Dissertation

submitted to the

Combined Faculties for the Natural Sciences and for Mathematics of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

Master of Science in Biology Stephan Alexander Gütgemann

born in: Bonn, Germany

Oral-examination:

Molecular basis for the lipid raft recruitment of NK cell receptors and development of a sialic acid-based Siglec-7 inhibitor

Referees: Prof. Dr. Walter Nickel

Prof. Dr. Carsten Watzl

“He who asks a question is a fool for 5 minutes;
he who does not ask a question remains a fool forever.“

- Ancient Chinese Proverb -

Table of Contents

Acknowledgements

First of all I want to thank my supervisor Prof. Dr. Carsten Watzl. Thank you for giving me the possibility to do this work in the first place, for your ever-helpful guidance and making the lab a nice place to be. Your advice was always available if sought and greatly helped me in finishing this work.

I also want to thank Prof. Dr. Walter Nickel for officially representing this work in the faculty of biosciences and his support in the organization of this work.

Thanks go to my colleagues, all the members of the (in)famous Watzl lab (past and present)! First for the tremendous amount of cake, second for your continuous help, creating a pleasant working atmosphere and making me laugh and laugh and laugh…. Thanks to you, I learned a vast amount of stuff (not always about science but always worth knowing).
I will dearly miss your company, especially that of:

Stephan 'Käpt'n' Meinke
Doris Urlaub
André 'Wingman' Cohnen
Maren 'Klaucs' Claus
Christian Schütz
Petra Netter
Steffen Killian
Sebastian Stark
Sabine Wingert
Martha-Lena Müller
Mina Sandusky
Stefanie Margraf
Wolfgang Merkt
Birgitta Messmer

I also sincerely want to thank our collaborators Prof. Dr. Brossmer and his ever-tireless technician Horst Prescher for their interest and contribution to this work. Thank you for the inhibitors and your help, both were essential to this work.

Moreover, I want to thank Dr. Martin Frank for permitting me the use of his Siglec-7 docking models. Your models greatly aided in the understanding of our work.

Furthermore I want to thank all members of the institute for immunology in Heidelberg for their constant and termless support. Whatever was asked for, it was given without hesitation.

Special thanks belong to my future wife! Thank you for your ever-lasting support, your patience and your love!

I also want to thank my family, especially my parents, my godfather and my siblings for motivating me, assisting me and helping me whenever they can.

Last but not least I want to thank my friends, just for being there when it counts.

Summary

The activation status of natural killer (NK) cells is determined by the integration of activating and inhibitory signals at the immunological synapse between the NK cell and a target cell. In this work we studied the activating and inhibitory signals of NK cells:

The presence of cholesterol-rich membrane domains at the activating immunological synapse and the absence of these membrane domains at inhibitory immunological synapses has supported the role of lipid rafts in the regulation of NK cell activation. Several activating NK cell receptors are known to localize into these lipid rafts upon ligation and thereby influence the functional outcome of a NK cell-target cell contact. How these receptors are recruited into these important membrane domains remains unknown though. In order to elucidate the molecular basis of these mechanisms we studied the structural requirements of the human activating NK cell receptors 2B4 and NKG2D for receptor recruitment into detergent-resistant membrane (DRM) fractions as a model for lipid raft association. Structural requirements in 2B4 for DRM recruitment could be identified at the luminal side of the transmembrane domain and in the CxC motif near the cytoplasmic side of the transmembrane domain. Similar to 2B4, we could determine a requirement for the NKG2D transmembrane domain for DRM recruitment. However, DRM recruitment of both receptors was independent from signal transduction. Thus our study presents data about potential molecular mechanisms involved in lipid raft recruitment.

In order to study NK cell inhibition, we developed inhibitors against the sialic acid-binding receptor Siglec-7 on NK cells. This receptor is known to exert inhibitory effects on the function of NK cells and is harnessed by certain pathogens and malignancies for immune evasion. The inhibitors were designed by collaborating biochemists based on the structure of unmodified sialic acid. We screened a broad collection of inhibitors and tested these in functional assays for their capacity to block Siglec-7 receptor-ligand interaction and their effect on NK cell activity. In conclusion, this collaborative study was able to generate the first inhibitor with high affinity against Siglec-7 and with a functional effect on the cytotoxicity of NK cells.

Zusammenfassung

Die Aktivierung von natürlichen Killer (NK)-Zellen resultiert aus der Integration von aktivierenden und inhibierenden Signalen an der immunologischen Synapse zwischen einer NK-Zelle und einer Zielzelle. Diese Arbeit behandelt diese aktivierenden sowie inhibierenden Signale in NK-Zellen:

Durch die Präsenz von Cholesterin-reichen Membrandomänen in aktivierenden Synapsen und die Abwesenheit dieser Membrandomänen in inhibierenden Synapsen wurde die Rolle von „Lipid Rafts“ in der Regulation der NK-Zellaktivierung gestärkt. Im Falle mehrerer aktivierender NK-Zell-Rezeptoren wurde eine Rekrutierung in diese Membrandomänen nach Zielzell-Bindung festgestellt was den funktionellen Ausgang solch eines Zellkontaktes maßgeblich beeinflussen kann. Wie diese Rezeptoren allerdings in die Membrandomänen rekrutiert werden ist bislang nicht bekannt. Um die molekularen Mechanismen für diese Rekrutierung aufzudecken haben wir die strukturellen Voraussetzungen für die Rekrutierung der NK-Zell-Rezeptoren 2B4 und NKG2D in Detergenz-resistente Membranen (DRM) als Model für die „Lipid Raft“-Rekrutierung untersucht. Strukturelle Voraussetzung in 2B4 für die DRM-Rekrutierung lagen an der luminalen Seite der Transmembrandomäne, sowie in einem CxC-Motiv an der zytoplasmatischen Seite der Transmembrandomäne. Auch NKG2D war strukturell für eine DRM-Rekrutierung auf die Transmembrandomäne angewiesen. Eine Signaltransduktion war jedoch bei beiden Rezeptoren nicht involviert. Diese Studie weist damit strukturelle Voraussetzungen und potenzielle Mechanismen für die „Lipid Raft“-Rekrutierung von Rezeptoren auf.

Um die NK-Zell Inhibition zu untersuchen wurden Inhibitoren gegen den Sialinsäure-bindenden Rezeptor Siglec-7 entwickelt. Dieser Rezeptor kann die Zellaktivierung von NK-Zellen hemmen und wird durch diverse Pathogene und in verschiedenen Krankheiten als Immunevasionsmechanismus missbraucht. Auf der Grundlage von unmodifizierter Sialinsäure wurde eine Kollektion von Inhibitoren von kollaborierenden Biochemikern entwickelt und von uns in funktionellen Assays auf die Fähigkeit die Rezeptor-Ligandenbindung zu blockieren sowie die NK-Zellaktivität zu beeinflussen getestet. In dieser gemeinschaftlichen Studie wurde der erste hoch-affine Siglec-7-Inhibitor mit Einfluss auf die NK-Zellfunktion entwickelt.

1 Introduction

1.1 Natural killer cells

Natural killer (NK) cells are a subset of lymphocytes belonging to the innate immune system of vertebrates and comprise 2-18 % of the lymphocytes in peripheral blood [1]. NK cells were first discovered in 1975 and named according to the observed function of these cells. The term “natural cytotoxicity” was coined as this population of cells performed cytotoxicity without the need for prior sensitization, unlike T or B cells [2-5]. Although initially thought to be nonspecific, the activity of NK cells is regulated, as was first predicted by the formulation of the “missing self hypothesis” in 1986 [6]. This notion was supported by the discovery of various inhibitory [7, 8] and activating [9-11] receptors on NK cells. These findings implied that unlike other lymphocytes like T cells or B cells, NK cells do not express an affinity maturated monoclonal antigen receptor on their surface. Instead they express a diverse array of germ-line encoded activating and inhibitory receptors [12]. This independence enables NK cells to respond faster to an infection than lymphocytes of the adaptive immune system as no somatic recombination and clonal expansion is required. Still, NK cells expand upon infection and migrate from the blood to the site of infection. In fact, NK cells are the major population of immune cells keeping viral infections at bay in the first days, until the more specific adaptive immune response can clear the infection, if still present [13, 14]. These are the main reasons why NK cells are commonly classified into the innate rather than the adaptive immune system. Similarities with other lymphocytes remain though, as NK cells originate from the same common lymphoid progenitor as T and B cells in the bone marrow. For example, NK cells share properties like common lymphoid signal cascades with T and B cells. In contrast to T cells, NK cells do not rely on the thymus though [15].

NK cells are not a population of simple killer cells, but include several subtypes of cells and overall fulfill a range of different tasks and functions in the body. Commonly described as $CD56^{+}CD3^{-}$ lymphocytes, NK cells nowadays are additionally characterized by their expression of NKp46 [16, 17]. Still, this marker set seems to be insufficient to completely describe NK cells, as $NKp46^{+}$ non-NK cells have already been described [18, 19]. NK cells are not a homogenous population of $NKp46^{+}$ $CD56^{+}$ $CD3^{-}$ lymphocytes though. There are two main populations defined by their level of CD56 surface expression: $CD56^{dim}$ and $CD56^{bright}$ NK cells. In addition, the NK cell population contains plenty of cells with completely different functions: The

already mentioned cytotoxic capacity of NK cells residues mostly in the $CD56^{dim}$ $CD16^{+}$ NK cells [20], which is the most prominent NK cell population in peripheral blood (about 90% of peripheral blood NK cells). NK cells are also capable producers of cytokines like interferon (IFN)-γ, tumor necrosis factor (TNF)-α and granulocyte macrophage colony stimulating factor (GM-CSF) [21]. This function resides in the $CD56^{bright}$ $CD16^{-}$ NK cells mostly, which are preferentially found in lymph nodes, secondary lymphoid tissues and only to a lesser extent in the peripheral blood [22]. The functional capacity of these subsets is not exclusive however, and each subset is able to perform the other's main function as well, although to a lesser extent [20]. This functional junction makes more sense in view of recent findings, which imply a developmental link between those subsets, and state that the $CD56^{bright}$ NK cells are the less mature NK cells and eventually develop into $CD56^{dim}$ NK cells [23, 24]. Other specialized subsets of NK cells with different functions were also found in the gut, the lung, the liver and the uterus [18, 25-27].

1.2 Functions of NK cells

The most prominent function of NK cells is their ability to lyse sensitive target cells, in most cases virally infected cells or transformed cells [28, 29]. NK cells are also involved in immune responses against other intracellular pathogens [30, 31]. A lack of NK cells or their functions has been shown to cause increased sensitivity towards infections and transformed cells [32]. Especially in the early course of an infection NK cells play a major role in protection, while the adaptive immune response is still developing [12, 21]. Once a contact between NK cell and target cell is established and an immunological synapse (IS) has formed, the NK cell gauges the target cell for its sensitivity. Whether or not a target cell is sensitive is defined by its expressed ligands for activating and inhibitory receptors that are described in more detail later on. If a target cell is sensitive, a NK cell possesses two different mechanisms to kill it: The first is the lysis by cytotoxic granules released upon the target cell in the IS. These granules contain different effector molecules like perforin, granzymes and granulysin, which interfere with the membrane integrity of the target cell and induce apoptosis e.g. by activation of the caspase cascades [33, 34]. Apart from this mechanism, NK cells also express ligands for so-called death receptors, like Fas-receptor or TRAIL receptor, and can trigger these receptors on the target cell, again resulting in apoptosis induction [34]. One has to keep in mind though that NK cells are not completely independent from adaptive immune responses, as e.g. NK cells can recognize bound antibodies with a Fc receptor and mediate killing of antibody-coated target cells, a process called antibody-dependent cellular cytotoxicity (ADCC)

[9]. These mechanisms are used to kill infected and/or transformed cells, but NK cells also harbor different links to adaptive immunity: It has previously been shown that NK cells are able to modulate immune responses, either by secretion of cytokines or by killing other immune cells. For example, T_H cells can be skewed toward a T_H1 response by the IFN-γ produced by NK cells [35]. On the other hand, a bidirectional influence between NK cells and dendritic cells (DCs) has been described: NK cells can activate DCs and are stimulated by DCs in return, but can kill immature DCs as well [36, 37]. These interactions can modulate the adaptive immune system; either by boosting an immune response by activating DCs, or a potential harmful immune response (e.g. against a self-antigen) can be prevented by killing immature DCs in the right place [36]. Another protective role against autoimmune pathologies has emerged in the last years: NK cells are able to recognize the activation status of macrophages [38] and eventually remove them. If this control is absent, hyperactivated cells could cause harm in the body, which is the case in certain kinds of hemophagocytic lymphohistiocytosis. A deficiency in perforin secretion, which abolishes the cytotoxic potential of NK cells, can result in the occurrence of hyperactivated macrophages and T cells that cause severe harm via different mechanisms [39-41].

Other functions of NK cell subsets or NK cell-related subsets include the control of mucosal homeostasis in mucosa-associated lymphoid tissues as well as regulation of angiogenesis in the decidua during pregnancy [18, 19, 42].

1.3 NK cell activation & inhibition

The recognition of target cells by NK cells, independent whether they are infected, transformed or hyperactivated, relies on the integration of signals from activating and inhibitory receptors. Both, activating and inhibitory receptors are present on NK cells in a vast array of different receptors in order to enable the NK cell to gauge the target cell by different means. In a first step the NK cell makes contact with its target cell, which involves the binding of adhesion molecules on the target cell by the NK cell integrins. This ligation of adhesion molecules induces first signals resulting in cytoskeletal reorganization at the contact site [43, 44]. During the establishment of a contact site an immunological structure is formed between the two cells: the IS. The IS is ordered and can be observed in different contacts of immune cells, but was first observed for T cells and antigen presenting cells [45]. Based on the original observation from T cells, the activating IS has been proposed to be divided into three distinct concentric rings: the central supramolecular activation cluster (cSMAC) contains the signaling receptors and eventually the degranulation site of the cytotoxic

granules. The cSMAC is surrounded by the peripheral SMAC (pSMAC) formed by adhesion molecules, like integrins, which tightly segregate the cSMAC from the outside. The pSMAC is encircled by the distal SMAC (dSMAC) that encloses the IS [45, 46]. Once contact is established, the NK cell gauges the target cell with its receptors by integrating activating and inhibitory signals, either resulting in activation or inhibition. An activating signal of one receptor is not enough to trigger resting NK cells, with the exception of the Fc receptor CD16, instead a combination of signals from different receptors is required to activate the NK cell. Most of the activating NK cell receptors are able to synergize in order to cross the required activation threshold [47, 48]. This led to the notion to call all NK cell receptors, except CD16, 'co-activating receptors'. Although NK cells are regarded as natural killers, an activation, e.g. by cytokines as IL-2 or IL-15, can lower this threshold to the level that the triggering of a single receptor is sufficient to activate the NK cells [48].

An activating signal causes a series of consecutive steps: An activating NK cell immune synapse (aNKIS) is established in an actin-dependent way and activating receptors are recruited into the IS together with lipid raft clustering in the synapse [45, 49]. Meanwhile, the microtubule-organizing center (MTOC) is reoriented towards the target cell. The cytotoxic granules are then polarized towards the target cell and their content is released upon the target in the synaptic cleft. Dominating inhibitory signals, in contrast, cause the formation of an inhibitory NK cell immune synapse (iNKIS), which is structurally distinct from the aNKIS [50]. This leads to a dephosphorylation of Vav-1, the essential guanine nucleotide exchange factor (GEF) for the small GTPase Rac1, which is crucial for actin remodeling and subsequent steps [51, 52]. This abolishes actin remodeling, receptor clustering and lipid raft clustering in the IS [45]. A dephosphorylation of Vav-1 thus causes an early block of signaling by activating receptors. This process is independent of the cytoskeleton and finally causes the NK cell to spare the target cell [52].

1.4 Inhibitory NK cell receptors

Since the formulation of the "missing self hypothesis" it has been clear that certain receptors on NK cells must be able to inhibit a cytotoxic response against target cells [6]. Several different groups of inhibitory receptors have been described on NK cells. The majority of these receptors recognize MHC class I molecules, but there are some receptors as well that recognize non-MHC ligands. These ligands can be influenced by cytokines, stress, viral infection or transformation and thus give a status of the target cell. For example, certain viruses cause a reduction of MHC class I molecules on the cell surface of infected cells in order to evade T cell responses

[53]. At the same time this decreases the inhibition of NK cells towards them by reducing inhibitory ligands, making them more susceptible for NK cell mediated lysis. Even inhibitory receptors are not structurally related in all species, their function is the same. All of them share the presence of immunoreceptor tyrosine-based inhibition motifs (ITIMs) or related signaling motifs in their cytoplasmic domain.

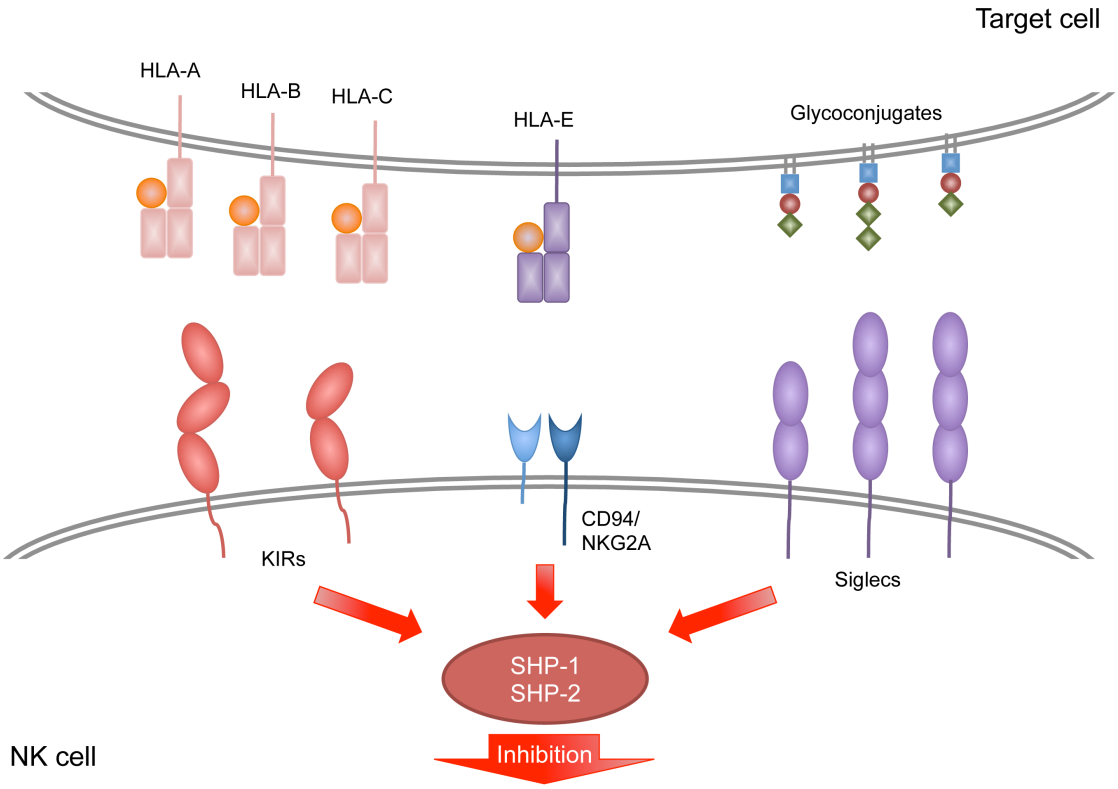


Figure 1: Simplified model of NK cell inhibition. A range of inhibitory receptors expressed on the NK cell surface recognizes diverse ligands on the target cell upon contact. Most inhibitory NK cell receptors (like Killer cell Immunoglobulin-like receptors or KIRs) recognize MHC class I molecules or related molecules, but some receptors recognize MHC-independent ligands, e.g. glycoconjugates as recognized by Siglec receptors. Upon ligation an inhibitory receptor is phosphorylated and recruits phosphatases (like SHP-1) that mediate downstream inhibition by dephosphorylating key substrates of activating signal pathways.

The common mechanism includes the phosphorylation of the ITIM followed by the subsequent recruitment of phosphatases like the protein tyrosine phosphatases SHP-1 and SHP-2 or the inositol-5-phosphatase SHIP-1 [54]. These phosphatases then abolish activating signals by dephosphorylating key substrates like Vav-1 [52]. A simplified model is depicted in Figure 1.

1.4.1 KIRs

The main group of inhibitory receptors on human NK cells is formed by the killer cell immunoglobulin-like receptors (KIRs), which recognize HLA molecules on the surface of target cells [55]. KIRs contain either two or three extracellular Ig-like domains and either a long cytoplasmic tail containing an ITIM or a short cytoplasmic tail without the ITIM. The ITIM-containing KIRs belong to the inhibitory receptors

whereas the KIRs without an ITIM associate with ‘DNAX-activating protein of 12 kDa’ (Dap12) and belong to the activating receptors [56, 57]. The extracellular domains are highly homologous in activating and inhibitory KIRs. The exact reason for this high homology between activating and inhibitory KIRs is still unknown. The functional homologues of the KIRs in the mouse are the Ly49 receptors, which belong to the C-type lectin-like receptors and are therefore structurally unrelated to the KIRs [58]. As with the KIRs, the Ly49 receptors bind MHC class I molecules and contain inhibitory as well as activating members.

1.4.2 CD94/NKG2

Another receptor group consists of the heterodimer of the lectin-like CD94 and members of the C-type lectin-like NKG2 family of receptors [59]. As with the KIRs, this receptor family contains activating and inhibitory receptors. The most important inhibitory receptor out of this group, CD94/NKG2A, recognizes the non-classical HLA allele HLA-E. HLA-E presents the leader peptides of other MHC class I molecules and thereby presents a range of MHC class I-derived ligands [60].

1.4.3 Other inhibitory receptors

The third group of MHC class I binding inhibitory receptors is formed by the leukocyte Ig-like receptors (LILRs) which are expressed on NK cells and recognize different HLA alleles [61].

Apart from these MHC class I recognizing receptors, NK cells also express a variety of receptors which have non-MHC ligands [54]: For example, the receptor NKR-P1 recognizes CLEC2D [62], LAIR-1 binds collagen [63, 64] and KLRG1 binds cadherins [65]. Even more inhibitory receptors with completely different ligand specificities are expressed enabling NK cells to gauge target cell condition through different means [54].

1.4.4 NK cell education

Receptors of the KIR and CD94/NKG2 family are randomly expressed on NK cells [66]. This phenomenon can lead to the occurrence of NK cells devoid of inhibitory receptors or at least inhibitory receptors able to recognize abundant self-MHC alleles [67]. In contrast, this could also lead to NK cells that have several functional inhibitory self-receptors. The lack of any inhibition together with the potential to recognize activating ligands should lead to autoreactivity of these NK cells against healthy cells. The apprehended autoreactivity of NK cells without inhibitory self-ligands does not occur though. NK cells which are unable to recognize inhibitory

ligands are hyporesponsive [68]. A process of education of maturing NK cells has been proposed, but the exact mechanism still remains unknown: One model states that NK cells may be stimulated by default, but enter the state of hyporesponsiveness without an occasional inhibitory signal [69]. In contrast, another model assumes that NK cells remain hyporesponsive until they are “licensed” by inhibitory signals resulting in responsive NK cells [70, 71]. The view of this education was changed when it was shown that the regulation of NK cells does not occur in a digital “on-off” modulation as an increasing amount of inhibitory self-ligands resulted in NK cells of higher responsiveness [72]. This observation led to the formulation of the Rheostat model, which can be applied to both previous education models [73]. This model states that the responsiveness of NK cells is tuned by a continuum that is defined by the amount of inhibitory input in the NK cells education. Thus, an NK cell without an inhibitory input becomes hyporesponsive whereas an NK cell with strong inhibitory input (e.g. through several KIRs) becomes very responsive. The hyporesponsive NK cells have been shown to be of importance despite their functional deficiency though: In a murine cytomegalovirus model it was shown that the actual immune response was dependent on the hyporesponsive NK cells. These hyporesponsive NK cells can regain function in this situation, which might be due to the completely absent inhibition of these cells [74]. Additionally, recent studies imply that the effect of education on the responsiveness of an NK cells is reversible and can change in a new environment [75]. Further research has to be performed to unravel this important issue.

1.4.5 Siglecs

An interesting group of sialic acid-binding lectin receptors is also present on NK cells [76]: Sialic acids are nine-carbon sugars derivatized from neuraminic acid via *N*- or *O*-substitution and are commonly attached to the non-reducing end of extracellular glycoconjugates, e.g. gangliosides [77, 78]. These sialosides have different functional roles and are involved in physiological, pathophysiological and pathological mechanisms. For example, the influenza virus uses sialic acids to bind to target cells via hemagglutinin (HA) and induces production of sialidase after infection in order to render the cell resistant to other viral particles [79]. The lectin family of proteins has evolved to recognize glycosylations and consists of several subgroups: The C-type lectins (e.g. selectins), the S-type lectins (Galectins) and the I-type (or, as members of the Ig-superfamily, Ig-like) lectins. The best-studied group of I-type lectins is classified as the sialic acid-binding Ig-like lectins (Siglecs) family and consists of 16 known members in humans [80]. These receptors are again

subgrouped into the conserved group 1 found in all mammals and the rapidly evolving CD33-related Siglecs, which show immense differences between species [80-82]. With two exceptions (Siglec-4 and Siglec-6), all Siglecs are expressed in the hematopoietic system and play an important role in the regulation of immune cells [83]. The Siglec receptors share features in structure: All receptors are type I transmembrane proteins and contain an amino-terminal V-set Ig-domain and a variable number of C2-set Ig-domains followed by a transmembrane domain [83]. The cytoplasmic part of the proteins differs in the conserved group 1 of Siglecs, but most of the CD33-related Siglecs harbor an ITIM proximal to the transmembrane domain followed by an ITIM-like motif [83]. These motifs are able to recruit protein tyrosine phosphatases such as SHP-1 and SHP-2 [83] and show inhibitory effects on the function and proliferation of cells, if the receptors are triggered [84, 85]. In contrast to other sialic acid-binding lectins, the ligand recognition of Siglecs by the V-set Ig-domain is not only based on the negative charge of the ligand, but it was shown that the ligand interacts through several specific interactions with the receptor [82, 86]. One crucial interaction common to Siglecs is the formation of a salt bridge between an arginine in the receptor and the carboxylate of the sialic acid [86, 87]. Though the ligand specificity is higher than in other I-type lectins, the binding affinity of Siglecs towards their ligands is low and binding relies on avidity [83]. The CD33-related Siglecs mainly differ in their V-set Ig-domain and thus in their specificity towards differently linked sialic acids. Usually, Siglec receptors are masked by *cis* interactions with sialic acids, but can be unmasked upon sialidase treatment or by cell activation [88].

On NK cells, the expression of three members of the Siglec family was shown, all belonging to the CD33-related group: Siglec-3 (or CD33) [89], Siglec-7 (CD328) [87, 90, 91] and Siglec-9 (CD329) [92-94]. CD33 contains only one C2-set Ig-domain and is partially expressed on NK cells as an isoform without V-set Ig-domain, which thus does not possess sialic acid-binding capacity [89]. Siglec-7 and Siglec-9 both contain a V-set Ig-domain and two C2-set Ig-domains. They are similar in structure in over 80%, but differ in their ligand specificity [92-95]. In addition, Siglec-7 is the major Siglec receptor abundant on NK cells, whereas it is also present on a subset of CD8+ T cells, to a lower extent on monocytes and at low levels on granulocytes [91]. Sialic acid specificities of these Siglecs vary as shown in Figure 2 [83]. Whereas Siglec-7 has an unusually high affinity for α2,8-disialyl residues and binds well to branched α2,6-sialyl residues, Siglec-9 (and Siglec-3 to a lesser extent) binds well to terminal α2,3- and α2,6-sialyl residues [83, 95, 96].

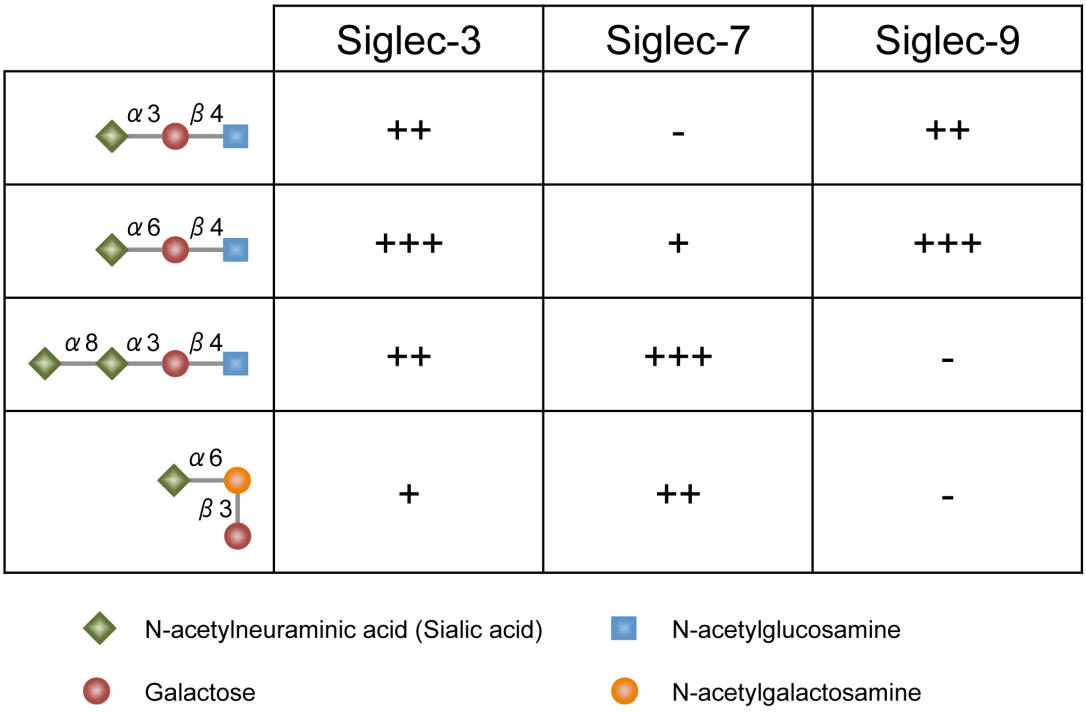


Figure 2: Specificities of NK cell expressed Siglec receptors. Specificities for the most common sialic acid residues and their linkages are displayed for the three NK cell Siglec receptors. The degree of specificity ranges from (-) no specificity, (+) low specificity, (++) moderate specificity and (+++) high specificity. Specificity data were adopted from studies employing glycoprobes to screen Siglec specificities [95, 96].

The best-studied Siglec expressed on NK cells is Siglec-7, which was investigated in structure [97-101] and function [102] in detail; however, much remains to be unraveled still. The α2,8-disialyl residues recognized by Siglec-7 are especially abundant on a group of gangliosides as GD3, GD1b and GT1b, also called the “b-series” gangliosides [78, 103]. Functional implications for ganglioside expression have been shown in several studies: For example, the expression of the ganglioside GD3 on P815 cells modulated the lysis mediated by NK cells [104] and the DSGb5 ganglioside (branched α2,6-disialic) on renal cell carcinoma cells also decreased the NK cell-mediated lysis [105]. The ‘b-series’ gangliosides are present in neuronal tissues [78] where such inhibitory interactions with Siglec receptors could prevent autoreactivity [106]. In addition, GD3 has been shown to be expressed strongly in melanomas [107], which might imply an immunoevasion mechanism applied by cancer cells. But Siglec-7 has also been shown to be involved in viral and bacterial control: HIV infection was shown to be associated with the appearance of Siglec-7-deficient NK cell subpopulations which can be used as progression markers of the disease [108, 109]. In addition, bacteria have been shown to acquire Siglec ligands in order to modify the host-pathogen interaction [110, 111].

