activation of neutrophils, since sialylated glycans on endogenous cells as well as pathogens have been shown to inhibit the formation of NET (12, 60–62). In the cervix, neutrophils and sperm have to penetrate the viscous mucus that consists mostly of mucins, glycoproteins that are frequently decorated with sialic acid residues. For this reason, we have investigated the inhibitory capabilities of bovine cervical mucins to modulate the activation of neutrophils.

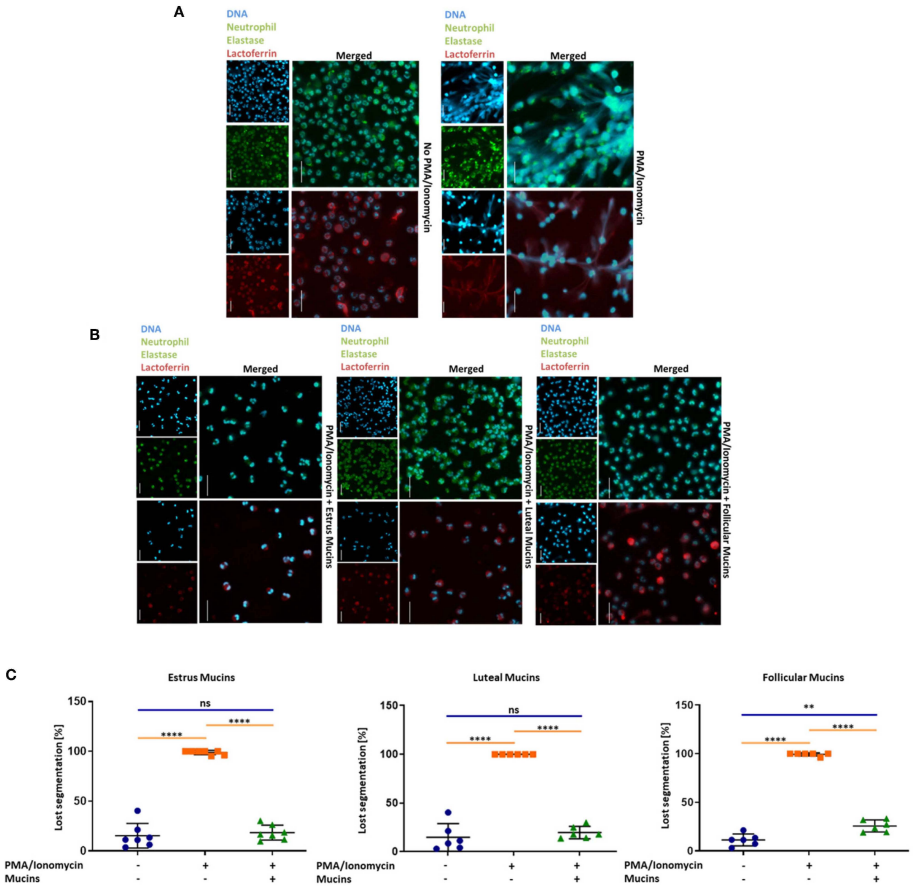

FIGURE 4 | Twenty microgram/microliter cervical mucins prevent NET release induced by a combination of 1.5 μM PMA and 3 μM ionomycin. **(A)** DNA fluorescence staining (DAPI) and staining of neutrophil elastase (Green) and lactoferrin (Red) of bovine neutrophils without any stimulation and stimulated with PMA/ionomycin. The term "merged" indicates the overlay of the nuclei staining with the staining of neutrophil elastase or lactoferrin, respectively. Scale bars: 20 μm. **(B)** DNA fluorescence staining and neutrophil elastase (Green) and lactoferrin (Red) of bovine neutrophils stimulated with PMA/ionomycin co-incubated with 20 μg/μL bovine cervical mucins from different stages of the estrus cycle. The term "merged" indicates the overlay of the nuclei staining with the staining of neutrophil elastase or lactoferrin, respectively. Scale bars: 20 μm. **(C)** The percentage of activated cells after 4 h of stimulation, without any stimulation, stimulated with PMA/ionomycin and stimulated with PMA/ionomycin co-incubated with 20 μg/μL bovine cervical mucins from different stages of the estrous cycle. Total cell number and cells with remaining segmented nuclei were counted and the percentage of activated neutrophils was calculated. Mean values and standard deviations are displayed in the diagrams (estrus mucins $n = 7$ different animals; luteal and follicular mucins $n = 6$ different animals). Paired ANOVA and a multiple-comparison Tukey test were applied. Statistically significant differences are given: ns, not significant; $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$. For higher magnification of the stained neutrophils please see **Supplementary Figure 2**.

In a first set of experiments, bovine PMNs were isolated and tested for the expression of Siglecs. Surprisingly, an inhibitory Siglec, that was assigned as Siglec-8 in cattle, is mainly expressed in the isolated cell fractions. In humans, Siglec-8 is assumed to be a mast cell/eosinophil specific receptor preferring the binding of 6′-S-sialyl-Le^x (41, 44, 63, 64). Sequence alignments and structure comparison between the glycan-binding domain of hSiglec-8 and the assumed binding domain of bovine Siglec-8

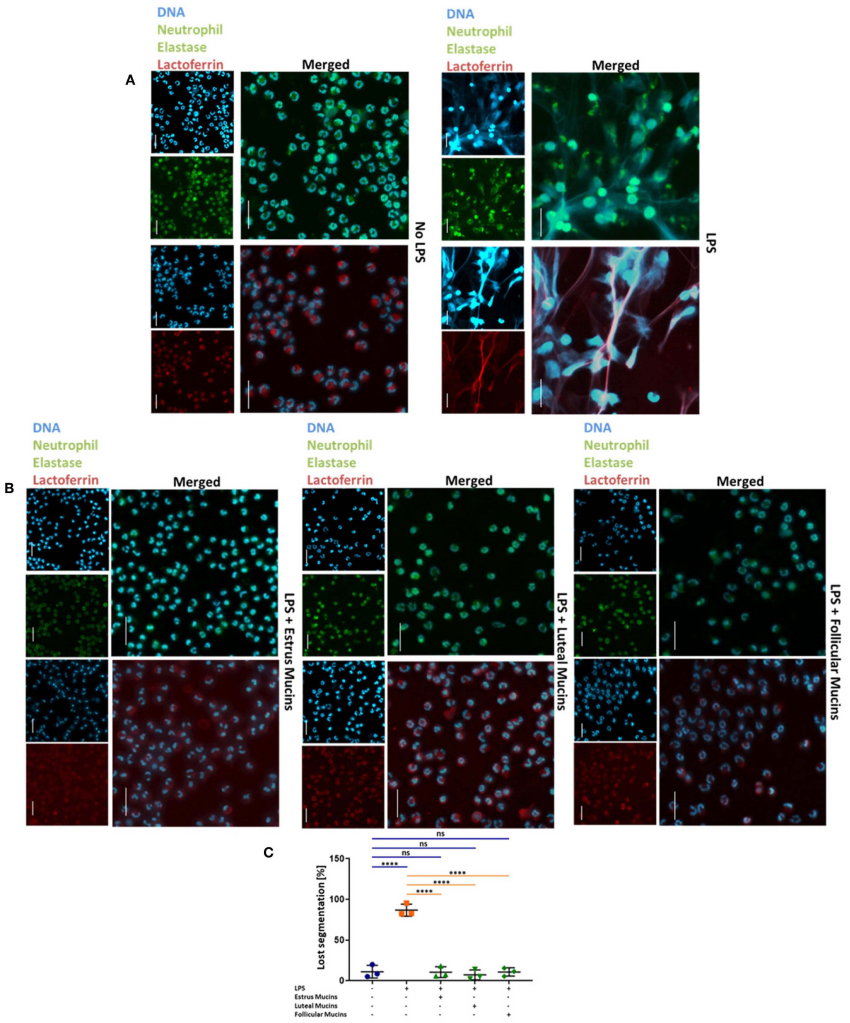

FIGURE 5 | Cervical mucins prevent NET release induced by 20 μg/mL LPS. **(A)** DNA fluorescence staining (DAPI) and staining of neutrophil elastase (Green) and lactoferrin (Red) of bovine neutrophils without any stimulation and stimulated with LPS from *pseudomonas aeruginosa*. The term "merged" indicates the overlay of the nuclei staining with the staining of neutrophil elastase or lactoferrin, respectively. Scale bars: 20 μm. **(B)** DNA fluorescence staining (DAPI) and staining of neutrophil elastase (Green) and lactoferrin (Red) of bovine neutrophils stimulated with 20 μg/mL LPS co-incubated with 20 μg/μL bovine cervical mucins. The term "merged" indicates the overlay of the nuclei staining with the staining of neutrophil elastase or lactoferrin, respectively. Scale bars: 20 μm. **(C)** The percentage of activated cells after 4 h of stimulation, without any stimulation, stimulated with LPS and stimulated with LPS co-incubated with 20 μg/μL bovine cervical mucins from different stages of the estrus cycle. Total cell number and cells with remaining segmented nuclei were counted and the percentage of activated neutrophils was calculated. Mean values and standard deviations are displayed in the diagrams ($n = 3$ independent isolations from 2 different animals). Paired ANOVA and a multiple-comparison Tukey test were applied. Statistically significant differences are given: ns, not significant; $^{****}p \leq 0.0001$. For higher magnification of the stained neutrophils please see **Supplementary Figure 2**.

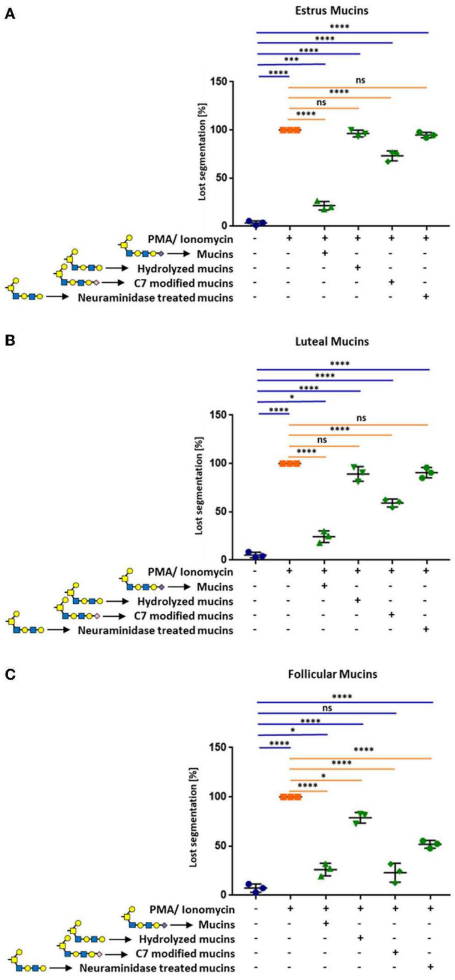

FIGURE 6 | Cervical mucins inhibit the release of NETs induced by 1.5 μM PMA in combination with 3 μM ionomycin by sialic acid on its surface. The experiments were performed with mucins from **(A)** estrus, **(B)** luteal, and **(C)** follicular samples. Untreated mucins, as well as hydrolyzed, C7 modified and neuraminidase treated mucins were applied to stimulated neutrophils in a final concentration of 20 μg/μL. The percentage of activated cells was calculated by counting the segmented nuclei as well as the total cell number. Mean values and standard deviations are displayed in the diagrams ($n = 3$ different animals). Paired ANOVA and a multiple-comparison Tukey test were applied. Statistically significant differences are given: ns, not significant, $^{*}p \leq 0.05$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$. Glycan illustration: Yellow square:

(Continued)

FIGURE 6 | N-acetylgalactosamine, Blue square: N-acetylglucosamine, Yellow circle: Galactose, Purple diamond: Sialic acid, rose diamond: C7 modified sialic acid.

exhibited structural differences suggesting that these changes are associated with alterations in glycan-binding properties and a loss of its binding preference for the 6′-S-Gal motif. Besides Siglec-8, Siglec-4 represents a further Siglec, which is commonly not expressed in neutrophils in humans. In contrast to Siglec-8, Siglec-4 does not contain ITIMs or ITIM-like sequences but a FYN kinase phosphorylation site in cattle (65, 66) and is predominantly known to be expressed in Schwann cells (67), as well as oligodendrocytes, stabilizing myelin-axon interactions (43). In contrast to the low copy numbers of the B-cell marker Siglec-2, which might be a contamination from lymphocytes, it is unlikely that the high mRNA values for Siglec-4 and Siglec-8 are from contaminating cells. More than 95% of the isolated cells show the characteristic lactoferrin and neutrophils elastase staining of PMNs, whereby neutrophil elastase is a marker for azurophilic granules in PMNs, lactoferrin is stored in specific granules (46, 47). However, it is impossible to fully exclude this possibility. Nevertheless, it is clear that prevalently inhibitory Siglecs were expressed in the isolated cell fractions.

To investigate the potential of cervical mucins to inhibit NET release, cervical mucins of different phases of the estrous cycle were isolated and applied to neutrophils, which were stimulated with LPS or PMA/ionomycin. The obtained results showed that not only estrus but also luteal and follicular mucins efficiently inhibited the formation of NET independent of the applied stimulus for NETosis. Remarkably, the chemical modification and release of the sialic acid residues on cervical mucins decrease the inhibitory capability. Therefore, a Siglec-dependent inhibition of NET release is more likely.

However, cervical mucins are not the only known immunomodulatory biomolecules in the female reproductive tract after mating. If NET is formed, DNases in the seminal plasma are able to degrade the released DNA framework and therefore, protect sperm from entrapment (1). Interestingly, it seems that a loss of DNase activity is associated with subfertility in the stallion (1). Nevertheless, DNases can only protect spermatozoa within a limited radius from the site of ejaculate deposition (the vagina). The same applies for sialic acid polymers in ejaculates, which can neutralize in a chain length dependent manner the cytotoxic characteristic of histones, without a neutralization of the antimicrobial capacity of lysine-rich histones (68, 69). Even though, few spermatozoa are polysialic acid positive (70), most of these protective sugar units against NET will get lost during the cervical transit. However, during the passage through the cervix, sperm penetrates a dense meshwork of sialylated mucins secreted by the cervical epithelium. Thus, as they come in contact with neutrophils in this area, we propose that mucin-associated sialic acid residues prevent an exaggerated NET formation. Similar to sialylated structures on circulatory erythrocytes, cervical mucins may counteract in a sialic acid dependent manner an over-active

neutrophil NET response so that more sperms can continue their journey toward the site of fertilization.

In addition to a possible regulatory effect of sialic acid residues on mucins, sialic acid residues are also evident on the spermatozoa glycocalyx. These play a role in protection against phagocytosis and sperm desialylation results in a loss of these protective effects (12). Thus, two powerful killing mechanisms of neutrophils (phagocytosis and NET) might be modulated in a sialic acid dependent manner by both the spermatozoan itself as well as cervical mucins to ensure the survival of sufficient numbers of spermatozoa in the female reproductive tract for the fertilization of the ovum.

In sum, the results demonstrated that cervical mucins inhibit the formation of NET. The inhibitory capacity strongly depends on the sialylation status of the mucins demonstrating once more the central role of these acidic sugar residues in reproductive biology (71–74). Since mucins are generated by epithelial cells throughout the body, comparable mucin dependent mechanisms may also play a role in other organs, such as the lung or the gastrointestinal tract. Thus, mucins as a natural and biodegradable product might be a further target to develop novel clinical applications for the modulation of NETosis.

DATA AVAILABILITY STATEMENT

The used datasets of Siglec 8 for this study were obtained from NCBI data bank (https://www.ncbi.nlm.nih.gov/). The datasets for the 3D-model of human Siglec-8 were obtained from Pröpster et al. (41) (PDB 2n7b). All novel datasets generated for this study are included in the manuscript **Supplementary Files.**

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because bovine cervices were only collected from animals sent to the abattoir for meat production. Bovine neutrophils were isolated from blood samples received from the slaughterhouse or from leftover blood samples of regulatory blood collections from other projects (7221.3-1-010/16-1). Both the slaughter processes and the blood sampling were performed in accordance with the guidelines, applicable laws and provisions for ethical regulations.

AUTHOR CONTRIBUTIONS

KB, AR, MG, TV, and KZ performed the experiments and analyzed in addition to CR and SG the data. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02478/full#supplementary-material

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Nanoparticles Equipped with α2,8-Linked Sialic Acid Chains Inhibit the Release of Neutrophil Extracellular Traps

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Abstract: Neutrophils can combat the invasion of pathogens by the formation of neutrophil extracellular traps (NETs). The NET mechanism is not only an effective tool for combating pathogens, but is also associated with diseases. Therefore, NETs are a potential target for combating pathologies, such as cystic fibrosis and thrombosis. We investigated the potential of nanoparticles, which were modified with α2,8-linked sialic acid chains, to modulate NET release during phorbol myristate acetate stimulation. Interestingly, when these nanoparticles were applied, the formation of reactive oxygen species was partly inhibited and the release of NET was counteracted. However, although the release of NET fibers was prevented, the nuclei still lost their characteristic segmented structure and became swollen, indicating that only the release, and not complete activation was suppressed. Intriguingly, coincubation of α2,8-sialylated particles with free sialic acid chains prevented the outlined inhibitory effects. Thus, the sialic acid chains must be attached to a linker molecule to generate an active bioconjugate that is able to inhibit the release of NET.

Keywords: neutrophil; NETosis; reactive oxygen species (ROS); innate immunity; sialic acids; siglecs; polysaccharide

1. Introduction

Neutrophil granulocytes are the most abundant leukocytes of the innate immune system representing the first line of defense against invading pathogens [1–3]. In the case of inflammation, these granulocytes exit the circulation system via trans-endothelial migration, a selectin-driven mechanism [4]. Arriving at the inflammation site, they release reactive oxygen species (ROS), as well as antimicrobial peptides and phagocytose pathogens, such as bacteria.

Moreover, in 2004, Brinkmann and colleagues discovered that neutrophils can undergo beneficial suicide, resulting in the release of neutrophil extracellular traps (NETs) [5]. The DNA fibers released are associated with numerous antimicrobial components [5–8]. The formation of NETs can be induced by pathogens, such as bacteria, fungi, and viruses, as well as by chemical stimuli, such as calcium ionophore A23187 or phorbol myristate acetate (PMA) [9–12]. When the formation of NETs is induced by PMA, calcium influx from the endoplasmic reticulum is induced and protein kinase C alpha (PKC) is activated. PKC initiates the activation of the Raf-MEK-ERK pathway. ERK1/2 seems to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase by phosphorylation, contributing to the activation of the NADPH oxidase enzyme complex [4,9,13–15]. This complex produces ROS, which are enzymatically converted to hypochlorous acid (HOCl) by myeloperoxidase [4,9]. This leads

to the transfer of neutrophil elastase to the nucleus, where neutrophil elastase degrades histones, such as histone H4, promoting the decondensation of DNA. During the last step, granular vesicles, in addition to plasma membranes, rupture and a mixture, which consists of antimicrobial biomolecules (e.g., myeloperoxidases, neutrophil elastases, lactoferrin, defensins, and cytotoxic histones) is released to combat the invading pathogens [9,16].

In addition to beneficial effects, the formation of NET is associated with diseases, such as infertility, small-vessel vasculitis, rheumatoid arthritis, preeclampsia, ulcerative colitis, Crohn's disease, systemic lupus erythematous, thrombosis, and cystic fibrosis [9,17–23]. Thus, the inhibition of an exaggerated NET release is an opportunity to combat these pathologies.

In mammals, several biomolecules have the capacity to modulate immunological mechanisms. Since all our cells are surrounded by a glycocalyx, consisting of glycolipids and glycoproteins, glycan-dependent mechanisms frequently take place during processes of innate and adaptive immunity [24]. In mammals, glycans are often terminated with sialic acid residues [25]. These sialylated structures can be recognized by immune cells using, for example, sialic acid-binding immunoglobulin–like lectins (siglecs), which are important immunoregulatory elements in vertebrates [26–29]. Intriguingly, inhibitory siglecs can counteract kinase-dependent activation of immune cells by recruiting SHP1 and SHP2 [28,30]. For example, Varki and colleagues described that, in the bloodstream, siglec-9 on neutrophils inhibits neutrophil activation by binding sialylated glycoproteins on erythrocytes [31]. Thus, siglecs became a target for modulating immunological events [32]. One possibility is to decorate nanoparticles with sialylated glycans. For instance, Spence et al. showed that nanoparticles coated with dimers of α2,8-linked sialic acid residues, which are the ligand for murine siglec-E, decreased inflammation driven by lipopolysaccharide (LPS) in murine macrophages [33]. Human neutrophils are known to express siglec-3, -5, -9, and -14, and interestingly, siglec-5 is able to recognize α2,8-linked sialic acid residues, such as siglec-E in mice [34–36].

Thus, we hypothesized that α2,8-linked sialic acid residues can be used to modulate the activation of neutrophils and NETosis. The obtained results demonstrate that the release of NETs is inhibited by the application of particles containing α2,8-linked sialic acid chains, indicating that α2,8-sialylated nanoparticles are a tool for manipulating the formation of NET.

2. Materials and Methods

2.1. Materials

Endoneuraminidase (endoN) was kindly provided by Martina Mühlenhoff (Medizinische Hochschule, Hannover, Germany) [37]. All reagents used were of analytical grade.

