CD33-Related Siglecs in Early Recognition of Tumour Cells & Viruses by Mononuclear Phagocytes

Dissertation

of

Sarang Suhas Limaye

Submitted to the Promotion Committee

of the Fachbereich 2 (Biology and Chemistry)

Universität Bremen

for obtaining a doctoral degree in Science

- Dr. rer. nat.-

Universität Bremen

Reviewers of the dissertation: Prof. Dr. rer. nat. Sørge Kelm

Prof. Dr. rer. nat. Andreas Dotzauer

Abstract

The sialic acid binding immunoglobulin-like lectins (siglecs) comprise a family of receptors that are differentially expressed on leukocytes and other immune cells. Their molecular properties and the presence of tyrosine-based motifs suggest that they could be involved in fine-tuning the immune responses. Peripheral blood derived monocytes express siglec-3, -5, -7, -9, and -10 (1-10% monocyte population). Upon differentiation to macrophages a general trend of decrease in siglec-3, -5 and -9 and increase for siglec-7 expression was observed. The level of expression and degree of change during differentiation varied between individual donors. It is of special interest that siglec expression on mononuclear phagocytes is further regulated by different cytokines (IFNγ: increased siglec-1 and decrease siglec-5, -7 and -9; IL-4: increased siglec-7 and 9 and decrease siglec-1). Six month monocyte surface siglec expression analysis of the 15 volunteers revealed that, siglec-9 shows the most diverse expression pattern as compared to the other monocyte limited siglecs, and these changes in expression pattern has direct relation with serum CRP levels. Furthermore, co-culturing monocytes with viruses revealed that mere presence of viral particles affects monocyte surface siglec expression profile. In addition, the viral dose and time of incubation contribute to these changes. The alteration in the siglec expression pattern in response to pathogens/tumour cells could be part of the host defence system. K562 erythroleukemia tumour cells were able to elicit early sialic acid (Sia) dependent cytokine secretion by mononuclear phagocytes. And this early cytokine secretion by mononuclear phagocytes upon co-culture with K562 surface Sia derivatives was confirmed with real time IL-6 mRNA synthesis. Furthermore, an accumulation of siglec-7 was observed at the cellular synapses between these two cell types. As siglecs are the only Sia recognising receptors present on innate immune system cells, and they could be involved in the observed Sia mediated activation processes. Here it is shown that siglec-7 could plays an important role in controlling the Sia mediated activation of mononuclear phagocytes by K562 tumour cells, as in presence of anti siglec-7 mAb activation of mononuclear phagocytes is reduced by 30-40%.

Key words: Sialic acid, siglecs, ITIM, ITAM, Monocytes, Macrophages, Cytokines, IL-6, IL-1β, Viruses, Influenza virus, HAV-7, Coxsackie virus, NDV, CMV, K562 tumour cells

Zusammenfassung

Die Sialinsäure bindenden Immunglobulin-ähnlichen Lektine (Siglecs) umfassen eine Familie von Rezeptoren, die unterschiedlich auf Leukozyten und anderen Immunzellen exprimiert werden. Ihre molekularen Eigenschaften und das Vorhandensein von Tyrosin-basierten Motiven sind Merkmale, die sie mit anderen Rezeptoren gemeinsam haben, was darauf hindeutet, dass sie an der Feinabstimmung angeborener Immunreaktionen beteiligt sind. Periphere Blut-Monozyten exprimieren Siglec-3, -5, -7, -9 und -10 (1-10% der Monozytenpopulation). Nach Differenzierung zu Makrophagen wurde ein allgemeiner Trend zur Abnahme der Expression von Siglec-3, -5 und -9 und zum Anstieg bei der Siglec-7 Expression beobachtet. Die Höhe der Expression und der Grad der Veränderung während der Differenzierung ist zwischen den einzelnen Spendern unterschiedlich. Es ist von besonderem Interesse, dass die Siglec-Expression auf mononukleären Phagozyten auch durch verschiedene Zytokine reguliert wird (IFN-γ reguliert Siglec-1 hoch und reguliert Siglec-5, -7 und -9 runter; IL-4: reguliert Siglec-7 und 9 hoch und Siglec-1 runter). Eine 6-monatige Analyse der Siglec-Expression auf der Oberfläche von Monozyten von 15 Freiwilligen hat gezeigt, dass Siglec-9 das verschiedenartigste Expressionsmuster im Vergleich zu den anderen Siglecs zeigt, was mit dem CRP-Spiegel im Serum korreliert. Eine Co-Kultivierung von Monozyten mit Viren ergab, dass die bloße Anwesenheit von viralen Partikeln das Siglec-Expressionsprofil auf der Monocytenoberfläche beeinflußt. Zudem tragen die virale Dosis und die Inkubationszeit zu diesen Modifikationen bei. Die Veränderung im Siglec-Expressionsmuster in Reaktion auf Krankheitserreger / Tumourzellen könnte ein Teil des Abwehrsystems sein. K562-Erythroleukämie-Zellen konnten Sia-abhängig die Zytokinsezernierung von mononukleären Phagozyten induzieren. Diese frühe Zytokinausschüttung wurde mit real time-IL-6 mRNA-PCR bestätigt. Darüber hinaus war eine Ansammlung von Siglec-7 auf den zellulären Synapsen zwischen diesen beiden Zelltypen zu beobachten. Da Siglecs die einzigen Sia-erkennen Rezeptoren auf den Zellen des angeborenen Immunsystems sind, könnten sie bei den beobachteten Aktivierungsprozessen eine Rolle spielen. In der vorliegenden Arbeit wird gezeigt, dass Siglec-7 bei der Sia-vermittelte Aktivierung von mononukleären Phagozyten durch K562-Tumourzellen eine Rolle spielt, da anti-Siglec-7 mAb die Aktivierung von mononukleären Phagozyten um 30-40% reduziert.

Acknowledgments

This is perhaps the easiest and hardest chapter that I have to write. It will be simple to name all the people that helped to get this done, but it will be tough to thank them enough. I will nonetheless try…

I would like to express my sincere gratitude towards my advisor, Prof. Dr. Sørge Kelm to offer the work in his lab on the topic “CD33-related siglecs in early recognition of tumour cells & viruses by mononuclear phagocytes” at university Bremen, and for his continuous support to my Ph.D. study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me immensely during research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study.

I am very grateful and wish to thank Prof. Dr. Andreas Dotzauer for being the second reviewer for my PhD thesis, despite of his busy schedule and for providing me opportunity to work on this project in his lab and for his constant encouragement and for his valuable suggestions.

A special gratitude to all my former and current fellow lab mates in AG Kelm; Frank, Hendrik, Ketan and Thaddeus for the simulating discussions and all the fun we had in the previous years. I specially like to tank Thaddeus and Mrs. Dotzauer for their valuable suggestions during my thesis writing. I wish to thank AG Kelm lab technicians Nazila, Petra, and Monica for their technical support through out my PhD work, specifically for the making media and buffers for my experiments. I would also like to thank all the members of ‘Virology Department at UFT, University of Bremen’, for the welcoming support and help with the virology experiments and technical assistance with the FACS. Thanks you Sabin for administration help that she had provided for all these years.

A special thanks to Red Cross Blood Bank, Hamburg for providing me blood every week, and would like to thank Mr. Raber for helping me in blood collection. Without your help the experiments would not have run so smoothly. I am very grateful to Dr. Mädler for allowing me to use real time PCR system, and Prof. Dringen for providing cell homogenisation system for my experiments.

It’s a pleasure to express gratitude to all my close friends, whom are too many to mention always stood by my side asking over and over again “When will you get it done? Next week? Next Month? When?”

Last but not the least I owe my deepest gratitude to my dear parents, my sister, and my "extended family", Apte’s and Puranik’s, because they were always there for me and have supported me and to one above all us God.
Juili, because you were always there and supported me always, I love you.

Declaration

This thesis is a presentation of my original research work and it has not been submitted anywhere for any award. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. The work was done under the guidance of Prof. Dr. Sørge Kelm, at the University of Bremen, Germany.

Bremen, 6th March 2012

_____

Sarang Suhas Limaye

Table of Content

List of Figures

List of Tables

List of Abbreviations

α-MEM	α-modified Eagle medium (cell culture medium)
Ab	antibodies
Amp	Ampicillin
Anti-huIgG	Anti-human IgG antibody directed against huIgG (Fc specific)
Anti-huIgG-AP	huIgG-AP conjugated with alkaline phosphatase
APS	Ammonium persulfate
sec	Second
Arg	Arginin
AS	Autologus serum
AUS	*Arthrobacter ureafaciens* sialidase
BCA-Assay	bicinchoninic acid protein assay
bp	base pair
BSA	Bovine serum albumin
BCR	B cell receptor
BC	Buffy-coats
CD	cluster of differentiation (cell surface molecules)
CDrSiglec	CD33-related siglec
d	domain(s)
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP´s	Deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbend assay
Em	Emission
Ex	Exitation
Fc	fragment crystallizable
FCS	Fetal Calf Serum
FDP	Fluorescein diphosphate, tetraammonium
FL	fluorescent light
FSC	Forward Scatter
g	Gram
Gal	galactose
GalNAc	N- N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GMP	glycomacropeptide
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hu	Human
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IL	Interleukin
ITIM	Immunoreceptor tyrosine-based inhibitory motif

ITAM	Immunoreceptor tyrosine-based activation motif
Kd	Equilibrium constant
kDa	kilodalton
L	Litter
LB	Luria-Bertani medium
MAG	myelin-associated glycoprotein
MAL II	Maackia amurensis lectin
min	Minute
MWM	molecular weight marker
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NHS	N-Hydroxysuccinimide
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PE	phycoerythrin
Pen/Strep	Penicillin/streptomycin
PNA	Peanut agglutinin
Pos	Position
RNA	ribonucleic acid
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute (cell culture medium)
RT	room temperature
SDS	Sodium dodecyl sulfate
sec	Second
SH	Src-Homology
Shc	Src homologous and collagen
SHP	SH2-domain-containing phosphatase
Sia	sialic acid
siglec-(Nr.)-Fc	Recombinant protein with the first three extracellular domains of respective human siglec and the Fc portion of human IgG with a 3C Protease cleavage site
SNA	Sambuccus nigra (elderberry shrub) bark lectin
Src	tyrosine kinase Rous sarcoma
SSC	Side Scatter
Taq	Thermus aquaticus
TEMED	N, N; N', N'-Tetramethylethylendiamin
Th	T-helper cell
Tris	tris (hydroxymethyl)-aminomethane
Triton X-100	polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
Trp	Tryptophan
Tween 20	Polyoxyethylene (20) sorbitan monolaurate
Tyr	Tyrosine

U	Units
VCS	*Vibrio cholerae sialidase*
V	Volt
v/v	volume ratio
W/v	weight to volume

The chemical elements are abbreviated with the usual symbols.

1. Introduction

1.1. Sialic acid binding immunoglobulin-like lectins

Sialic acid binding immunoglobulin-like lectins, siglecs, form a family of cell surface receptors expressed on immune cells that mostly mediate inhibitory signalling responses (Hanasaki et al., 1995; Brinkman-Van der Linden EC et al., 2002; O'Reilly and Paulson, 2009; Crocker and Redelinghuys, 2008). Like other important inhibitory immune receptor families such as killer-cell immunoglobulin-like receptor and leucocyte immunoglobulin-like receptor, siglecs are transmembrane molecules that contain inhibitory signalling motifs called immunoreceptor tyrosine based inhibitory motifs (ITIMs) in their cytoplasmic tails and immunoglobulin superfamily domains in their extracellular parts (Kelm et al., 1994; Sgroi et al., 1993; Crocker and Varki, 2001; Crocker et al., 2007; von G.S. and Bochner, 2008; Walker and Smith, 2008; Tedder et al., 2005; Crocker and Redelinghuys, 2008; Collins et al., 2006)(Figure 1.1). Compared to other immunoglobulin super-family proteins a unique feature of siglecs is their specificity towards sialylated carbohydrates, unlike other immune receptors that bind to protein determinants. Siglecs play a wide range of roles in the immune system. Sequencing of the human genome gave rise to the discovery of new members, expanding the group from the well-characterized conserved members, sialoadhesin, CD22, CD33 and myelin-associated glycoprotein (MAG), to the rapidly evolving CD33-related siglec (CD33rsiglec) subfamily that are expressed by different cell types (von G. S. and Bochner, 2008) (Figure1. 1 and Table 1.1).

1.2. CD33-related siglec and their importance in the immune responses

The CD33rsiglecs are mainly expressed by the cells of the innate immune system, such as neutrophils, eosinophils, monocytes, macrophages, NK cells, dendritic cells, and mast cells (Table. 1.1). CD33rsiglecs have been described to modulate the immune system, like inhibition of cellular proliferation (Vitale et al., 1999; Xiong et al., 2009), induction of apoptosis (Nutku et al., 2005; von G. S. et al., 2005), inhibition of cellular activation (Paul et al., 2000; Ulyanova et al., 2001; Avril et al., 2004; Ikehara et al., 2004; Avril et al., 2005), and induction of pro-inflammatory cytokine secretion (Lajaunias et al., 2005).

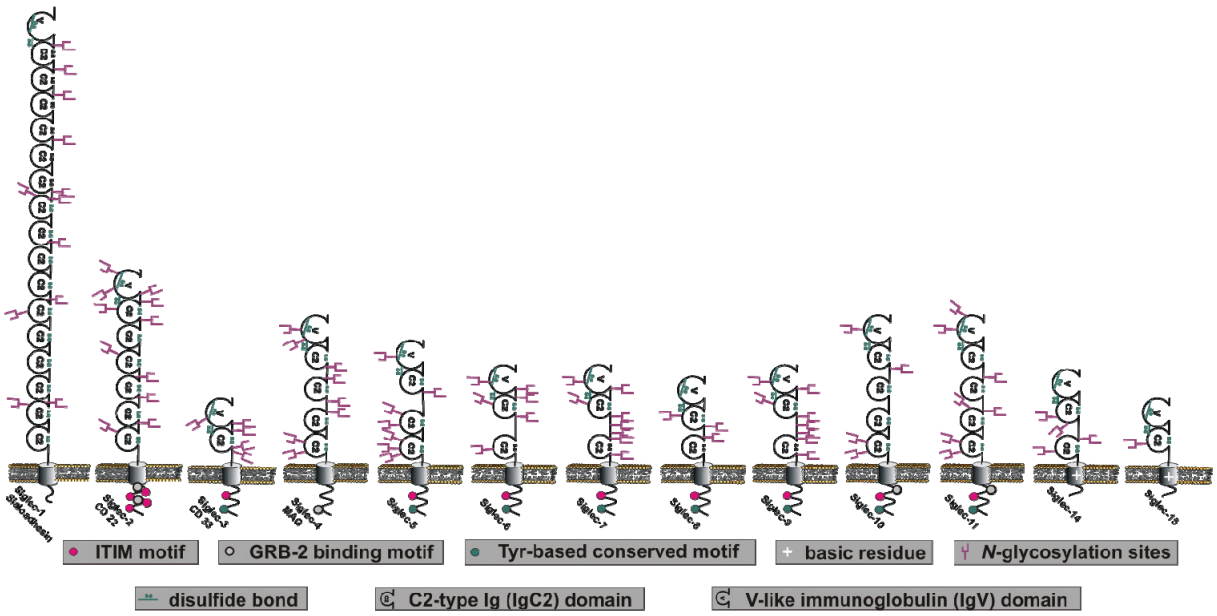


Figure 1.1: Nomenclature and key structural characteristics of human siglecs.
DAP12 domains are illustrated as a + in the transmembrane region, co-associating with siglec-14 and siglec-15. See key for symbols representing cytoplasmic signalling motifs and expression.

CD33 and siglec-7 have been shown to inhibit proliferation of haematopoietic cells and myeloid leukaemic cells in transfected Ba/F3 cells (Orr et al., 2007). Siglecs-8 and -9 induced cell-death (Nutku et al., 2003; von G. S. et al., 2005) is enhanced in the presence of cytokines that normally help cell survival. The above indicates a very complex interplay between cytokine receptor and siglec signalling pathways (von G. S. et al., 2006). CD33rsiglecs can also function as endocytic receptors in the clearance of sialylated antigens and/or in promoting or inhibiting antigen presentation (Lock et al., 2004; Avril et al., 2006; Walter et al., 2008; Nguyen et al., 2006; Biedermann et al., 2007)

1.3. CD33-related siglec attenuate innate immune responses

The ability of human siglecs to bind sialic acids (Sia) present on the same cell surfaces would expose humans to pathogens. This possibly reinvented Sia via convergent evolution, leading to inhibitory siglec function so as to dampen innate immune responses. Indeed, many microorganisms that express Sia (Neu5Ac) appear to be human-specific commensalisms, becoming pathogenic when circumstances allow (Vimr et al., 2004). For example, Group B *Streptococcus* expresses a Sia-containing capsule that engages human neutrophil (siglec-9), dampening its

responses (Weiman et al., 2009). Other sialylated pathogens that are recognized by siglecs (Jones et al., 2003), likely with similar outcomes (Khatua et al., 2009). Notably, such pathogens would be a strong selective force, because they often affect fetuses, infants and young adults and frequently cause lethal brain infections (Vimr et al., 2004)

Table 1.1: Siglecs expression on various human cells types (von G.S. and Bochner, 2008)

	Siglec												
Cell type	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**14***	**15**
B cell		+			±	±				+		+	
Basophile			+		+	+		±				+	
CD8+ T cell							+		+				
CD34+ cell¶			+		+				+	+		+	
Dendritic cell			+				+		+	+			
Eosinophils								+		±			
Epithelial cell	+												
Macrophage	+		+		+						+	+	+
Mast cell†		±	+		+	+		+				+	
Microglia cell												+	
Monocyte			+		+		+		+	+	+	+	+
Neutrophil			+		+				+			+	
NK cell							+		±	+			
Oligodendrocyte				+									
Placental trophoblast						+							
Schwann cell				+									

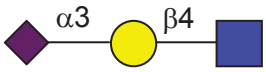
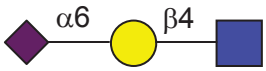
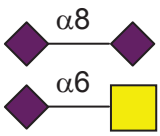
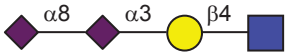
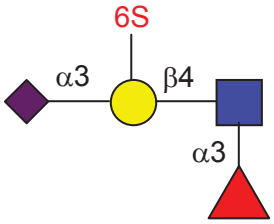
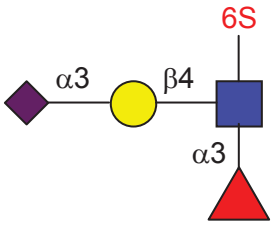
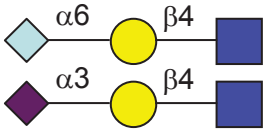
*** Expression is probably similar to siglec-5, but this has not yet been confirmed; ± Expressed intracellular or only weakly on the cell surface**

1.4. Siglecs and Sia binding specificities

Siglecs bind to Sia with different specificities based on the linkage and the underlying sugars (Strenge et al., 2001; Blixt et al., 2003) (Table 1.2). Although siglec ligands were identified using synthetic Sia probes, the *in vivo* natural ligands are yet to be characterized. Generally Sia binding sites of the siglecs are often masked by endogenous glycoproteins from the same cell surface making it difficult to determine the function of Sia binding in intact cells. One possibility to prevent Sia binding by

siglecs can be achieved by sialidase treatment or by cellular activation (Razi and Varki, 1999). Many different studies investigating the glycan specificities of different siglecs suggest that at least *in vitro*, both sialylated glycoprotein and/or gangliosides could function as potential binding partners (Collins et al., 1997; Crocker et al., 1991; Hanasaki, 1995; Kelm et al., 1994; Sawada et al., 1999; Sgroi et al., 1993; Strenge et al., 1999; Yamaji et al., 2002; Strenge et al., 2001).

Table 1.2: Siglecs preference for different sialoside structures (vonG.S. and Bochner, 2008)

siglec	Sialoside preference
siglec-1/Sn	
siglec-3/CD33	
siglec-5	
siglec-7	
siglec-8	
siglec-9	
siglec-10	

Colour coding: purple diamond, N-acetylneuraminic acid; pale blue diamond, N-glycolylneuraminic acid; yellow circle, galactose; yellow square, N-acetylgalactosamine; blue square, N-acetylglucosamine; red triangle, fucose; red S, sulphate.

1.5. Sialic acid and its biological role

In higher invertebrates the outer ends of glycan chains are often covered by Sia (Varki and Gagneux, 2009; Schauer, 2009). Biosynthetic pathways for these nine carbon backbone molecules likely evolved from those for ancestral nonulosonic acids (Lewis et al., 2009). Although Sias are rare in other taxa, they are ubiquitous on all vertebrate cell surfaces and are essential for embryonic development (Schwarzkopf et al., 2002). Indeed, they mediate many critical endogenous functions based on their physical properties and their recognition by intrinsic receptors (Varki and Gagneux, 2009; Schauer, 2009). Also, Sias are used by complement factor H (Pangburn et al., 2000) and by siglecs (Angata et al., 2006; Crocker and Redelinghuys, 2008) as signals for self recognition in the vertebrate innate immune system. However, given their location and abundance, Sias are equally targets for extrinsic receptors of numerous pathogens (Varki and Gagneux, 2009). Sias have been modified repeatedly via convergent evolution by microbes that interact with vertebrates (Weiman et al., 2010; Vimr and Steenbergen, 2006). Such imitation allows microorganisms to use Sias not only to mask themselves from the complement and adaptive immune systems (Schauer, 2009; Pangburn et al., 2000),. but also to engage siglecs, dampening the innate immune response (Carlin et al., 2009). For all these reasons, Sias are at the nexus of an evolutionary arms race between the vertebrate hosts and their pathogens. This competition may also explain why there are different kinds of Sias, each presented in several different linkages to the underlying monosaccharide, on a variety of different types of glycans (Varki, 2010; Schauer, 2009).

1.6. Regulation of siglec function with *cis*- and *trans*-ligands

Very little is known about the nature of physiologically important ligands and counter-receptors of the siglec family. This is because siglec binding sites are typically blocked by *cis*- interactions with other glycan ligands expressed on the same cell surface (Collins et al., 2004). In nature, *cis*-ligands may dominate over *trans*-ligands and thus modulate the biological activities of siglecs (Collins et al., 2006). CD22 on B cells gets delocalized at the contact site with the cells expressing CD22 ligands (Lanoue et al., 2002). The high-affinity synthetic Sia probes can overcome CD22 *cis*-ligands interaction (Nicoll et al., 2003). B-cell activation in response to antigen-presenting cells is suppressed if antigen and the CD22 ligand are expressed on the

same cell (Yeaman et al., 2002). Siglec-7 dependent reduction in NK-cell cytotoxicity was observed with target cells over-expressing α2–8-linked glycans. Cytotoxicity was increased by sialidase treatment, which cleaves the *cis*-interacting ligands from the cell surface (Ravetch and Lanier, 2000). The above examples illustrate the role played by *cis*- and *trans*-ligands in siglec function.

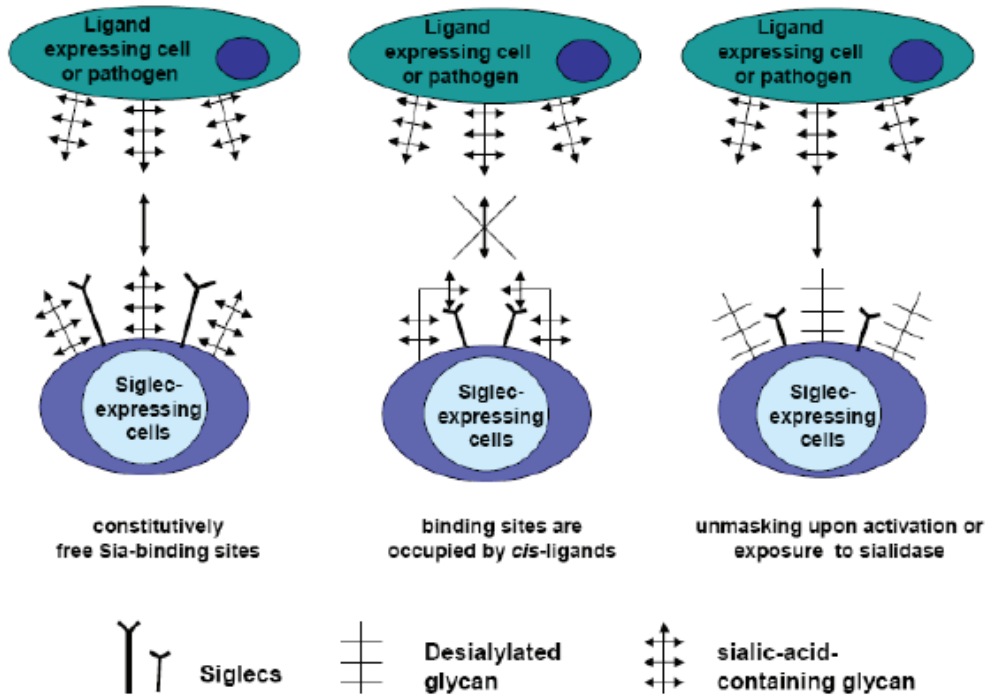


Figure 1.2: Schematic presentation of siglecs in native cis- and trans-interactions (adapted from Crocker P presentation)

Most siglecs are masked on the cell surface because they are involved in cis-interactions with Sia expressed on the same cell surface. Sialidase, which cleaves the cis-interacting siglec ligands, or in some cases cellular activation can also remove siglecs from cis-interaction, which allows them to make interactions with* trans*-ligands. Trans-interactions might occur during an encounter with another cell or a pathogen expressing higher affinity ligands that can compete with the cis-interactions.

1.7. The role of CD33rsiglecs in regulating cytokine production

Over-expression of siglec-9 in macrophage-like cell lines suppresses the Toll-like receptor (TLR) -dependent production of pro-inflammatory cytokines, tumour necrosis factor-α (TNFα) and IL-6, in macrophages followed by lipopolysaccharide (LPS) or peptidoglycan stimulation (Ando et al., 2008). These effects were abolished when the critical tyrosine residues in ITIM and ITIM-like motifs of siglec-9 were mutated (Ando et al., 2008). These observations are consistent with the studies of human monocytes in which siRNA-mediated knockdown of CD33, led to spontaneous secretion of pro-

inflammatory cytokines (Lajaunias et al., 2005) and collectively they indicate that ITIM-bearing CD33rsiglecs may restrain the pro-inflammatory functions of macrophages. Cross-talk between CD33rsiglecs and TLR signalling pathways was also demonstrated for siglec-H (Blasius and Colonna, 2006; Blasius et al., 2006). Following cross-linking of siglec-H expressed in pDC (Dendritic cells) with antibodies, type-I interferon production in response to TLR-9 ligation with CpG was strongly inhibited. This paradoxical inhibition of cytokine production via DAP12-coupled 'activating' receptors has been observed with several pDC expressed receptors and may be the result of a signalling pathway in pDCs shared with B cells that suppresses type 1 interferon production (Sjolin et. al, 2006).

Siglec-E is a typical inhibitory mouse siglec expressed on myeloid cells (Zhang et al., 2007; Yu et al., 2001). Boyd et al., (Boyd et al., 2009), demonstrated a TLR- and MyD88-dependent up-regulation of siglec-E on mouse bone-marrow-derived macrophages. Cross-linking siglec-E using specific antibodies shows reduction in production of nuclear factor-kB-dependent cytokines, TNFα and IL-6, in response to LPS stimulation. This suggests that siglec-E up-regulation on macrophages represents a negative feedback pathway that limits the inflammatory response to LPS signalling. A major drawback of receptor over-expression and the use of antibodies to cross-link siglecs is that they may trigger non-physiological signalling pathways. Siglecs are normally masked on the cell surface via *cis*-interactions with cell-expressed sialic acids, which limits the ability of exogenous *trans*-ligands to induce clustering at the cell surface.

1.8. Importance of ITIM-like motifs in siglec function

Most CD33rsiglecs have two conserved cytoplasmic tyrosine-based motifs, comprising a membrane-proximal ITIM and a membrane-distal ITIM-like motif. Achieving balance between positive and negative signals within the immune system is very important (Ikehara et al., 2004; Nguyen et al., 2006). Loss of inhibitory signalling is often associated with autoimmune reactivity and unchecked inflammatory responses, illustrating the essential role such systems play in the immune system (Paul et al., 2000; Ulyanova et al., 2001; Avril et al., 2004; Avril et al., 2005; Taylor et al., 1999; Yu et al., 2001).

The presence of ITIM-like motifs in the cytoplasmic regions of most of the siglecs strongly suggest that they play a role in siglec mediated cellular responses (Crocker

and Varki, 2001; Crocker and Varki, 2001). Regulation of ITAM-dependent immune responses by CD33rsiglecs has been shown in various cell types, including transfected T cells, mast cells and myeloid cells. Interestingly, both primary human T cells and Jurkat cells, which normally lack significant levels of CD33rsiglecs, showed decreased T-cell-receptor-dependent activation following over-expression of siglec-5, siglec-7 or siglec-9 (Avril et al., 2004; Avril et al., 2005; White et al., 2005; Taylor et al., 1999; Biedermann et al., 2007). Mutagenesis experiments with CD33rsiglecs have shown that the ITIM dominates over the ITIM-like motif, both for the recruitment of SHP1 and SHP2 and for inhibitory signalling functions (Jones et al., 2003; Monteiro et al., 2005; Nitschke, 2009; Avril et al., 2006). However, the ITIM-like motif was required for optimal recruitment of SHP1, but not of SHP2, and could therefore be important in fine-tuning downstream signalling from CD33rsiglecs. The ITIMs of CD33rsiglecs are important for other functions, including the suppression of siglec-dependent adhesion to sialylated ligands and endocytosis (Janicke and Mannel, 1990). In addition, robust binding to SHP1 and SHP2 requires tyrosine phosphorylation of both the ITIM and ITIM-like motif. It has also been suggested, that sialylated pathogens modulate leukocyte activation through ITIM-mediated signalling of CD33rsiglecs, thereby benefiting the pathogen by dampening inflammatory and immune responses (Putz and Mannel, 1995; Westenfelder et al., 1993).

Mononuclear phagocytes are innate immune cells with well-established roles in the primary response to pathogens, but also in tissue homeostasis, coordination of the adaptive immune response, inflammation, resolution, and repair mechanisms. These cells recognize danger signals through receptors capable of inducing specialized activation programs. The classically known macrophage activation is induced by IFNγ, which triggers a harsh pro-inflammatory response that is required to kill intracellular pathogens. Macrophages also undergo alternative activation by IL-4.

1.9. Sia-dependent functions of macrophages

Several studies investigating potential roles of Sia in macrophage functions have been addressed using the interaction of macrophages with tumour cells. Cameron et al. showed that macrophage-mediated cytotoxicity to tumour cells of patients having osteogenic sarcoma correlates with the degree of Sia expression on the tumour cells (Cameron, 1983). Only cells with high Sia content were susceptible to macrophage-mediated cytotoxicity. Several other studies then confirmed that macrophages

recognise tumour cells by sialylated structures and that this binding induces TNFα and IL-1 production by macrophages. The plasma membranes of Jurkat or K562 cells are able to induce TNFα production by monocytes indicating that the activating structure is exposed on the surface of the tumour cells (Westenfelder et al., 1993). The activating capacity of such tumour cell membranes was abolished with the destruction of sialylated glycans by treating the tumour cells with periodate, sialidase or PNGase F, prior to exposure to macrophages (Sawabe et al., 2000) but not with glutardialdehyde treatment (Hakomori, 1991; Ito et al., 2001; Saldova et al., 2008). Another important Sia function of monocytes and macrophages is the clearance of damaged and apoptotic cells by phagocytosis. Phagocytosis of oxidized T-cells and erythrocytes by human monocytic leukemia THP-1 cells differentiated into macrophages is Sia-dependent and can be inhibited by sialidase treatment of the oxidized cells (Colotta et al., 1992; Mantovani et al., 1992; Mattoli et al., 1991; Farrar et al., 1982).

1.10. Tumour associated macrophages

Phenotypic changes in malignancy often correlate with a dramatic transformation of cellular glycosylation pattern due to changes in the activity of one or more of the glycosyltransferases during the process of transformation from normal to tumour cells (Oberling, 1997). This may in turn be able to influence the tumour cell recognition by siglecs present on mononuclear phagocytes. As a result, there is increased cytokine secretion which finally may influence disease progression. Most malignant tumours contain numerous macrophages as a major component of their leukocytic infiltrate. The tumour microenvironment of tumour-associated macrophages (TAMs) can influence the tumour growth in two directions (1) they can co-exist with the malignant cells in a symbiotic manner and contribute to tumour metastasis and proliferation or (2) they can destroy neoplastic cells and present tumour-specific antigens to other leucocytes, leading to the induction of a specific immune response (Valdez and Perdigon, 1991). The monocyte derived macrophages can exhibit very high antibody-dependent and independent specific cytotoxicity for tumour cells; these macrophages are capable of phagocytosis of these cells and can present antigen on their surface (Gordon, 1998; Goerdt et al., 1999). Since mononuclear phagocytes are the first immune cells getting in contact with the tumour cells, siglecs could play an important role in tumour cell recognition and activation of the immune system.

TAMs are often in an activated state compared to normal tissue macrophages (Fenton et al., 1992; Dickensheets and Donnelly, 1997). Macrophages can be activated either by IFNγ, the dominant cytokine released by type 1 T-helper cells or by IL-4, the dominant cytokine released by type 2 T-helper cells. In general, activation of macrophages by IFNγ enhances the anti-microbial activity, whereas IL-4 induces an alternative activation which leads to efficient antigen presentation to B-cells (Cameron and Churchill, 1982). Interestingly, IL-4 can diminish the macrophage response to IFNγ (and other type I lymphokines), whereas IFNγ has comparable suppressive activity targeting the alternative response pattern induced by IL-4 (Putz and Mannel, 1995; Janicke and Mannel, 1990; Westenfelder et al., 1993). Several other studies have confirmed that the induced production of TNFα and IL-1β by macrophages is mediated by sialylated glycoconjugates, as indicated by plasma membranes of Jurkat or K562 cells being able to induce cell activation (Shrive et al., 1996; Oliveira et al., 1979).