1.5 Activating NK cell receptors

All NK cells express a range of different activating receptors granting them the ability to screen target cells for their respective ligands. Those ligands can either be upregulated upon stress or infection or be expressed physiologically by cells. Although several receptors are used by human and murine NK cells alike, even with identical ligands, other receptors are unique to individual species. This is a major reason why it is not possible to draw accurate conclusions from murine NK cell experiments for humans (and vice versa). The receptors can roughly be divided into three groups of receptor types with some exceptions based on their signaling properties [57].

1.5.1 ITAM-signaling receptors

The first group comprises receptors that signal through the interaction with signaling partner chains as the FcεRIγ chain, the CD3ζ chain or Dap12, which contain an immunoreceptor tyrosine-based activation motif (ITAM) to transduce a signal into the cell. Via a positively charged amino acid (a lysine or arginine) in the transmembrane region the receptor couples with a signal chain containing a negatively charged amino acid (commonly aspartate) in the transmembrane region [56]. These receptors include the natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 and the Fc receptor CD16. NKp46 and NKp30 are present on all human NK cells whereas NKp44 is only expressed on NK cells upon activation [112-114]. All of these receptors have been shown to bind viral ligands [115-117], but their cellular ligands remain less well understood. Only for the receptor NKp30 two cellular ligands have been postulated: the nuclear protein BAT3 and B7-H6 [118, 119]. Engagement of these receptors results in the phosphorylation of the ITAM of the signaling chain by an Src-family kinase. Phosphorylated ITAMs are then recognized and bound by the tyrosine kinases ‘spleen tyrosine kinase’ (SYK) or ’70 kDa zeta-chain associated protein’ (ZAP70), which then initiate a signaling cascade through adaptor proteins like ‘linker for activation of T cells’ (LAT), phosphatidyl-inositol-3-kinase (PI3K), phospholipase C γ (PLC) and Vav. This pathway is more or less identical for all ITAM-signaling receptors, including activating KIR receptors, e.g. as KIR2DS1, and activating members of the natural killer group 2 receptor family, as CD94/NKG2C [57].

1.5.2 SLAM-related receptors

The second group of activating receptors contains the signaling lymphocyte activation molecule (SLAM) -related receptors (SRRs) [120]. These type I

transmembrane receptors belong to the Ig-superfamily and consist of usually two extracellular Ig-domains: an amino-terminal V-set domain and a C2-set domain [120]. The cytoplasmic part contains several so-called immunoreceptor tyrosine-based switch motifs (ITSMs), which are able to bind different adaptors and can mediate both activation and inhibition, though in human activation dominates [120]. On human NK cells, only three SRRs are expressed: 2B4 (CD244) [10], CD2-like receptor activating cytotoxic cells (CRACC) [121] and NK, T and B cell antigen (NTB-A) [122]. Whereas CRACC and NTB-A are homophilic [120], 2B4 binds the glycosylphosphatidylinositol (GPI)-linked CD48 which is expressed on various hematopoietic cells [123]. All three receptors can mediate cytotoxicity by NK cells, but due to its wide ligand expression 2B4 is the best-studied SRR on NK cells. Upon ligation, 2B4 is recruited into lipid rafts at the immune synapse in an actin-dependent way [124] and the ITSMs become phosphorylated by Src-family kinases [125]. This results in the association with the adaptors 'SLAM-associated protein' (SAP) [126] and 'SH3 domain-binding protein 2' (3BP2) [127]. SAP association causes the recruitment of the Src-family kinase Fyn that mediates a stronger phosphorylation of 2B4 and adaptors. 3BP2 is phosphorylated after 2B4 ligation and recruits Vav-1, LAT and PLC-γ [57, 127]. This combinatorial signaling is crucial for NK cell activation and results in cytotoxicity and cytokine secretion by the NK cell. The presence of SAP and Fyn are essential for proper function of 2B4 as was shown in mice experiments [128]. In addition, defects in SAP result in fatal lymphoproliferative responses against EBV infection, often observed in patients with X-linked lymphoproliferative disease (XLP). In those patients the NK cell activation through 2B4 (and NTB-A) is impaired [129-133]. In addition to SAP, 2B4 and NTB-A also associate with the adaptor protein EAT-2 whereas CRACC only binds EAT-2 [120]. Situations in which these receptors mediate inhibitory instead of activating signals have been described [130] and a lack of stimulatory adaptor proteins like SAP was suggested to allow phosphatases to bind phosphorylated ITSMs [134]. Thus the decision whether SRRs act activating or inhibitory might be determined by the ratio of abundant receptor and adaptor [135].

1.5.3 NKG2D

A third group is formed by the type II transmembrane C-type lectin-like receptor NKG2D that, in human, signals via the signaling partner 'DNAX-activating protein of 10 kDa' or Dap10. In mouse, NKG2D can additionally associate with Dap12. Unlike other members of the NKG2 group that associate with CD94, NKG2D is expressed as a disulfide-linked homodimer associated with two disulfide-linked homodimers of Dap10 on NK cells and subsets of T cells [136, 137]. NKG2D relies on Dap10 for its

surface expression and signaling capacity, as the receptor itself only has a short cytoplasmic tail without signaling motifs [138]. As with other signaling chains, Dap10 associates via a negatively charged aspartate in its transmembrane domain with a positively charged arginine in the transmembrane domain of NKG2D [138]. NKG2D recognizes a broad range of ligands such as the MHC class-I polypeptide-related chain A (MICA) and B (MICB), and the group of cytomegalovirus UL16 binding proteins (ULBPs) [139]. These ligands have been shown to be induced upon stress, infection and transformation [140] and thus grant NKG2D a major role as co-activating receptor in immune responses in those circumstances [141]. Signaling of NKG2D is mediated by a special tyrosine-based signaling motif (YINM) in the cytoplasmic domain of Dap10. This motif can be activated by phosphorylation through Src-family kinases and is bound by both PI3K and Grb2 that mediate downstream signaling [142, 143]. NKG2D has also been shown to be recruited into lipid rafts at the IS upon ligation where the presence of Src-family kinases and other signaling adaptors assist signal transduction [144].

1.5.4 CD16

An important NK cell receptor by its own right is CD16, the low affinity FcγRIIIA receptor, which plays a special role in NK cell activation [9]: CD16 recognizes bound IgG antibodies on the surface of cells and therefore enables NK cells to recognize effector proteins of the adaptive immune system on target cells, eventually resulting in killing of these target cells. In addition, CD16 is the only receptor on resting NK cells that results in full effector mechanisms upon ligation without co-activation by other receptors [47, 48]. CD16 associates with homo- or heterodimers of the FcεRIγ chain and/or the CD3ζ chain and signals similar to other ITAM-associated receptors [9].

1.5.5 Integrins

Another essential group of co-activating NK cell receptors is formed by the integrins on NK cells. NK cells express several members of this family: The $ß_1$ integrin VLA-4 is expressed together with $ß_2$ integrins like lymphocyte function-associated antigen 1 (LFA-1). The main function of these receptors is to mediate adhesion with other cells by the ligation with their corresponding ligands, VCAM-1 (for VLA-4) and ICAM-1-5 (for LFA-1), and to assist in the proper formation of an immunological synapse [43, 45]. This is also achieved by their linkage through talin to the actin cytoskeleton [145]. Apart from this function, it has been shown that LFA-1 ligation is sufficient to induce the polarization of cytotoxic granules towards the immunological synapse and

therefore induces a crucial co-activating signal [47]. Integrins are able to signal ‘inside-out’ as well as ‘outside-in’ and are regulated by a complex signaling system [57].

1.5.6 Other activating receptors

Another activating receptor involved in adhesion is CD2: It is a member of the Ig-superfamily and binds CD58 as well as the 2B4 ligand CD48, although with a lower affinity [146]. It has been shown that CD2 enhances the adhesion of an NK cell with its target cells and is found together with LFA-1 in the pSMAC of an NK cell immune synapse [43, 45].

Further activating receptors on NK cells include the receptor DNAX accessory molecule 1 (DNAM-1). This receptor has been shown to be involved in the recognition of transformed cells as it recognizes the poliovirus receptor (CD155) and nectin-2 (CD112); both of these ligands may be upregulated on transformed cells [147, 148]. Other activating receptors include the less well-studied receptors CD160 which binds to MHC class I ligands [149] and the C-type lectin-like receptors NKp80, which binds activation induced C-type lectin (AICL) found on monocytes and macrophages [38, 150], and NKp65 which binds keratinocyte-associated C-type lectin (KACL) [151].

1.6 Lipid rafts

The main purpose originally described for the plasma membrane was the establishment of the cell as a separate unit from the surrounding environment [152]. Based on this function, the plasma membrane was regarded for a long time as a homogenous layer of lipids interspersed with proteins. This changed after the initial observations of phase separation in model membranes [153, 154].

These observations in model systems led to the formulation of a cholesterol-dependent lateral segregation of phases rich in cholesterol, sphingolipids and saturated lipids in the membrane. This segregation is based on different physical interactions between the lipids in the membrane: The rigid planar structure of cholesterol promotes interactions with adjacent straight and stiff hydrocarbon chains especially formed by saturated lipids; in contrast, the interaction with more kinked unsaturated hydrocarbon chains is disfavored [155]. The structure of cholesterol also drives adjacent hydrocarbon chains into their straight conformation, which results in a more ordered and thick membrane patch [156]. Lipids that cannot adopt a straight conformation, e.g. due to unsaturation, are displaced from the vicinity of cholesterol.

In addition, sphingolipids comprise several features that strengthen this association: Commonly, sphingolipids contain long saturated hydrocarbon chains. These favor association with cholesterol, but also enable the connection of the outer membrane leaflet with the inner leaflet via interdigitation [157]. In contrast to glycerophospholipids more commonly found outside these patches, sphingolipids also can accept as well as donate hydrogen bonds, which strengthen association with other sphingolipids and cholesterol as well [155].

In model systems, the associative properties of these lipid species can be observed in the segregation into two phases: an ordered lipid phase (L_o) comprising cholesterol, sphingolipids and long saturated glycerophospholipids and a disordered lipid phase (L_d) comprising mostly unsaturated phospholipids [158].

Based on these observations in model membranes, the possibility of membrane heterogeneity in living cells was discussed and their existence and composition was analyzed. Early studies on this topic made use of the resistance of such cholesterol-rich membrane patches to detergent extraction in order to isolate them from cellular membranes. In these assays, the disordered phases are dissolved and separated from the detergent-resistant ordered phases by density centrifugation. The low density of the cholesterol-rich membrane patches coined the term ‘lipid rafts’, which float atop of the soluble fractions. Nowadays the more correct term ‘detergent-resistant membranes’ (DRMs) is used for these isolated membrane fractions, which can be analyzed for their composition with various methods after isolation [159, 160].

Though these studies give information about possible lipid raft association of membrane components, they have the disadvantage that artifacts can arise and they do not resemble the membrane of living cells [161]. At the time these studies were performed lipid rafts could not be visualized, which is due to their nanoscale size [162], and this lack of direct proof of the existence of lipid rafts caused a large-scale debate whether obtained data were based on artifacts or biased [161].

Up to now, a broad range of proteins has been shown to be located in lipid rafts or at least be associated with them [162]. However, the physical interactions between lipids seem insufficient to explain membrane heterogeneity in cells: the introduction of lipid raft-associated proteins in the already mentioned model membranes resulted in the localization of these proteins in the disordered phase rather than the ordered phase [163, 164]. Therefore, another factor has to be involved in membrane heterogeneity. This might well be the presence of proteins itself as well as the interaction of proteins with lipids. Indeed, studies have shown that the cellular membrane is full of membrane-associated proteins [165] and several modifications of proteins have been identified so far, which mediate or favor lipid raft localization:

GPI-anchors have been shown to mediate lipid raft association as shown by the constitutive DRM association of GPI-linked proteins [166]. Moreover, the modification of proteins with the saturated 16-carbon fatty acid palmitate has been shown to increase lipid raft affinity. This was found for example in certain Src-family kinases like Fyn which are enriched in lipid rafts [167]. However, palmitoylation alone does not seem to be sufficient for lipid raft recruitment as there are several palmitoylated proteins absent from lipid rafts.
The association of proteins with certain types of lipids has also been shown [168]; dependent on the lipid this association can increase lipid raft affinity of the protein as well. Furthermore, the direct binding of cholesterol has been shown for certain proteins like caveolin-1 [169] and sphingolipids-binding sites have been described [170]; two additional types of interaction to increase the affinity for lipid rafts of proteins. The length of transmembrane proteins by itself has also been shown to influence localization of a membrane protein [171, 172]. Additionally, the interaction of non-raft membrane proteins with raft-associated membrane proteins might increase lipid raft association as well.

In the last years, more and more data supporting the existence of lipid rafts emerged [162]; these also include the visualization of membrane patches on cellular membranes [163, 173]. Therefor, the heterogeneity of cellular membranes and the ability to form lipid rafts has been supported repeatedly. The current model of lipid rafts describes the presence of small nanosized dynamic assemblies of lipid raft components in a resting state in the membrane. These nanosized rafts can be activated to coalesce to larger rafts or even membrane macrodomains upon multimerization. This can be induced by ligand binding of associated receptors, or by the action of the underlying cytoskeleton, which has been shown to be crucial for lipid raft assembly [174]. These coalesced lipid rafts are supposed to be more heterogeneous in their composition and more suited for the corresponding function. A simplified illustration of resting state rafts and clustered rafts is presented in Figure 3.

So far, lipid rafts have been implicated in several cellular functions like membrane sorting throughout the Golgi, signaling and endocytosis as well as more specific functions in specialized cells [162, 175]. Whereas the sorting function predominantly relies on the segregation of the membrane domains and subsequent divergent destinations [175], the signaling function is based on the association of several signaling components as receptors or kinases with lipid rafts. So far, protein tyrosine

kinases, adaptor proteins, different G proteins as well as phosphoinositides have been associated with lipid rafts [159, 167, 176]. In the coalesced lipid rafts, all these signaling components come together and probably are protected from the action of excluded phosphatases, effectively forming a potent signaling platform [159].

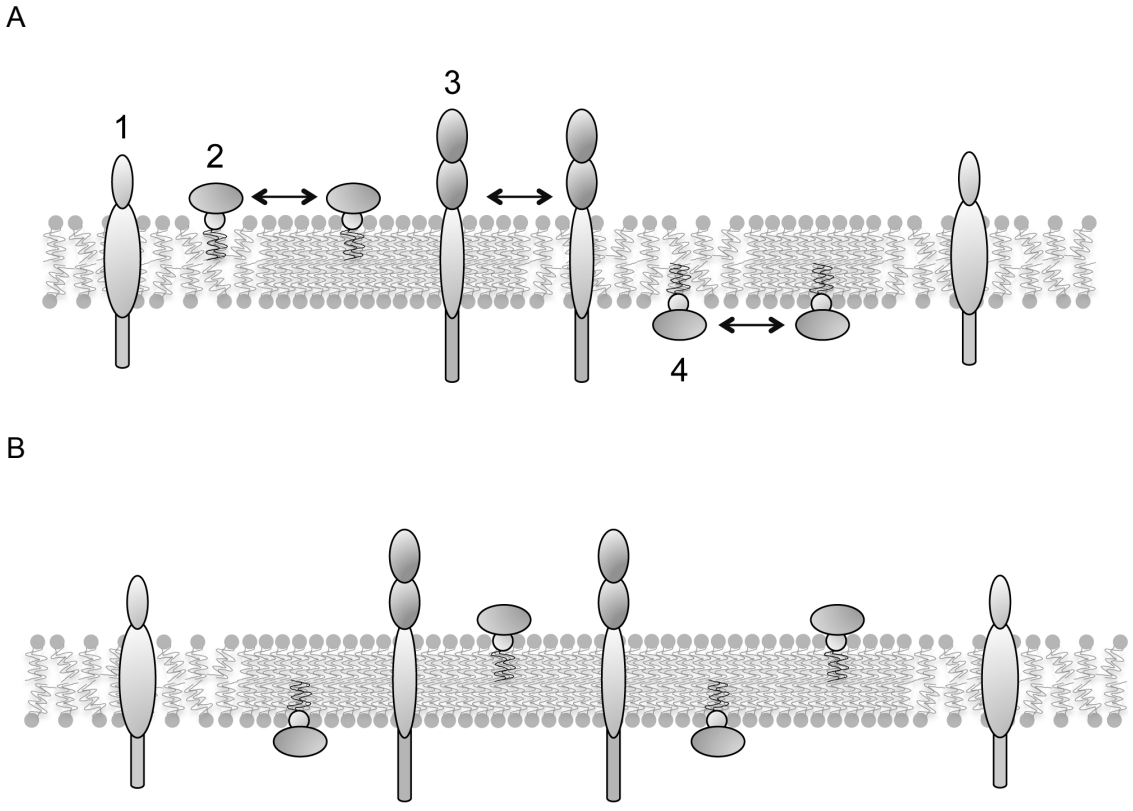


Figure 3: Simplified illustration of lipid raft organization [175]. (A) depicts small raft nanodomains in resting state. Non-raft proteins (1) reside in the disordered membrane, while diverse raft-associated proteins like GPI-anchored proteins (2), raft-associated transmembrane proteins (3) and palmitoylated proteins (4) are dynamically present in the nanodomains or the non-raft membrane. Residence in nanodomains might increase depending on a protein and its modifications. Upon ligation of nanodomain-resident receptors, the coalescence of small nanodomains can lead to larger domains finally resulting in a macrodomain or clustered lipid raft depicted in (B). This process is usually cytoskeleton-dependent but by means of simplicity it was omitted in the illustration. Raft-associated receptors are resident in the lipid rafts and are thus localized in a favoring environment to fulfill their function, e.g. signal transduction, while non-raft proteins as phosphatases are excluded. Dense membrane represents ordered cholesterol- and sphingolipids-rich membrane, whereas the unordered membrane represents membrane poor in cholesterol or sphingolipids, but rich in unsaturated glycerophospholipids.

1.6.1 Lipid rafts in NK cells

Upon the formation of an activating NKIS, lipid rafts are clustered at the contact site of NK cell and target cell [49]. Initial signals are required to mediate this clustering, as the inhibition of proximal protein tyrosine kinases abolishes the lipid raft clustering [49]. In addition, the inhibition of the cytoskeleton was also shown to abolish the lipid

raft clustering at the synapse indicating a possible involvement of the cytoskeleton in this process [177]. A similar dependency of lipid rafts on the cytoskeleton was observed in T cells [178].

Moreover, the clustering of lipid rafts is abolished if the NKIS is inhibitory instead of activating [177, 179]. It was shown that the lipid rafts were excluded in an active cytoskeleton-dependent mechanism from the inhibitory IS. Inhibitory receptors are excluded from lipid rafts but are able to signal independently from the cytoskeleton in order to displace the lipid raft-signaling platform from the NKIS. This was mediated through the activity of SHP-1, which is recruited to the IS by signaling inhibitory receptors [177, 179]. The involvement of lipid rafts during signal transduction in NK cells was also investigated in more detail for different NK cell receptors: For example, different activating NK cell receptors were associated with lipid rafts, either constitutively or upon ligation [124, 144, 180-182]: The activating NK cell receptor 2B4 was shown to be localized into DRMs upon ligation and this was dependent on intact cytoskeleton and Src-family kinases. The phosphorylation of 2B4, which is required for 2B4-mediated signal transduction, is not sufficient to induce raft recruitment. Instead it is more likely that 2B4 is recruited into lipid rafts in an actin-dependent way and phosphorylated there [124]. The receptor NKG2D was also shown to be localized into lipid rafts after receptor ligation or sensitive target cell contact [144]. These receptors are displaced from the lipid rafts in a resting state or by inhibition through inhibitory receptors, but accumulate in the lipid rafts in case of activation. Although the knowledge about the mechanism underlying the displacement of lipid rafts is increasing, the molecular mechanisms underlying the lipid raft recruitment of receptors upon stimulation are unknown.

Different studies also showed that the ability of NK cells to lyse sensitive target cells was decreased if the lipid rafts were disturbed, e.g. by methyl-ß-cyclodextrin (MßCD)-mediated depletion of cholesterol [49, 124]. Thus lipid rafts play an essential role in the function of NK cells by merging the ligated activating receptors with essential signaling components at the aNKIS.

1.7 Aim of the thesis

The research of the lipid raft theory is a growing field. It has been established that the cellular membrane is not just a homogenous barrier separating the cell from its surroundings. Instead, by applying heterogeneity in the membrane, a cell can orchestrate different functions. More and more components of these lipid rafts are identified; also several activating NK cell receptors have been shown to associate with this membrane microdomains upon ligation. However, the mechanisms underlying the recruitment of these receptors into the lipid rafts remain unknown. The present thesis therefore focused on the molecular mechanism involved in the recruitment of NK cell receptors: The SLAM-related receptor 2B4 and the C-type lectin-like receptor NKG2D were selected as representative NK cell surface receptors which are recruited into lipid rafts upon stimulation. These receptors were studied with detergent extraction methods for their lipid raft recruitment in order to identify crucial criteria for the recruitment mechanisms in the structure of the receptors.

The second part of the thesis deals with the inhibitory NK cell receptor Siglec-7 that recognizes sialic acid ligands and dampens NK cell activity. A range of studies presented evidence for a potentially harmful exploitation of this receptor by tumors and different pathogens. In order to provide means to break this exploitation and obtain a new therapeutic and scientific tool, the aim was to develop a component capable to inhibit the Siglec-7 ligand interaction. We therefore developed and applied a functional Siglec-7 binding assay to screen a range of substances based on the structure of sialic acid and tested promising candidates in functional NK cell assays.

2 Materials and Methods

2.1 Materials

2.1.1 Vectors

Name	Use	Source
pCR2.1-TOPO	Subcloning of PCR products	Invitrogen, Carlsbad, CA
pBABEplus	Mammalian expression vector	Addgene
pMaxCloning	Mammalian expression vector	Addgene
pMOW	Mammalian expression vector	Klingenmüller Lab
pMOWplus	Mammalian expression vector	Replaced pMOW-MCS with MCS from pBABEplus
pMXs-neo	Mammalian expression vector	Addgene

2.1.2 Constructs

Name	Tag	Size (bp)	Vector	Resistance	Source
2B4wt	HA	1267	pBABEplus	Puromycin	Eissmann [134]
2B4-CA	HA	1267	pBABEplus	Puromycin	J. Endt
2B4-D0	HA	1267	pBABEplus	Puromycin	Eissmann [134]
2B4-YF	HA	1267	pBABEplus	Puromycin	Eissmann [134]
2B4-LAT-TM	HA	1267	pBABEplus	Puromycin	Eissmann [134]
2B4wt	HA	1267	pMOWplus	Puromycin	This thesis
2B4-CA	HA	1267	pMOWplus	Puromycin	This thesis
2B4-D0	HA	1267	pMOWplus	Puromycin	This thesis
2B4-YF	HA	1267	pMOWplus	Puromycin	This thesis
2B4-LAT	HA	1267	pMOWplus	Puromycin	This thesis
2B4-FLVIIVIL-A	HA	1267	pMOWplus	Puromycin	This thesis

2B4-SALFLGT-A	HA	1267	pMOWplus	Puromycin	This thesis
2B4-LACFCV-A	HA	1267	pMOWplus	Puromycin	This thesis
2B4-FLV-A	HA	1267	pMOWplus	Puromycin	This thesis
2B4-IIV-A	HA	1267	pMOWplus	Puromycin	This thesis
2B4-ILS-A	HA	1267	pMOWplus	Puromycin	This thesis
DAP10wt	FLAG		pMxs-neo	G418	M. Sandusky
Dap10-YF	FLAG		pMxs-neo	G418	M. Sandusky
NKG2Dwt	-	767	pBABEplus	Puromycin	M. Sandusky
NKG2DΔcyt	-	767	pBABEplus	Puromycin	M. Sandusky
NKG2D-RA	-	767	pBABEplus	Puromycin	M. Sandusky
NKG2Dwt	-	767	pMOWplus	Puromycin	This thesis
NKG2DΔcyt	-	767	pMOWplus	Puromycin	This thesis
NKG2D-RA	-	767	pMOWplus	Puromycin	This thesis

2.1.3 Bacteria

E. coli strain	Use	Source
TOP10	Amplification of plasmids	Invitrogen, Carlsbad, CA

2.1.4 Cells

If not indicated otherwise, cells were grown with 10 % (v/v) FBS and 1 % (v/v) Penicillin/Streptomycin in addition to the below given culture media constituents.

Cell type	Origin	Culture medium
BA/F3	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol
BA/F3 2B4wt	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin

BA/F3 2B4-CA	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-D0	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-FLV-A	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-FLVIIVIL-A	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-IIV-A	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-ILS-A	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-LACFCV-A	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-LAT	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-SALFLGT-A	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 2B4-YF	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 µg/ml Puromycin
BA/F3 NKG2Dwt DAP10wt	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 mM G418, 1 µg/ml Puromycin
BA/F3 NKG2Dwt Dap10-YF	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 mM G418, 1 µg/ml Puromycin
BA/F3 NKG2DΔcyt DAP10wt	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 mM G418, 1 µg/ml Puromycin
BA/F3 NKG2DΔcyt Dap10-YF	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 mM G418, 1 µg/ml Puromycin
BA/F3 NKG2D-RA	Murine pro B cell line	RPMI, 0.5 mM 2-mercaptoethanol, 1 mM G418

Human peripheral blood mononuclear cells (PBMC)	Isolated from whole blood or buffy coats	
K562	Human erythroleukemic cell line	IMDM
Mel1106	Human melanoma cell line	RPMI
Primary human NK cells	Isolated by negative selection from PBMCs	IMDM, 10 % (v/v) human serum, 1 % (v/v) NEAA, 1 % (v/v) sodium pyruvate, 100 IU/ml IL-2
Phoenix ampho	Adenovirus-transformed 293T cell line	DMEM

2.1.5 Reagents

Name	Source
5-Bromo-4-chloro-3-indoxyl-ß-D-galactoside (X-Gal)	Roth, Karlsruhe, Germany
Agarose	Gibco, Invitrogen, Carlsbad, CA
Ampicillin	Roth, Karlsruhe, Germany
Bovine serum albumin (BSA)	Serva, Heidelberg, Germany
Cell culture media	Gibco, Invitrogen, Carlsbad, CA
Chromium-51 (^{51}Cr)	Hartmann Analytik, Braunschweig, Germany
100 bp DNA ladder	Invitrogen, Carlsbad, CA
1 kb DNA ladder	Invitrogen, Carlsbad, CA
Fetal bovine serum (FBS)	Gibco, Invitrogen, Carlsbad, CA
Kanamycin	Roth, Karlsruhe, Germany
LB-Broth	Invitrogen, Carlsbad, CA

Lipofectamine® 2000	Invitrogen, Carlsbad, CA
Lymphocyte separation medium (LSM)	PAA, Pasching, Germany
MOPS buffer, 20x	Invitrogen, Carlsbad, CA
Non essential Amino acids (NEAA)	Gibco, Invitrogen, Carlsbad, CA
Nonfat dry milk powder	Saliter, Obergünzburg, Germany
NuPAGE® 10% Bis-Tris Gels	Invitrogen, Carlsbad, CA
Opti-MEM®	Gibco, Invitrogen, Carlsbad, CA
Penicillin/Streptomycin	Gibco, Invitrogen, Carlsbad, CA
Phytohemagglutinin (PHA-P)	Sigma-Aldrich, St. Louis, MO
Polybrene	Sigma-Aldrich, St. Louis, MO
Polyvinylidene fluoride (PVDF) membrane	Millipore, Billerica, MA
Precision Plus Protein Standard	Bio-Rad, Hercules, CA
Protein G agarose	Invitrogen, Carlsbad, CA
Protein G dynabeads	Invitrogen, Carlsbad, CA
Puromycin	Sigma-Aldrich, St. Louis, MO
Sodium Pyruvate	Gibco, Invitrogen, Carlsbad, CA
SuperSignal West Dura	Thermo Fisher Scientific, Waltham, MA
TAE buffer, 10x	Invitrogen, Carlsbad, CA
TrypLE™ Express	Invitrogen, Carlsbad, CA
X-ray films	Pierce, Thermo Fisher Scientific, Waltham, MA

2.1.6 Inhibitors

All Siglec-7 inhibitors were produced in and obtained from the lab of Prof. Dr. Brossmer (Biochemistry Center, Heidelberg University, Heidelberg, Germany).

2.1.7 Buffers

Name	Components, Source	
DNA sample buffer	50 % (v/v)	Glycerol
	0.25 % (w/v)	Bromophenol blue
	10 mM	EDTA
FACS buffer	1x	PBS
	2 % (v/v)	FBS
Igepal lysis buffer	10 % (v/v)	TNE buffer, 10x
	10 % (v/v)	Igepal stock solution
	1 mM	PMSF
	1 mM	Vanadate
Igepal stock solution	10 % (v/v)	Igepal CA-630
MOPS buffer, 20x	Invitrogen, Carlsbad, CA	
PBS, 10x	1.37 M	NaCl
(pH 7.4)	81 mM	Na_2HPO_4
	27 mM	KCl
	15 mM	KH_2PO_4
PBS, 1x	Gibco, Invitrogen, Carlsbad, CA	
PBS-T	10 % (v/v)	PBS, 10x
	0.05 % (v/v)	Tween 20
PBS-T/NaCl	10 % (v/v)	PBS, 10x
	0.05 % (v/v)	Tween 20
	0.5 M	NaCl
RBC wash buffer	1x	PBS
	0.1 % (w/v)	BSA
	2 mM	EDTA
Reducing sample buffer, 5x	50 % (v/v)	Glycerol
(pH 6.8)	25 % (v/v)	2-mercaptoethanol
	10 % (w/v)	SDS
	0.1 % (w/v)	Bromophenol blue
	0.3125 mM	Tris-HCl

Sucrose stock solution, 85 %	85 % (w/v)	Sucrose
	10 % (v/v)	TNE buffer, 10x
	1 % (v/v)	Triton X-100 stock solution
TAE buffer, 10x	Invitrogen, Carlsbad, CA	
TNE buffer, 10x	1.5 M	NaCl
(pH 7.4)	50 mM	EDTA
	200 mM	Tris-HCl
Triton X-100 lysis buffer	10 % (v/v)	TNE buffer, 10x
	3 % (v/v)	Triton X-100 stock solution
	1 mM	PMSF
	1 mM	Vanadate
Triton X-100 stock solution	10 % (v/v)	Triton X-100
Western blot blocking buffer	1x	PBS-T
	5 % (w/v)	Nonfat dry milk powder
Western blot transfer buffer	129 mM	Glycine
	24 mM	Tris
	20 % (v/v)	Methanol

2.1.8 Mouse monoclonal antibodies

Name	Conjugate	Clone	Source, Reference
α-2B4	-	C1.7	Immunotech, Beckman Coulter, Brea, CA
α-2B4	PE	C1.7	BioLegend, San Diego, CA
α-CD45	-	69/CD45	Transduction Laboratories, BD, Franklin Lakes, NJ
α-FLAG	-	M2	Sigma-Aldrich, St. Louis, MO
α-Fyn	-	25	Transduction Laboratories, BD, Franklin Lakes, NJ
α-HA	-	2C16	MBL, Biozol, Eching, Germany
α-NKG2D	-	149810	R&D Systems, Minneapolis, MN

α-NKG2D	-	3.1.1.1	[144]
α-NKG2D	PE	1D11	BioLegend, San Diego, CA
α-Siglec-7	-	194212	R&D Systems, Minneapolis, MN
α-Siglec-7	PE	F023-420	Pharmingen, BD, Franklin Lakes, NJ
α-Siglec-9	-	191240	R&D Systems, Minneapolis, MN
α-Siglec-9	PE	191240	R&D Systems, Minneapolis, MN
IgG control	-	MOPC21	Sigma-Aldrich, St. Louis, MO
IgG control	PE	MOPC21	BioLegend, San Diego, CA

2.1.9 Secondary antibodies

Name	Conjugate	Source
Goat α-human IgG	Biotin	Jackson ImmunoResearch, West Grove, PA
Goat α-mouse F(ab')$_2$	PE	Jackson ImmunoResearch, West Grove, PA
Goat α-mouse IgG	HRPO	Jackson ImmunoResearch, West Grove, PA

2.1.10 Recombinant proteins

Name	Conjugate	Source
7-AAD	-	Pharmingen, BD, Franklin Lakes, NJ
Annexin-V	PE	Pharmingen, BD, Franklin Lakes, NJ
Human IL-15	-	R&D Systems, Minneapolis, MN
Human IL-2	-	Hemagen Diagnostics, Columbia, MD
Human Siglec-7-Fc fusion protein	-	R&D Systems, Minneapolis, MN

Human Siglec-9-Fc fusion protein	-	R&D Systems, Minneapolis, MN
Streptavidin	PE	Jackson ImmunoResearch, West Grove, PA

2.1.11 Enzymes

All enzymes were used in buffers provided by the manufacturer.