2.2. Human Neutrophils

All volunteers provided written informed consent and all samples were anonymized. The use of human neutrophils was approved by the local ethics office of the University of Giessen, School of Medicine (05/00).

2.3. Digestion and Fractionation of Sialic Acid Chains

10 mg colominic acid (Gerbu, Heidelberg, Germany) was digested via the use of endoN (4.46 µg mL^{-1}, 2 h, 37 °C, 250 rpm). Resulting cleavage products were separated and collected according to the degree of polymerization (DP) by anion exchange chromatography, as described earlier, to get sialic acid chains with chain lengths consisting of <9 N-acetylneuraminic acid (Neu5Ac) residues [38–40]. The retention time of specific sialic acid chains was determined by mildly labeling 10 mg colominic acid with 1,2-diamino-4,5-methylenedioxybenzene (DMB) (Dojindo, Kumamoto, Japan) under the following conditions: A total of 10 mg colominic acid was dissolved in 200 µL DMB reaction buffer (9 mM sodium hydrosulfite, 0.5 M β-mercaptoethanol, 20 mM trifluoroacetic acid (TFA), and 1.35 M DMB) and 200 µL MilliQ water, incubated overnight at 11 °C, and stopped by the addition of 100 µL

1 M NaOH [39,41–43]. The different chain lengths were separated with a DNAPac PAc-100 column (22 mm × 250 mm; 13 µm; Thermo Fisher Scientific, Waltham, MA, USA) using HPLC system (Smartline System, Knauer, Berlin, Germany). MilliQ water (E1) and 2 M ammonium acetate buffer (E2) were used as eluents at a flow rate of 2.5 mL min^{-1}. The following gradient was used for the separation, 0 min = 0% (*v*/*v*) E2, 20 min = 26% (*v*/*v*) E2, 30 min = 34% (*v*/*v*) E2, 45 min = 38% (*v*/*v*) E2, 85 min = 88% (*v*/*v*) E2, 110 min = 100% (*v*/*v*) E2, and 141 min = 0% (*v*/*v*) E2. DMB-labeled sialic acid chains were detected using a fluorescence detector at 372 nm for excitation and 456 nm for emission. In order to proof the degree of polymerization, aliquots of dried samples were mildly DMB labeled, as described above. Sample separation took place on an analytical DNAPac PAc-100 column (4 mm × 250 mm; 13 µm; Thermo Fisher Scientific). MilliQ water (E1) and 2 M ammonium acetate buffer (E2) were used as eluents at a flow rate of 1 mL min^{-1} using the following gradient, 0 min = 100% (*v*/*v*) E1, 5 min = 100% (*v*/*v*) E1, 15 min = 92% (*v*/*v*) E1, 20 min = 89% (*v*/*v*) E1, 30 min = 86% (*v*/*v*) E1, 55 min = 84% (*v*/*v*) E1, 100 min = 80% (*v*/*v*) E1, and 130 min = 77% (*v*/*v*) E1.

2.4. Quantification of Sialic Acids

To quantify the amount of N-acetylneuraminic acid, sample aliquots were hydrolyzed with 0.2 N TFA for 4 h at 80 °C. Dried samples, as well as appropriate standards, were then DMB-labeled under the following conditions: 40 µL DMB reagent + 40 µL MilliQ water, 2 h, 55 °C, 350 rpm. The labeling was stopped by the addition of 20 µL 0.2 M NaOH [44,45]. The quantification was performed with a LiCroCart 250-2 Merck and a SuperSpher 100 RP-C18 column, as described previously [44–47].

2.5. Coupling of Sialic Acids on Latex Beads

The separated sialic acid chains, as well as Neu5Ac (sialic acid monomers; monoSia) (Carbosynth, Compton, UK), were used for coupling reaction. Aliphatic amine latex particles (1% *w*/*v*, 0.1 µm; Thermo Fisher Scientific) were washed twice prior usage. A total of 150 µL latex particles were resuspended in 123 µL PBS and homogenization was performed by using ultrasonic. A total of 200 µg of dried sugars were dissolved in 25 µL PBS and then added to the latex particles. A total of 1.5 µL 5 M $NaCNBH_3$ was added and the coupling reaction took place for 2 h at 65 °C and 250 rpm. After this, sialic acid–coupled particles were washed twice and resuspended in 100 µL PBS. An aliquot was taken to quantify the coupling reaction, as described in the previous chapter. The used nanoparticles showed no cytotoxic characteristics as tested previously in [40]. Regarding the stability of the sialylated particles, it should be noted that the sialylation status is reduced by 30% when stored at 8 °C for one month, resulting in a decreased activity.

2.6. Isolation of Human Neutrophils

Human neutrophils were isolated as described previously by Saffarzadeh et al. [48]. Therefore, a density gradient using a Histopaque-1077 and a Histopaque-1119 was applied at 37 °C and 700 × g for 30 min. The neutrophil containing layer was washed with PBS. After erylysis (lysis buffer: pH 7.5; 0.15 M NH_4Cl, 0.1 mM EDTA, 1 mM $KHCO_3$) cells were washed with PBS. The erylysis was performed two times. As a final step, the received pellet was washed and resuspended in RPMI 1640 (Thermo Fisher Scientific) with 1% penicillin/streptomycin (PenStrep; Thermo Fisher Scientific) and 1% fetal bovine serum (FBS; Thermo Fisher Scientific). A total of 30,000 cells/well were dissolved in RPMI 1640 with 1% PenStrep, and 1% FBS and incubated 1 h at 37 °C and 5% CO_2 before the NETs were induced.

2.7. NETosis Stimulation and Inhibition Assay

The stimulation of neutrophils with 20 nM PMA took place in Poly-L-Lysine coated 12-well chamber slides for 2 h/4 h at 37 °C and 5% CO_2. To inhibit NETosis cells were incubated with PMA and a final concentration of 10 µg mL^{-1} sialic acid was coupled on latex particles. Coincubations of α2,8-sialylated particles with 30 µg mL^{-1} free sialic acid chains of an inherent chain length were performed under equal conditions.

2.8. Immunofluorescence Staining

After 2 h or 4 h of PMA stimulation cells were fixed with 4% paraformaldehyde (PFA) for 30 min at 4 °C. After washing, cells were incubated with 0.5% Triton X-100 for 1 min, followed by three further washing steps. Blocking was performed for 30 min at 37 °C with 2% IgG free bovine serum albumin (BSA) (Carl Roth, Karlsruhe, Germany) and anti-Neutrophil Elastase (Abcam, Cambridge, UK) was incubated overnight at 4 °C. After further washing steps, the secondary antibody (FITC anti-rabbit, 1 h, room temperature (RT)) was added before the nuclei staining with 4′,6-Diamidin-2-phenylindol (DAPI) was done (Carl Roth, 1 µg mL^{-1}). Fixation was performed with 2% PFA for 20 min at RT. Samples were then mounted and analyzed using fluorescence microscopy (Carl Zeiss confocal laser scanning microscope LSM 800). The determination of the NET area per cell was based on the release of decondensed DNA fibers during NETosis (DAPI staining) and was performed using Cellprofiler 2.2.0. Three pictures were randomly taken of each biological sample.

2.9. Measuring of the Production of Reactive Oxygen Species via Dihydrorhodamine 123 (DHR)

Since the production of reactive oxygen species reaches its maximum after 30 min of PMA incubation [49], 35,000 cells/well were seeded and DHR (Thermo Fisher Scientific) was coincubated with PMA with a final concentration of 1.445 µM diluted in RPMI 1640, 1% FBS, and 1% PenStrep for 30 min at 37 °C and 5% carbon dioxide. Measurement was performed with a Gallios, Beckman Coulter, at an excitation of 488 nm and with the emission at 525 nm ± 25 nm (argon laser).

2.10. Determination of the Membrane Potential with Bis-(1,3-Dibutylbarbituric Acid)trimethine Oxonol ($DiBAC_4$)

The determination of the membrane potential was performed with $DiBAC_4$. (Thermo Fisher Scientific). Therefore, 35,000 cells/well were seeded and NETosis was induced with 20 nM PMA for 3.5 h and cells were further incubated 30 min with 250 nM $DiBAC_4$. Measuring took place with a Gallios, Beckman Coulter, at an excitation of 488 nm and with the emission at 525 nm ± 25 nm (argon laser), as described previously by Löhrke et al. [50].

2.11. NETose-Microscopy of Living Cells

After the isolation of human neutrophils, 50,000 cells/well were seeded in a Poly-L-Lysine -coated 12-well chamber slide. To de-stress cells from the isolation procedure, the following step was an incubation in RPMI 1640 for 1 h at 37 °C and 5% carbon dioxide. Afterwards, the cells were washed twice with RPMI 1640 and stained with 1 µg mL^{-1} DAPI (Carl Roth) for 30 min before a further washing step was done. To stain the membrane, Deep Red Cell Membrane Stain (1:1000; Thermo Fisher Scientific) was added 15 min before NETosis induction, with 50 nM PMA. Pictures were taken at an interval of 2 per min.

2.12. Determination of Membrane Integrity via Propidium Iodide (PI) Staining

Membrane integrity was analyzed using PI (Miltenyi Biotec, Bergisch Gladbach, Germany; Annexin V-FITC Kit) in a final concentration of 1 µg mL^{-1}. After removing PI, nuclei were stained with Hoechst (Sigma Aldrich, St. Louis, MO, USA) in a final concentration of 1 µg mL^{-1}. Cells were directly analyzed using fluorescence microscopy (Carl Zeiss confocal laser scanning microscope LSM 800). Three pictures were randomly taken from three independently performed experiments. The total cell number and PI positive cells were counted.

2.13. Statistical Analysis

Data sets were analyzed with Graph Pad Prism 7.0 software using ANOVA and a multiple-comparison Tukey test or a *t*-test with Welch's correction, when the calculated values passed the Shapiro–Wilk normality test. Otherwise, the Kruskal–Wallis test in combination with

Dunn's test for multiple comparisons was applied. Differences were considered statistically significant at $p \leq 0.05$. Statistically significant differences are given the labels * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$.

3. Results

3.1. α2,8-Sialylated Particles Decrease the Production of Reactive Oxygen Species and Membrane Depolarization

NADPH oxidase represents a key enzyme in the signaling cascade during the formation of NET induced by PMA [51]. To test whether nanoparticles containing α2,8-linked sialic acid chains modulate the activation of neutrophils, ROS production was analyzed. To quantify ROS, DHR was used. In the presence of ROS, DHR is oxidized to cationic rhodamine 123, which exhibits green fluorescence. As shown in Figure 1A, in comparison to the neutrophils treated with PMA, unstimulated neutrophils assemble approximately 98% less ROS. When nanoparticles with sialic acid monomers were applied during the PMA stimulation, no effect on the PMA-induced ROS generation was observed. In contrast, the α2,8-sialylated nanoparticles statistically significantly decrease the ROS production that had been initiated by PMA. However, the production of ROS could not be completely abolished. Thus, chemically coupled α2,8-linked sialic acid chains seem to influence the activation of neutrophils.

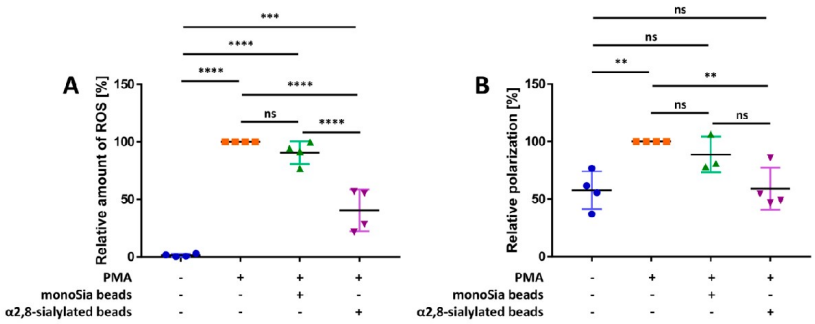

Figure 1. α2,8-sialylated particles reduce the production of ROS and the depolarization of the membrane when neutrophils are stimulated with PMA. (**A**) Scatter plot of the relative amount of ROS (%) produced after 30 min of stimulation with PMA. The determination of the relative amount of ROS (%) was performed using DHR. The values for PMA-treated cells were set to 100%. Four independent experiments were performed. (**B**) Scatter plot of the polarization status of the membrane. Determination of membrane depolarization was performed using $DiBAC_4$. The emitted fluorescence of the PMA-induced cells was set to 100%. Mean values ($n \geq 3$) and standard deviations are displayed in the diagrams. ANOVA and a multiple-comparison Tukey test were applied. Statistically significant differences are given as follows: ns, not significant, ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$.

The occurrence of membrane depolarization after activation of the NADPH oxidase complex was described in the 1980s [52–54]. Experiments focused on inhibition of NADPH oxidase complex–induced electron transport showed that the inhibition of electron transport leads to attenuated membrane depolarization [54]. In line with these experiments, neutrophils of patients with chronic granulomatous disease (CGD) showed no membrane depolarization after PMA stimulation, as this disease is characterized by an impaired NADPH oxidase complex [52,55,56]. Since α2,8-sialylated nanoparticles inhibit ROS production, we also examined whether membrane depolarization is influenced. To this end, the polarization status of the cells was determined with $DiBAC_4$, a substance that can enter depolarized cells. Here, $DiBAC_4$ can bind intracellular proteins exhibiting enhanced fluorescence and a

red spectral shift. In Figure 1B, it is apparent that the stimulation of neutrophils with PMA results in cell membrane depolarization. Statistically significant differences between non-treated and stimulated cells were detected, which could not be influenced by monosialylated nanoparticles. However, in line with the ROS experiments, α2,8-sialylated nanoparticles inhibited membrane depolarization considerably.

In sum, the outlined results of the cell assays suggested that the essential steps during PMA stimulation, which trigger the formation of NET, are impaired by α2,8-sialylated nanoparticles.

3.2. *The Swelling of Neutrophil Nuclei Is Not Influenced by α2,8-Sialylated Nanoparticles*

The formation of ROS is directly linked to the translocation of neutrophil elastase into the nucleus because ROS serves as a substrate for myeloperoxidase. Subsequently, the increase in HOCl initiates the detachment of neutrophil elastase from the myeloperoxidase/neutrophil elastase complex, enabling the transfer of neutrophil elastase into the nucleus and triggering decondensation of DNA [4,9]. The result is that the nuclei lose their typical segmented structure and swell.

When we compared the nuclear structure of unstimulated neutrophils with that of the activated neutrophils in the presence of α2,8-sialylated nanoparticles, we observed that nearly all nuclei lost their segmented structure and the nuclei were swollen 4 h after the beginning of the PMA stimulation (Figure 2). Thus, the results suggest that the decondensation of DNA could not be prevented.

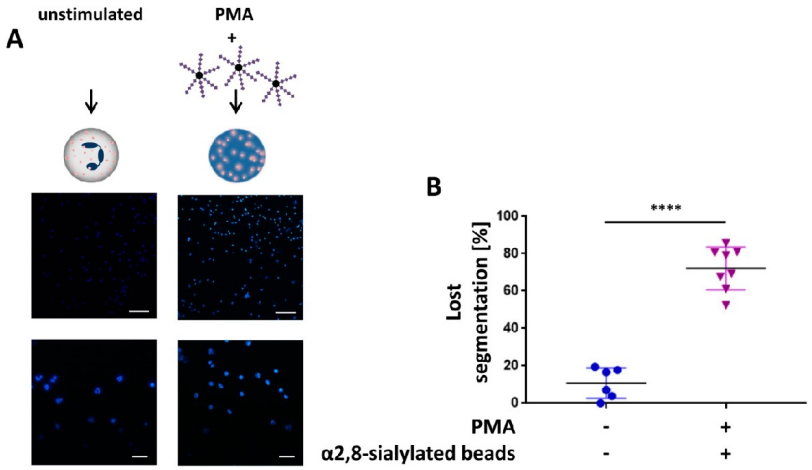

Figure 2. α2,8-sialylated nanoparticles do not prevent the loss of nucleus segmentation. (**A**) DNA was stained with DAPI (blue) in unstimulated neutrophils in addition to neutrophils, which were stimulated with PMA (20 nM PMA for 4 h) in the presence of α2,8-sialylated particles. Upper scale bar: 100 µm, lower scale bar: 20 µm. (**B**) Based on the fluorescence pictures, the total number of cells that lost their segmented structure was determined. Mean values ($n \geq 6$) and standard deviations are displayed. The statistical significance was calculated with the *t*-test with Welch's correction. The statistically significant difference is given as follows: **** $p \leq 0.0001$.

The translocation of neutrophil elastase to the nuclei is an important step during the decondensation of DNA and the formation of NETs [16]. To test whether the localization of neutrophil elastase was also unchanged due to the application of α2,8-sialylated nanoparticles, the enzyme was visualized using a polyclonal Ab against neutrophil elastase. Based on published data concerning the time point of the translocation of neutrophil elastase into the nucleus, immunofluorescence staining of neutrophil elastases was performed 2 h after the PMA treatment [16].

In contrast to the unstimulated neutrophils, neutrophil elastase was transferred into the nucleus after stimulation (Figure 3). In addition, the segmented structure of the nucleus was resolved. Comparable results were obtained using α2,8-sialylated particles. Thus, the reduction of ROS to approximately 40%, which was induced by α2,8-sialylated nanoparticles, does not prevent the transformation of the nuclei.

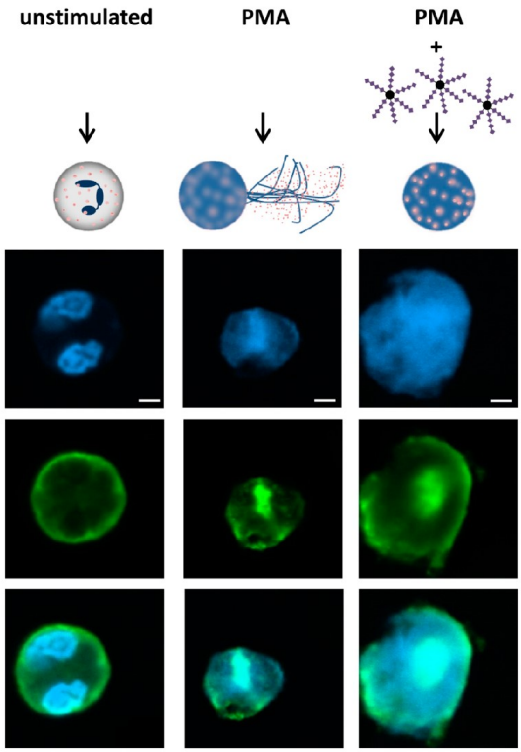

Figure 3. Neutrophil elastase also enters the nucleus during the application of α2,8-sialylated nanoparticles. Cells were incubated with 20 nM PMA for 2 h and were stained with a polyclonal antibody against neutrophil elastase (FITC; green). DNA was visualized using DAPI (blue). Scale bar: 2 µm.

3.3. α2,8-Sialylated Particles Inhibit the Release of NET

In addition, the release of NET was examined. The stimulation of NETosis leads to the formation of the DNA meshwork, which can be visualized with DAPI. As shown in Figure 4A,B, 4 h after PMA stimulation, the expected NET filaments are formed. In contrast, the unstimulated neutrophils retained their shape, including their segmented nuclei. However, the application of α2,8-sialylated nanoparticles prevented NET release although the nuclei were swollen. The calculation of the NET area shows that NET expansion was statistically significantly reduced.

The same experimental test setup was also performed with monosialylated beads, which showed no impact on the activation by PMA (Figure 5). In addition, experiments were performed to investigate whether free sialic acid chains have the same effect or whether sialic acid chains have to be attached to

a linker to inhibit the release of NETs. To this end, we used free sialic acid chains together with the α2,8-sialylated beads. Again, α2,8-sialylated nanoparticles had the capability to prevent NET release (Figure 6). However, this effect was inhibited by free sialic acid chains. Thus, a linker molecule is necessary to inhibit the release of NETs.

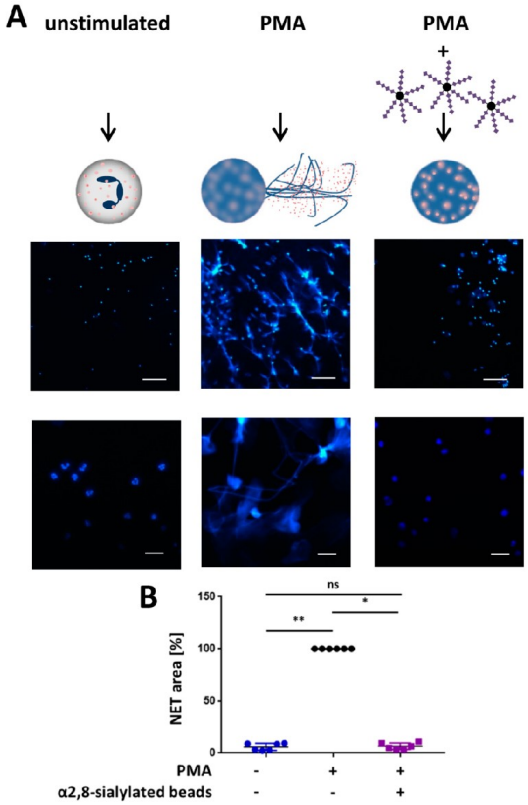

Figure 4. α2,8-sialylated particles inhibit NET release. (**A**) Fluorescence staining (DAPI; blue) of neutrophils without any stimulation of neutrophils, stimulated with 20 nM PMA for 4 h, and of neutrophils stimulated with 20 nM PMA coincubated with α2,8-sialylated particles for 4 h. Upper scale bar: 100 µm, lower scale bar: 20 µm. (**B**) Analysis of the NET area (%). The analysis was performed using Cell Profiler 2.2.0. The NETosis area (%) was calculated by determining the blue fluorescent areas (DAPI). PMA inducement was set to 100%. Mean values ($n = 6$) and standard deviations are displayed. The Kruskal–Wallis test and Dunn's test for multiple comparisons were applied. Statistically significant differences are given as follows: ns, not significant, * $p \leq 0.05$ and ** $p \leq 0.01$.