1.11. Siglecs as immuno-modulators and targets for pathogens

High Sn expression is seen in chronic inflammatory diseases such as rheumatoid arthritis (Crocker and Redelinghuys, 2008), atherosclerosis (Gijbels, et al., 1999) and models of inherited demyelinating diseases of the nervous system (Kobsar et al., 2006). In contrast with the CD33rSiglecs, there is currently little evidence that Sn mediates signalling functions via its *trans*-membrane tail or cytoplasmic region which lacks obvious signalling motifs. Furthermore, cell–cell and cell–matrix interactions are accentuated further by the extension of the *N*-terminal V-set domain beyond the reach of shorter *cis*-interacting inhibitory siglecs closer to the plasma membrane. Sn-deficient mice exhibit reduced CD4+ T-cell and inflammatory responses in a model of autoimmune uveoretinitis. They also exhibited reduced CD8+ T-cell and macrophage recruitment in models of inherited demyelinating neuropathy in both the central and peripheral nervous systems (Crocker et al., 2007). IFNγ, a potent antiviral cytokine and immune modulator, was shown to induce Sn expression in monocytes which normally do not express the receptor and also to increase Sn expression in macrophages (York et al., 2007). IFNγ produced by activated T-cells and NK cells has also been shown to induce Sn expression on monocytes (Rempel et al., 2008; Crocker and Redelinghuys, 2008).

Siglecs has also been shown to facilitate pathogen interactions. For example, Sn and siglec-5 can promote macrophage uptake of sialylated strains of *Neisseria meningitidis* (Crocker, 2005) and functions in endocytosis of the macrophage/monocyte-tropic porcine reproductive and respiratory syndrome virus (PRRSV)(Delputte et al., 2007; Delputte et al., 2007; Delputte and Nauwynck, 2006). During the acute period of HIV-1 infection, IFNγ is produced by NK cells and T-cells, and IFNγ is released by pDCs (plasmacytoid dendritic cells) as part of the antiviral response. This may lead to induction of Sn on monocytes, which in turn binds avidly to the virus in a sialic acid-dependent manner. This may permit the effective transinfection of permissive cells and the delivery and distribution of HIV-1 to target cells in the periphery. Several CD33rsiglecs can interact with sialic acids on *N. meningitidis*, *C. jejuni*, group B *Streptococcus* and *Trypanosoma cruzi* (Jones et al., 2003; Monteiro et al., 2005; Avril et al., 2006; Carlin et al., 2009). Siglec-dependent uptake of these pathogens could potentially benefit the host by promoting pathogen destruction and antigen presentation. It has also been suggested, but not proven, that sialylated pathogens modulate leukocyte activation through ITIM-mediated signalling of CD33rsiglecs, thereby benefitting the pathogen by dampening inflammatory and immune responses.

The newly evolved members of the human CD33rsiglecs group such as Siglec-14 and -16 have revealed the existence of paired inhibitory and activating receptors within this gene family. Although the primordial functions of the CD33rsiglecs are likely to be ITIM-dependent dampening of immune responses and endocytosis, the emergence of ITAM-coupled paired receptors points towards a counter-strategy of the host towards sialylated pathogens. Clearly there is an intricate interplay between pathogens and the multiple immune receptors that determines the outcome of the immune response, and siglecs are part of this complex network that also includes many other lectin-like receptors. It will be a related challenge to understand the impact of pathogen sialylation on siglec-mediated host immune responses, an issue that may give insights into the evolutionary pathways that have led to the diversification of this family. It will also be of interest to see whether the cytokine dependent induction of CD33rSiglecs has evolved primarily to promote macrophage–host cell interactions in adaptive immunity or whether this is a determining factor in host resistance and/or susceptibility to certain sialylated pathogens.

1.12. Aim of the project

The CD33rsiglecs are mainly expressed by the cells of hematopoietic system, and have been described to alter the immune responses. It's not very clear how these responses are triggered or controlled. The unique ability of siglecs, specificity towards sialylated carbohydrates may be either used to mask pathogen receptor sites or this specificity towards Sia derivatives could be exploited by pathogens to invade the immune system. This is because in native conditions siglec binding sites are typically blocked by *cis*-interactions with glycans expressed on the same cell surface. These *cis*-interactions can be overcome in presence of higher affinity binding partner present on adjacent tumour cells or pathogen. The resultant involvement in *trans*-interactions may result in trigging immune responses. These interactions and siglec specificities towards underneath glycane linkages could be better understated through binding and inhibition assays. As siglec is a trans-membrane protein for easier handling, the first three domains involving important Sia binding domain of siglecs (-5, -7, -8, -9 and -10) cloned into the pDEF vector and stably transfected into CHOLec1 cell. CHOLec1 mammalian cells cannot synthesize complex oligosaccharide which is advantageous for binding and inhibition assays which can be hampered by glycan heterogeneity. I*n-vivo* experiments with erythroleukemia K562 tumor cell and Sia free siglecs will help to identify and isolate trans-binding partners present on K562 cells. Furthermore these Sia free siglecs can be screened for potential synthetic or biological inhibitors in binding and inhibition assays. Sia free siglecs were screened for their quality and functionality before using them in above mentioned experiments.

As described earlier siglecs show complex expression pattern on hematopoietic cells. Some reports suggests that siglec over-expression in macrophage-like cell lines suppresses the receptor dependent production of pro-inflammatory cytokines. It will be interesting to investigate if changes in inflammatory marker levels influence the siglec expression. Preliminary data show that siglec expression level on monocytes isolated from different individuals is not same. Long-term monocytes surface siglec profiling will give a hint in this direction. The data obtained can be plotted against serum CRP levels, as CRP levels get elevated in response to immune response. This will enlighten the link between immune system and siglec expression.

The immune compromised system established by co-culturing purified virus (enveloped and non enveloped) together with monocytes in suspension culture. The

resultant time and virus concentration mediated changes in monocyte surface siglec expression will give direct evidence. These experiments with virus particles will help to assess the direct role of pathogen mediated changes on monocyte surface siglec expression.

Siglecs are also described to be involved in inhibition of cellular proliferation, induction of apoptosis, inhibition of cellular activation, induction of pro-inflammatory cytokine secretion. The primordial functions of theCD33rsiglecs are likely to be in ITIM dependent dampening of immune responses and endocytosis. Clearly there is an intricate interplay between pathogens and the multiple immune receptors that determines the outcome of the immune response, and siglecs are part of this complex network. A related challenge will be to understand the impact of pathogen sialylation on siglec-mediated host immune responses. It will also be of interest to see whether the cytokine dependent induction of CD33rSiglecs has evolved primarily to promote macrophage–host cell interactions in adaptive immunity or whether this is a determining factor in host resistance and/or susceptibility to certain sialylated pathogens.

Macrophages are known to interact with sialylated *trans*-ligands on tumour cells, which induce Sia-dependent activation of the macrophages. Since no Sia-binding lectins other than siglecs are known to be present on macrophages, it is likely that this activation is mediated by siglecs. Previous experiments showed that Siglec-7 and siglec-10 bind with high affinity to the K562 erythroleukemia cell line, indicating that these tumour cells express high affinity binding partners for these two siglecs. It will be interesting to see, if one tumour-specific siglec binding partner can be isolated from different tumour cells. Such an interaction could describe a new pathway through which macrophages can distinguish between normal and neoplastic cells. To prove this hypothesis, macrophage-mediated anti-tumour activity such as IL-6 or IL1β cytokine production can be monitored. It will be also of an interest to see, if after blocking the receptor siglec macrophage mediate the anti tumour response with same efficiency. In addition, it will be interesting to investigate which of the CD33rsiglecs mediate this interaction. The project aims to investigate “How do carbohydrates and siglecs regulate the Sia mediated immune response by monocytes and macrophages?”

2. Materials

2.1. Antibodies and plant lectins

The concentrations and dilutions for reagents are according to the guidelines mentioned on product data sheet.

2.1.1. Antibodies for flow cytometry

Name	Manufacturer	Concentration/Dilution
FITC-mouse anti human CD 14 monoclonal antibody	Immunostep research	1mg/mL/1:50
PE-conjugated Goat Anti-mouse IgG antibody	Jackson Immunoresearch Laboratories, INC.	1mg/mL/1:100
Mouse anti human siglec-(1, 3, 5, 7, 8, 10) monoclonal antibody	Generous gift from Prof. Dr. Paul Crocker (cell culture supernatant)	N.A./1:25
Mouse Anti human siglec 9 mAb	Generous gift from Prof. Dr. Paul Crocker (cell line)	1mg/mL/1:100
PE-Streptavidin		1mg/mL/1:100

2.1.2. Antibodies for immunodetection

Name	Manufacturer	Concentration/Dilution
POD-donkey anti mouse IgG	Affinipure	0.4mg/mL
Biotin-goat anti human IgG, Fcγ-Fragment	Affinipure	0.64µg/mL
Goat anti human siglec-(5, 7, 8, 9, 10) polyclonal antibody	AG Kelm	Different for each antibody

2.1.3. Plant lectins and detection reagents

Name	Manufacturer	Concentration/Cilution
biotinylated MAL II (Maackia amurensis lectin II)	Vector Laboratories	10g/mL in 0.2% BSA
biotinylated PNA (Peanut agglutinin)	Vector Laboratories	10g/mL in 0.2% BSA

biotinylated SNA (Sambucus nigra bark agglutinin)	Vector Laboratories	2g/mL in 0.2% BSA
Peroxidase conjugated) Vector Laboratories® ABC reagent (Vectastain®)	Vector Laboratories	1:1 in 0.2% BSA in TBS-T

2.2. Chemicals and consumables

2.2.1. General chemicals, enzymes, and consumables

All solutions were made up in doubly deionized water (ddH_2O), from an Ultra Clear® Inegra UV Plus water purification system (SG, Barsbüttel). Media and buffer solutions were autoclaved (30 min, 121°C) or sterile filtered through (0.22 µm) filters. Unless otherwise stated, chemicals are of analytical grade. Cell culture media and reagents were obtained from Gibco, Sigma etc; whereas materials and equipment for SDS-PAGE are from the BIO-RAD (Hercules, California, USA) company. DNA-modifying enzymes, DNA polymerases and molecular weights standards used were from MBI Fermentas, New England Biolabs, Promega and Gibco.

Serological pipettes, plastic tubes, cell culture dishes, pipette tips, and 1.5mL microfuge tubes were purchased from Sarstedt, 0.2mL PCR tubes were from Biozym. Multichannel pipettes 12- and 8-channel, multichannel pipette tips (20-300µL), and cuvettes (220-1600nm) were purchased from Eppendorf, Hamburg, Germany.

2.3. Materials for the affinity chromatography

Name	Manufacturer
Chromatography columns Poly Prep®	BIO-RAD(Hercules, California, USA)
NHS-Sepharose Fast Flow (capacity 35mg protein/mL matrix	Amersham Bioscience
Protein A-Sepharose Fast Flow (capacity 35mg IgG/mL matrix)	Amersham Bioscience

2.4. Special materials and reagents

Name	Manufacturer
Acetonitrile (methyl cyanide)	Sigma
Base pair-/Molecular weight standards (80-10000 bp)	MBI Fermentas
BSA (fraction V)	PAA Laboratories
Cellline CL350 or CL1000 fermenter	Integra
Dialysis tubing (Visking ® 8/32, exclusion limit 10 kD)	Serva
ExGen500 in vitro transfection reagent	MBI Fermentas
FDP (fluorescein diphosphate)	MoBiTec (Gibco)
Hygromycin	PAA Laboratories
Skimmed milk	Heirler GmbH
X-ray film Hyper film ECL	Amersham Bioscience
Protein molecular weight standard (pre-stained + unstained)	MBI Fermentas
PVDF membrane Hybond-P	Amersham Bioscience
PAA-α2,3-sialyllactose	Gift from Dr. Strenge
PAA-α2,6-sialyllactose	Gift from Dr. Strenge
PAA-Sialyl-Lewis^{x}	Gift from Dr. Strenge
PAA-Sialyl-Lewis^{a}	Gift from Dr. Strenge
PAA-6´-O-sulfo-lactose	Gift from Dr. Strenge
Trypsin (Sequencing Grade Modified)	Promega
Vibrio cholera sialidase (VCS) 1 unit/mL	Dade Behring
Bode Korsolex® Basic	Bode Chemie Hamburg
Bode Baktobod®	Bode Chemie Hamburg
Minisar®Sterile-EO single use (0.20 µm)	Sartorius Biotech, Göttingen
Vivaspin 6	Sartorius Biotech, Göttingen
Vectaspin Micro, 0.2µm	Whatman® Schleicher & Schuell

2.5. Kits

Name	Manufacturer
BCA Protein Assay Reagent	Pierce
ECL Western blotting detection reagent	Amersham Bioscience
FastPlasmid Mini Kit™	Qiagen
HiSpeed Plasmid Midi Kit™	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit™	Qiagen
Vectastain® ABC Kit	Vector Laboratories

2.6. Bacteria, viruses and cell lines

2.6.1. Bacterial and virusstrains

Name	Manufacturer/donor
αDH5 *Escherichia coli*	Dr. Klipp Bochum
XL1-blue *Escherichia coli*	Stratagene
influenza virus strain A/PR8/34	Kind gift from Prof. Andreas Dotzauer
Coxsackie B2 virus	Kind gift from Prof. Andreas Dotzauer
Newcastle Disease Virus (NDV)	Kind gift from Prof. Andreas Dotzauer
Cytomegalovirus (CMV)	Kind gift from Prof. Andreas Dotzauer
human hepatitis A virus-7 (HAV-7)	Kind gift from Prof. Andreas Dotzauer
human hepatitis A virus-glioblastoma (HAV-GBM)	Kind gift from Prof. Andreas Dotzauer

2.6.2. Cell lines

Name	Manufacturer/donor
CHOK1 cell line (Subclone of Chinese Hamster Ovary, CHO Cells)	DSMZ, Heidelberg
CHOLec1 cell line (Subclone of Chinese Hamster Ovary cells)	DSMZ, Heidelberg
Foetal Rhesus monkey Kidney cell line (FRhK-4 cell line)	DSMZ, Heidelberg
K8 mouse hybridoma cell line producing anti hu siglec-9 monoclonal antibody	Kind gift from Prof. Dr. Paul Crocker

2.7. Media

Name	Components
LB medium (With Ampicillin)	15g tryptone, 5g yeast extract 5g NaCl makeup volume to 1L ddH_2O autoclaved, (+ 60µg/mL ampicillin)
LB-agar plates (+Amp)	15g agar in 1L LB medium (+ 60g/mL ampicillin)
RPMI medium	RPMI-1640 liquid medium (without glutamine)
α-MEM medium	10.17g α-MEM powder 2.2g $NaHCO_3$. fill up to1L ddH_2O, sterile filter, store at 4°C
DMEM medium (Dulbecco's modified Eagle's medium) Supplement list	1% (v/v) nonessential amino acid solution (MEM) 1mM sodium pyruvate MEM 0.07% L-glutamine 0.05mM 2-mercaptoethanol 100U/mL penicillin 100U/mL streptomycin 10-10% (v/v) FCS, heat inactivated

2.8. Physiological buffers

Name	Components
HBS (HEPES buffered saline)	10mM HEPES pH 7.4/150mM NaCl
HBS-T (HBS with Tween20)	HBS with 0.05% (w/v) Tween 20
HBA (HBS with BSA)	HBS with 0.5% (w/v) BSA
PBS (phosphate buffered saline)	137mM NaCl/2.7mM Na_2HPO_4, 1.5mM K_2HPO_4
PBA (PBS with BSA)	PBS with 0.5% (w/v) BSA
HBS-/PBS-Azid	HBS/ PBS with 0.02% (v/v) sodium azide (2% (w/v))
TBS (Tris buffered saline)	10mM Tris-HCl pH 7.4/150mM NaCl
TBS-T (TBS with Tween20)	TBS with 0.15% (w/v) Tween

TBE buffer (1L 10× solution)	108g Tris/55g boric acid/40mL 0.5M EDTA pH 8/ad 1L ddH_2O
TMF buffer (100mM $CaCl_2$×2H_2O/40mM $MnCl_2$×4H2O/50mM RbCl ad ddH_2O)	100mM CaCl2 ×2H2O/40mM MnCl2 ×4H_2O/50mM RbCl ad ddH_2O
0.1M bicarbonate buffer, pH 8.3	0.1M $NaHCO_3$, 0.5M NaCl
AoAB (Alsevier's medium without antibiotics)	7.94g sodium citrate/NaCl 4.5g/20.56g glucose pH 6, 1 ad 1L ddH_2O
4-fold separation gel buffer	1.5M Tris-HCl pH 8.7 with 0.4% SDS
4-fold stacking buffer	0.5M Tris-HCl pH 6.8 with 0.4% SDS
2-fold-reducing sample buffer	125mM Tris-HCl pH 6.8/20% (w/v) glycerol/0.05% (w/v) bromophenol blue/4% (w/v) SDS/10mM DTT
electrophoresis running buffer	25mM Tris/192mM Glcyin/0.1%/10% SDS
blotting buffer	25mM Tris/192mM glycine/20% (v/v) methanol
Loading buffer	250mM Na_2EDTA, 87% (v/v) glycerol, 0.5mg/mL (w/v) bromophenol blue
Coomassie Brilliant Blue solution	0.5% (w/v) SERVA Blue R/40% (v/v) methanol/10% (v/v) acetic acid/Solution then filtered through a folded filter
Coomassie distaining solution	40% (v/v) methanol/10% (v/v) acetic acid/Solution recovered with the activated carbon filter
Fixing solution (SDS-PAGE)	30% (v/v) ethanol/10% (v/v) acetic acid
Sensitizing solution (Sodium thiosulfate solution)	0.2% (w/v) sodium thiosulfate×5H_2O/0.5M sodium acetate×3H2O/30% (v/v) ethanol/0.5% (v/v) glutaraldehyde (25% (w/v) solution)
Silver nitrate solution	0.1% (w/v) silver nitrate/0.02% (v/v) formaldehyde (37% (w/v) solution)
Developer solution	2.5% (w/v) sodium carbonate pH 11.3/0.01% (V/V) formaldehyde (37%

	(w/v) solution)
Stop solution (Silver staining)	0.05M EDTA pH 8.0
blocking reagent (PVDF membrane)	5% skim milk/BSA in TBS-T

2.9. SDS-polyacrylamide gel

2.9.1. Separation gel solution

Component	Volume
4 – fold separation gel buffer	2.5mL
Acryl-/Bisacrylamid	8-15% (depending on gel)
ddH_2O	ad 10mL
TEMED	5µL
10% (w/v) APS solution	50µL

2.9.2. Stacking gel solution

Component	Volume
4-fold Stacking buffer	1mL
Acryl-/Bisacrylamid	533µL
ddH_2O	2.46mL
TEMED	2.4µL
10% (w/v) APS solution	21µL

2.10. Agarose gel

1% agarose gel: 0.5g agarose (Eurogentec); 50mL TBE buffer; boil; cool slightly; 5µL ethidium bromide (10mg/mL solution).

2.11. Oligonucleotides

All oligonucleotides for $Siglec_{d1-3}$-Fc chimera were obtained from the Eurofins MWG Operon, Ebersberg, Germany.

Name	Sequence (5'→3')	Description
OSK695	CGTCTAGAATGCTGCCCCTGCTGCTGCTGCCCCTGC	XbaI/ Hu Sig-5 Sense
OSK1066	GCAGATCTACTTACCTGTTGAGAGATTCAGAAAAAT	BglII/Hu Sig-5 antisense

OSK697	CGTCTAGAATGCTGCTGCTGCTGCTGC	XbaI/ Hu Sig-7 Sense
OSK1069	GCAGATCTACTTACCTGTGGAGAGGTTCAGGGAAAC	BglII/Hu Sig-7 antisense
OSK699	CGTCTAGAATGCTGCTGCTGCTGCTGCTGCCC	XbaI/ Hu Sig-8 Sense
OSK1070	GCAGATCTACTTACCTGTGGAGAGGCTCAGGGAAAT	BglII/Hu Sig-8 antisense
OSK703	CGTCTAGAATGCTACTGCCACTGCTGCTGTC	XbaI/ Hu Sig-10 Sense
OSK1071	GCAGATCTACTTACCTGTAGAGAGGTCCAGGGCTCG	BglII/Hu Sig-10 antisense

2.12. Machines and special software

2.12.1. Centrifuges

Name	Company
Mini Spin	Eppendorf
Centrifuge 5810 R	Eppendorf
Centrifuge 5804 R	Eppendorf
Optima™LE-80K Ultracentrifuge	Beckman Coulter
LE-70 Ultracentrifuge	Beckman Coulter

2.12.2. Fluorescence assisted cell-sorting machine

Name	Company
Coulter·Epics XL-MCL Expo 32	Beckman Coulter
FLOWCHECK™ Fluorospheres	Beckman Coulter

2.12.3. Photometer and flurometer

Name	Company
MultiScan Acent photometer	Thermo Scientific
MultiScan Acent flurometer	Thermo Scientific
Photometer	Fermentas
Nanodrop	Applied biosciences
ABC real time PCR	Applied biosciences

2.12.4. Microscope

Name	Company
Olympus CK2	Olympus

2.12.5. Software

Name	Company
EXPO 31 ADC Analysis	Beckman Coulter
EXPO 31 ADC XL 3 Color	Beckman Coulter

3. Methods

3.1. Cell culture/tissue culture techniques

3.1.1. Monocyte Isolation from human buffy-coats or peripheral blood

In humans, monocytes form approximately 1-10% of total leukocytes and are widely used for in vitro generation of macrophages. Several methods for monocyte isolation from peripheral blood exist, such as adhesion and negative and positive immunoselection. Many protocols for monocyte isolation were devised based on density gradient centrifugation (Almeida et al., 2000; Lehner and Holter, 2002). A cost-effective method was used to obtain highly enriched, monocyte suspension cultures (Repnik et al., 2003).

3.1.1.1. Ficoll density gradient centrifugation

Human blood samples were obtained from a blood donation unit in Hamburg as Buffy-coats or from collected from peripheral vein in the arm of human volunteers. Blood was collected into 10mL collection syringe containing citrate buffer to prevent coagulation. Blood sample was diluted 1:1 with RPMI media. 35mL of blood suspension was overlaid over 15mL of Ficoll solution. Tubes were centrifuged at RT (Eppendorf Centrifuge 5810R bench centrifuge) for 15min at 950g (acceleration=0/brakes=0). The upper serum layer was carefully transferred into fresh falcon tubes in order to prepare autologous serum or CRP analysis. The PBMC layer was collected into separate falcon tube, and washed 3 times with 45mL RPMI (350 g/ 7min /break=3). The cells were stained with Trypan blue and counted using the Neubauer-counting chamber.

3.1.1.2. Isolation of monocytes using cell adhesion to plastic surface

Monocytes were isolated by adhesion to the plastic tissue culture plate surface. PBMCs were resuspended in RPMI (10% autologous serum/ Penicillin-Streptomycin) and 2-3 million PBMC/well were distributed into 24-well tissue culture plates and incubated for 1h at 37°C. After incubation non adherent lymphocytes were removed by washing the wells with PBS warmed at 20°C. With this method pure mononuclear phagocyte cultures could be obtained.

3.1.1.3. Isolation of monocytes by Percoll gradients

Unattached monocytes in suspension cultures obtained with combined Ficoll/Percoll gradient (Repnik, 2003). In the hyper-osmotic percoll gradient, 3mL of the PBMC suspension having approximately 50-70 millioncells/mL was then overlaid onto the 10mL of the hyper-osmotic percoll gradient solution and centrifuged (Eppendorf Centrifuge 5810R bench centrifuge) at RT for 15min at 580g (acceleration=0 /break=0). The monocytes at the interface were collected and washed 3 times with 45mL RPMI (350g /7min /break=3). After counting, the cells were re-suspended in RPMI medium to a density of 50-70 millioncells/mL. 3mL of this monocyte enriched suspension was then overlaid on 3mL of iso-osmotic Percolll gradient solution and centrifuged (Eppendorf Centrifuge 5810R bench centrifuge) at RT for 15min at 350g (acceleration=0 /break=0). The supernatant was discarded and the monocytes pallet was re-suspended carefully in less than 1mL of RPMI medium and cells were washed 3 times with 45mL RPMI (350g/ 7min/ break=3). Cells were then counted before further use.

3.1.1.4. Negative isolation of monocytes from PBMC's

Dynal® monocyte negative isolation kit is used for the negative isolation method, 5-10$\times10^7$cells/mL PBMC were re-suspended in 0.1% BSA/PBS solution. After adding 20µL/1$\times10^7$ PBMC of blocking reagent, cells were incubated for 10min at 4°C. Afterwards 20µL antibody mix (provided with the kit) was added per 1$\times10^7$ PBMC and incubated for 10min at 4°C. Cells were centrifuged for 8min at 500g with 1mL of PBS/0.1% BSA per 1-5$\times10^7$ PBMC in Eppendorf Centrifuge 5810R bench centrifuge. Cells were then re-suspended in 0.9mL of PBS/ 0.1% BSA/1$\times10^7$ PBMC. 10µL of washed Dynabeads were then added to the cell suspension per 1$\times10^7$ of cells and incubated for 15min at 4°C with gentle tilting and rotation. Rosettes were re-suspended by pipetting 5-6 times and 1-2mL of PBS/ 0.1% BSA per 1$\times10^7$ PBMC was added. The tube was placed in the Dynal MPC magnet for 2min and monocytes containing supernatant were transferred to a fresh tube. The cells were counted before further use.

3.1.2. Cell counting: Neubauer counting chamber

Cells were counted using the Neubauer counting chamber. Staining with trypan blue ensures that the total number of viable cells in a cell suspension can be counted as

trypan blue stains the dead cells with its characteristic blue colour. Such cells are therefore not included during counting. Cells were mixed in 1:1 proportion with 2× Trypan-blue solution and 10µL was applied to a Neubauer counting chamber. The total number of cells in one of the big nine squares of the Neubauer chamber was counted under phase contrast microscope. At least three large squares were counted and average numbers used in calculating the total number of cells. The number of cells per/mL was obtained by multiplying the average cell count in the large squares with 10^4.

3.1.3. Human monocytes and monocyte-derived macrophage culture

Macrophages can be obtained by culturing monocytes for 4-6 days in the presence of 2ng/mL recombinant human M-CSF (Gordon, 1995; Montaner et al., 1994; Montaner et al., 1999). Monocyte and monocyte-derived macrophage cells were cultured in either special high binding plates (SARSSTED-24 or 6 well plates) or maintained in suspension culture (6 well CORNIG plates). Cells were maintained in RPMI/ 2.5% autologous serum/ Penicillin-Streptomycin.

3.1.3.1. Activation of monocytes and monocyte-derived macrophages

Mononuclear phagocytes can be pre-activated by adding 20ng/mL recombinant human IL-4 overnight. For IFNγ activation cells were primed initially for 4h with 1 U/mL recombinant human IFNγ followed by overnight incubation in the presence of 100 U/mL recombinant human IFNγ, under 5% CO_2 in H_2O saturated atmosphere at 37°C.

3.1.4. Cultivation of FRhK-4 cells

FRhK-4 cells were maintained in DMEM medium (DMEM/ 10% heat-inactivated FCS/ Penicillin-Streptomycin /L-glutamine) under 5% CO_2 in H_2O saturated atmosphere at 37°C. The cells were grown to 90% confluency before passaging onto fresh tissue culture flasks.

3.1.5. Virus cultivation and purification

3.1.5.1. HAV-7/ Coxsackie B virus cultivation and purification

FRhK-4 cells were infected with 1mL of seeding supernatant containing HAV-7 or Coxsackie B2 virus and incubated at 37°C for 14 days. Infected cells with medium

were freeze-thawed three times. Cell debris was removed by centrifugation at 2000 rpm for 15 min. The supernatant was transferred to 50mL falcon tubes and 50mM Tris-HCl (pH 7.5)/ 10mM $MgCl_2$/ DNaseI 20µg/mL was added and incubated at 37°C for 30min. The following detergents (0.4% DOC, 1% NP-40/ 0.5% Sarcosine/ 50mM EDTA) were then added and incubated for 1h at RT (Approx. 20mL of this solution was dispensed per 50mL falcon tube). DNA was removed by adding an equal volume of chloroform to the cell culture supernatant at 4°C for 30 min. The tubes were centrifuged for 20min at 2500 rpm. Chloroform extraction step was repeated for approx. 3 times until clear inter-phase was obtained. Trace amounts of chloroform were removed from aqueous phase by vacuum-drying for 1h.

Sucrose gradient centrifugation was performed on the aqueous phase with 0.4% DOC/40% sucrose/0.5% Sarcosine/1mM Tris-HCl (pH 7.5)/ 100mM NaCl/ 50mM EDTA (pH 8.0)/ 1% NP-40 gradient solution. The supernatant was removed with a pipette without disturbing the pellet. Pellet was resuspended in 1mL PBS and 20µL aliquots were stored at -80°C in sterile reaction cups.

3.1.5.2. Determination of tissue culture infectious dose50

FRhK-4 cells were diluted 1:8 using DMEM/ 1% FCS and distributed in two 96 well flat bottom micro titre plates. 10^{-2} to 10^{-12} viral dilutions were prepared in DMEM/ 1% FCS. 100µL of diluted viral sample was distributed in respective wells and plates were incubated at 37°C at 5% CO_2 for approximately 7 days. The plates were observed under light microscope to determine the cytopathic activity (Coxsackie B2 virus) or after 14 day incubation immunocytochemistry assay was performed for HAV-7 virus. Tissue culture infectious dose50 (TCID50) was calculated using equation developed by KÄRBER formula (Spearman et al, 1908, Karber et al., 1931).

3.1.5.3. Cultivation and purification of influenza virus strain A/PR8/34

Propagation of influenza virus was carried out in pathogen-free chicken eggs after 11-12 days of fertilization. The egg was placed in front of a light source to locate a non-veined area of the allantoic cavity just below the air sac, which was marked with a pencil. After sterilization with 70% ethanol, a small nick was made in the shell using a jeweler's scribe. Next, a hole was drilled at the top of the egg. The allantoic cavity of the egg was inoculated with the virus using a 1mL tuberculin syringe fitted with a 1/2 inch, 27gauge needle. The two holes in the shell were sealed with parafilm. The

eggs were then incubated for 2 to 3 days in temperature and humidity controlled incubators so as to effect viral infection under optimal conditions. Incubation was stopped by rapid refrigeration at 5 ±3°C. The virus was harvested by removing the part covering the air sac on the top of the egg shell. The shell membrane and chorioallantoic membrane were pierced with a pipette which was then used to remove about 10mL allantoic fluid per egg. The allantoic fluid containing virus was centrifuged at 3000 rpm, for 1h at 4°C. After this step, 1.04 - 1.16 g/mL of cleared allantonic fluid was then placed on the top of a sucrose gradient (for sucrose cushion Optima™LE-80K Ultracentrifuge with, SW20 Ti Rotor, 15,000 rpm 1h and then 40,000 rpm overnight). The supernatant was removed with a pipette without disturbing the pellet. Pellet was resuspended in 1mL PBS and 20µL aliquots were stored at -80°C in sterile reaction cups.

3.1.6. Maintenance of K562 human chronic myeloid leukaemia cells

K562 cells were cultured in H_2O saturated atmosphere at 37°C under 5% CO_2 condition in RPMI (RPMI/ 10% heat-inactivated FCS) medium. For passaging, 3 fresh culture dishes were made from a single plate that was grown to 90% confluence.

3.1.6.1. Co-culture of human mononuclear phagocyte with K562 tumour cell or viruses

For co-culture experiments K562 cells grown to 70% confluent were harvested and with 45mL RPMI (350g/ 7min/ break=3) and added to the mononuclear phagocytes in 1:5 ratios. Cells were incubated in RPMI cell culture media from 30min to 20h at 37°C at 5% CO_2. K562 erythrolukemia cells were either kept untreated or AUS-treatmetn was carried out before added to co-culture system. In the same manner K562 membrane preparations were also co-cultured with mononuclear phagocytes. In some experiments, mononuclear phagocytes were pre-activated with the typical Type-I or Type-II T-helper cell cytokines (IFNγ and IL-4). For inhibition of Sia mediated activation of mononuclear phagocyte experiments anti siglec antibody was added 1h before adding K562 cells to co-culture system.

For cytokine analysis co-culture supernatant was then collected and centrifuged down for 2min at 2000rpm at RT. After changing the reaction tube supernatant was again centrifuged for 15min at 14000 rpm at 4°C and stored immediately at -80°C until further use.

To monitor changes in monocyte surface limited siglec expression, monocytes in suspension culture were co-cultured with either enveloped viruses (Influenza virus, NDV, CMV) or non-enveloped viruses (HAV-7, HAV-GBM, viruses) in RPMI cell culture media from 10min to 20h at 37°C at 5% CO_2. In case of Influenza and Coxsackie B2 virus co-culture experiments (10µL of undiluted sample) and for NDV, CMV, HAV-7 and HAV-GBM (100µL of undiluted sample) were directly added to monocytes. Three different dilutions (1:100, 1:1000, and 1:10000) were also tested for time verses virus concentration experiments.

3.1.7. Development of chemically competent bacteria cells

αDH5 and XL1-blue strains of *Escherichia coli* were made competent by the chemical method. A 1mL overnight starter culture of the desired *E. coli* strain was added to 100mL of preheated LB medium containing 2mL of 1M Mg^{2+} solution. The culture was incubated at 37°C on a shaker at 240rpm. At regular intervals the optical density of the culture was measured at a wavelength of 600nm until a value of 0.5 OD was attained. The culture was centrifuged for 10min in 50mL tubes at 4000rpm at 4°C (Eppendorf table centrifuge 5810). The supernatant was discarded by decanting the tube. Cell pellets were resuspended in 25mL TMF buffer and kept on ice for 1h. The cells were again centrifuged and resuspended in 5mL fresh TMF buffer plus 1mL of glycerol. 200µL aliquots in 1.5mL reaction tubes were prepared and immediately stored at -80°C.