Name	Use	Source
Calf intestinal alkaline phosphatase (CIAP)	Dephosphorylation of DNA	New England Biolabs, Ipswich, MA
Neuraminidase (Arthrobacter ureafaciens α2-3,6,8,9-Neuraminidase)	Desialylation of cells	Calbiochem, Merck, Darmstadt, Germany
Restriction endonucleases	Cutting of DNA	New England Biolabs, Ipswich, MA
T4 DNA ligase	Ligation of DNA	New England Biolabs, Ipswich, MA
Taq DNA polymerase	PCR	New England Biolabs, Ipswich, MA

2.1.12 Oligonucleotides

All oligonucleotides were synthesized by Thermo Fisher Scientific (Waltham, MA).

Name	Use	Sequence (5'-3')
2B4-FLV-A fwd	Cloning of 2B4-FLV-A	AAT TCA GAT TTT GGC CGG CAG CAG CAA TCA TCG TGA TTC TAA GCG CAC TGT TCC TTG GCA CCC TTG CCT GCT TCT GTG TGT GGA GGA GAA AGA GGC

2B4-FLV-A rev	Cloning of 2B4-FLV-A	TCG AGC CTC TTT CTC CTC CAC ACA CAG AAG CAG GCA AGG GTG CCA AGG AAC AGT GCG CTT AGA ATC ACG ATG ATT GCT GCT GCC GGC CAA AAT CTG
2B4-FLVIIVIL-A fwd	Cloning of 2B4-FLVIIVIL-A	AAT TCA GAT TTT GGC CGG CAG CAG CAG CAG CAG CAG CAG CAA GCG CAC TGT TCC TTG GCA CCC TTG CCT GCT TCT GTG TGT GGA GGA GAA AGA GGC
2B4-FLVIIVIL-A rev	Cloning of 2B4-FLVIIVIL-A	TCG AGC CTC TTT CTC CTC CAC ACA CAG AAG CAG GCA AGG GTG CCA AGG AAC AGT GCG CTT GCT GCT GCT GCT GCT GCT GCT GCC GGC CAA AAT CTG
2B4-IIV-A fwd	Cloning of 2B4-IIV-A	AAT TCA GAT TTT GGC CGT TTT TGG TGG CAG CAG CAA TTC TAA GCG CAC TGT TCC TTG GCA CCC TTG CCT GCT TCT GTG TGT GGA GGA GAA AGA GGC
2B4-IIV-A rev	Cloning of 2B4-IIV-A	TCG AGC CTC TTT CTC CTC CAC ACA CAG AAG CAG GCA AGG GTG CCA AGG AAC AGT GCG CTT AGA ATT GCT GCT GCC ACC AAA AAC GGC CAA AAT CTG
2B4-ILS-A fwd	Cloning of 2B4-ILS-A	AAT TCA GAT TTT GGC CGT TTT TGG TGA TCA TCG TGG CAG CAG CAG CAC TGT TCC TTG GCA CCC TTG CCT GCT TCT GTG TGT GGA GGA GAA AGA GGC
2B4-ILS-A rev	Cloning of 2B4-ILS-A	TCG AGC CTC TTT CTC CTC CAC ACA CAG AAG CAG GCA AGG GTG CCA AGG AAC AGT GCT GCT GCT GCC ACG ATG ATC ACC AAA AAC GGC CAA AAT CTG
2B4-LACFCV-A fwd	Cloning of 2B4-LACFCV-A	AAT TCA GAT TTT GGC CGT TTT TGG TGA TCA TCG TGA TTC TAA GCG CAC TGT TCC TTG GCA CCG CAG CAG CAG CAG CAG CAT GGA GGA GAA AGA GGC

2B4-LACFCV-A rev	Cloning of 2B4-LACFCV-A	TCG AGC CTC TTT CTC CTC CAT GCT GCT GCT GCT GCT GCG GTG CCA AGG AAC AGT GCG CTT AGA ATC ACG ATG ATC ACC AAA AAC GGC CAA AAT CTG
2B4-SALFLGT-A fwd	Cloning of 2B4-SALFLGT-A	AAT TCA GAT TTT GGC CGT TTT TGG TGA TCA TCG TGA TTC TAG CAG CAG CAG CAG CAG CAG CAC TTG CCT GCT TCT GTG TGT GGA GGA GAA AGA GGC
2B4-SALFLGT-A rev	Cloning of 2B4-SALFLGT-A	TCG AGC CTC TTT CTC CTC CAC ACA CAG AAG CAG GCA AGT GCT GCT GCT GCT GCT GCT GCT AGA ATC ACG ATG ATC ACC AAA AAC GGC CAA AAT CTG
2B4 Seq fwd	Sequencing	CTC ATC AAG GCA GCT CAG C
2B4 Seq rev	Sequencing	CAA TCG AGC AGG GTT CTG G
pMOW Seq fwd	Sequencing	GAT CCT CCC TTT ATC CAG C
pMOW Seq rev	Sequencing	GCA ACA TAG TTA AGA ATA CC

2.1.13 Kits

Name	Use	Source
First strand cDNA synthesis kit	RT-PCR	Roche Diagnostics, Basel, Switzerland
Gel extraction kit	DNA fragment purification	Qiagen, Hilden, Germany
NK cell negative isolation kit	Isolation of human NK cells	Invitrogen, Carlsbad, CA
Plasmid Midi kit	Plasmid DNA purification	Qiagen, Hilden, Germany
Plasmid Mini kit	Plasmid DNA purification	Qiagen, Hilden, Germany
RNeasy Mini kit	RNA isolation	Qiagen, Hilden, Germany

2.2 Methods

2.2.1 Molecular biology

Isolation of RNA

RNA was isolated from BA/F3 cells using the RNeasy Mini kit according to the instructions of the manufacturer.

Reverse Transcription of RNA

The First strand cDNA synthesis kit was applied according to the manufacturers instructions in order to generate cDNA from RNA.

Polymerase chain reaction (PCR)

DNA sequences were amplified from cDNA via PCR. For each respective task, different conditions and primers were applied.

Ligation of DNA fragments

Insert and vector DNA were mixed at an approximate ratio of 5:1 in the corresponding ligation buffer. Prior to incubation for 1 h at room temperature or 16 h at 16°C, 2 units of T4 DNA ligase were added to the mixture. The ligation product was then used for transformation of competent TOP10 bacteria.

Transformation of bacteria

Competent TOP10 bacteria were used for transformation according to the manufacturers instructions. Transformed bacteria were grown on LB agar plates at 37°C for 24h with the appropriate selection medium.

Plasmid DNA isolation

TOP10 bacteria were grown in 1x LB medium overnight at 37°C stirring. Appropriate selective media were added to the medium prior. After overnight growth, bacteria were harvested by centrifugation at 3’500 x g for 5 min. Subsequently, plasmid DNA was isolated using the plasmid Mini or Midi kit according to the manufacturers instructions.

DNA restriction digest

For DNA digests, between 1 and 2 µg of DNA were incubated with 2 units of the respective DNA restriction endonucleases for at least 1 h at 37°C. The reactions were performed in the reaction buffers recommended by the manufacturer.

Agarose gel electrophoresis

DNA fragments were separated using 1 – 2 % (w/v) agarose gels prepared in TAE buffer and 0.0001 % (v/v) ethidium bromide. Prior to loading on a gel, the DNA was mixed with DNA loading buffer. For size reference of the DNA fragments, 100 bp and/or 1 kb DNA ladders were run in the gels.

Isolation of DNA fragments from agarose gels

DNA fragments were excised from the gel and processed using the gel extraction kit according to the manufacturers instructions.

2.2.2 Cell biology

Cell Culture

All cells were grown at 37°C and 5 % CO_2 in a humidified incubator under sterile conditions. Cell lines were split regularly either by medium exchange (suspension cells) or trypsinization (adherent cells) every 2 – 3 days and cell culture flasks were replaced regularly. Cryostocks of cells were prepared by freezing the cells in 10 % (v/v) DMSO in FBS at 75°C. For long-term storage, cryostocks were put to liquid nitrogen. Cell lines were thawed regularly or prior to a batch of experiments. FBS and human serum were heat inactivated by incubation for 30 min at 56°C prior to use.

Red blood cell (RBC) isolation

RBCs were obtained from whole blood or buffy coats: 2 – 3 ml blood were washed with 10 ml PBS and centrifuged at 3’000 x g for 10 min at room temperature. 2 ml of the pelleted RBCs were harvested and then washed twice with 13 ml of RBC wash buffer and centrifuged at 2’000 x g for 10 min at room temperature. With the final pellet of RBCs a 0.1 % (v/v) RBC solution in RBC wash buffer was prepared and stored at 4°C for further experiments.

NK cell isolation

Human polyclonal NK cells were isolated from whole blood or buffy coats. First, PBMCs were separated from the remaining cells by a density gradient centrifugation of the blood over lymphocyte separation medium (LSM). The PBMCs were harvested from the gradients and washed. NK cells were isolated from the PBMCs by the use of a NK cell negative isolation kit. NK cell yield ($NKp46^+$, $CD3^-$, $CD56^+$ cells) ranged from 90 – 99 % of the purified cells. Purified cells were maintained in primary NK cell medium at a concentration of 2 x 10^6 cells/ml.

NK cell stimulation

NK cell were stimulated with 100 IU/ml hIL-2 24 h prior to use in experiments.

Transduction and transfection of BA/F3

2 x 10^6 Phoenix ampho cells were seeded in a 25 cm^2 culture flask in the corresponding medium and incubated overnight at 37°C and 5 % CO_2. Phoenix cells were then transfected with 10 µg of pMOWplus or pMXs-neo vector using Lipofectamine® 2000 and Opti-MEM® according to the manufacturers instructions. Phoenix cells were incubated for another 24 h at 37°C with 5 % CO_2. The next day, the medium was replaced with BA/F3 medium and Phoenix cells were incubated for another 24 h. The supernatant then was harvested and used for the transduction of BA/F3 cells. 5 x 10^5 BA/F3 cells were resuspended in 2 ml supernatant containing retroviral particles together with 5 µg/ml polybrene. Cells were then centrifuged with 1'350 x g for 90 min at room temperature in order to bring cells and viral particles together. After centrifugation, the cells were incubated at 37°C with 5 % CO_2 overnight. The next day, the medium was replaced with fresh BA/F3 medium. 24 h after medium exchange, the vector-specific selection medium was added to the culture and further on used in culture of the transduced cells.

BA/F3 stimulation

In order to stimulate the receptors on BA/F3 cells for lipid raft assays, the receptors were cross-linked with antibodies: 1 x 10^8 cells were resuspended in 1 ml cold PBS and incubated for 10 min on ice with 10 µg/ml of primary murine antibody. Cells were then washed with 500 µl cold PBS, pelleted by centrifugation at 480 x *g* for 5 min at 4°C and resuspended in 1 ml cold PBS with 10 µg/ml goat-α-mouse secondary antibody. Negative controls were incubated for 10 min on ice, whereas positive controls and samples were stimulated for 10 min at 37°C. Cells were then washed with 500 µl cold PBS and pelleted by centrifugation as done before.

BA/F3 cell lysis

For lipid raft assays, 1 x 10^8 stimulated BA/F3 were resuspended in 1 ml 0.3 % Triton X-100 lysis buffer and incubated for 30 min on ice. Lysates were then homogenized with 10 strokes of a glass dounce homogenizer (loose-fit).

Flow cytometry

Surface staining of cells was performed in 96-well V-bottom plates. 1 x 10^5 cells were resuspended in 50 µl fluorescence-activated cell sorting (FACS) buffer with 10 µg/ml of the corresponding unlabeled primary antibody or a concentration recommended by the manufacturer in case of labeled primary antibodies. In case of unlabeled primary antibodies, the cells were incubated for 20 min on ice, then washed with 150 µl FACS buffer and pelleted by centrifugation at 480 x g for 5 min at 4°C. These cells were then resuspended in 50 µl FACS buffer with 0.5 % fluorophore-labeled secondary antibody. Cells in presence of fluorophore-labeled antibodies (both primary and secondary) were incubated again for 20 min at 4°C but in exclusion of light in order to protect the light-sensitive fluorophores. Cells were then washed again with 50 µl FACS buffer, pelleted by centrifugation at 480 x g for 5 min at 4°C and then resuspended in 150 µl FACS buffer containing 2 % formaldehyde for fixation. Cells were then analyzed using a BD FACScalibur device and results were evaluated and presented with the FlowJo software from Treestar. All histograms were presented as % of Max.

FACS-based Siglec inhibition assay

To assess the inhibitory potential of the Siglec inhibitors, Siglec fusion proteins were incubated with the inhibitors and subsequently tested for their ability to bind sialic acids on RBCs: 50 µl of a 0.2 µg/ml Siglec-Fc fusion protein solution in PBS was incubated with various concentrations of the corresponding siglec inhibitors for 10 min at room temperature to allow inhibitor binding. Meanwhile, 50 µl of the previously prepared 0.1 % RBC solution were pelleted by centrifugation at 2'000 x *g* for 5 min at 4°C. After the incubation, the RBCs were resuspended in the inhibitor/Siglec-Fc mixture and incubated for 20 min on ice to allow residual binding of the Siglec-Fc fusion protein to the sialic acids on the RBCs. RBCs were then washed with 150 µl FACS buffer and centrifuged at 2'000 x *g* for 5 min at 4°C. For detection of Siglec binding, the RBCs were resuspended in 50 µl FACS buffer with 10 µg/ml biotin-conjugated goat-α-human antibody and incubated for 20 min on ice. The RBCs were then washed and pelleted as before, resuspended in 50 µl FACS buffer with 1:200 Streptavidin-PE and incubated for 20 min on ice in exclusion of light due to the

fluorophore. Finally, the RBCs were again washed and pelleted, then resuspended in 150 µl FACS buffer with 2 % formaldehyde and analyzed on a BD FACScalibur device. Data were evaluated using the FlowJo software. Percent inhibition of Siglec binding by inhibitors was calculated from the mean fluorescence intensity (MFI) values with the following equation:

$$\%\ inhibition\ =\ 100 - \frac{(MFI\ stained\ RBCs\ with\ inhibitor - MFI\ unstained\ RBCs)}{MFI\ stained\ RBCs} x\ 100$$

Structures of inhibitors were illustrated with the ISIS/Draw software from MDL Information systems.

FACS-based viability assay

IL-2 activated human NK cells were assessed for their viability after Siglec inhibitor treatment: 1 x 10^5 human IL-2 activated NK cells were incubated for 24 h at 37°C and 5 % CO_2 with NK cell medium in the presence of Siglec inhibitors at various concentrations. The NK cells were then washed with fresh medium and pelleted by centrifugation at 480 x *g* for 5 min at 4°C. Subsequently, the NK cells were stained with 7-AAD and Annexin-V-PE according to the manufacturers instructions in Annexin-V-binding buffer. NK cells were then analyzed for their viability using a BD FACScalibur device and the FlowJo software.

Chromium release assay

Mel1106 or K562 were used as target cells in chromium release assays. The target cells were grown to mid-log phase, then 5 x 10^5 cells were harvested, resuspended in assay medium (IMDM medium containing 10 % FBS and 1 % Penicillin/Streptomycin) and labeled for 1 h at 37°C with 100 µCi ^{51}Cr (3 MBq). Target cell were then washed twice and resuspended in fresh medium with a concentration of 5 x 10^4 target cells/ml. IL-2-activated human NK cells were used as effector cells in all chromium release assays. Antibody and Siglec inhibitor dilutions were prepared in 96-well U-bottom plates and effector cells were added 15 min prior to target cells in dependence of the final effector to target ratio to the 96-well plate. All combinations were tested in triplicates. 5 x 10^3 target cells were then added to all wells and the plates were incubated for 4 h at 37°C with 5 % CO^2. For maximum ^{51}Cr release, labeled target cells were incubated with 1 % Triton X-100 and for spontaneous ^{51}Cr release labeled target cells were incubated with medium alone. After the 4 h incubation, supernatants were harvested and chromium release was measured in a

γ-counter. The percentage of specific release in the assay was calculated with the following equation:

$$\%\ specific\ release = \frac{(experimental\ release - spontaneous\ release)}{(maximum\ release - spontaneous\ release)}\ x\ 100$$

2.2.3 Protein biochemistry

Lipid raft assay

BA/F3 lysates were mixed 1:1 with cold 85 % sucrose solution, resulting in a 42.5 % sucrose/lysate mixture. With the lysate/sucrose mixture and sucrose solutions of various concentrations, a discontinuous gradient was prepared in an ultracentrifuge tube. The lysate mix was used to overlay 500 µl 85 % sucrose solution. The lysate mix was then overlaid with 6 ml 35 % sucrose lysis buffer and this phase was overlaid with 3.5 ml 5 % sucrose lysis buffer. This gradient was then centrifuged in an ultracentrifuge at 200'000 x *g* for 16 – 20 h at 4°C. After centrifugation, the gradients were harvested and fractionated into 2 ml fractions resulting in 6 fractions from top to bottom: 1 (discarded), 2/3, 4/5, 6/7, 8/9 and 10/11. Detergent-resistant membrane (DRM) fractions were directly processed for immunoprecipitation or SDS-PAGE and then stored at -20°C.

Immunoprecipitation

For the analysis of 2B4 raft recruitment, an immunoprecipitation step was included in order to remove unspecific signals from the lipid raft fractions. To this end, 500 µl of each fraction were mixed with 500 µl Igepal lysis buffer with either 20 µl Protein G agarose or 10 µl Protein G dynabeads together with 1 µg primary antibody. The mixture was then incubated with gentle agitation for 1 h at 4°C. Subsequently, beads were either washed by centrifugation (agarose) or with magnets (dynabeads) for at least 3 times with Igepal lysis buffer. Beads were then further on processed for western blot analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Lipid raft assays fraction samples (untreated or immunoprecipitated) were mixed with reducing sample buffer and boiled at 95°C for 10 min. Samples were then centrifuged for 1 min at 20'000 x *g* and loaded on NuPAGE® 10% Bis-Tris gels. In

addition, Precision Plus Protein Standard was loaded as a size reference. For separation, the gels were run for 75 min at 150 V in MOPS buffer.

Western blot

Following SDS-PAGE, the proteins were transferred unto polyvinylidene difluoride (PVDF) membranes: PVDF membranes were activated with methanol and then placed on the gels. Subsequently, protein transfer was performed for 100 min at 200 mA in western blot transfer buffer. After western blotting, the membranes were blocked for 1 h with gentle agitation in western blot blocking buffer and subsequently washed at least 3 times with PBS-T. Membranes were then incubated overnight at 4°C and with gentle agitation with primary antibody. Membranes were washed at least 3 times with PBS-T/NaCl and then incubated for 1 h with horseradish-peroxidase (HRPO)-conjugated goat-α-mouse secondary antibody at a dilution of 1:5'000-20'000 in western blot blocking buffer with gentle agitation at room temperature. Membranes were again washed at least 3 times with PBS-T and developed using SuperSignal West Dura and X-ray films. Films were scanned using a GS-800 densitometer from Bio-Rad.

3 Results

3.1 Mechanisms of lipid raft recruitment of NK cell receptors

3.1.1 Lipid raft recruitment of 2B4 (CD244)

It has been previously shown, that the human NK cell surface receptor 2B4 (h2B4) is recruited into lipid rafts by a cytoskeleton-dependent mechanism upon receptor cross-linking [124]. The exact nature of this mechanism so far remains unknown. In order to elucidate this mechanism, we generated h2B4 mutants to inactivate certain domains and motifs in 2B4 that might be responsible for the raft recruitment of this receptor. The role of the transmembrane domain was to be analyzed by the exchange of the 2B4 transmembrane domain with the LAT transmembrane domain, as this protein has been reported to be constitutively present in lipid rafts (mutant 2B4-LAT) [183]. This mutant should allow for constitutive lipid raft recruitment of 2B4. In another attempt to achieve impaired raft recruitment, we inactivated all four ITSMs of 2B4 by mutating the phosphotyrosines to phenylalanines (mutant 2B4-YF) in order to block adaptor binding and analyze the influence of these adaptors on the raft recruitment of 2B4. We also deleted the cytoplasmic domain of 2B4 by insertion of a stop codon in order to abolish any influence by this domain (mutant 2B4-D0). These mutants are depicted in Figure 4.

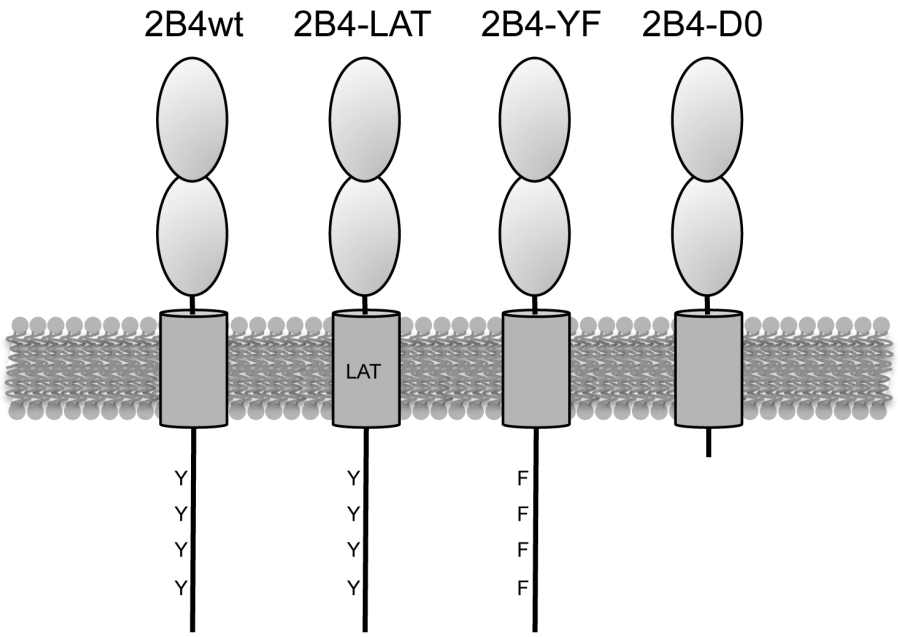


Figure 4: Schematic representation of h2B4 mutations. Constructs include 2B4wt (wildtype), 2B4-LAT (2B4 transmembrane domain replaced with LAT transmembrane domain), 2B4-YF (phosphotyrosines mutated into alanines) and 2B4-D0 (deleted cytoplasmic domain).

These 2B4 mutants were transduced into BA/F3 cells and these BA/F3 cells were sorted for their 2B4 expression levels by flow cytometry, which resulted in BA/F3 cells with a stable expression of all 2B4 mutants on the cell surface (Figure 5A). BA/F3 cells were used to analyze the lipid raft recruitment of NK cell receptors, because this cell line shares the lymphoid lineage of NK cells but is devoid of other NK cell-related receptors that might influence the lipid raft behavior additionally.

The cells were stimulated by antibody cross-linking of 2B4, then lysed and separated for raft (DRM) and non-raft fractions with a lipid raft density sucrose gradient. The fractions were subsequently immunoprecipitated for 2B4 or directly applied to western blot analysis. All constructs were first tested in this way whether a stimulus resulted in increased DRM recruitment in comparison to an unstimulated sample of the same construct (data not shown). Unstimulated and stimulated wildtype 2B4 were used as a reference and control for proper DRM recruitment in each assay. Figure 5 shows representative results for wildtype 2B4 DRM recruitment. In all lipid raft assays, the protein tyrosine phosphatase CD45 serves as a non-raft marker and the palmitoylated Src-family kinase Fyn as a 'lipid raft' marker in order to enable the distinction between those two phases. As shown in Figure 5, wildtype 2B4 is only recruited towards the DRM fraction upon stimulation of the receptor. Due to the variability of these assays and the saturation level of the blots, we neglected quantification and relied on qualitative differences for all experiments.

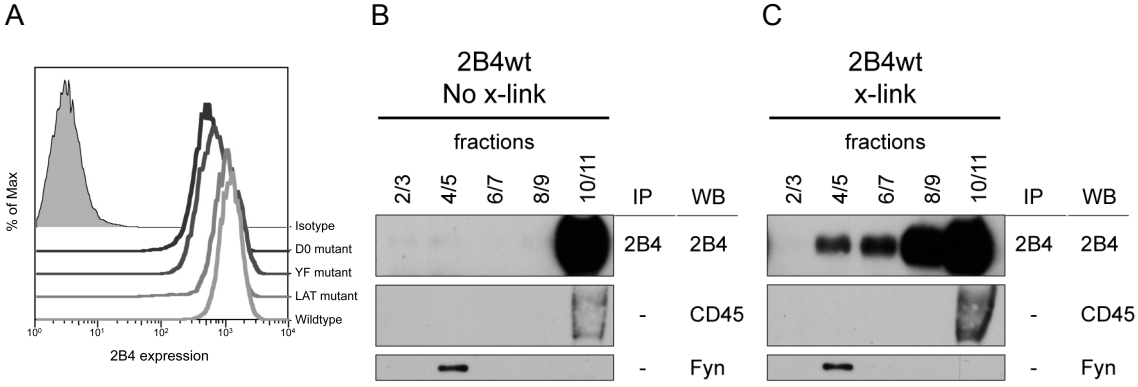


Figure 5: Surface expression of 2B4 constructs on BA/F3 cells and representative Western blots of sucrose density gradient fractions (n=4). (A) BA/F3 cells were stained with a 2B4-specific antibody (bold lines) or with an isotype matched control (shaded). All histograms are presented as % of Max. (B-C) Wildtype unstimulated (B) and stimulated (C) 2B4 constructs were tested for DRM recruitment. Samples were either stimulated by 2B4 crosslinking for 10 min at 37°C (x-link) or left unstimulated with a control antibody at 4°C (no x-link). Cells were then lysed and applied to the sucrose density gradient. Gradients were fractionated, either applied to 2B4-IP first or directly applied to Western blotting. Membranes were analyzed for 2B4 (38 kDa + glycosylation), Fyn (59 kDa) as lipid raft marker and CD45 (147 kDa) as non-raft marker with specific antibodies.

All mutants were then tested with such lipid raft assays; representative results are shown in Figure 6. For practical reasons, control samples are only presented for wildtype 2B4; all 2B4 mutants are completely excluded from DRM fractions if unstimulated (data not shown).

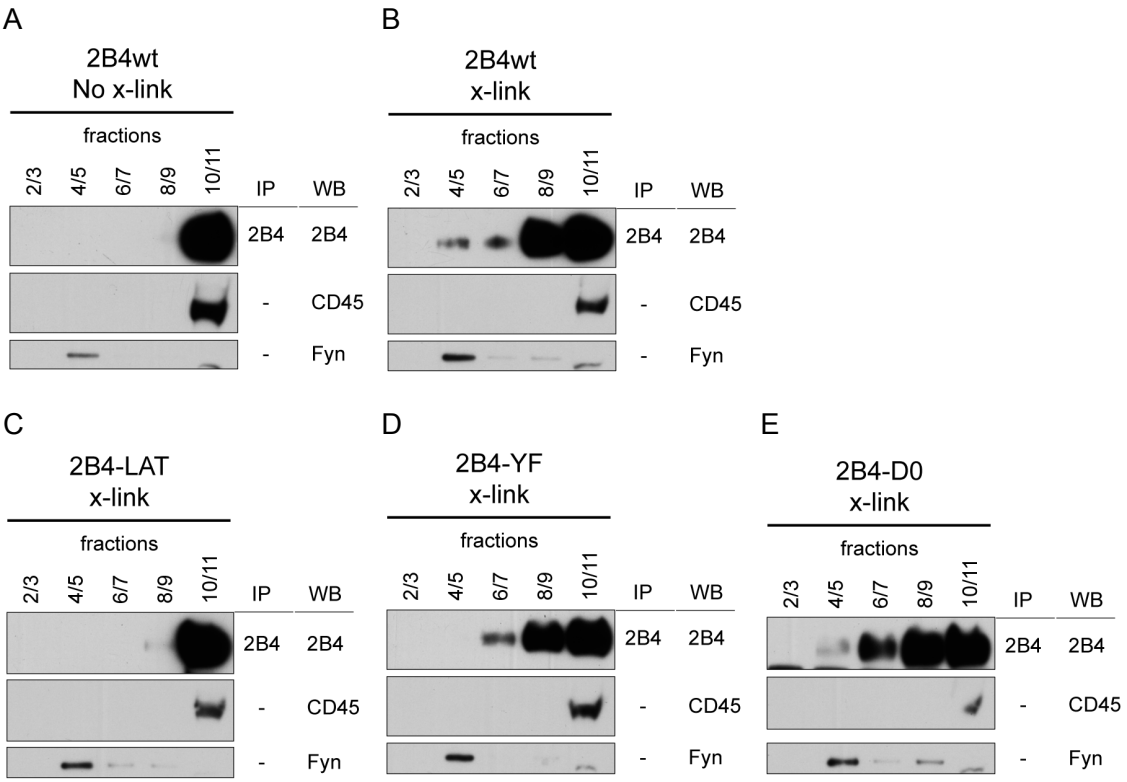


Figure 6: Representative Western blots of sucrose density gradient fractions (n=4). Unstimulated h2B4 wildtype (A) and stimulated wildtype (B), 2B4-LAT (C), 2B4-YF (D) and 2B4-D0 (E) constructs were tested. Samples were either stimulated by 2B4 crosslinking for 10 min at 37°C (x-link) or left unstimulated with a control antibody at 4°C (no x-link). Cells were then lysed and applied to the sucrose density gradient. Gradients were fractionated, either applied to 2B4-IP first or directly applied to Western blotting. Membranes were analyzed for 2B4, Fyn as lipid raft marker and CD45 as non-raft marker with specific antibodies.