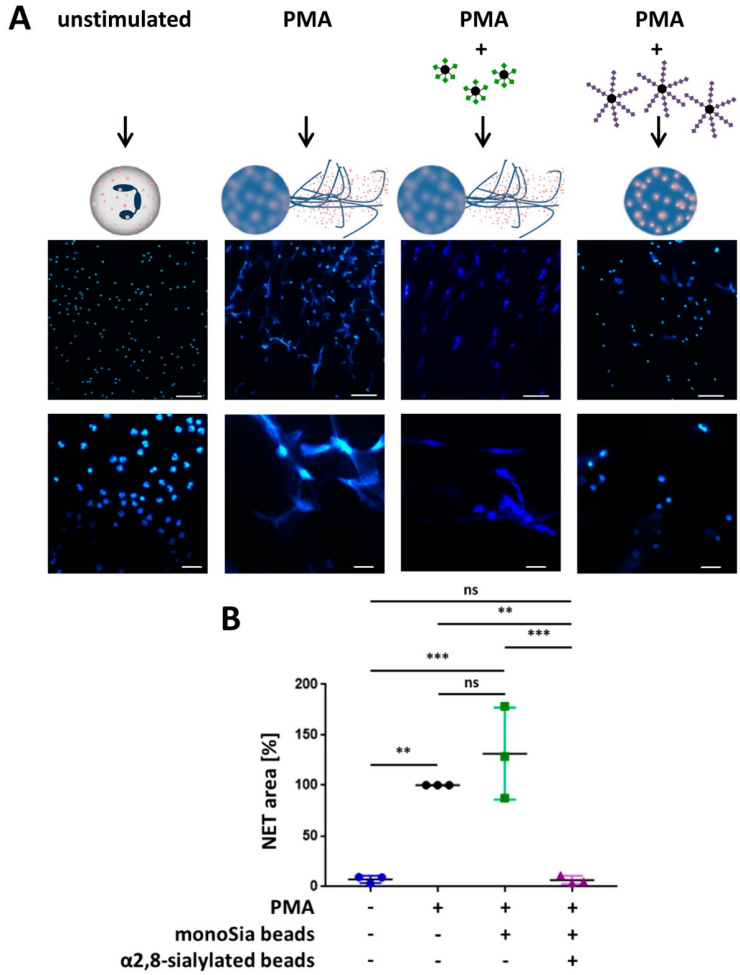

Figure 5. α2,8-sialylated particles inhibit NET release. (**A**) Fluorescence staining (DAPI; blue) of neutrophils without any stimulation of neutrophils, stimulated with 20 nM PMA for 4 h, and of neutrophils stimulated with 20 nM PMA coincubated with monosialylated particles or α2,8-sialylated particles for 4 h. Upper scale bar: 100 µm, lower scale bar: 20 µm. (**B**) Analysis of the NET area (%). The analysis was performed using Cell Profiler 2.2.0. The NETosis area (%) was calculated by determining the blue fluorescent areas (DAPI). PMA inducement was set to 100%. Mean values (n = 3) and standard deviations are displayed. ANOVA and multiple comparison Tukey test were applied. Significant differences are given as follows: ns, not significant, ** $p \leq 0.01$ and *** $p \leq 0.001$.

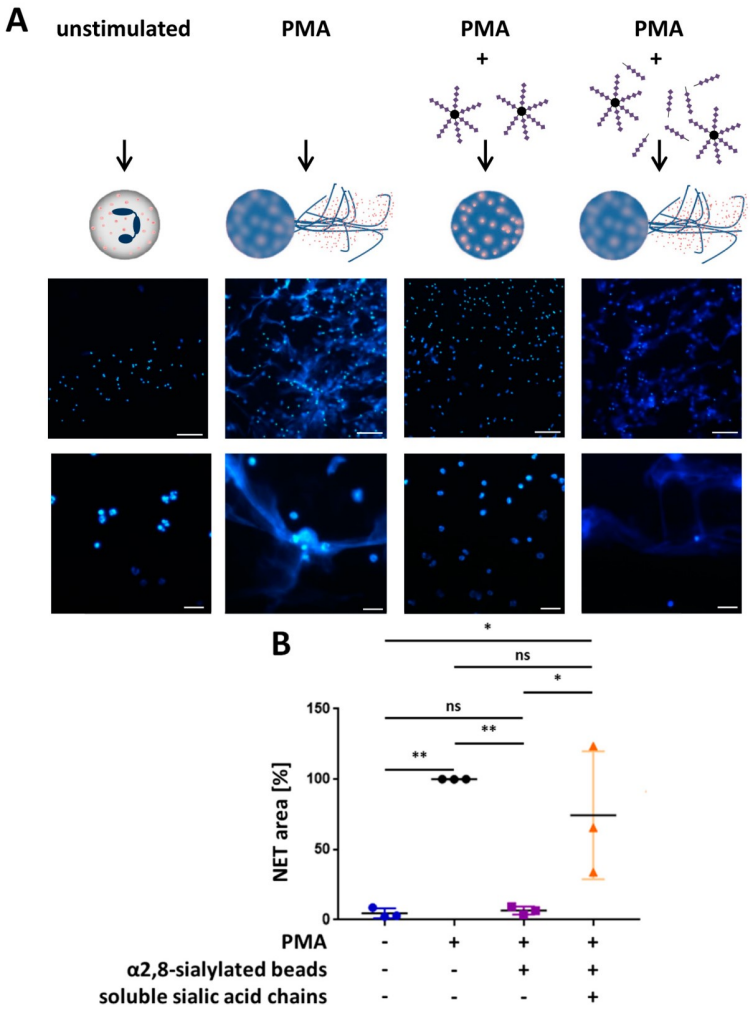

Figure 6. Free sialic acid chains prevent the outlined inhibitory effects of α2,8-sialylated particles. (**A**) Fluorescence staining (DAPI; blue) of isolated human neutrophils stimulated with 20 nM PMA and coincubated with α2,8-sialylated particles or free sialic acid chains in combination with α2,8-sialylated nanoparticles. Upper scale bar: 100 µm, lower scale bar: 20 µm. (**B**) Analysis of the NET area (%). Analysis was performed on three different experimental approaches using Cell Profiler 2.2.0. NETosis area (%) was calculated by determining the blue fluorescent areas (DAPI). PMA stimulation was set to 100%. Mean values and standard deviations are displayed in the diagrams. ANOVA and multiple comparison Tukey test were applied. Significant differences are given as follows: ns, not significant, * $p \leq 0.05$ and ** $p \leq 0.01$.

3.4. α2,8-Sialylated Particles Prevent Cell Membrane Perforation

For the release of NETs, cell membrane rupture is necessary [9]. Using Deep Red Cell Membrane Staining, it is possible to visualize the cellular membrane during NETosis and its explosive bursts to release the DNA network (illustrated in Figure 7 and Supplemental 1; video). The resulting hole allowed the release of intracellular substances and the penetration of molecules into the cell. Interestingly, the intensity of the membrane staining became stronger during the alteration of the nuclear structure, indicating that the membrane properties of the neutrophils had changed before the release of NETs.

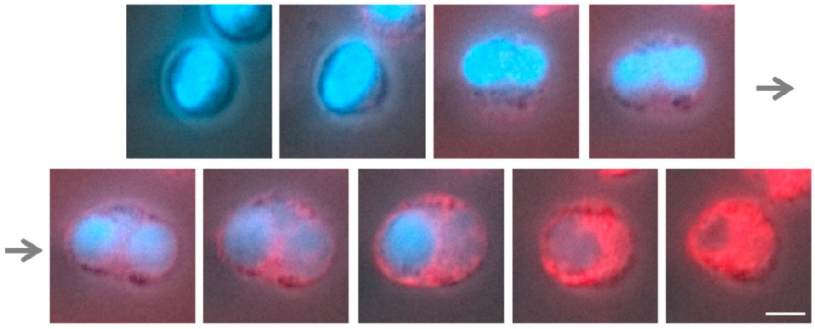

Figure 7. Release of NETs. NETosis stimulation was performed with 50 nM PMA. At the very beginning of PMA induction, a healthy cell with an intact nucleus is visible. Then DNA decondensation takes place, followed by disintegration of the nuclear membrane and a fusion of DNA with the released cytoplasmic proteins. Thus, at this time point, the cell dimension expands, leading to a rupture of the outer membrane, along with the release of the DNA-protein mixture into the fluid surrounding the cell. The empty cell membrane remains behind. The cell membrane (red) was stained with Deep Red and DNA (blue) with DAPI. Scale bar: 5 µm.

In order to test whether the outer membranes were still intact we used PI, a substance that can pass only perforated, not intact, biomembranes. Once PI enters the cell, it interacts with DNA, resulting in a red fluorescent staining. The calculation was performed by counting the total number of cells compared to the number of PI-positive cells. The results indicate that, compared to untreated neutrophils, PMA led to an increasing number of perforated cells (Figure 8). Similar results were obtained when monosialylated beads were added. However, the application of α2,8-sialylated beads resulted in a significantly reduced number of PI-positive cells, demonstrating that α2,8-sialylated beads inhibit the perforation of the biomembrane, and thus, the release NET.

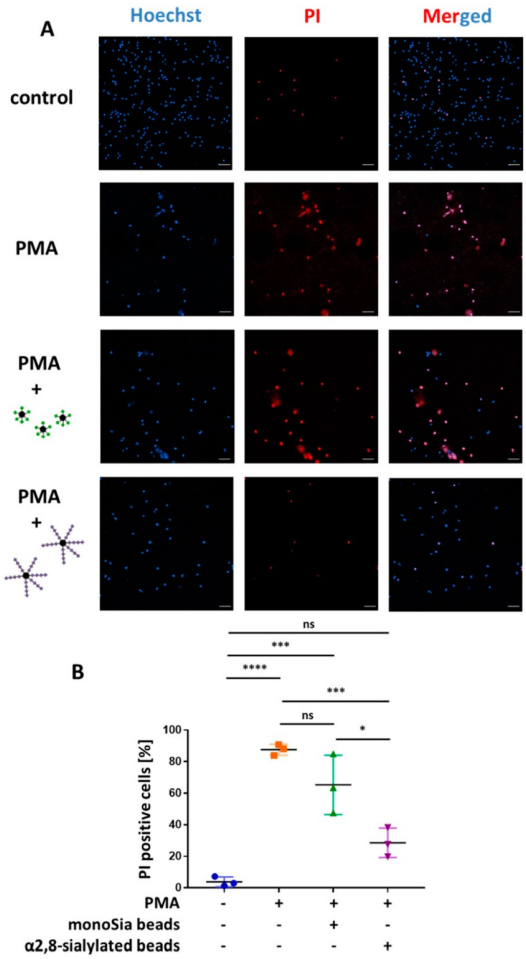

Figure 8. Coincubation of PMA-stimulated neutrophils with α2,8-sialylated particles significantly decreases the number of PI-positive cells. (**A**) Isolated neutrophils stimulated with 20 nM PMA and isolated neutrophils stimulated with 20 nM PMA coincubated with α2,8-sialylated particles or monosialylated particles were stained with PI (red) and Hoechst (blue) to determine membrane permeability. Unstimulated isolated neutrophils served as a control. Scale bars: 50 µm. (**B**) Based on the fluorescence images the total number of cells was determined and was set in relation to the number of PI-positive cells. Mean values (n = 3) and standard deviations are displayed in the diagrams. ANOVA and a multiple-comparison Tukey test were applied. Significant differences are given as follows: ns, not significant, * $p \leq 0.05$; *** $p \leq 0.001$; and **** $p \leq 0.0001$.

4. Discussion

In 2015, Spence et al. discovered that nanoparticles coated with the murine siglec-E ligand, α2,8-linked sialic acid residues, decrease macrophage-driven inflammation [33]. Based on this

discovery, we decided to investigate the potential of α2,8-sialylated particles to inhibit the release of NETs. Interestingly, the application of α2,8-sialylated particles decreased the production of ROS in PMA-stimulated neutrophils, while monosialylated particles showed no effect. In line with the reduced ROS, depolarization was also inhibited by these sugar-coated nanoparticles. Since the activation of the NADPH oxidase complex includes PKC and the Raf-MEK-ERK pathway, resulting in the formation of ROS [14,57], and siglecs are known to inhibit the immune response by counteracting kinases via the recruitment of the phosphatases SHP1 and SHP2 [28,30], the involvement of siglecs may trigger these observations. As siglec-5 is known to prefer α2,8-linked sialic acids, decreased ROS production and depolarization might be the result of the interaction between α2,8-linked chains and siglec-5 of neutrophils [34–36]. However, the impaired ROS production and depolarization did not prevent swelling of the nuclei. This indirectly indicates that neutrophil elastases still translocate to the nuclei, because neutrophil elastase is necessary for decondensation of DNA and, therefore, for nuclei swelling as described previously [16].

Nevertheless, the release of NETs was retained and the α2,8-sialylated particles prevented the perforation of the outer membrane (Figure 8). Interestingly, a comparable effect was described for lactoferrin [58]. In line with α2,8-sialylated beads, elastase translocates to the nucleus in the presence of lactoferrin and nucleus swelling takes place. In addition, in the case of lactoferrin, the last step, the release of NET is inhibited. Okubo and colleagues observed that lactoferrin accumulates on the surface of neutrophils [58]. They suggested a physical blocking barrier, which is mediated via a "lactoferrin-shell". The first released DNA aggregates with lactoferrin forming a plug, which prevents the release of NETs [58]. As recently shown, polysialic acid (polySia) can interact with lactoferrin and supports the effect of lactoferrin to inhibit NET release [59]. However, whether the membrane was still intact was not tested.

In addition to lactoferrin, α2,8-linked polymers of sialic acids are known to induce membrane interactions, because these polymers can interact with phospholipids [60]. This interaction usually takes place in ordered regions of membranes, such as lipid rafts [61]. The staining of cell membranes with Deep Red during NETosis (Figure 7) suggested that fluid and dynamic processes occur in the biomembrane, which might mediate such an interaction. Thus, we suggest that the membrane is stabilized by the multivalency of α2,8-sialylated nanoparticles, which might act as "cross struts". Furthermore, perhaps an interaction with membrane proteins, such as siglec-5, supports the stabilization of the cell membrane in a comparable fashion. These hypotheses are supported by the observation that free sialic acid chains inhibit the effects of the α2,8-sialylated nanoparticles.

However, in contrast to lactoferrin, α2,8-sialylated beads influence ROS production [58], which is essential for the formation of NETs induced by PMA. The observed inhibition of ROS formation might be the result of an activation of siglec-5 by the α2,8-sialylated beads. The lower ROS content might additionally reduce the dynamic of the neutrophil burst. Presumably, the combination of these two effects (the "cross struts" effect and lower ROS levels) leads to stabilization of the biomembrane, which prevents the cell membrane from rupturing.

Although the exact mechanism of action has been unknown until now, α2,8-sialylated nanoparticles are a promising option for modulating NETosis during NET-related pathologies. Furthermore, for application as a pharmacological tool, the α2,8-sialylated nanoparticles must be tested in different in vivo models where NETs are the main cause that induces pathology.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-4991/9/4/610/s1, Supplemental 1: Visualization of NETosis.

Author Contributions: K.F.B., T.V. and A.K. performed the experiments and analyzed in addition to S.P.G. the data. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflicts of Interest: The authors have no conflicts of interest to declare.

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MINI REVIEW
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Glycans as Modulators for the Formation and Functional Properties of Neutrophil Extracellular Traps: Used by the Forces of Good and Evil

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A very common mechanism to trap pathogens is the release of DNA. Like flies in a spider's web, pathogens are enclosed in a sticky chromatin meshwork. Interestingly, plants already use this mechanism to catch bacteria. In mammals, especially neutrophils release their DNA to prevent an invasion of bacteria. These neutrophil extracellular traps (NETs) are equipped with antimicrobial molecules, including, for instance, histones, antimicrobial peptides, lactoferrin, and neutrophil elastase. Thus, in a defined area, pathogens and toxic molecules are directly adjacent. However, several of these antimicrobial substances are also cytotoxic for endogenous cells. It is, therefore, not surprising that distinct control mechanisms exist to prevent an exaggerated NETosis. Nevertheless, despite these endogenous control instruments, an extraordinary NET release is characteristic for several pathologies. Consequently, NETs are a novel target for developing therapeutic strategies. In this review, we summarize the roles of glycans in the biology of NETs; on the one hand, we focus on the glycan-dependent strategies of endogenous cells to control NET formation or to inactivate its cytotoxic effects, and, on the other hand, the "sweet" tricks of pathogens to inhibit the release of NETs or to prevent NET-mediated killing mechanisms are examined. Understanding both, the forces of good and evil, allows the development of novel glycan-based approaches to combat the harmful side of NETs during distinct pathologies.

Keywords: NETosis, Siglecs, polysialic acid, histones, glycosaminoglycans

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INTRODUCTION

Neutrophil granulocytes possess a panel of different mechanisms to combat invading pathogens. As first line of defense neutrophils can combat pathogens by phagocytosing them, by releasing antimicrobial peptides or reactive oxygen species (ROS) and by the release of neutrophil extracellular traps (NETs), a process first described in 2004 by Brinkmann et al. (1, 2). NET release can be induced by bacteria, viruses, fungi or non-physiological stimuli, such as ionophores and phorbol-myristate acetate (PMA) (3, 4). It seems to be that different pathways can be used to induce the formation of NET and even more than 13 years after the first description of NETs several mechanisms are controversially discussed and numerous open questions still need to be answered [excellently summarized in Boeltz et al. (5)]. What we know is that NET consists of a meshwork of decondensed DNA fibers, cytotoxic histones, and antimicrobial peptides and has the ability to catch and render invading pathogens harmless (3, 5, 6). Since NET contains several biomolecules,

which are also cytotoxic for endogenous cells, in addition to the desired antimicrobial effects, NETs are associated with numerous pathologies (7–16). Therefore, an exaggerated release of NET has to be prevented, leading to the necessity of control mechanisms to regulate the formation of NETs.

Within the last few years, interest in glycans and how they modulate different functions has increased immensely (17–19). Since all of our cells are surrounded by a glycocalyx consisting of highly glycosylated proteins and lipids, it is obvious that glycan-dependent processes occur frequently (19, 20). Indeed, glycans have essential roles within various biological processes, such as cell proliferation, cell differentiation, the development of organs, and within the immune system (19, 21). In the field of immunology, glycans drive diverse mechanisms, ranging from the discrimination of the self and non-self, using for instance sialic acid-binding immunoglobulin-like lectins (Siglecs), to glycosaminoglycan (GAG)-mediated chemokine presentation (22).

GAGs are a number of long, unbranched polysaccharides composed of repeating disaccharide units, whereby, the repeating disaccharides consist of uronic acid or galactose and an amino sugar, which can be additionally modified. Prominent members of the GAG family are: hyaluronic acid, heparin/heparan sulfate, chondroitin sulfate/dermatan sulfate, and keratin sulfate (23). Besides their role in chemokine presentation, GAGs are also involved in several other biological processes, like cell signaling, angiogenesis (24), metastasis, tumor progression (25, 26) and coagulation (27, 28).

In addition to GAGs, sialylated glycans play an important role during immunological events. In mammals, glycans are frequently terminated by these sugar residues (29). Sialic acids are negatively charged and modulate both immunological processes and organ development (30–33). Sialylated structures can be recognized by Siglecs (34–36), which are transmembrane receptors expressed in different cells of vertebrates that can mainly either inhibit or activate the immune response (35, 37–39). Although Siglecs are meant to be a valuable tool to distinguish between self-associated molecular patterns (SAMPs) and pathogen-associated molecular patterns (PAMPs), several pathogens are already known to elude the immune system by mimicking host sialylation and, therefore, masking themselves as SAMPs.

This review is therefore focusing on the role of glycoconjugates of endogenous cells in controlling NET releases and impairing the negative outcome of NETs as well as on the role of glycoconjugates of pathogens that influence NET formation or that directly influence NET release and the biological activity of NETs.

THE INTERACTION BETWEEN GLYCANS AND NEUTROPHILS: A PHYSIOLOGICAL CONTROL SYSTEM IN CIRCULATION

An impaired NET release and missing NET clearance are, for instance, associated with the formation of a thrombus (7, 40, 41). Not only do the NET fibers serve as a scaffold for the formation of a thrombus. Furthermore, neutrophil elastase is released in the extracellular area and inhibits among others the anticoagulants antithrombin and tissue factor pathway inhibitor. In addition, extracellular histones are known to increase the thrombin generation, causing platelet activation and coagulation (40–43). Therefore, it is not surprising that an impaired NET release within circulation needs to be prevented.