3.1.7.1. Transformation of chemically competent bacteria

The heat- shock treatment or method was used to achieve transformation of bacteria cells. For this, a 200µL aliquot of frozen competent cells was thawed and 50µL of ligation mixture was added. The reaction mixture was shaken gently and incubated for 30min on ice. Subsequently, the bacteria were placed in a water bath (heat shock) for 2min at the 42°C, chilled briefly on ice and 700 µL of SOC medium was added immediately and incubated in a shaker for 45min at 37°C and 240 rpm. The bacterial suspensions were centrifuged for 1min at 1000rpm and the supernatant was removed. The pellet was resuspended in 100µL SOC medium, and then spread on a LB/Amp agar plate and incubated at 37°C overnight.

3.1.8. Production of stable cell lines

3.1.8.1. Cultivation and maintenance of adherent eukaryotic cells

CHO-K1/Lec1 cells were grown in α-MEM medium with 10% heat-inactivated FCS. Incubation was at 37°C under 5% CO_2 in H_2O saturated atmosphere. The cells were grown to 90% confluency before they were passaged onto several 10cm cell culture dishes. For passaging, the following steps were carried out. Cell culture dishes were washed 2× with 7mL 1×PBS and then 1mL trypsin-EDTA solution (per 10cm plate) was added to the cells. The solution was immediately aspirated and the cell culture dishes were incubated for 2-5min in the incubator. The detached cells were resuspended in 10mL culture medium and evenly distributed on the cell culture dishes.

3.1.8.1.1. FCS Immunoglobulin-depletion

To avoid contamination of secreted siglec Fc chimeras with immunoglobulin from FCS that is contained in the cell culture media, 20% FCS in the culture medium (RPMI or DMEM) was incubated overnight at 4°C with protein A-sepharose beads (GE Healthcare). The immunoglobulin Fc part binds to Protein A and thus Ig's can be removed (Bebbington, 1991). Protein A beads were removed by passing the media over a column with membrane and then a sterile filter before storing at -20°C.

3.1.8.2. Stable transfection of CHOLec1 cells

The pDEF vector in which $Siglec_{d1-3}$ coding sequences was inserted contained -(3c)-Fc, in addition to the ampicillin resistance gene, the hygromycin B resistance gene which could be used as marker in selecting stable cell lines. Only successful transfected CHO cells could survive the antibiotic hygromycin B because of the hygromycin B phospho-transferase (HygR) resistance activity impacted upon them from the pDEF vector upon successful uptake of the plasmid. For stable transfection cells were cultured in αMEM with 10% FCS until they were 70-80% confluent. The cell culture medium was removed and the cells were washed once with PBS. Transfection was carried out with the cationic polymer ExGen500 (MBI Fermentas) according to the protocol provided by the manufacturer. This reagent forms complexes with the DNA which are then deposited by gravity on the cells and are absorbed probably by endocytosis from the cell. For confluent cells on a 100mM cell culture dish, 58µg of DNA was added to an appropriate volume of sterile 150mM

NaCl solution and was mixed gently and centrifuged briefly. Then 192µL ExGen500 was added, bringing the total volume to 1mL, mixed by vortexing for 10 sec and then incubated for 10min at RT. The transfection mix was added to a cell culture dish filled with 14mL serum-free αMEM medium. After 3-4h old culture medium was removed and replaced with fresh 20mL pen/strep containing cell culture medium.

3.1.8.3. Selection of stably transfected cells

The cells were trypsinized and resuspended in 5mL medium after a 24h incubation period. To determine the cell density, cells were counted with Neubauer-counting chamber. The cell density was adjusted to 5×10^5cells/mL. For selection of successfully transfected cells, hygromycin B was added in different dosages starting with at least 350 g/mL concentration. The outer rows were filled with copper sulphate solution to prevent contamination. After 3-5 days, plates were checked under phase contrast microscope to ascertain degree of cell survival. Media was changed on a weekly basis. After 2-3 weeks the hygromycin-resistant cell populations were screened for production and secretion of siglecs by sandwich ELISA. Highest producing cells were selected and transferred to 6-well culture plates. Subsequently they were transferred to 10cm culture plates.

3.1.8.4. Recombinant Fc-chimeras production

Successful expression of siglec Fc-chimeras was determined by sandwich ELISA. Recombinant Fc-chimeras was harvested every 2 or 3 days, cells in suspension culture were centrifuged for 7min at 700 rpm to harvest the supernatant. Cells were resuspended in fresh medium and were further incubated. The pH of the harvested supernatant was adjusted with 1M HEPES, and centrifuged for 20min at 9000 rpm (Eppendorf Centrifuge 5810R bench centrifuge) to remove any cell debris. The supernatant was then sterile filtered (0.2µM) to remove any contaminants and then stored at -20°C until purification.

3.1.8.5. Adaption of CHOLec1 cells to EX-CELL® Chemically defined CHO Serum-free media

EX-CELL® Chemically defined Hydrolysate Fusion media is an animal product free, chemically defined media. This synthetic media is specially optimized for Chinese Hamster Ovary (CHO) cell lines. Cells grown in this media do not adhere but rather

are in suspension. To avoid immunoglobulin contamination from FCS and to increase the production of siglecs, cells were adapted for EX-CELL® CD CHO serum free medium. Culture supernatant was harvested every other day and pH was adjusted with 1M HEPES. Supernatant was centrifuged for 10min at 900rpm to remove dead cells and other debris.

3.1.9. K562 membrane protein preparation

K562 cells were pelleted at 300g and washed twice in Dulbecco's PBS (DPBS) and then resuspended in a cold solution containing 100mM KCl, 5mM NaCl, 3mM MgCl2, 50mM Hepes, pH 7.4, and 1mM dithiothreitol (DTT) (lysis buffer) and placed on ice for 20 min. The suspension was centrifuged at 500g and 4°C for 10 min, to remove nuclei and unbroken cells, and the supernatant then spun at 14000g and 4°C for 10 min. The resulting pellet was resuspended in lysis buffer containing 1mM EGTA and then homogenized in a Teflon-glass homogenizer with 10 strokes of the pestle. Following a further spin at 14000g and 4°C for 10 min, the crude membrane fractions were resuspended in 50mM Tris/HCl, pH 7.0, at approx. 2mg/ml and stored at -80°C till use. Protein determinations were by the BSA assay method using BSA as standard.

3.2. Assays

3.2.1. Flow cytometry analysis

Flow cytometry analysis of a single cell suspension yields multi-parameter data corresponding to Forward Light Scatter (FSC), 90° Light Scatter- Side scatter (SSC), and FL1-FL4. This information allows identifying and characterizing various subpopulations of cells. Scattered and emitted light from cells is converted to electrical pulses by optical detectors. The electrical pulses generated from light are then processed by a series of linear and log amplifiers. Logarithmic amplification is most often used to measure fluorescence in cells. The instrument used here was the Beckman-Coulter XL instrument, bench-top flow cytometer analyzers (COULTER® EPICS®XL™).

With flow cytometry the expression levels of distinct cells surface structures can be analyzed. Fluorescent labelled antibodies used to measure cell surface expression of distinct proteins and polyvalent oligosaccharides probes to analyse the presence of corresponding glycane structures on the cells.

For staining, approx 100000–200000 cells were liquated per well in 96 well round bottom micro titter plates in 100µL staining buffer. Staining was achieved by incubating cells first with the primary reagent (i.e. specific antibodies, or sugar probes) for 6h or overnight at 4°C. After washing the cells 3× with 200µL staining buffer, the fluorescent labelled secondary detection reagent was added and cells were incubated for 4h at 4°C. Cells were washed and fixed by adding 150µL 0.08% paraformaldehyde (PFA) in staining buffer. Flow cytometry analysis was then performed.

3.2.1.1. Binding of siglec-Fc chimera to K562 cells

1-2x10^5 untreated or sidalidase-treated K562 tumour cells were resuspended in 100µL staining buffer (10mg/mL BSA, 10mM PBS pH 7.4, 0.01% NaN_3). Incubated with 2µg of Siglec-Fc chimeras (Siglec-1, -3, -5, -7, -8, -9, -10 either purified from CHOK1 or Lec1 cells) for 1h on ice. Cells were washed 3× with 350µL of staining buffer and either fixed directly with 0.8% paraformaldehyde in PBS and stained for another hour on ice with the 2µL PE-labelled anti hu-IgG-Fc antibodies prior to additional washing and fixation. Flow cytometry data were acquired using a flow cytometer.

3.2.1.2. Determination of PAA-biotin glycan binding

1x10^5 cells were resuspended in 100µL staining buffer (10mg/mL BSA, 10mM PBS pH 7.4, 0.01% NaN_3) and incubated with 1µg PAA-biotin glycan probes for 1h on ice. Cells were washed twice with 1mL of staining buffer and either fixed directly with 0.8% paraformaldehyde in PBS and stained for another hour on ice with the PE-streptavidin prior to additional washing and fixation. Flow cytometry data were acquired using a flow cytometer.

3.2.1.3. Detection of monocytes from PBMC's

1-2x10^5 PBMC's were resuspended in 100µL staining buffer and 8µL of fluorescent labelled (FITC) anti CD14 antibody was added to the sample and incubated for 6h at 4°C. After washing the cells 3× with 350µL FACS buffer and fixed directly with 0.8% paraformaldehyde in PBS, cells were analysed by flow cytometer.

3.2.1.4. Siglec detection on monocytes

1-2x10^5 monocyte enriched sample or monocyte-virus co-culture samples were resuspended in 100µL staining buffer, and 1-5µL monoclonal mouse anti hu-Siglec antibodies was added to the sample and incubated for 6h-20h at 4°C. After washing the cells 3× with 350µL FACS buffer and fixed directly with 0.8% paraformaldehyde in PBS, 2µL PE-labelled anti mouse IgG Fc antibodies was added for another 4h on ice to detect siglec expression by flow cytometer.

3.2.2. Enzyme-linked immunoadsorbent assay (ELISA)

3.2.2.1. Human Interleukin-6 and -1β ELISA

The ELISA performed was the two-step sandwich ELISA. Released pro-inflammatory cytokines from monocyte-K562 tumour cell co-culture supernatants were analysed using an ELISA kit. Microwells were filled with 100µL of IL-6 or IL1β capture antibody diluted in bicarbonate coating buffer. Plates were sealed with parafilm and incubated overnight at 4°C. On the following day, wells were aspirated and washed 3× with ~300µL /well wash buffer consisting of 2% FCS. To avoid unspecific binding, plates were blocked with ~200µL/well assay buffer. Incubation was carried out at RT for 1h. Then, the plates were aspirated and washed with ~300µL /well with wash buffer. 100µL of each standard, sample, and control were pipetted into appropriate wells and after sealing with parafilm incubated for 2h at RT. Wells were washed 5 times with ~300µL wash buffer /well and 100µL of working detector solution (Detection Antibody + SAv-HRP reagent) was added to each well. The sealed plate was incubated for 1h at RT and washed 7 times with ~300µL /well wash buffer (in this final wash step, soak wells in wash buffer for 30 sec for each wash). 100µL of substrate solution was added to each well and the plate was incubated without plate sealer for 30min at RT in the dark. 50µL of stop solution was added to each well before reading the absorbance at 450nm after 30min of stopping reaction.

3.2.2.2. Quantitative sandwich ELISA for detection of produced Fc chimeras

Purified proteins or cell culture supernatants of Siglec-Fc chimeras were detected in a sandwich ELISA with antibodies against the human Fc portion. The micro titter plate was coated at 4°C overnight with 10µL of anti-human IgG solution (capture antibody) in bicarbonate buffer. Next day, the capture antibody solution was

discarded by flipping the plate on paper towels and the plate was washed 3 times with TBS/Tween20 solution. Then 5µL of an anti-human IgG-AP solution mixed with 5µL of Siglec-Fc solution in a 1:2 dilution series was added. For calibration 1:5 serial dilution series in TBS/Tween20 starting from 2µg/mL of only Fc-part was used. TBS/Tween20 was used as a negative control. The cell culture supernatants from stable cell lines grown in Excel media were previously diluted at least 1:10 before a 1:2 dilution series was made. For each dilution of the Fc-chimera, triplicate determinations were performed. The Fc chimeras/anti-human IgG-AP (secondary antibody) mix were applied and incubated for 4h at 4°C to allow the Fc part to bind to the immobilized antibody. After incubation, the supernatant was discarded and the plate was washed 3× with TBS/Tween20. By adding an enzyme substrate, in this case 20µL FDP solution, Fc-chimeras concentration can be determined. The signal was quantified by a Fluroscan Ascent (Thermo Life Sciences) fluorescence plate reader at Ex/Em = 485 ±20 / 528 ±20nm. The concentration of the samples was calculated using the established calibration curve.

3.2.2.3. Solid phase cell binding assay

The Sia-specific binding of Siglec-Fc chimeras was verified by a solid phase assay with human erythrocytes as target cells whose surfaces glycans carry α2,3- and α2,6-linkages for Sia. Blood was obtained from a volunteer donor with a serum collection syringe which contained citrate as anticoagulant. The blood was centrifuged for 10min at 2000rpm in an Eppendorf 5810R bench centrifuge. The plasma and the leucocytes were removed by suction, the red cell pellet was washed 3× with HBS and resuspended in AoAB (Alsevier's Buffer) and stored at 4°C. Buffer was changed on a daily basis.

As a control for nonspecific, non-Sia-dependent binding sialidase treated erythrocytes were used; the Sias were removed with sialidase enzyme from *Vibrio cholerae* (VCS). The solid phase assay was performed similarly to the sandwich ELISA (instead of secondary antibody, human erythrocytes were used). Captured Siglec-Fc chimeras were incubated with 50µL of the 0.5% solution of erythrocyte suspension in HBA. Cells were incubated for 1h at RT. Unbound erythrocytes were resuspended by gently shaking the plate and removed by aspiration. The bound erythrocytes were fixed by adding 100µL 0.25% glutaraldehyde solution in HBS with

gentle shaking of the plate. After fixation, the wells were washed twice with HBA and cell-formations observed under the microscope.

3.2.2.4. C-reactive protein (CRP) assay

The assay is based on the double antibody sandwich format where bound anti-CRP antibodies capture CRP from serum. This bound CRP can be detected with an enzyme that conjugates anti-CRP antibody after adding chromogenic enzyme substrate to the wells. This reaction is stopped with EDTA and the optical density measured to reveal levels of CRP. CRP concentrations are read off the standard curve and, when corrected by the dilution factor, give the CRP concentration in the serum sample.

Samples and standards were prepared to an appropriate dilution for each specimen to be tested according to the manufacturers' instructions. 50µL of each CRP standard and diluted test sample was dispensed into designated microwells. Then they were mixed briefly on a plate shaker, covered, and incubated at RT for 60min. After washing the micro tittre wells with 350µL of working-strength wash solution, 100µL CRP Tracer solution was added and mixed briefly before incubated for 60min at RT. After washing, 100µL of substrate solution was added and incubated uncovered at RT for 30min. To stop the reaction 100µL EDTA solutions was added and the OD was measured at 405nm in MultiScan Acent Photometer.

3.2.3. BCA (bicinchoninic acid) protein assay

With the help of the BCA protein test based on bicinchoninic acid, the amount of protein in solution was measured (BCA™ ProteinAssayKit, Pierce). For calibration, BSA concentration standards ranging from 0.025-2.0mg/mL were used. 5µL protein solution was pipetted into the micro titter plate wells in duplicate. 100µL of the reaction mixture from the BCA kit was then added and briefly mixed; the micro titter plate was then covered with parafilm layer and incubated for 30min at 37°C. After incubation, the OD was measured at 562nm in a Multiscan Ascent (Thermo Life Sciences) photometer. Using the BSA calibration series, the protein concentration was determined for each sample.

3.2.4. Immunocytochemistry of monocytes and K562 tumour cells

Monocytes were allowed to adhere in 24-well plates before tumour cells were added to the cultures, and then they were co-cultured for 6-18h. After co-culture, cells were washed carefully twice with PBS followed by fixation of the cells for 10min using 4% paraformaldehyde in PBS. Fixed cells were washed twice with PBS and the remaining free aldehyde groups were blocked by incubation with PBS/10% FCS for 1h at RT. Free Fc-receptors of monocytes were then blocked for 30min by adding 10µg/mL purified human Fc-fragments diluted in PBS/3% bovine serum albumin (BSA). Cells were then stained with mAb antiSiglec-7 hybridoma cell culture supernatants diluted 1:5 in PBS/3% BSA, followed by detection of bound antibodies using TRITC-labelled anti mouse IgG antibodies.

3.3. Protein biochemistry

3.3.1. Ultracentrifugation of cells culture supernatant

The cell culture supernatant was thawed and sodium azide solution was added (0.02% (v/v)) to prevent any bacterial contamination. Approximately 65mL of supernatant was dispensed in each ultracentrifuge tube and the tubes were carefully balanced to a difference of not more than 0.05g, as recommended by the manufacturer of the instrument (Beckmann Coulter). Ultracentrifugation was carried out at 4°C at initially at 7800rpm for 15min and then at 40000rpm for 45min in TI-45 rotor (Beckmann Coulter). The supernatant was taken out carefully without disturbing the pellet. 500µL aliquot was taken from supernatant for SDS-PAGE, ELISA and was stored at -20°C.

3.3.2. Purification of Fc chimeras and antibodies using Protein A beads

Purification of antibody/Siglec-Fc chimera was carried out at 4°C. 1mL Protein A beads were equilibrated with Wash buffer I (WB I). The equilibrated beads were added to the cell culture supernatant and incubated overnight at 4°C (batch process) on a rotary shaker. The following day, beads treated supernatant was passed over column with membrane in order to retain protein A-Sepharose beads for elution of bound protein. Beads were first washed with 100mL WB I and then with 100mL Wash buffer II (WB II). The elution from beads was done by pH shock by applying 500µL Elution buffer I (EB I). Every time, 15-20 samples were collected in 1.5mL reaction tube. To prevent conformational changes of the protein due to the pH shock,

neutralization of the eluted samples was done immediately by adding 75µL Neutralization buffer (NB). The cups were vortexed immediately for better mixing. Protein A beads were regenerated using 20mL elution buffer II followed by 5mL NB. Washing was done by 20mL WB II and column was stored in 20mL 20% ethanol in WB II. Absorbance of all the fractions was measured by a photometer. Fractions with O.D at 280 that were > 0.05 were pooled together. Sodium azide was added to a final concentration of 0.02% and the reaction cups were kept for 30min at 4°C.

3.3.3. Buffer exchange using PD-10 column

Buffer exchange was carried out by gel permeation chromatography using PD-10 column (Sephadex 25, GE Healthcare) against 1× HBS. The column was cleaned with 2 column volumes of ddH_2O followed by 2 column volumes 0.1M NaOH/1% SDS, then with 2 column volumes of 0.1M HCl and with 2 column volumes of double distilled water. The PD-10 columns used here accommodated a maximum volume of 2mL. Therefore, larger protein volumes were concentrated using Vivaspin 6. 2mL sample was loaded and elution was done with 1× HBS. 30 fractions of 500µL each were collected. Absorbance was measured and fractions with O.D at 280 of 0.05 were pooled. Sterile filtration was done using Vectaspin Micro, 0.2 µm. Centrifugation was done for 2min at 2000rpm. The final protein content was determined by BCA assay. All the aliquots were stored at 4°C.

3.3.4. Quality control and characterization of purified proteins

3.3.4.1. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed in mini gel apparatus from BIO-RAD (Hercules, California, USA) with the buffer system (Tris-glycine-HCl) according to Laemmli (Laemmli, 1970) under denaturing and non-denaturing conditions.

The protein samples were prepared by adding an equal volume of 2-fold reducing/non-reducing sample buffer before boiling for 5min at 95°C. The mini gels were loaded with the denatured proteins and with a molecular weight standard and electrophoresis was performed at 15mA/gel for approximately 1-1.5h. For western blotting pre-stained protein standards were used.

3.3.4.2. Coomassie brilliant blue staining

The proteins in the SDS PAGE gel can be visualized with Comassie Brilliant Blue G250 (SERVA Blue R; SERVA, Heidelberg), triphenyl methane dye. After washing the separation gel with ddH_2O in order to remove SDS, it was incubated for 30-50min in Coomassie Brilliant Blue solution. The separation gel was washed briefly in ddH_2O before gradually distained with distaining solution.

In later experiments Coomassie-based protein staining solution PageBlue™ from MBI Fermentas was used. PageBlue™ solution has a higher sensitivity and contains no methanol and can be decolorized with ddH_2O.

3.3.4.3. Silver staining

SDS-gels are fixed with 50mL fixing solution. Then 50mL of sensitising sodium thiosulfate solution was added and incubated for 30min. After washing gels 3× in ddH_2O for 20min, 50mL of silver nitrate solution was added and incubated for 30min. The gels were washed three times quickly with ddH_2O. To visualize the protein 50mL of developer solution was added. If the developer solution turned brown, fresh developer solution was added till we observed clear protein bands. To stop the reaction 50mL stop solution was added and incubation was performed for 15min. Before handling gels were rinsed with ddH_2O.

3.3.4.4. Western Blotting

The transfer of proteins from electrophoresis gels to an immobilizing membrane is called the protein or Western blotting (Towbin et al., 1979).Here, the protein transfer took place under an electric field (Kyhse-Andersen, 1984) in a blotting chamber (Mini Trans-Blot® Electrophoretic Transfer chamber, to a polyvinylidene difluoride membrane (PVDF membrane). The PVDF membrane was first soaked in 100% methanol and then dampened in ddH_2O, before it was put in the threshing cassettes together with gel and all other components in blotting buffer. According to the manufacturer's instructions the components were placed together in the gel cassettes without any air bubbles because it may interfere with blotting process. The transfer took place in the chamber filled with Blotting buffer with a constant 100V for 1h at 4° C. After successful transfer the pre-coloured (pre-stained) protein standard will be visible on the membrane, the gel side of the PVDF membrane was marked and the membrane was washed for 10min in TBS-T buffer.

3.3.4.4.1. Immunodetection of proteins

For the immunodetection of proteins on PVDF membrane, membrane was incubated overnight at 4°C in blocking reagent. The membrane was washed five times for 5min in TBS-T buffer. The primary antibody solutions were pipetted onto the protein side on membrane and incubated for 1-1.5h at RT. After washing the membrane as described before, it was placed in 20mL of the secondary peroxidase (POD)-conjugated antibody containing solution and incubated for 1h at RT under slightly shaking conditions. The detection was carried out with the ECL (Enhanced Chemoluminescence) reagent. 1mL substrate solution was prepared according to the manufacturer instructions and membrane was incubated on parafilm for 5min. Membrane was exposed with an X-ray film for 0.5 to 10min and then the exposed film was developed in an Agfa Curix60 film developer; the machine was set according to manufacturer's instructions.

3.4. Sialidase treatment

3.4.1. Vibrio cholerae sialidase treatment

The enzyme sialidase from *Vibrio cholerae* (VCS) removes Sias from protein surface. For the VCS-treatment 190µL packed (previously centrifuged) human erythrocytes were washed three times with HBA with 2mM $CaCl_2$, and then in 310µL calcium-HBA (HBA/2mM $CaCl_2$) then 40mU/mL (40µL) VCS was added and incubated for 3h at 37°C. After incubation, the cells were washed five times with three times more volume of HBA and finally resuspended for storage in AoAB.

3.4.2. Arthrobacter ureafaciens sialidase treatement

To remove Sia from cell surface under mild physiological conditions, 1-6x10^6 cells were washed twice with 1mL RPMI/50mM HEPES pH 6.9, resuspended in 1mL RPMI/ 50mM HEPES pH 6.9 containing 20mU *Arthrobacter ureafaciens* sialidase (AUS), incubated at RT for 30min and washed 5 times with the buffer used in the following steps.

3.5. Molecular biology methods

3.5.1. Human Interleukin-6 real time polymerase chain reaction (TaqMan)

Real-time polymerase chain reaction (RT-PCR) enables both detection and quantification of one or more specific sequences in a DNA sample as absolute

number of copies or relative amount when normalized to DNA input or additional normalizing genes. To perform RT-PCR, total RNA was isolated from mononuclear phagocytes and after DNase treatment to remove contamination DNA (Promega DNAse) RNA concentration in each sample was quantified. RNA was then reversely transcribed to make cDNA and then PCR was performed to quantify IL-6 mRNA copy number with calibrated sample.

3.5.1.1. peqGOLD Isolation Systems TriFast™

Total RNA from mononuclear phagocytes after co-culture with K562 cells was extracted, by using peqGOLD Isolation Systems with TriFast™ solution from PEQLAB Biotechnologie GmbH. Cells were directly lysed in culture dish by addition of TriFast™ and by passing the cell lysate several times through a pipette. The amount of TriFast™ needed is based on the area of the culture dish (1mL per 10cm^2) and not on the number of cells. 0.2mL of chloroform was added and incubated for 15sec and reagent tubes were shaken vigorously by hand and again incubated for 3-10min at RT. After centrifugation at 12000g the RNA containing aqueous phase was collected in fresh reaction tube. The RNA was precipitated with 0.5mL of isopropanol per 1mL of Tri-Fast™ samples by centrifuge for 10min at 4°C at 12.000g max. The RNA pellet from bottom was carefully collected and washed twice with 75% ethanol by vortexing and subsequent centrifugation for 8min at 7500g at 4°C. The excess isopropanol from the RNA pellet was air-dried and the RNA pellet was resuspended in Rnase-free water before storing at -20°C until further use. Aliquote was taken to check RNA quantity and quality with photometer.

3.5.1.2. Preparation of DNA-free RNA

DNaseI is an endonuclease that digests single- and double-stranded DNA. The enzyme activity is strictly dependent on Ca^{2+} and is activated by Mg^{2+} or Mn^{2+} ions. To an RNase-free tube 1µg total RNA was added with 1µL 10× reaction buffer with $MgCl_2$, 1µL (1U) DNaseI, and filled up to 10µL with DEPC-treated Water, incubated at 37°C for 30min. To deactivate DNase activity, 1µL 50mM EDTA was added and incubated at 65°C for 10min. This RNA preparation was used as a template for reverse transcriptase.

3.5.1.3. cDNA Synthesis for RT-PCR

RevertAid™ Hminus Reverse Transcriptase (RT) is a genetically modified M-MuLV RT. The enzyme possesses RNA-dependent and DNA dependent polymerase activity, but lacks RNase H activity due to point mutation in the RNase H domain. To generate first-strand cDNA for use in two-step RT-PCR, 100ng of total RNA in a sterile, nuclease-free tube was mixed with Oligo(dT)18 primers, filled up to 12.5µL with DEPC-treated water, mixed, briefly centrifuged and incubated at 65°C for 5min, chilled on ice, again briefly centrifuged and placed on ice. 4µL of 5× Reaction Buffer, 0.5µL (20U) RiboLock™ RNase Inhibitor, 2µL (1mM final concentration) dNTP (10mM) Mix, and 1µL (200U) RevertAid™ Hminus Reverse Transcriptase was added to this solution to obtain a total volume of 20µL. The reaction tube was gently shaken and centrifuged briefly before being incubated for 60min at 42°C. The reaction was terminated by heating at 70°C for 10min. The reverse transcription reaction product was then stored at -20°C. 2µL aliquot of the reaction mix was taken to perform control PCR with Tubuline primers.

3.5.1.4. TaqMan® IL-6 real time PCR

To quantify the expression of human IL-6, commercial TaqMan® Pre-Developed Assay Reagents for Gene Expression (PE) were used, and reactions were performed according to the protocol included. The specificity of PCR primers was tested under normal PCR conditions in a thermocycler (Eppendorf Mastercycler Gradient). As an internal control, we used commercially available Tubulin primers, internal control primers and probe were added at 50nM concentrations. Step One Plus Real time PCR system Thermal cycling proceeded with 50 cycles at 95°C for 15Sec and 60°C for 1min. Data were analyzed using a Step One software V2.0 program from Applied Biosciences. Input RNA amounts were calculated with a multiple comparative method for the mRNAs of interest and Tubuline. Analyses were performed in triplicate for each data point. The results are expressed as ratio of given mRNA/Tubulin.

3.5.2. Cloning of siglec$_{d1-3}$ incorporated pDEF vector in CHO lec1 cell line

In order to create stable cell lines for some CD33rSiglecs, the coding sequence for the first three C-terminus siglec domains (CD33, -5, -7, -8, and -10) including V-set domain was amplified from pCDM8 vector. This particular expression vector has no rhinovirus 3c protease proteolytic cleavage site and has no selection pressure marker

for creating stable cell lines. The coding sequence for siglec(s) d1-3 was cloned in the pDEF expression vector. The coding sequence contains internal restriction enzyme sites for SpeI/XbaI and BglII. So the amplified product from pCDM8 vector will also have these restriction endonuclease sites incorporated at the 5' and 3'-ends, respectively. Thus, the amplified product can have the same cohesive ends as at the interfaces on the expression vector. pDEF vector has multiple cloning site including for XbaI and BamHI. The sense primer amplification product will have either SpeI or XbaI at the 5'-end and the antisense primer amplified product will have BglII interface at the 3'-end. So after restriction digestion, the amplification product cut with SpeI/XbaI and BglII and the expression vector cut with XbaI and BamHI will have the same cohesive ends.

3.5.2.1. Amplification of DNA by PCR

The source of the DNA used as template can be purified plasmid DNA or an entire colony of bacteria. If a colony is to be used as a template, a single colony was picked-up with a sterile toothpick from an agar plate in a PCR reaction tube, and then replica plate was prepared. All components were used as master mix and 50µL was added to each selected template DNA or bacteria colony.

3.5.2.2. PCR with Taq polymerase

A 0.2mL reaction tube (Biozym) was used for preparing the PCR mix with the Taq polymerase (MBI Fermentas).

Component	Stock	Amount
Template DNA or a bacterial colony picked		1ng
Primer 1 (20 pmol/µL)	100 pmol	2µL
Primer 2 (for the opposite strand) (20 pmol/µL)	100 pmol	2µL
2mM dNTP mix (0.5mM dATP, dCTP, dGTP and dTTP)	2.5mM	5µL
25mM MgCl2	0.2mM	5µL
Taq reaction buffer 10-fol	1-fold	5µL
Taq polymerase (1 unit/µL)	1 Unit	1µL
ddH2O		ad 50µL

The PCR was carried out under the following conditions in a thermal cycler from Eppendorf.

Process	Temperature	Duration	Cycle
initial denaturation: inactivation of nucleases and complete denaturation	95°C	30 sec	
denaturation of the DNA	95°C	30 sec	30
annealing (Tm)	56-65°C	30 sec	
polymerase reaction (continuous renewal/extension)	72°C	240 sec	
final extension	72°C	1300 sec	
Cooling/hold	4°C		

3.5.2.3. PCR with pfu polymerase

The Pfu polymerase was used provides an additional 3'-5' exonuclease activity which is effectively a correction activity (Proof Reading).

Component	Stock	Amount
Template DNA		1ng
Primer 1 (20 pmol/µL)	100 pmol	2µL
Primer 2 (for the opposite strand) (20 pmol/µL)	100 pmol	2µL
0.2mM dNTP mix (0.5mM dATP, dCTP, dGTP and dTTP)	10mM	1µL
25mM MgCl2	0.2mM	5µL
Reaction buffer MgSO4-10-fold	1-fold	5µL
Pfu polymerase (2.5 unit/µL)	1.25 Unit	0.5µL
ddH2O		ad 50µL

The PCR was carried out under the following conditions in a thermal cycler from Eppendorf.

Process	Temperature	Duration	Cycle
initial denaturation: inactivation of nucleases and complete denaturation	95°C	30 sec	
denaturation of the DNA	95°C	30 sec	30
annealing (Tm)	56-65°C	30 sec	
polymerase reaction (continuous renewal/extension)	72°C	240 sec	
final extension	72°C	1300 sec	
Cooling/hold	4°C		

To control the PCR reaction 5µL of the reaction mixture was analyzed on an agarose gel. PCR product was purified either using the QIAquick PCR Purification Kit from Qiagen or from the agarose gel using QIAquick Gel Extraction Kit from Qiagen.

3.5.2.4. Purification of PCR products

The purification was performed with the QIAquick™ PCR Purification Kit (Qiagen). 5-fold guanidine hydrochloride-containing buffer (PB) was added to the PCR product and then the PCR mixture was pipetted on the spin columns provided with the kit and centrifugation for 13000rpm at RT in Eppendorf MiniSpin. The flow through was discarded and the bound DNA was washed with 750µL of the ethanol-containing buffer (PE) by centrifugation. Then, 50µL EB buffer (10mM Tris-HCl pH 8.5) was applied to the column and incubated for 1min. Then the DNA was eluted into a 1.5mL reaction tube by 1min centrifugation. For control 2µL of DNA solution were analyzed on an agarose gel.

3.5.2.5. Separation and extraction of DNA

3.5.2.5.1. Agarose gel electrophoresis of DNA

In an agarose gel electrophoresis, DNA fragments are separated according to size. The ethidium bromide intercalates with DNA and fluoresces under UV light, so that the DNA bands are visible after electrophoresis with UV light. Depending on the future use, a preparative comb (120µL volume) or an analytical comb (12µL volume) was used. Thereafter, 100µL or 10µL of sample was pipetted together with 15µL or 1.5µL loading buffer and separated; the molecular weight standard was also added on the same gel. The electrophoresis was carried out for 30-60min at 100 volts. Then, the visualization of DNA bands was carried out on a UV-table at 405nm, using Herolab-Geldokumentations systems.

