THE REGULATION OF NK CELL DETACHMENT FROM TARGET CELLS AS A KEY FACTOR FOR SERIAL KILLING AND EFFECTOR FUNCTION

Dissertation

submitted to the

Faculty of Chemistry and Biological Chemistry

at the Technische Universität Dortmund

for the degree of

Doctor of Natural Sciences

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Date of Disputation   02.06.2017, Dortmund

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- Mark Janssen, Youtube

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Acknowledgements

Als erstes möchte ich Carsten Watzl für die Möglichkeit danken hier meine Doktorarbeit zu schreiben. Vielen Dank für die super Betreuung und Dein Vertrauen in mich und meine Arbeit – in Dortmund und auf all den Konferenzen. Dein Enthusiasmus und Glauben in die Forschung ist ansteckend und all die Diskussionen haben mir sehr geholfen.

Außerdem vielen Dank an Prof. Jan Hengstler für die Übernahme der Zweitkorrektur und seine Begeisterung für die Forschung. Dies macht das IfaDo zu einem hervorragenden Ort der Wissenschaft.

Besonderen Dank an Maren und Doris für die großartige Hilfe über all die Jahre und dass sie letztendlich ihren inneren Rechtschreib-Monk an meiner Arbeit ausgelebt haben. Und natürlich an Biene und Nicole mit denen auch der ödeste Assay noch Spaß gemacht hat. Aber vor allem für die wunderbare Zeit mit euch in und außerhalb des Labors, für all den Spaß den wir hatten, das kollektive Dehydrieren und eure Freundschaft.

Außerdem möchte ich allen Mitgliedern der AG Watzl danken. Die gute Stimmung in den Büros, an den *Benches* und beim essen machen dieses Labor zu einem Ort wo man morgens gerne hinfährt. Es waren schöne 3,5 Jahre.

Asmaa Adel Rayan Mohammed	Kevin Bode	Nicole Dychus
Aylin Yurtseven	Laura Salinas Tejedor	Peter Bröde
Doris Teutsch	Linda Drenkelforth	Petra Netter
Doris Urlaub	Madeleine Dorsch	Sabine Wingert
Frank Fasbender	Maren Claus	Samantha Schaffner
Ilka Neumann	Martin Obholzer	Silvia Capellino
Isabel Prager	Max Erpenbach	Simone Reipschläger
Janine Duppach	Melanie Ebel	Stefan Gütgemann
Jürgen Damaschke	Mina Sandusky	

Und auch die anderen Mitarbeitern des IfaDo waren immer hilfsbereit. Besonderen Dank an Rosie, Agata und Sonja mit denen ich einige Projekte bearbeiten durfte.

Natürlich will ich meiner Familie und meinen Freunden für die Unterstützung und all die schönen Erlebnisse der letzten Jahre danken (Und für die guten Tipps: „Schick dein Paper doch einfach an *Nature*!"). Ich hab es sehr genossen in eurer Nähe zu sein, mit euch zu feiern und Zeit zu verbringen. Ohne euch geht es nicht! Und letztendlich vielen Dank an Jacqueline. Dafür, dass du immer für mich da bist, mich unterstützt und aufheiterst wenn es mal seien muss.

Abstract

Natural Killer cells efficiently recognize and kill infected or malignant cells and can eliminate several of these cells one after the other in a process called serial killing. The activation of NK cells and how they form conjugates with target cells is well studied, but for the efficient killing of multiple cells, NK cells also have to detach from the previous target cell. The process of detachment is still poorly understood; therefore analyzing the regulation of this process in more detail is important for the investigation of NK cell biology. I used flow cytometry based assays to analyze the adhesion and the detachment of NK cells and target cells.

The data show, that the detachment was different between fresh, IL-2 activated and cultured NK cells while the formation of conjugates was almost unchanged. Interestingly, blocking NK cell cytotoxicity inhibited detachment. Reducing target cell death by increasing their resistance against NK cells also reduced the detachment of NK cells and increased the production of the cytokines IFN-γ and TNF-α. Induction of NK cell independent target cell death had the opposite effect. The loss of ligands for activating NK cell receptors and the majority of adhesion molecules on the target cell membrane during cell death could be responsible for the NK cell detachment. Additionally, NK cells seem to sense soluble factors released by dying cells, like apoptotic bodies, which accelerated the detachment.

This demonstrates, that the detachment of NK cells is a highly regulated process, which depends on the activation status of the NK cell, ongoing receptor-ligand interactions and changes in the protein profile of the cell membrane after death of the target cell. Regulation of detachment is not only necessary for the efficiency of serial killing but also influences other NK cell effector functions like cytokine production.

Zusammenfassung

Natürliche Killerzellen (NK Zellen) erkennen sehr effizient kanzerogene oder virus-infizierte Zellen und können mehrere dieser Zellen nacheinander töten. Während die Aktivierung und der Aufbau eines Konjugats mit der Zielzelle besonders gut erforscht und verstanden sind, ist der Prozess des Ablösens von der Zielzelle noch wenig untersucht. Dabei ist das Ablösen der NK Zelle nach dem Tod der Zielzelle essenziell um mehrere Zielzellen schnell und effektiv nacheinander zu töten. In dieser Arbeit wurde die Regulation des Ablösens von NK Zellen detailliert untersucht. Mittels Durchflusszytometrie analysierte ich die Bildung und auch den Zerfall von Konjugaten der NK Zellen mit den Zielzellen.

Wir konnten zeigen, dass sich frische, IL-2 aktivierte und kultivierte NK Zellen unterschiedlich schnell von ihren Zielzellen ablösen. Dabei gibt es nur kleine Unterschiede in der Bildung der Konjugate zwischen den unterschiedlich aktivierten NK Zellen. Wurde die Zytotoxizität der NK Zellen inhibiert, dann reduzierte das den Zerfall von Konjugaten deutlich. Denselben Effekt konnten wir beobachten wenn die Resistenz der Zielzellen gegen NK Zellen erhöht wurde. Das langsamere Ablösen der NK Zellen führte in beiden Fällen zu einer Überproduktion der proinflammatorischen Zytokine TNF-α und IFN-γ. Den gegenteiligen Effekt, nämlich eine Beschleunigung des Zerfalls der Konjugate, konnten wir zeigen, wenn in den Zielzellen unabhängig von den NK Zellen Apoptose induziert wurde. Die Zielzellen verlieren nach dem durch NK Zellen induzierten Tod viele Adhäsionsproteine sowie einige ihrer aktivierenden Liganden. Dies könnte zum Ablösen der NK Zelle von der Zielzelle führen. Außerdem scheinen NK Zellen lösliche Faktoren wahrzunehmen, die von den sterbenden Zielzellen abgegeben werden und den Zerfall der Konjugate beschleunigen.

Ich konnte zeigen, dass das Ablösen von NK Zellen ein stark regulierter Prozess ist. Er ist abhängig von der Voraktivierung der NK Zelle, anhaltenden Rezeptor-Liganden Interaktionen und Veränderungen in der Proteinzusammensetzung der Zielzellmembran nach dem Tod der Zielzelle. Die Regulation des Ablösens der NK Zelle von der Zielzelle ist nicht nur für effizientes *Serial Killing* nötig, sondern beeinflusst auch andere Effektorfunktionen wie die Zytokinproduktion.

1 Introduction

1.1 A short introduction into NK cell biology

During hematopoiesis the common lymphoid progenitor (CLP) develops into one of three different lymphocytes: T, B or Natural Killer (NK) cells. T and B lymphocytes are classified as members of the adaptive immune system due to their ability to express a wide range of different receptors, gained through somatic recombination and hypermutation of genes (Boehm and Swann, 2014). NK cells in contrast are considered to be part of the innate immune system. Their receptors are germ line encoded and recognize specific molecular patterns on the outer cell membrane of other cells and need no prior sensitization. However, in the last 10 years it became more and more clear that NK cells have additional features of the adaptive immune system and can generate long-lived memory-like cells after contact to haptens, cytokines or virus-induced proteins (Cooper et al., 2009; O'Leary et al., 2006; Sun et al., 2009). NK cells are mainly present in the blood and constitute approximately 5-15% of all peripheral blood lymphocytes (PBLs). In case of an inflammation NK cells can migrate into organs and detect and kill diseased cells through direct cellular contact or the secretion of cytokines and attract other immune cells through the release of chemokines. Additionally, all organs harbor tissue resident NK cells, which can have distinctive features regarding the expression of receptors or effector proteins (Lysakova-Devine and O'Farrelly, 2014). NK cells are a heterogeneous group of cells defined by the expression of surface proteins. Commonly NK cells are defined as negative for the T cell receptor complex CD3 and positive for glycoprotein CD56 as well as the natural cytotoxicity receptor NKp46 (combined: $CD3^-CD56^+NKp46^+$ lymphocytes). Though, in the past decade many studies revealed that NK cells from different compartments of the body can have an individual protein composition, which makes the classification of cells complicated. NK cells are further divided into $CD56^{bright}$ cells, which are the main producer of cytokines and $CD56^{dim}$ cells, which express much more perforin and are more cytotoxic (Caligiuri, 2008; Vivier et al., 2008).

NK cells express two opposing types of receptors: 1) Activating receptors that bind different ligands on the cell surface, some of these are induced or upregulated by stressed and diseaseed cells. 2) Inhibitory receptors, most of which bind to MHC class I molecules on healthy cells and send an inhibiting signal to reduce NK cell activation. The activating and inhibiting signals are integrated and the outcome decides whether the cytotoxicity of the NK cell is induced or not. The secretion of perforin and Granzymes into the immunological synapse between the NK and target cell mainly mediates cytotoxicity. Additionally, NK cells can kill via stimulating death receptors on the target cell with TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) and the secretion of cytokines like Tumor Necrosis Factor (TNF)-α and Interferon (IFN)-γ (Campbell and Hasegawa, 2013).