Lizcano et al. investigated the reason why isolated neutrophils are more susceptible to undergoing NETosis than neutrophils within circulation (44). They determined that glycophorin A, a sialoglycoprotein that is located on the surface of erythrocytes, is a candidate that might be responsible for this effect. It is able to bind Siglec-9 on the surface of neutrophils, inhibiting neutrophil activation within circulation (**Figure 1A**). Remarkably, modification of the sialic acid on the surface of the erythrocytes prevents the outlined inhibitory effects (44). Interestingly, also cancer cells, which are characterized by hypersialylation, seem to use Siglec-5 and Siglec-9 to evade entrapment by activated neutrophils [excellently reviewed by Adams et al. (45) and Rodrigues and Macauley (46)].

Besides sialylated glycoconjugates, heparin, a well-known anticoagulant, inhibits neutrophil degranulation and aggregation *in vitro* (47). Furthermore, the influence of low molecular weight heparin, unfractionated heparin, O-desulfated heparin, hyaluronic acid, dextran sulfate, and poly-L-glutamic acid on neutrophil activation was investigated (48). The activation of neutrophils with different stimuli induced the release of neutrophil elastase. However, the application of the different stimuli in combination with low molecular weight heparin as well as dextran sulfate inhibited neutrophil activation and, therefore, the release of neutrophil elastase (**Figure 1A**) (48, 49). In contrast, the non-sulfated dextran and poly-L-glutamic acid showed no effect on neutrophil activation, leading to the assumption of a sulfate-dependent process. Furthermore, Xu et al. investigated the role of heparan sulfate in the biology of NETs (50). In heparan sulfate uronyl 2-O-sulgotransferase deficient mice less NET is formed after stimulation with group B streptococcus (GBS). Remarkably, when NET was treated with heparan lyase, its antimicrobial activity decreased (50). Thus, the formation and the activity of NETs seem to be modulated by heparan sulfate.

Besides heparan sulfate, other glycans are known to influence the biological activity of NETs. In this context, Brown et al. focused on neutrophil elastase and neutrophil-induced human bronchial epithelia cell detachment (48). Whilst hyaluronic acid had no effect, low-molecular-weight heparin, unfractionated heparin, O-desulfated heparin, and dextran sulfate significantly inhibited the neutrophil elastase-induced detachment (48). Furthermore, Fuchs et al. published that the treatment of NETs with heparin destroys their scaffold and prevents the formation of a thrombus (**Figure 1B**). Heparin has a high-charge-dependent affinity to histones (51, 52) and is able to release histones from chromatin fibers, therefore, destabilizing NETs (**Figure 1B**) (40). Since histones that are released during NETs are able to damage negatively charged cell membranes, histones are often described as antimicrobial peptides (AMPs) that are released during NETs alongside with other antimicrobial biomolecules like lactoferrin

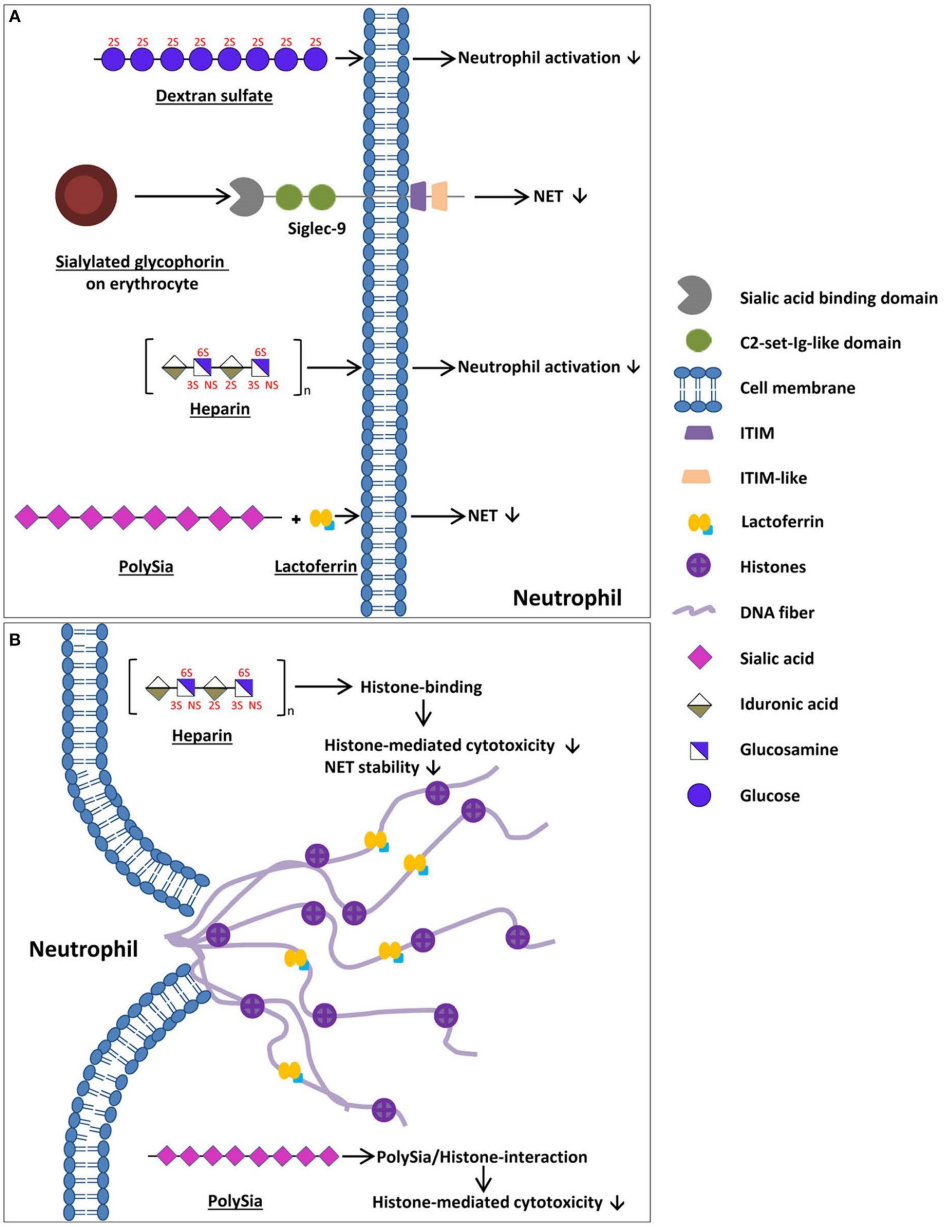

FIGURE 1 | Mechanisms of endogenous cells and naturally occurring components to enhance bacterial entrapment, to decrease impaired NET release, and to decrease the cytotoxic outcome of NETs. **(A)** Natural occurring components preventing NET release/neutrophil degranulation. Glycophorin is a sialoglycoprotein located at the surface of erythrocytes that inhibits NET release/neutrophil activation via sialic acid binding to Siglec-9 within circulation. Furthermore, the GAG heparin as well as dextran sulfate inhibit neutrophil activation in a sulfate-dependent manner. The interaction of lactoferrin with polySia increases the inhibition of NET releases. **(B)** Mechanisms to decrease the cytotoxic outcome of NETs for a body's own cells as well as to increase bacterial entrapment. PolySia as well as GAGs, such as heparin, bind to released histones, reducing histone-mediated cytotoxicity. The cartoons of all polysaccharides show only exemplary parts of these polymers and may differ from the actual structure (e.g., chain length and composition).

and neutrophil elastase (1, 6, 53–56). Unfortunately, these properties of all histones (H1, H2A, H2B, H3, and H4) are toxic not only for pathogens but also for endogenous cells (57–60). Within the plasma, the cytotoxicity of histones is reduced by the inter-alpha-inhibitor-protein (IAIP) associated with high molecular weight hyaluronic acid and chondroitin sulfate. IAIP as well as high molecular weight hyaluronic acid and chondroitin sulfate bind recombinant histone H4, contributing to reduced histone mediated cytotoxicity (**Figure 1B**) (61).

In addition to GAGs, such as hepain (52), another linear carbohydrate, polysialic acid (polySia), is a naturally occurring inhibitor of the cytotoxic effects of histones (59, 62, 63). Remarkably, polySia was detected in the plasma of different species, from fish to humankind, and may represent a natural buffer system for the inactivation of the cytotoxicity of extracellular histones in blood (**Figure 1B**) (64). PolySia influences histone-mediated cytotoxicity in a concentration as well as in a chain-length-dependent manner (65, 66). In line with that, polysialylated nanoparticles and *in vitro* polysialylated cervical mucins represent tools to counteract histone-mediated cytotoxicity during an exaggerated NET formation (65, 66). Interestingly, quite recently, Kühnle et al. (67, 68) published that polySia interacts with lactoferrin. Lactoferrin is known to inhibit a NET release by forming a "lactoferrin-shell" around the activated neutrophils (69). *In vitro* experiments suggested that the efficiency of lactoferrin in preventing the release of NETs was enhanced in the presence of polySia (67).

Thus, the presented examples show endogenous glycan-dependent ways to control the release of NETs in addition to decreasing their damaging effects, indicating, once more, the widespread function of glycosylation within the field of immunology. However, it has to be considered that a medal always has two sides. Some pathogens exploit the previously described mechanisms for their own purposes, as described in the next chapter.

THE GLYCOSYLATION OF PATHOGENS: A POWERFUL TOOL TO ESCAPE NETS

Bacteria

During their evolution, several pathogens have "learned" to use carbohydrate-dependent mechanisms to modulate the immune system. For instance, distinct bacteria strains target Siglecs to circumvent the release of NETs by neutrophils. *Pseudomonas aeruginosa (P. aeruginosa)*, for example, can use sialic acids from its hosts to decorate their glycoconjugates with sialic acids. On the surface of *P. aeruginosa*, *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and 9-O-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac2) have been found, enabling *P. aeruginosa* to inhibit neutrophil activation via the activation of Siglec-9 (70). According to Khatua et al. *P. aeruginosa* directly binds to the neutrophil via Siglec-9, stimulating the production of cytokine IL-10 and TGF-β (71). The generation of ROS is inhibited, and a decrease of the release of neutrophil elastase is detected. Since ROS production can be an initial step of NETosis, it is not surprising that the release of NETs is inhibited by the binding partners of Siglec-9 on the surface of *P. aeruginosa* (**Figure 2**) (72, 73).

However, *P. aeruginosa* is not the only pathogen using Siglec-9 to circumvent the activation of neutrophils. Carlin et al. (74) also showed that GBS is able to inhibit neutrophil activation through Siglec-9. GBS eluded the human immune system, causing invasive infections in human newborns by hosting the common terminus of human glycoproteins, Siaα(2,3)Galβ(1,4)GlcNAc, on their capsular polysaccharide. Here, the sialic acid binding of GBS to neutrophil Siglec-9 also initiated the production of the NET-suppressive cytokine IL-10 (74).

In addition to these findings, glycans of GBS interact with Siglec-5 and Siglec-14 (75). These Siglecs are an example of an antagonistic interplay between Siglecs. The sialic acid binding of Siglec-14 counteracts the pathogen-induced suppression of neutrophil activation (**Figure 2**). Intriguingly, the absence of Siglec-14 due to Siglec-14 null-polymorphism in humans leads to the increased susceptibility of neutrophils to GBS. The relevance of this pathogen-induced inhibition of neutrophil activation becomes apparent through the discovery of the Siglec-5 and Siglec-14 expressions on amniotic epithelium (75). These epithelial cells are the site of the initial contact area of the fetus and the pathogens. Ali et al. suggested that Siglec-14 null-polymorphism might relate to the risk of prematurity during GBS invasion.

In 2016, a surprising discovery was made by Secundino et al. (76). While investigating GBS and its capability to bind and activate Siglec-9 via its sialylated glycans, they observed that Siglec-9 also bound high molecular weight hyaluronan, consisting of repeating disaccharide units of *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA), with alternating β1,4- and β1,3-linkages. Intriguingly, they detected a new, specific binding site, apart from the V-set Ig-like domain. Since the capsular polysaccharide of group A streptococcus (GAS) contains high-molecular-weight hyaluronan units, NETs' formation and oxidative bursts were prevented (**Figure 2**) (76). Remarkably, a single inhibitory Siglec recognizes two different glycan motifs as SAMPs, leading to the suppression of neutrophil activation.

Although GBS seems to be the best studied pathogen regarding sialic acids and NETs inhibition, several more pathogens, like *Campylobacter jejuni*, *Neisseria gonorrhoeae*, and *Escherichia coli* K1, are also able to synthesize sialylated glycans, leading to the assumption that comparable strategies are also used here to escape NETs (70, 77–79). Since polySia is also able to modulate NET formation and the activity of NETs, it seems likely that polySia-positive bacteria, like *Escherichia coli* K1 and distinct *Neisseria meningitides* strains, are able to trigger polySia-dependent mechanisms (80, 81). However, until now, no study has examined the impact of polySia during their invasion in the context of NETs.

Interestingly, obstructing NET release is not the only tool of bacteria, to elude the immune system. Several pathogens are able to circumvent NET-mediated killing. *Streptococcus pneumoniae* (*S. pneumoniae*) for instance, which is one of the major causes of mortality and morbidity, circumvents NET-mediated

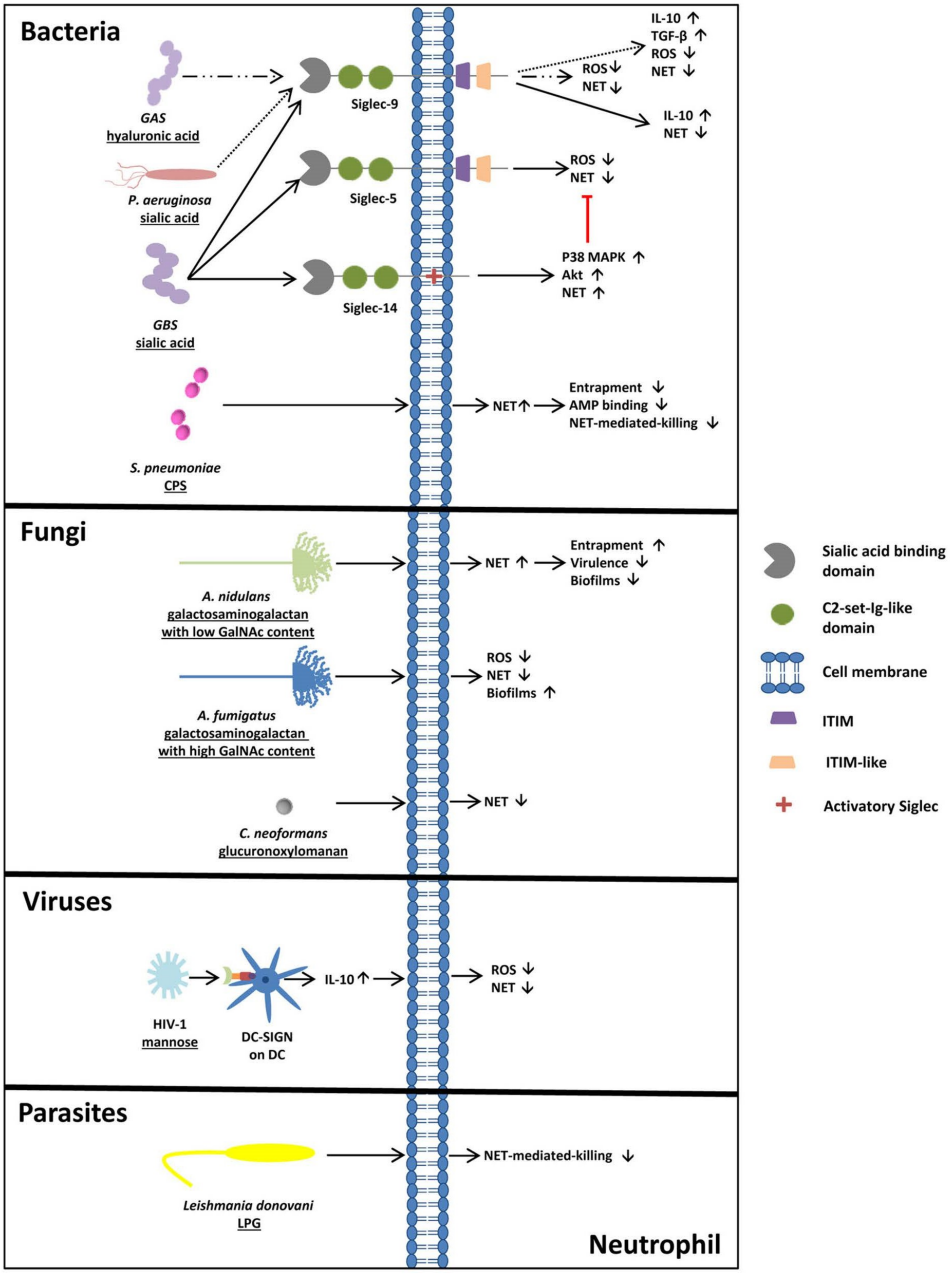

FIGURE 2 | Glycosylation of pathogens- a powerful tool to circumvent NET-mediated entrapment and killing. Bacteria circumventing NET release or NET-mediated killing. *S. pneumoniae* induces NET release, but NET-mediated killing and entrapment is bypassed due to the capsular polysaccharide (CPS). Pathogens like GBS,

(Continued)

FIGURE 2 | GAS, and *P. aeruginosa* exploit Siglecs for their own purposes. Via sialic/hyaluronic acid binding to Siglecs, IL-10 as well as TGF-β production are upregulated and NET release is inhibited. Also fungi circumvent NET release/ NET entrapment by glycans. Compared to the less pathogenic *A. nidulans*, surrounded by galactosaminogalactan with a low amount of GalNAc, *A. fumigatus*, surrounded by galactosaminogalactan with a high GalNAc content, inhibits NET release and ROS production and forms more adherent biofilms, explaining the differences in virulence of these two fungi strains. In addition, the capsular strain of *C. neoformans* inhibits the release of NETs due to its glucuronoxylomannan coating, whereas acapsular strains of *C. neoformans* induce NETosis. In addition, sialylated viruses like HIV-1 prevent ROS-dependent NET release by DC-SIGN engagement and parasites, like *Leishmania donovani* circumvent NET mediated killing by lipopeptidoglycan (LPG) on its surface.

killing through its polysaccharide capsule (82). Encapsulated *S. pneumoniae* strains show significantly reduced trapping by NETs compared to non-capsulated strains (**Figure 2**). Furthermore, *S. pneumoniae* contain positively charged lipoteichoic acid on their surface, which increases the electrochemical repulsion of antimicrobial peptides (82).

Fungi

Moreover, it is not only bacteria that circumvent NET-mediated entrapment and killing. In their research, Rocha et al. (83) detected that the fungus *Cryptococcus neoformans* (*C. neoformans*) is surrounded by capsular polysaccharides containing glucuronoxylomannan. The wild type of strain inhibits the release of NETs, whereas acapsular mutants or mutants surrounded by glucuronoxylomannogalactan induce NETosis (**Figure 2**) (83).

Furthermore, *Aspergillus fumigatus* (*A. fumigatus*), which accounts up for around 80% of all invasive *Aspergillus* infections, shows resistance against NET-induced damage due to the production of cell-wall-associated galactosaminogalactan and a secreted form of galactosaminogalactan. Galactosaminogalactan consists of galactose and N-acetylgalactosamine (GalNAc) residues. Since galactosaminogalactans play a certain role in host-pathogen interactions, as they are required for biofilm formation, a galactosaminogalactan-deficient mutant of *A. fumigatus* exhibited reduced virulence (**Figure 2**) (84–88).

Interestingly, *Aspergillus nidulans* (*A. nidulans*), a strain producing galactosaminogalactans with a lower content of GalNAc residues in comparison to *A. fumigatus*, was found to be less pathogenic, formed less-adherent biofilms, and was, therefore, more susceptible to NET-induced damage. Since *A. nidulans* is only known to induce pathologies in patients with chronic granulomatous disease (CGD), characterized by an impaired NADPH oxidase complex, further investigations concerning the influence of NADPH oxidase revealed that cell-wall-bound galactosaminogalactans in *A. fumigatus* enhance resistance against NADPH-oxidase-dependent neutrophil extracellular damage. This might explain the increased virulence of *A. nidulans* in CGD patients (88).

Viruses

Interestingly, also viruses are able to evade immune control mechanisms. The HIV-1 virus, for instance, counteracts NET formation by engaging the C-type lectin DC-SIGN (CD209) on dendritic cells via its envelope glycoprotein containing more high mannose than complex N-glycan structures. The binding induces the production of IL-10, contributing to the inhibition of ROS-dependent NET release upon TLR7 and TLR8 engagement (89) (**Figure 2**). This study let suggest that also glycans of other viruses can target DC-SIGN, such as Ebola virus, the Japanese encephalitis virus and of the hepatitis C virus, that may also influence in an indirect way the formation of NET (90–92).

Parasites

Leishmania donovani, a protozoan parasite, contains lipopeptidoglycan on its surface and mutants lacking lipopeptidoglycan show less survival in NET in comparison to the wild type strain (**Figure 2**) (93). In addition, virulent strains of *Leishmania donovani* can contain high amount of sialylated glycans representing binding partners for Siglec-5 leading to an inhibition of ROS production in macrophages (94). Comparable mechanism of these *Leishmania donovani* glycans, which are terminated with α2,3- and α2,6-linked sialic acid residues, may also take place on neutrophils and might also be used by other sialic acid positive parasites.