3.5.2.5.2. DNA extraction from agarose gel

On the UV table, the corresponding DNA bands were concisely cut from the agarose gel with a scalpel and transferred to 1.5mL reaction tubes. The weight of the gel piece (100µL≈100mg) was determined (maximum 400mg) and the guanidine thiocyanate-containing buffer QG was added three times to the volume of gel piece. Then it was incubated for 10-minutes at 50°C mixed well for 2-3 times during incubation or until the gel had dissolved completely. The other steps were done similar to the QIAquick™ PCR Purification Kit.

3.5.2.6. Plasmid

The pDEF (Fc-3c) vector (pDEF) was provided by Dr. Crocker (Dundee, UK). The resistance gene for hygromycin B (HygR) under control of the SV40 late promoter allows a high copy number of the plasmid in transfected cells. This expression vector is for eukaryotic cells and has the eukaryotic transcription factor EF-1. With pDEF vector the transfected eukaryotic cells can be selected by resistance to hygromycin B and thus permanently stably transfected cells can be obtained. Furthermore, the vector contains also an origin of replication for bacteria (ori), for the reproduction of bacteria, and a gene for resistance to ampicillin, which allows the positive selection of transformed bacteria. The pDEF vector also includes the human IgG1 Fc portion, which includes the hinge region (hinge-CH2-CH2-CH3). This region has been modified and a proteolytic cleavage site is included for the rhinovirus 3c protease: pDEF (Fc-3c).

In this vector, the coding sequences for the extracellular siglec domains were cloned, which are then referred to as siglec-Fc chimeras for expression. The expressed siglec-Fc chimeras can be separated by the protease interface after the successful expression of the Fc-part and three monomeric siglec-(Nr.)d1-3 parts. The vector contains interfaces for Xba*I* and Bam*HI* in its multiple cloning site. The coding sequence of siglec-(Nr.)d1-3 (Rutherford et al., 1993; Gessani et al., 1993) was amplified from the expression vector pCDM8, together with the genomic sequence of the Fc portion of human IgG1.

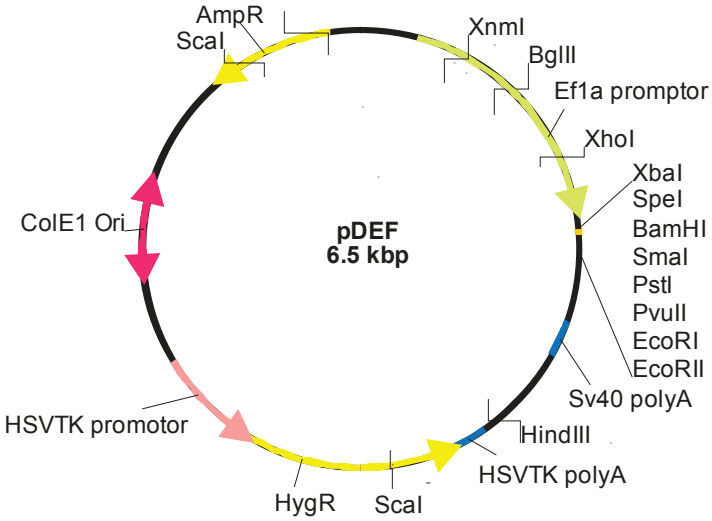


Figure 3.1: pDEF vector

3.5.2.7. Enzymatic modification of DNA

3.5.2.7.1. Restriction digestion of DNA

Using appropriate restriction endonucleases plasmid DNA is cut into defined fragments. These restriction endonucleases require certain buffers of the respective companies offered in 10-fold concentrated. In a digestion mix with two restriction endonucleases, the buffer suitable for both enzymes should be determined and used. The so-called MED-restriction buffer (10x buffer: 2.92% (w/v) NaCl/1.21% (w/v) Tris-HCl pH 7.5/2.03% (w/v) $MgCl_2$ +1mg/mL (w/v) BSA) can be used with most DNA-modifying enzymes.

Each reaction mix (10-50µL) contains the following components:

1µg DNA
20-10 units of each enzyme
1/10µL of the specified volume of the corresponding 10-fold buffer
ad 10-50µL ddH_2O.

The reaction mix is incubated for 2h at 37°C. The inactivation of the restriction endonucleases was achieved by heating the reaction mix to 70°C for 10min. The digested plasmids can be stored at -20°C until use.

3.5.2.7.2. Ligation of DNA fragments

Each ligation mixture (50µL) contains the following components:

20ng vector DNA
3-5 fold molar excess DNA (restriction digested)
2 units T4 DNA ligase
1/5µL of the specified volume of 5×buffer
ad 50µL ddH_2O

The digested mix was incubated for 16h at 18°C. After incubation the ligation mixture was transformed into chemically competent *E. coli*.

3.5.3. Plasmid DNA isolation and purification

3.5.3.1. Plasmid ‘mini’ preparation of DNA

QIAprep™miniprep Kit (Qiagen) was used for the preparation of small quantities of plasmid DNA. For this, the corresponding clone was grown in 2mL LB/Amp medium overnight at 37°C in shaker at 240rpm. 1.5mL of the overnight culture was centrifuged for 1min in an EppendorfminSpin. The bacteria pallet was resuspended in

250µL of P1-buffer (50mM Tris-HCl pH 8.0/10mM EDTA) with 250µL P2-lysis buffer (200mM NaOH/1% SDS), mixed by repeated inversion, and incubated for maximum 5min at RT. The cell lysate was neutralized with 350µL P3-buffer (3M potassium acetate neutralized pH 5.5). After 10min centrifugation at RT (13,000rpm, EppendorfminiSpin), the supernatant was subjected to the provided silica columns for 1min at RT and centrifuged (13,000rpm, EppendorfminiSpin). The eluate was discarded and the bound plasmid DNA was washed with 750µL of the ethanol-containing PE-Buffer. Then the DNA was eluted with 50µL ddH_2O. For control 2µL of DNA solution was analyzed on an agarose gel.

3.5.3.2. Plasmid midi-preparation for DNA extraction

For preparation of larger plasmid quantities HiSpeed™ midiprep Kit (Qiagen) was used, a 200mL of overnight transformed *E. coli* culture was prepared. According to the manufacturer protocol plasmid DNA was purified after alkaline lysis of overnight bacterial culture using the supplied ion-exchange columns.

The buffers P1-P3 correspond to those of the QIAprep™ midiprep Kit. The overnight culture was transferred to a 50mL plastic tube and centrifuged for 10min at 4°C and 4000rpm (Eppendorf table centrifuge 5810R). After decanting the supernatant the pellet was resuspended in a total of 6mL of P1 buffer, and then 6mL of lysis buffer P2 was added to the mixture, mixed by inverting several times and incubated for 5min at RT. The cell lysate was neutralized by adding 6mL of P3 with repeated inverting. The lysate was added to an enclosed filter cartridge in which the cell material could be retained during 10min incubation. 4mL QBT buffer (750mM NaCl/50mM MOPS pH 7.0/15% (v/v) isopropanol/0.15% (v/v) Triton X-100) was added to equilibrate silica gel column and then the lysate was filtered. The column was then washed with 20mL washing buffer QC (1M NaCl/50mM MOPS pH 7.0/15% (v/v) isopropanol) and the DNA was eluted from the column with 5mL elution buffer QF (1.15M NaCl/50mM Tris-HCl pH 8.5/15% (v/v) isopropanol). Then the DNA was precipitated with 3.5mL isopropanol by incubating for 5min with at RT and pressed through the QIAprecipitator™ filter. The DNA was eluted with 1mL TE buffer (10mM Tris-HCl pH 8.0/1mM EDTA) from the filter.

3.5.3.3. Determination of the plasmid DNA concentration

The concentration and purity of a DNA-containing sample can be determined photometrically. The average absorption maximum of nucleic acids is at a wavelength of 260nm and the average maximum absorption of proteins at a wavelength of 280nm. From the ratio of E260/E280, which should be above 1.7, the purity of the DNA was determined. The absorbance at 260nm gives information about the concentration of DNA in a sample. Absorbance of 1 corresponds to approximate 1mg/mL of double-stranded DNA. For the measurement of 1µL sample was diluted 1:100 with TE buffer. DNA solution was measured at 260nm and 280nm.

4. Results

4.1. Characterization of CHOLec1 produced siglec Fc-chimeras

The glycocalyx covering the nucleated cells through its Sia derivatives along with siglecs is involved in various biological processes (Varki and Lowe, 2009).The possible immunomodulator roles of CD33rsiglecs have been discussed in the introductory chapter. These immunomodulator functions of CD33rsiglecs could be investigated through various Sia specific binding and inhibitory assays. As Sia expressed on the same cell/protein surface may influence these studies. Desialylated siglecs were derived from CHO cell line. This section will discuss siglec synthesis and quality control measures performed to get functionally active desialylated siglecs.

4.1.1. Siglec Fc-chimera producing CHOLec1 cell lines

CHOLec1 cell line is stably transfected with pDEF plasmids containing the first three domains of siglec-5, -7, -8, and -10, respectively, which are linked to the Fc part of human IgG (3.1.8.2.). Initially stably transfected cells were maintained in FCS containing αMEM cell culture media (3.1.8.3). Stably transfected CHOLec1 cells secrete siglec Fc-chimera in cell culture media. Siglecs were purified using Protein A beads (3.3.2), as Protein A has specificity towards the Fc part. IgG depletion of FCS was necessary to avoid FCS-derived IgG contaminations in the produced proteins. However, this is a time consuming and expensive procedure (3.1.8.1.1). Moreover, total elimination of IgGs is not possible. To overcome these problems stably transfected CHOLec1 cell lines were adapted to chemically define synthetic EXCell® media (3.1.8.5).

The optimal CHOLec1cell density for maximum siglec Fc-chimeras production was found to be 2-3$\times 10^4$/mL. This optimal cell density varies with the siglec Fc-chimera such cells are producing. The most effective production with highest cell density was obtained for siglec-7, where ~4mg/L of siglec-7 Fc chimeras was purified from 1L harvested cell culture supernatant. Every batch of supernatant harvested was tested using ELISA to check for secretion of siglec Fc-chimera and levels of production (3.2.2.2).

4.1.2. Quality control for purified siglec protein

Purified siglec Fc chimeras were run under reducing conditions in 10% SDS-PAGE (3.3.4.1). Proteins were visualised by silver staining for any contaminates (3.3.4.3), or

they were detected with anti siglec antibody in western blot (3.3.4.4.1). Under reducing conditions all the purified siglec Fc-chimeras ran at about 70 kDa (Figure 4.1). Comparing the running behaviour of CHOLec1 cell line synthesised proteins verses CHOK1 cell line synthesised proteins, CHOK1 synthesised glycosylated siglec Fc-chimera ran slower. Whereas CHOLec1 produced desialylated (lacking complex *N*-glycane structures) siglecs run faster. Also the CHOK1 produced siglec Fc-chimera showed several unspecific protein bands.

The nature of these extra bands was deduced with anti hu IgG antibody. Using this antibody, only a single band around 70 kDa was detected (Figure 4.1). Also the anti-hu IgG directed against the Fc part does not bind to any of these (contaminating extra bands) proteins (data not shown).

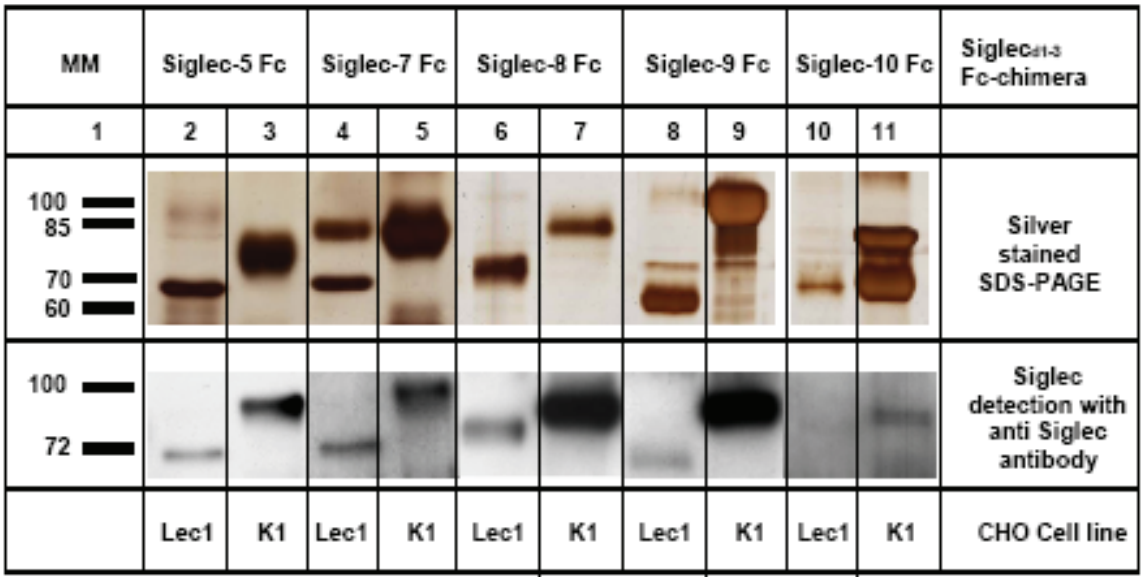


Figure 4.1: Silver staining and western blot detection of CHOLec1/K1 purified siglec Fc-chimeras

SDS-PAGE under reducing conditions: 2µg of CHOLec1/K1 produced siglec Fc-chimera loaded in each lane. Siglec Fc chimera bands visualized by silver staining. Siglec Fc-chimera band runs at around 72 kDa. CHOLec1 produced siglecs run faster as compared to CHOK1 produced siglecs. Anti siglec polyclonal antibody detects siglecs with high specificity in western blot analysis only. Anti siglec antibody detects CHOK1 produced siglecs more efficiently.

4.1.2.1. Solid phase cell adhesion assay as a functionality test

Siglecs can bind to naturally occurring Sia derivatives through their V-set domain. Erythrocytes surface is very rich with Sia derivatives; Sia dependent binding will give proof of the functionally active siglecs (3.2.2.3). The highest immobilized siglec concentration which is able to exert very compact erythrocyte binding (honey comb

structure) is described as being above the threshold concentration (Figure 4.2) of functionally active siglecs. In the subsequent dilution step this compact binding is lost and erythrocytes start regaining their original shape this concentration is referred to as being below the threshold concentration (Figure 4.2). This transformation is sudden and after this, the number of erythrocytes bound per well reduces rapidly with successive dilution steps (Figure 4.2). The erythrocytes free of Sia (3.4.1) were unable to exert any binding with the CHOLec1/K1 produced siglecs (Figure 4.2).

Table 4.1: Threshold concentration (µg/mL) levels for CHOLec1/K1 produced and purified siglec Fc-chimeras in erythrocyte solid phase cell adhesion assay.

Siglec	Cell lines	
	CHOK1	CHOLec1
siglec-5_{d1-3}Fc	0.2	0.2
siglec-7_{d1-3}Fc	0.1	0.05
siglec-8_{d1-3}Fc	0.8	0.8
siglec-9_{d1-3}Fc	0.2	0.1
siglec-10_{d1-3}Fc	0.4	0.4

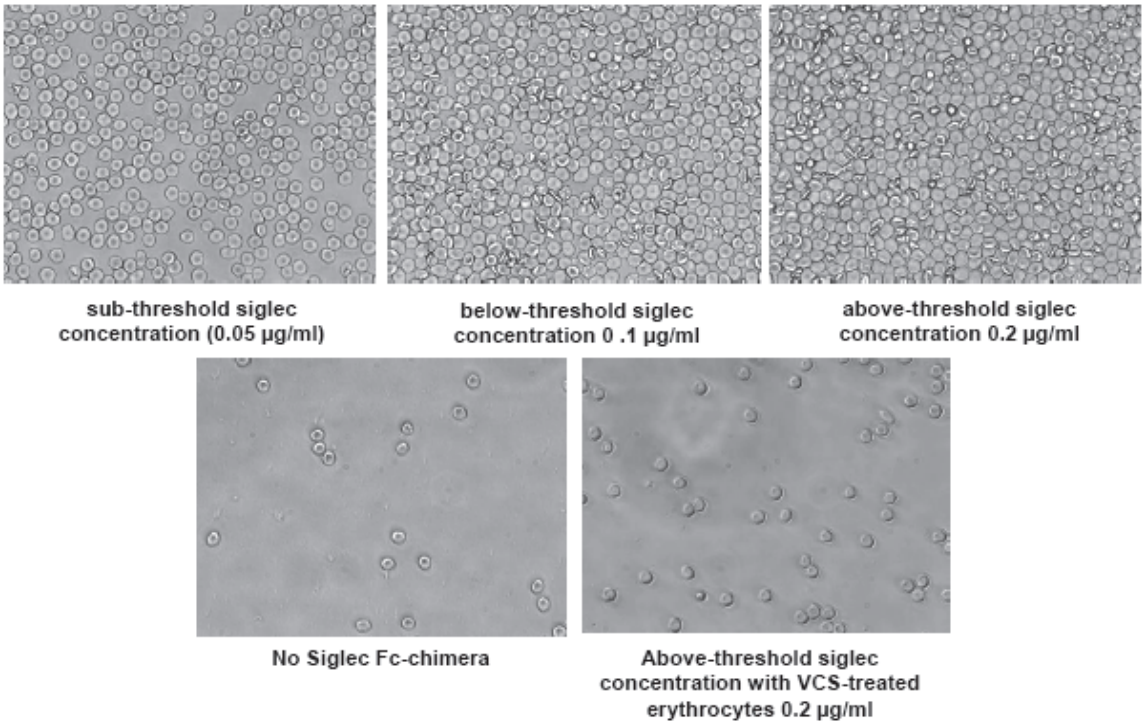


Figure 4. 2: Solid phase cell adhesion assay performed with CHOLec1 produced and purified siglec-5 Fc chimera

Starting with 0.8µg/mL a serial dilution of CHOLec1 produced, siglec-5 Fc-chimeras were immobilized on high binding plastic plate. In solid phase cell adhesion assay up to 0.2µg/mL of siglec-5 could exert above-threshold Sia mediated binding towards untreated erythrocytes. VCS-treated erythrocytes fail to show any binding with above threshold level immobilized siglec Fc-chimeras. Unspecific binding of erythrocytes to plastic plate surface was not observed.

Among the CHOLec1/K1 produced siglecs, siglec-7 (0.05µg/mL) exerts the strongest binding towards untreated erythrocytes, whereas siglec-8 showed the weakest binding (Table 4.1). Upon comparing CHOK1 and CHOLec1 produced siglecs, CHOLec1 produced siglec-7 and -9 showed more efficient binding towards erythrocytes. As siglecs exhibit low affinity towards naturally occurring Sia derivatives (Blixt et al., 2003b), the uncomplexed siglec binding towards erythrocyte confirms that the siglecs produced in CHOK1 and Lec1 cell lines are functionally active and can be used in future binding and/or inhibition experiments.

4.2. Heterogeneity in siglec expression

Siglecs are cell-surface proteins and are expressed on hematopoietic cell surface. Siglec shows a distinct hematopoietic cell specific expression pattern *(von G.S. and Bochner, 2008)*. Some siglecs are broadly expressed while the expression of others is limited to few cell types (von G.S. and Bochner, 2008). Detailed analysis of siglecs expression on the cells of hematopoietic system is important to understand their exact role in immune responses. As myeloid progenitors, monocytes and macrophages are the cells to come first in contact with pathogens evading immune system. This chapter will elucidate the variations in siglec expression on mononuclear cells under different circumstances. Siglec expression analysis performed with flow cytometry.

4.2.1. Efficiency of Ficoll/Percoll combined gradient

Mononuclear phagocytes were obtained with buffy-coats obtained from human volunteers with Ficoll/Percoll combined density gradient (3.1.1). Table 4.2, shows the average monocyte-to-lymphocyte ratios over Ficoll/Percoll combined density gradient, with the average monocyte yield and recovery. The values showed here were obtained from 56 independent isolations experiments performed over a period of three years. The cell suspension composition was evaluated by flow cytometry with forward scatter (FSC) vs. side scatter (SSC) (3.2.1). Figure 4.3, shows enrichment of monocytes population with successive gradient steps (denoted by enclosed circle).

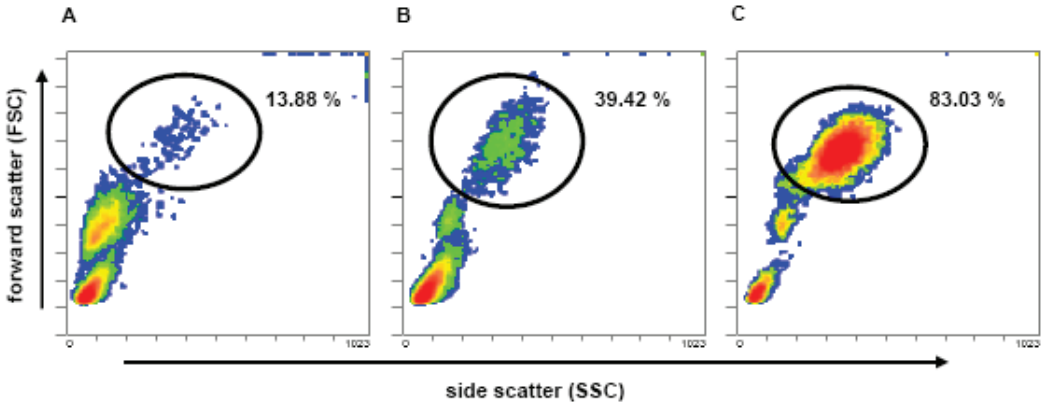


Figure 4. 3: Enrichment of monocyte from total BC with Ficoll-Percoll gradient

Typical leucocyte distribution on a FSC vs. SSC density plots. Monocytes have higher FSC values than lymphocytes and comprise a larger interval of SSC values. The indicated percentages of cells (monocytes) are relative to the total number of cells analysed (indicated by a enclosed circle). (A) ~14% enriched monocyte after first Ficoll gradient; (B) most

lymphocyte free monocyte-enriched culture after the hyper-osmotic Percoll gradient; (C) ~83 % enriched platelets and dead cells free monocytes population after iso-osmotic Percoll gradient.

The mean percentage of monocytes in the starting MNC suspensions was 11±6%. The suspensions were highly contaminated with platelets, regardless of the number of washing steps. The ratio of monocytes to lymphocytes increased significantly after separation on hyper-osmotic percoll, and was further increase after iso-osmotic percoll gradient. The average purity of monocytes in the final suspensions was 77±10% (Figure 4.3). The contaminating MNC were mainly T lymphocytes and NK cells. The separation on the iso-osmotic Percoll was crucial for eliminating platelets and dead cells although it resulted in some loss of monocytes.

Table 4.2: The average monocyte yield and recovery obtained with the isolation of monocytes on a Ficoll/Percoll density gradient (MNC: mononuclear cells, SD: standard deviation, n: number of samples)

	Starting MNC suspension	**Final monocyte-Enriched suspension**	**MNC yield**	**Monocyte**	
	Monocytes (%)	**Monocytes (%)**	**MNC**	**Yield ($\times 10^6$)**	**Recovery (% MNC)**
Mean value	11	75	703	77	13.5
SD (n = 56)	6	10	238	36	6

The viability of monocytes in the final suspension was 96% (data not shown). The average final cell yield was 13.5% based on the starting MNC numbers, as assessed by cell counting (3.1.2). This was also the approximate value of the average percentage of monocytes in the starting MNC suspensions, as assessed by flow cytometry. The average monocyte ratios in the starting and in the final cell suspensions were determined after calculating the average monocyte yield and recovery (Figure 4.3). Based of these values, the final monocyte recovery is ~75%. Moncyte percentage was derived with respect to total cells only after anti CD14 antibody binding.

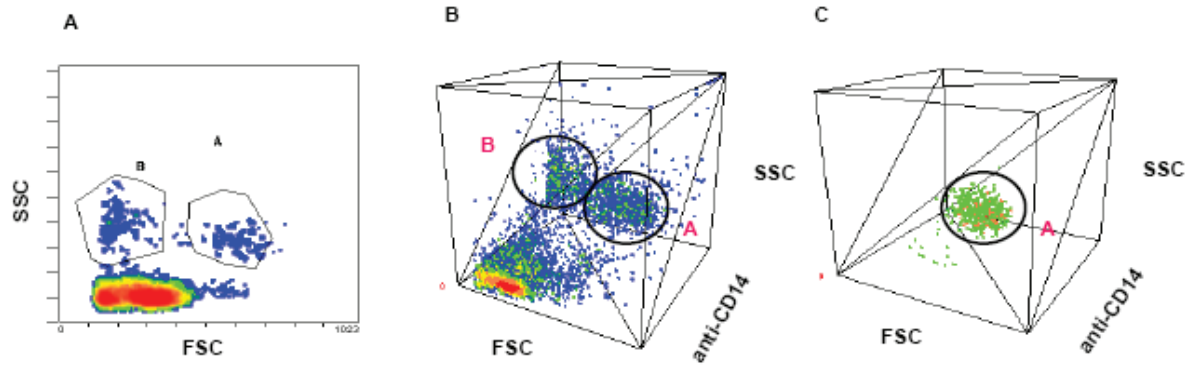


Figure 4.4: Gating monocytes in monocyte enriched PBMC sample

A: 2-D density plot showing leukocyte distribution, monocytes having higher FSC values than lymphocytes and comprising a larger interval of SSC values (gate A) whereas lymphocytes having lower FSC value (gate B); B: 3-D density plot showing cells stained with anti-CD14 antibody; (C) CD14 positive monocytes distribution on 3-D density plot (gate A).

The isolated cells (monocytes) were stained with anti-CD14 antibody (monocyte specific surface antigens) and analysed by flow cytometry (3.2.1.3). As monocytes isolated with Ficoll/Percoll gradient were not very pure for siglec expression analysis it was necessary to locate them from total analysed cells. A homogeneous monocyte population (gate A) can be observed after anti-CD14 antibody binding (Figure 4.4). The efficiency of monocyte isolation is largely dependent on preparation of the Percoll gradients and errors in sample handling.

4.2.2. siglec expression on mononuclear phagocytes

Expression of siglec-1, -3, -5, -7, -9, and -10 was analysed on isolated mononuclear phagocytes. Siglec expressing monocytes was identified after double staining the cells with anti-CD14 (monocyte specific) and anti-siglec antibody (3.2.1.4)(Figure 4.5). Peripheral blood-derived monocytes express up to five (siglec-3, -5, -7, -9, and -10) of the known CD33rSiglecs on their surfaces. Figure 4.5 shows distribution of total PBMCs in a 3D density plot. All the cells do not express siglecs with the same efficiency. Monocytes expressing siglecs were typed only after found to be double positive (siglec and CD14)(Figure 4.5A and B). Sigelc-8 which is not expressed on monocytes was used as internal negative control (Figure 4.5C). The anti-CD14 (monocyte) and anti-siglec antibody (siglec) positive double positive cells will cover larger distances in both the FL1 (FITC) and FL2 (PE) channels (Figure 4.5A and B).

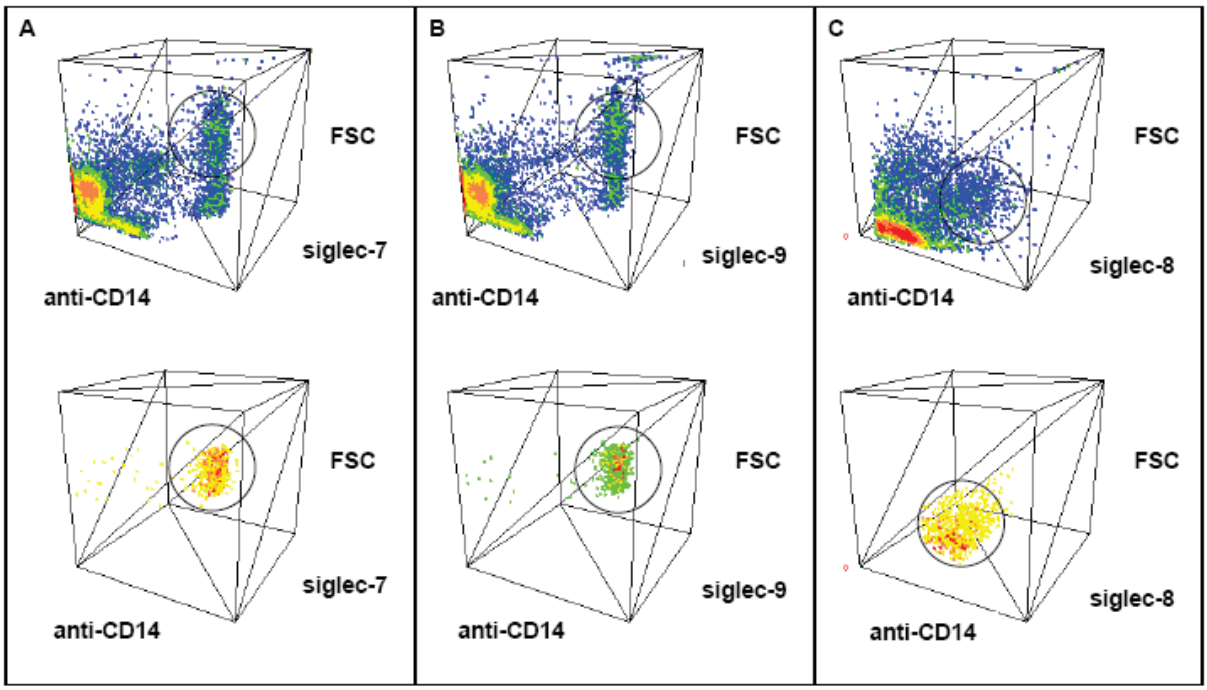


Figure 4.5: Siglec expression on monocyte

Monocyte specific siglec expression was typed using mouse anti-human siglec monoclonal antibodies (primary) together with PE-labelled donkey anti-mouse IgG antibodies (secondary). monocytes detected with FITC-labelled CD14 antibody. Cells were analysed using flow cytometry; (A, B and C) show 3D density of monocytes for, siglecs-7, -9 and -8 respectively. Double positive monocytes are indicated by enclosed circle.

4.2.3. Siglecs expressed on human monocytes

Monocytes isolated with percoll gradient method express siglec-3, -7, -5, -9 and -10 (small population) (3.2.1.4)(Figure 4.6). In native state, siglecs are usually involved in *cis*-interactions with Sia derivatives present on same cell surface. Siglec-3 being the shortest is always covered by glycocalyx and hence could not be detected until after sialidase treatment of these cells. Siglec-9 showed highest level of expression on monocyte surfaces while siglec-5 and -7 showed intermediate expression levels. Siglec-10 expression is limited to small monocyte populations (1–10%) with high expression levels. Siglec-1 and -8 which are not expressed on monocytes do not show signals above the noise level. Monocytes do not express every siglec with same intensity.

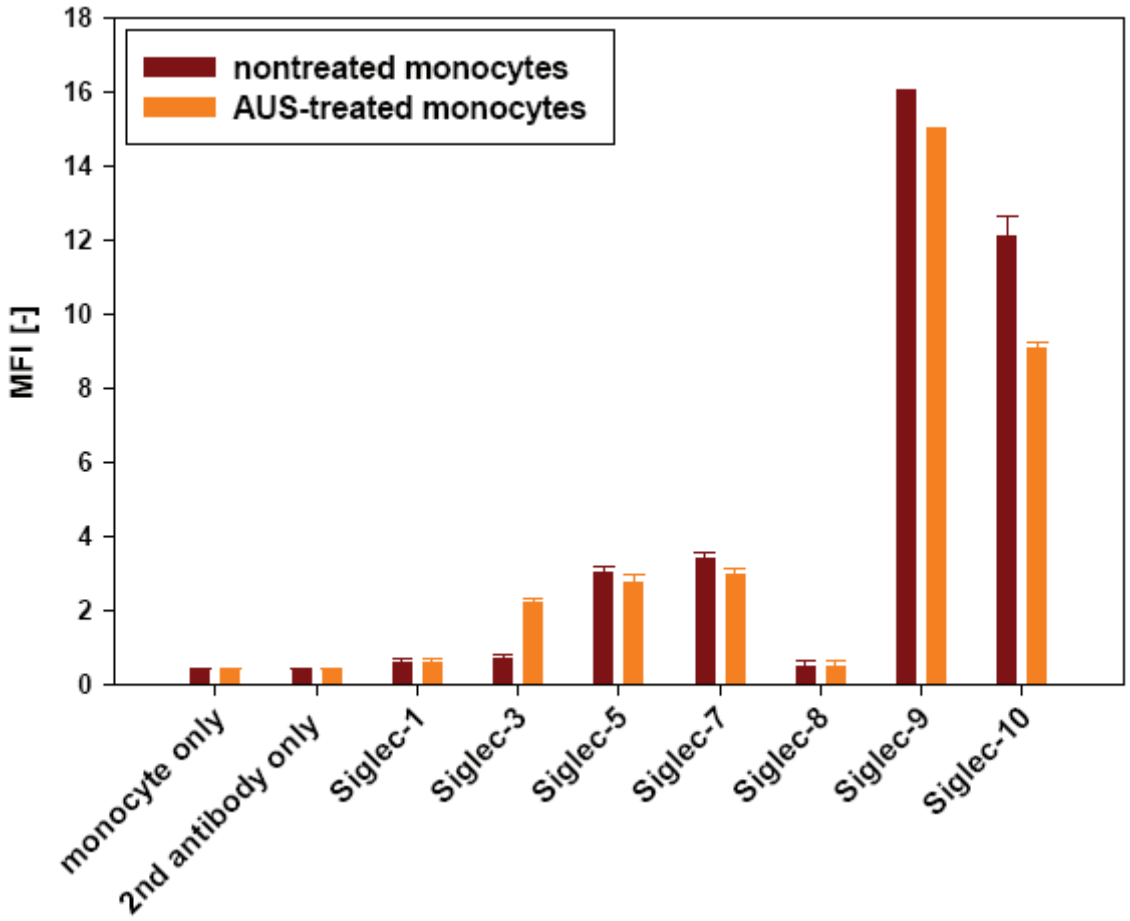


Figure 4.6: Siglecs expression on native verses AUS-treated monocytes

Mean fluorescence intensity (MFI) for siglec expression was measured with flow cytometry on FL2 log scale. Cells were stained with FITC-monoclonal mouse anti human siglec antibody and detected with PE-labelled anti mouse IgG antibodies. All cells analysed were positive for CD14, anti siglec-8 antibodies were used as a isotype matched control for monoclonal antibody. MFI values in bar chart represent the expression of the siglecs on human peripheral blood-derived monocytes before and after sialidase treatment.