Unexpectedly, the mutation replacing the transmembrane domain of 2B4 with the transmembrane domain of LAT (2B4-LAT) did not result in the constitutive recruitment of the receptor into the DRM fraction (Figure 6C). In contrast, the 2B4-LAT receptor was completely excluded from the DRM fraction even under stimulating conditions. Interestingly, the mutation of the ITSMs of 2B4 (2B4-YF) did not cause a major impairment in DRM recruitment (Figure 6D). This was supported by the results of the cytoplasmic deletion mutant (2B4-D0), which did not show any change in DRM recruitment compared to the wildtype controls (Figure 6E).

Besides the replacement of the transmembrane domain of h2B4 with the transmembrane domain of LAT, we replaced the transmembrane domain with a

longer fraction of LAT as well as a mutated transmembrane domain of FcγRIIA described to mediate constitutive lipid raft association [184]. Both of these mutants were constitutively excluded from the DRM fractions (data not shown). Thus, although the exact reason for the 2B4-LAT mutant exclusion from DRM fractions was unknown, it was clear that the transmembrane domain of 2B4 has an important influence on the recruitment into lipid rafts.

To this end, we generated new mutants of h2B4: The transmembrane domain of 2B4 consists of 21 amino acids: FLVIIVILSALFLGTLACFCV (in single letter code). In order to identify the crucial parts within the transmembrane domain, we divided the domain into three parts and produced a substitution mutant with alanines for each part, resulting in the mutants 2B4-FLVIIVIL-A, 2B4-SALFLGT-A and 2B4-LACFCV-A (Figure 7).

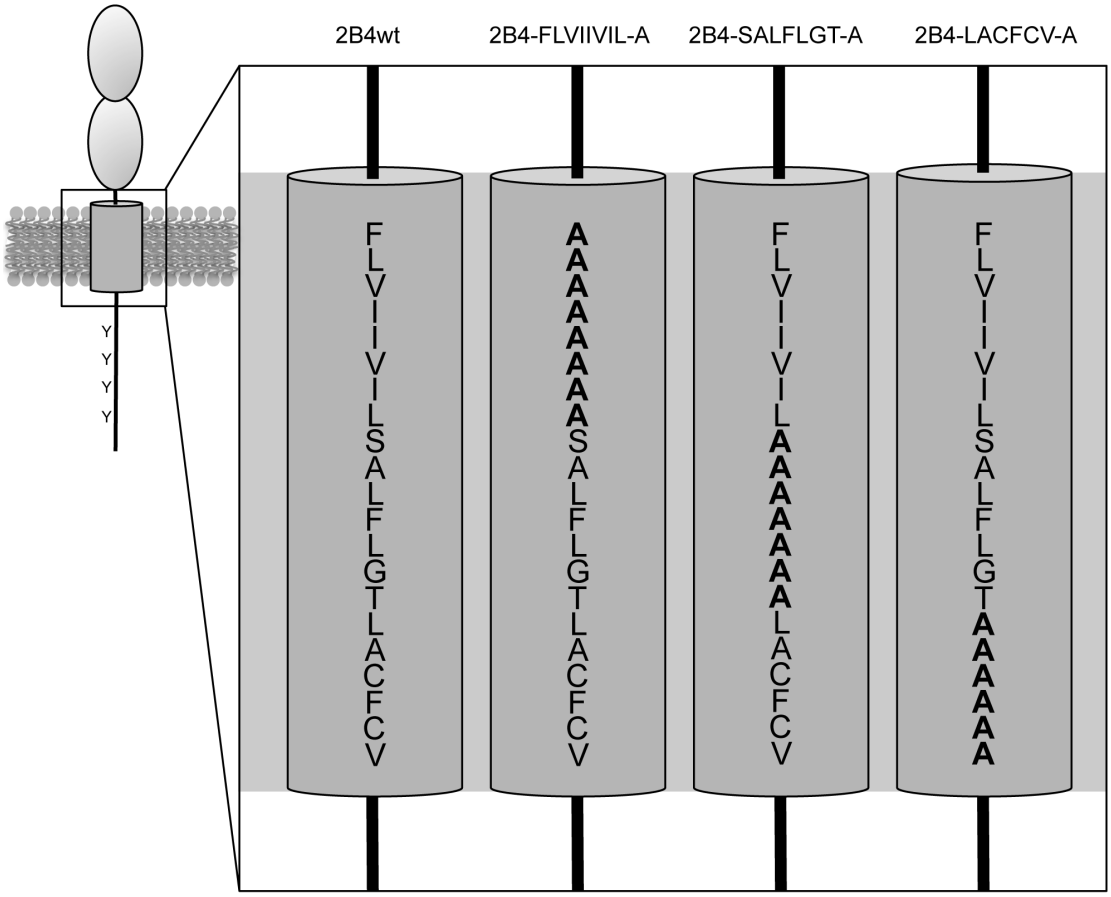


Figure 7: Schematic representation of h2B4 mutations. Constructs include 2B4wt (wildtype), 2B4-FLVIIVIL-A (FLVIIVIL of transmembrane domain mutated to alanines), 2B4- SALFLGT-A (SALFLGT of transmembrane domain mutated to alanines) and 2B4-LACFCV-A (LACFCV of transmembrane domain mutated to alanines).

Again those mutants were transfected into BA/F3 cells and stable clones were sorted by flow cytometry. As can be seen in Figure 8A, the surface expression of these constructs on BA/F3 cells was uniform.

These 2B4 mutants were then tested for lipid raft recruitment with similar sucrose density gradients as done with the previous mutants (Figure 8).

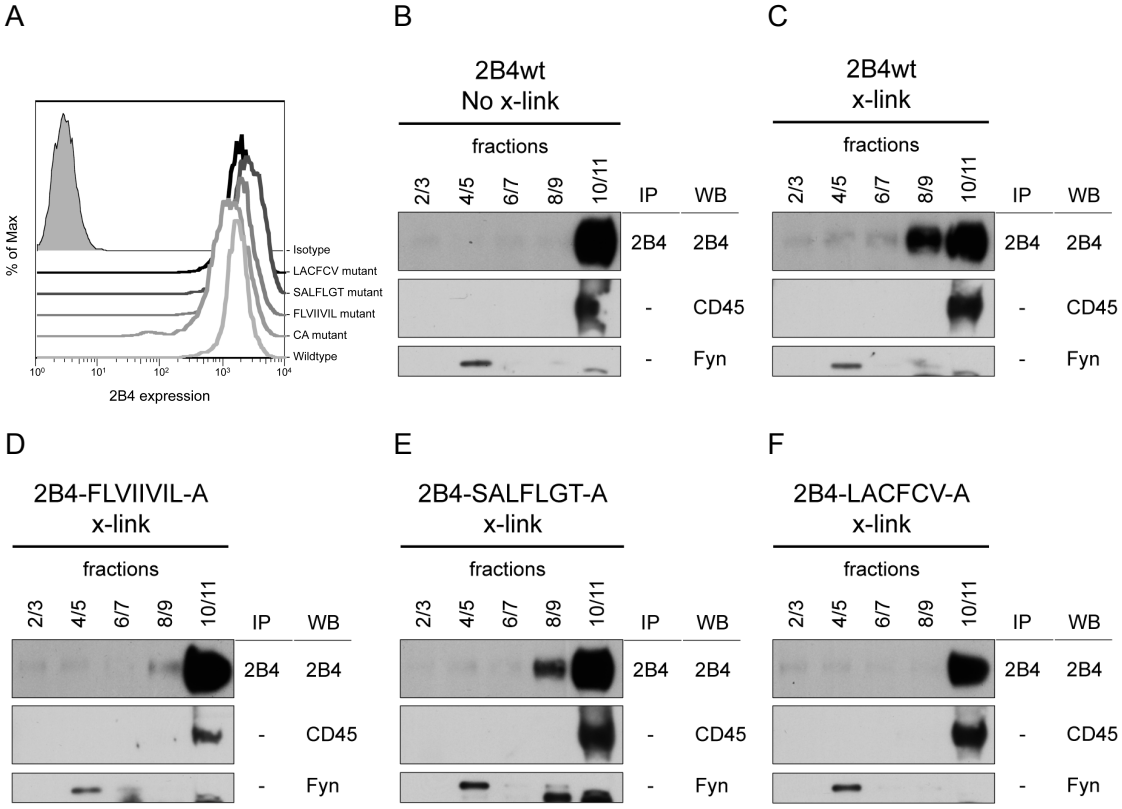


Figure 8: Surface expression of 2B4 constructs on BA/F3 cells and representative Western blots of sucrose density gradient fractions (n=5). (A) BA/F3 cells were stained with a 2B4-specific antibody (bold lines) or with an isotype matched control (shaded). All histograms are presented as % of Max. (B-F) Unstimulated h2B4 wildtype (wt) (B) and stimulated wildtype (C), FLVIIVIL-A- (D), SALFLGT-A- (E) and LACFCV-A- (F) 2B4 constructs were tested. Samples were either stimulated by 2B4 crosslinking for 10 min at 37°C (x-link) or left unstimulated with a control antibody at 4°C (no x-link). Cells were then lysed and applied to the sucrose density gradient. Gradients were fractionated, either applied to 2B4-IP or directly applied to Western blotting. Membranes were analyzed for 2B4, Fyn as lipid raft marker and CD45 as non-raft marker with specific antibodies.

The mutation of the three parts of the 2B4 transmembrane domain had different effects: While the mutation of the FLVIIVIL caused a moderate impairment in DRM recruitment upon stimulation (Figure 8D), the mutation of SALFLGT did not cause a major impairment (Figure 8E). In contrast to the SALFLGT substitution, the LACFCV substitution caused a severe impairment of DRM recruitment (Figure 8F). Overall, the effects were decreasing in strength with the LACFCV the most crucial domain, then followed by FLVIIVIL and finally SALFLGT with a minor role. These results

hinted toward the importance of the LACFCV part of the transmembrane domain for the raft recruitment of 2B4. In fact, the two cysteines in this domain create a ‘CxC’ motif that has been previously reported to influence the behavior of murine 2B4 and the lytic function of murine NK cells [185]. Additionally, this CxC motif is recognized as a positive hit by the palmitoylation prediction software CSS-Palm 3.0 [186].

In order to check for the importance of this CxC motif, we generated a mutant substituting both cysteines in this motif with alanines as shown in Figure 9 (2B4-CA).

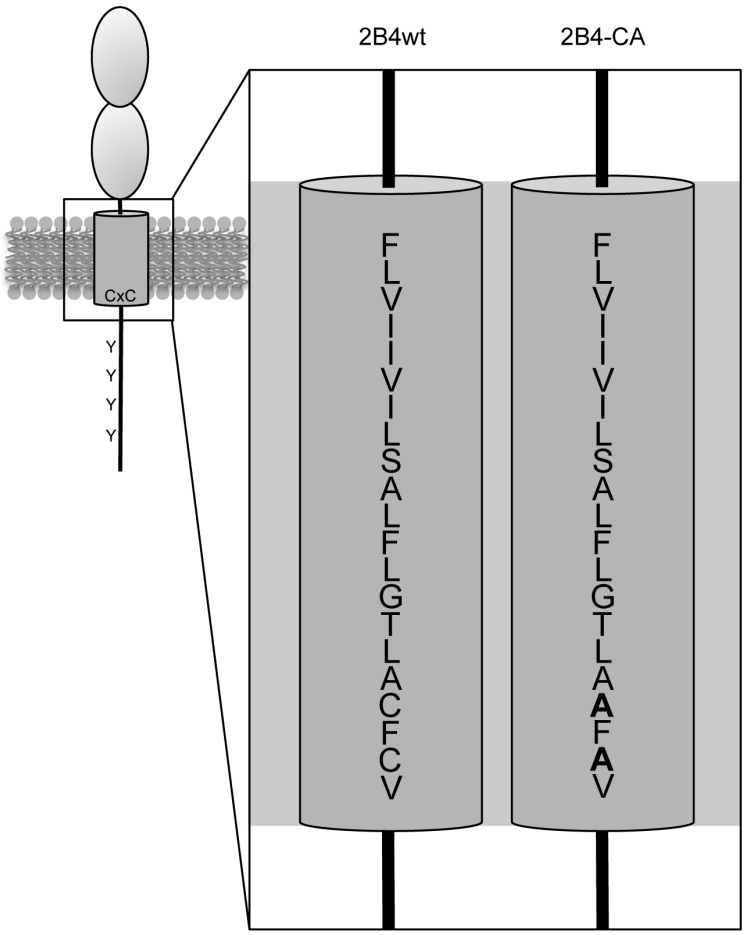


Figure 9: Schematic representation of h2B4 mutations. Constructs are 2B4wt (wildtype) and 2B4-CA (CxC motif in transmembrane domain substituted with AxA).

The surface expression of this mutant was similar to that of the other 2B4 constructs as shown in Figure 8A. In accordance with the assumed importance of the CxC motif, the DRM recruitment of the 2B4-CA mutant upon stimulation was drastically abolished (Figure 10).

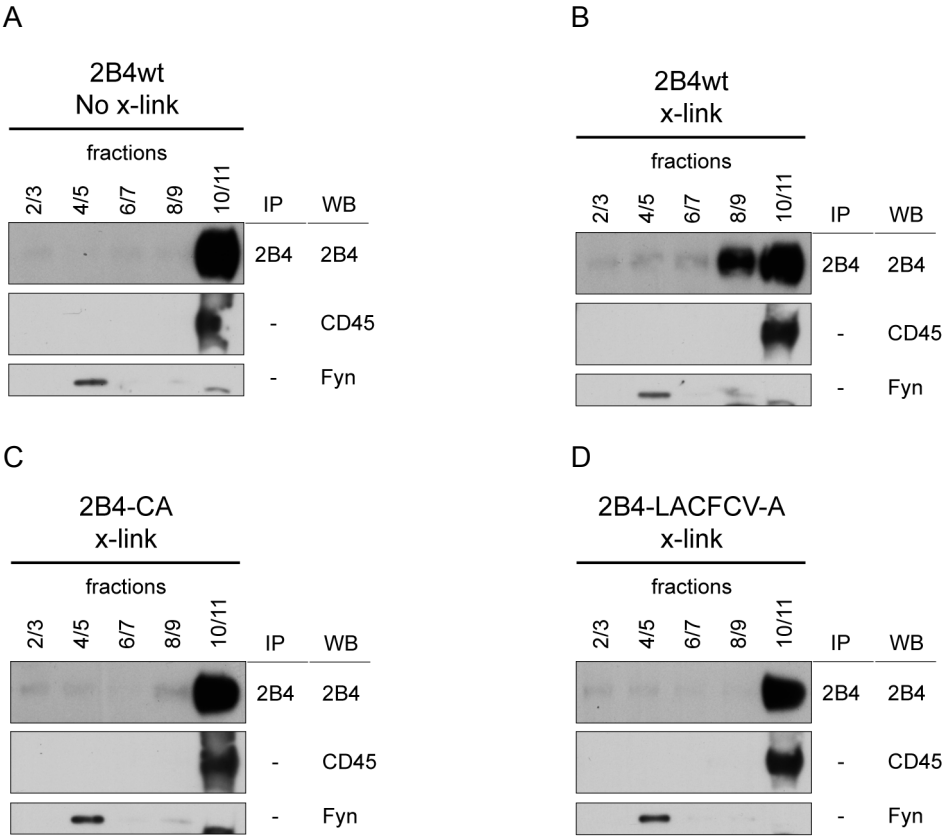


Figure 10: Representative Western blots of sucrose density gradient fractions (n=5). Unstimulated h2B4 wildtype (wt) (A) and stimulated (B), 2B4-CA (C) and 2B4-LACFCV-A (D) constructs were tested. Samples were either stimulated by 2B4 crosslinking for 10 min at 37°C (x-link) or left unstimulated with a control antibody at 4°C (no x-link). Cells were then lysed and applied to the sucrose density gradient. Gradients were fractionated, either applied to 2B4-IP or directly applied to Western blotting. Membranes were analyzed for 2B4, Fyn as lipid raft marker and CD45 as non-raft marker with specific antibodies.

From these data it can be concluded that the CxC motif located in the LACFCV sequence in the transmembrane domain of 2B4 is essential for the lipid raft recruitment of 2B4.

In order to elucidate whether palmitoylation is the underlying mechanism of 2B4 raft recruitment, we applied the palmitoylation inhibitor 2-bromopalmitate to block palmitoylation of wildtype 2B4 in BA/F3 cells. However, the inhibitor did not show any effect on 2B4 nor on our palmitoylation control Fyn (data not shown) and thus did not result in reliable data.

Apart from the effect the LACFCV part and the CxC motif mediate on the raft recruitment, we observed an effect of the FLVIIVIL part as well. Therefore, we constructed three partial mutants of this sequence as well, resulting in the mutants 2B4-FLV-A, 2B4-IIV-A and 2B4-ILS-A, which are depicted in Figure 11.

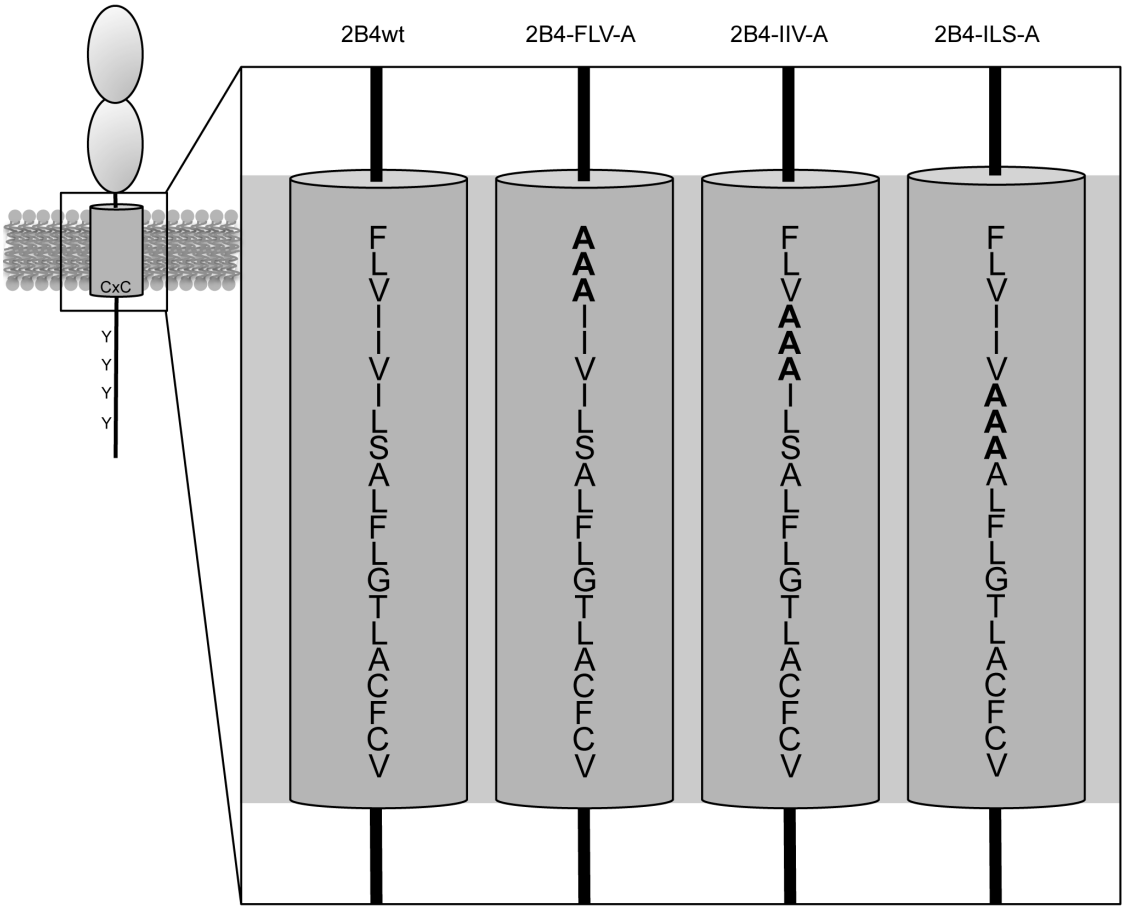


Figure 11: Schematic representation of h2B4 mutations. Constructs include 2B4wt (wildtype), 2B4-FLV-A (FLV of transmembrane domain substituted with alanines), 2B4-IIV-A (IIV of transmembrane domain substituted with alanines) and 2B4-ILS-A (ILS of transmembrane domain substituted with alanines).

These mutants were transduced into BA/F3 cells as described for the previous mutants and BA/F3 2B4 cells were selected for the stable expression of the 2B4 constructs. The 2B4 constructs were expressed to a similar extent by all BA/F3 cells (Figure 12A).

Again, the mutated receptors were assayed for their DRM recruitment with sucrose density gradients, shown in Figure 12.

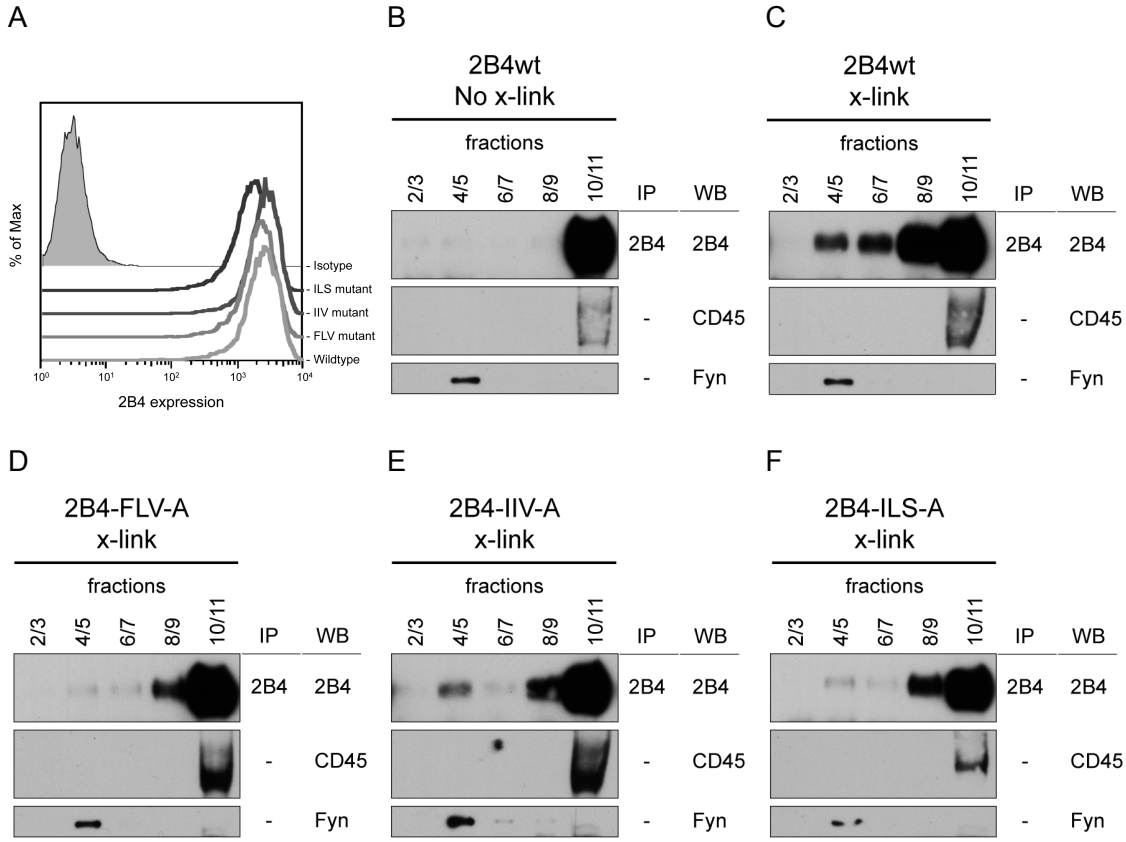


Figure 12: Surface expression of 2B4 constructs on BA/F3 and representative Western blots of sucrose density gradient fractions (n=5). (A) BA/F3 cells were stained with a 2B4-specific antibody (bold lines) or with an isotype matched control (shaded). All histograms are presented as % of Max. (B-F) Unstimulated h2B4 wildtype (B) and stimulated wildtype (C), FLV-A- (D), IIV-A- (E) and ILS-A- (F) 2B4 constructs were tested. Samples were either stimulated by 2B4 crosslinking for 10 min at 37°C (x-link) or left unstimulated with a control antibody at 4°C (no x-link). Cells were then lysed and applied to the sucrose density gradient. Gradients were fractionated, either applied to 2B4-IP first or directly applied to Western blotting. Membranes were analyzed for 2B4, Fyn as lipid raft marker and CD45 as non-raft marker with specific antibodies.

As with the previous mutants of the 2B4 transmembrane domain, the substitutions of different parts of the FLVIIVIL sequence led to differential effects on the DRM recruitment of 2B4: The substitution of IIV caused a moderate impairment in DRM recruitment in all experiments (Figure 12E). In contrast, the effect of both the FLV and ILS substitution caused variant, yet more severe impairments of the 2B4 DRM recruitment than the IIV substitution (Figure 12D, F).

3.1.2 Lipid raft recruitment of NKG2D

We also investigated the structural basis for lipid raft recruitment of the NK cell surface receptor NKG2D. Unlike 2B4, NKG2D surface expression is strongly dependent on the presence of an adaptor protein. On human NK cells this adaptor protein is exclusively Dap10 [138] whereas Dap12 is bound by murine NKG2D as well. In a previous study, Endt *et al.* [144] showed that wildtype human NKG2D was

recruited to lipid rafts upon receptor stimulation. In order to check for the dependency of human NKG2D on its cytoplasmic domain and the interaction with Dap10, we generated three BA/F3 cell lines with different receptor/adaptor combinations depicted in Figure 13. The first NKG2D construct contained a deleted cytoplasmic domain in combination with wildtype Dap10 (NKG2DΔcyt DAP10wt). The second construct contained a NKG2D with deleted cytoplasmic domain in combination with a Dap10 containing an inactivated signaling motif (NKG2DΔcyt Dap10-YF) and the third NKG2D variant was mutated in its transmembrane domain: arginine 66 was substituted with alanine abolishing the interaction of NKG2D and Dap10 (NKG2D-RA). Additionally, Dap10 was not transfected in the BA/F3 NKG2D-RA mutants in order to reduce possible adaptor interactions.

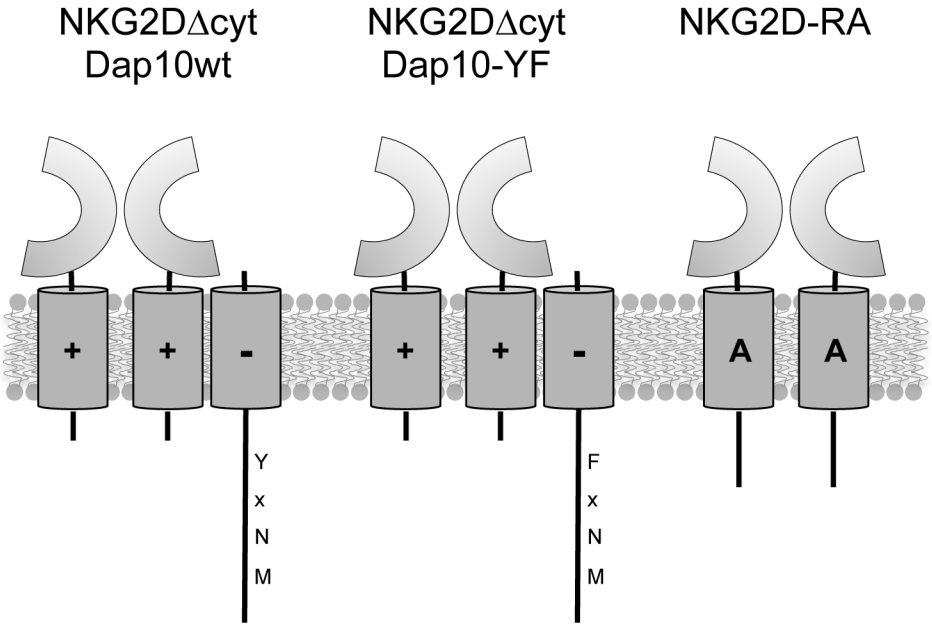


Figure 13: Schematic representation of human NKG2D/Dap10 constructs on BA/F3 cells. Constructs include a NKG2D variant with deleted cytoplasmic domain (NKG2DΔcyt) and a NKG2D variant with an arginine to alanine substitution in its transmembrane domain, which is crucial for NKG2D-Dap10 interaction (NKG2D-RA). In addition, Dap10 constructs include a wildtype variant (DAP10wt) and a Dap10 variant with an inactivating tyrosine to phenylalanine substitution in the signaling motif (Dap10-YF).

Both NKG2DΔcyt constructs were highly expressed on transfected BA/F3 cells (Figure 14A). In contrast, the expression of NKG2D-RA on the cell surface of BA/F3 cells was reduced, as was expected by the abolished NKG2D-Dap10 interaction. Nevertheless, we performed sucrose density gradients with these cells and checked for proper DRM recruitment. The results of these assays are shown in Figure 14.