CONCLUSION

This review gives a short summary concerning the impact of glycans to modulate the formation and activity of NET describing glycan dependent mechanisms of endogenous cells to prevent the activation of neutrophils or to inactivate cytotoxic molecules of NET, which are, however, also used as an escape strategy by distinct pathogens. All of the outlined examples show that glycans play a key role in the biology of NET and that they have great potential as a therapeutic tool in NETs-associated pathologies.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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5. Discussion

5.1 The evolution of Siglecs

Within the last few years knowledge about the role of Siglecs in mammals increased significantly, mainly through the investigation of knockout mice. Knocking out the gene for Siglec-G in mice increased autoimmunity (Müller et al. 2015), while Siglec-F -/- mice develop neurodegenerative diseases (Siddiqui et al. 2019) and the depletion of the gene encoding for MAG causes a reduced preservation of myelin integrity and regulation of neuronal growth and is associated with multiple sclerosis (Lehmann et al. 2004, Li et al. 1996, DeBellard et al. 1996). In addition, Wu *et al*. detected that Siglec-E deficient dendritic cells fail to internalize TLR-4 upon *Escherichia coli* infection, resulting in increased tumor necrosis factor α (TNFα) and IL-6 release, contributing to an increased immune response upon infection (Wu, Ren and Chen 2016). Even if these distinct examples show that in mammals the balance between pathogen defense and pathophysiological manifestations is modulated by Siglecs, knowledge about the evolution of Siglecs will further support the deciphering of their functional roles in health and diseases. However, studies in lower vertebrates are missing. Thus, with the aim to increase the knowledge about the evolutionary role of Siglecs and to contribute to the organization of the ever-growing number of complex immune regulatory receptors, we performed blast researches, sequence alignments and phylogenetic analysis and discussed the results obtained in the context of housing situations and physiological changes occurring during the evolution of different species. In addition, as until now only the expression of MAG and Siglec-15 in teleost's was described (Lehmann et al. 2004, Angata et al. 2007), we profiled the expression of the genes encoding Siglec-1, CD22, MAG and Siglec-15 in two different salmonid fishes (maraena whitefish, rainbow trout) and one percid fish (pikeperch) and investigated the expression of Siglecs and Siglec-associated genes of maraena whitefish after acute handling stress. Acute stress was provoked by chasing and netting fishes for a period of one minute, coming along with air exposure. Since for CD22, MAG and Siglec-15 specific functional characteristics, like highly conserved cysteines or glycosylation sites are well known (Ereño-Orbea et al. 2017, Wasim et al. 2019, Angata et al. 2007, Pronker et al. 2016), we also analyzed the sequences and compared the sialic acid binding domain of murine MAG (pdb: 5LF5) and the first Ig-domain of maraena whitefish MAG as well as the sialic acid binding domain of human CD22 with the first Ig-domain of maraena whitefish CD22 using YASARA (19.9.17). However, although the sequences of Siglec-1, CD22, MAG and Siglec-15 from pikeperch, shared indeed high sequence identity to

the counterpart sequences of yellow perch, rather low identity with the sequences of the other fish species was detectable. To take into account the possibility of a deficient annotation, sequences of pikeperch were excluded from sequence analysis.

5.1.1 Siglec-1, CD22, MAG and Siglec-15 emerged first 400 million years ago

Data bank research revealed, that the sequences of Siglec-1, CD22, MAG and Siglec-15 appeared first during the evolution from cartilaginous fish (e.g. shark) to bony fish and are therefore conserved since around 400 million years, when the separation of tetrapods (human) and teleosts (fish) took place, indicating an evolutionary pressure on these genes, independent of the habitat and independent of physiological conditions (**Figure 11**) (Cao and Crocker 2011, Bornhöfft et al. 2018, Lehmann et al. 2004, Angata et al. 2007). Since jawless fishes are the only vertebrate without myelin, those lack the gene encoding for MAG, the Siglec which contributes to axon-myelin stabilization in human (Knowles 2017, Zalc 2006, Sun et al. 2004). In addition, they are CD22 negative as in mammals CD22 is responsible for the downregulation of the auto-reactivity of the BCR and jawless fishes are BCR negative (Boehm et al. 2012, Nitschke 2005, Tedder et al. 2005). Siglec-15 contributes to the development of bones and is therefore firstly found during the evolution from cartilaginous fish to bony fishes, assumed to be involved in the strengthening of cartilages (Sato et al. 2018, Bornhöfft et al. 2018) (**Figure 11**).

Interestingly, no sequences encoding CD33r-Siglecs were detectable in fish, while human are characterized by the highest amount of CD33r-Siglecs (Bornhöfft et al. 2018). Since compared to fish, where the innate immunity is of supreme importance for health (Uribe et al. 2011), in human the adaptive immune system is well-developed and indispensable (Alberts B 2002), it is assumed that the high amount of CD33r-Siglecs is a human feature to curb auto-reactivity. Remarkably, according to Varki *et al.* the loss of the ability of human to synthesize the self-marker, Neu5Gc, coming along with an excessive production of, the “new” self-marker, Neu5Ac on endogenous cells, while also pathogens are decorated with Neu5Ac, caused an increased susceptibility of human to autoimmune diseases. For instance, Neu5Ac on foreign structures like LPS or the influenza virus might cause the production of auto-antibodies, resulting in excessive autoimmune reactions and damage of endogenous tissues (Varki 2017a, Ang et al. 2002).

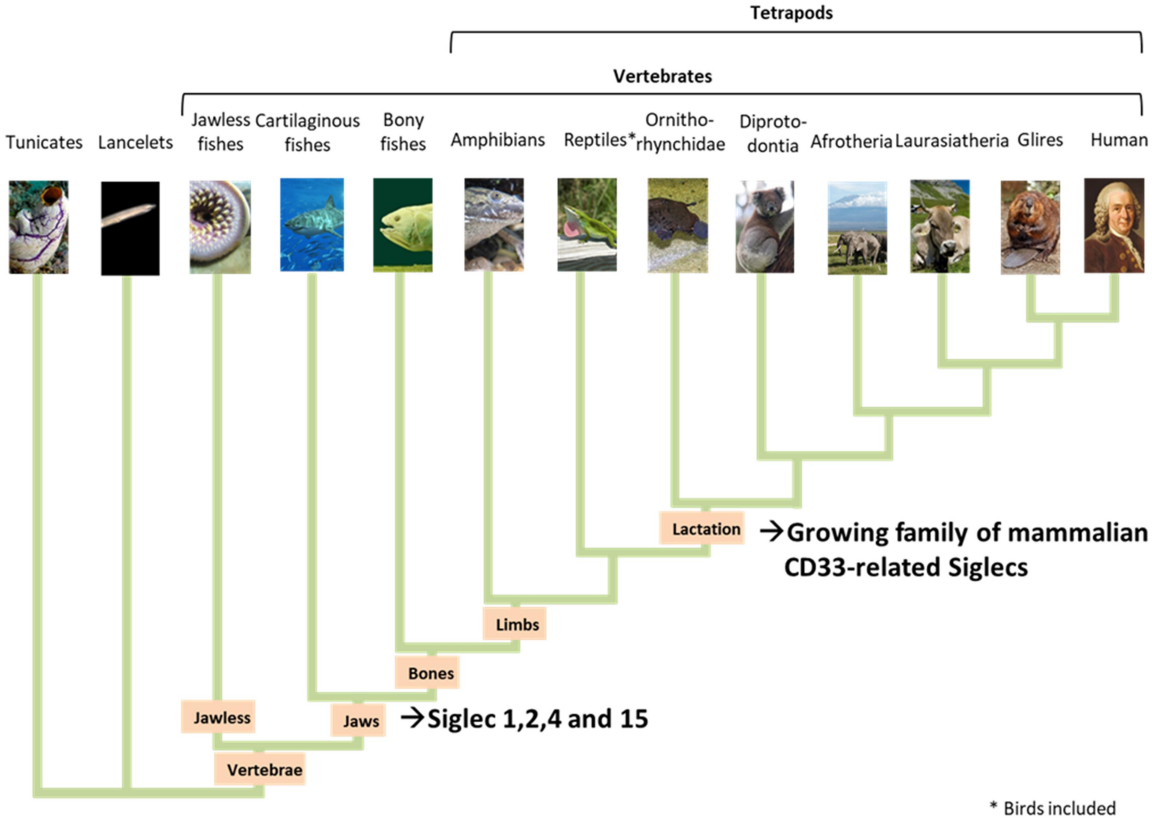


Figure 11: Milestones of the evolution of Siglecs. With the emergence of jaws, Siglec-1, CD22, MAG and Siglec-15 occurred, while the group of CD33-related Siglecs expanded with the onset of lactation (Bornhöfft et al. 2018) CC-BY.

5.1.2 The indispensable role of Siglecs: From lower vertebrates to human

While investigating the expression of those four highly conserved receptors in different tissues of the investigated fishes, similar to humans and independent of the investigated fish, we found high levels of Siglec-15 and Siglec-1 in tissues housing, mainly macrophages like the spleen and the head kidney (O'Reilly and Paulson 2009, Crocker et al. 2007, Macauley et al. 2014, Bornhöfft et al. 2020). Highest levels of CD22 were detected in spleen of pikeperch and in the head kidney of the investigated salmonids, while we detected an unexpected expression pattern across the investigated fish for the gene encoding for MAG. We discovered MAG expression in lymphoid tissue. MAG was most highly expressed in the gills of maraena whitefish and rainbow trout, while in pikeperch highest expression levels for MAG were detected in spleen. Since the results indicate, that MAG expression in fishes is not restricted to cells of the nervous system, in a next step we analyzed the expression of Siglecs of specific, enriched cells of maraena whitefish. Using a percoll-gradient-centrifugation we separated erythrocytes from leukocytes. Following that the leukocytes fraction was further processed using flow cytometry, generating fraction I (lymphocytes, monocytes,

macrophages) and fraction II (granulocytes). Surprisingly, regarding all four investigated receptors, highest expression levels were detectable in the erythrocyte fraction (**Figure 12**) (Bornhöfft et al. 2020).

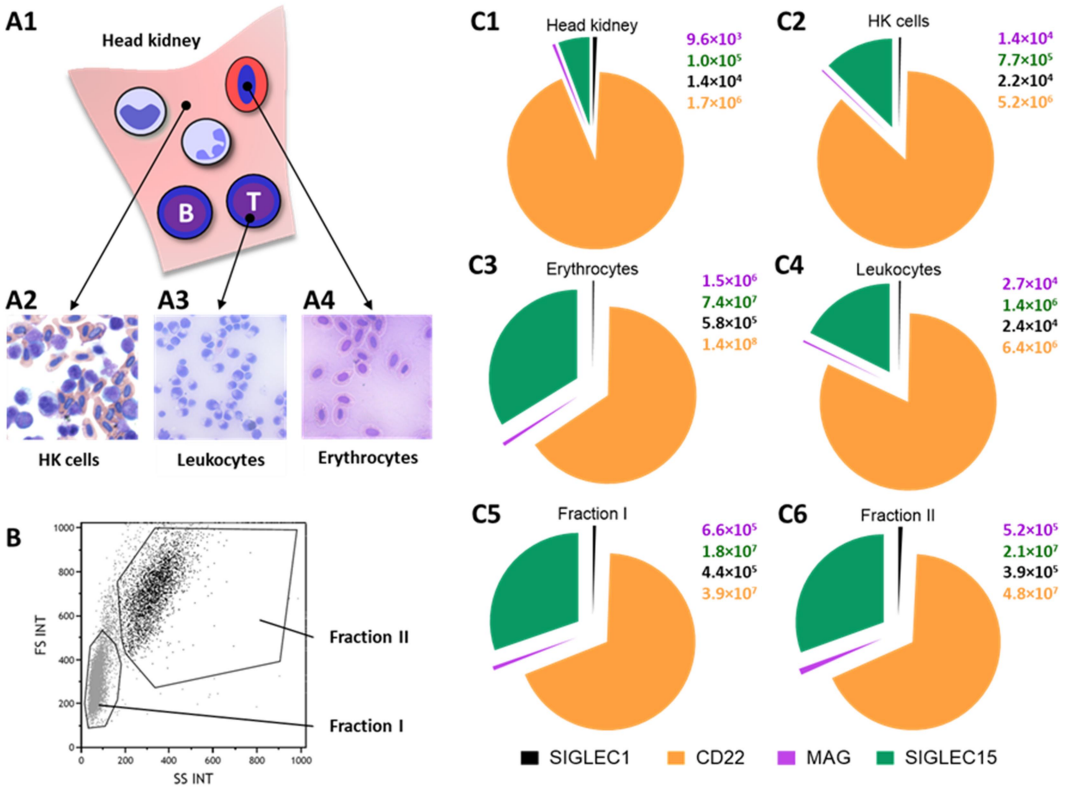
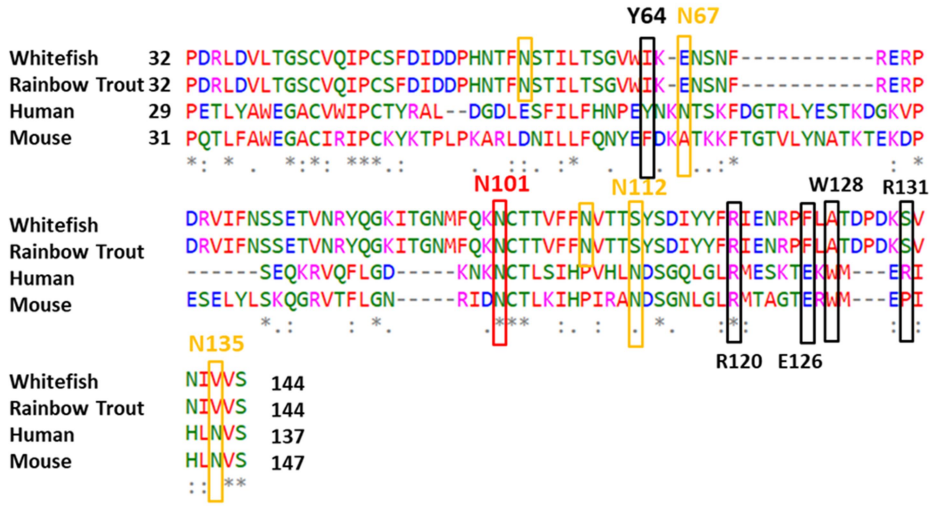


Figure 12: Pie chart of the copies/µg for Siglec-1, CD22, MAG and Siglec-15. A) Gruenwald-Gimsa staining of the isolated cell types. **B**) Exemplary flow cytometric isolation of fraction I and fraction II **C1-C6**) Expression of Siglec-1, CD22, MAG and Siglec-15 is shown for whole head kidney, extracted head-kidney cells, erythrocytes, a heterogeneous leucocyte suspension, a fraction (I) mainly containing lymphocytes and monocytes/macrophages as well as fraction (II) enriched with granulocytes (n = 5). (Bornhöfft et al. 2020) CC-BY.

In mammals, erythrocytes are mainly responsible for the transportation of oxygen. They are produced in the bone marrow; dividing stem cells differentiate to nucleated erythroblasts, which differentiate to reticulocytes without nuclei, while retaining their cell organelles. Those reticulocytes are than released into circulation, where they represent 0.5-2.5% of the red blood cells and further mature to organelle-less erythrocytes (Raja-Sabudin et al. 2014, Ney 2011). However, since erythrocytes in fishes are nucleated, exerting immunological functions like phagocytosis, antigen presentation and modulation of leukocyte activity (Passantino et al. 2007, Puente-Marin et al. 2018, Morera et al. 2011, Workenhe et al. 2008), an involvement of Siglec-sialic acid interaction in host pathogen interaction in fish is likely. Interestingly, unlike in mammals,

CD22 expression was further detectable in all the other investigated cell fractions. This leads to the question whether or not the expanded expression of CD22 might compensate the lack of CD33-related Siglecs in fishes (Bornhöfft et al. 2020) ,since in mammals, CD22 is mainly known to reduce the auto-reactivity of the BCR by the binding of sialic acids in cis or/and trans direction (Müller et al. 2013). Thus, in order to further elucidate the functionality of CD22 in fishes, we performed sequence alignment and created 3D models using YASARA. According to Orbea *et al.* CD22 possesses twelve N-glycosylation sites, while only six are in close proximity to the V-set Ig-like, the sialic acid binding domain (N_{67}; N_{101}; N_{112}; N_{135}; N_{164}; N_{231}). While N_{67}, N_{112}, N_{135}, N_{164}, N_{231} can be exchanged by an A residue without losing functionality, exchanging N_{101} leads to the disruption of the protein expression probably since N_{101} is essential for the correct folding of the protein (Ereño-Orbea et al. 2017). Interestingly, sequence alignment of CD22 from maraena whitefish and rainbow trout revealed the presence of all N-glycosylation sites, but N_{135} (**Supplement 1**), although they are at marginally different locations compared to human. However, several amino acids known to be involved in sialic acid binding are absent in maraena whitefish and rainbow trout CD22, indicating differences in the sialic acid binding properties of mammalian and fish CD22. This suggestion was enhanced by the detected differences in the 3D structures of the V-set Ig-like domain of human and maraena whitefish CD22 (**Figure 13**). In addition, only CD22 from rainbow trout was ITIM positive, while CD22 of maraena whitefish lacked an ITIM. Sequence alignments of CD22 orthologs from rainbow trout and maraena whitefish revealed a high degree of sequence identity, while the intracellular area of CD22 of maraena whitefish was missing. Thus, the presence of an ITIM cannot be excluded (**Supplement 1**) (Bornhöfft et al. 2020).

A)



B)

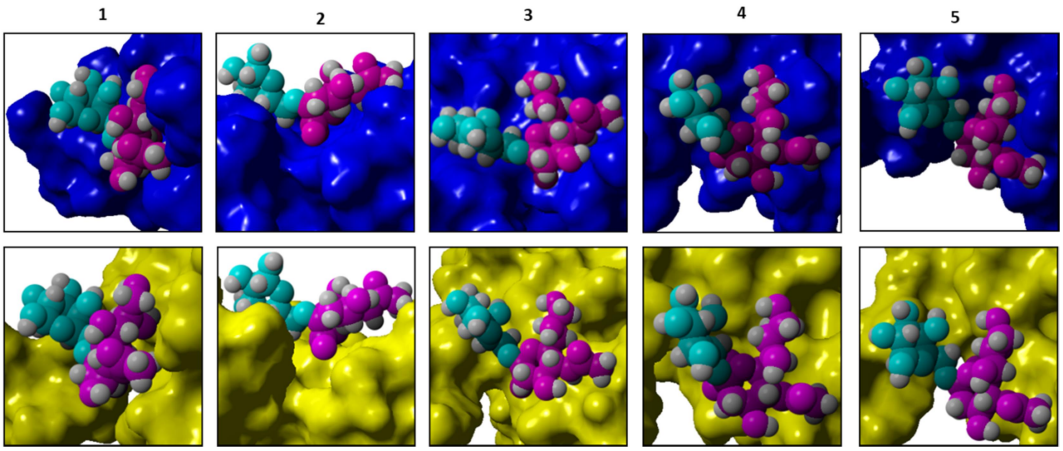


Figure 13: Sequence and structural comparison of CD22. A) Sequence alignment of the V-set Ig-like domain of CD22 from maraena whitefish, rainbow trout, human and mouse. Sequences of CD22 form human and mouse were available in the NCBI database (human CD22: NP_001762.2, mouse CD22: NP_033975.3). The V-set, Ig-like domains of human and murine CD22 were determined by the SMART program. The first Ig domain of maraena whitefish and rainbow trout was determined by sequence alignment. The different colors indicate the chemical properties of the amino acids as follows: red: small hydrophobic/aromatic amino acids; blue: acidic amino acids; magenta: basic amino acids; green: hydrophilic, polar, and small amino acids. Black boxes indicate the amino acids that are essential for sialic acid binding, while the red boxes show the conserved N-glycosylation site, N_{101}. In addition, the orange boxes indicate further amino acids known to be a target for N-glycosylation. Numbering is based on the human sequence of CD22. Alignments were performed using the Clustal Omega tool of EMBL-EBI. **B)** The 3D modelling was performed using YASARA. Modelling of human CD22 was based on pdb 5VKM, including 5 point mutations (N_{67}A, N_{112}A, N_{135}A, N_{164}A, N_{231}A, bound α2,6 sialyllactose (Ereño-Orbea et al. 2017), while the sequence of the V-set Ig-like domain of maraena whitefish CD22 was based on sequence alignments. Sequence of human CD22 was shortened to the first Ig domain. CD22 models of human and

maraena whitefish CD22 are shown in blue and yellow, respectively. Bound glycans are specifically labeled as follows: galactose: cyan, sialic acid: pink [Caption and Figures are slightly modified from: (Bornhöfft et al. 2020) CC-BY].

Siglec-15 expression was detected in all investigated cell types, too. Sequence analysis of the two salmonid fishes revealed that the lysine responsible for the interaction of Siglec-15 with DAP molecules and cysteines, known to contribute to the functional tertiary structure, are as conserved from mammals to vertebrates as R_{143} known to be essential for sialic acid binding (**Supplement 2**). However, researchers investigating the sialic acid binding potential of the cloned ortholog from Siglec-15 from zebrafish detected no strong binding to the glycans tested *ex vivo*, independent of the availability of cysteine residues (Angata et al. 2007, Bornhöfft et al. 2020).