4.2.4. Changes in siglec expression upon differentiation and activation of monocytes and monocyte derived macrophages

4.2.4.1. siglec expression on monocytes and monocyte derived macrophages

Monocytes isolated from peripheral blood express siglec-3, -5, -7, and -9. This expression however changed upon adherence of the cells to culture dishes and *in vitro* differentiation to macrophages (3.1.3). Upon differentiation to macrophages, the level of cell surface siglec-3, -5, and -9 was strongly reduced whereas siglec-7 was increased (Figure 4.7). Interestingly, cell surface siglec-5 expression was reduced just after 15min of adherence, whereas changes of the other siglecs were observed only after several hours (data not shown).

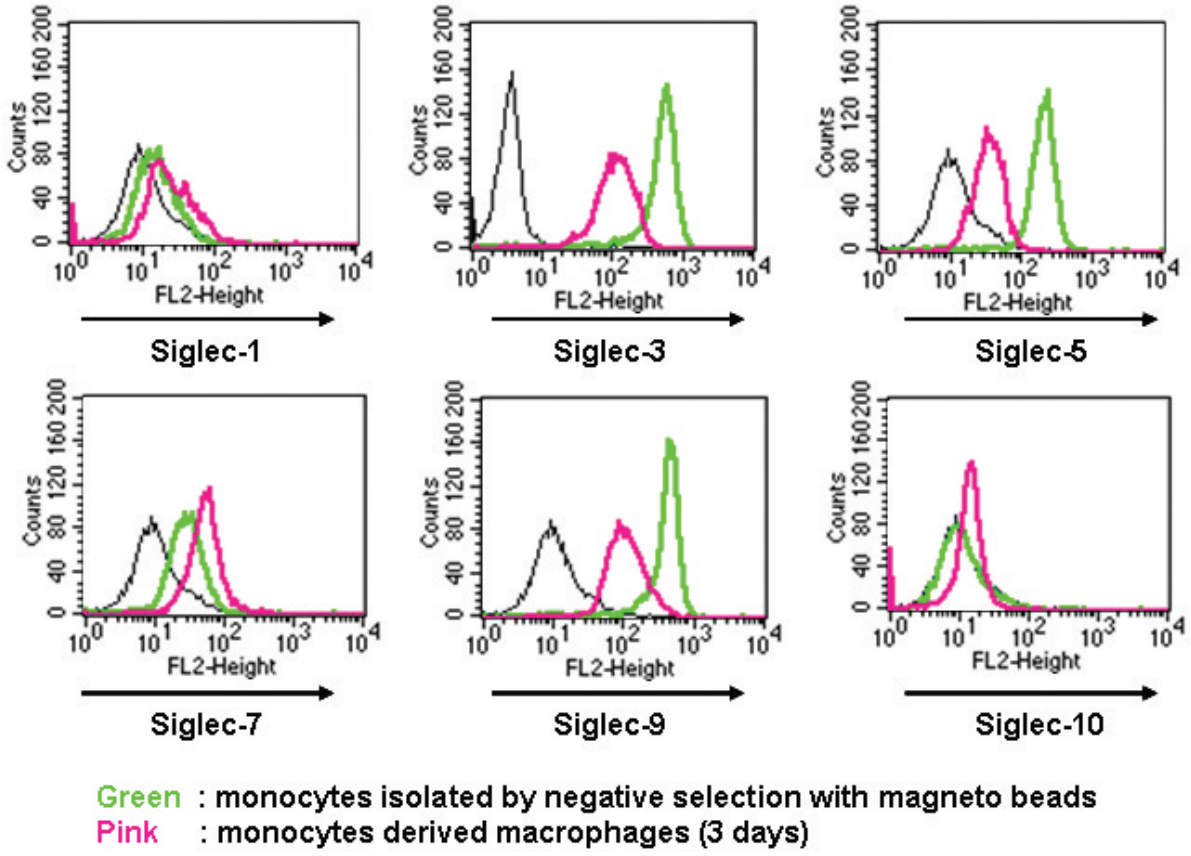


Figure 4.7: Siglecs expression on human monocytes and monocyte-derived macrophages
Peripheral blood-derived monocytes express siglec-3, -5, -7, -9, and -10. Cells were stained with FITC-monoclonal mouse anti siglec polyclonal antibodies followed by staining with PE-labelled anti mouse IgG Fab-fragments. Antibody binding was analyzed by flow cytometry. All cells shown were positive for CD14. Anti siglec-8 antibodies were used as isotype matched control.

These recorded changes in siglec expression were highly reproducible and independent of the isolation method for the monocytes (isolation by negative selection using Miltenyi or Dynal negative selection kits (3.1.1.4), combined Ficoll/Percoll gradients (3.1.1.3) or adhesion to plastic surface (3.1.1.2)) and source, which could be either from BC or fresh peripheral blood in the presence of autologous serum or fetal calf serum (data not shown).

4.2.4.2. Changes in siglec expression upon activation of mononuclear phagocytes with IFNγ and IL-4

Isolated monocytes were allowed to differentiate for 3-6 days to macrophages under M-CSF (3.1.3). The monocyte derived macrophages were activated in *vitro* by cytokines, IFNγ and IL-4 (3.1.3.1). IL-4 (type-II) and IFNγ (type-I) cytokine induced opposite changes in siglec expression (Figure 4.8). Activation with IL-4 leads to

upregulation of siglec-7, -9 and –10, while IFNγ induces an upregulation of siglec-1 and downregulation of siglec-7 and -9 (Table 4.3). All changes observed were reproducible in several independent experiments with cells from different healthy individuals.

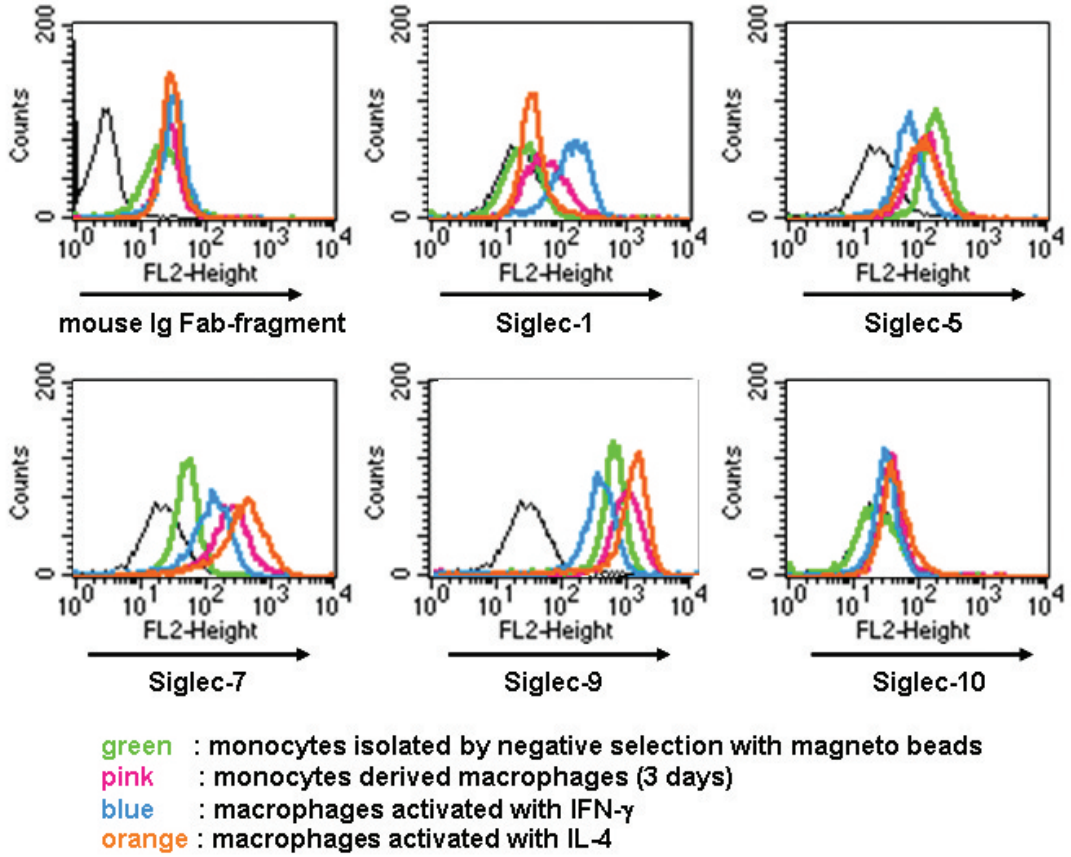


Figure 4.8: Siglecs expression on human monocytes and monocyte-derived macrophages

Activation with IL-4 leads to an up regulation of siglec-7, -9 and –10, the IFNγ induces an up regulation of siglec-3 and down regulation of siglec-7 and -9. Cells were stained with monoclonal mouse anti siglec polyclonal antibodies followed by staining with PE-labelled anti mouse IgG Fab-fragments. Antibody binding was analyzed by flow cytometry. Anti siglec-8 antibodies were used as isotype matched control.

Table 4.3: Changes in siglec expression upon activation with IFNγ and IL-4

	Siglec-1/Sn	Siglec-3/CD33	Siglec-5	Siglec-7	Siglec-9	Siglec-10
IFN-γ	↑	n.c.	↓	↓	↓	n.c.
IL-4	↓	n.c.	n.c.	↑	↑	n.c.

n.c.= not changed. ↑ upregulation ↓ downregulation

4.2.4.3. Availability of siglec Sia binding sites on unactivated and activated mononuclear phagocytes

The availability of Sia binding sites for *trans*-interactions as well as their involvement in *cis*-interactions is important in determining siglecs functions. This raises the question whether siglec Sia binding sites on mononuclear phagocytes are available for *trans*-interactions. Therefore, the binding of polyvalent PAA-derivatives carrying sialylated oligosaccharides as probes to monocytes and macrophages was analysed, using the corresponding 6´-O-sulfo-lactose derivative as negative control (3.2.1.2). Binding of the sialylated probes carrying α2,3-sialyllactose, α2,6-sialyllactose, Sialyl-Lewis^x or Sialyl-Lewis^a was observed in AUS desialylated cells (Figure 4.9).

The Sia binding sites of siglec-3, -5, -7, and –9 present on monocular phagocyte cells are involved in *cis*-interactions. Robust binding was obtained with probes carrying α2,3-linked Sia (α2,3-sialyllactose, Sialyl-Lewis^x, followed by Sialyl-Lewis^a), whereas probes bearing α2,6-sialyllactose were barely bound (Figure 4.9), reflecting the glycan specificities of siglecs present on these cells. Furthermore, differentiation to macrophages or activation of these cells with either IL-4 or IFNγ for 5min, 15min, 1h, 3h, 1d, 2d, 3d did not result in unmasking of siglec Sia binding sites (data not shown).

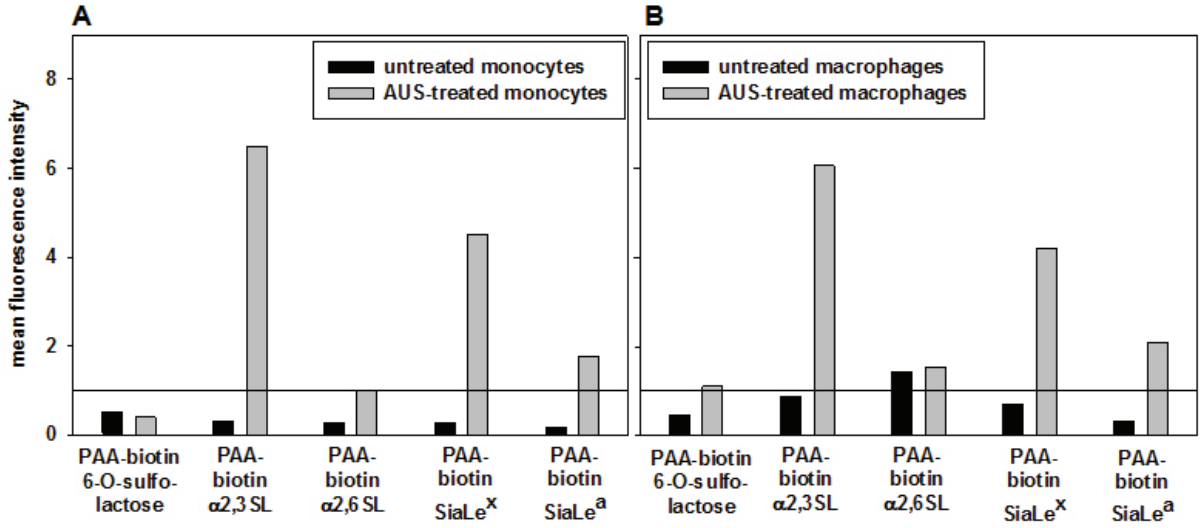


Figure 4.9: The availability of siglec Sia binding sites on human monocytes and monocyte-de<rived macrophages.

Cells were stained with biotinylated PAA-oligosaccharide probes followed by detection of bound probes with PE-labelled streptavidin. Probe binding was analyzed by flow cytometry. As a negative control, 6´-O-sulfo lactose probe was used. Also in order to see whether binding sites are occupied by cis-interactions, control cells were treated with sialidase prior to binding. With the native control no binding above noise level has been obtained with monocytes for any

of the tested probes viz. α2,3-sialyllactose, α2,6-sialyllactose, Sialyl Lewis^x or Sialyl Lewis^a. After sialidase treatment binding of α2,3-sialyllactose and Sialyl Lewis^x was higher than binding of α2,6-sialyllactose or Sialyl Lewis^a.

4.2.5. Siglec expression on monocytes is donor dependent

The studied population showed that monocyte siglecs do not have a same expression level. The studied healthy adult individuals exhibit differential levels of every siglec expression. It was observed that Siglec-9 is the most diversely expressed siglec on monocytes (Figure. 4.10). Siglec-10 also has a very wide range of expression but its expression is limited to a small monocyte population (1-10%). Siglec-3, -5, and -7 showed moderate variations in their expression levels whereas sigelc-1 and -8 were not detected on monocytes.

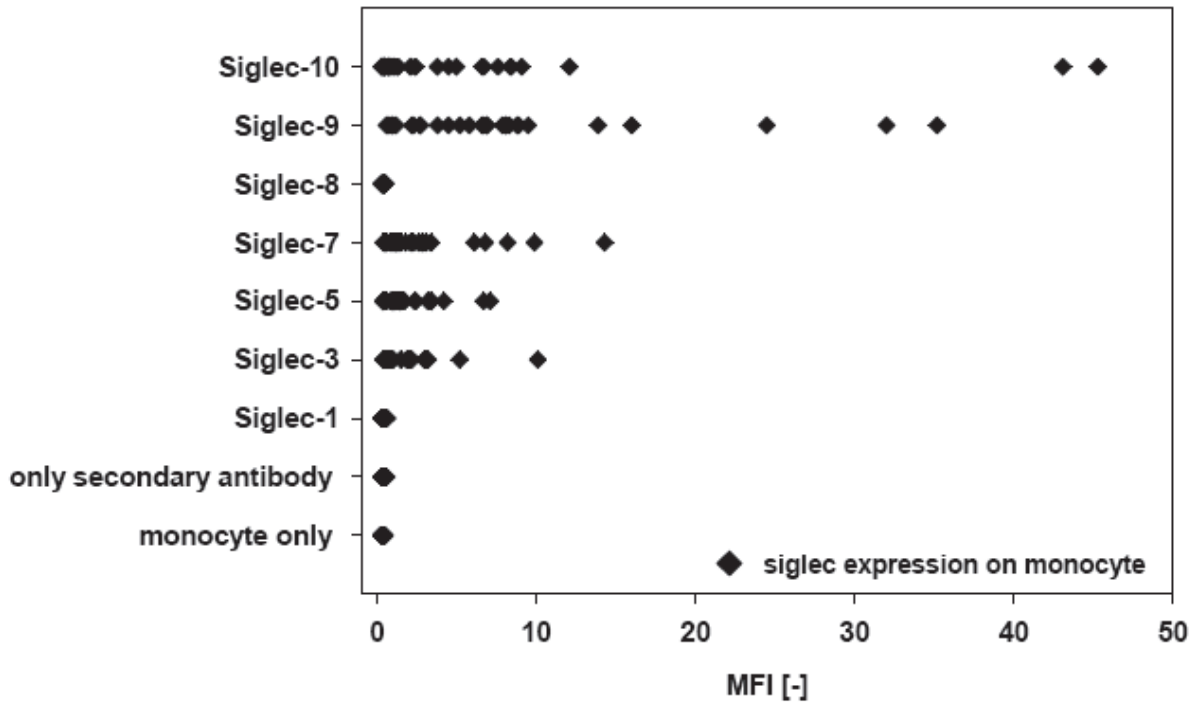


Figure 4.10: siglec expression on monocytes is donor dependent

Monocytes were isolated from buffy-coats and monocyte surface siglec expression profile was created for 52 individuals. Mean fluorescence intensity was determined with flow cytometer (FL1) and plotted on a dot plot. Each dot represents particular siglec expression for individual donor.

4.2.6. Six month siglec expression profile on monocytes

Regularity in siglec expression on monocytes from individual volunteers was determined; blood samples were collected from the same 15 donors for six months every 2 weeks. And six month monocyte siglec expression profile was created for

each individual. Siglec-7 and -9 expression levels were monitored for their consistency in expression on monocytes. Siglec-8 expression was also monitored as negative control (data not shown).

Six month siglec profiling revealed that for a volunteer at any given time, siglec-9 shows the most diverse expression. Siglec-7 showed moderate deviation from its basal expression levels. Volunteers also reported if they were ill at the time of blood collection. Donors who did not had any apparent signs of illness showed moderately consistent siglec expression levels (Figure 4.11A). Most of the cases where the volunteer reported ill, siglec-9 expressions showed larger deviation from its basal expression level (Figure 4.11B).

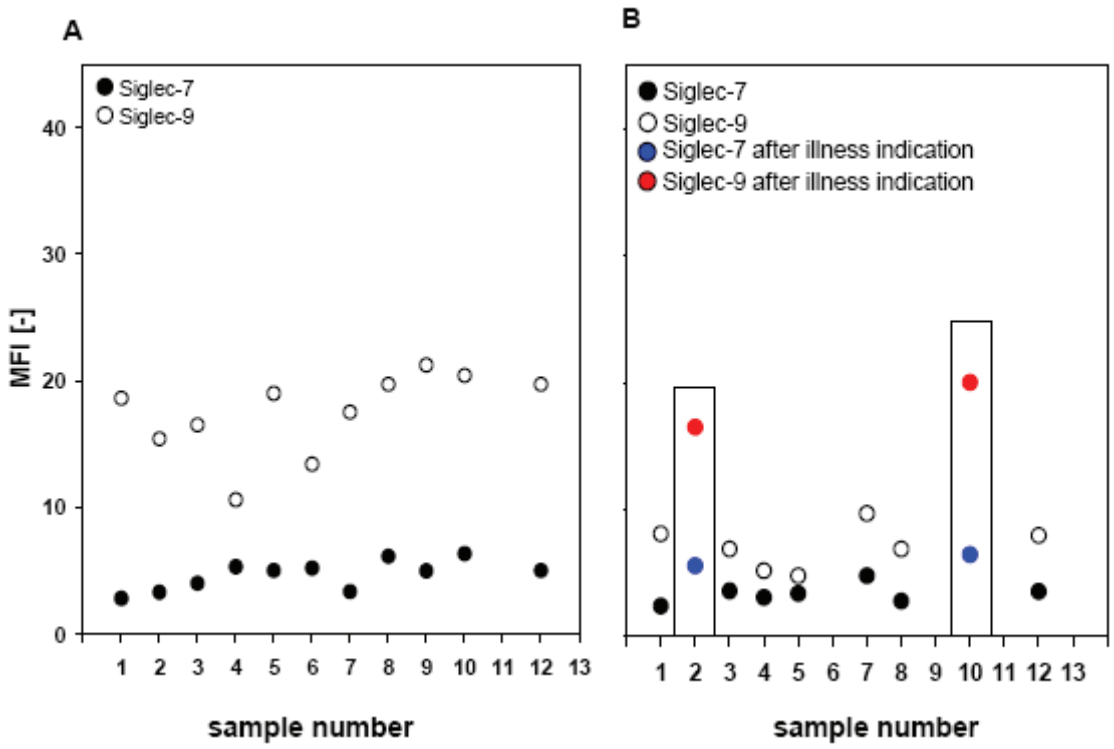


Figure 4.11: Six months siglec expression profile for two individuals

Samples were collected from 2 different donors every 15 days for six months. Siglec-7 and-9 expressions profile for six month. (A) Donor not reported ill shows relatively consistent siglec expression profile. (B) Donor with illness indication at the time of sample collection.

4.2.7. Co-relation between siglec and CRP-expression levels

Under normal circumstances human CRP has low expression level (0.1–0.5 µg/mL) but under inflammation condition the CRP levels are elevated approximately a 1000-fold (Simmons and Seed, 1988). CRP levels in blood serum samples collected from the volunteers were measured with CRP-Assay (3.2.2.4). CRP levels were plotted against the siglec expression level for particular time point for each volunteer. In most

of the cases where the volunteer reported an apparent sign of illness, the high siglec-9 levels coincided with higher serum CRP levels (Figure 4.12). In a case where the volunteer received vaccination against seasonal Influenza virus during the course of study, showed elevated siglec-9 expression levels coinciding with higher CRP levels. This elevation in either siglec-9/CRP level is comparable with siglec-9/CRP expression level when individuals reported ill.

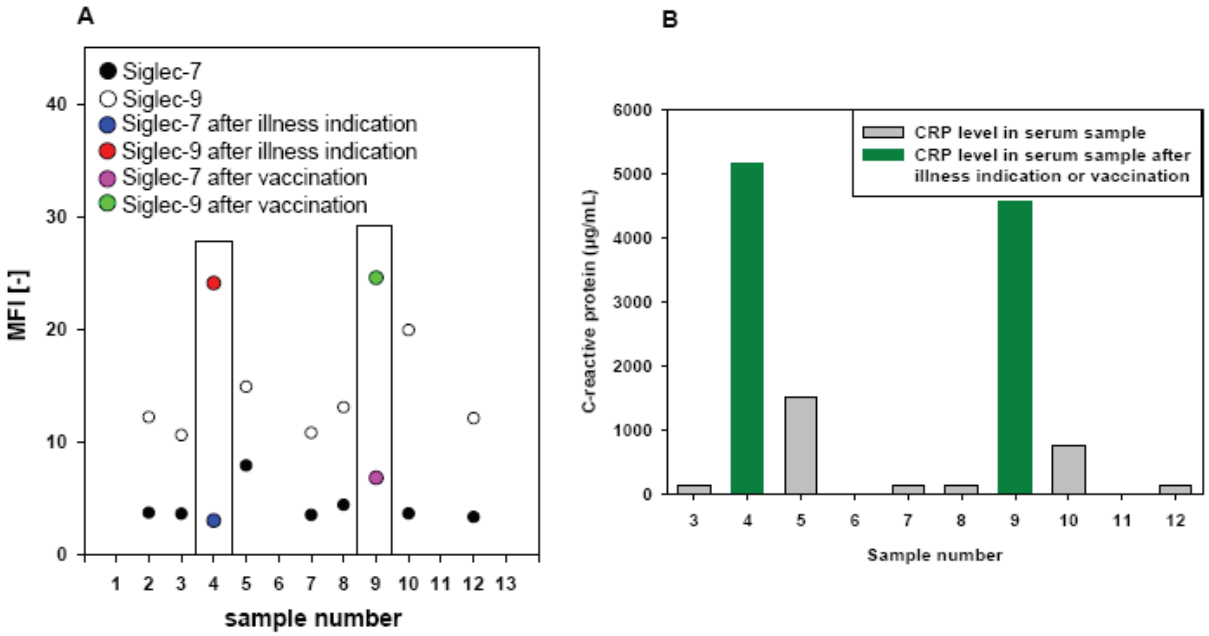


Figure 4.12: Siglec-9 expression on monocytes and its relation with serum CRP levels

(A) Siglec-7 and-9 expressions profile for six month: red and blue dot represent that donor reported ill at the time of sample collection while green and pink dot represents collections made after vaccination. (B) Serum CRP levels profile (sample no 1, 2, and 13 are missing).

4.2.8. Differences in siglec expression between different ethnic groups

Among the studied volunteers, individuals belonging to the same ethnic group found to have more or less the same siglec expression levels. The most diversely expressed siglec, siglec-9, has a comparatively higher expression level in volunteers with Caucasian origin than in volunteers with Asian origin. Siglec-7 expression is more or less the same in both ethnic groups (Figure 4. 13).

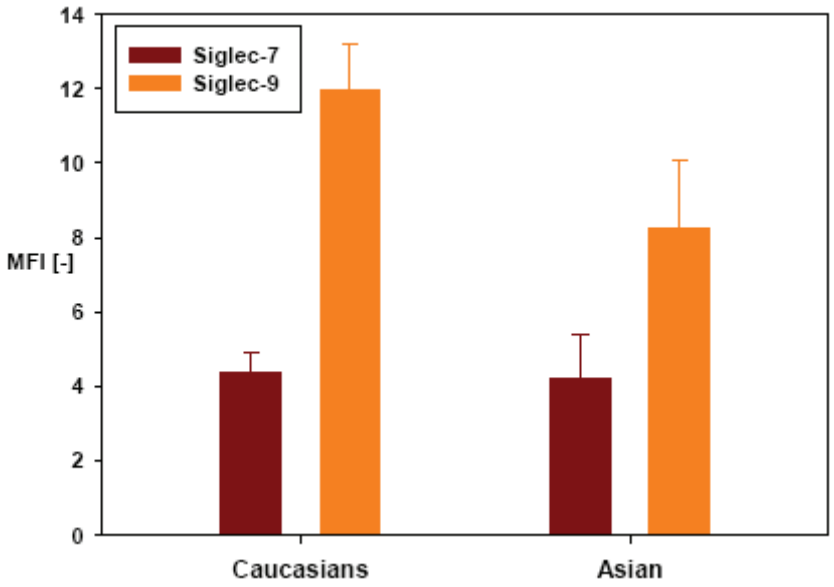


Figure 4.13: Differences between Caucasians vs. Asian ethnic groups in monocyte surface siglec-7 and -9 expressions.

4.3. Influence of viruses on siglecs expression on monocyte surfaces

Monocyte surface siglec expression is donor dependent and expression levels were found to vary over time and immune status of an individual. After correlating siglec expression levels with CRP levels, indicate that variations in siglec expression could be a result of changes in immune status. Some reports showed that pathogens use siglecs Sia recognising ability to invade immune system or vies a versa. This could either lead to elimination of the pathogen or disease progression. Role of siglec in early pathogen recognition stages was investigated after co-culturing monocytes in suspension cultures with different viruses. Changes in monocyte surface siglec expression were monitored and analysed with flow cytometry.

4.3.1. Quantification of viruses

Different viruses were cultivated and purified as described in the methods section (3.1.5). The hemagglutination activity of Influenza virus strain A/PR8/34 was tested for functionality. Serial dilutions of influenza were incubated with chicken erythrocytes. Up to a dilution of 1:640 virus was able to elicit rosettes formation. The HA titer value for influenza virus strain A/PR8/34 was determined to be 640.

Functional activity for HAV-7 (immunocytochemical analysis) and Coxsackie viruses (cytopathic activity) was tested. HAV-7 infects and propagates in host cells without destroying them. TCID50 value for HAV-7 was therefore determined with the help of immunocytochemical analysis. FRHK4 cells were infected with serial dilutions of HAV-7 virus and incubated for two weeks before immunocytochemical analysis for virus detection. TCID50 for HAV-7 was determined to be 10^{5mL}. In the case of Coxsackie B virus cytopathatic activity exerted on FRhK4 cells was determined and was found to be 10^{8mL}. CMV and HAV-GBM virus preparations were available as non purified cleared cell lysates and purified NDV was available with a titer of 4×10^{6}.

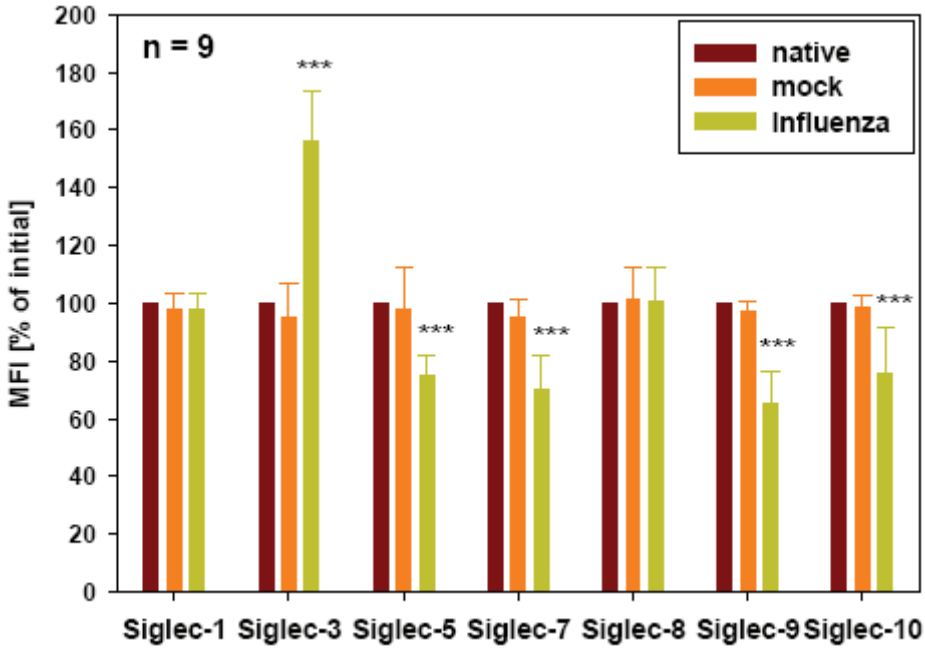
4.3.2. Siglec expression on monocyte surfaces is altered upon co-culture with viruses

4.3.2.1. Co-culture of monocytes with enveloped viruses

Monocytes in suspension culture were co-cultured with enveloped viruses (Influenza virus, NDV, and CMV) for 24h at 37°C (3.1.6.1). Presence of these viruses in co-culture system influence siglec expression. Influenza virus which recognises Sia derivatives on host cell surface for infection and disease progression affected siglec

expression on monocytes the most. Significant decrease in siglec-5, -7, -9, and -10 expression levels was recorded, and a significant increase in siglec-3 expression was observed (Figure 4.14). Siglec-3 detection was very unusual as under normal conditions sialidase treatment is required for siglec-3 detection. This is because Siglec-3 is usually covered by the cell's glycocalyx.

NDV which has a similar envelope structure as Influenza virus doses not affect siglec expression similarly. Co-culture with NDV leads to significant increase in monocytes siglec-7 and -9 surface expressions and shows a significant decrease in monocyte siglec-10 surface expression (Figure 4.14). Except siglec-9 whose expression decreased upon co-culture with CMV, all other siglecs expression remained unaffected with regard to this very virus. (Figure 4.14).