1.2 Activation of NK cells by cytokines

The fight against diseased cells, pathogens and tumors involves the interaction of a broad range of different immune cells. They communicate via cytokines to control and synchronize the immune response. The cytokines Interleukin-2 for short time pre-activation and Interleukin-2, Interleukin-15 and Interleukin-21 for long time culture of the NK cells were used in this study.

Interleukin-2 (IL-2) is part of the common γ-chain cytokine family and a main activator of NK cells. It is mostly produced by activated T cells and activates NK cells, but also $CD4^+/CD8^+$ T cells and B cells. IL-2 is additionally produced by dendritic cells (DCs) at an early time point of an infection and induces proliferation of NK cells and increases cytotoxicity as well as the production of cytokines like IFN-γ (Granucci et al., 2001; London et al., 1986; Trinchieri et al., 1984; Young and Ortaldo, 1987). IL-2 binds to the IL-2 receptor, which consists of an α- and β-subunit and the common cytokine receptor γ-chain. $CD56^{dim}$ and $CD56^{bright}$ NK cells have different affinities for IL-2. While $CD56^{dim}$ NK cells express the intermediate-affinity receptor, $CD56^{bright}$ NK cells express the high affinity IL-2 receptor and can therefore already be affected by low-doses of IL-2 (Fehniger et al., 2003). After IL-2 binds to its receptor, JAK1/JAK3 signaling proteins attached to the intracellular domains of the receptor initiate activating signals. Different signaling pathways including PI3K, STAT and MAPK pathways are activated by IL-2 (Boyman and Sprent, 2012).

Interleukin-15 (IL-15) stimulates NK cell survival and can enhance the proliferation (Ferlazzo et al., 2004; Kennedy et al., 2000). In an inflammatory environment IL-15 is produced by many members of the innate immune system like DCs, monocytes and macrophages but also by epithelial cells (Fehniger and Caligiuri, 2001). IL-15 prolongs the survival of NK cells by enhancing the expression of anti-apoptotic protein Bcl-2, which counteracts cellular stress through inactivation of different caspases (Cory et al., 2003; Ranson et al., 2003). IL-15 exists as a soluble molecule and membrane bound on the surface of the expressing cell types. The stimulation via membrane bound IL-15 dramatically increases the proliferation of NK cells. Therefore in the *ex vivo* culture of NK cells, irradiated PBMCs or genetically modified K562, which express membrane bound IL-15, are added as feeder cells (Cho and Campana, 2009).

Interleukin-21 (IL-21) is, like IL-2 and IL-15, part of the IL-2 cytokine family. It is mainly produced by activated $CD4^+$ T cells and stimulates the expansion of NK cells and proliferation of T cells and B cells (Parrish-Novak et al., 2000). Additionally, IL-21 increases the production of perforin, Granzyme A and B and thereby increases the cytotoxicity against target cells like K562 (Skak et al., 2008). IL-21 treatment can double the expansion of *ex vivo* cultivated NK cells compared to cultivation with IL-2 and IL-15 alone (Granzin et al., 2016).

1.3   NK cell receptors

1.3.1   Balance of receptor signaling

The potential capacity of NK cells to bind and kill autologous cells makes it indispensable to highly regulate this cytotoxic process. If NK cells do not receive enough activating signals by stressed, malignant or infected cells, they fail to detect and kill them. This could cause a disease to spread and result in a menace for the whole organism. On the other hand overactive NK cells that cannot be inhibited by healthy cells are able to harm the body as well. Under these circumstances NK cells can damage tissues and provoke auto-immune diseases.
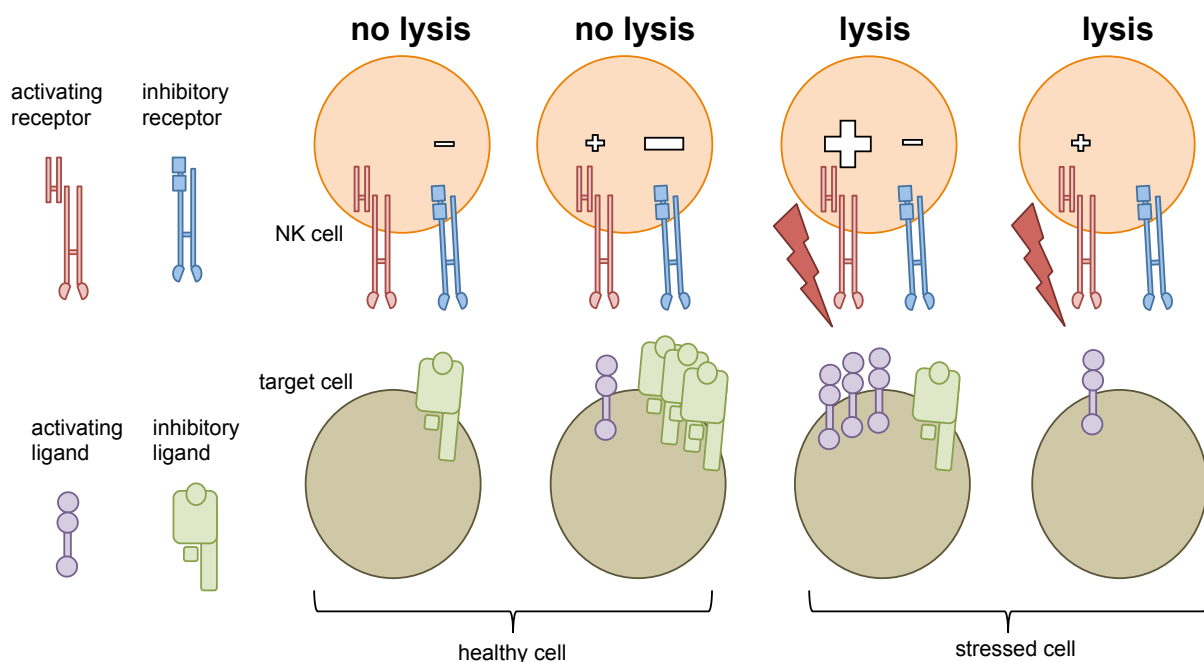
Figure 1. Schematic illustration of NK cell regulation. NK cells are regulated by germ-line encoded activating or inhibitory receptors. After ligation with its specific ligand, the receptors induce activating or inhibiting signals. Depending on which signal predominates the NK cell is activated and kills the target cell or this is inhibited. The absence of activating ligands or the presence of many inhibiting ligands leads to an inhibition of the NK cell and prevents the target cell lysis. In contrast, the overexpression of activating ligands or the loss of inhibiting ligands stimulate the NK cell and induce the target cell lysis.

The activity of NK cells is regulated by means of a complex system of activating and inhibiting signals. The signals are integrated and the outcome determines if the NK cell attempts to lyse the target cell or not. These signals are initiated by germ-line encoded receptors on the cell surface, which signal through their intracellular domains or adaptor proteins. They are discriminated in activating and inhibitory receptors even though there are overlaps. Stressed and diseased cells induce or increase the surface expression of ligands for activating receptors. Healthy cells have less of those activating ligands but express high amounts of MHC class I proteins, which are recognized by inhibitory NK cell receptors (Fogel et al., 2013; Long et al., 2013; Watzl, 2014). As depicted in Figure 1 the abundance of ligands for activating and inhibiting receptors is essential for the decision about NK cell activation. Target cells which lack ligands for activating receptors but express inhibitory MHC class I molecules

do not activate the NK cell and are left unaffected. This detection of healthy cells is entitled self-recognition. The presence or overexpression of activating ligands can induce NK cell cytotoxicity if the activating signal overwhelms the inhibitory signal. In accordance with the missing-self theory the loss or down-regulation of MHC class I by malignant or virus infected cells diminishes the inhibitory signal and induces lysis of the target cell (Raulet, 2006).

1.3.2 Activating NK cell receptors

NK cells have a broad range of germline encoded activating receptors. They recognize soluble ligands, surface proteins on potential target cells and cytokines. These receptors are associated intracellularly with adaptor proteins and signal upon ligation through the phosphorylation of downstream signaling molecules, which initiates NK cell effector functions. NK cell receptors can be grouped by the activation-motif they or their adaptors utilize. Natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 and the Fc receptor FcγRIIIA (CD16) are coupled to immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptor proteins FcεRIγ chain, CD3ζ chain or DNAX-activating protein of 12 kDa (DAP12). In humans the Natural Killer Group 2D (NKG2D) receptor associates exclusively with the YxxM tyrosine-based motif bearing DNAX-activating protein of 10 kDa (DAP10) adaptor protein and Signaling lymphocytic activating molecule (SLAM) family receptors 2B4, NTB-A and CRACC contain immunoreceptor tyrosine-based switch motifs (ITSM) that can couple to SLAM-associated protein (SAP) or EAT-2 (Long et al., 2013; Vivier et al., 2011; Watzl, 2014). Phosphorylation of these adaptor proteins by kinases leads to binding/activation of downstream signaling molecules like AKT, ERK and others. The detailed functions of the receptors, which are investigated in this thesis, are described in the following chapters.