Interestingly, unlike in human, MAG was also detected on immune cells. As this indicated a potential further function of MAG in lower vertebrates, we analyzed the sequences of the genes encoding for MAG in the two salmonids (salmon, maraena whitefish). Notably, sequence analysis revealed, that all sequences comprise an intracellular 'immune receptor tyrosine-based inhibition motif' (ITIM), whereas the mammalian ortholog lack these motifs (**Supplement 3**). In addition, all N-glycosylation sites within the sialic acid binding domain, which are essential for the specific axon-myelin spacing (9-12 nm) were conserved throughout the investigated salmonids (Pronker et al. 2016, Bornhöfft et al. 2020). The presence of intracellular ITIMs in MAG was already published 2004 by Lehmann *et al*.. They investigated zebrafish and pufferfish and detected ITIMs, and assumed that the conservation of those motifs, might contribute to essential biological functions which differ from those described for mammalian MAG (Lehmann et al. 2004). Therefore, the presence of the ITIM, in combination with the conservation of all N-glycosylation sites as well as its expression on immune cells, encourages us to hypothesize that in salmonids and cyprinids MAG acquire an immune regulatory function (Bornhöfft et al. 2020).The ITIM motif seems to be a hereditary feature of Siglecs, which is consistent in most Siglecs, but got lost during the evolution of higher vertebrates (Bornhöfft et al. 2018, Lehmann et al. 2004). Since, nevertheless copy numbers of MAG within the brain of maraena whitefish exceeded those in lymphoid tissue (Bornhöfft et al. 2020), the key task of MAG seems to be the maintenance of axon-myelin spacing within the nervous system, as it is the case in human. In human, MAG expression is restricted to Schwann cells and oligodendrocytes and as a cell-adhesion and bi-directional molecule, which interacts

with specific neuronal glycolipids, MAG contributes to the axon-myelin spacing (Cagnoni et al. 2016, Quarles 2007, Sun et al. 2004).

Since for both, the successful maintenance of axon-myelin spacing as well as for the inhibition of an immune response, the binding of sialic acid is essential, in a further step, we investigated the sialic acid-binding properties of the gene encoding for MAG in maraena whitefish. Remarkably, all amino acids known to be involved in sialic acid binding (Pronker et al. 2016) were conserved from mammals to lower vertebrates (**Figure 14 A**), indicating that the preservation of the ability to bind sialic acid is likely. With the aim to confirm that, we performed 3D modelling analysis of the sialic acid binding domain of maraena whitefish MAG using YASARA. Regarding **Figure 14 B** showing the comparison between murine and maraena whitefish MAG, it becomes apparent, that the binding pocket of of Neu5Ac-α2,3-Gal-β1,3-GalNAc is almost identical, indicating similar sialic acid binding properties of MAG in fish and mammals (Bornhöfft et al. 2020).

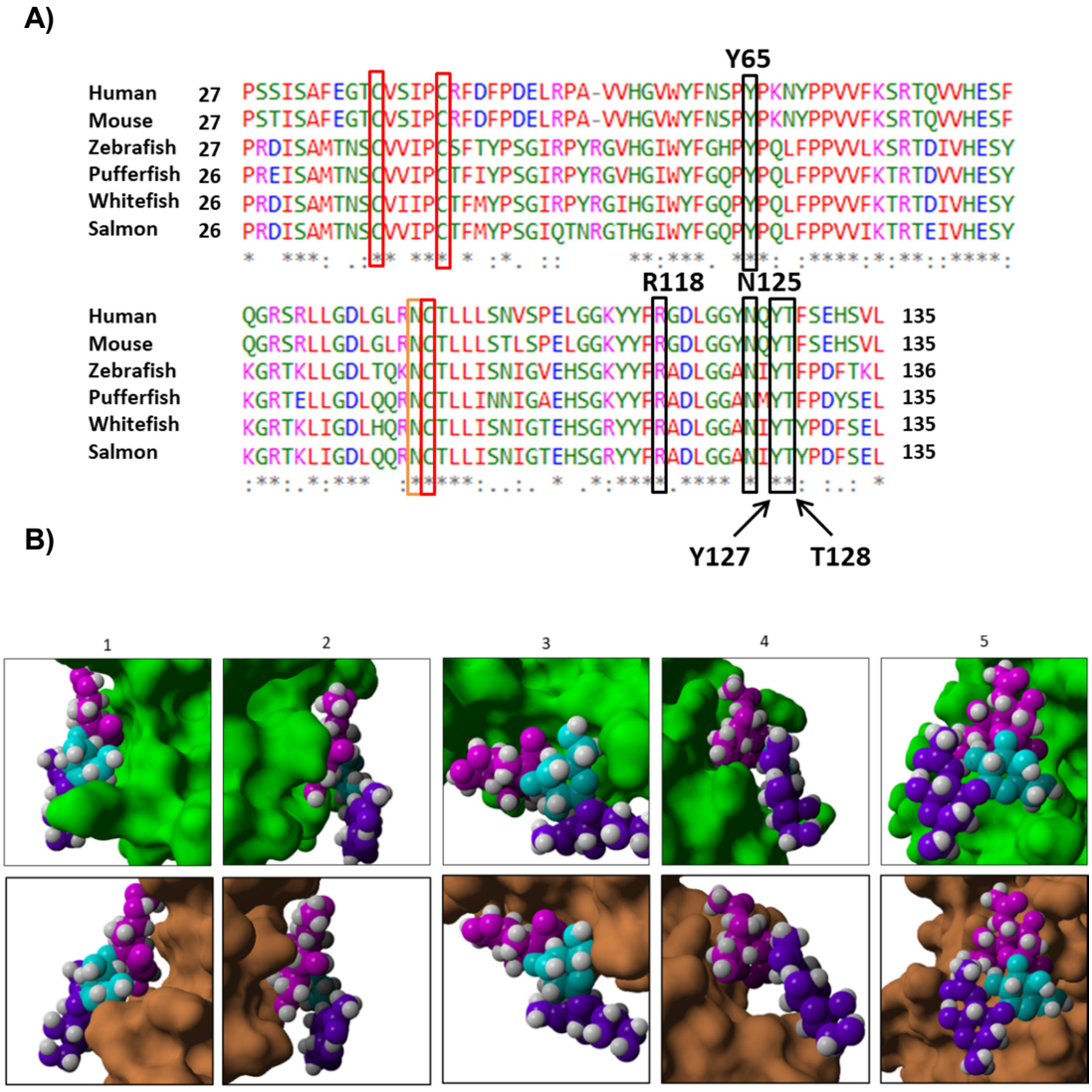


Figure 14: Sequence and structural comparison of murine MAG and maraena whitefish MAG. A)The sequence of mouse, human, zebrafish and pufferfish MAG were available in the NCBI data bank and listed in the following: Zebrafish MAG: XP_021337068.1; Pufferfish MAG: XP_011616490.1; Mouse MAG: XP_030098048.1; Human MAG: AAB58805.1. The V-set Ig-like domain was determined by analyzing the pdb sequence of murine MAG (pdb 5LF5) as well as the human sequence with SMART. For pufferfish, zebrafish, maraena whitefish and salmon V-set-Ig-like domains were determined by sequence alignment. Alignments were performed using the Clustal Omega tool of EMBL-EBI. The different colors indicate the properties of the amino acids. Red: small hydrophobic/aromatic amino acids; blue: acidic amino acids; magenta: basic amino acids; green: hydrophilic, polar and small amino acids. Black boxes label amino acids involved in sialic acid binding, while the orange box shows an amino acid known to be a target for N-glycosylation. Amino acid labeling is based on the human sequence. **B**) The modeling of murine and maraena whitefish MAG was performed using YASARA. By inserting amino acid exchanges to the published murine MAG (pdb 5LF5; green) the 3D model of maraena whitefish MAG was designed (brown). Both sequences were shortened to the V-set Ig-like domain. The sialic acid binding domain of maraena whitefish MAG and murine MAG are shown in 5 different perspectives. Bound glycans are specifically labeled: Galactose is labeled in cyan, N-acetyl-D-galactosamine is labeled in purple and sialic acid is labeled in pink [Caption and Figures are slightly modified from: (Bornhöfft et al. 2020) CC-BY].

Furthermore, in order to determine the influence of handling stress on the expression of Siglecs in different tissues of maraena whitefish, we exposed the fishes to acute handling stress coming along with air exposure. Remarkably, expression profiles changed in a tissue-specific manner. The expression of Siglec-1 and CD22 increased within the brain, while expression levels decreased in the heart and in the spleen (Siglec-1) or in muscle (CD22). Interestingly, in all the other tissues the expression levels were unaffected by acute handling stress. MAG expression in the brain remained unchanged but decreased in lymphoid tissues like the spleen and head kidney, supporting the assumption of an immunological role of MAG in fishes. Moreover, less MAG transcripts as well as Siglec-15 transcripts were detectable in the gills, the respiratory organ of fishes upon acute stress. As the gills might be directly affected by air exposure taking place during the handling procedure, this decrease might prevent tissue damage caused by an exaggerated immune response triggered by handling stress (Bornhöfft et al. 2020).

Overall these results provide the answer to the first question formulated in the objectives: “Did the evolution of immunological mechanisms comes along with the development of distinct Siglecs and does stress, linked with increased susceptibility to infections, influence the expression pattern of Siglecs?”. The analysis showed that the first emergence of CD22, MAG and Siglec-15 comes along with the evolution of physiological key elements, like the adaptive immune system. In addition, the data obtained demonstrate that a basal expression of Siglec-1, CD22, MAG and Siglec-15 is conserved from fish to human, indicating that already in lower vertebrates Siglecs play an indispensable role in the maintenance of equilibrium between pathogen defence and pathophysiological manifestations. In addition, the data let suggest that also in mammals stress might influence the expression pattern of immunomodulatory Siglecs. However, so far this hypothesis is only a speculation and has to be proofed in future.

5.2 Sialylated glycoconjugates inhibit the release of NETs potentially through Siglec-interaction

Several studies imply that pathogens within the ejaculate as well as sperm themselves (based on *in vitro* experiments) induce NET release, contributing to decreased fertility (Alghamdi et al. 2009, Zambrano et al. 2016, Hahn et al. 2012). Thus, the maintenance of a balance between pathogen defence and the protection of fertility by preventing sperm from entrapment by NETs, is essential. Already in the 1980s DNase I was detected in human seminal plasma (Singer et al. 1985), probably decreasing the amount of sperm entrapped by NETs (Zambrano et al. 2016), and polysialic acid was

detected in semen, representing a cytoprotective component (Simon et al. 2013). But both mechanisms are limited in their sphere of activity. We assume that in order to ensure fertility Siglec-sialic acid mediated interactions are needed to decrease neutrophils immune response and thereby protect sperm from entrapment. Therefore, we wanted to investigate the potential of naturally occurring cervical mucins of different stages of oestrous cycle, as highly glycosylated and sialic acid carrying naturally occurring glycoconjugates (Galuska et al. 2017b), to inhibit NET release via the interaction with Siglecs. To this end a bovine *in vitro* model was used simulating the physiological presence of mucins in the cervix. Cattle were selected, since in contrast to rodents the passage of spermatogonia through the cervix is comparable in humans (Suarez and Pacey 2006). For the precise purification of mucins, stages of the oestrous cycle were determined. The isolation was performed by the combination of a density gradient ultra-centrifugation with a size exclusion chromatography and desalting. Luteal, estrus and follicular mucins were added to PMA/Ionomycin or LPS stimulated neutrophils and NET release was investigated. Furthermore, in order to determine the role of sialic acid within the inhibitory effect of cervical mucins, chemically and enzymatically modified cervical mucins were incubated with neutrophils (Bornhöfft et al. 2019a).

In a further step we wanted to determine the effect of a distinct sialic acid-motif present on the surface of mucins. To this end, we generated nanoparticles coated with oligomers of α2,8-linked sialic acid residues and investigated the potential of those oligosialylated conjugates to inhibit NET release (Bornhöfft et al. 2019b).

5.2.1 Cervical mucins inhibit neutrophils activation in a sialic acid-dependent manner

In order to examine the capability of bovine cervical mucins to prevent the release of NET, we supplemented different concentrations of luteal, estrus and follicular cervical mucins to PMA/Ionomycin or LPS (from *Pseudomonas aeruginosa*) stimulated neutrophils. While 1 µg/µL of cervical mucins was sufficient to inhibit NET release induced by LPS, the addition of 10 µg/µL mucins reduced NET release induced by PMA/Ionomycin by only around 40% (Bornhöfft et al. 2019a), although both - PMA/Ionomycin and LPS stimulation activate the Raf-MEK-ERK pathway, finally stimulating the production of ROS by the NADPH oxidase complex, contributing to the translocation of NE and MPO to the nucleus, DNA decondensation as well as histone H3 citrunillation (Sorvillo et al. 2019, de Bont et al. 2018). Differences in the efficiency of the applied mucins on NET release might be the effect of the non-physiological

PMA/Ionomycin concentrations used during the experiments. Only when 20 µg/µL cervical mucins were applied, NET release was prevented, independent of the stimuli used. Bovine cervical mucins seem to inhibit neutrophil activation completely, as nuclei retain their segmented structure and granularity remained, independently of the used stimuli and independent of the stage of oestrous cycle they were from (**Figure 15**; for a higher magnification see **Supplement 4**) (Bornhöfft et al. 2019a).

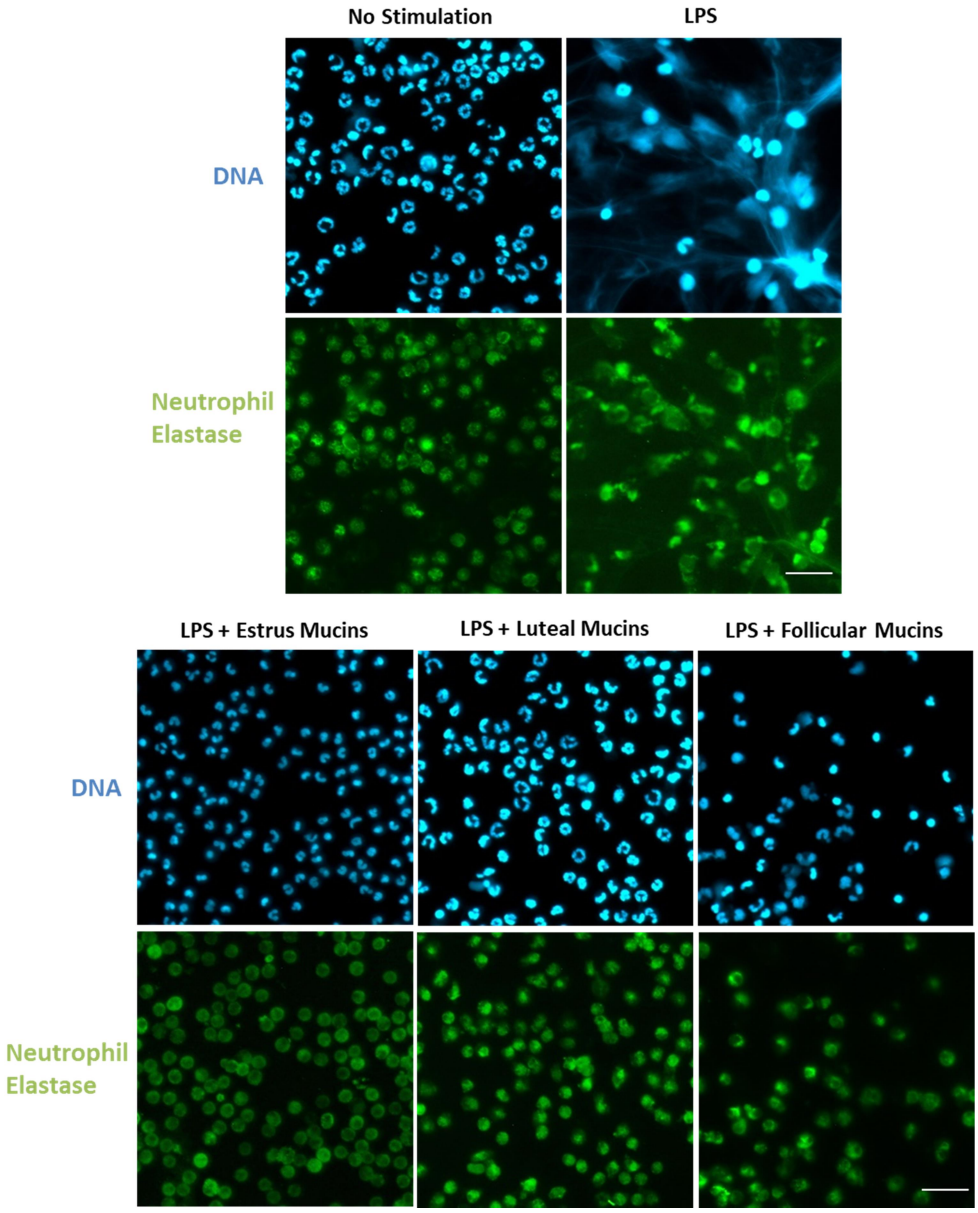


Figure 15: Sialic acid carrying bovine cervical mucins completely inhibit neutrophil activation stimulated with LPS. Neutrophils were isolated and unstimulated neutrophils, neutrophils stimulated with 20 µg/mL LPS from *Pseudomonas aeruginosa* and cells stimulated with LPS co-incubated with mucins were stained with DAPI (blue) and anti-neutrophil elastase antibody (green). Nuclei retain their segmented structure and granularity remains, when mucins were applied [modified (Bornhöfft et al. 2019a) CC-BY] Scale bar: 20 µm.

Since previous work of our research group detected sialic acid (monomers, dimers, trimers of Neu5Ac) on bovine cervical mucins (Galuska et al. 2017b) and Lizcano *et al*. described a Siglec-sialic acid dependent mechanism to prevent an exaggerated release of NETs within circulation (Lizcano et al. 2017), we further questioned, whether or not also in this case, sialic acid is responsible for the inhibitory effects of mucins.

To this end we modified sialic acid via mild periodate oxidation or removed the sialic acids by neuraminidase digestion as well as by hydrolysis. Interestingly, when hydrolyzed follicular and estrus cervical mucins were applied, no remaining inhibitory function of mucins was detectable and NET release occurred, while in the case of luteal mucins, the reduced sialyation was not sufficient to allow NET release and only a loss of the segmented nucleus structure was observed. Since the hydrolytic conditions (80°C, 1 N acetic acid) may result in additional, undesired structural changes of the protein backbone, we further oxidized the terminal sialic acid residues (Bornhöfft et al. 2019a). The oxidation with sodium periodate alters the exocyclic side chain of the terminal sialic acid residues. The C9 and C8 are released, leaving a C7 body with a highly reactive aldehyde group behind. This aldehyde is in the following reduced, resulting in a hydroxyl group at the C7 of the terminal sialic acid (Inoue and Inoue 2003). Remarkably, the conversion of the C9-sugar to a C7-sugar prevented the inhibitory effects of estrus and luteal mucins, but not of follicular mucins. The inhibition of NET release still occurs. In addition sialic acids were enzymatically released with neuraminidases. The enzymatic treatment of estrus and luteal mucins completely prevent the inhibitory effects, whereas in the case of follicular mucins a lower impact was detectable. Around 50% of the neutrophils still do not undergo NETosis. This might be a consequence of an incomplete release of α2,6-linked sialic acid, as confirmed by the agarose gel blot (Bornhöfft et al. 2019a).

Thus, the terminal sialic acid residues seem to be essential for the inhibitory effects of bovine cervical mucins on neutrophil activation. As furthermore, RT-qpCR analysis of Siglecs expressed by bovine neutrophils revealed the presence of CD22, MAG, Siglec -5, -8 and -14, also here, similar to the process described to take place within circulation; Siglec-sialic acid interactions might be involved in the inhibition of NET release (Bornhöfft et al. 2019a). Probably due to the broad diversity of sialic acid derivatives and therefore the possible engagement of several different Siglecs, neutrophil activation is completely inhibited by cervical mucins- nuclei retained their segmented structure, granularity remained and the rupture of the plasma membrane was prevented (Bornhöfft et al. 2019a) (**Figure 16**).