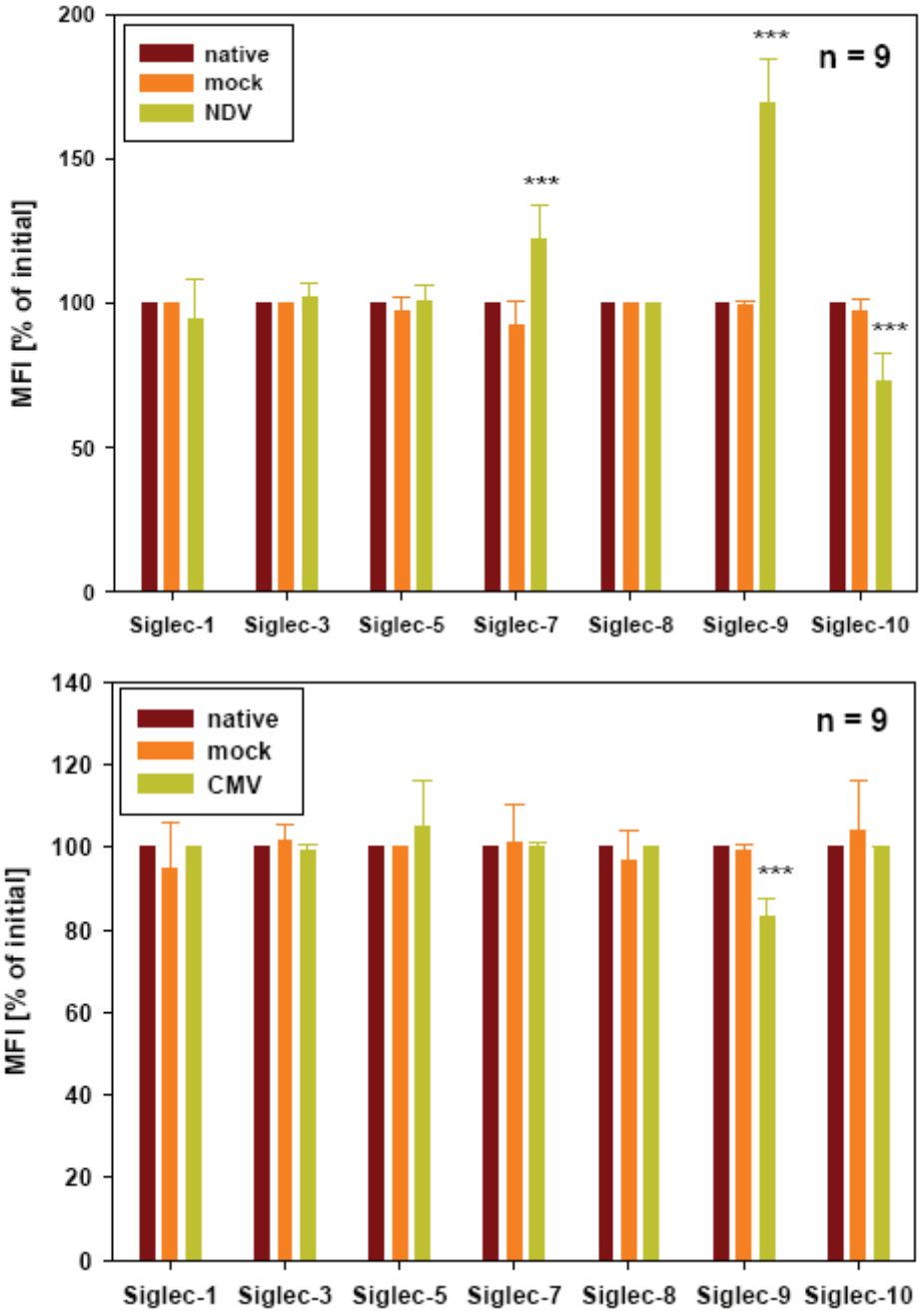
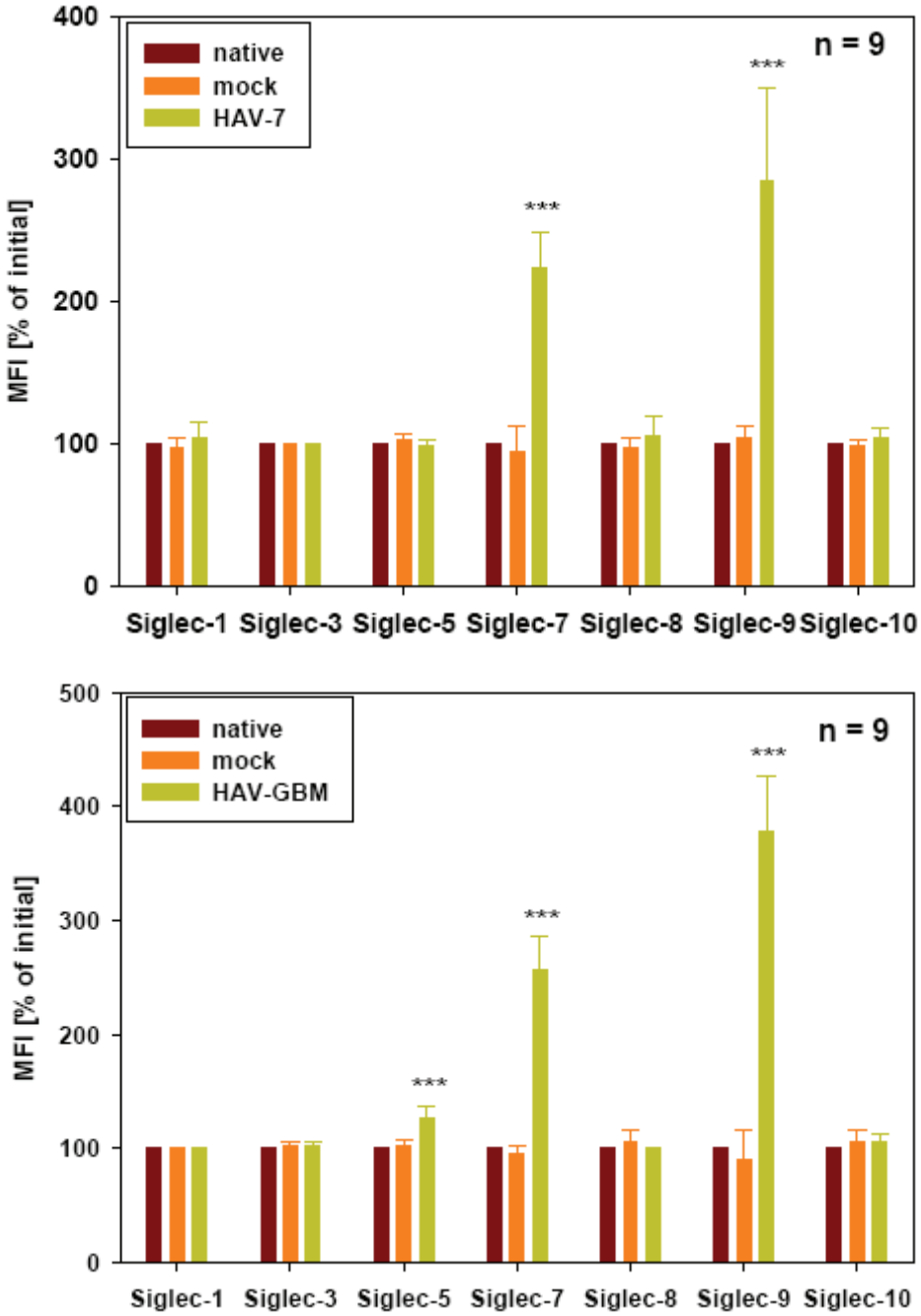


Figure 4.14: Changes in siglec expression upon infection with enveloped viruses

Each enveloped virus affects siglec expression differentially; siglec-9 expression is affected by all three viruses. Siglec-1 and -8 were used as internal negative control as they are not expressed on monocytes. (A, B, and C) Influenza, NDV, and CMV virus were co-cultured individually with monocytes in suspension culture for 20h at 37°C under 5% CO_2. Cells were then stained with monoclonal anti siglec antibody before analysing with flow cytometry. Changes in siglec expression [MFI] were calculated as % of initial siglec expression from 9 independent experiments for statistical significance (n = 9).

4.3.2.2. Influence of non-enveloped viruses on monocytes siglec expression

Two strains of HAV virus were used to co-culture with monocytes in suspension. Increase in siglec-7 and -9 on monocytes' surface expression was detected (Figure 4.15) in presence of both the HAV strains. Additionally HAV-GBM co-culture leads to a significant increase in siglec-5 expression level on monocytes (Figure 4.15). Co-culturing Coxsackie B2 virus with monocytes resulted in a significant decrease of siglec-7, -9, and -10 expression levels. Siglec-1 and -8 which are not expressed on monocytes showed no changes in their expression and therefore were considered as internal negative controls.



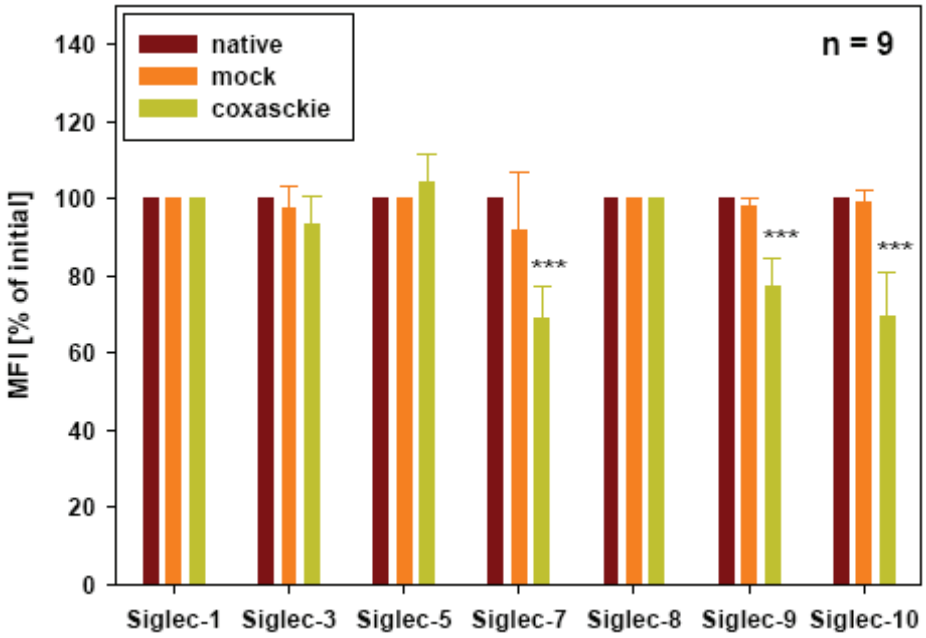


Figure 4.15: Effect of non-enveloped virus co-culture on monocyte surface siglec expression

Non-enveloped viruses affect monocyte surface siglec expression. (A, B, and C) Coxsackie B2 virus, HAV-7, and HAV-GBM virus were co-cultured individually with monocytes in suspension culture for 20h at 37°C under 5% CO_2. Cells were then stained with monoclonal anti siglec antibody before analysing with flow cytometry. Changes in siglec expression [MFI] were calculated as % increase or decrease of initial siglec expression from 9 independent experiments for statistical significance (n = 9).

Sigelc-9 surface expression on monocytes was affected by all the viruses. There is no connecting link between type of viruses used for co-culture and changes observed on monocyte surface siglec expression, as each virus affects siglec expression differently. Changes in monocyte surface siglec expression are summarised in Table 4.4.

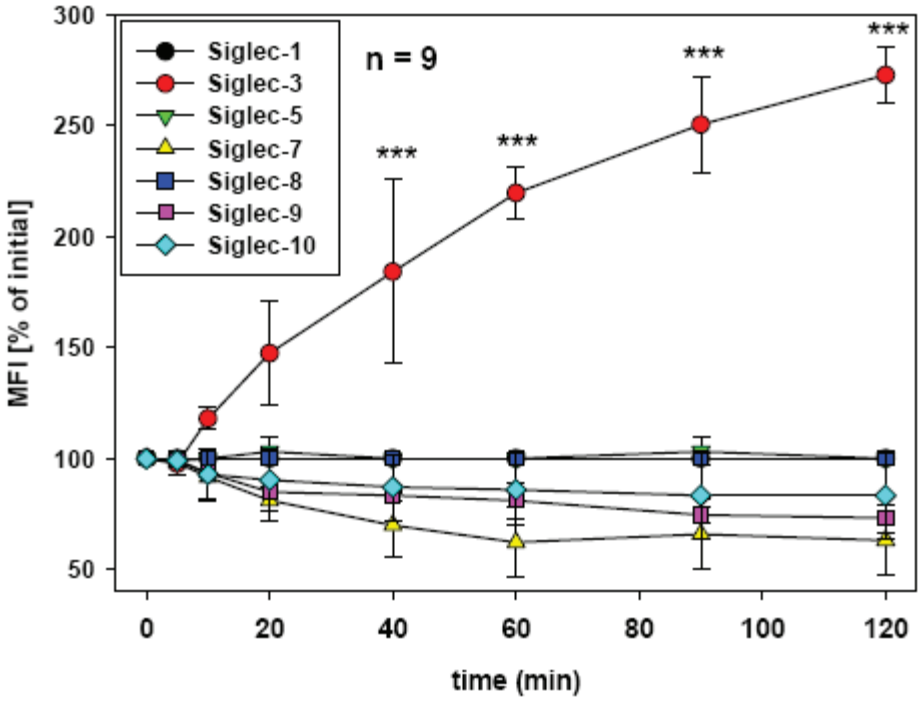
Table 4.4: siglec expression changes on monocytes upon co-culture with viruses

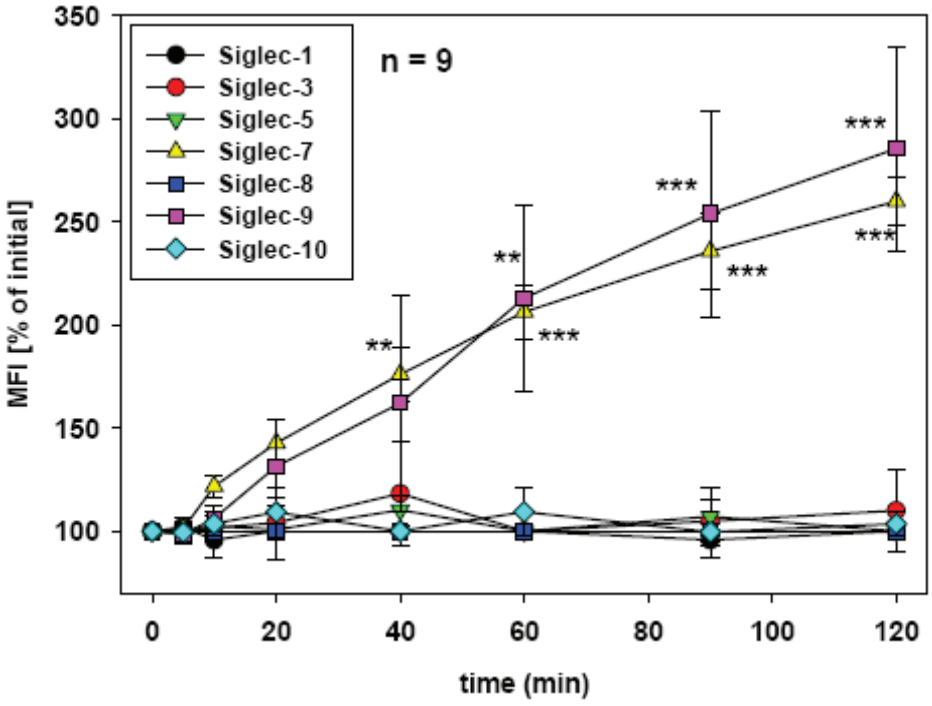
Virus	Siglecs						
	1	3	5	7	8	9	10
Influenza	N.C.	↑	↓	↓	N.C.	↓	↓
NDV	N.C.	N.C.	N.C.	↑	N.C.	↑	↑
CMV	N.C.	N.C.	N.C.	N.C.	N.C.	↓	N.C.
Coxsackie B2	N.C.	N.C.	N.C.	↓	N.C.	↓	↓
HAV-7	N.C.	N.C.	N.C.	↑	N.C.	↑	N.C.
HAV-GBM	N.C.	N.C.	↑	↑	N.C.	↑	N.C.

Keyset: N.C. = not changed; ↑ = increased expression; ↓ = decreased expression

4.3.3. Effect of co-culture time on virus mediated monocyte surface siglec expression

Influenza, HAV-7 and Coxsackie B2 seem to affect a wider range of siglecs on monocytes compared with the other viruses. These viruses were selected to investigate further for their influence on siglec expression. Monocytes in suspension culture were co-cultured with these viruses between 0-120 min. Such a short time exposure of monocytes to viruses leads to changes in the expression of siglecs on monocytes surfaces. Changes in siglec expression were detected as early as after 10min of co-culture. Results are in accordance with previous experiment. A significant increase in siglec-3 and a decrease in siglec-5, -7, -9, and -10 expression was recorded. HAV-7 co-culture resulted in rapid increase in monocyte siglec-7 and -9 expression level. Co-culturing monocytes with Coxsackie B2 virus showed very complex siglec expression changes. Monocytes initially showed a rapid increase in siglec-9 expression which was eventually lowered down over co-culture time, whereas initial low expression level of Siglec-7 showed a sharp increase after 40min which was then lowered to reach its native expression levels at the end of 120min co-culture time. These changes in siglec expression had very large standard deviation, and do not have any statistical significance. Mock-treated and native monocytes do not show any significant deviation from their native siglec expression levels.


Siglec-1
Siglec-3
Siglec-5
Siglec-7
Siglec-8
Siglec-9
Siglec-10
n = 9
MFI [% of initial]
time (min)
300
250
200
150
100
50
0
20
40
60
80
100
120
***


Siglec-1
Siglec-3
Siglec-5
Siglec-7
Siglec-8
Siglec-9
Siglec-10
n = 9
MFI [% of initial]
time (min)
350
300
250
200
150
100
0
20
40
60
80
100
120
**
***

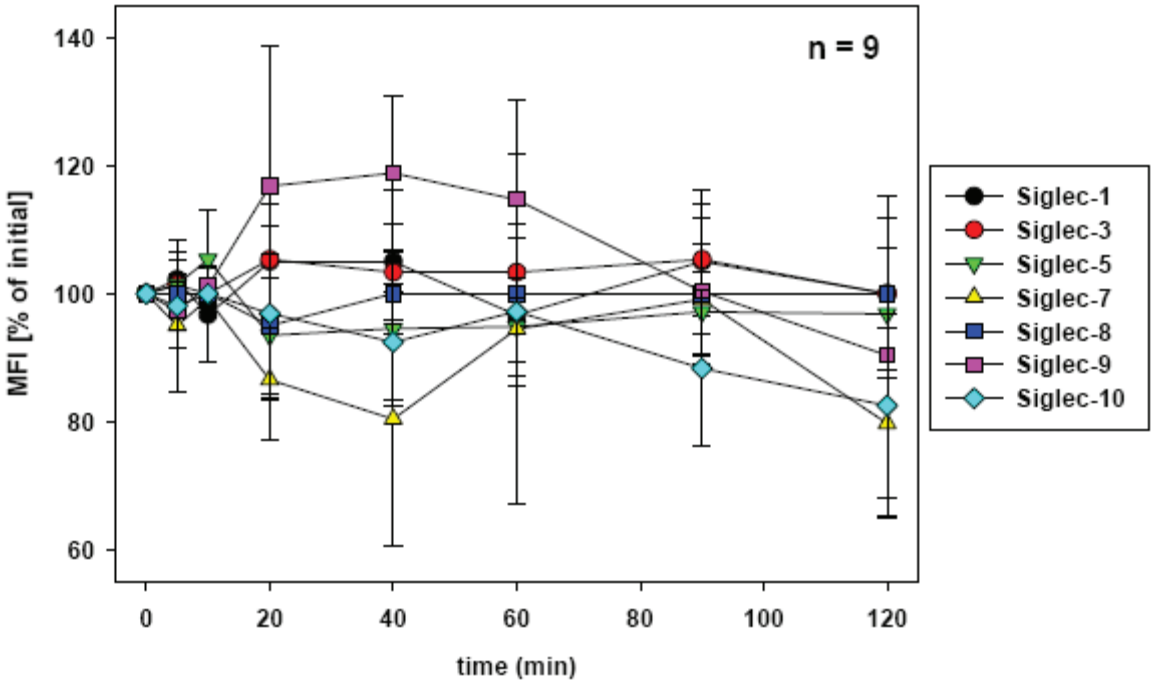


Figure 4.16: Time mediated siglec expression changes exerted on monocyte.

Effect of co-culture time on virus mediated monocyte surface siglec expression. (A, B, and C) Influenza, HAV-7, and Coxsackie B virus were co-cultured individually with monocytes in suspension culture between 0-120min at 37°C under 5% CO_2. Cells were then stained with monoclonal anti siglec antibody before analysing with flow cytometry. Changes in siglec expression [MFI] were calculated as % increase or decrease of initial siglec expression from 9 independent experiments for statistical significance (n = 9).

4.3.4. Effect of time vs. virus concentration on monocyte siglec-7 and -9 expressions

Presence viral particles in monocyte co-culture system leads to changes in monocyte surface siglec expression. The number of viral particle in co-culture with these cells could be critical as in most cases virus dose is the limiting factor for successful invasion and disease progression. Influenza virus and HAV-7 viruses were selected for these experiments. Three different viral dilutions (1:100, 1:1000, and 1:10000) along with undiluted sample were tested. Changes in siglec-7 and -9 expressions on monocytes surface were monitored.

Lowering the virus concentration in co-culture system showed a direct influence on siglec expression. The monocyte siglec expression remains unaffected at lower virus concentration (Figure 4.17). As virus concentration increases in the co-culture system, the effect exerted (changes in siglec expression) becomes more prominent. Results are in accordance with time-dependent siglec expression changes experiments. As Influenza virus and monocytes co-culture time progresses, a steady

decrease in siglec-7 and siglec-9 expression on monocyte surface was observed (Figure 4. 17) and this effect was more prominent with undiluted virus sample verses 1:10000 diluted samples where siglec expression changes are not very evident.

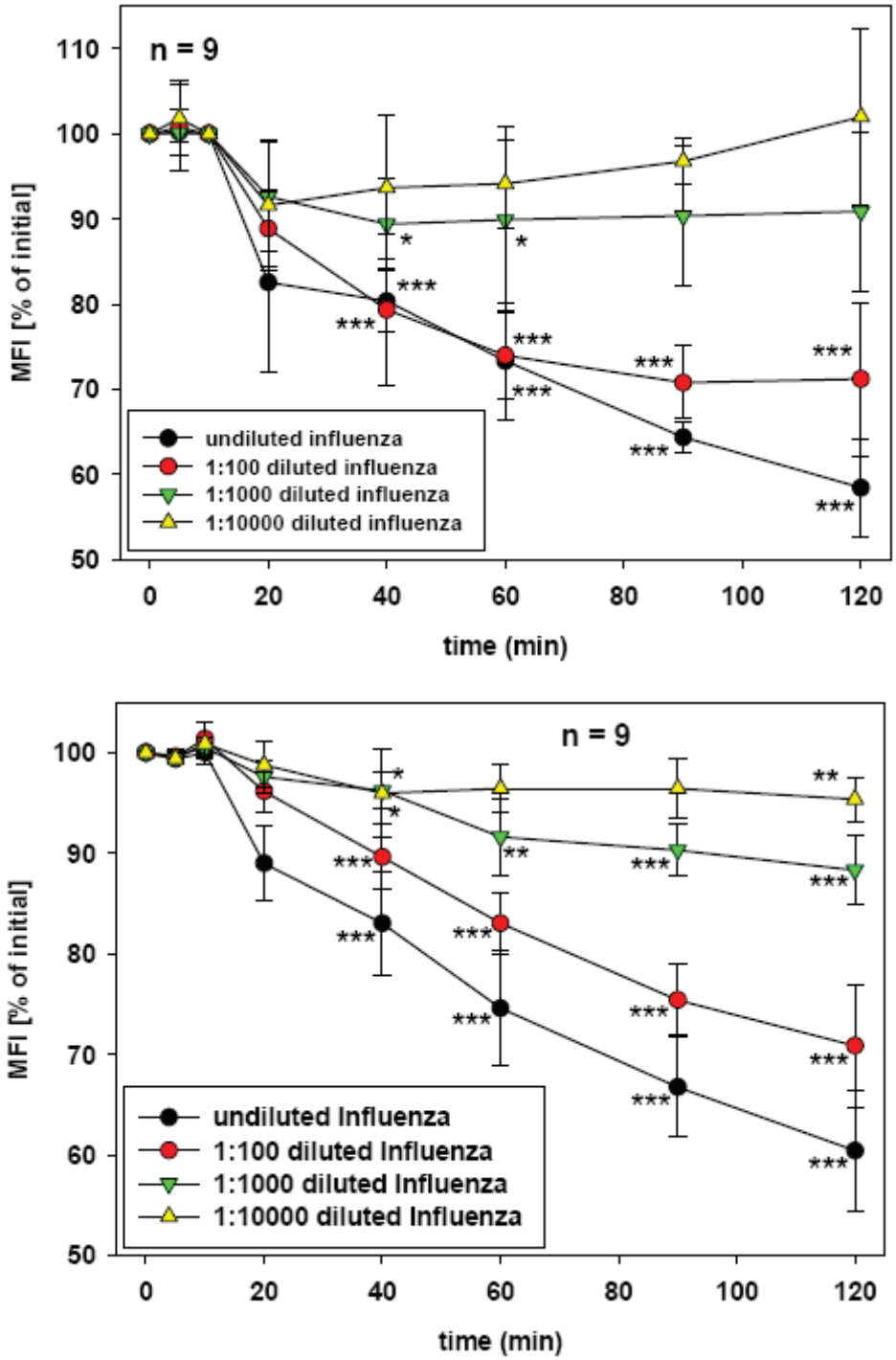
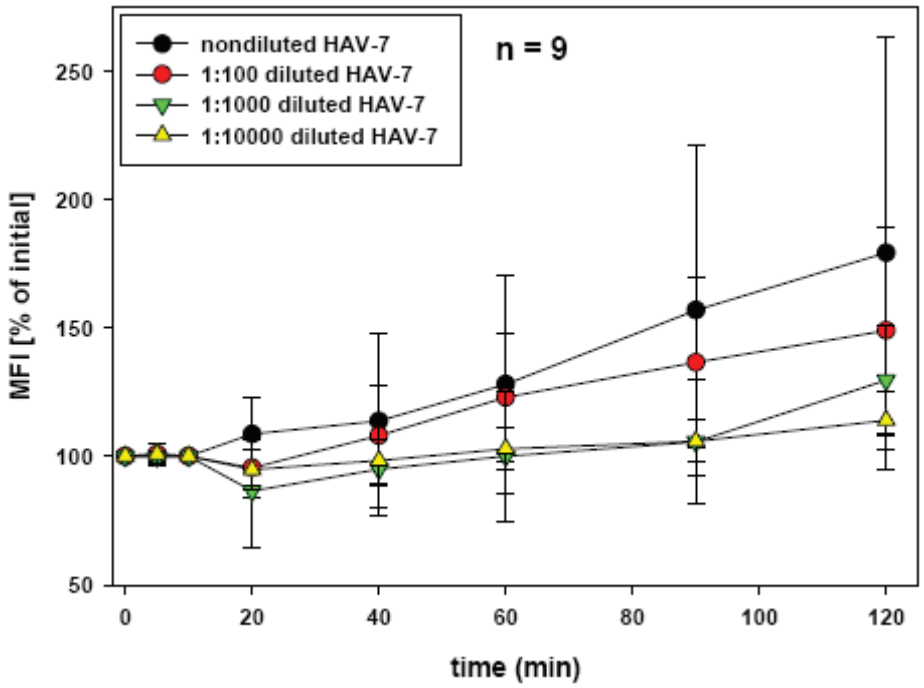


Figure 4.17: Effect of Influenza virus dose and co-culture time on monocyte surface siglec-7 and -9 expressions

Influenza virus was co-cultured with monocytes in suspension culture between 0-120min at 37°C under 5% CO_2. Cells were then stained with monoclonal anti siglec antibody before analysing with flow cytometry. Changes in Siglec-7 (A) and -9 (B) expression [MFI] were

calculated as % increase or decrease of initial siglec expression from 9 independent experiments for statistical significance (n = 9).

Co-culturing monocytes with HAV-7 virus also resulted in a steady time verses virus concentration-dependent increase in siglec-7 and siglec-9 expression on monocyte surface (Figure 4. 18). This suggests that there is direct relation between numbers of virus particle getting in contact with the monocytes over time. Changes observed in siglec-7 and -9 expressions were reproducible in several independent experiments with cells from different healthy donors.



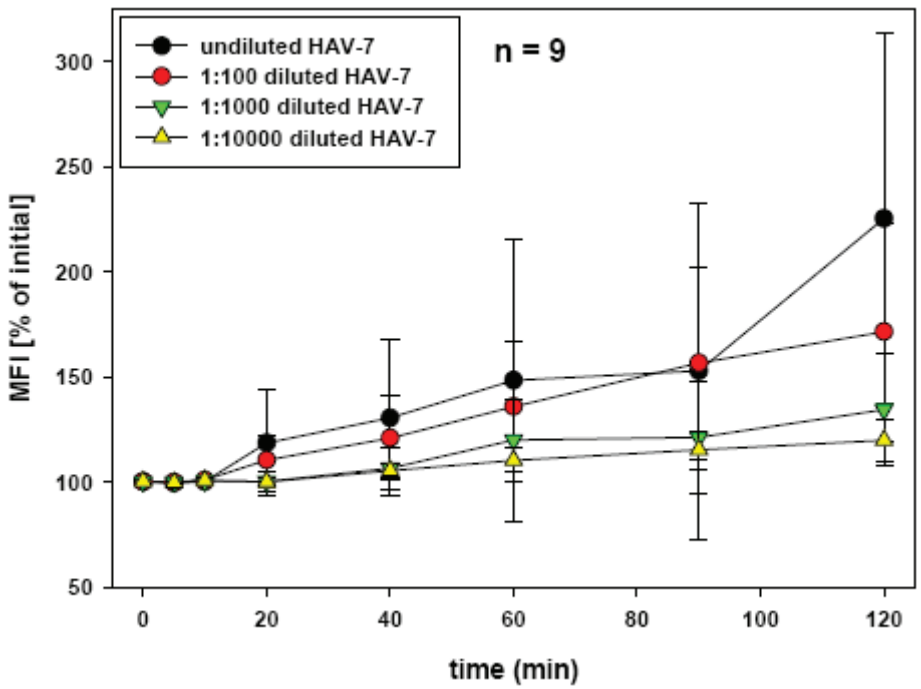


Figure 4.18: Effect of HAV-7 virus dose and co-culture time on monocyte surface siglec-7 and -9 expressions.

HAV-7 virus was co-cultured with monocytes in suspension culture between 0-120min at 37°C under 5% CO_2. Cells were then stained with monoclonal anti siglec antibody before analysing with flow cytometry. Changes in Siglec-7 (A) and -9 (B) expression [MFI] were calculated as % increase or decrease of initial siglec expression from 9 independent experiments for statistical significance (n = 9).

4.4. Siglec interaction with tumour cells

The phenotypic change in malignancy often correlates with a dramatic transformation of cellular glycosylation due to changes in the activity of one or more glycosyltransferases, during the process of transformation from normal to tumour cells (Oberling, 1997a). This may in turn be able to influence the tumour cell recognition by siglecs presented on mononuclear phagocytes. It has been shown that the contact of tumour cells with siglecs leads to increased cytokine secretion, and could finally influence disease progression. It will be interesting to investigate the time kinetics of cytokine secretion in presence of K562 erythroleukemia tumour cells. Inhibition of these activation processes in presence of inhibitory monoclonal anti siglec antibody will also be investigated.

4.4.1. Binding of siglec-Fc chimera to K562 tumour cells

Along with cytokines, cell-cell contacts can activate monocytes or macrophages. Sialylated carbohydrate structures on tumour cell membranes, i.e. on K562 erythroleukemia cells induce activation of monocytes (Valdez and Perdigon, 1991a). Siglecs could be involved in this activation, since no other Sia specific lectins are present on mononuclear phagocytes. This hypothesis predicts the presence of siglec binding partners on the corresponding tumour cells. To check this hypothesis that binding partners for siglecs exists on tumour cells, the Sia dependent binding of CHOLec1 produced Fc-chimera to K562 tumour cells was analysed (3.2.1.1). The highest binding was observed for siglec-7 Fc, followed by siglec-10 Fc, and siglec–1 Fc, whereas siglec-5, -8, and -9 Fc chimera did not show any detectable binding. In most cases siglec-9 Fc binding was very low, in some experiments a small population (1-10 %) of K562 tumour cells bound siglec-9 Fc at levels between siglec-7 Fc and siglec-10 Fc (data not shown). For all siglecs the binding observed was Sia-dependent as confirmed by cells treated with AUS leading to reduced signal levels.

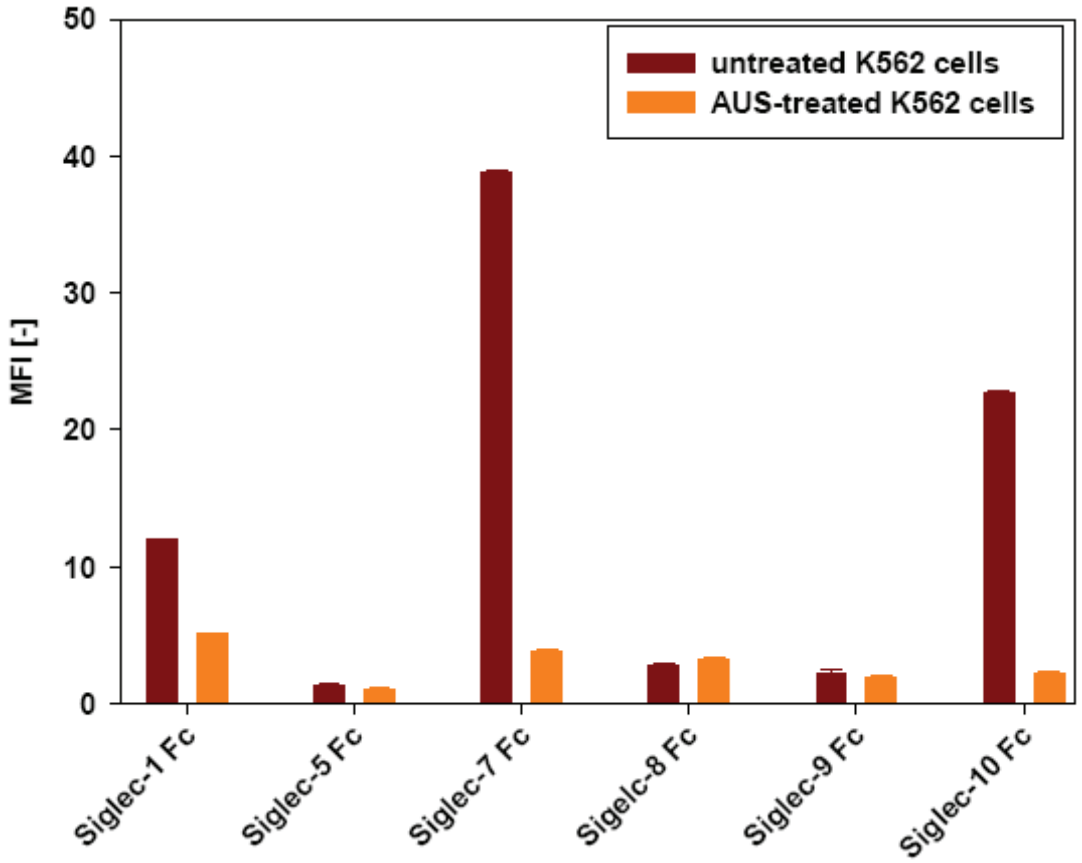


Figure 4.19: Binding of non-complexed CHOLec1 produced siglec Fc-chimeras to K562 tumour cells.