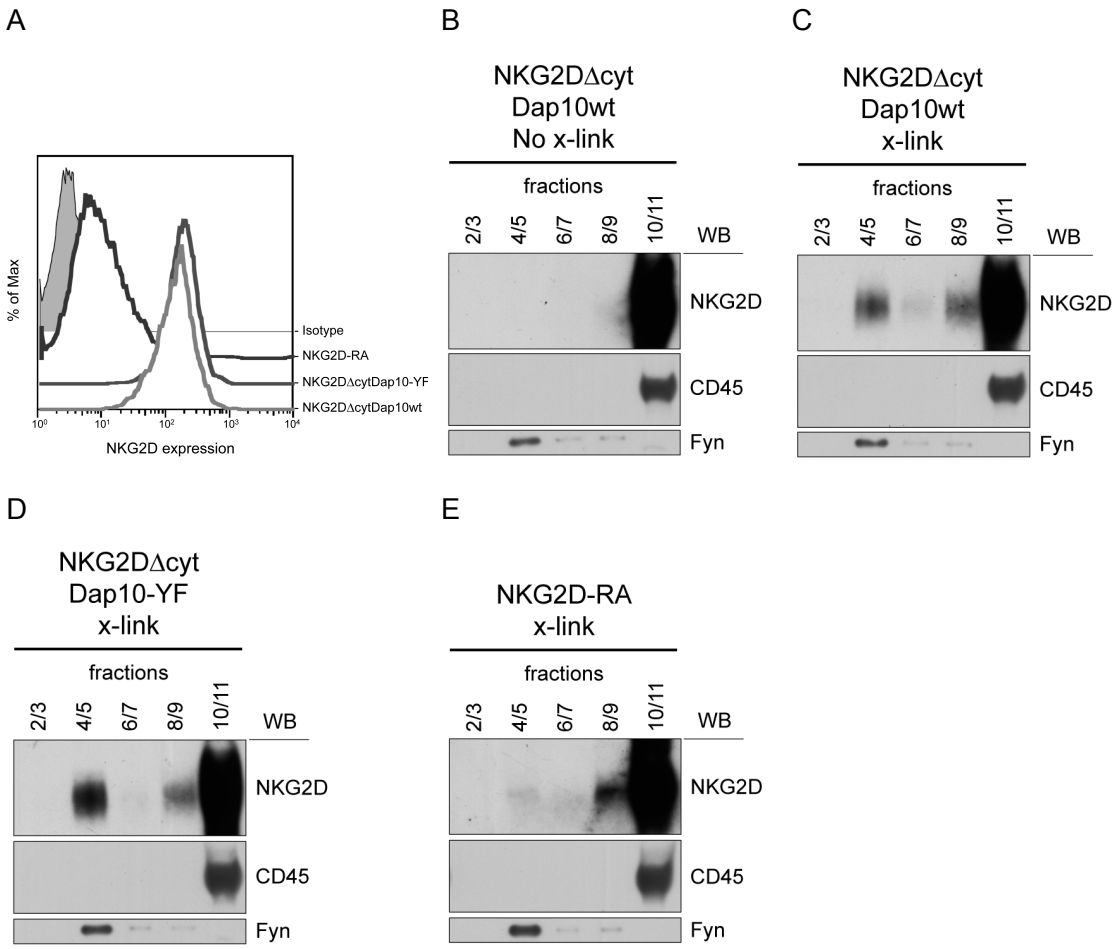


Figure 14: Surface expression of NKG2D constructs on BA/F3 cells and representative Western blots of sucrose density gradient fractions (n=4). (A) BA/F3 cells were stained with an NKG2D-specific antibody (bold lines) or with an isotype matched control (shaded). All histograms are presented as % of Max. (B-E) Unstimulated NKG2D∆cyt DAP10wt (B) and stimulated NKG2D∆cyt DAP10wt (C), NKG2D∆cyt Dap10-YF stimulated (D) and NKG2D-RA stimulated (E) constructs were tested DRM recruitment. Samples were either stimulated by NKG2D crosslinking for 10 min at 37°C (x-link) or left unstimulated with a control antibody at 4°C (no x-link). Cells were then lysed and applied to the sucrose density gradient. Gradients were fractionated and then applied to Western blotting. Membranes were analyzed for NKG2D, Fyn as lipid raft marker and CD45 as non-raft marker with specific antibodies.

As with 2B4, NKG2D is excluded from the DRM fractions if unstimulated, but is recruited into the DRM fractions upon stimulation [144]. These results could be confirmed. The same is true for NKG2D with a deleted cytoplasmic domain, but functional signaling motif in Dap10, which is recruited into the DRMs upon stimulation (Figure 14B, C). Even with a disabled signaling motif in Dap10 in combination with a deleted NKG2D cytoplasmic domain, NKG2D can be recruited into DRMs upon stimulation (Figure 14D).

Thus, neither the cytoplasmic domain of NKG2D nor the signaling functions of Dap10 seem crucial for lipid raft recruitment of NKG2D. In addition, although weak, NKG2D can be detected in the DRM fraction without interaction with Dap10 (Figure 14E),

indicating, that other factors, apart from Dap10 interaction, mediate raft recruitment of NKG2D. The reduced signal might be due to the very low surface expression of NKG2D in case of NKG2D-RA (Figure 14A). Future studies will have to clarify if amino acids within the NKG2D cytoplasmic tail are involved in raft recruitment of this molecule.

3.2 Inhibition of Siglec-7 by sialic acid-based inhibitors

3.2.1 Development of Siglec-7 inhibitor

Siglec receptors are inhibitory receptors recognizing sialic acid residues. They have been implicated in regulation of immune cells, including NK cells, and shown to be involved in several malignancies. Siglec-7 is the major Siglec receptor on NK cells, but so far, no functional inhibitor for this receptor is available. Therefore, in collaboration with the lab of Prof. Brossmer (Biochemistry Center, Heidelberg University, Heidelberg, Germany) our aim was to develop an effective Siglec-7 inhibitor. The inhibitors were synthesized in the laboratory of Prof. Brossmer and subsequently tested by us for the ability to inhibit the interaction of Siglec-7 with its ligands. In order to detect the interaction of Siglec-7, we made use of a Siglec-7 RBC binding assay previously described [97] and modified it to fit our purpose.

RBCs were used as sialic acid presenting platforms and Siglec-7-Fc chimeras were used to bind the Siglec-7 ligands among these sialic acids (Figure 15A). RBCs could be stained for Siglec-7 ligands as shown in Figure 15B. This staining was specific for the binding ability of Siglec-7, as shown by the decrease of Siglec-7-Fc staining mediated by a block with Siglec-7-specific antibody (Figure 15C). Inhibitors were designed on the basis of the structure of 2-alpha-O-methyl sialic acid (Figure 15D), referred to in this work as unmodified sialic acid or compound 37 (see tables) for simplicity reasons. Briefly, potential inhibitors were tested by pre-incubation with the Siglec-7-Fc chimera prior to incubation of the mixture with RBCs (step I, Figure 15A). Bound Siglec-7-Fc chimeras were detected by incubation with biotin-labeled goat-α-human IgG (step II, Figure 15A), followed by incubation with PE-labeled streptavidin (step III, Figure 15A). If the inhibitor was able to impair the Siglec-7 interaction with its ligands, the staining in the assay was decreased, as shown in Figure 15E.

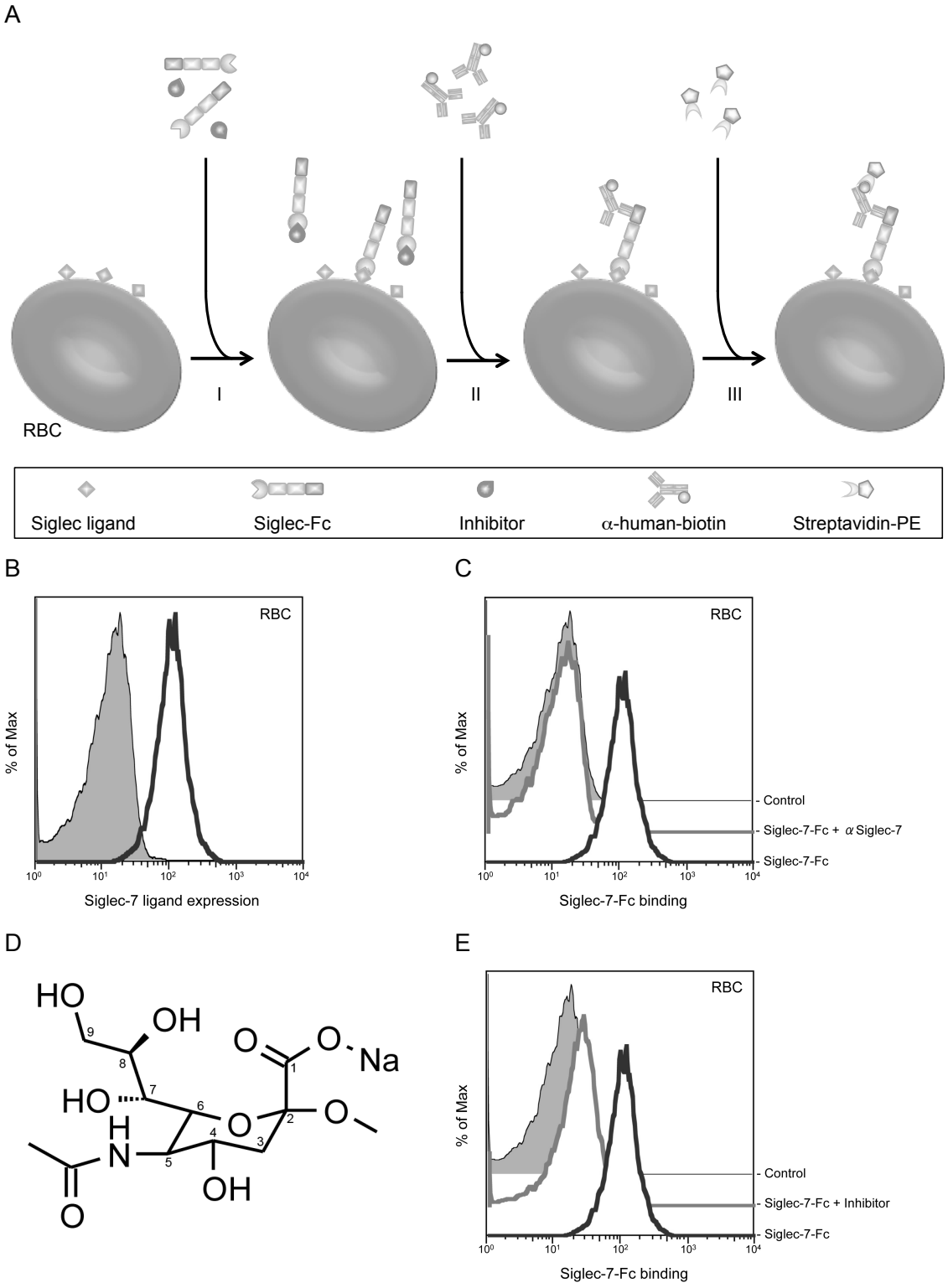


Figure 15: Basis of Siglec-7 inhibition assay on RBCs. (A) Schematic representation of Siglec RBC binding assay. Reagents are depicted below the scheme. (B) Expression of Siglec-7 ligands on red blood cells (RBCs). Effect of α-Siglec-7 antibody (C) or a Siglec-7 inhibitor (E) on Siglec-7-Fc binding to RBCs. Shaded histograms represent an unstained control without Siglec-7-Fc protein. (D) Structure of 2-alpha-O-methyl sialic acid (2-alpha-O-methyl-N-Acetylneuraminic acid, alpha-O-Me Neu5Ac). All histograms are presented as % of Max.

By testing different modifications of the original structure, we were able to improve the potential of the inhibitors stepwise. The modification at position 9 of sialic acid with different residues led to an increase in inhibitory potential of the inhibitors as shown in Figure 16. One of the most effective members of the inhibitors modified at position 9 is shown in blue in Figure 16A and C and table 2. Compared to unmodified sialic acid (shown in red) this inhibitor was up to ten times more efficient in the inhibition assays and showed IC_{50} values in the lower millimolar range.

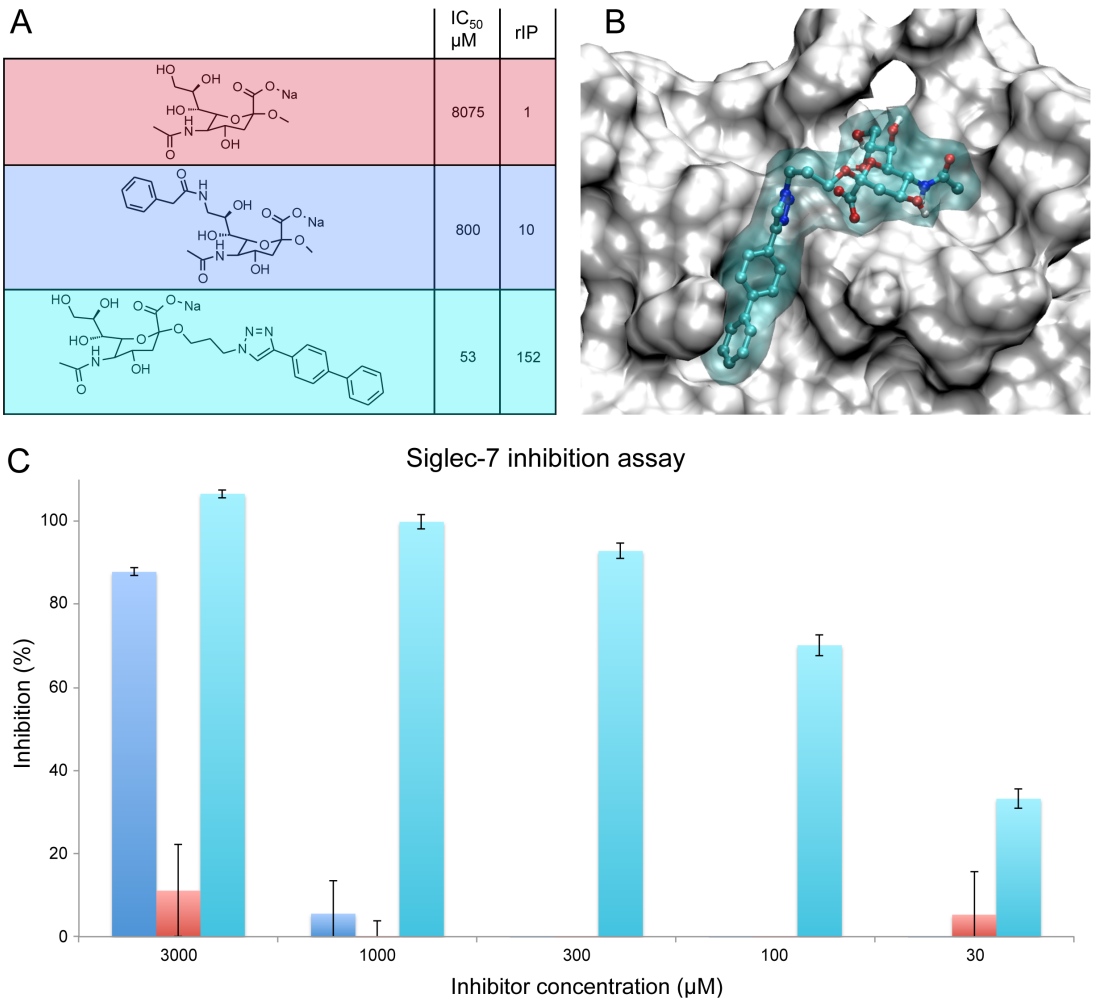


Figure 16: Development of Siglec-7 inhibitors, part I. (A) presents early substances used to inhibit Siglec-7. Unmodified sialic acid (red), compound 27 (blue) and compound 112 (cyan) are depicted in structure together with IC_{50} values and relative inhibitory potential (rIP) compared to unmodified sialic acid. (B) shows compound 112 at the ligand-binding pocket of Siglec-7 in a modeling study performed by Dr. Martin Frank (Biognos AB, Göteborg, Sweden). (C) presents the mean inhibition (± SD) of Siglec-7 ligand interaction at different concentrations mediated by all three compounds in RBC binding assays. Data are representative of at least three independent experiments. % inhibition was calculated as explained in Materials and Methods.

Further modification at this position, including those of the published oxamido inhibitor [97], led to improvements of inhibitory potentials (table 3). Later modifications were performed at position 2, as this position was predicted to enable a good fit of the inhibitor into the binding site. The most efficient modification at position 2 was the addition of a biphenyltriazolpropyl residue (Figure 16, cyan) that caused a 152-fold increase in inhibitory potential compared to sialic acid and achieved an IC_{50} in the micromolar range (Figure 16A and C, table 4). Docking studies supported the notion that this long and flexible modification enabled the inhibitor to fit into the cavity of tyrosine 64 within the binding groove of Siglec-7, as shown in Figure 16B.

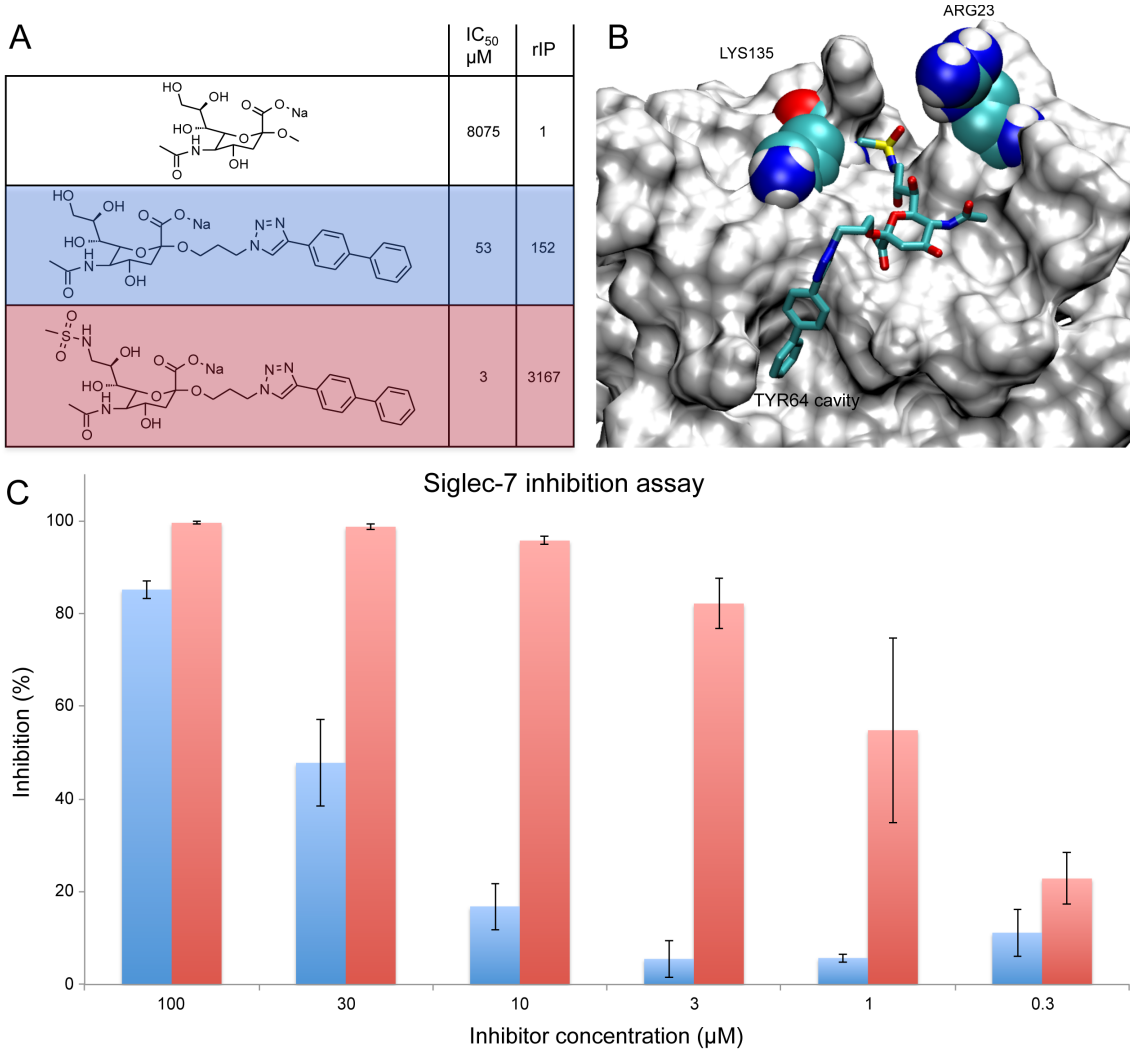


Figure 17: Development of Siglec-7 inhibitors, part II. (A) presents late substances used to inhibit Siglec-7. Unmodified sialic acid, compound 112 (blue) and compound 118 (red) are depicted in structure together with IC_{50} values and relative inhibitory potential (rIP) compared to unmodified sialic acid (white). (B) shows compound 118 at the ligand-binding pocket of Siglec-7 in a modeling study performed by Dr. Martin Frank (Biognos AB, Göteborg, Sweden). The mesyl group of compound 118 is shown in neighborhood of lysine 135 and arginine 23 of Siglec-7 and the biphenyl tail is shown in the tyrosine 64 cavity. (C) presents the mean inhibition (± SD) of Siglec-7 ligand interaction at different concentrations mediated by the compounds 112 and 118 in RBC binding assays. Data are representative of at least three independent experiments.

Additional improvements in inhibitory potential were sought through the modification of this substance at position 9 of the sialic acid structure. One of the most potent substances was modified at position 9 with a mesyl group as shown in red in Figure 17A and table 11. This substance achieved an IC_{50} in the lower micromolar range, which corresponds to a 3167-fold increase in inhibitory potential compared to unmodified sialic acid (Figure 17A and C). This improvement was most likely due to the possible formation of interactions between the mesyl group of the inhibitor and lysine 135 and arginine 23 of the receptor as shown in Figure 17B, which depicts docking of the mesyl group of the inhibitor between lysine 135 and arginine 23.

Although we generated more potent inhibitors by now (table 10), we decided to test compound 118 for functional effects on human NK cells at this point. Thus, in the further course of this thesis, the Siglec-7 inhibitor used refers to this compound. An excerpt of all substances tested so far is presented in tables 1 - 11 with their relative inhibitory potentials.

Table 1. Relative inhibitory potencies (rIPs) of α-O-Me Neu5Ac and 9-N-oxamido α-O-Me Neu5Ac

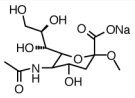
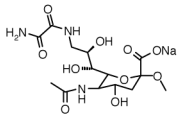
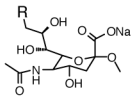
Compound	R	IC 50	rIP
37		8075 ± 1075	1
3		1642 ± 480	4,1

Table 2. Relative inhibitory potencies (rIPs) of 9-N-substituted derivatives of α-O-Me Neu5Ac



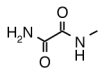
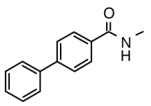
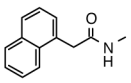
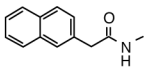
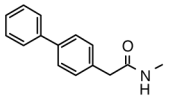
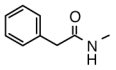
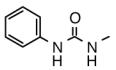
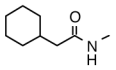
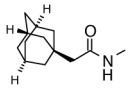
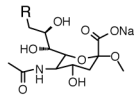
Compound	R	rIP
37	OH	1
3		4,1
35		< 1,5
32.2		< 1,5
83		< 1,5
55		8,2
45		4,5
27		10
19		< 1
98		< 1,5
97		< 1,5

Table 3. Relative inhibitory potencies (rIPs) of substituted 9-N-oxamido α-O-Me Neu5Ac derivatives



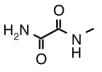
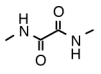
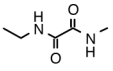
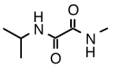
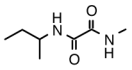
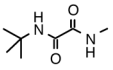
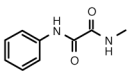
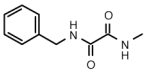
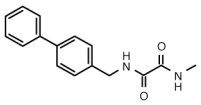
Compound	R	rIP
37	OH	1
3		4,1
154		< 2
156		< 2
15		< 2
14		< 2
16		< 2
18		58
4		8
6		8

Table 4. Relative inhibitory potencies (rIPs) of α-O-Me Neu5Ac and 2-α-O-(biphenyl-triazol-propyl) Neu5Ac

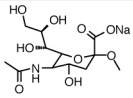
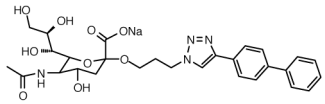
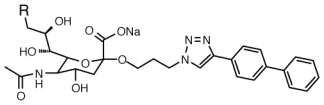
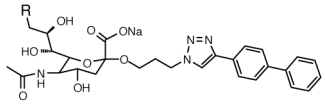
Compound	R	IC 50	rIP
37		8075 ± 1075	1
112		53 ± 30	152

Table 5. Relative inhibitory potencies (rIPs) obtained by derivatives with an replaced hydroxyl group



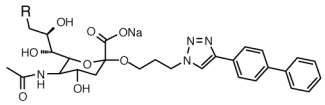
Compound	R	rIP
112	OH	1
126	H	0,15
125	NH_2	0,24
122	SH	0,52

Table 6. Relative inhibitory potencies (rIPs) of acylated and alkylated amino derivatives at position C-9



Compound	R	rIP
112	OH	1
120		0,73
153		0,28
142		< 0,5
143		< 0,5

Table 7. Relative inhibitory potencies (rIPs) of derivatives obtained by combination of residues at postion 2 and 9



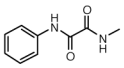
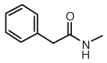
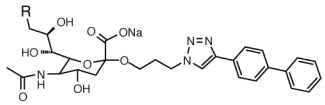
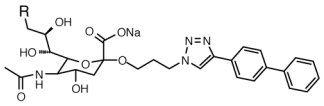
Compound	R	rIP
112	OH	1
136		3,5
160		6,8
113		0,60

Table 8. Relative inhibitory potencies (rIPs) of derivatives with an acidic residue in position 9



Compound	R	rIP
112	OH	1
130		3,6
115		21

Table 9. Relative inhibitory potencies (rIPs) of sulfonamide derivatives



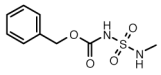
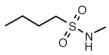
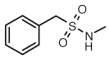
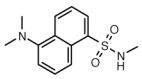
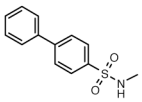
Compound	R	rIP
130		1
118		0,81
137		0,33
138		0,37
141		0,80
140		0,22
127		1,7
128		0,27
129		0,13
121		0,13
119		0,40
151		0,07
152		unsoluble

Table 10. Relative inhibitory potencies (rIPs) of α-O-Me Neu5Ac and 9-N-ethylsulfamido 2-α-O-(biphenyl-triazol-propyl) Neu5Ac

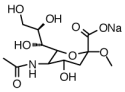
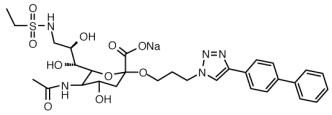
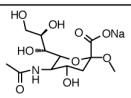
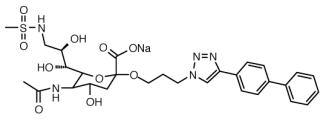
Compound	R	IC 50 microM	rIP
37		8075 ± 1075	1
127		1,55 ± 0,19	5210

Table 11. Relative inhibitory potencies (rIPs) of α-O-Me Neu5Ac and 9-N-methylsulfamido 2-α-O-(biphenyl-triazol-propyl) Neu5Ac

Compound	R	IC 50 microM	rIP
37		8075 ± 1075	1
118		2,55 ± 0,53	3167

3.2.2 Functional effects of sialic acid-based inhibitor on NK cells

In the next step, we wanted to know whether the Siglec-7 inhibitor showed effects on the function of human NK cells. We choose ^{51}Cr release assays in order to analyze NK cell activity, the killing of target cells. First, we screened target cell lines for a suitable expression of Siglec-7 ligands. From this screening, the two cell lines, the erythroleukemic K562 and the melanoma cell line Mel1106 were selected due to their expression profile (Figure 18A-C).

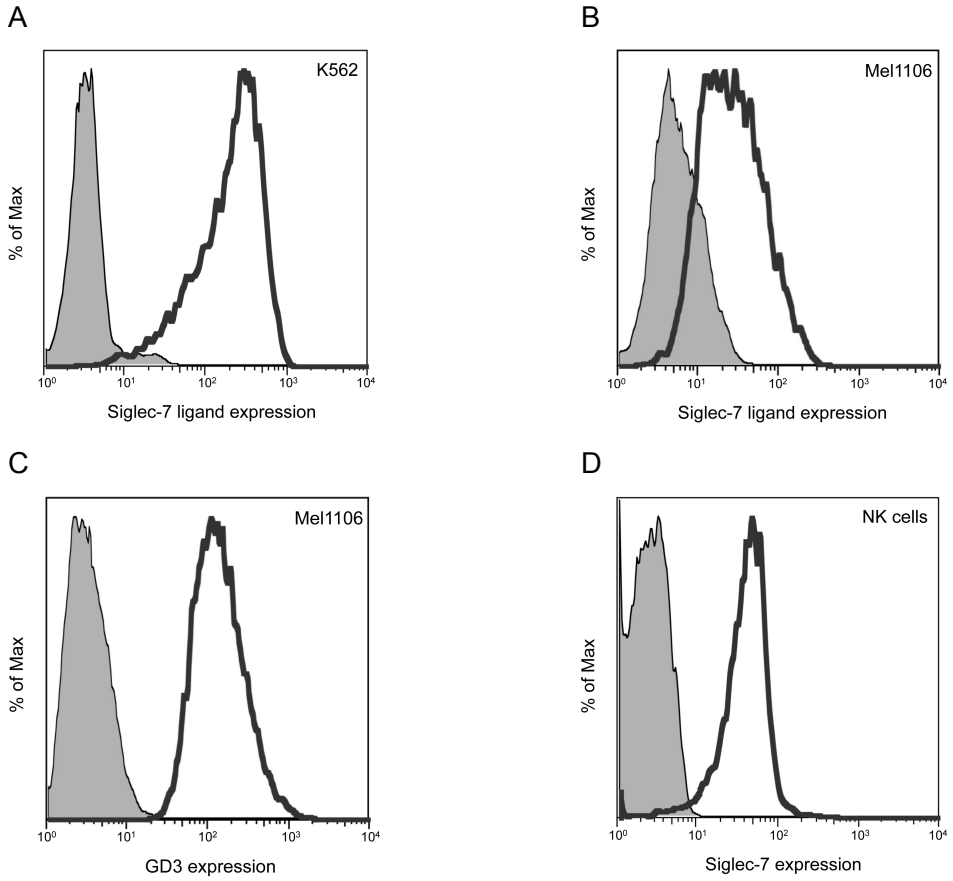


Figure 18: Expression of Siglec-7 ligands and Siglec-7 on cells used in ^{51}Cr release assays. Siglec-7 ligand expression on K562 (A) and Mel1106 (B) cells. High affinity Siglec-7 ligand GD3 expression is shown in C. Expression of Siglec-7 on NK cells is shown in D. Shaded histograms represent control stainings without Siglec-7-Fc fusion protein (A-B) or isotype controls (C-D). All histograms are presented as % of Max.

K562 cells express a moderate amount of Siglec-7 ligands (Figure 18A) whereas Mel1106 cells express Siglec-7 ligands to a lower extent (Figure 18B). In accordance with the literature [104, 107], Mel1106 express the high affinity Siglec-7 ligand GD3 (Figure 18C). In addition, all NK cells used for the ^{51}Cr release assays were tested for Siglec-7 expression, a representative staining is shown in Figure 18D.

K562 cells were first tested as target cells in the ^{51}Cr release assay due to their higher Siglec-7 ligand expression and their common use as target cells for NK cells in these assays.