1.3.2.1 Natural Killer Group 2D (NKG2D) receptor

One of the best-characterized NK cell receptors is the Natural Killer Group 2D (NKG2D) receptor. After it had been discovered, NKG2D could be identified as an activating receptor in NK cells (Houchins et al., 1991; Wu et al., 1999), which is encoded by the KLRK1 gene and consists of just two alleles (Glienke et al., 1998). Besides all NK cells NKG2D is expressed by CD8$^+$ T cells, NKT and γδ T cells and binds to ligands from two families related to MHC class I molecules. One ligand family consists of MHC class I polypeptide-related sequence A and B (MICA and MICB) and one of UL16 binding proteins 1-6 (ULBP1-6). All ligands are stress-induced and usually absent on healthy cells. The surface expression is only induced after infections with pathogens, transformation or another damage of the cell (Lopez-Larrea et al., 2008). In humans the NKG2D homodimer associates intracellularly with four chains of the transmembrane adaptor protein DAP10 (Garrity et al., 2005). This hexameric NKG2D:DAP10 structure initiates signal transduction through the YxxM tyrosine-based motif in the cytoplasmic tails of the DAP10 proteins, which can bind and activate further

signaling molecules. The following phosphorylation cascades are necessary for NK cell effector functions. DAP10 can bind the p85α subunit of phosphatidylinositol-3 kinase (PI3K) after phosphorylation (Wu et al., 1999), which then can phosphorylate and activate extracellular signal-regulated kinase (ERK) and ERK activator Mitogen-activated protein kinase kinase (MEK) (Upshaw and Leibson, 2006). Both molecules are crucial for NK cell cytotoxicity. But this signaling pathway alone is not sufficient to trigger NK cell cytotoxicity. The phosphorylation of VAV1 is mediated by the adaptor protein Growth factor receptor-bound protein 2 (Grb2) binding to DAP10 and is needed and crucial for the reorganization of the actin cytoskeleton and polarization of the microtubule-organizing center (MTOC) (Upshaw et al., 2006).

1.3.2.2 DNAX Accessory Molecule-1 (DNAM-1)

The surface protein DNAX accessory molecule (DNAM-1, CD226) is an activating receptor, which binds nectin and nectin-like molecules. Triggering of DNAM-1 by its ligands Nectin-2 (CD112) and PVR (CD155) initiates NK cell cytotoxicity and the production of IFN-γ (de Andrade et al., 2014). CD112 and CD155 are ubiquitously expressed but upregulated by solid tumors, lymphomas and virus-infected cells (Lenac Rovis et al., 2016; Qu et al., 2015; Sanchez-Correa et al., 2012). After ligation DNAM-1 is phosphorylated at its cytoplasmic tail via Src family kinases and associates with the adaptor protein Grb2. The signaling molecules VAV1, PI3K and Phospholipase C γ1 (PLCγ1) are activated and phosphorylate ERK, AKT and induce calcium flux (Zhang et al., 2015). These pathways result in the reorganization of the cytoskeleton by polymerization of actin, granule polarization and the production of IFN-γ. In the past, DNAM-1 and adhesion molecule lymphocyte function-associated antigen 1 (LFA-1) were considered to be a functional unit, which promotes adhesion to the target cell (Shibuya et al., 1999). But this association is less clear, since it was discovered recently, that LFA-1 is not critical for DNAM-1 signaling (Zhang et al., 2015). It is important to remark, that the DNAM-1 ligands CD112 and CD155 are also recognized by the inhibitory receptor T cell immunoreceptor with Ig and ITIM domains (TIGIT). Triggering of this receptor leads to the disruption of granule polarization and decreases the cytotoxicity of NK cells (Stanietsky et al., 2013; Stanietsky et al., 2009).

1.3.2.3 Natural Cytotoxicity Receptors

Natural cytotoxicity receptors (NCRs) are a group of three activating NK cell receptors. They include NKp30, NKp44 and NKp46, which all signal through ITAM bearing adaptor proteins. The first discovered member was NKp46 the expression of which is restricted mostly to NK cells (Sivori et al., 1997; Walzer et al., 2007). The cellular ligand for NKp46 remained elusive for a long time. Recently Vivier and colleagues discovered Properdin (alias Complement factor P; CFP) to be a ligand; a protein that is involved in the complement system and opsonizes diseased and apoptotic cells (Vivier, 2016; Xu et al., 2008). The receptor NKp44 was discovered shortly after NKp46 and is only expressed on

activated NK cells (Vitale et al., 1998). Three cellular ligands for NKp44 are known up to now: The proliferating cell nuclear antigen (PCNA) is overexpressed in cancer cells and inhibits NK cell function through activation of the cytoplasmic NKp44-ITIM motif (Rosental et al., 2011). The NKp44-L and the recently discovered platelet derived growth factor D (PDGF-D) activate NK cells through ITAM-signaling and are mainly expressed on virus infected or transformed cells (Baychelier et al., 2013; Colonna, 2016). The activating receptor NKp30 is expressed on all NK cells and binds to B7H6 and BAT-3 on stressed and infected cells (Brandt et al., 2009; Pende et al., 1999; Pogge von Strandmann et al., 2007). All three NCRs signal through ITAM motifs but use different adaptor proteins. After ligation, Src family kinases phosphorylate the FcεRIγ and CD3ζ chain linked to NKp46 and NKp30, or DAP12 linked to NKp44. The following assembly of a signaling complex involves the recruitment of the kinases ZAP70 and SYK, transmembrane adaptors like LAT and NTAL as well as cytosolic adaptor proteins like SLP76. Next, PI3K, PLC-γ1/2 and VAV2/3 can bind and are activated through phosphorylation and finally start the cytotoxic machinery (Long et al., 2013; Vivier et al., 2011; Watzl, 2014).

1.3.2.4   NK-T-B-antigen (NTB-A)

The co-activating receptor NTB-A belongs to the SLAM family receptors and is expressed by all NK, T and B cells. It binds homophilic to NTB-A proteins on the surface of potential target cells and stimulates cytotoxicity and the production of pro-inflammatory cytokines (Bottino et al., 2001; Flaig et al., 2004). A recent study suggests that NTB-A can additionally bind to viral hemagglutinin (HA) and co-stimulate the anti-viral activity of NK cells (Duev-Cohen et al., 2016). Upon ligation, NTB-A associates intracellularly with the adaptor proteins SAP and EAT-2 (Bottino et al., 2001; Eissmann and Watzl, 2006), which bind and activate downstream signaling molecules and induce the production of IFN-γ and TNF-α and boost NK cell cytotoxicity (Falco et al., 2004).

1.3.2.5   2B4-CD48

2B4 is another co-activating receptor of the SLAM receptor family and expressed on CD8^{+} T cells and NK cells (Nakajima et al., 1999; Tangye et al., 1999). It binds to CD48, which is ubiquitously expressed on hematopoietic cells but is upregulated on diseased cells (McArdel et al., 2016). The infection with Epstein-Barr virus (EBV) as well as the transformation of cells increases the expression of CD48 (Hosen et al., 2012; Thorley-Lawson et al., 1982). After ligation with CD48, 2B4 is phosphorylated at its intracellular ITSM domain and can bind the adaptor proteins SAP and EAT-2 . Dependent on the availability of adaptor proteins and together with a second activating signal, triggering of 2B4 induces the cytotoxicity and production of cytokines like IFN-γ (Johnson et al., 2000; Johnson et al., 2003; Messmer et al., 2006; Sivori et al., 2000). Recruitment of the Src-family kinase FynT starts a phosphorylation cascade, which activates LFA-1 through inside-out signaling and increases the

adhesion to the target cell (Hoffmann et al., 2011). As mentioned before, CD48 is also expressed on NK cells themselves and 2B4 is able to bind it *in cis* on the same cell. This *cis*-interaction diminishes the binding to CD48 *in trans* on the target cell and represents another mechanism of NK cell regulation (Claus et al., 2016b). Interestingly in absence of functional SAP, the phosphatases Src homology region 2 domain-containing phosphatase (SHP)-1/2 and SH2-containing Inositol 5'-Phosphatase (SHIP-1), can bind to the ITSM domains of 2B4. This leads to an inhibiting signal, which counteracts ITAM-mediated activation of the NK cell and weakens the immune response through dephosphorylation of activating signaling molecules like VAV1 (Meazza et al., 2014; Stepp et al., 1999b; Watzl and Claus, 2014).

1.3.3 Inhibitory Receptors

Inhibitory receptors on NK cells deliver signals, which can block activating signals and prevent lysis of the target cell. Most potent inhibitory receptors are receptors of the KIR2DLx or KIR3DLx family and NKG2A/CD94, which all bind to MHC class I proteins on the target cell. Other inhibitory receptors recognize non-MHC class I ligands like collagen (LAIR1), sialic acids (SIGLEC-3/7/9) or cadherins (KLRG1). Their cytoplasmic tail contains one or two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). After triggering of the receptor, Src family kinases phosphorylate these ITIMs and enable phosphatases SHIP-1, SHP-1 and SHP-2 or others to bind, which then catalyze the dephosphorylation and thereby inactivation of activating signaling molecules like VAV1/2/3, PI3K, AKT or SYK. NK cells express an individual repertoire of inhibitory receptors. It is essential that NK cells that lack inhibitory receptors for self-MHC class I and therefore are deficient in one mechanism to distinguish between healthy and diseased cells have a reduced activity. A process called education determines the responsiveness of NK cells depending on their inhibitory receptors (Brodin et al., 2009; Long et al., 2013; Watzl and Long, 2010).