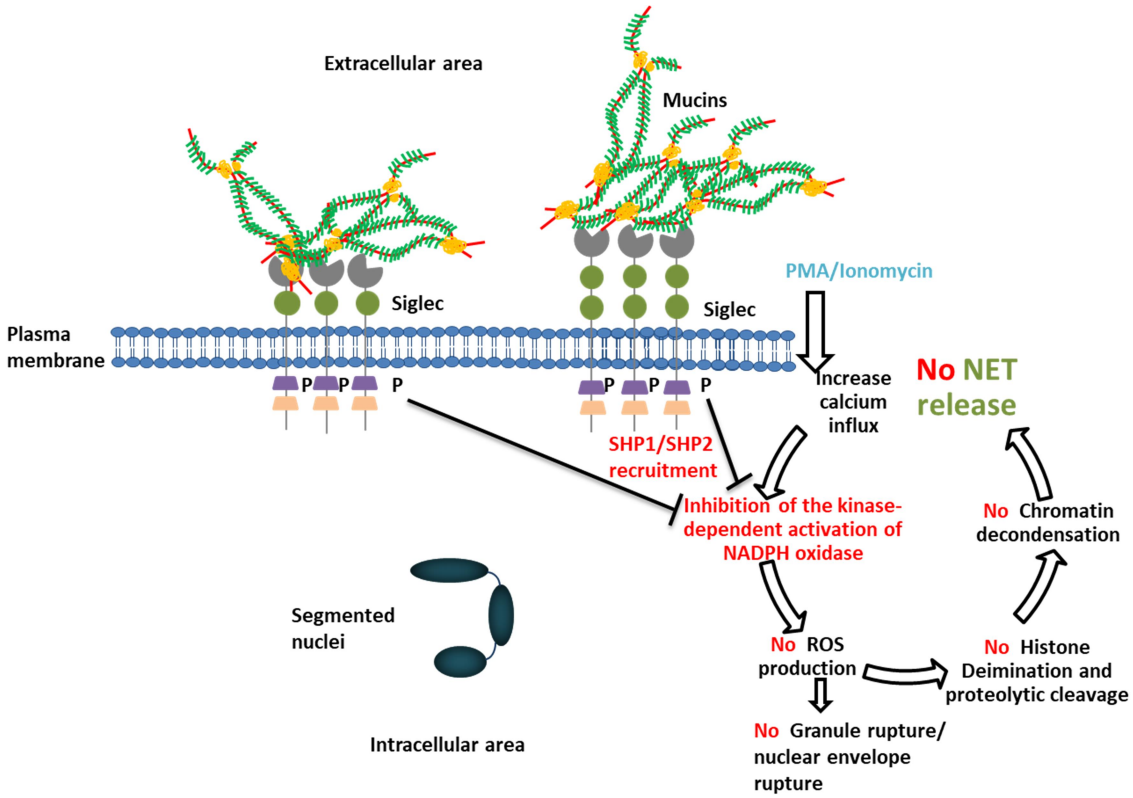


Figure 16: Assumed mechanism behind the inhibition of NET release by bovine cervical mucins. Since bovine cervical mucins are highly glycosylated proteins we assume that the engagement of several Siglecs by different sialic acid derivatives lead to the inhibition of NET release.

Finally, these results allow us to answer the second question formulated in the objectives: “Could cervical mucins protect sperm from entrapment by NETs by inhibiting the release of NETs within the cervix through sialic acid on its surface and if so, is the involvement of Siglecs likely?”. Bovine cervical mucins have the potential to inhibit NET release *in vitro* in a sialic acid-dependent and concentration-dependent manner. Probably similar to the binding of sialic acid on the surface of erythrocytes to Siglecs on neutrophils, sialic acid binding to Siglecs induces receptors clustering, contributing to the inhibition of the kinase-dependent onset of NET formation (Lizcano et al. 2017, Bornhöfft et al. 2019a). Since bovine as well as human mucins carry sialic acid residues (Andersch-Björkman et al. 2007, Galuska et al. 2017b) and the bovine and human oestrous cycle share common features like, the side of sperm deposition as well as the emerge of follicular waves, the number of follicular waves, the selection of the dominant follicle and the ovulation of a single follicle, we assume similar effects of cervical mucins within human reproductive tracts (Baerwald, Adams and Pierson 2003, Adams and Pierson 1995). Therefore, besides the presence of DNase I within the seminal plasma as well as sialic acid polymers on the surface of sperm, causing

degradation of NETs and decrease cytotoxic effects of histones, mechanisms which are both limited in their sphere of activity, mucins which line the female reproductive tract might contribute to sperm survival by engaging Siglecs (Zlatina and Galuska 2019, Zlatina et al. 2018, Simon et al. 2013, Alghamdi and Foster 2005, Bornhöfft et al. 2019a, Zambrano et al. 2016).

5.2.2 Oligosialylated particles inhibit NET release but not neutrophil activation

In order to investigate, whether or not artificially sialylated particles have a similar potential to inhibit the activation of neutrophils and might therefore serve as a future target for novel clinical applications to modulate the release of NETs, we coupled sialic acid oligomers to latex particles and incubated those with PMA stimulated human neutrophils. In contrast to mucins - containing numerous differently linked sialylated glycan motifs -, the nanoparticles were only coated with α2,8-linked Neu5Ac residues. Interestingly, the results were different to those obtained by applying cervical mucins: although NET release was prevented nuclei swelling occurred, indicating that the translocation of neutrophil elastase to the nuclei, which contributes to DNA decondensation still takes place (**Figure 17**) (Bornhöfft et al. 2019b).

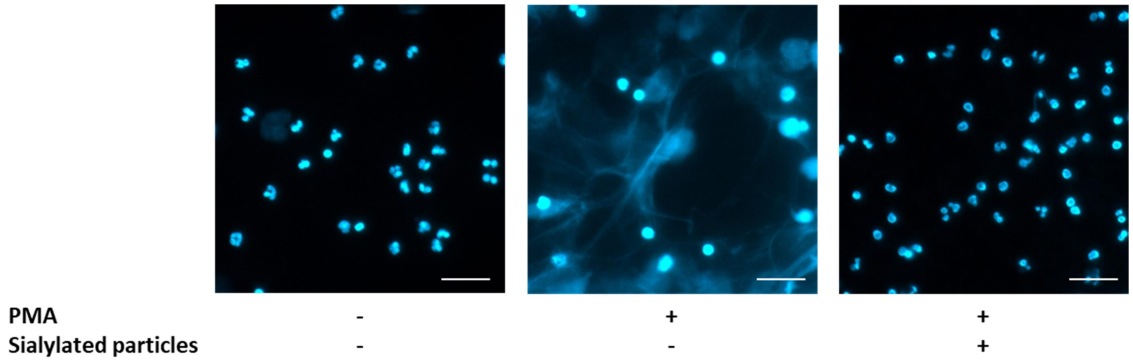


Figure 17: Oligosialylated particles inhibit NET release but neutrophils are activated. Unstimulated neutrophils, neutrophils stimulated with 20 nM PMA and cells stimulated with PMA co-incubated with oligosialylated particles were stained with DAPI (blue). Scale bars indicate 20 µm.

As ROS produced by the NADPH oxidase complex are assumed to contribute to the translocation of NE to the nuclei as well as to nuclear envelope and plasma membrane rupture (Brinkmann and Zychlinsky 2012, Nel et al. 2016, Desai et al. 2016, Dwivedi and Radic 2014, Brinkmann 2018), in a first set of experiments we tested whether or not sialylated particles modulate ROS production. To this end dihydrorhodamine 123 (DHR123) was used. DHR123 is oxidized in the presence of ROS to the cationic rhodamine 123, exhibiting green fluorescence measurable by flow cytometry. As expected the stimulation of neutrophils with PMA caused an increased production of

ROS, which decreased significantly by the addition of oligosialylated particles. However, as previously mentioned, NE still translocate to the nucleus, indicating that the decreased ROS production is not sufficient to inhibit neutrophil activation (Bornhöfft et al. 2019b). As it was shown in the 1980s, that activation of the NADPH oxidase occurs along with membrane depolarization, this was further investigated (Henderson, Chappell and Jones 1987). Therefore, Bis(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC) was used. DiBAC is able to enter depolarized cells and exhibits enhanced fluorescence when binding to intracellular proteins. Membrane depolarization failed, when oligosialylated particles were applied, although cells stimulated with PMA showed an increased depolarization of the membrane. Thus, these results suggest, that the decreased ROS production might be caused by an impaired activation of the NADPH oxidase complex due to the addition of oligosialylated particles (Bornhöfft et al. 2019b). Interestingly, these results fit with knowledge about patients suffering from chronic granulomatous disease (CGD): As this disease is characterized by an impaired activation of the NADPH oxidase, patients show no membrane polarization after PMA stimulation, contributing to the inability to form NETs (Seligmann and Gallin 1980, Cohen et al. 1981, Castranova et al. 1981).

Interestingly, Siglec-5, -9, and -14 are expressed in human neutrophils (O'Reilly and Paulson 2009, Angata, Nycholat and Macauley 2015, Macauley et al. 2014). As the inhibitory Siglec-5 binds α2,8-linked sialic acid chains and the activation of the NADPH oxidase complex includes kinase-dependent pathways (Dwivedi and Radic 2014, Hakkim et al. 2011), an involvement of Siglecs may trigger these inhibitiory effects (Bornhöfft et al. 2019b).

Interestingly, free sialic acid oligomers showed no effect on NET release, indicating that sialic acids have to be immobilized to modulate NET release. Furthermore, the additional supplementation of free sialic acid oligomers to neutrophils stimulated with PMA and co-incubated with sialylated particles prevented the outlined inhibitory effects. Probably, the binding of the free unconnected sialic acid chains prohibited Siglec clustering, necessary for the inhibitory function of Siglecs (Bornhöfft et al. 2019b).

For lactoferrin a similar effect on NET release is described. In line with the effects of oligosialylated particles, although NET release is inhibited in the presence of lactoferrin, neutrophil elastase translocates to the nucleus and nuclear swelling occurs. According to Okubo *et al*. this effect can be ascribed to the accumulation of lactoferrin on the surface of neutrophils, forming a "lactoferrin-shell", acting as a physical blocking

barrier. It is assumed, that the first released DNA forms aggregates with lactoferrin, which inhibits the release of NETs (Okubo et al. 2016). In sum, the results observed lead to the response of the last question needed to be answered: “Could nanoparticles conjugated with α2,8-linked sialic acid residues trigger similar inhibitory effects on NET release and if so, is also here the involvement of Siglecs likely?”. We showed that the release of NETs was prevented by applying sialylated molecules, although nuclei swelling occurred. As Neubert *et al*. defined chromatin swelling as “point of no return” of NET release, we assume that in our case at least two district mechanisms are responsible for the inhibitory effects of sialylated nanoparticles (Neubert et al. 2018). On the one hand, oligosialylated particles might interact with the phospholipid double layer, stabilizing the membrane and therefore prevent plasma membrane rupture (**Supplement 5**) and on the other hand, sialic acid interactions with Siglec-5 may contribute to NET inhibition by decreasing ROS production via the decreased activation of the NADPH oxidase complex (**Figure 18**) (Bornhöfft et al. 2019b).

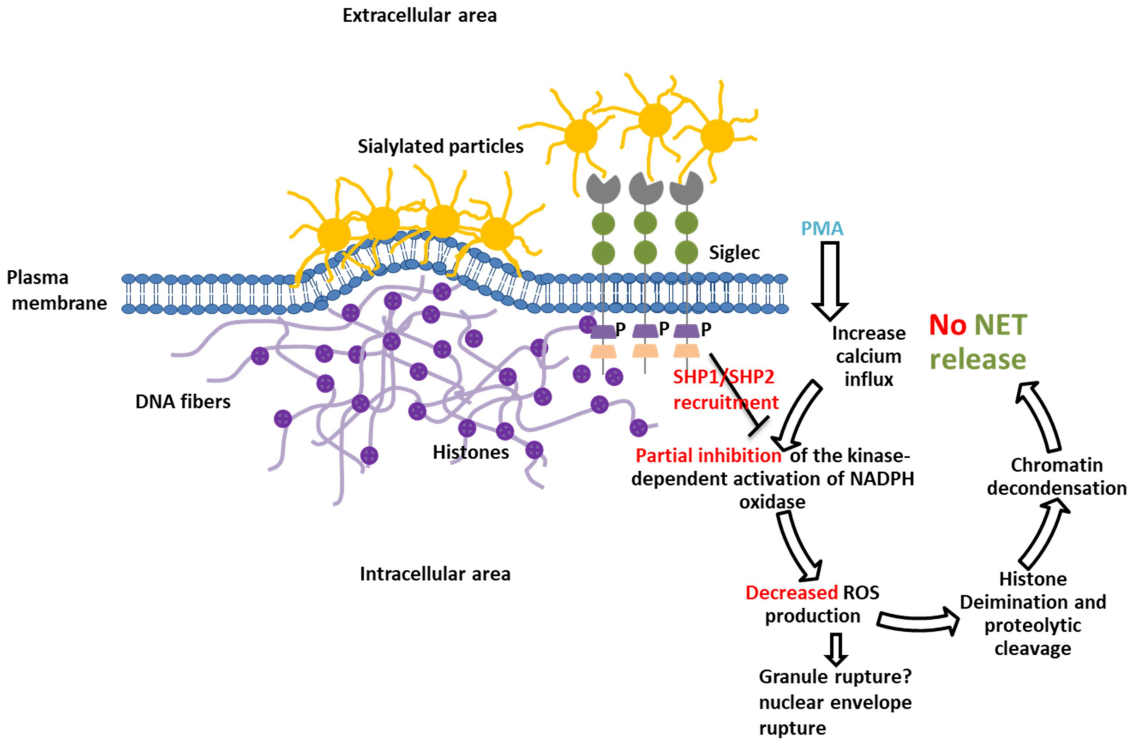


Figure 18: The assumed mechanism behind the inhibition of NET release via α2,8 sialylated particles. PMA induced NET release is initiated by an increased calcium influx, causing the activation of PKC, essential for the activation of the ROS producing NADPH oxidase. When sialylated particles were applied, those are assumed to bind to Siglec-5 on the surface of neutrophils, leading to the phosphorylation of the ITIMs, coming along with the recruitment of two phosphatases SHP1/SHP2, which inhibit the kinase (PKC)-dependent activation of the NADPH oxidase, contributing to decreased ROS production. As DNA decondensation and the translocation of NE to the nuclei still occurred, in addition an interaction of the sialylated particles with the plasma membrane is assumed, stabilizing the membrane and preventing its rupture.

However, since the particles coupled with α2,8-linked sialic acid oligomers did not completely inhibit neutrophil activation, while highly sialylated mucins did, we assume, that the complexity of differently sialylated structure is responsible for the engagement of different Siglecs, resulting in the complete inhibition of neutrophil activation. Therefore, a future promising approach might be the coupling of different sialic acids to nanoparticles. The potential medical benefit for humanity is further highlighted in the upcoming chapter.

5.2.3 Potential relevance to the field of medicine of artificial/ natural occurring sialylated structures

In human around 5% of the cases of infertility have immunological causes (Archana et al. 2019) and in some cases the reason remains unexplained (Elhussein et al. 2019). Regarding the involvement of neutrophils in immunologically caused infertility, the

current hypothesis implies that, the released NETs not only serve as a trap for pathogens but also for sperm themselves, contributing to infertility (Hahn et al. 2012). However, based on our results, we assume that mucins lining the female reproductive tract prevent sperm from entrapment by inhibiting an exaggerated release of NETs. We suggest that a very high level of stimuli would have to be introduced to initiate NET release under physiological conditions within the cervix (Bornhöfft et al. 2019a). Once further experiments have confirmed a correlation between the amount, composition or glycosylation pattern of mucins and fertility, mucins might serve as a biomarker/diagnostic tool for the determination of fertility/infertility. In addition, for instance, heparin-induced-thrombocytopenia (HIT) often comes along with the formation of a thrombus, due to an exaggerated NET formation. According to Perdomo *et al*. a combination of non-heparin anticoagulants with anti-NET therapy is forward looking. Thus, sialalyted conjugates might be a potential tool to modulate the release of NET associated with the formation of a thrombus in HIT coming along with high motility and morbidity rates (Perdomo et al. 2019).

Since in general, several autoimmune diseases are associated with exaggerated NET release or a missing NET clearance, like systemic lupus erythematosus (SLE), CF and rheumatoid arthritis, the interaction of sialylated structures with Siglecs might be a target to develop novel clinical applications for the modulation of NET release (He, Yang and Sun 2018). However, one has to consider, the challenging task of a targeted, highly specific application of those sialylated structures to the desired site of action, the challenging task of ensuring a controlled depletion, once they are required no more as well as possible side effects caused by the application of highly negatively charged molecules to the system.

6. Summary

It has been known since 2004 that neutrophil granulocytes are able to release so-called neutrophil extracellular traps (NETs). NETs consist of decondensed DNA decorated with antimicrobial molecules and cytotoxic histones, which can not only capture pathogens but also render them harmless. This process, initially classified as "beneficial suicide", has been getting more and more attention. To date, the actual mechanism of the release of NETs is not fully understood, but it is already clear that NETs also have a dark side. The exaggerated release of NETs seems to be associated with numerous diseases including infertility. Nowadays, it has been suggested that NETs serve as trap for sperm, contributing to infertility. Interestingly, there are physiological mechanisms known that influence the release of NETs. For example, sialylated glycans on the surface of erythrocytes interact with Siglecs on the surface of neutrophils leading to the inhibition NET formation within the circulation, which might be an important mechanism in vertebrates to prevent an exaggerated activation of the immune system. Intriguingly, our analysis demonstrated that the genes encoding for Siglec-1, CD22, MAG and Siglec-15 are conserved since approximately 400 million years and that, for instance, stress influence the expression pattern of these regulative receptors in fish. These results indicate that the first Siglec-dependent mechanisms were already established in lower vertebrates. Siglec-mediated mechanisms may also take place in the female reproductive tract of mammals. Here highly sialylated mucins are produced and released in extraordinary quantities. We detected that cervical mucins might be important to maintain the balance between pathogen defence and pathophysiological manifestations. Our studies showed that, in line with our hypothesis, cervical mucins ensure fertility by inhibiting the release of NETs as well as the activation of neutrophils via their sialic acid residues. Therefore, we assume that a very high level of stimuli would have to be introduced to initiate NET release under physiological conditions within the cervix and that the inhibition of NETosis might contribute to fertility. Furthermore, α2,8-linked oligosialylated nanoparticles were investigated to mimic the effects of a distinct sialic acid-motif, which is present on cervical mucins. These particles were able to inhibit NET release. However, although the release of NETs could be prevented, neutrophils were activated. The DNA decondensed, neutrophil elastase translocated into the cell nucleus and the nuclear and granular membrane ruptured, but the plasma membrane remained intact. Thus, for a complete inhibition differently attached sialic acid residues (α2,3- and/or α2,6-linked sialic acid resides) on nanoparticles might be necessary as mucins contain all these different sialic acid-motifs.

In sum, this work shows that from lower vertebrates to mammals, Siglec-sialic acid interactions are needed to maintain the balance between pathogen defence and pathophysiological manifestations and that the interaction of sialylated structures with Siglecs can be exploited to develop new clinical applications for the modulation of NET release.

7. Zusammenfassung

Seit 2004 ist bekannt, dass Neutrophile Granulozyten in der Lage sind, sogenannte *Neutrophil Extracellular Traps*, kurz NETs, freizusetzen. Diese bestehen aus dekondensierter DNS dekoriert mit antimikrobiellen Molekülen und zytotoxischen Histonen, wodurch Pathogene nicht nur gefangen, sondern auch unschädlich gemacht werden können. Dieser zunächst als „profitabler Selbstmord“ eingestufter Prozess, bekam innerhalb der letzten Jahre mehr und mehr Aufmerksamkeit. Bis heute sind die genauen Mechanismen der Freisetzung von NETs nicht vollständig aufgeklärt, doch bereits heute ist klar, dass NETs bei weitem nicht nur positive Effekte mit sich bringen. So ist die überschüssige NET Freisetzung assoziiert mit zahlreichen Erkrankungen, einschließlich Infertilität. Heutzutage gilt die Vermutung, dass NETs für Spermien zu Fallen werden und somit zur Infertilität führen. Interessanterweise sind physiologische Mechanismen bekannt, die die Freisetzung von NETs beeinflussen. Beispielsweise interagieren sialylierte Glykane auf der Oberfläche von Erythrozyten mit Siglecs auf Neutrophilen und hemmen somit die Freisetzung von NETs innerhalb der Zirkulation. Dies könnte ein wichtiger Mechanismus von Wirbeltieren sein, um überschlüssige NET Freisetzung zu inhibieren. Unsere Analyse zeigte, dass die für Siglec-1, CD22, MAG und Siglec-15 kodierenden Gene seit ungefähr 400 Millionen Jahren konserviert sind und dass beispielsweise Stress das Expressionsmuster dieser regulativen Rezeptoren in Fischen beeinflusst. Diese Ergebnisse deuten darauf hin, dass die ersten Siglec-abhängigen Mechanismen bereits bei niederen Wirbeltieren etabliert wurden. Siglec-vermittelte Mechanismen können auch im weiblichen Fortpflanzungstrakt von Säugetieren stattfinden. Hier werden hochsialylierte Muzine hergestellt und in außergewöhnlichen Mengen freigesetzt. Unsere Experimente zeigten, dass zervikale Muzine wichtig sein könnten, um das Gleichgewicht zwischen der Abwehr von Krankheitserregern und pathophysiologischen Manifestationen aufrechtzuerhalten. Unsere Studien zeigten, dass zervikale Muzine, entsprechend unserer eingangs aufgestellten Hypothese, die Aktivierung von Neutrophilen sowie die Freisetzung von NETs über Sialinsäure-Interaktionen inhibieren. Dementsprechend, gehen wir davon aus, dass ein sehr hohes Maß an Stimuli eingeführt werden muss, um in der Zervix unter physiologischen Bedingungen NET-Freisetzung zu initiieren. Darüber hinaus haben wir den Einfluss von α2,8-verknüpfte oligosialylierten Nanopartikeln auf die Freisetzung von NETs untersucht, um die Auswirkungen eines spezifischen Sialinsäuremotifs zu imitieren, welches auf zervikalen Muzinen vorkommt. Die Freisetzung von NETs konnte inhibiert werden, jedoch nicht die Aktivierung der Neutrophilen. Die DNS dekondensierte, Neutrophile Elastase translozierte in den

Zellkern und die Kern- sowie die Granularmembran platzte-nicht jedoch die Plasmamembran. Für eine vollständige Hemmung können daher unterschiedlich gebundene Sialinsäurereste (α2,3- und / oder α2,6-verknüpfte Sialinsäurereste) auf Nanopartikeln erforderlich sein, da zervikale Muzine alle diese unterschiedlichen Sialinsäuremotive enthalten.