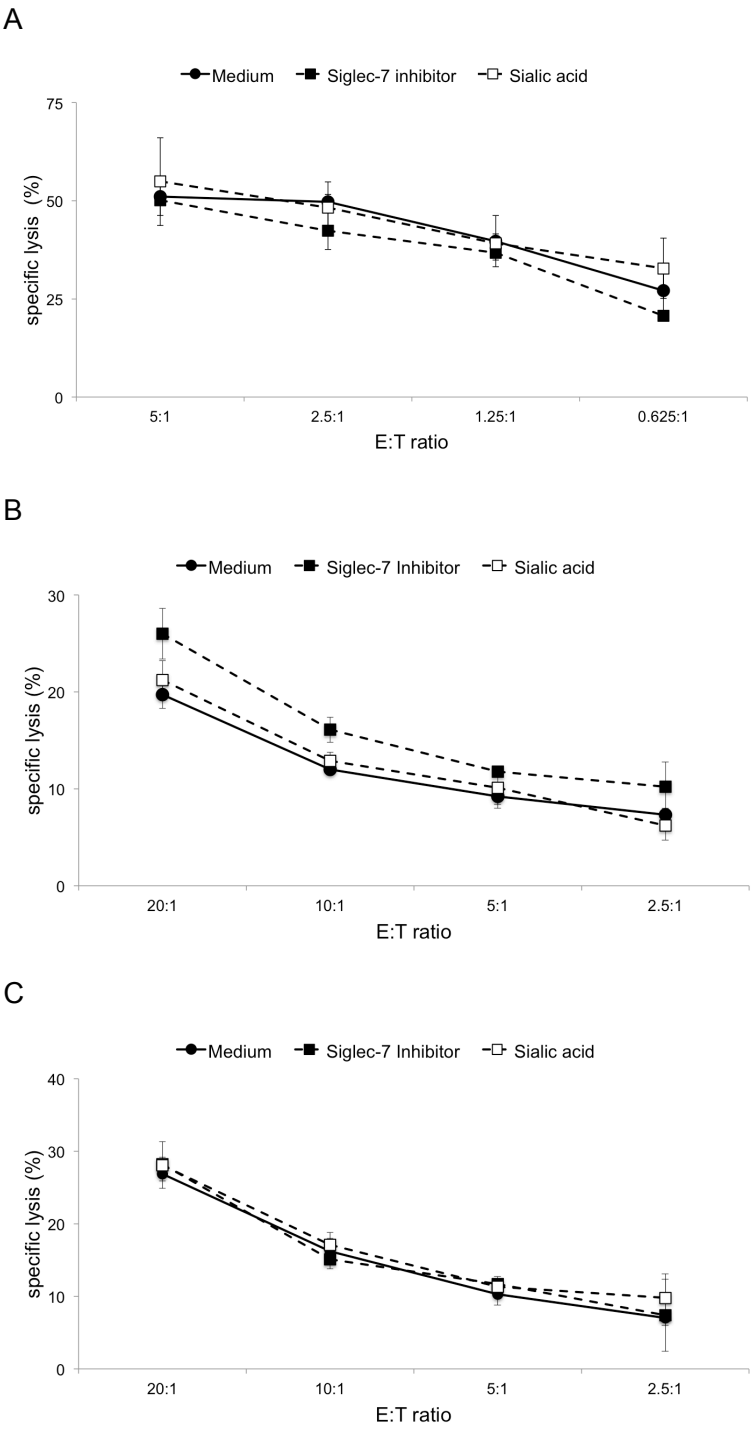


Figure 19: ^{51}Cr release assays with Siglec-7 inhibitor. IL-2-activated human NK cells were pre-incubated with 0.3 mM Siglec-7 inhibitor or unmodified sialic acid or medium alone preceding coincubation for 4 h at 37°C with ^{51}Cr-labeled target cells. Values represent mean specific lysis ± SD of triplicates of representative ^{51}Cr release assays. A representative assay with K562 target cells is shown in A (n = 3). Representative assays with Mel1106 target cells are shown in B and C (n = 5).

The inhibitor (compound 118, table 11) was tested for its efficacy in the assay. Unmodified sialic acid was used as a control (compound 37). IL-2-activated primary human NK cells were used as effector cells throughout the ^{51}Cr release assays. To

our surprise, the Siglec-7 inhibitor did not increase the lysis of K562 cells in comparison to sialic acid alone or a medium control (Figure 19A).

In order to exclude a cell line specific effect, as K562 cells are extraordinary stimulatory for NK cells and thus might override weak inhibition signals, we performed the ^{51}Cr release assays with the Mel1106 cells, which are much more resistant to NK cell mediated lysis. As expected, the lysis of Mel1106 target cells was lower than that of K562 cells. Interestingly, we found a small increase in lysis of the Mel1106 target cells as shown in Figure 19B, but this was only the case in about half of the experiments. The other assays with the Mel1106 cells with the same conditions did not show any effect on the killing of the target cells (Figure 19C). Thus the inhibitor was able to achieve an improved lysis of the target cells although not in all experiments.

3.2.3 Factors influencing Siglec-7 inhibitor efficacy

It has been previously published that Siglec-7 shares some ligand specificity with its relative Siglec-9 that shares 84 % structure homology with Siglec-7 [91, 94, 95]. Both of these Siglec receptors have been described to be expressed by human NK cells. Thus, the questions arose whether the developed Siglec-7 inhibitors affected Siglec-9 as well and whether this interaction was the basis for observed functional effects. Human NK cells were analyzed via flow cytometry for their expression profiles of Siglec-7 and Siglec-9 (Figure 20A, B). In all experiments, the staining of Siglec-7 on the NK cells was higher than that of Siglec-9. The antibodies used were assessed via ELISA for their affinity towards Siglec-7 and Siglec-9 in order to enable a correlation of the flow cytometry data with the amount of Siglec expression. In those assays the α-Siglec-9 antibody showed a higher affinity (data not shown), which supports the conclusion drawn from the FACS data that Siglec-7 is more abundant on the surface of human NK cells. In another attempt to investigate the effect of the Siglec-7 inhibitor on Siglec-9, we applied a modified Siglec-7 inhibition assay. Instead of RBCs, which only express Siglec-7 ligands, K562 were used as sialic acid platforms as they expressed ligands for both, Siglec-7 and Siglec-9 (Figure 20C). Siglec-7-Fc and Siglec-9-Fc fusion proteins were incubated with the Siglec-7 inhibitor and, as with the previous Siglec-7 inhibition assays, the reduction of binding was determined by flow cytometry. Those experiments showed that the interaction of Siglec-7 with its ligands was about 100-fold more sensitive to the inhibitor than the interaction of Siglec-9 with its ligands (Figure 20D). These data supported our hypothesis that the observed effects were due to an inhibition of Siglec-7 and not Siglec-9.

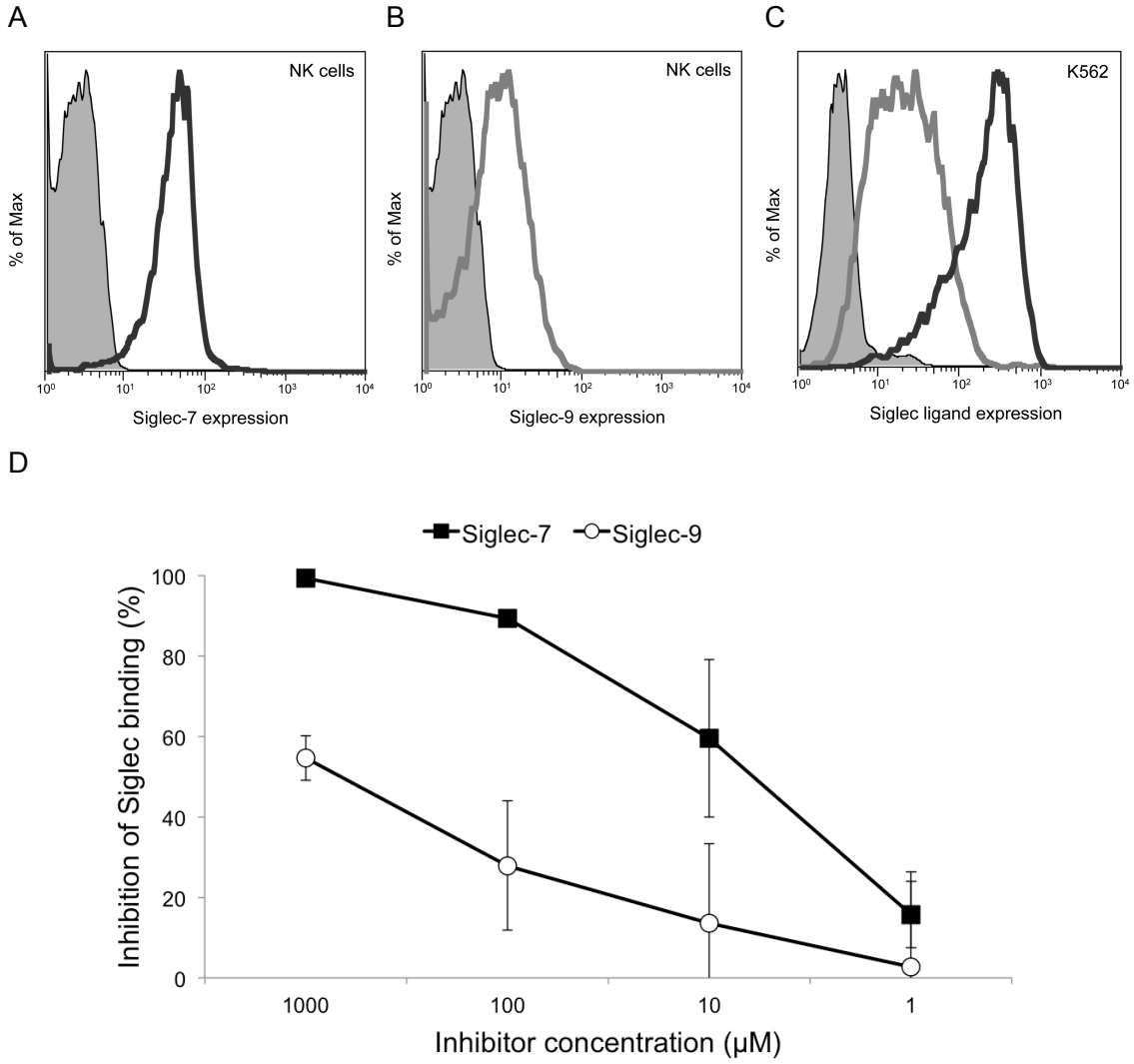


Figure 20: Specificity of compound 118 against Siglec-7-Fc & Siglec-9-Fc. Expression of Siglec-7 (A) or Siglec-9 (B) on NK cells used in ^{51}Cr release assays. Shaded histograms represent isotope controls. (C) Expression of Siglec-7 ligands (black) and Siglec-9 ligands (gray) on K562 used for comparison of inhibitor specificity. Shaded histogram represents unstained control without Siglec-Fc. All histograms are presented as % of Max. (D) Inhibition of Siglec binding by different concentrations of the Siglec-7 inhibitor (n=3).

Another possibility for inaccurate results of Siglec-7 inhibition on NK cells is a potential toxicity of the inhibitor that might influence the lysis of cells during the ^{51}Cr release assays.

In those assays, a lysis of target cells in the presence of the inhibitor alone could not be detected (data not shown), but the toxicity towards the NK cells could not be assessed with these assays. To this end, NK cells were incubated in the presence of the inhibitor at various concentrations and afterwards analyzed for apoptosis by a living/dead analysis using Annexin-V and 7-AAD (Figure 21A-C). Annexin-V binds to phosphatidylserine that is only present on the cell surface, if the cell enters apoptosis

and is unable to sustain membrane integrity. 7-AAD is a membrane-impermeable DNA-intercalating agent, which stains DNA once the cellular membranes disintegrate. From these stainings, cells can be classified as healthy (Annexin-V$^-$/ 7-AAD$^-$), early apoptotic (Annexin-V$^+$/ 7-AAD$^-$) or late apoptotic (Annexin-V$^+$/ 7-AAD$^+$). The incubation of the NK cells with the inhibitor did not result in an increased amount of apoptotic cells at the applied concentrations as shown in Figure 21.

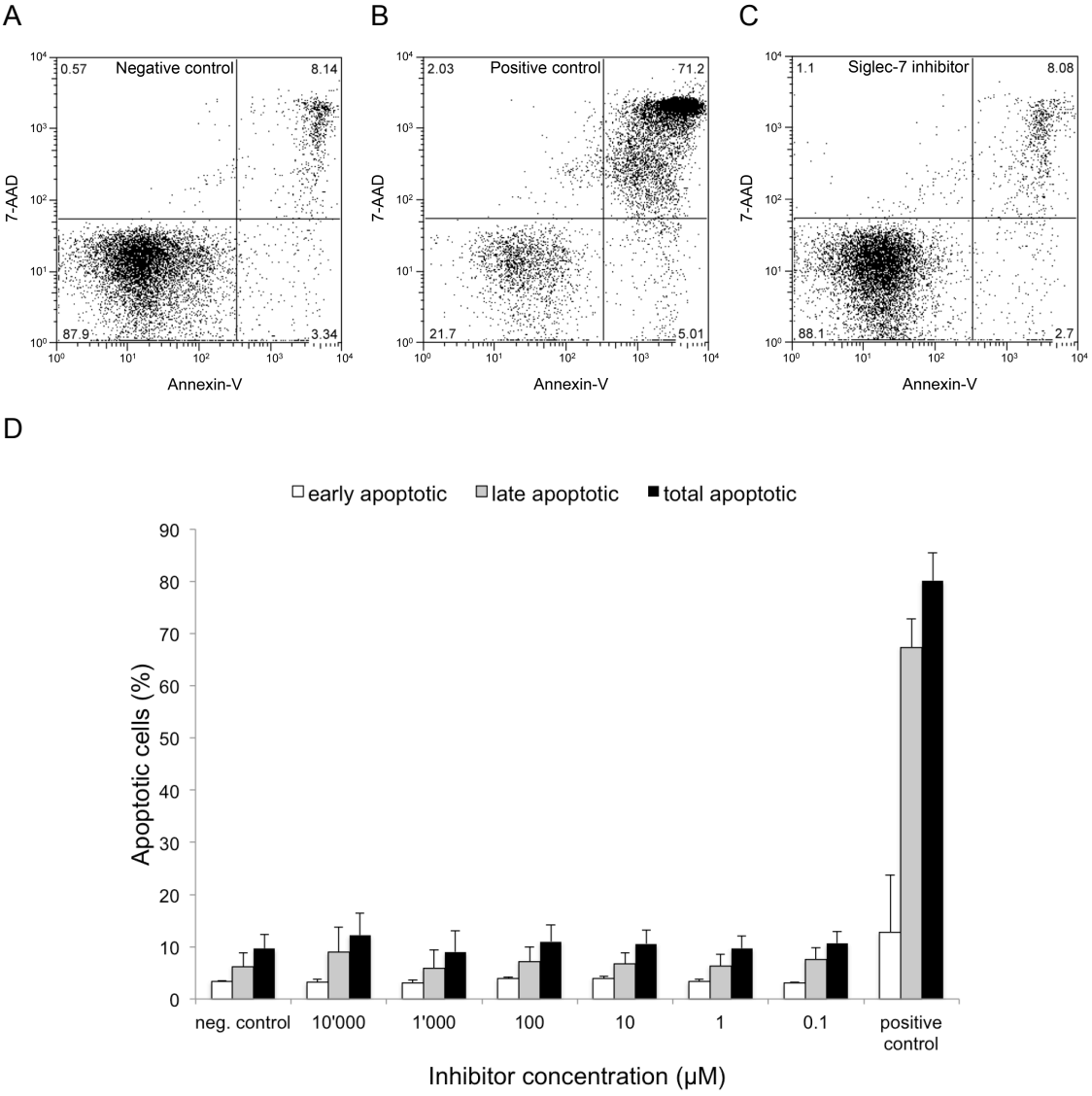


Figure 21: Toxicity of Siglec-7 inhibitor on primary human IL-2 activated NK cells. NK cells were incubated for 24 h at 37°C with medium alone (neg. control) or different concentrations of the Siglec-7 inhibitor or an ATP-depleting apoptosis-inducing agent (positive control). NK cells were then stained for Annexin-V binding and 7-AAD and classified into early apoptotic (AnnexinV$^+$/7-AAD$^-$) or late apoptotic (AnnexinV$^+$/7-AAD+) events. Representative blots of negative control, positive control and 10'000 µM Siglec-7 inhibitor are shown in (A-C). (D) presents statistics of three independent experiments.

According to these data, a side effect of toxicity could be dismissed. Thus, we generated a specific inhibitor capable of a potent block of the ligand binding of Siglec-7.

4 Discussion

4.1 Lipid raft recruitment of activating NK cell receptors

4.1.1 Lipid raft recruitment of the SLAM-related receptor 2B4

Using a mutagenesis approach we analyzed the DRM recruitment of the human 2B4 for its molecular requirements. The implication of these observations has to be regarded with care as the data were obtained with a sucrose density gradient and cannot be translated directly to an identical role in the physiology of NK cell lipid rafts.

Indeed, there has been an intense debate over the existence of lipid rafts and the methods applied to study them [161]. These critical remarks concerning lipid rafts are not without basis. Several points have to be regarded concerning the applied detergent extraction with sucrose density gradients. During the detergent extraction, the membranes are kept at 4°C which is not the physiological temperature for cellular membranes. As lipid membranes are temperature sensitive, this reduction in temperature might cause changes in the obtained membrane fractions by itself. In addition, the use of the detergent Triton X-100 to solubilize non-raft membranes is not without cruces as it is not entirely sure, whether the detergent only removes the non-raft part of the membrane.

Several studies reported observations that might challenge this assumption of simple phase separation [161]: For example, the two leaflets might mix upon detergent extraction and thereby change the composition of the obtained DRMs; the detergent resistance of outer and inner leaflet may vary and cause a membrane solubilization divergent from the predicted one. Moreover, only particular proteins might be extracted depending on their physical properties from the membrane and this might change the DRM composition [187]. There is a whole range of factors that might alter the final composition of the obtained DRMs; therefore the DRM fractions should not be mistaken for an exact equivalent of lipid rafts in the living cell. Additionally, as can be seen from the DRM fractions in Figure 6 and Figure 8, the assays can vary drastically in their appearance; thereby hindering the exact quantification of such assays.

On the other hand, data obtained from these assays should not be regarded as false or unfeasible. Obviously, the localization of membrane components in these assays is not random: GPI-anchored proteins were shown to be enriched in DRMs [166] as are other proteins with different modifications, e.g. palmitoylation [163]. Therefore, specific interactions seem to underlie these observations of membrane segregation.

With increasing data supporting the presence of lipid rafts [162], also the data obtained by detergent extraction become more meaningful.

Altogether, residence of proteins in DRMs should be regarded as indication for a possible association of the proteins with lipid rafts. Whether this is the case has to be confirmed with experiments in more physiological membranes or preferentially in living cells. The fact that small changes in the studied mutant receptors caused severe and reproducible changes in the DRM recruitment of these molecules supports the significance of these data.

The observation of a complete loss of DRM recruitment upon the exchange of the 2B4 transmembrane domain with the transmembrane domain of LAT is surprising, as lipid raft association similar to the constitutive palmitoylation-dependent lipid raft localization of LAT was expected. The obvious difference might be due to a lack of sequences regulating the palmitoylation of the LAT transmembrane domain as a successful palmitoylation also depends on the surrounding structure of the target amino acid. For example, the common presence of hydrophobic amino acids and prenylation or myristoylation sites adjacent to the palmitoylation site has been described [188]. The reason why our construct does not localize into the DRM fraction might be due to a too short replacement domain from LAT, which is devoid of these regulatory structures for palmitoylation. In order to circumvent this problem we exchanged a bigger portion of the LAT transmembrane domain or a transmembrane domain of a FcγRIIA mutant described to constitutively localize into lipid rafts [184]. Neither of these mutants was localized into the DRM fraction (data not shown). Either these mutants were also insufficient to enable palmitoylation, or palmitoylation of h2B4 alone is insufficient to mediate DRM recruitment. Still, the complete loss of DRM recruitment upon transmembrane domain exchange highlights the importance of this domain for the lipid raft recruitment of h2B4.

In addition, we observed a crucial requirement of a CxC motif in the transmembrane domain of human 2B4 for the recruitment of the receptor into DRMs after cross-linking (Figure 10). This requirement of the h2B4 is consistent with published data for the murine 2B4 (m2B4), which is also dependent on this motif for DRM recruitment [185]. In contrast to the transmembrane domain, the cytoplasmic domain of h2B4 (including the signaling motifs) was dispensable for the recruitment into DRMs after cross-linking (Figure 6). Previous studies proposed that h2B4 is first recruited into lipid rafts and then phosphorylated [124]. Our observation that the deletion of the cytoplasmic domain of h2B4 does not affect the DRM recruitment supports the notion that lipid raft recruitment is independent of receptor phosphorylation and occurs first.

Thus, the possibility that a signaling partner, recruited after h2B4 phosphorylation, mediates the lipid raft recruitment of h2B4 is very unlikely.

The exact properties of the CxC motif are not resolved though. Different modifications and interactions can recruit a protein to lipid rafts: Cholesterol- and sphingolipid-binding and/or association have been described to affect lipid raft association [168-170]. These sphingolipids modifications are unlikely in case of the CxC motif, though, as sphingolipids mainly reside in the outer leaflet of the membrane whereas the CxC motif is located at the boundary of the cytoplasmic side. The same is true for GPI-anchors, which are enriched in the outer leaflet [189]. Cholesterol is present in the inner leaflet of the plasma membrane as well, and thus might be harnessed by binding or association though. Still, modifications or interactions at the inner leaflet of the membrane appear more reasonable due to the location of the CxC motif in the transmembrane domain. One modification, which has been shown to increase association with lipid rafts through the inner leaflet, is S-acylation, more specifically the palmitoylation of proteins [163] as found in the CxxC motif of LAT [183] or the CxC motif of Lck [190] which is intriguingly similar to the CxC motif of h2B4.

Using the palmitoylation prediction software CSS-Palm [186], we could confirm the CxC motif as a potential palmitoylation site in h2B4. However, our attempt to inhibit palmitoylation with 2-bromopalmitate, an established palmitoylation inhibitor [191], did not result in feasible data (data not shown). The labeling of h2B4 with radioactive palmitate did not result in feasible data either ([124], data not shown and personal communication). A better way to study possible protein palmitoylation has emerged in the last years in the field of click chemistry [192] which enables the specific detection of palmitoylated proteins.

Click chemistry includes the modification of substances with simple, small units. The most prominent example of such a Click reaction is the Huisgen cycloaddition. In this reaction, an azide-labeled substance reacts with an alkyne and forms a triazole ring that covalently links both original substances [193]. These reactions and their products are stereospecific, physiologically stable and non-toxic. For example, azide-labeled palmitates could be fed to cells in order to be incorporated by those. Subsequently, the cells would be lysed and immunoprecipitated for the protein of interest. In another step, the Click reaction could be performed with an alkyne-labeled detection reagent in order to determine palmitoylation of the immunoprecipitated protein. The application of such methods will surely provide further inside into the modifications of lipid raft-associated proteins.

Interestingly, a palmitoylation of m2B4 could not be detected in a previous study (data not shown) [185]. This should not be taken into account towards a similar behavior of h2B4 though, as differences between lipid raft behavior of h2B4 and m2B4 emerged in previous studies: For example, h2B4 was shown to localize into DRM only after cross-linking ([124] and Figure 5) whereas m2B4 was constitutively resident in DRMs [185]. In general, palmitoylation should not be regarded as an exclusively permanent modification that results in constitutive effects though. Palmitoylation of proteins has also been described to be of transient nature [194] and crosslinking h2B4 might induce its palmitoylation lasting only for a given time, e.g. the duration of a stimulatory cell contact. Although this might also be an effect caused by the used cell lines (e.g. due to different extents of *cis*-interactions of 2B4 with CD48), rather than the receptor, the behavior of m2B4 should not be put on the same level as the behavior of h2B4. Further analysis is thus required to elucidate the underlying causes.

Another possibility is that h2B4 associates with another protein via the CxC motif, and that this association mediates lipid raft recruitment of h2B4. Such an association would occur prior to the phosphorylation of the ITSMs, as raft recruitment is independent of ITSM phosphorylation (see 2B4-YF in Figure 6), and therefore signal transduction of h2B4 as well. The association either has to be induced prior to ITSM phosphorylation or be constitutive.

Interestingly, in this context, two previous studies described a constitutive association of LAT with h2B4 [195, 196]. This association may either be direct or mediated by the shared residence in lipid rafts. These studies immunoprecipitated LAT with h2B4, but the possibility that the association observed in these studies is mediated by a shared residence in resistant membrane fractions still remains. A similar observation of association with LAT was made for m2B4 and this association of m2B4 with LAT was dependent on the CxC motif [185]. Therefore, h2B4 might associate with LAT via this CxC motif as well.

In support of this notion, an interaction of LAT with the co-receptors CD4 and CD8 via a similar CxC motif in the co-receptors has been shown [197]. Additionally, an interaction of both co-receptors with the protein tyrosine kinase Lck mediated by the same motif has been described previously [198, 199]. Therefore, such a CxC motif might be used for protein association in the immune cells. The existence of such an association with LAT via the CxC motif in h2B4 surely has to be studied in detail and hopefully will shed light into the combination of lipid raft recruitment and signal transduction of 2B4.

If this interaction between h2B4 and LAT holds true, this raises the question, whether h2B4 might mediate other associations with this motif as well. As mentioned previously, LAT can associate with CD4 and CD8 via this motif while both co-receptors can associate with Lck. Therefore, an association of h2B4 with Src-family kinases with a similar CxC motif might be possible. The associative potential and regulation of interactions via this CxC motif in h2B4 have to be resolved in future studies.

The question arises whether LAT is required for the lipid raft recruitment of 2B4. All previous studies determining the DRM recruitment of 2B4, including this one, made use of cell lines expressing LAT [124, 185] which is consistent with the physiological situation as 2B4 is predominantly expressed in LAT expressing cells. A potential dependency of lipid raft recruitment could be analyzed either by LAT deficient cells or by the use of gene knockdown methods. Support for the importance of LAT for 2B4 is found in murine lymphokine-activated killer (LAK) cells, where LAT deficiency was shown to cause a reduction in 2B4-mediated cytotoxicity [185]. Whether such functional impacts are also the case for 2B4-mediated cytotoxicity in human NK cells is unknown.

Of course, other mediators of lipid raft recruitment might associate with h2B4 via the CxC motif as well. Although there is no data about signal-independent associations of proteins with h2B4 apart from LAT, it is known that the lipid raft recruitment is dependent on an intact cytoskeleton [124].

A crucial role of the cytoskeleton for lipid raft regulation has been also shown in other cell types as T cells [174] and cytoskeleton-related proteins like ezrin, radixin, moesin (ERM) proteins are present in the DRM fractions of immune cells, e.g. of T cells [174, 200]. It is not known though, whether the requirement of an intact cytoskeleton lies on the active recruitment of the receptors into lipid rafts and thus the formation of lipid rafts, or whether the cytoskeleton is only required to stabilize the lipid raft. The fact that an h2B4 mutant without cytoplasmic domain is able to migrate into the DRM fraction makes an interaction of the cytoskeleton or linking proteins through this part of h2B4 unlikely. It has to be considered though that the crosslinking of h2B4 with antibodies on the extracellular side might substitute for a cytoskeleton-mediated arrangement of h2B4 in our assays. The cytoskeleton should thus not be neglected as a potential mediator in initial lipid raft recruitment of h2B4.

Additionally, the possibility remains that a protein associating via the CxC motif mediates a connection to the cytoskeleton. The regulation of lipid raft stability by the cytoskeleton has been shown in different studies and models: A picket-fence model was proposed in which an actin network below the plasma membrane influences the

organization and stability of lipid rafts [201]. In addition, an intact cytoskeleton was required for the exclusion of lipid rafts from iNKIS [179]. Moreover, the reorganization of the actin fibers at the NKIS is one of the first steps induced during synapse formation [44] and this reorganization might affect the coalescence of lipid rafts in active or passive ways. Therefore, the cytoskeleton seems to hold lipid rafts together and at the required location; whether it also mediates the active recruitment of lipid raft-associated receptors remains to be seen.

Another observation made from our experiments was that the amino acids of the h2B4 transmembrane domain most likely resident at the extracellular side of the membrane also influence the DRM recruitment of h2B4 (Figure 8); although this effect was not as strong as the one observed for the CxC motif.
Upon further examination we could pinpoint this effect to the FLVIIVILS sequence in the transmembrane domain (Figure 12). The strongest effect on DRM recruitment by mutation in this region was mediated by the FLV and ILS sequences. These amino acids are not adjacent but separated by the amino acids IIV. The most common structural element used by proteins to span a membrane is the α-helix [202]. It is preferential in its orientation as it directs the hydrogen bonds of the peptide backbone to the internal side. Thereby it reduces the extent of polar groups at the lipid-protein interface, which might interfere with the alignment of the transmembrane domain. In an α-helix, every N-H group forms a hydrogen bond with every C=O group four amino acids earlier in the helix and therefore every four amino acids are placed on top of each other. This fits with the gap formed by the IIV between FLV and ILS. Therefore, the positioning of the FLV one helix turn over the ILS might form a structure important for the DRM recruitment of h2B4.
It is also of note that this segment is mostly formed by hydrophobic amino acids. A similar requirement for the presence of hydrophobic amino acids in the luminal side of the transmembrane domain for lipid raft association has been observed for the viral transmembrane protein hemagglutinin (HA) [203].
In fact, the association of proteins with membrane lipids has previously been described as a means to increase lipid raft association and is regarded as a fundamental interaction for membrane heterogeneity [162]. This so-called ‘wetting’ or ‘lubrication’ describes the association of a protein (but not necessarily the binding) with a lipid. The lipid-facing surface of a membrane protein is formed by grooves and protrusions which can provide low specificities for certain lipids [204]. Although transmembrane domains of proteins are regarded to reside stiffly in the membrane, it was proposed that lipids can be distorted to better fit the specific structure of a

transmembrane domain [172]. Therefore, certain lipids would show a better associative capacity than others, which is underscored by the vast repertoire of different lipids abundant in cellular membranes [205].

This specificity could even be improved by the combination of different proteins with lipid associative potential. For example, receptor clustering or oligomerization could capture matching lipids, which then would influence the lipid raft association of the receptor cluster [162, 204]. This behavior has been shown for caveolin that, as an oligomer, contains a cholesterol-binding groove [169]. The reduced capability of h2B4 to localize into DRM fractions upon mutation of specific amino acids at the luminal side of the transmembrane domain might be due to a decreased association potential of the transmembrane domain with raft-associated membrane lipids or due to unknown protein interactions at the outer leaflet. Although the 2B4 structure has been analyzed in previous studies, the focus of these studies was on the extracellular ligand binding domains [206, 207]; there is no data available about the structure of the 2B4 transmembrane domain that might allow a more detailed analysis of possible lipid-receptor association.

In addition, the binding of lipids as cholesterol or sphingolipids to a protein can influence membrane localization as well. The modification of a protein with cholesterol and palmitates can link a protein to a membrane, as seen in the protein family hedgehog [175, 208]. Additionally, a range of sphingolipids-binding domains has been described in the last years that allow a transmembrane protein to bind sphingolipids and thereby alter their lipid raft association [168, 170]. Such binding sites might exist in the transmembrane domain of h2B4 and assist in the lipid raft recruitment of h2B4 upon ligation, but whether the FLV and ILS sequences in the transmembrane domain of h2B4 form such a cholesterol- or sphingolipids-binding site has to be solved in future work.

It is also a compelling finding that the cytoplasmic domain of h2B4 with replaced extracellular and transmembrane domains (and therefore without the CxC motif and FLV/ILS) is sufficient for phosphorylation of 2B4 and subsequent signaling [209]. If an association of h2B4 with lipid rafts occurs via the transmembrane domain, this would indicate an interesting divergence between the mechanism for lipid raft recruitment and the mechanism for signal transduction. As mentioned previously, LAT deficiency in murine LAK cells was shown to impair 2B4-mediated cytotoxicity [185]. Whether this is due to impaired raft recruitment or the lack of a signaling component remains to be shown. It was previously described that LAT is phosphorylated after the phosphorylation of h2B4 [195].