1.3.3.1 The inhibitory receptor CD300a

The inhibitory receptor CD300a is part of the CD300 receptor family. It is expressed on all NK cells and a majority of other hematopoietic cells. The receptor consists of an IgV-like domain and an intracellular tail with four ITIMs (Bachelet et al., 2005; Cantoni et al., 1999). Phosphatidylserine (PS) and phosphatidylethanolamine (PE) were identified as ligands for CD300a. Both are not present on the outer side of the membrane of healthy cells but translocate to the cell surface of apoptotic and dying cells and act as an eat-me signal for phagocytes (Nakahashi-Oda et al., 2012; Simhadri et al., 2012). After ligation tyrosine residues in the ITIM motifs are phosphorylated by Src family tyrosine kinases, which enable phosphatases SHP-1 and SHP-2 to bind and being phosphorylated (Cantoni et al., 1999). Lankry *et al*. could show that CD300a on NK cells interacts with PS on the target cell. Blocking of this interaction with milk fat globule-EGF factor 8 protein (MFG-E8) increased the NK cell

cytotoxicity. MFG-E8 is a glycoprotein, which is secreted by activated macrophages and binds PS on apoptotic cells. This enables phagocytes to bind MFG-E8 via alpha(v)beta(3) integrin and engulf and eliminate the target cell (Hanayama et al., 2002). PS was also found to be overexpressed on several tumor cell lines and this was proposed to be a mechanism of tumor immune escape (Lankry et al., 2013).

1.3.4 NK cell secreted cytokines and chemokines

NK cell-mediated cellular cytotoxicity is not the only participation of NK cells to the human immune response. NK cells also secrete a broad range of cytokines and chemokines to interact with other immune cells or which directly attack the target cell. The best-known NK cell derived cytokines are Interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which are described in detail in this chapter. Interleukins secreted by NK cells after activation are the eosinophil activating IL-5, the anti-inflammatory IL-10 and the B cell activating IL-13 (Hoshino et al., 1999; Mehrotra et al., 1998; Warren et al., 1995). NK cells also produce the hematopoietic growth factor and immune modulator GM-CSF (Levitt et al., 1991). As part of the early immune response NK cells produce and secrete several chemokines, which guide other immune cells to the site of inflammation. The macrophage inflammatory protein (MIP)-1α/β, IL-8 and CCL5 (also RANTES) are released shortly after NK cell stimulation and attract other NK cells, macrophages, neutrophils and T cells (Fauriat et al., 2010; Griffith et al., 2014).

NK cells secrete IFN-γ upon stimulation with IL-12/18 or direct contact to target cells (Fauriat et al., 2010). Particularly the infection of target cells with viruses, bacteria or parasites stimulates the production of IFN-γ. It influences the immune response in two ways: It acts on the target cells directly and stimulates various other immune cells. On the target cell, binding of IFN-γ to the two IFN-γ receptor subunits activates janus kinase 1/2 (JAK1/2) and the MAPK pathway, which leads to the phosphorylation of the transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT3 (Kovarik et al., 1999). These enhance the expression of MHC class I proteins, which render the diseased cells more susceptible for T cell-mediated killing. The transcription of other proteins like death receptor CD95 or apoptosis inducing caspase-1 is also induced by STAT1 and STAT3 and make these cells more susceptible for immune surveillance (Lin et al., 2017; Shankaran et al., 2001; Shin et al., 2001). As an activator for immune cells IFN-γ can stimulate the proliferation and survival of CD4^{+} T cells (Reed et al., 2008) and contribute to the activation of cytotoxic CD8^{+} T lymphocytes (CTLs) (Giovarelli et al., 1988). The activation of macrophages by IFN-γ is essential for the clearance of bacteria (Nathan et al., 1984).

Within the first 3 h after target cell contact resting NK cells secrete soluble sTNF-α (Fauriat et al., 2010) and also express membrane bound mTNF-α on their surface (Caron et al., 1999). The ligation of

TNF-α with the death receptors TNF receptor 1 or 2 can induce different cellular responses. In the absence of anti-apoptotic protein TNF receptor-associated factor 2 (TRAF2), TNF-α induces apoptosis through caspase activation and thereby participates directly in killing of target cells (Micheau and Tschopp, 2003). Immune cells express TRAF2 and therefore TNF-α receptor 1 and 2 induce transcription factor NFκB activation and stimulate expression of anti-apoptotic and pro-inflammatory proteins (Cabal-Hierro and Lazo, 2012).

1.4 The immunological synapse

Cytotoxic lymphocytes can mediate cellular cytotoxicity inducing target cell apoptosis via delivery of cytotoxic proteins or stimulation of death receptors. The NK cell needs physical contact with the target cell. This specialized contact area is called immunological synapse (IS) and serves on the one hand as firm connection, which brings NK cell receptors and their ligands in close proximity and facilitates their interaction. On the other hand the IS functions as a sealed space into which lytic granules are secreted. The process of NK cell killing can be divided into 3 main steps: The formation of the IS, the mediation of cytotoxicity and the termination of the conjugate (Mace et al., 2014).

1.4.1 Formation of the immunological synapse

NK cells circulate in the peripheral blood or are resident in lymphoid or non-lymphoid tissues. Like other lymphocytes, NK cells are able to move directed and migrate in and through different tissues to monitor tissue cells and detect diseased ones. NK cells are attracted to the areas of interest by cytokines like IL-15 (Allavena et al., 1997). In the mouse, also NKG2D ligand Rae-1b seems to influence NK cell motility (Deguine et al., 2010). This attraction leads to a first approach to potential target cells. Binding of adhesion molecules like LFA-1, CD2 or L-Selectin (CD62L) can initiate a loose adhesion to these cells without ligation of other activating receptors. This adhesion arrests the cell and allows the binding of additional NK cell receptors to their ligands. Engagement of activating NK cell receptors NKG2D, DNAM-1 or 2B4 or adhesion molecule CD2 results in inside-out signaling. This activates further LFA-1 molecules and increases the binding to its ligands ICAM-1/2/3, which stabilizes the adhesion to the target cell (Bryceson et al., 2009). Other mechanisms discussed to be involved in the initial adhesion of NK cells to their targets are NK cell nanotubes that can connect NK to target cells over larger distances. They contain NKG2D, the adaptor protein DAP10 and signaling molecule VAV1 and could guide the NK cell movement towards the target cell (Chauveau et al., 2010). In resting NK cells, triggering of LFA-1 alone leads to the convergence of lytic granules at the MTOC and their transport to the immunological synapse but not to degranulation (Bryceson et al., 2005). Degranulation and killing of the target cell need the signal of additional activating receptors (Bryceson et al., 2009; Hsu et al., 2016) or the pre-activation with cytokines like IL-2 (Barber et al., 2004).

LFA-1 activation is an important trigger for the NK cell to start the formation of the IS. After activation, the adaptor protein Talin switches from its inactive to its active state and binds to the intracellular tail of the ß2 chain (CD18) of LFA-1 (Martel et al., 2001). This binding leads to conformational rearrangements of the extracellular integrin domain, which results in an increased affinity to the LFA-1 ligands ICAM-1/2/3 (Tadokoro et al., 2003). Additionally, the interaction of LFA-1 and Talin also is important for outside-in signaling and leads to the activation of signaling molecules, which results in the rearrangement of the actin cytoskeleton – a key event in the formation of the IS (Mace et al., 2009). The binding of Talin to LFA-1 activates two signaling pathways. On the one hand Talin and the Arp2/3 complex associate with the protein vinculin and cluster at the intracellular tail of LFA-1. On the other hand Talin forms a complex with type Iγ phosphatidylinositol 4-phosphate 5-kinase (PtdIns(4)P 5-kinase). This complex binds to LFA-1 and synthesizes Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2). The increased concentration of PtdIns(4,5)P_2 results in the recruitment of Wiskott–Aldrich Syndrome protein (WASp), which activates the actin nucleation activity of Arp2/3. Both pathways together promote the accumulation and reorganization of the actin cytoskeleton at the site of active LFA-1 (Mace et al., 2009; Mace et al., 2010). The lytic granules are present continuously and the activation of LFA-1 or the pre-activation with IL-2 results in the convergence of these lytic granules at the MTOC using the motor protein dynein to move along microtubules. The convergence of lytic granules is important for the following directed degranulation towards the target cell, because this increases the killing efficiency and decreases bystander killing (Hsu et al., 2016). At this point, IS formation is susceptible to signals from inhibitory receptors that can stop target cell lysis. As described in part 1.3.3, ligation of inhibitory receptors activates phosphatases SHP-1/2 and SHIP-1, which then dephosphorylate signaling proteins important for activation. Important inhibitory steps are the dephosphorylation of VAV1 by SHP-1, which blocks actin-dependent activation signals and the phosphorylation of CRK. This prevents the association of CRK in activating signaling complexes and thereby diminishes the NK cell activation (Peterson and Long, 2008; Stebbins et al., 2003).

1.4.2   Maintenance of the immunological synapse and killing of the target cell

Without inhibitory signals, the NK cell initiates the target cell lysis. To this end the cytoskeleton is rearranged and the content of the lytic granules is released into the IS, which induces apoptosis of the target cell.

After NK cell activation, phosphorylated signaling molecules like the Guanine exchange factor (GEF) VAV1 initiate the rearrangement of the cytoskeleton. The Arp2/3 complex is recruited to the IS and polymerizes new F-actin filaments. It is activated by Wiskott-Aldrich-Syndrom-Protein family members WASp and WAVE2. The expression of these proteins can be enhanced by pre-activation

with IL-2 (Mace et al., 2010; Orange et al., 2002; Orange et al., 2011). Small GTPases downstream of VAV1 like Rac1 and Cdc42 as well as other GEFs are involved in this process (Billadeau et al., 1998; Carlin et al., 2011; Krzewski et al., 2006). The polymerization of new actin filaments is indispensable for the stability of the NK:Target cell contact (Netter, 2014). It is needed to create a tight actin-mesh, which stabilizes the IS and creates a dense and sealed space between the cells. The rearrangement of the cytoskeleton also enables receptors to form microclusters (Abeyweera et al., 2011; Treanor et al., 2006). Finally the presence of energy in form of ATP is needed during the whole time of conjugation to maintain IS stability (Netter, 2014).