Zusammenfassend zeigt diese Arbeit, dass von niederen Wirbeltieren bis zu Säugetieren Siglec-Sialinsäure-Wechselwirkungen erforderlich sind, um das Gleichgewicht zwischen der Abwehr von Krankheitserregern und pathophysiologischen Manifestationen aufrechtzuerhalten, und dass die Wechselwirkung von sialylierten Strukturen mit Siglecs genutzt werden kann, um klinische Anwendungen für die Modulation der NET-Freisetzung zu entwickeln und somit NET-assoziierte Erkrankungen zu therapieren.

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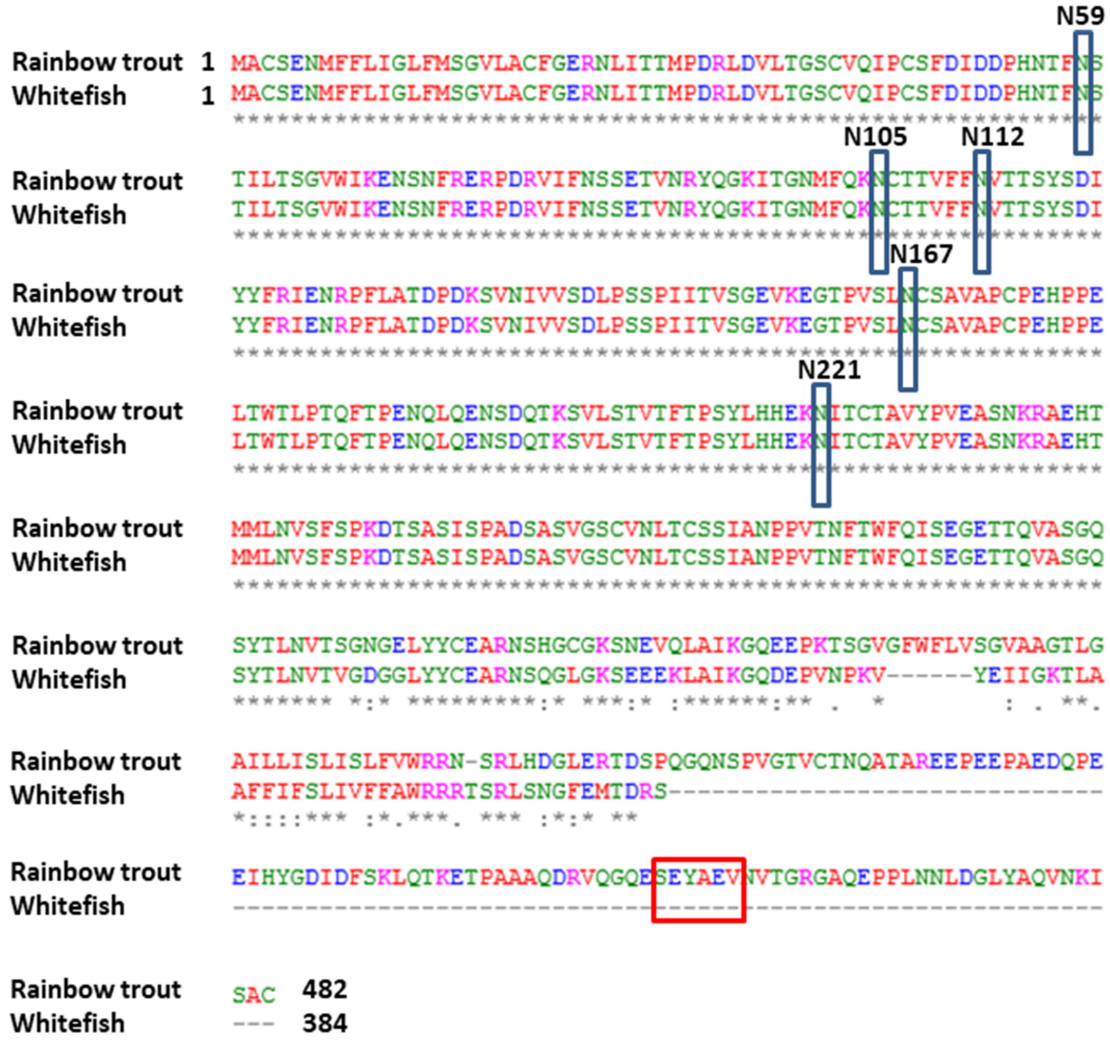
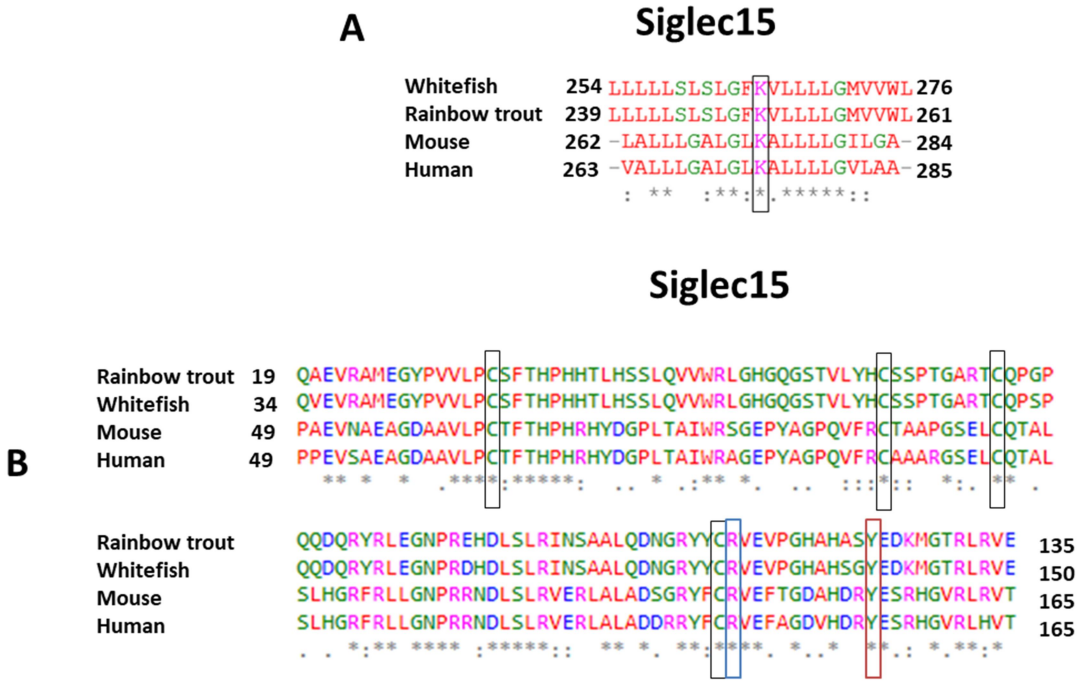
9. Appendix

9.1 Supplementary Material



Supplement 1: Sequence alignment of the genes encoding CD22 from rainbow trout and maraena whitefish. Alignments were performed by using the Clustal Omega tool of EMBL-EBI. The different colours show the different properties of the amino acids. Red: small hydrophobic/aromatic amino acids; blue: acidic amino acids; magenta: basic amino acids; green: hydrophilic, polar and small amino acids. The red box labels the ITIM motif of CD22 (SEYAEV), while the blue boxes show N-glycosylation sites. Sequence of maraena whitefish is suggested to be incomplete; allowing us to speculate that the ITIM motif might be present [Figure and caption are from (Bornhöfft et al. 2020) CC-BY, caption is party modified].



Supplement 2: Sequence alignment of different areas of Siglec-15. A) Sequence alignment of the transmembrane domain of Siglec-15. The location of the transmembrane domain of human and murine Siglec-15 were retrieved from Uniprot, while the sequences of maraena whitefish and rainbow trout were forecasted using SMART (http://smart.embl-heidelberg.de/). Black boxes indicate the conserved lysine residue, responsible for the interaction with DAP10/12. **B)** Sequence alignment of the first Ig-domain of Siglec-15. Shown are the conserved cysteine residues of Siglec-15, known to contribute to the functional tertiary structure (black boxes). The alignments were performed with the Clustal Omega tool of EMBL-EBI. The different colours label the chemical properties of the amino acids. Red: small hydrophobic/aromatic amino acids; blue: acidic amino acids; magenta: basic amino acids; green: hydrophilic, polar and small amino acids [Figures and caption are slightly modified and are from (Bornhöfft et al. 2020), CC-BY].

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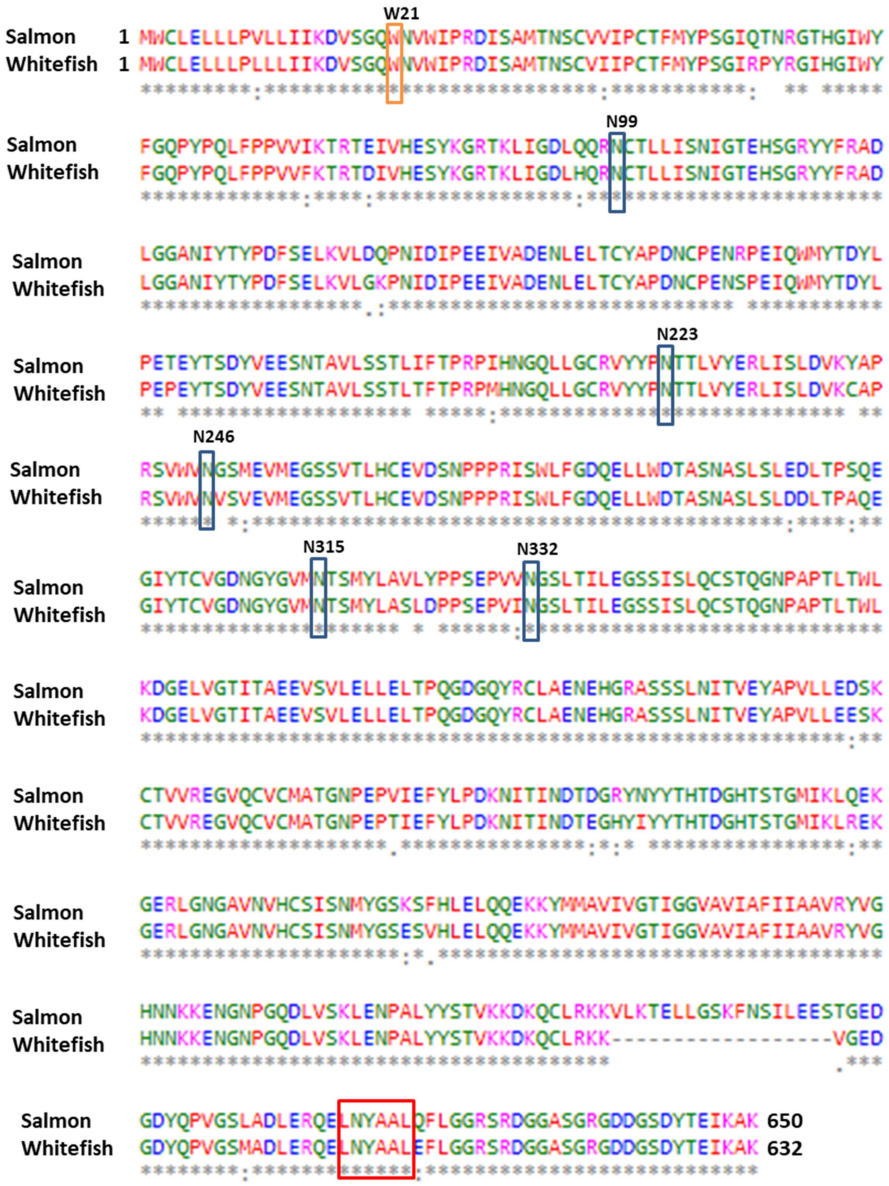
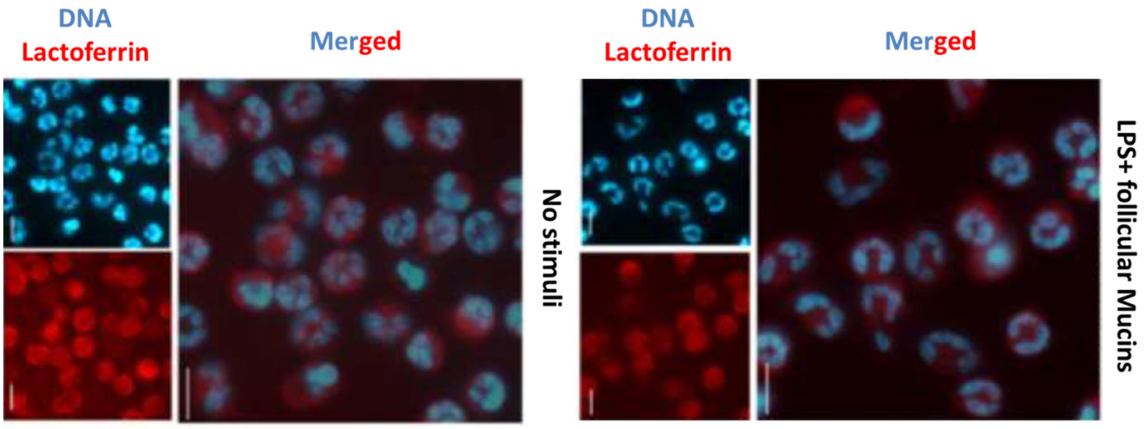
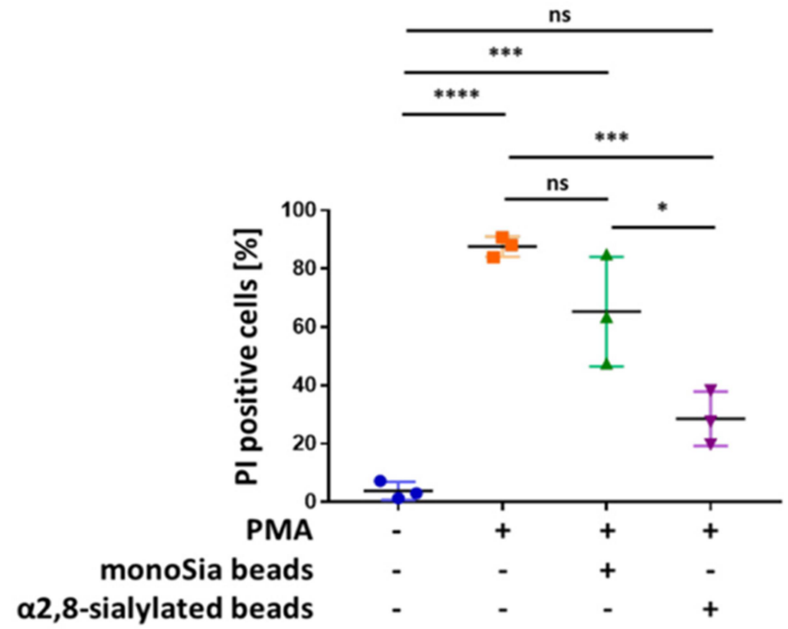


Supplement 3: Partial sequence alignment of MAG from salmon and maraena whitefish. Blue boxes indicate the conserved N-glycosylation sites, while the orange box shows the conserved W22, while the red box shows the ITIM sequence (LNYAAL). Alignments were performed using the Clustal Omega tool of EMBL-EBI. The different colours indicate the properties of the amino acids. Red: small hydrophobic/aromatic amino acids; blue: acidic amino acids; magenta: basic amino acids; green: hydrophilic, polar and small amino acids (Bornhöfft et al. 2020) CC-BY.



Supplement 4: Inhibition of NET release by bovine cervical mucins. DNA fluorescence staining (DAPI, blue) and staining of lactoferrin (red) of neutrophils without any stimulation and stimulated with 20 µg/mL LPS from *P. aeruginosa* co-incubated with 20 µg/µL follicular mucins. Nuclei retain their segmentation and granularity endured. The term "merged" indicates the overlay of the nuclei staining with the staining of lactoferrin. Scale bars: 10 µm [modified (Bornhöfft et al. 2019a) CC-BY].



Supplement 5: Amount of PI-positive cells. Based on the fluorescence images the total number of cells was determined and was set in relation to the amount of PI-positive cells. PI staining was performed of untreated human neutrophils, of human neutrophils induced to form NETs with 20 nM PMA and neutrophils stimulated with 20 nM PMA, co-incubated with monosialylated particles and oligosialylated particles (α2,8 sialylated beads). Mean values (n=3) and standard deviations are shown. ANOVA and multiple comparison Turkey test were applied. Significant differences are given as follows: ns, not significant, * $p \leq 0.05$; *** $p \leq 0.001$; and **** $p \leq 0.0001$ [modified (Bornhöfft et al. 2019b) CC-BY].

9.2 Publications

Kim F. Bornhöfft, Tom Goldammer, Alexander Rebl, Sebastian P. Galuska: 'Siglecs: A journey through the evolution of sialic acid-binding immunoglobulin-type lectins'; Developmental& Comparative Immunology, Volume 86, p. 219-231, doi: 10.1016/j.dci.2018.05.008, September 2018.

Kim F. Bornhöfft, Torsten Viergutz, Andrea Kühnle, Sebastian P. Galuska. 'Nanoparticles equipped with α2,8-linked sialic acid chains inhibit the release of neutrophil extracellular traps'; MDPI Nanomaterials 9 (4), doi: 10.3390/nano9040610, April 2019.

Kim F. Bornhöfft, Sebastian Galuska: 'Glycans as modulators for the formation and functional properties of neutrophil extracellular traps: used by the forces of good and evil'; Front. Immunol.10:959, doi: 10.3389/fimmu.2019.00959, May 2019.

Kim F. Bornhöfft, Alexander Rebl, Mary E. Gallagher, Torsten Viergutz, Kristina Zlatina, Colm Reid, Sebastian P. Galuska: 'Sialylated cervical mucins inhibit the activation of neutrophils to form neutrophil extracellular traps in bovine *in vitro* model'; Front. Immunol. 10:2478, doi: 10.3389/fimmu.2019.02478, November 2019.

Kim F. Bornhöfft, Joan Martorell Ribera, Torsten Viergutz, Marzia Tindara Venuto, Ulrike Gimsa, Sebastian Peter Galuska, Alexander Rebl: 'Characterization of sialic acid-binding immunoglobulin-type lectins in fish reveals teleost-specific structures and expression pattern', Cells, pii: E836, doi: 10.3390/cells9040836, March 2020.

Christina E. Galuska, Jan A. Dambon, Andrea Kühnle, **Kim F. Bornhöfft**, Gerlinde Prem, Kristina Zlatina, Thomas Lütteke and Sebastian P. Galuska; 'Artificial Polysialic Acid Chains as Sialidase-Resistant Molecular-Anchors to Accumulate Particles on

Neutrophil Extracellular Traps'; Front. Immunol. 8:1229, doi: 10.3389/fimmu.2017.01229, September 2017.

Andrea Kühnle, Thomas Lütteke, **Kim F. Bornhöfft**, Sebastian P. Galuska; 'Polysialic acid modulates the binding of external lactoferrin in neutrophil extracellular traps'; MDPI Biology 8 (2), doi: 10.3390/biology8020020, March 2019.

9.3 Conference contributions

28th Joint Glycobiology Conference, Aachen, Germany (09/2017):

Poster: ‘Siglecs: Indispensable Immune-Regulatory Receptors since the Period of Silurian 430 Million Years ago?’

British Society of Immunology, Brighton, Great Britain (12/2017):

Poster: ’Molecular Evolution of Siglecs’.

29th Joint Glycobiology Conference, Ghent, Belgium (10/2018):

Poster: ‘Oligosialylated nanoparticles prevent the release of neutrophil extracellular traps’.

30th Joint Glycobiology Conference, Lille, France, (10/2019):

Talk: ‘Bovine cervical mucins inhibit NET release in a sialic acid-dependent manner’.

9.4 Declaration of honour

“I hereby declare that I have completed this work independently and without inadmissible assistance or the use of other than the resources quoted. All texts that have been quoted verbatim or by analogy from published and non-published writings and all details based on verbal information have been identified as such. In the analyses that I have conducted and to which I refer in this thesis, I have followed the principles of good scientific practice, as stated in the Statute of Justus Liebig University Giessen for Ensuring Good Scientific Practice, as well as ethical principles and those governing data protection and animal welfare. I give my assurance that third parties have not received from me, either directly or indirectly, any financial remuneration for work in connection with the content of this doctoral thesis and that the work presented has not been submitted in the same or a similar form to another assessment authority in Germany or elsewhere for the purpose of being awarded a doctorate or another assessment procedure. All material taken from other sources and other persons and used in this thesis or to which direct reference is made has been identified as such. In particular, all those who took part directly and indirectly in the production of this study have been named. I agree to my thesis being subjected to scrutiny by plagiarism detection software or by an internet-based software program.”

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Kim Bornhöfft

9.5 Ehrenwörtliche Erklärung

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Gießen, 12.03.2021_____

Kim Bornhöfft

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