Additionally, phosphorylated h2B4 was only detected in DRM fractions and DRM recruitment of h2B4 is Src-family kinase dependent [124]. As the DRM recruitment of h2B4 is Src-family kinase dependent and h2B4 does not rely on its cytoplasmic domain for DRM recruitment, the phosphorylation of other mediators is necessary to mediate the lipid raft localization of h2B4. This might either be the regulation of lipid raft stability by the cytoskeleton, in this case maybe ERM proteins or related proteins, or the active recruitment of h2B4 by a yet unidentified component. Taken together, these data imply that LAT is phosphorylated in the lipid rafts after h2B4 has been recruited there.

From our data we draft the following hypothesis based on the current lipid raft model [175]:
In the resting state, the receptor 2B4 is resident in the non-raft phase of the membrane (Figure 22A). The affinity towards more ordered nanodomains may be increased by weaker forces, e.g. by a lipid modification at the luminal transmembrane domain sequence FLVIIVILS or by the association of this domain with lipids. These nanodomains are probably not big enough to withstand detergent extraction though. Upon ligation of the receptor, these nanodomains may be clustered leading to larger nanodomains (Figure 22B). Subsequently, these will coalesce into a macrodomain (Figure 22C), if the stimulus is sufficient (e.g. by an aNKIS). The recruitment of the smaller nanodomains containing 2B4 into this macrodomain is cytoskeleton-dependent [124]; either due to active transport or to stabilization of the lipid raft. The recruitment additionally is dependent on the CxC motif in the transmembrane domain of 2B4. The underlying cause might be a modification, as palmitoylation, or the association with a lipid raft associated component, e.g. LAT. The effects of lubrication and the CxC motif are probably not mutually exclusive though. The CxC motif might assist in small nanodomain association whereas the lubrication might also favor large nanodomain formation. The transit from small nanodomains into larger domains might also mark the development of a detergent resistance and include a positive feedback loop. Such a feedback loop could involve initial signals (e.g. via Vav-1), which induce or stabilize nanodomains recruitment by means of cytoskeletal remodeling. This would cause larger nanodomains to recruit more small nanodomains in order to form a macrodomain. In these macrodomains or so-called lipid rafts, 2B4 is phosphorylated by resident Src-family kinases. This step then initializes the signal transduction through 2B4 by recruitment of SAP and 3BP2 and subsequent signaling partners

[57]. During this signaling process, LAT is phosphorylated as well [195]. Ultimately, this process will then result in cytotoxicity and/or cytokine secretion of the NK cell.

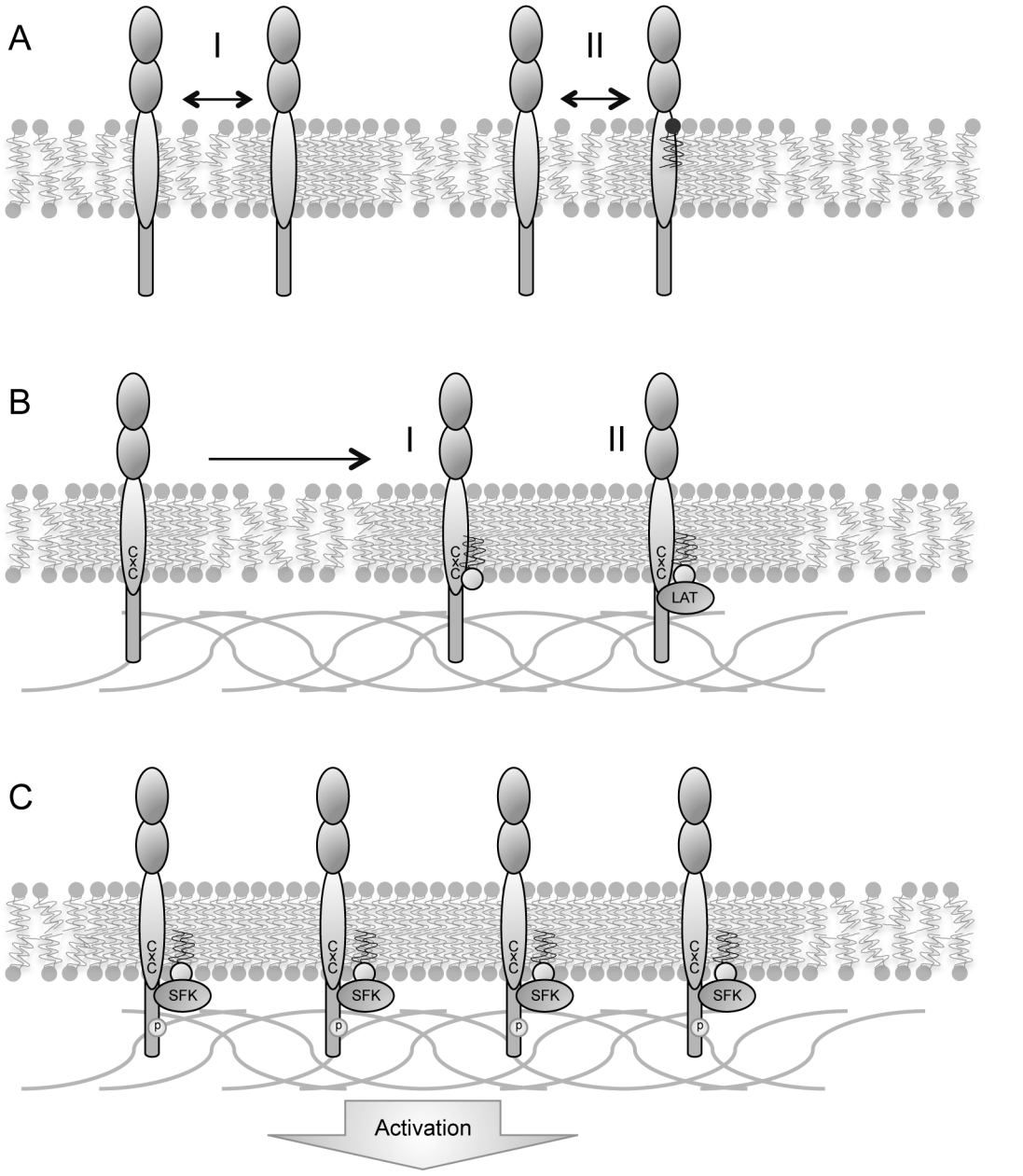


Figure 22: Schematic illustration of our model of 2B4 lipid raft recruitment. (A) presents the resting state. 2B4 dynamically moves in and out of small raft nanodomains (A-I). The association with such nanodomains may be increased by modifications of 2B4, as lubrication (A-II). (B and C) depict mechanisms induced by receptor ligation. (B) Small raft nanodomains coalesce into larger nanodomains upon 2B4 ligation. This is dependent on an intact cytoskeleton and the CxC motif at the cytoplasmic transmembrane domain of 2B4. The dependence on the CxC motif might be due to palmitoylation (B-I) or association with a raft-resident molecule as LAT (B-II). (C) Raft macrodomains are formed from the nanodomains at the aNKIS. 2B4 is phosphorylated in this domain by raft-resident Src-family kinases and transduces an activating signal into the NK cell.

Especially the lack of detailed data for the transmembrane domain of 2B4 should be approached in order to obtain a better understanding of mechanism mediated by this domain. The involvement of the cytoskeleton in the initial lipid raft recruitment of h2B4 should be clarified. A more physiological approach with cell-cell contacts should be applied to circumvent potential effects mediated by the extracellular clustering via antibodies. Several modifications are also known to influence lipid raft association of a membrane protein and these should be studied more closely: A putative palmitoylation of h2B4 has to be resolved, e.g. by click chemistry. Potential interaction via the CxC motif with other membrane components has to be analyzed. Additionally, the importance of the CxC motif could be analyzed via a knock-in mutant with a mutated LACFCV domain but functional CxC motif. The study of the binding capacity to different components with mutants of the CxC motif seems reasonable; additionally the capacity of h2B4 to migrate into DRMs in dependency of these components should be analyzed. The effect of the luminal transmembrane sequence FLVIIVILS should be studied as well as lipid associations and modifications of proteins at the outer membrane leaflet have been described to mediate lipid raft association. Therefore, modification of the 2B4 transmembrane domain with lipid species should be analyzed applying chemical methods as click chemistry or bioinformatic approaches. The putative association of lipids could be studied by a better resolution of the 2B4 structure, with a focus on the transmembrane domain, and modeling studies to gauge the surface of the 2B4 transmembrane domain for grooves and protrusions that allow putative association with specific lipid species.

Overall, this work has expanded the knowledge about the 2B4 transmembrane domain and provides the basis for more elucidated work on the mechanisms of lipid raft recruitment. In addition, this work was also able to support previous notions about mechanisms and requirements of 2B4 lipid raft recruitment.

4.1.2 Lipid raft recruitment of the C-type lectin-like receptor NKG2D

The function of lipid rafts has previously been described to be obligatory for NKG2D-mediated activation of NK cells [144] (and data not shown), in agreement with other studies showing the importance of lipid rafts for NK cell activation [49, 124]. In addition to 2B4, we therefore investigated the basis of the lipid raft recruitment of NKG2D. Similar to our approach to analyze molecular mechanisms of 2B4 recruitment into lipid rafts, we applied a mutagenesis approach with sucrose density

gradients to investigate the mechanisms underlying the previously observed lipid raft recruitment of NKG2D [144]. The methods applied for this were the same as with the 2B4 study and therefore every concern mentioned for the 2B4 results also applies for the NKG2D data and will not be discussed again. In addition, due to a failure in the study no feasible data for the wildtype NKG2D were obtained and thus only observations possible from the obtained data are discussed.

Our data show that NKG2D can be recruited into DRM fractions despite the lack of the NKG2D cytoplasmic domain and the signaling capacity of Dap10 (Figure 14). In addition, although devoid of an interaction with Dap10 and at far lower surface expression levels, minimal amounts of NKG2D could be detected within the DRM fraction (Figure 14E). These observations give important clues for the mechanism underlying NKG2D raft recruitment: NKG2D is distinct from the above-discussed SRR 2B4 as it is a type II transmembrane receptor and does not contain signaling motifs as 2B4, but instead associates with Dap10 [136, 138]. Despite these differences, NKG2D lipid raft recruitment seems to be independent from signal transduction mediating cellular activation, as the tyrosine-based signaling motif of Dap10 and the whole cytoplasmic domain were dispensable. This releases the raft recruitment of NKG2D from the need to associate with Dap10, PI3K or Grb2, which is phosphotyrosine-dependent and required for NKG2D-mediated cytotoxicity [143]. Therefore, similar to the data for 2B4, the lipid raft recruitment of NKG2D is likely to occur prior to the signal transduction of Dap10. This leaves several alternatives for discussion: The transmembrane domain of NKG2D, the extracellular part of NKG2D or each domain of Dap10 might assist the lipid raft recruitment. The overall influence of Dap10 seems to be rather low as trace amounts of NKG2D could be detected in the DRM fractions in absence of Dap10 and with about 30-fold decreased surface expression levels of NKG2D. This reduces the possibility of Dap10-mediated effects, such as associations with other proteins or modifications as palmitoylation or lubrication [162], that might influence NKG2D raft recruitment. Dependent on these data, a putative role of Dap10 should not be excluded though yet. The impact of Dap10 should be analyzed further to exclude a false interpretation of these sparse data.

Apart from oligomerization upon ligation, a direct effect of the extracellular part of NKG2D is unlikely. The transmembrane domain of NKG2D might harbor different possibilities for lipid raft recruitment: The first possibility is palmitoylation. Indeed, CSS-Palm [186] predicted a positive hit at cysteine 57 in the transmembrane domain of NKG2D. It is important to note that such a positive hit does not guarantee a palmitoylation at such site. So far, a palmitoylation of NKG2D has not been

described. Another possibility is the association with proteins which mediate lipid raft recruitment, for example cytoskeleton-associated proteins [174], such as the ERM proteins. Apart from the interaction with Dap10 via the arginine at position 66 in the transmembrane domain of NKG2D and the aspartate at position 57 of Dap10 [138], no direct interaction of NKG2D mediated by its transmembrane domain is known. Potential binding motifs as the CxC motif of 2B4 have not been identified in NKG2D and NKG2D seems to be regulated independent of transmembrane adaptors, e.g. as LAT [142]. A more likely influence on the lipid raft recruitment of NKG2D might be mediated by the nonpolar amino acids in the transmembrane domain of NKG2D that are located at the interface to the lipids of the membrane. These amino acids define possible association or binding sites for lipids and these can influence lipid raft recruitment [168]. This alone can influence the lipid raft association of NKG2D, but is even emphasized by the quaternary structure of NKG2D: A NKG2D homodimer is formed through disulfide-bridges, which associates with two Dap10 homodimers [138]. Thereby a single unligated NKG2D receptor complex comprises six transmembrane domains with a potential lubrication of each. Such a complex alone could trap certain lipids (as seen with other membrane proteins, e.g. caveolin-1 [168, 169]) and thereby obtain a lipid raft affinity. This would even increase upon ligation of the receptor as more ordered membrane is included into this raft by the clustering of the receptors. Such an effect of receptor complexes could be analyzed by the mutation of the cysteines forming intermolecular disulfide-bridges to alanines, thereby breaking the NKG2D complex down into trimers. The DRM recruitment of such mutants could be analyzed with the here-applied methods.

It is also interesting to note that previous studies observed a requirement for an intact cytoskeleton for NKG2D (similar to 2B4) to transduce activating signals [144]. Therefore, the mechanism underlying NKG2D raft recruitment might be similar to that of 2B4 in a general scheme: By the ligation of NKG2D, smaller NKG2D-containing nanodomains might be clustered to larger nanodomains with putative first signaling capacity. Signaling at this point could influence the cytoskeleton in order to recruit more NKG2D-containing nanodomains. In dependence of the cytoskeleton and probably a positive feedback loop, a macrodomain with full signaling capacity is formed. This macrodomain mediates NK cell activation at the cell contact site in case of an aNKIS.

Interestingly, Endt *et al* describe a basal recruitment of NKG2D into DRM fractions in absence of stimuli in some of their experiments and speculate for a higher lipid raft affinity of NKG2D compared to other raft-associated receptors [144]. We observed a similar effect in some experiments with NKG2D (data not shown). Indeed, the

NKG2D complex could result in a higher affinity towards lipid rafts in case of lubrication as compared to other receptors as 2B4, which could only harbor one lubricated transmembrane domain. Thus, NKG2D-containing nanodomains might coalesce more easily into detergent-resistant membrane domains.

Overall our data predict the main molecular mechanism of NKG2D recruitment into lipid rafts to reside in its transmembrane domain. The exact nature of this mechanism has to be resolved in future work, which will have to clarify whether NKG2D is palmitoylated and/or whether the homodimer of NKG2D is lubricated, e.g. by the association with sphingolipids.

4.2 Development of a Siglec-7 inhibitor

The sialic acid-binding receptor Siglec-7 is highly involved in the modulation of the immune system and has been implicated in immune surveillance against cancer [104, 105, 210], viral infections [108, 109] as well as bacterial infections [110, 111]. Additionally, it plays potential roles in tolerance against cells without high amounts of inhibitory ligands for other receptors, like erythrocytes or neuronal tissues.
The development of an inhibitor that can be of advantage for scientific as well as therapeutic approaches thus appears more and more reasonable.

In this work we discovered an inhibitor for Siglec-7 by screening for receptor-ligand affinity in a RBC inhibition assay, employing the described ability of Siglec-7 to bind RBCs [87, 91]. By a stepwise modification, inhibitors with a >3000-fold higher affinity compared to that of unmodified sialic acid (tables 10-11) were obtained. We assessed the ability of the inhibitor (compound 118, table 11) to affect NK cell function in ^{51}Cr release assays, but we could only observe functional effects in a fraction of the experiments. The lack of any effect of the inhibitor on K562 might be due to the high stimulation given by K562 to NK cells [211]. As activating and inhibitory signals in NK cells are integrated, a high activating stimulus by the K562 might override a weak inhibitory signal by Siglec-7 and thus result in full activation of the NK cells. Therefore, the presence of an inhibitor might not be detectable in this setting.
The fact that we only observed low effects in a fraction of the Mel1106 killing assays might be due to different underlying causes:
According to the literature, Siglec-7 can occur in two isoforms: One isoform with a V-set Ig-domain and one C2-set Ig-domain [87] and another isoform with a V-set Ig-

domain and two C2-set Ig-domains [91]. The expression of both isoforms on NK cells cannot be excluded so far. Although the C2-set domains have some implication for the ligand binding by Siglec-7 [86], the specificity of the inhibitor should not be altered by such differences. In contrast, the size of extracellular domains of NK cell receptors was shown to influence the signal integration [212]. The kinetic segregation model [213] states, that at the contact site of cells, the size of receptors can influence their segregation from this site. For example, very big receptors are unable to remain in the narrow synaptic cleft and are segregated from it. Thus, Siglec-7 isoforms with differing sizes might be segregated differentially and affect the integration of inhibitory signal by this receptor. It remains to be elucidated whether different cell types express only one isoform and whether isoform expression is donor-specific.

Binding of ligands on other cells by Siglec receptors is also dampened by their common *cis* interaction with ligands, which results in masking of the receptor [88]. Siglec receptors are capable to bind sialic acids on the same cell they are expressed on, resulting in *cis* interaction. Such binding decreases the capacity of a receptor to bind a ligand on a different cell, namely a *trans* interaction. Such *cis* masking might have decreased the potential interaction of Siglec-7 with ligands on the target cells; thereby abolishing Siglec-7 mediated inhibition [214]. However, *cis* masking was shown to be decreased after sialidase treatment or cell activation [88]. We exclusively used IL-2 stimulated NK cells in the ^{51}Cr release assays. This might have influenced the *cis* masking of Siglec-7. Previous studies with different Siglec receptors used phorbol ester and calcium ionophore stimulation to induce activation-mediated unmasking of the receptors [88] and it remains to be tested, whether the stimulation of NK cells with IL-2 alone is sufficient to induce unmasking in a similar fashion. A complete dependency on unmasking of the receptor is also unlikely, as studies described the *trans* interaction of Siglec-2 despite *cis* masking [215] and a Siglec-7-dependent inhibition of GD3-expressing target cells was detectable without prior sialidase-treatment [104]. These findings either imply that Siglecs can recognize *trans* ligands despite *cis* masking, which might involve the low affinity of most Siglec ligands and could be broken by high affinity ligands as GD3 on target cells. Or a fraction of Siglecs remains unmasked and able to bind *trans* ligands; the latter might be achieved by a segregated localization of Siglecs and *cis*-ligands in the plasma membrane. In fact, a segregation of Siglec-2 in certain membrane domains is known [83], though there are no such data for Siglec-7 yet.

Our data are also dependent on the inhibitory potential of Siglec-7 on NK cells. In our assays our inhibitor did only cause weak increases in lysis, but this small change might just resemble the whole capacity of Siglec-7 to influence NK cell activation. So

far, the direct inhibitory potential of Siglec-7 has not been related to the inhibitory potential of other receptors on NK cells, as KIRs for example. Siglec-7 might be limited in its inhibitory potential on NK cells and therefore the inhibition of Siglec-7 might not result in stronger effects.

Additionally, the experimental setting applied in our assays might not fulfill the optimal conditions for the observation of Siglec-7-mediated inhibition. In contrast to K562, Mel1106 cells are less stimulatory and have a higher load of inhibitory ligands. Maybe a different combination of activating and inhibitory ligands on the target cell would be more suited for this task. The perfect condition would include an inhibition of NK cells by the target cells only dependent on Siglec-7 whereas the activating stimuli should be weak enough for Siglec-7 to influence the overall NK cell activation. Future screenings of target cells might result in a better model for the analysis of Siglec-7 inhibitors.

Finally, although our inhibitor is >3000-fold more potent than the unmodified sialic acid, the affinity of the inhibitor might still be insufficient to cause a potent effect on the inhibition mediated by Siglec-7 in this experimental setup. Therefore, the further improvement of the inhibitor remains an important focus of future work.

The effects observed might in addition be caused by Siglec-7 independent mechanisms: As was shown in several studies so far, all CD33-related Siglecs and especially Siglec-7 and Siglec-9 are highly homologous and mainly differ in their ligand-binding V-set Ig-domain [80]. This homology between Siglec-7 and Siglec-9 is so high that the exchange of only a few amino acids in the C-C loop of the V-set Ig-domain was sufficient to completely exchange ligand specificities of the two receptors [95]. In addition, previous ligand screening studies for Siglec-7 and Siglec-9 showed certain redundancies in specificity, e.g. against α*2,3*- and α*2,6*-sialyl residues [95, 96]. Therefore, the effect observed in the killing assays could also be due to a Siglec-9 effect. This possibility appears unlikely though. First, on NK cells, Siglec-7 is more abundant than Siglec-9 ([87, 91-94] and our data). Second, the specificity of the inhibitor towards Siglec-7 is higher than for Siglec-9.

According to our data, side effects of toxicity mediated by the inhibitor seem unlikely as well. The possibility of a drastic effect caused by the Siglec-7 inhibitor in such a killing assay stays a matter of debate: Although the structure of Siglec-7 has been well established since its discovery and inhibitory function of Siglec-7 has been shown [90, 104], the signaling after receptor ligation remains less well understood. According to the presence of a membrane-proximal ITIM in the Siglec receptor, a similar role as with other inhibitory receptors on NK cells was assumed. Indeed, it

was shown that Siglec-7 is able to recruit the tyrosine phosphatases SHP-1 and SHP-2 upon cross-linking [102], although to a lower extent than other receptors [216]. However, a second signaling motif is present membrane-distal to the ITIM, which was assumed to be an ITSM in early studies but was recognized to be a distinct ITIM-like motif later on [83, 217]. The exact function of this ITIM-like motif still remains unknown, apart from the fact that mutation of the membrane proximal ITIM motif abolishes SHP-1 association but not SHP-2 association with the membrane-distal ITIM-like motif [102]. In another study, Siglec-7 was targeted by the 'Suppressor of cytokine signaling 3' (SOCS3) for degradation once phosphorylated. SOCS3 belongs to a family of negative feedback proteins that attenuate cytokine-induced signals. The common mechanism causes the ubiquitination of the signaling molecules and results in their degradation. Siglec-7 degradation occurred in an unusual mechanism degrading SOCS3 as well in the process [218]. Siglec-7 was even shown to mediate an non-apoptotic cell death without the involvement of the known signaling motifs [219]. These data show homologies to the inhibitory pathways of other inhibitory receptors on NK cells, but several discrepancies remain. Additionally, all these signaling studies were performed in model cell lines distinct from primary NK cells and therefore exact statements about the signaling processes downstream of Siglec-7 triggering should be regarded with care. Taken all these together, the exact regulation of Siglec-7 is not yet unraveled and therefore, its ligation and the inhibition of this ligation with an inhibitor might result in yet ambiguous data.

Overall, our inhibitor effectively blocks Siglec-7 ligand interaction and is the first inhibitor able to modulate the activity of human NK cells. This study hopefully will promote the further development of Siglec inhibitors by providing information about the structural requirements of a Siglec-7 inhibitor.

In conclusion, this study further unraveled the molecular bases of lipid raft recruitment of the SLAM-related receptor 2B4 and the C-type lectin-like receptor NKG2D. Both of these receptors are crucially dependent on the structure of their transmembrane domain, but independent from signal transduction, for their localization into lipid rafts after ligation. In case of 2B4, the receptor is especially dependent on a CxC motif at the cytoplasmic side of the transmembrane domain and to a lesser extent dependent on the amino acids at the luminal side of transmembrane domain. These findings give profound information about the possible mechanisms leading to lipid raft recruitment and will enable a more specific analysis of these mechanisms.

Additionally, this study led to the development of the first specific Siglec-7 inhibitor with the potential to affect the activity of human NK cells. This outcome will aid in the future development of scientific and therapeutic tools in the field of Siglec research.

References

1. Vivier, E., et al., *Functions of natural killer cells.* Nature immunology, 2008. **9**(5): p. 503-10.
2. Herberman, R.B., et al., *Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells.* International journal of cancer. Journal international du cancer, 1975. **16**(2): p. 230-9.
3. Herberman, R.B., M.E. Nunn, and D.H. Lavrin, *Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity.* International journal of cancer. Journal international du cancer, 1975. **16**(2): p. 216-29.
4. Kiessling, R., et al., *"Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell.* European journal of immunology, 1975. **5**(2): p. 117-21.
5. Kiessling, R., E. Klein, and H. Wigzell, *"Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype.* European journal of immunology, 1975. **5**(2): p. 112-7.
6. Karre, K., et al., *Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy.* Nature, 1986. **319**(6055): p. 675-8.
7. Moretta, A., et al., *Receptors for HLA class-I molecules in human natural killer cells.* Annual review of immunology, 1996. **14**: p. 619-48.
8. Long, E.O., *Regulation of immune responses through inhibitory receptors.* Annual review of immunology, 1999. **17**: p. 875-904.
9. Trinchieri, G. and N. Valiante, *Receptors for the Fc fragment of IgG on natural killer cells.* Natural immunity, 1993. **12**(4-5): p. 218-34.
10. Valiante, N.M. and G. Trinchieri, *Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes.* J Exp Med, 1993. **178**(4): p. 1397-406.
11. Moretta, A., et al., *Inhibitory and activatory receptors for HLA class I molecules in human natural killer cells.* Chemical immunology, 1996. **64**: p. 77-87.
12. Raulet, D.H., *Interplay of natural killer cells and their receptors with the adaptive immune response.* Nature immunology, 2004. **5**(10): p. 996-1002.
13. Salazar-Mather, T.P., J.S. Orange, and C.A. Biron, *Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1alpha (MIP-1alpha)-dependent pathways.* The Journal of experimental medicine, 1998. **187**(1): p. 1-14.
14. Dokun, A.O., et al., *Specific and nonspecific NK cell activation during virus infection.* Nature immunology, 2001. **2**(10): p. 951-6.
15. Blom, B. and H. Spits, *Development of human lymphoid cells.* Annual review of immunology, 2006. **24**: p. 287-320.
16. Walzer, T., et al., *Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(9): p. 3384-9.

17. Walzer, T., et al., *Natural killer cells: from CD3(-)NKp46(+) to post-genomics meta-analyses.* Current opinion in immunology, 2007. **19**(3): p. 365-72.
18. Cella, M., et al., *A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity.* Nature, 2009. **457**(7230): p. 722-5.
19. Sanos, S.L., et al., *RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells.* Nature immunology, 2009. **10**(1): p. 83-91.
20. Colucci, F., M.A. Caligiuri, and J.P. Di Santo, *What does it take to make a natural killer?* Nature reviews. Immunology, 2003. **3**(5): p. 413-25.
21. Biron, C.A., et al., *Natural killer cells in antiviral defense: function and regulation by innate cytokines.* Annual review of immunology, 1999. **17**: p. 189-220.
22. Caligiuri, M.A., *Human natural killer cells.* Blood, 2008. **112**(3): p. 461-9.
23. Freud, A.G., et al., *Evidence for discrete stages of human natural killer cell differentiation in vivo.* The Journal of experimental medicine, 2006. **203**(4): p. 1033-43.
24. Romagnani, C., et al., *CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation.* Journal of immunology, 2007. **178**(8): p. 4947-55.
25. Burt, B.M., et al., *The lytic potential of human liver NK cells is restricted by their limited expression of inhibitory killer Ig-like receptors.* Journal of immunology, 2009. **183**(3): p. 1789-96.
26. Gregoire, C., et al., *The trafficking of natural killer cells.* Immunological reviews, 2007. **220**: p. 169-82.
27. Manaster, I. and O. Mandelboim, *The unique properties of uterine NK cells.* American journal of reproductive immunology, 2010. **63**(6): p. 434-44.
28. Cerwenka, A. and L.L. Lanier, *Natural killer cells, viruses and cancer.* Nature reviews. Immunology, 2001. **1**(1): p. 41-9.
29. Zamai, L., et al., *NK cells and cancer.* Journal of immunology, 2007. **178**(7): p. 4011-6.
30. Kaufmann, S.H., *Immunity to intracellular bacteria.* Annual review of immunology, 1993. **11**: p. 129-63.
31. Korbel, D.S., O.C. Finney, and E.M. Riley, *Natural killer cells and innate immunity to protozoan pathogens.* International journal for parasitology, 2004. **34**(13-14): p. 1517-28.
32. Wood, S.M., H.G. Ljunggren, and Y.T. Bryceson, *Insights into NK cell biology from human genetics and disease associations.* Cellular and molecular life sciences : CMLS, 2011. **68**(21): p. 3479-93.
33. Lieberman, J., *The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal.* Nat Rev Immunol, 2003. **3**(5): p. 361-70.
34. Chavez-Galan, L., et al., *Cell death mechanisms induced by cytotoxic lymphocytes.* Cellular & molecular immunology, 2009. **6**(1): p. 15-25.
35. Moretta, A., et al., *NK cells at the interface between innate and adaptive immunity.* Cell death and differentiation, 2008. **15**(2): p. 226-33.

36. Ferlazzo, G., et al., *Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells.* The Journal of experimental medicine, 2002. **195**(3): p. 343-51.
37. Vitale, M., et al., *NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKp30 triggering receptor.* Blood, 2005. **106**(2): p. 566-71.
38. Welte, S., Kuttruff, S., Waldhauer, I., and Steinle, A., *Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction.* Nat. Immunol., 2006. **7**(12): p. 1334-1342.
39. Bryceson, Y.T., et al., *Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients.* Blood, 2007. **110**(6): p. 1906-15.
40. Rudd, E., et al., *Spectrum, and clinical and functional implications of UNC13D mutations in familial haemophagocytic lymphohistiocytosis.* Journal of medical genetics, 2008. **45**(3): p. 134-41.
41. Jessen, B., et al., *Subtle differences in CTL cytotoxicity determine susceptibility to hemophagocytic lymphohistiocytosis in mice and humans with Chediak-Higashi syndrome.* Blood, 2011. **118**(17): p. 4620-9.
42. Tabiasco, J., et al., *Human decidual NK cells: unique phenotype and functional properties -- a review.* Placenta, 2006. **27 Suppl A**: p. S34-9.
43. Orange, J.S., et al., *The mature activating natural killer cell immunologic synapse is formed in distinct stages.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(24): p. 14151-6.
44. Riteau, B., D.F. Barber, and E.O. Long, *Vav1 phosphorylation is induced by beta2 integrin engagement on natural killer cells upstream of actin cytoskeleton and lipid raft reorganization.* The Journal of experimental medicine, 2003. **198**(3): p. 469-74.
45. Orange, J.S., *Formation and function of the lytic NK-cell immunological synapse.* Nat Rev Immunol, 2008. **8**(9): p. 713-25.
46. Dustin, M.L., *Modular design of immunological synapses and kinapses.* Cold Spring Harb Perspect Biol, 2009. **1**(1): p. a002873.
47. Bryceson, Y.T., et al., *Activation, coactivation, and costimulation of resting human natural killer cells.* Immunol Rev, 2006. **214**: p. 73-91.
48. Bryceson, Y.T., et al., *Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion.* Blood, 2006. **107**(1): p. 159-66.
49. Lou, Z., et al., *A balance between positive and negative signals in cytotoxic lymphocytes regulates the polarization of lipid rafts during the development of cell-mediated killing.* The Journal of experimental medicine, 2000. **191**(2): p. 347-54.
50. Vyas, Y.M., et al., *Spatial organization of signal transduction molecules in the NK cell immune synapses during MHC class I-regulated noncytolytic and cytolytic interactions.* J Immunol, 2001. **167**(8): p. 4358-67.
51. Billadeau, D.D., et al., *The Vav-Rac1 pathway in cytotoxic lymphocytes regulates the generation of cell-mediated killing.* J Exp Med, 1998. **188**(3): p. 549-59.