The next step in NK cell activation is the opening of ion channels and influx of calcium. As described in chapter 1.3, ligation of activating NK cell receptors leads to the binding and activation of adaptor proteins to their intracellular domain. Important for calcium signaling is the activation of PLCγ1. This enzyme catalyzes the hydrolysis of membrane bound $PtdIns(4,5)P_2$ to inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). Thereupon $InsP_3$ binds to $InsP_3$ calcium channels in the membrane of the endoplasmic reticulum (ER) and initiates the release of calcium into the cytosol (Patterson et al., 2005). The specialized calcium sensors stromal interaction molecule 1/2 (STIM1/2) in the membrane of the ER recognize the depletion of ER calcium stores and open calcium-release-activated calcium (CRAC) channels like ORAI1 in the cell membrane (Maul-Pavicic et al., 2011; Shaw and Feske, 2012). The resulting influx of calcium activates calcium-dependent enzymes and regulatory proteins like calmodulin or calcineurin, which both stimulate the exocytosis of lytic granules (Figure 2) (Pores-Fernando et al., 2009; Rochette-Egly and Tovey, 1984). Additionally calcium activates transcription factors NFAT, CREB and NFκB and thereby enhances the expression of cytotoxic proteins (Feske, 2007).

To study calcium signals, cells can be treated with different drugs, which can initiate or block the release of calcium into the cytosol. This study focuses on the ionophore Ionomycin and the SERCA pump inhibitor Thapsigargin. Ionomycin is a calcium salt produced by the bacterium *Streptomyces conglobatus*. It forms pores into the cell membrane and thereby allows extracellular calcium to diffuse into the cytosol (Liu and Hermann, 1978). Thapsigargin in contrast is an inhibitor for the sarco/endoplasmic reticulum Ca^{2+}-ATPase. This prevents the transport of calcium into the ER, which also results in an increase of the cytosolic calcium level (Lytton et al., 1991).
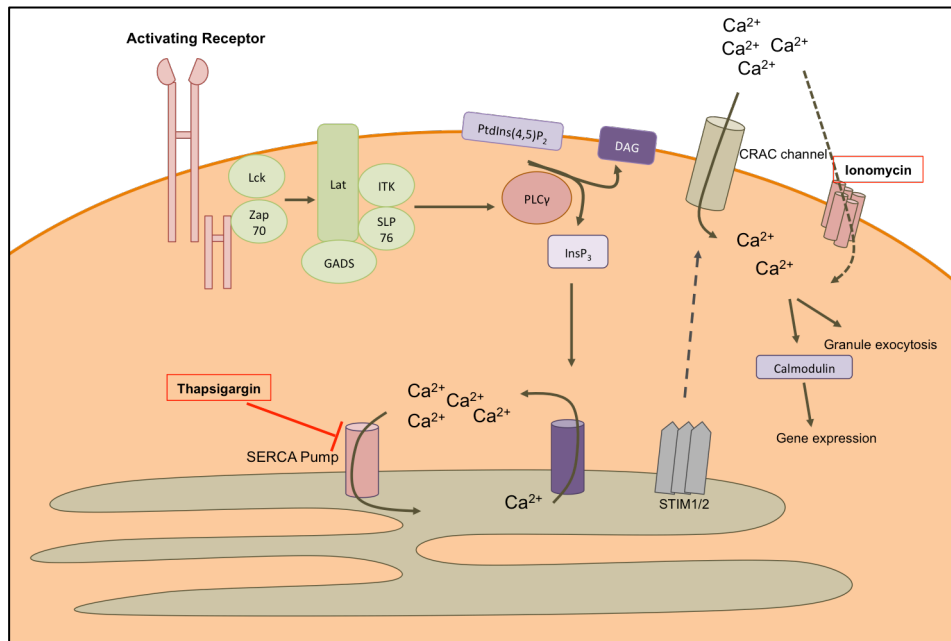
Figure 2. Schematic illustration of calcium signaling. Activation of NK cell receptors leads to the recruitment of the kinases Lck and Zap70. Activation of adaptor protein LAT initiates recruitment and activation of SLP76, GADS and ITK, which phosphorylate PLCγ. Activated PLCγ catalyzes the hydrolysis of PtdIns(4,5)P$_2$ to InsP$_3$ and DAG. InsP$_3$ opens Ca^{2+} channels in the ER membrane and releases calcium into the cytosol. Depleted calcium stores are recognized by STIM1/2 calcium sensors, which then open CRAC channels. Extracellular calcium diffuses into the cell and activates downstream signaling via calmodulin and initiates granule exocytosis. The drugs Ionomycin and Thapsigargin can raise cytosolic calcium levels. Ionomycin forms calcium permeable pores into the cell membrane, Thapsigargin blocks the SERCA pump and inhibits calcium transport into the ER.

Another family of second messengers involved in NK cell cytotoxicity are reactive oxygen species (ROS), which include superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH·). They are produced by NADPH oxidase (NOX) in the cell membrane and superoxide dismutase 2 (SOD2) in the mitochondrial membrane (Schieber and Chandel, 2014). Activation of NK cells by triggering of activating receptors leads to a rise of intracellular ROS, mainly produced by mitochondrial SOD2 enzyme. This initiates downstream signaling and activates transcription factor NFκB to enhance cytotoxicity of NK cells against target cells (Valle Blazquez et al., 1997). NFκB is known to be sensitive for redox signaling and activated by a rise of the ROS concentration in the cell. ROS increases the DNA binding and transactivation of NFκB, which leads to the expression of genes involved in inflammation and cell survival (Meyer et al., 1993). Additionally, protein tyrosine phosphatases (PTPs) like SHP-2 are inactivated by ROS, which decreases inhibitory signaling (Meng et al., 2002). The impact of ROS on NK cell cytoskeleton is not fully understood. ROS was reported to directly regulate actin dynamics in activated neutrophils by reduction of F-actin polymerization, which impaired polarization, chemotaxis, adhesion, and phagocytosis (Sakai et al., 2012). On the other hand, treatment of cells with a redox scavenger also resulted in the reduction of F-actin, which caused actin assembly inhibition and reduced retrograde actin flow (Munnamalai and Suter, 2009). It

is most likely that the concentration and composition of ROS is very important for the observed effects on the cell.

The following key aspect of NK cell cytotoxicity is the reorganization of the microtubule cytoskeleton, the polarization of the MTOC and the release of lytic granules. In cytotoxic T cells MTOC movement and polarization is dependent on Dynein motor proteins (Yi et al., 2013). In contrast it could be shown that MTOC polarization in NK cells works differently and seems to be based on the interaction of motor protein Kinesin-1 with the Arl8b ATPase. Additionally, together with the small GTPase Rab27a, Kinesin-1 and Arl8b are most likely responsible for the movement of lytic granules from the MTOC to the membrane at the IS (Tuli et al., 2013). Moreover the Golgi apparatus is polarized to the IS most likely for rapid secretion of lytic granules towards the IS (Kupfer et al., 1983). To maintain calcium and redox signals and probably to supply ATP, mitochondria are also polarized towards the IS after triggering of activating receptors (Abarca-Rojano et al., 2009). After polarization of the MTOC the associated lytic granules are secreted by moving from the MTOC through the actin mesh at the IS and attaching to the cell membrane. The movement through the actin network is dependent on the actin motor protein myosin IIA, which navigates the lytic granules through hypodense areas (Andzelm et al., 2007; Rak et al., 2011). The small GTPase Rab27a and the adaptor protein Munc13-4 are recruited to the lytic granules directly after activation of the NK cell (Wood et al., 2009). Rab27a is important for vesicle trafficking and Munc13-4 was shown to regulate membrane docking and fusion of lytic granules with the cell membrane (Menager et al., 2007; Stinchcombe et al., 2001). Other proteins involved in the docking of lytic granules with the cell membrane are SNARE proteins like VAMPs and syntaxin-11 (Krzewski and Coligan, 2012).

The fusion of lytic granules with the cell membrane finally leads to the secretion of cytotoxic proteins into the IS and towards the target cell. The main proteins involved in NK cell-mediated killing are perforin and Granzymes and both are found in NK cells as well as CTLs (Dennert and Podack, 1983; Masson et al., 1986; Podack and Dennert, 1983). The 70 kDa protein perforin consists of several domains including a targeting domain for sublocalization into the lytic granules and the N-terminal MACPF domain, which is important for its integration into the target cell membrane (Brennan et al., 2011; Rochel and Cowan, 1996). Additionally, the C-terminal C2 domain can bind to membranes in a calcium dependent manner (Voskoboinik et al., 2005). The mechanism how perforin works in NK cells is not completely investigated. However, the big similarities between perforin and the bacterial cholesterol-dependent cytolysins (CDCs) suggest an identical process. CDCs also have a membrane binding and a membrane attack MACPF domain. They form a pre-pore, composed of multiple CDC proteins, which binds to the target membrane. The transmembrane β-hairpins TMH1 and TMH2 of the MACPF domain from each protein insert into the target membrane and form a mature pore

(Voskoboinik et al., 2015). New data from microscopy experiments confirm that perforin works in a comparable way as CDCs (Leung et al., 2017). To prevent perforin from damaging the NK cell membrane it is only active at pH 7 and high concentrations of calcium. The acidic conditions inside the lytic granules affect its conformational state and thus prevent perforin from harming membranes before it is released (Praper et al., 2010). After degranulation perforin becomes active inside the IS. The lysosomal protein CD107a protects the NK cell surface inside the IS against the insertion of active perforin. The negatively charged residues of CD107a probably obstruct the also negatively charged C2 domain of perforin to bind to the NK cell membrane (Cohnen et al., 2013). It is not conclusively clarified how perforin enables the Granzymes to enter the target cell, but combination of both is necessary to efficiently induce apoptosis. Granzymes most likely attach to the target cell membrane. It is controversial whether Granzymes enter through perforin pores in the plasma membrane or if the pores induce repair processes that then lead to the internalization of membrane together with Granzymes (Keefe et al., 2005; Metkar et al., 2002). Inside the cell, perforin would mediate the release of Granzymes from the endosome into the cytosol, where it induces apoptosis by cleavage and activation of caspases.