Siglec Fc-chimeras were allowed to bind to K562 tumour cells and were detected with PE-labelled anti human IgG-Fc antibodies. Sia-independent binding is shown by desialylation of K562 tumour cells by AUS. Binding was analyzed by flow cytometry. Siglec-1, -7, and 10 Fc shows binding to K562 tumour cells, whereas siglec-5 -8, and-9 Fc bind with less efficiency or show no binding to K562 tumour cells.

4.4.2. Accumulation of siglec-7 at contact sites between two cell types

Since siglec-7 binding to K562 tumour cells was higher than the other siglecs tested, this study is focused on siglec-7 involvement in direct cell-cell interaction between mononuclear phagocytes and K562 tumour cells. If siglec-7 plays a role in mononuclear phagocyte activation by tumour cells, accumulation of this siglec at contact sites between these cells can be expected. This question was addressed by performing an immunocytochemical analysis of K562-monocyte co-cultures (3.2.4). It was observed that siglec-7 accumulated at the contact site between the two cell types, whereas siglec-7 was evenly distributed over monocytes which are not in contact with K562 tumour cells (Fig. 4.20). This suggests that the interaction of

siglec-7 with glycoconjugates present on K562 cell surfaces is strong enough to cap the binding partners at the contact sites.

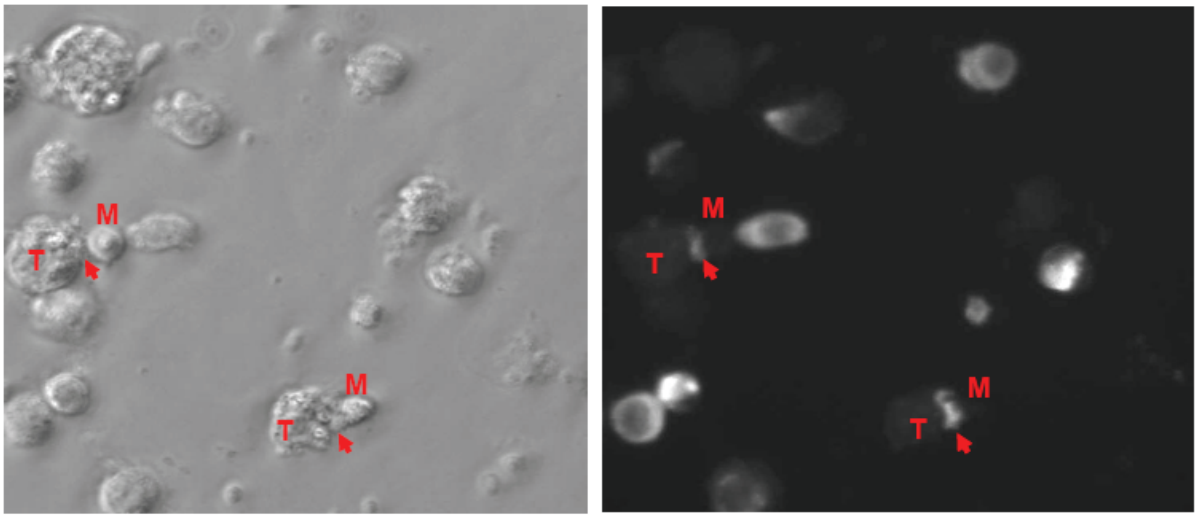


Figure 4.20: Accumulation of siglec-7 at the cellular synapse between monocytes and K562 tumour cells.

Monocytes (M) in co-culture with K562 tumour cells (T) were co-cultured in RPMI / 2.5% autologous serum for 8h at 37°C under 5% CO_2. Cells were fixed before staining anti Siglec-7 monoclonal antibody; shown here is siglec-7 accumulation at the contact site between the two cell types (arrowheads).

4.4.3. Cell surface sialoglycoconjugates of K562 tumour cells induce IL-6 production in monocyte derived macrophages

The potential of tumour cells to induce cytokine secretion by marophages was reinvestigated before addressing the question about the role of siglecs in this process.

4.4.3.1. Cytokine secretion by mononuclear phagocytes in presence of K562 tumour cell

Monocyte-derived macrophages and K562 tumour cells were selected as model cells, since K562 tumour cells are highly sialylated and bind several siglecs found on macrophages as shown above (4.4.1). Induction of IL-6 secretion required the presence of K562 tumour cells (3.1.6.1). If the tumour cells have been desialylated before the co-culture with macrophages (3.4.2), heterogeneous results were observed. Upon co-culture with IFNγ pre-treated macrophages and desialylated K562 tumour cells had no effect on its activation, but markedly reduced levels of IL-6

secretion by unactivated macrophages in the presence of desialylated K562 tumour cells were observed in some experiments. However, the level of reduced IL-6 secretion varied between 10 and 60% of that obtained with untreated K546 tumour cells (data not shown)(3.2.2.1). IL-4 pre-activated macrophages failed to respond to K562 tumour cell preparations. These observations suggest that K562 tumour cells may be able to induce IL-6 secretion by macrophages through sialylated cell surface glycoconjugates, but this effect may depend on the activation status of the macrophages.

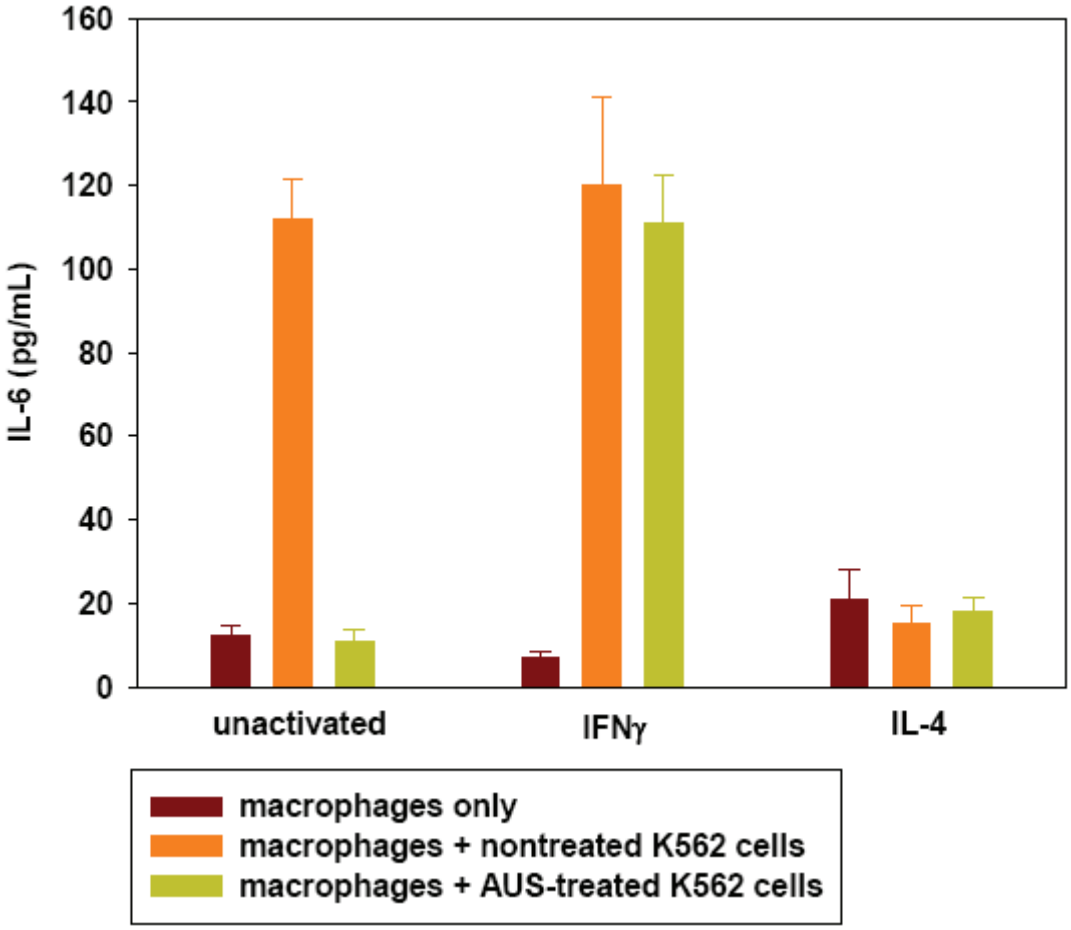


Figure 4.21: IL-6 release by pre-activated macrophages upon co-culture with sialidase-treated/ untreated K562 tumour cells

Macrophages were co-cultured with untreated K562 tumour cells in RPMI / 2.5% autologous serum overnight at 37°C under 5% CO_2; macrophages were either pre-activated with IFNγ or IL-4 or were non-activated. Non-activated macrophages produced ~110pg/mL of IL-6 upon co-culture with K562 tumour cells, whereas sialidase-treated K562 tumour cells failed to activate macrophages. Macrophages pre-activated with IFNγ produced similar amount of IL-6 in the presence of K562 tumour cells, however failed to show Sia dependent activation. Cells pre-activated with IL-4 were not activated upon co-culture with K562 tumour cells.

4.4.3.2. Time kinetics of IL-6 release by macrophages in the presence of K562 tumour cells

The activation process induced by the tumour cells was closely monitored for the time course of IL-6 release by macrophages in the presence of untreated or sialidase-treated K562 tumour cells. Surprisingly, ~10pg/mL of IL-6 was detected after 30min in co-cultures with untreated K562 tumour cells, whereas in the co-culture supernatants with AUS-treated tumour cells, no detectable amounts of IL-6 were released within the first 6 hours. In control experiments using LPS to activate the macrophages, detectable IL-6 levels (>2pg/mL) were found only after 1 hour of incubation (3.2.2.1).

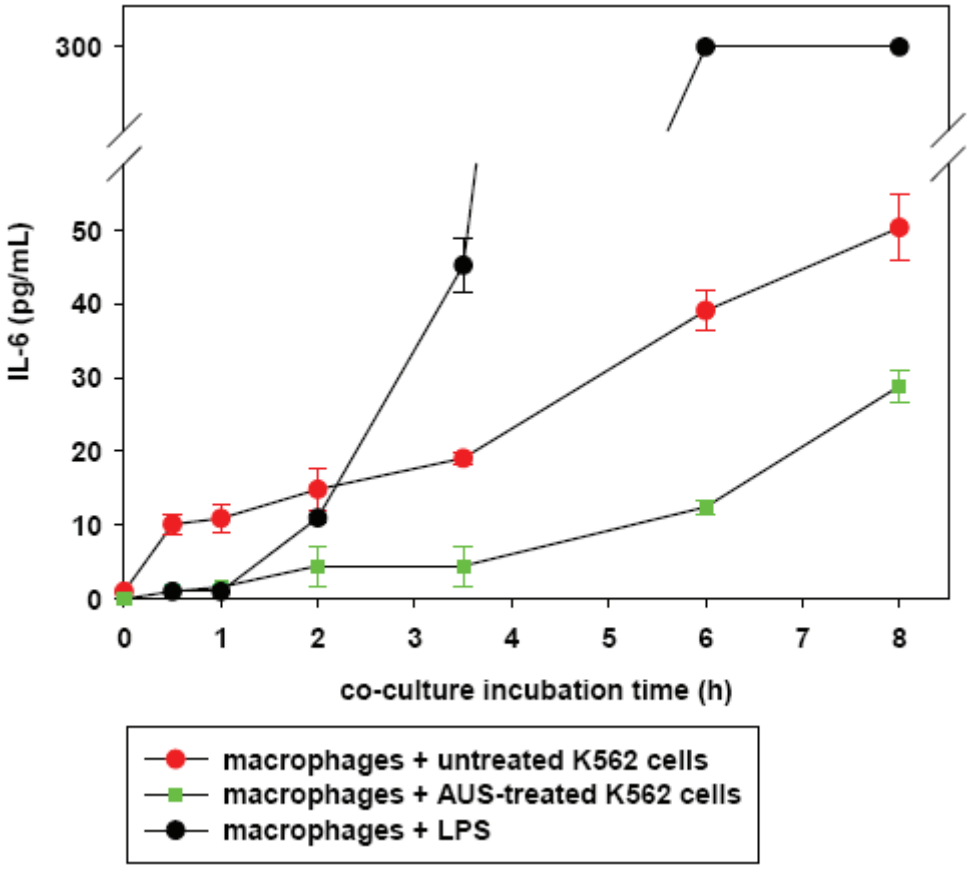


Figure 4.22: Time-dependent IL-6 production of macrophages after co-culture with K562 tumour cells.

Monocyte-derived macrophages were co-cultured with sialidase treated/untreated K562 tumour cells, IL-6 elicit was estimated. Untreated K562 tumour cells activated macrophages rapidly and even after 30min of co-culture ~10pg/mL of IL-6 can be measured in cell culture supernatant. Sialidase treated K562 tumour cells took much longer. The positive control for macrophage activation by LPS took a longer time.

4.4.3.3. K562 tumour cells surface sialoglycoconjugate induced IL-6 production

To investigate whether the quick release of IL-6 is caused by the sialylated glycoconjugates present on tumour cell surfaces, crude membrane preparations of K562 tumour cells were used to activate the macrophages. The results obtained were consistent with the results obtained from intact K562 tumour cells co-culture experiments (3.1.9). After 30 min, ~4pg/mL of IL-6 was detected in co-culture supernatants of macrophages incubated with K562 cell membranes (Figure 4.23). Macrophages were not activated if membranes had been sialidase treated prior to co-culture with macrophages (data not shown). The overall response was weaker than that elicited by intact K562 tumour cells, since membrane preparations were expected to take longer time to get in contact with macrophages as compared to intact K562 tumour cells. The induction of cytokine secretion elicited by the membrane preparations was faster and stronger than with LPS during the first two hours.

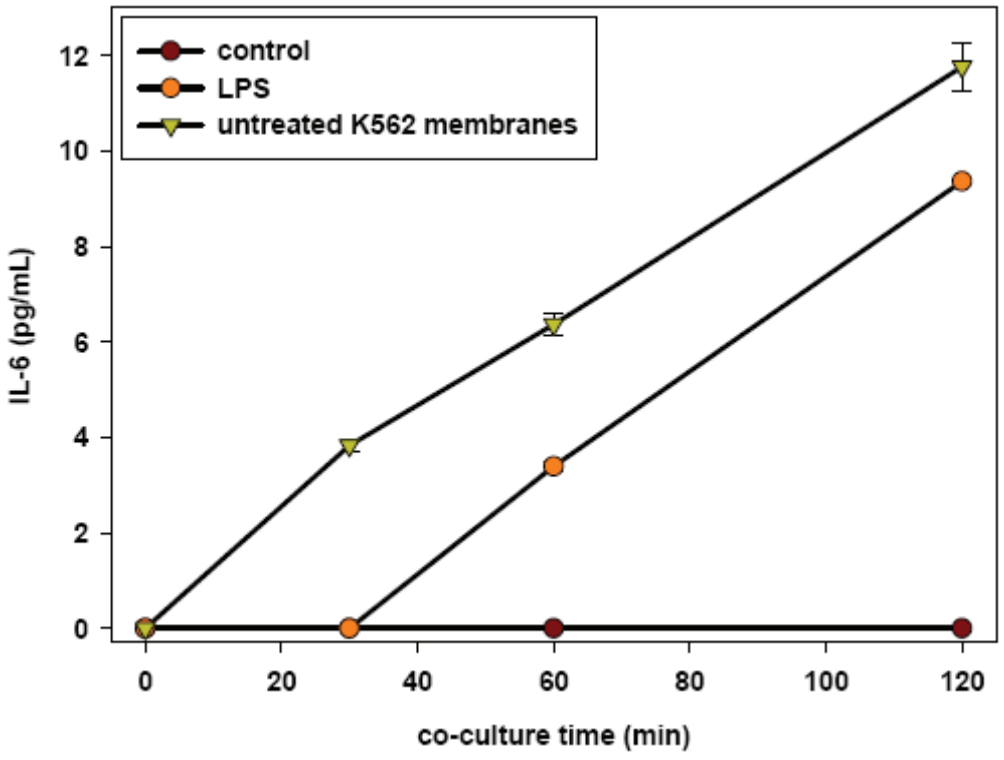


Figure 4.23: Time dependent IL-6 secretion by macrophages in presence of K562 tumour cell membrane preparations.

Only the K562 membrane preparations were able to activat macrophages. Upon co-culture with undiluted K562 tumour cell-membrane preparations, macrophage became activated and even after 30min of incubation ~4pg/mL of IL-6 was measured in the culture supernatant. Positive controls for macrophage activation, using LPS, also took a longer time.

4.4.3.4. K562 tumour cells vs. K562 tumour cell surface sialoglycoconjugate induced IL-6 production

Overall K562 tumour cell surface sialoglycoconjugate-induced IL-6 production was weaker than that elicited by intact K562 tumour cells. It could well be dependent on the time taken by membrane preparations to get in contact with macrophages. Results from parallel experiments were consistent with previous data. Untreated K562 tumour cells/membrane preparations were able to induce IL-6 production very early upon co-culture with either K562 tumour cells or K562 tumour cell membrane preparations; whereas LPS mediated IL-6 release response took a longer time.

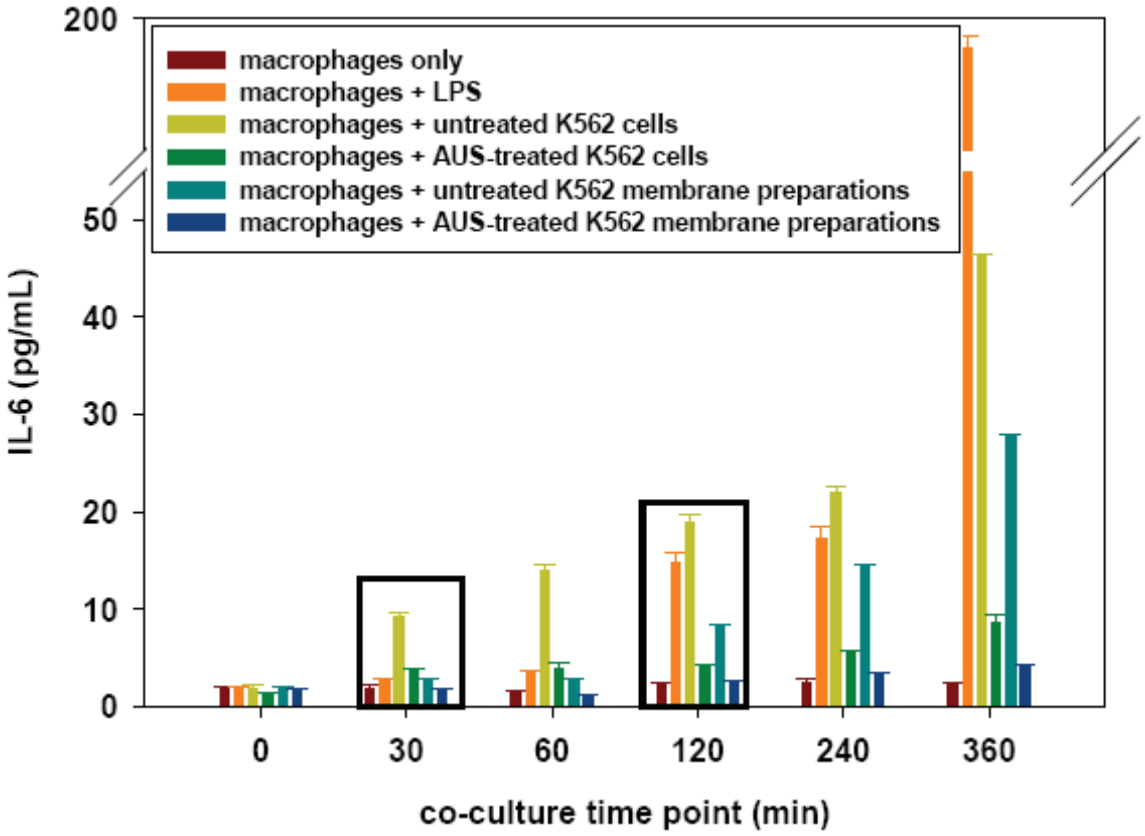


Figure 4.24: Time dependent IL-6 secretion by macrophages in presence of K562 tumour cell and membrane preparations

Macrophages were co-cultured with untreated and sialidase treated K562 tumour cell/membrane preparations and early Sia dependent activation was recorded. Presence of untreated K562 tumour cells macrophages released ~10pg/mL of IL-6 in co-culture after the first 30 min, whereas untreated K562 tumour cell-membrane preparations took 120min to release approximately the same amount of IL-6. A partial Sia dependency was obtained in time with AUS-treated K562 tumour cells and membrane preparations. LPS was used as positive control for macrophage activation.

4.4.4. Sialylated K562 tumour cells induce early IL-6 mRNA synthesis

To investigate if sialylated k562 tumour cells induce early IL-6 mRNA, real time PCR was carried out with the macrophage samples after co-cultured with K562 tumour cells (3.5.1). The K562 tumour cell surface sialoglycoconjugate induces early IL-6 mRNA synthesis. Figure 4.25 demonstrates that 30min of co-culture of macrophages with K562 tumour cells lead to synthesis of IL-6 mRNA. IL-6 mRNA synthesis was measured against the house keeping tubulin gene. An initial 400-fold increase of IL-6 mRNA levels was enhanced 4000-fold after 120 min. The LPS induced macrophages took longer to synthesise any detectable IL-6 mRNA. Similar results were obtained for IL-1β mRNA (data not shown). K562 membrane preparations took a longer time to elicit the IL-6 mRNA synthesis as they took longer to get in contact with macrophages (data not shown).

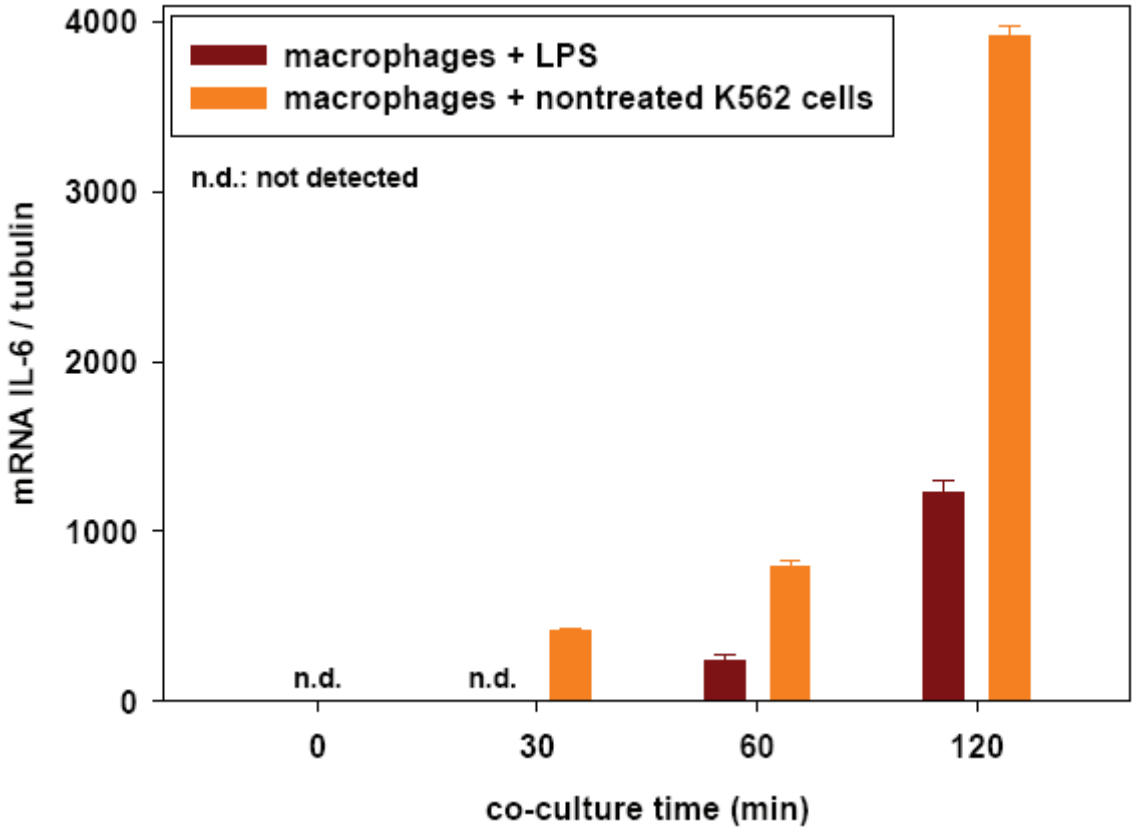


Figure 4.25: IL-6 real time PCR: relative quantity plot compared to the baseline sample for IL-6 mRNA from monocyte-K562 tumour cell co-culture system.

RNA samples from untreated K562 tumour cells and macrophages co-culture system were used to determine IL-6 mRNA levels. 30 co-culture sample showed increased level of IL-6 mRNA, whereas LPS activated sample took longer.

4.4.5. Secretion of IL-6 and IL-1β by mononuclear phagocytes is induced by K562 tumour cells possibly through interaction with siglec-7

As demonstrated above, cell surface glycoconjugates of K562 tumour cells can induce Sia dependent activation of mononuclear phagocytes suggesting that siglecs are involved in these processes. Furthermore, siglec-7 appeared to be the most likely candidate, since (1) it showed the highest binding to K562 tumour cells, (2) it accumulated at the contact sites between two cell types, and (3) it affinity-precipitated more potential binding partners from K562 tumour cells surface than other siglecs (master thesis by Sarang Limaye). To address this hypothesis, the cytokine secretion by mononuclear phagocyte was blocked using anti siglec-7 mAb before co-culture with K562 tumour cells. This antibody does not activate the macrophages Siglec-7 on mononuclear phagocyte surface was blocked using monoclonal anti siglec-7 mAb before co-culture with K562 tumour cells. The efficiency of Sia dependent cytokine elicitation by macrophages was monitored. In the presence of anti siglec-7 mAb, ~30% inhibition in IL-6 elicitation was recorded (Figure 4.21). The AUS treated K562 tumour cells were unable to activate macrophages.

Indeed, macrophages produced up to 40% less K562-induced cytokines in the presence of anti siglec-7 mAb. This indicates that the K562-mediated activation of the macrophages may be mediated at least to some extent through interaction of siglec-7 with sialylated glycoconjugates on the K562 tumour cells.

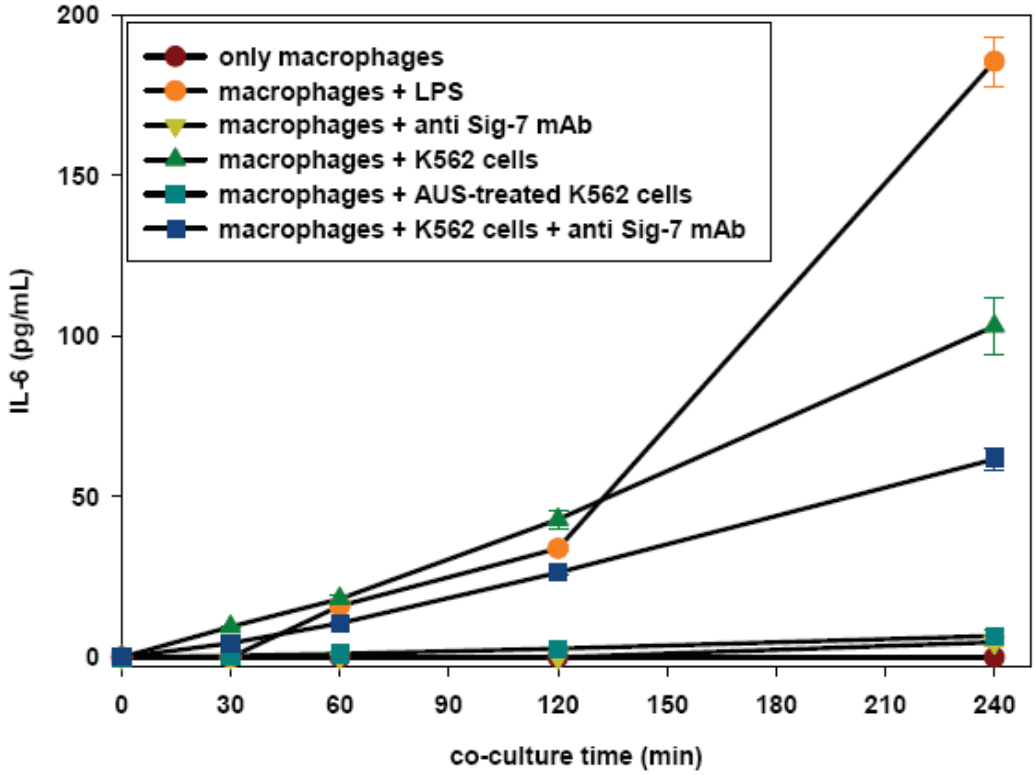


Figure 4.26: IL-6 production by macrophages upon co-culture with K562 tumour cells in the presence of blocking monoclonal sheep anti siglec-7 antibody.

K562 tumour cell-exerted Sia-dependent activation of macrophages is mediated by siglec-7. Siglec-7 is blocked by anti-siglec-7-mAb and the IL-6 secretion was monitored. After 30min co-culturing macrophages and untreated K562 tumour cells in the presence of anti-siglec-7-mAb a ~30% decrease in activation can was detected. AUS-treated K562 tumour cells failed to activate macrophages. Just the anti-siglec-7-mAb was not able to elicit activation of macrophages.

5. Discussion

Siglecs show a very high hematopoietic cell type restricted expression pattern (Table 1.1), in particular the CD33rsiglecs have more complex expression patterns on mononuclear phagocytes (O'Reilly and Paulson, 2009). Siglecs also have the ability to recognise distinct and varied sialoside sequences on glycoprotein and glycolipid glycans which are expressed on the same cell (in *cis*-) or on pathogen (bacteria, viruses), or tumour cells (in *trans*-) (Crocker and Redelinghuys, 2008). They also take part in immune processes through cytoplasmic ITIM and ITAM. To investigate further their role in immune system modulator processes. Complex siglec expression patterns on mononuclear phagocytes were analysed. Nearly untouched monocytes were used for this analysis. Monocytes and monocyte-derived macrophages were obtained by Ficoll/Percoll density gradient method and results were compared with other monocyte isolation protocols like adhesion to plastic surface and negative selection method. Variable cell counts were obtained with the adherence to plastic surface method and monocyte count was inversely proportional to the purity of the monocytes. Major drawback of the adherence to plastic surface method is they need to be detached before flow cytometry analysis. The negative immuno-selection monocyte isolation method is advantageous for the therapeutic applications but very expensive for research work. The monocytes isolated with Ficoll/Percoll isolation procedure have a similar purity to that obtained by the negative immuno-selection monocyte isolation method. The only disadvantage of Ficoll/Percoll procedure is platelets contamination, which could not be removed efficiently by low-speed centrifugation. The Ficoll/Percoll method is a simple, reliable, and cost-effective for isolating monocytes either from buffy-coats or whole blood samples. The yield of ~75% and the purity of ~75% are comparable to the other isolation methods.

Peripheral blood derived monocytes express siglec-3, -5, -7, -9, and -10 (1-10%). Upon differentiation to macrophages a general trend of decrease in siglec-3, -5 and -9 and increase for siglec-7 expression was observed in all experiments, the level of expression and degree of change during differentiation varied between individual donors. A comparison of siglec expression on monocytes isolated from the same donor at different time intervals over several months provided evidence that siglecs expression levels are variable, and these changes are probably refection of the immune status at that instance. A comparison of siglec expression with CRP levels in serum sample provided further supported this hypothesis. In most of the instances

where volunteers reported apparent sign of illness elevated CRP levels co-related with high siglec-9 expression levels. In a particular instance where volunteer was vaccinated with seasonal Influenza virus vaccine higher Siglec-9 expression levels matched with higher CRP levels. Serum CRP levels are used as biomarker for general inflammatory responses. Suggesting a direct relation between changes in immune system get directly reflected in monocyte surface siglec expression.

In-vivo experiments where monocytes were co-cultured with purified viruses', mere presence of virus particles (Influenza A, NDV, CMV, HAV-7, HAV-GBM, and Coxsackie B2 virus) resulted into changes in monocyte surface siglec expression. These changes in siglec expression were found not virus type dependent but virus dependent. Viruses belonging to either enveloped or non-enveloped group do not affect the siglecs expression similarly. Observed changes in monocyte siglec expression were regardless of the class of virus they incubated with. Such, changes in siglec expression in response to pathogens could be part of the host defence system as many pathogens are known to incorporate Sia-derivatives present on their surface for pathogenesis.

The changes in siglec expression on monocyte cell surface in response to viruses are very dynamic as even 30min of incubation with viruses resulted into changes in siglec expression. Viral (Influenza A and HAV-7) dose and time of contact also contributed to these observed changes in siglec expression, viral load and incubation time is directly proportional with siglec expression changes on the monocyte cell surface. The incorporation of Sia into pathogens could subvert the host immune defence by engaging inhibitory siglecs (Varki and Angata, 2006). Such a race against the pathogen could have played a crucial role in evolving alternative activating siglecs from the inhibitory genes (Varki and Angata, 2006). The rapidly evolving siglec family could have developed a host defence system against these pathogens. It is also possible that incorporation of Sia into pathogens could destabilize the host immune defence by engaging inhibitory siglecs (Varki and Angata, 2006). In this way the siglecs could play a critical role in evolving siglec mediated immune system modulator processes. To confirm this hypothesis further work is needed.

In an interesting siglec comparison between Causations volunteers verses Asians volunteers included in the study (15 individuals), shows that the Causations volunteers have a higher Siglec-9 expression level in comparison with the Asian volunteers. The study population is very little to conclude anything. It will be intresting

to investigate siglec expression among different races. This will put more light on the fact why some races are vulnerable to some pathogens while other show higher resistance.

It was found out that the siglec expression on human mononuclear phagocytes alters in response with either Type-I or Type-II cytokines. It was also observed that siglec expression is further regulated by different cytokines (IFNγ: increased siglec-1 and decreased siglec-5, -7 and -9; IL-4: increased siglec-7 and 9 and decreased siglec-1). The exact mechanism of these siglec expression changes in response to cytokines needs to be elucidated.