52. Stebbins, C.C., et al., *Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity.* Mol Cell Biol, 2003. **23**(17): p. 6291-9.
53. Zhou, F., *Molecular mechanisms of viral immune evasion proteins to inhibit MHC class I antigen processing and presentation.* International reviews of immunology, 2009. **28**(5): p. 376-93.
54. Long, E.O., *Negative signaling by inhibitory receptors: the NK cell paradigm.* Immunol Rev, 2008. **224**: p. 70-84.
55. Moretta, L. and A. Moretta, *Killer immunoglobulin-like receptors.* Current opinion in immunology, 2004. **16**(5): p. 626-33.
56. Lanier, L.L., *Up on the tightrope: natural killer cell activation and inhibition.* Nat. Immunol. , 2008. **9**(5): p. 495-502.
57. Watzl, C. and E.O. Long, *Signal transduction during activation and inhibition of natural killer cells.* Curr Protoc Immunol, 2010. **Chapter 11**: p. Unit 11 9B.
58. Lanier, L.L., *NK cell receptors.* Annu Rev Immunol, 1998. **16**: p. 359-93.
59. Borrego, F., et al., *The CD94/NKG2 family of receptors: from molecules and cells to clinical relevance.* Immunologic research, 2006. **35**(3): p. 263-78.
60. Borrego, F., et al., *Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis.* The Journal of experimental medicine, 1998. **187**(5): p. 813-8.
61. Brown, D., J. Trowsdale, and R. Allen, *The LILR family: modulators of innate and adaptive immune pathways in health and disease.* Tissue antigens, 2004. **64**(3): p. 215-25.
62. Aldemir, H., et al., *Cutting edge: lectin-like transcript 1 is a ligand for the CD161 receptor.* Journal of immunology, 2005. **175**(12): p. 7791-5.
63. Lebbink, R.J., et al., *Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1.* The Journal of experimental medicine, 2006. **203**(6): p. 1419-25.
64. Meyaard, L., et al., *LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes.* Immunity, 1997. **7**(2): p. 283-90.
65. Ito, M., et al., *Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity.* The Journal of experimental medicine, 2006. **203**(2): p. 289-95.
66. Raulet, D.H., R.E. Vance, and C.W. McMahon, *Regulation of the natural killer cell receptor repertoire.* Annual review of immunology, 2001. **19**: p. 291-330.
67. Orr, M.T. and L.L. Lanier, *Natural killer cell education and tolerance.* Cell, 2010. **142**(6): p. 847-56.
68. Fernandez, N.C., et al., *A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules.* Blood, 2005. **105**(11): p. 4416-23.
69. Gasser, S. and D.H. Raulet, *Activation and self-tolerance of natural killer cells.* Immunological reviews, 2006. **214**: p. 130-42.

70. Kim, S., et al., *Licensing of natural killer cells by host major histocompatibility complex class I molecules.* Nature, 2005. **436**(7051): p. 709-13.
71. Yokoyama, W.M. and S. Kim, *Licensing of natural killer cells by self-major histocompatibility complex class I.* Immunological reviews, 2006. **214**: p. 143-54.
72. Brodin, P., et al., *The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells.* Blood, 2009. **113**(11): p. 2434-41.
73. Brodin, P., K. Karre, and P. Hoglund, *NK cell education: not an on-off switch but a tunable rheostat.* Trends in immunology, 2009. **30**(4): p. 143-9.
74. Orr, M.T., W.J. Murphy, and L.L. Lanier, *'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection.* Nature immunology, 2010. **11**(4): p. 321-7.
75. Joncker, N.T., et al., *Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment.* The Journal of experimental medicine, 2010. **207**(10): p. 2065-72.
76. Lehmann, F., E. Tiralongo, and J. Tiralongo, *Sialic acid-specific lectins: occurrence, specificity and function.* Cellular and molecular life sciences : CMLS, 2006. **63**(12): p. 1331-54.
77. Traving, C. and R. Schauer, *Structure, function and metabolism of sialic acids.* Cellular and molecular life sciences : CMLS, 1998. **54**(12): p. 1330-49.
78. Lopez, P.H. and R.L. Schnaar, *Gangliosides in cell recognition and membrane protein regulation.* Curr Opin Struct Biol, 2009. **19**(5): p. 549-57.
79. Gamblin, S.J. and J.J. Skehel, *Influenza hemagglutinin and neuraminidase membrane glycoproteins.* The Journal of biological chemistry, 2010. **285**(37): p. 28403-9.
80. Cao, H. and P.R. Crocker, *Evolution of CD33-related siglecs: regulating host immune functions and escaping pathogen exploitation?* Immunology, 2011. **132**(1): p. 18-26.
81. Angata, T., et al., *Large-scale sequencing of the CD33-related Siglec gene cluster in five mammalian species reveals rapid evolution by multiple mechanisms.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(36): p. 13251-6.
82. Varki, A. and T. Angata, *Siglecs--the major subfamily of I-type lectins.* Glycobiology, 2006. **16**(1): p. 1R-27R.
83. Crocker, P.R., J.C. Paulson, and A. Varki, *Siglecs and their roles in the immune system.* Nature reviews. Immunology, 2007. **7**(4): p. 255-66.
84. Crocker, P.R. and A. Varki, *Siglecs, sialic acids and innate immunity.* Trends in immunology, 2001. **22**(6): p. 337-42.
85. Vitale, C., et al., *Engagement of p75/AIRM1 or CD33 inhibits the proliferation of normal or leukemic myeloid cells.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(26): p. 15091-6.
86. Varki, A. and P.R. Crocker, *I-type Lectins*, in *Essentials of Glycobiology*, A. Varki, et al., Editors. 2009: Cold Spring Harbor (NY).

87. Angata, T. and A. Varki, *Siglec-7: a sialic acid-binding lectin of the immunoglobulin superfamily.* Glycobiology, 2000. **10**(4): p. 431-8.
88. Razi, N. and A. Varki, *Cryptic sialic acid binding lectins on human blood leukocytes can be unmasked by sialidase treatment or cellular activation.* Glycobiology, 1999. **9**(11): p. 1225-34.
89. Hernandez-Caselles, T., et al., *A study of CD33 (SIGLEC-3) antigen expression and function on activated human T and NK cells: two isoforms of CD33 are generated by alternative splicing.* Journal of leukocyte biology, 2006. **79**(1): p. 46-58.
90. Falco, M., et al., *Identification and molecular cloning of p75/AIRM1, a novel member of the sialoadhesin family that functions as an inhibitory receptor in human natural killer cells.* The Journal of experimental medicine, 1999. **190**(6): p. 793-802.
91. Nicoll, G., et al., *Identification and characterization of a novel siglec, siglec-7, expressed by human natural killer cells and monocytes.* J Biol Chem, 1999. **274**(48): p. 34089-95.
92. Angata, T. and A. Varki, *Cloning, characterization, and phylogenetic analysis of siglec-9, a new member of the CD33-related group of siglecs. Evidence for co-evolution with sialic acid synthesis pathways.* J Biol Chem, 2000. **275**(29): p. 22127-35.
93. Foussias, G., G.M. Yousef, and E.P. Diamandis, *Identification and molecular characterization of a novel member of the siglec family (SIGLEC9).* Genomics, 2000. **67**(2): p. 171-8.
94. Zhang, J.Q., et al., *Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes.* J Biol Chem, 2000. **275**(29): p. 22121-6.
95. Yamaji, T., et al., *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, 2002. **277**(8): p. 6324-32.
96. Blixt, O., et al., *Sialoside specificity of the siglec family assessed using novel multivalent probes: identification of potent inhibitors of myelin-associated glycoprotein.* The Journal of biological chemistry, 2003. **278**(33): p. 31007-19.
97. Attrill, H., et al., *The structure of siglec-7 in complex with sialosides: leads for rational structure-based inhibitor design.* Biochem J, 2006. **397**(2): p. 271-8.
98. Attrill, H., et al., *Siglec-7 undergoes a major conformational change when complexed with the alpha(2,8)-disialylganglioside GT1b.* J Biol Chem, 2006. **281**(43): p. 32774-83.
99. Dimasi, N., et al., *Structure of the saccharide-binding domain of the human natural killer cell inhibitory receptor p75/AIRM1.* Acta Crystallogr D Biol Crystallogr, 2004. **60**(Pt 2): p. 401-3.
100. Dimasi, N., et al., *Expression, crystallization and preliminary crystallographic analysis of the extracellular IgV-like domain of the human natural killer cell inhibitory receptor p75/AIRM1.* Acta Crystallogr D Biol Crystallogr, 2003. **59**(Pt 10): p. 1856-8.
101. Alphey, M.S., et al., *High resolution crystal structures of Siglec-7. Insights into ligand specificity in the Siglec family.* J Biol Chem, 2003. **278**(5): p. 3372-7.

102. Avril, T., et al., *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, 2004. **173**(11): p. 6841-9.
103. Rapoport, E., et al., *Ganglioside binding pattern of CD33-related siglecs.* Bioorg Med Chem Lett, 2003. **13**(4): p. 675-8.
104. Nicoll, G., et al., *Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms.* Eur J Immunol, 2003. **33**(6): p. 1642-8.
105. Kawasaki, Y., et al., *Ganglioside DSGb5, preferred ligand for Siglec-7, inhibits NK cell cytotoxicity against renal cell carcinoma cells.* Glycobiology, 2010. **20**(11): p. 1373-9.
106. Backstrom, E., et al., *Direct NK cell-mediated lysis of syngenic dorsal root ganglia neurons in vitro.* Journal of immunology, 2000. **165**(9): p. 4895-900.
107. Urmacher, C., C. Cordon-Cardo, and A.N. Houghton, *Tissue distribution of GD3 ganglioside detected by mouse monoclonal antibody R24.* The American Journal of dermatopathology, 1989. **11**(6): p. 577-81.
108. Brunetta, E., et al., *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, 2009. **114**(18): p. 3822-30.
109. Brunetta, E., K.L. Hudspeth, and D. Mavilio, *Pathologic natural killer cell subset redistribution in HIV-1 infection: new insights in pathophysiology and clinical outcomes.* J Leukoc Biol, 2010. **88**(6): p. 1119-30.
110. Avril, T., et al., *Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on Campylobacter jejuni lipooligosaccharides.* Infect Immun, 2006. **74**(7): p. 4133-41.
111. Khatua, B., et al., *Sialic acids acquired by Pseudomonas aeruginosa are involved in reduced complement deposition and siglec mediated host-cell recognition.* FEBS Lett, 2010. **584**(3): p. 555-61.
112. Pessino, A., et al., *Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity.* J Exp Med, 1998. **188**(5): p. 953-60.
113. Cantoni, C., et al., *NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily.* J Exp Med, 1999. **189**(5): p. 787-96.
114. Pende, D., Parolini, S., Pessino, Sivori, S., Augugliaro, R., Morelli, L., Marcenaro, E., Accame, L., Malaspina, A., Biassoni, R., Bottino, C., Moretta, L., and Moretta, A., *Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells.* J. Exp. Med., 1999. **190**(10): p. 1505-1516.
115. Arnon, T.I., et al., *Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus.* Nature immunology, 2005. **6**(5): p. 515-23.
116. Arnon, T.I., et al., *Recognition of viral hemagglutinins by NKp44 but not by NKp30.* Eur J Immunol, 2001. **31**(9): p. 2680-9.

117. Arnon, T.I., G. Markel, and O. Mandelboim, *Tumor and viral recognition by natural killer cells receptors.* Seminars in cancer biology, 2006. **16**(5): p. 348-58.
118. Brandt, C.S., et al., *The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans.* J Exp Med, 2009. **206**(7): p. 1495-503.
119. Pogge von Strandmann, E., Simhadri, V. R., von Tresckow, B., Sasse, S., Reiners, K. S., Hansen, H. P., Rothe, A., Böll, B., Simhadri, V. L., Borchmann , P., McKinnon, P. J., Hallek, M. and Engert, A., *Human leukocyte antigen-B associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells.* Immunity, 2007. **27**: p. 965-974.
120. Claus, M., et al., *Regulation of NK cell activity by 2B4, NTB-A and CRACC.* Front Biosci, 2008. **13**: p. 956-65.
121. Bouchon, A., et al., *Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family.* Journal of immunology, 2001. **167**(10): p. 5517-21.
122. Bottino, C., et al., *NTB-A [correction of GNTB-A], a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease.* The Journal of experimental medicine, 2001. **194**(3): p. 235-46.
123. Elishmereni, M. and F. Levi-Schaffer, *CD48: A co-stimulatory receptor of immunity.* The international journal of biochemistry & cell biology, 2011. **43**(1): p. 25-8.
124. Watzl, C. and E.O. Long, *Natural killer cell inhibitory receptors block actin cytoskeleton-dependent recruitment of 2B4 (CD244) to lipid rafts.* J Exp Med, 2003. **197**(1): p. 77-85.
125. Watzl, C., C.C. Stebbins, and E.O. Long, *NK cell inhibitory receptors prevent tyrosine phosphorylation of the activation receptor 2B4 (CD244).* J Immunol, 2000. **165**(7): p. 3545-8.
126. Tangye, S.G., et al., *Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP.* Journal of immunology, 1999. **162**(12): p. 6981-5.
127. Saborit-Villarroya, I., et al., *The adaptor protein 3BP2 binds human CD244 and links this receptor to Vav signaling, ERK activation, and NK cell killing.* Journal of immunology, 2005. **175**(7): p. 4226-35.
128. Bloch-Queyrat, C., et al., *Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn.* The Journal of experimental medicine, 2005. **202**(1): p. 181-92.
129. Tangye, S.G., et al., *Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome.* Journal of immunology, 2000. **165**(6): p. 2932-6.
130. Parolini, S., et al., *X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells.* The Journal of experimental medicine, 2000. **192**(3): p. 337-46.

131. Nichols, K.E., et al., *Molecular and cellular pathogenesis of X-linked lymphoproliferative disease.* Immunological reviews, 2005. **203**: p. 180-99.
132. Nakajima, H., et al., *Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity.* European journal of immunology, 2000. **30**(11): p. 3309-18.
133. Benoit, L., et al., *Defective NK cell activation in X-linked lymphoproliferative disease.* Journal of immunology, 2000. **165**(7): p. 3549-53.
134. Eissmann, P., et al., *Molecular basis for positive and negative signaling by the natural killer cell receptor 2B4 (CD244).* Blood, 2005. **105**(12): p. 4722-9.
135. Chlewicki, L.K., et al., *Molecular basis of the dual functions of 2B4 (CD244).* Journal of immunology, 2008. **180**(12): p. 8159-67.
136. Bauer, S., et al., *Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA.* Science, 1999. **285**(5428): p. 727-9.
137. Garrity, D., et al., *The activating NKG2D receptor assembles in the membrane with two signaling dimers into a hexameric structure.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(21): p. 7641-6.
138. Wu, J., et al., *An activating immunoreceptor complex formed by NKG2D and DAP10.* Science, 1999. **285**(5428): p. 730-2.
139. Vivier, E., E. Tomasello, and P. Paul, *Lymphocyte activation via NKG2D: towards a new paradigm in immune recognition?* Current opinion in immunology, 2002. **14**(3): p. 306-11.
140. Stern-Ginossar, N. and O. Mandelboim, *An integrated view of the regulation of NKG2D ligands.* Immunology, 2009. **128**(1): p. 1-6.
141. Guerra, N., et al., *NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy.* Immunity, 2008. **28**(4): p. 571-80.
142. Billadeau, D.D., et al., *NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway.* Nature immunology, 2003. **4**(6): p. 557-64.
143. Upshaw, J.L., et al., *NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells.* Nature immunology, 2006. **7**(5): p. 524-32.
144. Endt, J., et al., *Inhibitory receptor signals suppress ligation-induced recruitment of NKG2D to GM1-rich membrane domains at the human NK cell immune synapse.* J Immunol, 2007. **178**(9): p. 5606-11.
145. Cantor, J.M., M.H. Ginsberg, and D.M. Rose, *Integrin-associated proteins as potential therapeutic targets.* Immunological reviews, 2008. **223**: p. 236-51.
146. Davis, S.J., et al., *CD2 and the nature of protein interactions mediating cell-cell recognition.* Immunological reviews, 1998. **163**: p. 217-36.
147. Bottino, C., et al., *Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule.* J Exp Med, 2003. **198**(4): p. 557-67.
148. Shibuya, A., et al., *DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes.* Immunity, 1996. **4**(6): p. 573-81.

149. Le Bouteiller, P., et al., *CD160: a unique activating NK cell receptor.* Immunology letters, 2011. **138**(2): p. 93-6.
150. Vitale, M., et al., *Identification of NKp80, a novel triggering molecule expressed by human NK cells.* Eur J Immunol, 2001. **31**(1): p. 233-42.
151. Spreu, J., et al., *Interaction of C-type lectin-like receptors NKp65 and KACL facilitates dedicated immune recognition of human keratinocytes.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(11): p. 5100-5.
152. Gorter, E. and F. Grendel, *On Bimolecular Layers of Lipoids on the Chromocytes of the Blood.* The Journal of experimental medicine, 1925. **41**(4): p. 439-43.
153. Ipsen, J.H., et al., *Phase equilibria in the phosphatidylcholine-cholesterol system.* Biochimica et biophysica acta, 1987. **905**(1): p. 162-72.
154. Lentz, B.R., D.A. Barrow, and M. Hoechli, *Cholesterol-phosphatidylcholine interactions in multilamellar vesicles.* Biochemistry, 1980. **19**(9): p. 1943-54.
155. Simons, K. and W.L. Vaz, *Model systems, lipid rafts, and cell membranes.* Annu Rev Biophys Biomol Struct, 2004. **33**: p. 269-95.
156. Garcia-Saez, A.J., S. Chiantia, and P. Schwille, *Effect of line tension on the lateral organization of lipid membranes.* The Journal of biological chemistry, 2007. **282**(46): p. 33537-44.
157. Niemela, P.S., M.T. Hyvonen, and I. Vattulainen, *Influence of chain length and unsaturation on sphingomyelin bilayers.* Biophysical journal, 2006. **90**(3): p. 851-63.
158. Veatch, S.L. and S.L. Keller, *Miscibility phase diagrams of giant vesicles containing sphingomyelin.* Physical review letters, 2005. **94**(14): p. 148101.
159. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 31-9.
160. Lingwood, D. and K. Simons, *Detergent resistance as a tool in membrane research.* Nat Protoc, 2007. **2**(9): p. 2159-65.
161. Munro, S., *Lipid rafts: elusive or illusive?* Cell, 2003. **115**(4): p. 377-88.
162. Lingwood, D. and K. Simons, *Lipid rafts as a membrane-organizing principle.* Science, 2010. **327**(5961): p. 46-50.
163. Levental, I., et al., *Palmitoylation regulates raft affinity for the majority of integral raft proteins.* Proc Natl Acad Sci U S A, 2010. **107**(51): p. 22050-4.
164. Sengupta, P., et al., *Structural determinants for partitioning of lipids and proteins between coexisting fluid phases in giant plasma membrane vesicles.* Biochimica et biophysica acta, 2008. **1778**(1): p. 20-32.
165. Engelman, D.M., *Membranes are more mosaic than fluid.* Nature, 2005. **438**(7068): p. 578-80.
166. Brown, D.A. and J.K. Rose, *Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface.* Cell, 1992. **68**(3): p. 533-44.
167. Cherukuri, A., M. Dykstra, and S.K. Pierce, *Floating the raft hypothesis: lipid rafts play a role in immune cell activation.* Immunity, 2001. **14**(6): p. 657-60.

168. Contreras, F.X., et al., *Specificity of intramembrane protein-lipid interactions.* Cold Spring Harbor perspectives in biology, 2011. **3**(6).
169. Murata, M., et al., *VIP21/caveolin is a cholesterol-binding protein.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(22): p. 10339-43.
170. Fantini, J., *How sphingolipids bind and shape proteins: molecular basis of lipid-protein interactions in lipid shells, rafts and related biomembrane domains.* Cellular and molecular life sciences : CMLS, 2003. **60**(6): p. 1027-32.
171. Kaiser, H.J., et al., *Lateral sorting in model membranes by cholesterol-mediated hydrophobic matching.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(40): p. 16628-33.
172. Mouritsen, O.G., *Model answers to lipid membrane questions.* Cold Spring Harbor perspectives in biology, 2011. **3**(9): p. a004622.
173. Lingwood, D., et al., *Plasma membranes are poised for activation of raft phase coalescence at physiological temperature.* Proc Natl Acad Sci U S A, 2008. **105**(29): p. 10005-10.
174. Chichili, G.R. and W. Rodgers, *Cytoskeleton-membrane interactions in membrane raft structure.* Cellular and molecular life sciences : CMLS, 2009. **66**(14): p. 2319-28.
175. Simons, K. and J.L. Sampaio, *Membrane organization and lipid rafts.* Cold Spring Harb Perspect Biol, 2011. **3**(10): p. a004697.
176. Hope, H.R. and L.J. Pike, *Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains.* Molecular biology of the cell, 1996. **7**(6): p. 843-51.
177. Fassett, M.S., et al., *Signaling at the inhibitory natural killer cell immune synapse regulates lipid raft polarization but not class I MHC clustering.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(25): p. 14547-52.
178. Villalba, M., et al., *Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells.* The Journal of cell biology, 2001. **155**(3): p. 331-8.
179. Masilamani, M., et al., *CD94/NKG2A inhibits NK cell activation by disrupting the actin network at the immunological synapse.* Journal of immunology, 2006. **177**(6): p. 3590-6.
180. Chakrabandhu, K., et al., *Palmitoylation is required for efficient Fas cell death signaling.* The EMBO journal, 2007. **26**(1): p. 209-20.
181. Kondadasula, S.V., et al., *Colocalization of the IL-12 receptor and FcgammaRIIIa to natural killer cell lipid rafts leads to activation of ERK and enhanced production of interferon-gamma.* Blood, 2008. **111**(8): p. 4173-83.
182. Rabot, M., A. Bensussan, and P. Le Bouteiller, *Engagement of the CD160 activating NK cell receptor leads to its association with CD2 in circulating human NK cells.* Transplant immunology, 2006. **17**(1): p. 36-8.
183. Zhang, W., R.P. Trible, and L.E. Samelson, *LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation.* Immunity, 1998. **9**(2): p. 239-46.

184. Garcia-Garcia, E., E.J. Brown, and C. Rosales, *Transmembrane mutations to FcgammaRIIA alter its association with lipid rafts: implications for receptor signaling.* Journal of immunology, 2007. **178**(5): p. 3048-58.
185. Klem, J., et al., *2B4 is constitutively associated with linker for the activation of T cells in glycolipid-enriched microdomains: properties required for 2B4 lytic function.* J Immunol, 2002. **169**(1): p. 55-62.
186. Ren, J., et al., *CSS-Palm 2.0: an updated software for palmitoylation sites prediction.* Protein engineering, design & selection : PEDS, 2008. **21**(11): p. 639-44.
187. Heerklotz, H., *Triton promotes domain formation in lipid raft mixtures.* Biophysical journal, 2002. **83**(5): p. 2693-701.
188. Salaun, C., J. Greaves, and L.H. Chamberlain, *The intracellular dynamic of protein palmitoylation.* The Journal of cell biology, 2010. **191**(7): p. 1229-38.
189. Orlean, P. and A.K. Menon, *Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycophospholipids.* Journal of lipid research, 2007. **48**(5): p. 993-1011.
190. Shenoy-Scaria, A.M., et al., *Cysteine3 of Src family protein tyrosine kinase determines palmitoylation and localization in caveolae.* The Journal of cell biology, 1994. **126**(2): p. 353-63.
191. Webb, Y., L. Hermida-Matsumoto, and M.D. Resh, *Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids.* The Journal of biological chemistry, 2000. **275**(1): p. 261-70.
192. Martin, B.R. and B.F. Cravatt, *Large-scale profiling of protein palmitoylation in mammalian cells.* Nature methods, 2009. **6**(2): p. 135-8.
193. Kolb, H.C., M.G. Finn, and K.B. Sharpless, *Click Chemistry: Diverse Chemical Function from a Few Good Reactions.* Angewandte Chemie, 2001. **40**(11): p. 2004-2021.
194. Resh, M.D., *Palmitoylation of ligands, receptors, and intracellular signaling molecules.* Science's STKE : signal transduction knowledge environment, 2006. **2006**(359): p. re14.
195. Bottino, C., et al., *Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells.* European journal of immunology, 2000. **30**(12): p. 3718-22.
196. Chuang, S.S., P.R. Kumaresan, and P.A. Mathew, *2B4 (CD244)-mediated activation of cytotoxicity and IFN-gamma release in human NK cells involves distinct pathways.* Journal of immunology, 2001. **167**(11): p. 6210-6.
197. Bosselut, R., et al., *Association of the adaptor molecule LAT with CD4 and CD8 coreceptors identifies a new coreceptor function in T cell receptor signal transduction.* The Journal of experimental medicine, 1999. **190**(10): p. 1517-26.
198. Shaw, A.S., et al., *Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the*

p56lck tyrosine protein kinase. Molecular and cellular biology, 1990. **10**(5): p. 1853-62.
199. Turner, J.M., et al., *Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs.* Cell, 1990. **60**(5): p. 755-65.
200. von Haller, P.D., et al., *Mass spectrometric characterization of proteins extracted from Jurkat T cell detergent-resistant membrane domains.* Proteomics, 2001. **1**(8): p. 1010-21.
201. Ritchie, K., et al., *The fence and picket structure of the plasma membrane of live cells as revealed by single molecule techniques (Review).* Molecular membrane biology, 2003. **20**(1): p. 13-8.
202. Almen, M.S., et al., *Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin.* BMC biology, 2009. **7**: p. 50.
203. Scheiffele, P., M.G. Roth, and K. Simons, *Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain.* The EMBO journal, 1997. **16**(18): p. 5501-8.
204. Lee, A.G., *Lipid-protein interactions in biological membranes: a structural perspective.* Biochimica et biophysica acta, 2003. **1612**(1): p. 1-40.
205. Yetukuri, L., et al., *Informatics and computational strategies for the study of lipids.* Molecular bioSystems, 2008. **4**(2): p. 121-7.
206. Ames, J.B., et al., *NMR structure of the natural killer cell receptor 2B4 (CD244): implications for ligand recognition.* Biochemistry, 2005. **44**(17): p. 6416-23.
207. Velikovsky, C.A., et al., *Structure of natural killer receptor 2B4 bound to CD48 reveals basis for heterophilic recognition in signaling lymphocyte activation molecule family.* Immunity, 2007. **27**(4): p. 572-84.
208. Porter, J.A., K.E. Young, and P.A. Beachy, *Cholesterol modification of hedgehog signaling proteins in animal development.* Science, 1996. **274**(5285): p. 255-9.
209. Chen, R., et al., *Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors.* Molecular and cellular biology, 2004. **24**(12): p. 5144-56.
210. Miyazaki, K., et al., *Loss of disialyl Lewis(a), the ligand for lymphocyte inhibitory receptor sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7) associated with increased sialyl Lewis(a) expression on human colon cancers.* Cancer Res, 2004. **64**(13): p. 4498-505.
211. Nishimura, M., et al., *Protection against natural killer cells by interferon-gamma treatment of K562 cells cannot be explained by augmented major histocompatibility complex class I expression.* Immunology, 1994. **83**(1): p. 75-80.
212. Kohler, K., et al., *Matched sizes of activating and inhibitory receptor/ligand pairs are required for optimal signal integration by human natural killer cells.* PloS one, 2010. **5**(11): p. e15374.
213. Davis, S.J. and P.A. van der Merwe, *The kinetic-segregation model: TCR triggering and beyond.* Nature immunology, 2006. **7**(8): p. 803-9.
214. Avril, T., et al., *Probing the cis interactions of the inhibitory receptor Siglec-7 with alpha2,8-disialylated ligands on natural killer cells and other leukocytes using glycan-specific antibodies and by analysis of*

alpha2,8-sialyltransferase gene expression. J Leukoc Biol, 2006. **80**(4): p. 787-96.
215. Lanoue, A., et al., *Interaction of CD22 with alpha2,6-linked sialoglycoconjugates: innate recognition of self to dampen B cell autoreactivity?* European journal of immunology, 2002. **32**(2): p. 348-55.
216. Yamaji, T., et al., *Characterization of inhibitory signaling motifs of the natural killer cell receptor Siglec-7: attenuated recruitment of phosphatases by the receptor is attributed to two amino acids in the motifs.* Glycobiology, 2005. **15**(7): p. 667-76.
217. Crocker, P.R. and A. Varki, *Siglecs in the immune system.* Immunology, 2001. **103**(2): p. 137-45.
218. Orr, S.J., et al., *SOCS3 targets Siglec 7 for proteasomal degradation and blocks Siglec 7-mediated responses.* J Biol Chem, 2007. **282**(6): p. 3418-22.
219. Mitsuki, M., et al., *Siglec-7 mediates nonapoptotic cell death independently of its immunoreceptor tyrosine-based inhibitory motifs in monocytic cell line U937.* Glycobiology, 2010. **20**(3): p. 395-402.

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AICL	Activation induced C-type lectin
ARG	Arginine
BSA	Bovine serum albumin
CD	Cluster of differentiation
Dap10	DNAX-activating protein of 10 kDa
Dap12	DNAX-activating protein of 12 kDa
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DRM	Detergent-resistant membrane
E. coli	*Escherichia coli*
ERM	Ezrin, radixin, moesin
E:T ratio	Effector to target ratio
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallizable
GD3	Disialoganglioside 3
GEF	Guanine nucleotide exchange factor
GM1	Monosialoganglioside 1
GPI	Glycosylphosphatidylinositol
h2B4	Human 2B4
HA	Hemagglutinin
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL	Interleukin
IP	Immunoprecipitation
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
KACL	Keratinocyte-associated C-type lectin
kDa	Kilo Dalton
KIR	Killer cell Ig-like receptor
LAK cell	Lymphokine-activated killer cell

LAT	Linker for activation of T cells
LILR	Leukocyte Ig-like receptor
LSM	Lymphocyte separation medium
LYS	Lysine
m2B4	Murine 2B4
MßCD	Methyl-ß-cyclodextrin
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIC	MHC class-I polypeptide-related chain
min	Minutes
MTOC	Microtubule organizing center
NCR	Natural cytotoxicity receptor
NEAA	Non-essential amino acids
NK cell	Natural killer cell
NKIS	NK cell immunological synapse
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PHA-P	Phytohemagglutinin
PI3K	Phosphatidyl-inositol-3-kinase
PLC	Phospholipase C
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
rIP	Relative inhibitory potential
SAP	SLAM-associated protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Siglec	Sialic acid-binding Ig-like lectins
SLAM	Signaling lymphocyte activation molecule
SOCS3	Suppressor of cytokine signaling 3
SRR	SLAM-related receptor
SYK	Spleen tyrosine kinase
TYR	Tyrosine
ULBP	UL16-binding protein
WB	Western blot
wt	wildtype

XLP	X-linked lymphoproliferative disease
x-link	Cross-link
ZAP-70	70 kDa zeta-chain associated protein