Granzymes are a family of serine proteases that consists of the Granzymes A, B, H, M and K. Granzyme B is the family member that has been studied in most detail and possesses the strongest cytotoxic activity. It can cleave and activate or inactivate many different substrates in the target cell. It has been proposed that over three hundred proteins could be affected by Granzyme B (Rousalova and Krepela, 2010). Granzyme B can directly cleave procaspase-3 and procaspase-7 and probably other procaspases, which initiates apoptosis by the activation of the caspase cascade (Darmon et al., 1995; Gu et al., 1996). Additionally, Granzyme B can cleave the pro-apoptotic Bcl-2 family member BH3 interacting-domain death agonist (BID) (Waterhouse et al., 2005). BID starts the permeabilization of the mitochondrial membrane causing the release of cytochrome C, which can activate different caspases and thereby induces apoptosis. The Serpin Proteinase Inhibitor 9 (SerpinB9) inhibits Granzyme B by binding to its catalytic region and prevents the cleavage of its substrate (Bird et al., 1998). SerpinB9 can be overexpressed in different types of tumors like lung or prostate cancer, probably to prevent NK/CTL-mediated killing (Ray et al., 2012; Soriano et al., 2012).

The activation of caspases leads to induction of apoptosis in the target cell, which has a large influence on the cell morphology. The process of programmed cell death involves shrinkage of the cell and pyknosis (Kerr et al., 1972). Proteases cleave and degrade actin filaments and thereby destroy the intracellular stability (Mashima et al., 1997). Additionally, caspases target a number of cell adhesion and cytoskeletal proteins. Activated caspase-3 and -7 are known to cleave focal adhesion kinase (FAK) by this means disrupting the formation of focal adhesion complexes (Wen et

al., 1997). E- and P-cadherins are degraded by activated caspases and VE-cadherin is probably shed by metalloproteinases (Herren et al., 1998; Schmeiser and Grand, 1999). These effects decrease the cell-cell and cell-matrix adhesion of apoptotic cells.

Another hallmark of apoptosis is membrane blebbing, which results in the release of apoptotic bodies. This heterogeneous group of 50-4000 nm big vesicles contains genetic material and organelles (Akers et al., 2013). Apoptotic bodies are recognized and engulfed by different cells of the immune system. Especially macrophages and dendritic cells detect these vesicles by "eat me" signals on their surface and eliminate them by endocytosis (Depraetere, 2000; Erdosova et al., 2002; Kranich et al., 2008). Nevertheless, apoptotic bodies can influence the activation of other immune cells as well. The uptake of apoptotic bodies can suppress CTLs by the presentation of membrane-bound immune regulatory TGF-ß1 on the vesicle surface (Xie et al., 2009).

1.4.3 Detachment of the NK cell and serial killing

Once the target cell has been killed, the NK cell terminates the contact and detaches. This process seems to be regulated, as NK cells clearly detach faster if the target cell has been killed successfully (Netter, 2014; Vanherberghen et al., 2013). The process of NK cell detachment is not completely investigated. At the beginning of the IS termination the NK cell is in a state of relative inactivity (Mace et al., 2014). Decreasing the activation status is mainly a result of activator down-regulation (Claus et al., 2016b; Roda-Navarro and Reyburn, 2009; Sandusky et al., 2006). It has also been discussed, if the collapse of target cell membrane integrity prevents the ongoing adhesion of the NK cell at a late point of apoptosis. Patients with *familial hemophagocytic lymphohistiocytosis* 2 (FHL-2) lack perforin in NK cells and CTLs. This reduces their ability to kill target cells enormously and leads to a clearly decelerated detachment and a massive secretion of pro-inflammatory cytokines (Jenkins et al., 2015).

It has been known for a long time that an important effector function of cytotoxic lymphocytes is *serial killing*. After they successfully killed a target, NK cells and CTLs can kill additional cells one by one and thereby effectively reduce the number of diseased cells (Ullberg and Jondal, 1981; Zagury, 1975). Most studies dealing with serial killing use microscopic approaches to track single NK cells and analyze their killing capability (Bhat and Watzl, 2007; Choi and Mitchison, 2013; Rothstein et al., 1978; Vanherberghen et al., 2016). Using this technique it could be revealed that not all NK cells are serial killers. Many NK cells successfully form a conjugate with target cells but fail to induce apoptosis. Other NK cells kill one to 14 target cells in a row. Some end up exhausted, not able to kill another target and few NK cells are active over the whole examination time (Bhat and Watzl, 2007; Choi and Mitchison, 2013; Vanherberghen et al., 2013). To prevent exhaustion NK cells are able to recycle lytic granules and effector proteins and synthesize effector molecules *de novo* (Li et al., 2011;

Liu et al., 2011; Liu et al., 2005). NK cells lacking the ability to efficiently recycle lytic granules, have a reduced ability to lyse multiple targets (Capuano et al., 2012). Noticeably the time period between the individual killing events differs. NK cells that kill multiple targets need more time for the first than for the following kills. It has been proposed that NK cells can keep activating stimuli from earlier targets and thereby overcome partial inhibition by the new one. This kinetic priming accelerates the killing of neighboring diseased cells (Choi and Mitchison, 2013).

Immunotherapy describes the use of autologous or allogeneic immune cells, stimulated with cytokines or by therapeutic antibodies and has become a serious alternative to conventional therapies against cancer or virus infections. Some of these interventions have influence on serial killing: The use of therapeutic antibodies against B lymphoblastoid cells can increase serial killing by NK cells (Bhat and Watzl, 2007). Additionally, NK cells can be genetically modified to express chimeric antigen receptor (CAR)–constructs. The transfection of NK cells with a ErbB2/HER2-specific CAR was shown to increase serial killing and decrease the tumor burden (Schonfeld et al., 2015). Understanding the regulation of NK cell detachment as a key factor for serial killing and NK cell effector functions could help to increase the efficiency of NK cell immunotherapies.

2   Aim of the thesis

Serial killing is a key feature of NK cell effector functions and facilitates the elimination of successive target cells by one single effector cell. The aim of the thesis was the investigation of NK cell detachment as a key factor for serial killing.

It is known how cytokine stimulation and ligation of individual receptors can induce activation in NK cells. Using a flow cytometry based approach to measure the decay of NK:target cell contacts, I wanted to link the dynamics of detachment directly to the type of NK cell activation. Ongoing signaling by activating receptors is important for the maintenance of the IS. I analyzed the impact of calcium and redox signaling on NK cell effector functions by treatment with interfering drugs and using a modified detachment assay I aimed to determine the calcium levels in NK cells during conjugation with a susceptible target cell. The recogniton of target cell death appears to be the main initiator of NK cell detachment and therefore I investigated how NK cells detect apoptotic target cells. Finally I compared the protein composition of the target cell membrane prior to and after NK cell-induced apoptosis to detect alterations which could initiate the detachment.

By investigation of the NK cell detachment the aim of this thesis was to gain better understanding how the termination of the NK cell IS is regulated and how NK cells successfully recognize the target cell death as early as possible.

3 Material and Methods

3.1 Materials

3.1.1 Primary monoclonal Antibodies

Name (Clone)	Conjugate	Source
CD3 (HIT3a)	PE	BioLegend, San Diego, California, US
CD56 (NCAM16.2)	BV421	BioLegend, San Diego, California, US
Serpinb9 (7D8)	/	Abcam, Cambridge, UK
CD107a (H4A3)	FITC, APC	BioLegend, San Diego, California, US
CD48 (BJ40)	PE	BioLegend, San Diego, California, US
ULBP1 (170818)	/	R&D Systems, McKinley, Minneapolis, USA
ULBP2 (165903)	/	R&D Systems, McKinley, Minneapolis, USA
B7H6 (875001)	PE	R&D Systems, McKinley, Minneapolis, USA
NTB-A (NT-7)	PE	BioLegend, San Diego, California, US
perforin (dG9)	FITC	BioLegend, San Diego, California, US
CD15 (MEM-158)	PE	Immunotools, Friesoythe, Germany
CD54 (H458)	PE	BD, Franklin Lakes, New Jersey, USA
CD58 (MEM-63)	/	Immunotools, Friesoythe, Germany
CD59 (MEM-43)	/	Immunotools, Friesoythe, Germany
CD102 (CBR-IC2/2)	PE	BioLegend, San Diego, California, US
CD62-E (hCD62E)	/	Immunotools, Friesoythe, Germany
Nectin-1	R1.302	BioLegend, San Diego, California, US
MICA (159227)	/	R&D Systems, McKinley, Minneapolis, USA
MICB (236511)	/	R&D Systems, McKinley, Minneapolis, USA