This may be relevant in tumour biology, because TAMs are often in an activated state compared to normal tissue macrophages (Putz and Mannel, 1995; Janicke and Mannel, 1990; Westenfelder et al., 1993). Therefore, it is feasible that cytokines present in the tumour environment modulate the Sia-dependent interaction of TAMs with tumour cells by regulating the siglec expression on these macrophages. Besides the expression pattern of siglecs, the competition of *cis*- and *trans*-interactions could also play an important role with regard to their function on TAMs, as described for siglec-2/CD22 on B-cells (O'Reilly et al., 2008). Indeed, the siglecs on mononuclear phagocytes are also found to be involved in cis-interactions.

Many tumour markers have turned out to consist of carbohydrate structures. In particular, sialylated glycoconjugates have been discussed to be involved in the activation of TAMs (Brunetta et al., 2009). However, the nature of these corresponding Sia specific lectins present on the mononuclear phagocytes has not been elucidated. The siglec specificities for sialylated glycoconjugates and the presence of inhibitory signalling motifs in their cytoplasmic tails suggest that they could be involved in regulating Sia dependent activation of TAMs. A rapid release of cytokines by mononuclear phagocytes upon co-culture with K562 tumour cells was observed. Further investigation revealed that this activation is Sia dependent. Nevertheless, siglec-7 appears to bind glycoconjugates on K562 erythroleukemia cells in *trans*-, as it is clustered at contact sites with these cells. These glycoconjugates seem to bind with high affinity, since the siglec-Fc chimeras bind to K562 cells without creating multivalent binding sites by complexing with anti-Fc Ab to overcome the relatively low affinity of siglecs towards sialylated glycans and to obtain stable binding of siglec-Fc chimeras to potential target cells (Wu et al., 2009). The interaction of K562 glycoproteins was specific for each siglec. The highest amount of

bound glycoproteins was obtained with siglec-7, strongly suggesting that siglec-7 plays a major role in the tumour cells recognition by TAMs. Since siglec-7 preferentially binds to α2,8-linked Sias (Ikemoto et al., 2003), it can be speculated that glycans containing this structure are present on these glycoproteins. Only little information is available on glycan structures on K562 surface glycoproteins and a detailed analysis of K562 cells glycosylation would be necessary to identify the recognition determinant(s) for siglec-7 in order to speculate on the functional consequences for cellular interactions.

TAMs appear to be often in an activated state, since they release cytokines like IL-6 (Terzidis-Trabelsi et al., 1992; Putz and Mannel, 1995; Janicke and Mannel, 1990). For example, TAMs isolated from renal cell carcinoma patients produced IL-6, tumour necrosis factor alpha (TNFα) and IL-1β without LPS stimulation, while monocytes isolated from the same patients hardly produced these cytokines without LPS stimulation (Ando et al., 2008). In agreement with previous reports (Strenge et al., 1999b; Kelm et al., 2002; Varki and Angata, 2006b; Blixt et al., 2003) it is demonstrated that sialylated glycoconjugates on K562 cell surfaces induce secretion of cytokines by monocyte-derived macrophages in a Sia dependent manner. Furthermore it is shown that the release of IL-6 and IL-1β is rapid and involves the induction of mRNA synthesis. Apparently, this process does not appear to involve all parts of the well described Toll-like receptor pathway, since the induction of cytokine secretion by LPS takes longer. Usually, CD33rSiglecs have been considered to regulate immunological reactions by acting as inhibitory proteins.

Interestingly, exposure to K562 cells had different effects on macrophages activated either with IL-4 or IFNγ. Whereas macrophages treated with IL-4 did not secrete IL-6 if stimulated by K562 cells, macrophages activated with IFNγ responded with similar IL-6 secretion if co-cultured with K562 cells. However, the response of IFNγ treated macrophages was Sia independent. One reason could be that these macrophages may recognise K562 cells by a different Sia dependent pathway.

It is very likely that siglec-7 plays a role in Sia mediated activation of mononuclear phagocytes by K562 tumour cells, since anti siglec-7 mAb reduced the activation of mononuclear phagocyte by 30-40 %. Possibly, either the monoclonal antibody could not prevent the interaction between siglec-7 and sialoglycoconjugates on K562 cells completely or other siglecs present on mononuclear phagocytes are involved in the remaining activation processes. In addition, differential expression of corresponding

glycan structures on tumour cells, which can also differ in the linkage of the Sia and/or the structure of the underlying glycans, is possible. Furthermore it is known that modifications of functional groups of the Sia can significantly change the affinities of siglecs to these glycans. It is therefore possible that specific high affinity binding partners exist which mediate signalling through siglecs, although many potential glycosylated binding partners are present on tumour cells.

In short, it can be said that siglec-7 is involved in the interaction between K562 tumour cells and human mononuclear phagocytes. In future it will interesting to address the open questions of Sia-dependent activation of macrophages which may be mediated through other siglecs, like siglec-1 or 10, and whether this mechanism is also applicable for other human tumour cells. This could be of special interest, since tumour cells are often higher sialylated than corresponding healthy cells. A siglec-mediated recognition of tumour cells by mononuclear phagocytes could describe a new mechanism of how phagocytes distinguish between healthy cells and tumour cells.

In conclusion CD33rsiglecs represent a significant component of Ig superfamily proteins expressed in the innate immune system. Their molecular properties and the presence of tyrosine-based motifs are features that are shared with other receptors involved in fine tuning innate responses. This work suggests that siglecs control immunity by acting as inhibitory receptors. Data demonstrate that at least some members of the siglec family are also able to transduce inhibitory signals in myeloid cells. The restricted expression of siglecs on myeloid and lymphoid cells and the rapid progress in understanding their roles as cell signalling and endocytic receptors have made them attractive targets for cell-directed therapeutics. Siglec-specific antibodies have been the primary tool for targeting siglecs in vivo, but glycan-based probes of siglecs show promise as an alternative method for targeting these receptors. Success with ongoing clinical trials and animal models will probably spur increased interest in the development of therapeutics targeting this class of receptors. Challenges for the future are to elucidate the precise functions of siglecs in immune responses using genetic approaches where feasible to understand how *cis* and *trans* interactions with sialylated glycoconjugates contribute to their functions and to unravel the relevant signalling pathways.

6. Bibliography

1. Ando, M., W.Tu, K.Nishijima, and S.Iijima. 2008. Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs. *Biochem. Biophys. Res. Commun.* 369:878-883.

2. Angata, T. 2008. [Functions and molecular evolution of Siglecs, a family of sialic acid receptor proteins]. *Tanpakushitsu Kakusan Koso* 53:733-738.

3. Angata, T., T.Hayakawa, M.Yamanaka, A.Varki, and M.Nakamura. 2006. Discovery of Siglec-14, a novel sialic acid receptor undergoing concerted evolution with Siglec-5 in primates. *FASEB J.* 20:1964-1973.

4. Angata, T., E.H.Margulies, E.D.Green, and A.Varki. 2004. Large-scale sequencing of the CD33-related Siglec gene cluster in five mammalian species reveals rapid evolution by multiple mechanisms. *Proc. Natl. Acad. Sci. U. S. A* 101:13251-13256.

5. Avril, T., H.Attrill, J.Zhang, A.Raper, and P.R.Crocker. 2006. Negative regulation of leucocyte functions by CD33-related siglecs. *Biochem. Soc. Trans.* 34:1024-1027.

6. Avril, T., H.Floyd, F.Lopez, E.Vivier, and P.R.Crocker. 2004. The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells. *J. Immunol.* 173:6841-6849.

7. Avril, T., S.D.Freeman, H.Attrill, R.G.Clarke, and P.R.Crocker. 2005. Siglec-5 (CD170) can mediate inhibitory signaling in the absence of immunoreceptor tyrosine-based inhibitory motif phosphorylation. *J. Biol. Chem.* 280:19843-19851.

8. Biedermann, B., D.Gil, D.T.Bowen, and P.R.Crocker. 2007. Analysis of the CD33-related siglec family reveals that Siglec-9 is an endocytic receptor expressed on subsets of acute myeloid leukemia cells and absent from normal hematopoietic progenitors. *Leuk. Res.* 31:211-220.

9. Blasius, A.L., M.Cella, J.Maldonado, T.Takai, and M.Colonna. 2006. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood* 107:2474-2476.

10. Blasius, A.L. and M.Colonna. 2006. Sampling and signaling in plasmacytoid dendritic cells: the potential roles of Siglec-H. *Trends Immunol.* 27:255-260.

11. Blixt, O., B.E.Collins, d.N.van, I, P.R.Crocker, and J.C.Paulson. 2003. Sialoside specificity of the siglec family assessed using novel multivalent probes: identification of potent inhibitors of myelin-associated glycoprotein. *J. Biol. Chem.* 278:31007-31019.

12. Boyd, C.R., S.J.Orr, S.Spence, J.F.Burrows, J.Elliott, H.P.Carroll, K.Brennan, G.J.Ni, W.A.Coulter, C.Jones, P.R.Crocker, J.A.Johnston, and C.A.Jefferies. 2009. Siglec-E is up-regulated and phosphorylated following lipopolysaccharide stimulation in order to limit TLR-driven cytokine production. *J. Immunol.* 183:7703-7709.

13. Brinkman-Van der Linden EC, J.L.Sonnenburg, and A.Varki. 2002. Effects of sialic acid substitutions on recognition by Sambucus nigra agglutinin and Maackia amurensis hemagglutinin. *Anal. Biochem.* 303:98-104.

14. Brunetta, E., M.Fogli, S.Varchetta, L.Bozzo, K.L.Hudspeth, E.Marcenaro, A.Moretta, and D.Mavilio. 2009. The decreased expression of Siglec-7 represents an early marker of dysfunctional natural killer-cell subsets associated with high levels of HIV-1 viremia *Blood* 114:3822-3830.

15. Cameron, D.J. 1983. Macrophage-mediated tumor cytotoxicity: role of macrophage surface sialic acid. *J. Surg. Oncol.* 22:77-80.

16. Cameron, D.J. and W.H.Churchill. 1982. Specificity of macrophage mediated cytotoxicity: role of target cell sialic acid. *Jpn. J. Exp. Med.* 52:9-16.

17. Carlin, A.F., Y.C.Chang, T.Areschoug, G.Lindahl, N.Hurtado-Ziola, C.C.King, A.Varki, and V.Nizet. 2009. Group B Streptococcus suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. *J. Exp. Med.* 206:1691-1699.

18. Collins, B.E., O.Blixt, A.R.DeSieno, N.Bovin, J.D.Marth, and J.C.Paulson. 2004. Masking of CD22 by cis ligands does not prevent redistribution of CD22 to sites of cell contact. *Proc. Natl. Acad. Sci. U. S. A* 101:6104-6109.

19. Collins, B.E., O.Blixt, S.Han, B.Duong, H.Li, J.K.Nathan, N.Bovin, and J.C.Paulson. 2006. High-affinity ligand probes of CD22 overcome the threshold set by cis ligands to allow for binding, endocytosis, and killing of B cells. *J. Immunol.* 177:2994-3003.

20. Collins, B.E., M.Kiso, A.Hasegawa, M.B.Tropak, J.C.Roder, P.R.Crocker, and R.L.Schnaar. 1997. Binding specificities of the sialoadhesin family of I-type lectins. Sialic acid linkage and substructure requirements for binding of myelin-associated glycoprotein, Schwann cell myelin protein, and sialoadhesin. *J. Biol. Chem.* 272:16889-16895.

21. Colotta, F., M.Sironi, A.Borre, W.Luini, F.Maddalena, and A.Mantovani. 1992. Interleukin 4 amplifies monocyte chemotactic protein and interleukin 6 production by endothelial cells *Cytokine* 4:24-28.

22. Crocker, P.R. 2005. Siglecs in innate immunity. *Curr. Opin. Pharmacol.* 5:431-437.

23. Crocker, P.R., S.Kelm, C.Dubois, B.Martin, A.S.McWilliam, D.M.Shotton, J.C.Paulson, and S.Gordon. 1991. Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages. *EMBO J.* 10:1661-1669.

24. Crocker, P.R., J.C.Paulson, and A.Varki. 2007. Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* 7:255-266.

25. Crocker, P.R. and P.Redelinghuys. 2008. Siglecs as positive and negative regulators of the immune system. *Biochem. Soc. Trans.* 36:1467-1471.

26. Crocker, P.R. and A.Varki. 2001a. Siglecs in the immune system. *Immunology* 103:137-145.

27. Crocker, P.R. and A.Varki. 2001b. Siglecs, sialic acids and innate immunity. *Trends Immunol.* 22:337-342.

28. Delputte, P.L. and H.J.Nauwynck. 2006. Porcine arterivirus entry in macrophages: heparan sulfate-mediated attachment, sialoadhesin-mediated internalization, and a cell-specific factor mediating virus disassembly and genome release. *Adv. Exp. Med. Biol.* 581:247-252.

29. Delputte, P.L., B.W.Van, F.Barbe, R.K.Van, and H.J.Nauwynck. 2007a. IFN-alpha treatment enhances porcine Arterivirus infection of monocytes via upregulation of the porcine Arterivirus receptor sialoadhesin. *J. Interferon Cytokine Res.* 27:757-766.

30. Delputte, P.L., B.W.Van, I.Delrue, C.Oetke, P.R.Crocker, and H.J.Nauwynck. 2007b. Porcine arterivirus attachment to the macrophage-specific receptor sialoadhesin is dependent on the sialic acid-binding activity of the N-terminal immunoglobulin domain of sialoadhesin. *J. Virol.* 81:9546-9550.

31. Dickensheets, H.L. and R.P.Donnelly. 1997. IFN-gamma and IL-10 inhibit induction of IL-1 receptor type I and type II gene expression by IL-4 and IL-13 in human monocytes. *J. Immunol.* 159:6226-6233.

32. Farrar, W.L., K.D.Elgert, and C.J.Burger. 1982. Tumor-induced impairment of T-cell-independent B-cell blastogenesis and DNA polymerase activity *Proc. Soc. Exp. Biol. Med.* 169:127-134.

33. Fenton, M.J., J.A.Buras, and R.P.Donnelly. 1992. IL-4 reciprocally regulates IL-1 and IL-1 receptor antagonist expression in human monocytes *J. Immunol.* 149:1283-1288.

34. Gessani, S., U.Testa, B.Varano, M.P.Di, P.Borghi, L.Conti, T.Barberi, E.Tritarelli, R.Martucci, D.Seripa, and . 1993. Enhanced production of LPS-induced cytokines during differentiation of human monocytes to macrophages. Role of LPS receptors. *J. Immunol.* 151:3758-3766.

35. Goerdt, S., O.Politz, K.Schledzewski, R.Birk, A.Gratchev, P.Guillot, N.Hakiy, C.D.Klemke, E.Dippel, V.Kodelja, and C.E.Orfanos. 1999. Alternative versus classical activation of macrophages. *Pathobiology* 67:222-226.

36. Gordon, S. 1995. The macrophage *Bioessays* 17:977-986.

37. Gordon, S. 1998. The role of the macrophage in immune regulation. *Res. Immunol.* 149:685-688.

38. Hakomori, S. 1991. Possible functions of tumor-associated carbohydrate antigens. *Curr. Opin. Immunol.* 3:646-653.

39. Hanasaki, K. 1995. [CD22: a cell adhesion molecule that displays alpha 2-6-linked sialic acid-binding lectin activity]. *Seikagaku* 67:1040-1047.

40. Hanasaki, K., A.Varki, and L.D.Powell. 1995. CD22-mediated cell adhesion to cytokine-activated human endothelial cells. Positive and negative regulation by alpha 2-6-sialylation of cellular glycoproteins. *J. Biol. Chem.* 270:7533-7542.

41. Ikehara, Y., S.K.Ikehara, and J.C.Paulson. 2004. Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. *J. Biol. Chem.* 279:43117-43125.

42. Ikemoto, S., N.Yoshida, K.Narita, S.Wada, T.Kishimoto, K.Sugimura, and T.Nakatani. 2003. Role of tumor-associated macrophages in renal cell carcinoma 22. *Oncol. Rep.* 10:1843-1849.

43. Ito, A., S.B.Levery, S.Saito, M.Satoh, and S.Hakomori. 2001. A novel ganglioside isolated from renal cell carcinoma *J. Biol. Chem.* 276:16695-16703.

44. Janicke, R. and D.N.Mannel. 1990. Distinct tumor cell membrane constituents activate human monocytes for tumor necrosis factor synthesis. *J. Immunol.* 144:1144-1150.

45. Jones, C., M.Virji, and P.R.Crocker. 2003. Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Mol. Microbiol.* 49:1213-1225.

46. Kelm, S., J.Gerlach, R.Brossmer, C.P.Danzer, and L.Nitschke. 2002. The ligand-binding domain of CD22 is needed for inhibition of the B cell receptor signal, as demonstrated by a novel human CD22-specific inhibitor compound. *J. Exp. Med.* 195:1207-1213.

47. Kelm, S., R.Schauer, J.C.Manuguerra, H.J.Gross, and P.R.Crocker. 1994. Modifications of cell surface sialic acids modulate cell adhesion mediated by sialoadhesin and CD22. *Glycoconj. J.* 11:576-585.

48. Kobsar, I., C.Oetke, A.Kroner, C.Wessig, P.Crocker, and R.Martini. 2006. Attenuated demyelination in the absence of the macrophage-restricted adhesion molecule sialoadhesin (Siglec-1) in mice heterozygously deficient in P0. *Mol. Cell Neurosci.* 31:685-691.

49. Lajaunias, F., J.M.Dayer, and C.Chizzolini. 2005. Constitutive repressor activity of CD33 on human monocytes requires sialic acid recognition and phosphoinositide 3-kinase-mediated intracellular signaling. *Eur. J. Immunol.* 35:243-251.

50. Lanoue, A., F.D.Batista, M.Stewart, and M.S.Neuberger. 2002. Interaction of CD22 with alpha2,6-linked sialoglycoconjugates: innate recognition of self to dampen B cell autoreactivity? *Eur. J. Immunol.* 32:348-355.

51. Lewis, A.L., N.Desa, E.E.Hansen, Y.A.Knirel, J.I.Gordon, P.Gagneux, V.Nizet, and A.Varki. 2009. Innovations in host and microbial sialic acid biosynthesis revealed by phylogenomic prediction of nonulosonic acid structure. *Proc. Natl. Acad. Sci. U. S. A* 106:13552-13557.

52. Lock, K., J.Zhang, J.Lu, S.H.Lee, and P.R.Crocker. 2004. Expression of CD33-related siglecs on human mononuclear phagocytes, monocyte-derived dendritic cells and plasmacytoid dendritic cells. *Immunobiology* 209:199-207.

53. Mantovani, A., F.Bussolino, and E.Dejana. 1992. Cytokine regulation of endothelial cell function *FASEB J.* 6:2591-2599.

54. Mattoli, S., F.Colotta, G.Fincato, M.Mezzetti, A.Mantovani, F.Patalano, and A.Fasoli. 1991. Time course of IL1 and IL6 synthesis and release in human bronchial epithelial cell cultures exposed to toluene diisocyanate *J. Cell Physiol* 149:260-268.

55. Montaner, L.J., R.P.da Silva, J.Sun, S.Sutterwala, M.Hollinshead, D.Vaux, and S.Gordon. 1999. Type 1 and type 2 cytokine regulation of macrophage

endocytosis: differential activation by IL-4/IL-13 as opposed to IFN-gamma or IL-10 *J. Immunol.* 162:4606-4613.

56. Montaner, L.J., P.Griffin, and S.Gordon. 1994. Interleukin-10 inhibits initial reverse transcription of human immunodeficiency virus type 1 and mediates a virostatic latent state in primary blood-derived human macrophages in vitro. *J. Gen. Virol.* 75 (Pt 12):3393-3400.

57. Monteiro, V.G., C.S.Lobato, A.R.Silva, D.V.Medina, M.A.de Oliveira, S.H.Seabra, S.W.de, and R.A.DaMatta. 2005. Increased association of Trypanosoma cruzi with sialoadhesin positive mice macrophages. *Parasitol. Res.* 97:380-385.

58. Nguyen, D.H., E.D.Ball, and A.Varki. 2006a. Myeloid precursors and acute myeloid leukemia cells express multiple CD33-related Siglecs. *Exp. Hematol.* 34:728-735.

59. Nguyen, D.H., N.Hurtado-Ziola, P.Gagneux, and A.Varki. 2006b. Loss of Siglec expression on T lymphocytes during human evolution. *Proc. Natl. Acad. Sci. U. S. A* 103:7765-7770.

60. Nicoll, G., T.Avril, K.Lock, K.Furukawa, N.Bovin, and P.R.Crocker. 2003. Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. *Eur. J. Immunol.* 33:1642-1648.

61. Nitschke, L. 2009. CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions. *Immunol. Rev.* 230:128-143.

62. Nutku, E., H.Aizawa, S.A.Hudson, and B.S.Bochner. 2003. Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. *Blood* 101:5014-5020.

63. Nutku, E., S.A.Hudson, and B.S.Bochner. 2005. Mechanism of Siglec-8-induced human eosinophil apoptosis: role of caspases and mitochondrial injury. *Biochem. Biophys. Res. Commun.* 336:918-924.

64. O'Reilly, M.K., B.E.Collins, S.Han, L.Liao, C.Rillahan, P.I.Kitov, D.R.Bundle, and J.C.Paulson. 2008. Bifunctional CD22 ligands use multimeric immunoglobulins as protein scaffolds in assembly of immune complexes on B cells. *J. Am. Chem. Soc.* 130:7736-7745.

65. O'Reilly, M.K. and J.C.Paulson. 2009. Siglecs as targets for therapy in immune-cell-mediated disease. *Trends Pharmacol. Sci.* 30:240-248.

66. Oberling, F. 1997a. Monocyte/macrophages as effector cells in cancer immunotherapy *Transfus. Sci.* 18:243-250.

67. Oliveira, E.B., C.Gotschlich, and T.Y.Liu. 1979. Primary structure of human C-reactive protein. *J. Biol. Chem.* 254:489-502.

68. Orr, S.J., N.M.Morgan, R.J.Buick, C.R.Boyd, J.Elliott, J.F.Burrows, C.A.Jefferies, P.R.Crocker, and J.A.Johnston. 2007. SOCS3 targets Siglec 7 for proteasomal degradation and blocks Siglec 7-mediated responses. *J. Biol. Chem.* 282:3418-3422.

69. Pangburn, M.K., K.L.Pangburn, V.Koistinen, S.Meri, and A.K.Sharma. 2000. Molecular mechanisms of target recognition in an innate immune system: interactions among factor H, C3b, and target in the alternative pathway of human complement. *J. Immunol.* 164:4742-4751.

70. Paul, S.P., L.S.Taylor, E.K.Stansbury, and D.W.McVicar. 2000. Myeloid specific human CD33 is an inhibitory receptor with differential ITIM function in recruiting the phosphatases SHP-1 and SHP-2. *Blood* 96:483-490.

71. Pepys, M.B. 2005. CRP or not CRP? That is the question. *Arterioscler. Thromb. Vasc. Biol.* 25:1091-1094.

72. Pepys, M.B. 2008. C-reactive protein is neither a marker nor a mediator of atherosclerosis. *Nat. Clin. Pract. Nephrol.* 4:234-235.

73. Pepys, M.B. and G.M.Hirschfield. 2001. C-reactive protein and its role in the pathogenesis of myocardial infarction. *Ital. Heart J.* 2:804-806.

74. Pepys, M.B. and G.M.Hirschfield. 2003. C-reactive protein: a critical update. *J. Clin. Invest* 111:1805-1812.

75. Putz, E.F. and D.N.Mannel. 1995a. Monocyte activation by tumour cells: a role for carbohydrate structures associated with CD2. *Scand. J. Immunol.* 41:77-84.

76. Ravetch, J.V. and L.L.Lanier. 2000. Immune inhibitory receptors *Science* 290:84-89.

77. Razi, N. and A.Varki. 1999. Cryptic sialic acid binding lectins on human blood leukocytes can be unmasked by sialidase treatment or cellular activation. *Glycobiology* 9:1225-1234.

78. Rempel, H., C.Calosing, B.Sun, and L.Pulliam. 2008. Sialoadhesin expressed on IFN-induced monocytes binds HIV-1 and enhances infectivity. *PLoS. One.* 3:e1967.

79. Rutherford, M.S., A.Witsell, and L.B.Schook. 1993. Mechanisms generating functionally heterogeneous macrophages: chaos revisited. *J. Leukoc. Biol.* 53:602-618.

80. Saldova, R., M.R.Wormald, R.A.Dwek, and P.M.Rudd. 2008. Glycosylation changes on serum glycoproteins in ovarian cancer may contribute to disease pathogenesis *Dis. Markers* 25:219-232.

81. Sawabe, T., S.Shiokawa, K.Sugisaki, T.Tsuda, and K.Yamamoto. 2000. Accumulation of common clonal T cells in multiple lesions of sarcoidosis *Mol. Med.* 6:793-802.

82. Sawada, N., H.Ishida, B.E.Collins, R.L.Schnaar, and M.Kiso. 1999. Ganglioside GD1 alpha analogues as high-affinity ligands for myelin-associated glycoprotein (MAG). *Carbohydr. Res.* 316:1-5.

83. Schauer, R. 2009. Sialic acids as regulators of molecular and cellular interactions *Curr. Opin. Struct. Biol.* 19:507-514.

84. Schwarzkopf, M., K.P.Knobeloch, E.Rohde, S.Hinderlich, N.Wiechens, L.Lucka, I.Horak, W.Reutter, and R.Horstkorte. 2002. Sialylation is essential for early development in mice. *Proc. Natl. Acad. Sci. U. S. A* 99:5267-5270.

85. Sgroi, D., A.Varki, S.Braesch-Andersen, and I.Stamenkovic. 1993. CD22, a B cell-specific immunoglobulin superfamily member, is a sialic acid-binding lectin. *J. Biol. Chem.* 268:7011-7018.

86. Shrive, A.K., G.M.Cheetham, D.Holden, D.A.Myles, W.G.Turnell, J.E.Volanakis, M.B.Pepys, A.C.Bloomer, and T.J.Greenhough. 1996. Three dimensional structure of human C-reactive protein *Nat. Struct. Biol.* 3:346-354.

87. Simmons, D. and B.Seed. 1988. Isolation of a cDNA encoding CD33, a differentiation antigen of myeloid progenitor cells *J. Immunol.* 141:2797-2800.

88. Strenge, K., R.Brossmer, P.Ihrig, R.Schauer, and S.Kelm. 2001. Fibronectin is a binding partner for the myelin-associated glycoprotein (siglec-4a). *FEBS Lett.* 499:262-267.

89. Strenge, K., R.Schauer, and S.Kelm. 1999a. Binding partners for the myelin-associated glycoprotein of N2A neuroblastoma cells. *FEBS Lett.* 444:59-64.

90. Taylor, V.C., C.D.Buckley, M.Douglas, A.J.Cody, D.L.Simmons, and S.D.Freeman. 1999. The myeloid-specific sialic acid-binding receptor, CD33, associates with the protein-tyrosine phosphatases, SHP-1 and SHP-2. *J. Biol. Chem.* 274:11505-11512.

91. Tedder, T.F., J.C.Poe, and K.M.Haas. 2005. CD22: a multifunctional receptor that regulates B lymphocyte survival and signal transduction. *Adv. Immunol.* 88:1-50.

92. Terzidis-Trabelsi, H., J.P.Lefevre, J.Bignon, and C.R.Lambre. 1992. Decreased sialidase activity in alveolar macrophages of guinea pigs exposed to coal mine dust *Environ. Health Perspect.* 97:103-107.

93. Ulyanova, T., D.D.Shah, and M.L.Thomas. 2001. Molecular cloning of MIS, a myeloid inhibitory siglec, that binds protein-tyrosine phosphatases SHP-1 and SHP-2. *J. Biol. Chem.* 276:14451-14458.

94. Valdez, J.C. and G.Perdigon. 1991b. Piroxicam, indomethacin and aspirin action on a murine fibrosarcoma. Effects on tumour-associated and peritoneal macrophages. *Clin. Exp. Immunol.* 86:315-321.

95. Varki, A. 2010. Colloquium paper: uniquely human evolution of sialic acid genetics and biology. *Proc. Natl. Acad. Sci. U. S. A* 107 Suppl 2:8939-8946.

96. Varki, A. and T.Angata. 2006. Siglecs--the major subfamily of I-type lectins. *Glycobiology* 16:1R-27R.

97. Varki, A. and P.Gagneux. 2009. Human-specific evolution of sialic acid targets: explaining the malignant malaria mystery? *Proc. Natl. Acad. Sci. U. S. A* 106:14739-14740.

98. Vimr, E.R., K.A.Kalivoda, E.L.Deszo, and S.M.Steenbergen. 2004. Diversity of microbial sialic acid metabolism. *Microbiol. Mol. Biol. Rev.* 68:132-153.

99. Vimr, E.R. and S.M.Steenbergen. 2006. Mobile contingency locus controlling Escherichia coli K1 polysialic acid capsule acetylation. *Mol. Microbiol.* 60:828-837.

100. Vitale, C., C.Romagnani, M.Falco, M.Ponte, M.Vitale, A.Moretta, A.Bacigalupo, L.Moretta, and M.C.Mingari. 1999. Engagement of p75/AIRM1 or CD33 inhibits the proliferation of normal or leukemic myeloid cells *Proc. Natl. Acad. Sci. U. S. A* 96:15091-15096.

101. von, G.S. and B.S.Bochner. 2008. Basic and clinical immunology of Siglecs. *Ann. N. Y. Acad. Sci.* 1143:61-82.

102. von, G.S., A.Schaub, M.Vogel, B.M.Stadler, S.Miescher, and H.U.Simon. 2006. Immunologic and functional evidence for anti-Siglec-9 autoantibodies in intravenous immunoglobulin preparations. *Blood* 108:4255-4259.

103. von, G.S., S.Yousefi, M.Seitz, S.M.Jakob, T.Schaffner, R.Seger, J.Takala, P.M.Villiger, and H.U.Simon. 2005. Siglec-9 transduces apoptotic and nonapoptotic death signals into neutrophils depending on the proinflammatory cytokine environment. *Blood* 106:1423-1431.

104. Walker, J.A. and K.G.Smith. 2008. CD22: an inhibitory enigma. *Immunology* 123:314-325.

105. Walter, R.B., B.W.Raden, R.Zeng, P.Hausermann, I.D.Bernstein, and J.A.Cooper. 2008. ITIM-dependent endocytosis of CD33-related Siglecs: role of intracellular domain, tyrosine phosphorylation, and the tyrosine phosphatases, Shp1 and Shp2. *J. Leukoc. Biol.* 83:200-211.

106. Weiman, S., S.Dahesh, A.F.Carlin, A.Varki, V.Nizet, and A.L.Lewis. 2009. Genetic and biochemical modulation of sialic acid O-acetylation on group B Streptococcus: phenotypic and functional impact. *Glycobiology* 19:1204-1213.

107. Weiman, S., S.Uchiyama, F.Y.Lin, D.Chaffin, A.Varki, V.Nizet, and A.L.Lewis. 2010. O-Acetylation of sialic acid on Group B Streptococcus inhibits neutrophil suppression and virulence. *Biochem. J.* 428:163-168.

108. Westenfelder, U., B.Schraven, and D.N.Mannel. 1993. Characterization of monocyte-activating tumour cell membrane structures. *Scand. J. Immunol.* 38:388-394.

109. White, M.R., E.Crouch, E.M.Van, M.Hartshorn, L.Pemberton, I.Tornoe, U.Holmskov, and K.L.Hartshorn. 2005. Cooperative anti-influenza activities of respiratory innate immune proteins and neuraminidase inhibitor. *Am. J. Physiol Lung Cell Mol. Physiol* 288:L831-L840.

110. Wu, C., U.Rauch, E.Korpos, J.Song, K.Loser, P.R.Crocker, and L.M.Sorokin. 2009. Sialoadhesin-positive macrophages bind regulatory T cells, negatively controlling their expansion and autoimmune disease progression. *J. Immunol.* 182:6508-6516.

111. Xiong, Y.S., Y.H.Zhou, G.H.Rong, W.L.Wu, Y.Liang, Z.X.Yang, H.L.Geng, and R.Q.Zhong. 2009. Siglec-1 on monocytes is a potential risk marker for monitoring disease severity in coronary artery disease. *Clin. Biochem.* 42:1057-1063.

112. Yamaji, T., T.Teranishi, M.S.Alphey, P.R.Crocker, and Y.Hashimoto. 2002. A small region of the natural killer cell receptor, Siglec-7, is responsible for its preferred binding to alpha 2,8-disialyl and branched alpha 2,6-sialyl residues. A comparison with Siglec-9. *J. Biol. Chem.* 277:6324-6332.

113. Yeaman, G.R., J.E.Collins, and G.A.Lang. 2002. Autoantibody responses to carbohydrate epitopes in endometriosis. *Ann. N. Y. Acad. Sci.* 955:174-182.

114. York, M.R., T.Nagai, A.J.Mangini, R.Lemaire, J.M.van Seventer, and R.Lafyatis. 2007. A macrophage marker, Siglec-1, is increased on circulating monocytes in patients with systemic sclerosis and induced by type I interferons and toll-like receptor agonists. *Arthritis Rheum.* 56:1010-1020.

115. Yu, Z., M.Maoui, L.Wu, D.Banville, and S.Shen. 2001. mSiglec-E, a novel mouse CD33-related siglec (sialic acid-binding immunoglobulin-like lectin) that recruits Src homology 2 (SH2)-domain-containing protein tyrosine phosphatases SHP-1 and SHP-2. *Biochem. J.* 353:483-492.

116. Zhang, T., Y.Li, Y.J.Wang, X.Wang, M.Young, S.D.Douglas, and W.Z.Ho. 2007. Natural killer cell inhibits human immunodeficiency virus replication in chronically infected immune cells. *Antiviral Res.* 73:132-139.