3.1.2 Secondary Antibodies (Polyclonal)

Name (Clone)	Conjugate	Source
Goat-anti Mouse IgG	Cy3	Jackson ImmunoResearch, West Grove, Pennsylvania, USA
Goat-anti Human IgG	PE	Jackson ImmunoResearch, West Grove, Pennsylvania, USA

3.1.3 IgG Control antibodies

Name (Clone)	Conjugate	Source
IgG1 (MOPC21)	PE	BioLegend, San Diego, California, USA
IgG1 (MOPC21)	FITC	BioLegend, San Diego, California, USA
IgG1 (MOPC21)	APC	BioLegend, San Diego, California, USA

3.1.4 Buffers and Media

4 % Paraformaldehyde (PFA)	PBS
	4 % Paraformaldehyde (PFA)
FACS Buffer	PBS
	2 % FCS
LC-AA Buffer	PBS
	0.5 % BSA
CTL Medium	IMDM
	10 % FCS
	1 % Penicillin-Streptomycin (10,000 U/mL) (P/S)
Ba/F3 Medium	RPMI 1640
	10 % FCS
	1 % P/S
	50 µM 2-Mercaptoethanol

HEK293T/HeLa Medium	DMEM
	10 % FCS
	1 % P/S

Calcium washing buffer	IMDM without phenol red
	2.5 mM Probenecid solution (fresh, in NaOH)
	5 mM HCl
	10% FCS
	1% P/S

Calcium assay buffer	IMDM without phenol red
	2.5 mM Probenecid solution
	5 mM HCl
	10 mM Hepes
	2% FCS

3.1.5 Cells

Composition of Media see 3.1.3

Name	Transfected with	Origin	Medium
Primary NK cells	*untransfected*	Whole Blood from healthy humans	CTL or *vericyte*
HEK293T	*untransfected*	Human embryonic kidney	DMEM
Phoenix Ampho	Constructs for amphotrophic virus gag-pol & envelope protein	Human embryonic kidney	DMEM
Ba/F3	GFP	Mouse pro B-Cells	RPMI
Ba/F3	NTB-A	Mouse pro B-Cells	RPMI
Ba/F3	B7H6	Mouse pro B-Cells	RPMI
Ba/F3	ULBP2	Mouse pro B-Cells	RPMI
Ba/F3	CD48	Mouse pro B-Cells	RPMI
K562	*untransfected*	CML	CTL
K562	CD107a	CML	CTL
K562	SerpinB9	CML	CTL
K562	CD107a-SerpinB9	CML	CTL
K562	mbIL15-41BBL	CML	CTL
One Shot® TOP10 Chemically Competent *E. coli*	Unknown	*E.coli*	LB

3.1.6 Inhibitors

Brefeldin A	Sigma-Aldrich, St. Louis, Missouri, USA
Thapsigargin	Abcam, Cambridge, UK
Concanamycin A	Sigma-Aldrich, St. Louis, Missouri, USA
Ionomycin	Merck Millipore, Billerica, Massachusetts, USA
N-Acetylcysteine	Sigma-Aldrich, St. Louis, Missouri, USA

3.1.7 Kits

Dynabeads untouched human NK Kit	Invitrogen/LifeTechnologies, Darmstadt, Germany
ELISA IFN-γ	BioLegend, San Diego, California, USA
ELISA TNFα	BioLegend, San Diego, California, USA
Hi Speed Plasmid Kit	Qiagen, Venlo, Netherlands

3.1.8 Reagents

PBS	Thermo Fisher Scientific, Rockford, IL, USA
F(ab)2-Fragment	Jackson ImmunoResearch, West Grove, Pennsylvania, USA
Lipofectamine 2000	Thermo Fisher Scientific, Rockford, IL, USA
Penicillin-Streptomycin	Thermo Fisher Scientific, Rockford, IL, USA
Fetal calf serum	Thermo Fisher Scientific, Rockford, IL, USA
Puromycin	Calbiochem/Merck Millipore, Billerica, MA, USA
G418 / Geneticin?	Thermo Fisher Scientific, Rockford, IL, USA
Dimethyl sulfoxide (DMSO)	Avantor (J.T.Baker), Center Valley, PA, USA
Lymphocyte Separation Medium (LSM)	PAN Biotech, Aidenbach, Germany
IL-2	NIH Cytokine Repository, Frederick MD, USA
IL-21	Miltenyi Biotech, Bergisch Gladbach, Germany
IL-15	Pan-Biotech, Aidenbach, Germany
Paraformaldehyde (PFA)	Sigma-Aldrich, St. Louis, Missouri, USA
Polybrene	Sigma-Aldrich, St. Louis, Missouri, USA
BD FACS Permeabilizing Solution 2	BD, Franklin Lakes, New Jersey, USA
Cell tracker orange	Thermo Fisher Scientific, Rockford, IL, US
Cell tracker violet	Thermo Fisher Scientific, Rockford, IL, US
CFSE	Thermo Fisher Scientific, Rockford, IL, US
ICAM-1-Fc	R&D Systems, McKinley, Minneapolis, USA
$MgCl_2$	AppliChem, Darmstadt, Germany
EGTA	Sigma-Aldrich, St. Louis, Missouri, USA
Fluo-8	Thermo Fisher Scientific, Rockford, IL, US
Probenecid	Sigma-Aldrich, St. Louis, Missouri, USA
IMDM	Thermo Fisher Scientific, Rockford, IL, US
RPMI1640	Thermo Fisher Scientific, Rockford, IL, US
IMDM without phenol red	Thermo Fisher Scientific, Rockford, IL, US
Hepes	Thermo Fisher Scientific, Rockford, IL, US

Chromium-51	Hartmann Analytic, Braunschweig, Germany
Triton-X-100	Carl Roth, Karlsruhe, Germany
Hydrogen Peroxide (H_2O_2)	AppliChem, Darmstadt, Germany
MFG-E8	R&D Systems, McKinley, Minneapolis, USA
Zombie-aqua	BioLegend, San Diego, California, USA
Vericyte medium	Medicyte, Heidelberg, Germany

3.1.9 Software

FlowJo v.8.8.7	*FlowJo*, LLC, Ashland ,USA
GraphPad PRISM v. 5.0f	GraphPad, La Jolla ,USA
BD FACSDiva	BD, Franklin Lakes, New Jersey, USA
BD CellQuest	BD, Franklin Lakes, New Jersey, USA

3.1.10 Plasmids

SerpinB9-pIRES-puro	(Liesche, 2014)
pBABE-SerpinB9	
pBABE-CD107a	

3.1.11 Equipment

BD FACSCalibur	BD, Franklin Lakes, New Jersey, USA
BD LSRFortessa	BD, Franklin Lakes, New Jersey, USA
Tecan M200 pro	Tecan, Männedorf, Switzerland
BD FACSJazz	BD, Franklin Lakes, New Jersey, USA

3.2 Methods

3.2.1 Cell biology

3.2.1.1 Cell culture

Cell lines and primary NK cells were incubated in a humidified incubator at 37°C and 5 % CO_2. Cells were maintained in appropriate medium (see *cell lines*) and stably transfected cells were selected with puromycin (0.5-1 µg/ml). Cell lines were split all 2-3 days and grown in cell culture flasks, which were exchanged every 2-3 weeks. Cells were slowly frozen in freeze-medium (FCS + 10 % DMSO) at -80°C and long-term stored at -170°C in liquid nitrogen. FCS and human serum were heat inactivated (56°C, 30 minutes) before use.

3.2.1.2 Isolation of primary NK cells

Blood was received from healthy donors and peripheral blood mononuclear cells (PBMCs) were obtained via density centrifugation over Lymphocyte Separation Medium (LSM). LSM was overlayed with blood and centrifuged at 1025xg for 25 minutes. NK cells were isolated from PBMCs with *Dynabeads® Untouched™ Human NK Cells Kit* (Thermo Fischer) via negative isolation according to the manufacturer's instructions. NK cells were defined as CD3⁻CD56⁺ lymphocytes and had a purity of 90-99%. Purified NK cells were either used fresh or pre-activated. For pre-activation, NK cells were treated over night with 200 IU/mL recombinant human IL-2 or cultivated for 2-3 weeks with feeder cells (see 3.2.1.3) or in *vericyte NK Cell Growth Medium* from Medicyte GmbH (see 3.2.1.4).

3.2.1.3 Expansion of NK cells with feeder cells

After isolation purified NK cells were maintained in CTL medium (IMDM + 10 % FCS + 1 % P/S see 0) supplemented with 200 IU/mL recombinant human IL-2 and 100 ng/ml recombinant human IL-21. The cells were then mixed with 30 Gy irradiated K562mbIL15-41BBL feeder cells at a ratio of 2:1 (NK:K562mb15) and plated in 96-U wells at a density of $1x10^6$ - $2x10^6$ cells/ml. NK cells were restimulated after 7 days with the same amount of IL-2 and feeder cells and incubated for additional 7 days prior to first use. The medium was exchanged all 3 – 4 days and cells were splitted after reaching a density of $3x10^6$ cells / ml.

3.2.1.4 Cultivation of NK cells in vericyte medium

As an alternative to feeder cells, NK cells were maintained in *vericyte NK Cell Growth Medium* from Medicyte GmbH (see 2.1.4) containing 10 % FCS, 1000 IU/ml recombinant human IL-2, 10 ng/ml recombinant human IL-15 and a so called *vericyte factor 115.* After isolation NK cells were cultured in *vericyte* medium at a density of $1x10^6$ and split 1:2 if they reached $2x10^6$ cells/ml. Cells were used after a minimum of two weeks.

3.2.1.5 Retroviral transfection of K562 cells

K562 cells were stably transfected using retroviruses produced by Phoenix-AMPHO cells. The genes of interest were subcloned in pBABE vector and purified using *Hi speed Plasmid Maxi Kit* (Qiagen). $1x10^6$ Phoenix-AMPHO cells were plated in a small tissue culture flask (T25) and transfected with Lipofectamine 2000 according to the manufacturer's instructions. After 24 h medium was exchanged with CTL medium and after an additional day, retrovirus-containing supernatant was harvested. $0.5x10^6$ K562 were resuspended in the virus supernatant and 5 µg/ml Polybrene was added prior to centrifugation (1.5 h at 1350 g, 37 °C). After 24 h incubation, medium was exchanged with fresh CTL medium and 0.5 µg/ml Puromycin was added for selection.

3.2.1.6 Fluorescence activated cell sorting

After transfection with two different plasmids containing the same resistance, K562 were sorted to enrich double positive cells. Cells were stained with Alexa Fluor 488-labeled mAb against the transfected protein, which was expressed by fewer cells (see 3.2.2.1) and sorted with a *BD FACSJazz™.* To obtain highly purified double positive cells FACS was adjusted to Sort objective "Purify" and Sort mode "2.0 Drops Pure". After sorting, cells were ~ 97% positive for both proteins.

3.2.2 Flow cytometry staining and functional assays

3.2.2.1 Cell staining for flow cytometry

For flow cytometry analysis, cells were stained in 96-V plates or microtubes. For intracellular staining cells were fixed with 2 % PFA, permeabilized with BD FACS Permeabilizing Solution 2 buffer for 10 minutes at RT prior staining. A minimum of $0.2x10^6$ cells was resuspended in 50 µl cold FACS buffer (PBS + 2% FCS) containing a respective amount of antibody. The antibody was either unconjugated or directly conjugated with a fluorophore. After 20 minutes incubation on ice, cells were washed and if necessary stained with a secondary antibody. After staining the cells were resuspended in FACS buffer and analyzed on a *BD LSR Fortessa^{TM}*.

3.2.2.2 Cell labeling for conjugate and detachment assay

Staining NK cells and target cells with different fluorescent cell dyes permits distinction of free cells and conjugates. Cells were stained with *Cell tracker orange* or *violet* or with *Carboxyfluorescein*

succinimidyl ester (CFSE) (all from Thermo Fisher). 1x10^6 NK cells were stained with 10 µM *cell tracker orange*, 1x10^6 K562, HeLa-CD48 or Ba/F3 cells were stained with 25 µM *cell tracker violet,* 10 µM *cell tracker orange* or 5 µM *CFSE* in PBS (depending on the experiment) for 30 minutes at 37°C. Afterwards 1 ml heat inactivated FCS was added for 10 minutes at room temperature to stop the reaction by binding of free dye. The cells were washed with CTL medium and incubated for at least 30 minutes to allow unbound dye to leak out. After a last washing step, cells were resuspended in a concentration of 1x10^6 NK cells or 2x10^6 target cells per mL and used for functional assays (see below).

3.2.2.3   Degranulation assay

NK cells and target cells (K562 or Ba/F3) were stained with cell dyes in different colors (see 3.2.2.2). Cells were coincubated at a ratio of 1:2 (NK:Target cell) for 2 h in the presence of α-CD107a antibody. If mentioned, inhibitor was added directly before coincubation. In the end cells were fixed with 2% paraformaldehyde for 10 minutes at room temperature, washed and resuspended in FACS-buffer. Cells were directly analyzed in a flow cytometer and the amount of CD107a$^+$ cells in all NK cells was evaluated using *FlowJo* software.

3.2.2.4   Plate bound detachment LC-AA Assay

To compare the LFA-1 activation from untouched and freshly detached NK cells with NK cells in conjugates, a modified Ligand-complex-based adhesion assay (LC-AA) was performed. 0.8x10^5 HeLa-CD48 were plated in 48-well plates one day before the experiment and stained with CFSE (see 3.2.2.2). ICAM-1-Fc chimera (50µg/ml) and PE-labeled goat anti-human F(ab)2 fragments (40µg/ml) were mixed in LC-AA buffer (PBS + 0.5% BSA) for 30 minutes at room temperature to prepare ICAM-1-Fc complexes. 0.7x10^6 fresh, unstimulated NK cells were resuspended in ICAM-1-Fc complexes containing buffer and added to the HeLa-CD48. After 30 minutes the unbound NK cells were removed and the plate was washed two times with PBS. 100 µl new ICAM-1-Fc complexes were added to the remaining cells and incubated for additionally 30 minutes. The freshly detached NK cells were removed and the remaining NK:HeLa-conjugates were detached using a cell scraper. All cells were fixed with 2% PFA after removal and directly analyzed with a BD LSRFortessa. The binding of ICAM-1-Fc to the NK cells was defined as positive for active LFA-1, quantified using *FlowJo* software and illustrated using *GraphPad PRISM*. NK cells were treated with 10 mM Mg^{2+} and 1 mM EGTA as positive control.

3.2.2.5   Conjugate Assay

NK cells and target cells were stained with fluorescent cell dyes in different colors (see 3.2.2.2). 0.5x10^5 fresh, IL-2 activated or cultivated NK cells were mixed with 1x10^6 target cells in a volume of 100 µl and centrifuged for one minute at 20xg to get cells in close proximity. Cells were coincubated

at 37°C for 0, 5, 10, 20, 40, 60 and 90 minutes. If mentioned in the experiment, an inhibitor was added directly at the beginning of coincubation. The reaction was stopped by vortexing the cells and fixing them with 2% PFA on ice. Cells were directly analyzed with a *BD LSRFortessa* and data was analyzed with *FlowJo* software. Conjugates were determined as double positive events within all NK cells and illustrated using *GraphPad PRISM* software.

3.2.2.6 Detachment assay (after Culley et al. 2011)

NK cells and target cells were stained with fluorescent cell dyes in different colors as described in 3.2.2.2 to distinguish free cells and conjugates. 0.5×10^6 NK cells and 1×10^6 target cells were mixed in 650 µl medium, centrifuged for one minute at 20xg and coincubated for 30 minutes at 37°C to allow formation of conjugates (pre-coincubation). Cells were diluted 1:25 with CTL medium and if mentioned an inhibitor was added. The cell mix was aliquoted in six equal samples and rotated at 37°C, which allows detachment of NK cells but prevents the formation of new conjugates. The reaction was stopped after 0, 10, 20, 40, 60 and 90 minutes by vortexing and fixation with 2% PFA on ice. As controls, NK cells and K562 were mixed and diluted with medium without prior pre-coincubation. The cells were directly analyzed with a *BD LSRFortessa* and data was analyzed with *FlowJo* software. Conjugates were determined as double positive events within all NK cells. The decay and half-life of conjugates was determined using *GraphPad PRISM* and the following formula for a one-phase exponential decay:

$$\tau = \frac{\ln\left(\dfrac{Y_{50} - Plateau}{Span}\right)}{-K}$$

τ	-	Half-life [min]
ln	-	Natural logarithm
Y	-	% of remaining conjugates [%]
Span	-	Span between the exponentially determined maximum and minimal amount of remaining conjugates
Plateau	-	Exponentially determined minimal amount of remaining conjugates [%]
K	-	Rate constant [min^{-1}]

3.2.2.7 Calcium Assay

Cells were stained with fluorescent cell dyes (see 3.2.2.2) to distinguish free NK cells and NK cells in conjugates. To measure intracellular calcium signal, NK cells were then additionally stained with fluorescent calcium binding dye fluo-8. 1×10^6 cells were loaded with 4.8 µM fluo-8 in wash medium (IMDM without phenol red, 2.5 mM Probenecid solution, 5 mM HCl, 10 % FCS, 1 % P/S) for 30 minutes at 37°C. Cells were washed twice and incubated for additional 30 minutes to allow unbound fluo-8 to leak out. After a last washing step, NK cells were resuspended in a concentration of 1×10^6 cells/ml in assay medium (IMDM without phenol red, 2,5 % Probenecid solution, 5 mM HCl, 10 mM Hepes, 2 % FCS). A Kinetik of the calcium signal was measured for NK cells alone or after coincubation with K562 in a BD FACSCalibur. The data was analyzed with *FlowJo* software. Conjugates were determined as double positive events within all NK cells.

3.2.2.8 Calcium Detachment Assay

To measure the calcium signal during NK cell detachment, K562 were stained with *Cell tracker orange* to distinguish between free K562 and NK:K562 conjugates (see 3.2.2.2). NK cells were stained with fluorescent calcium binding dye fluo-8 (see 0) and the NK cells were preincubated either with concanamycin A (50 nM) or DMSO for 2 h. 0.5×10^6 NK cells and 1×10^6 target cells were mixed in 650 µl of assay medium (IMDM without phenol red, 2,5 % Probenecid solution, 5 mM HCl, 10 mM Hepes, 2 % FCS), centrifuged for one minute at 20xg and coincubated for 30 minutes at 37°C to allow formation of conjugates (pre-coincubation). Cells were diluted 1:25 in assay medium, aliquoted in 22 equal samples and rotated at 37°C to facilitate NK cell detachment but prevent the formation of new conjugates. All 22 samples were measured successively over a time period of 88 minutes and every four minutes the current sample was exchanged with a new one. Using *FlowJo* software, NK cells and K562 were plotted against each other and conjugates were defined as double positive events. The calcium signal for free NK cells and NK cells in conjugates were analyzed over time. A regression line was calculated for the measured calcium data and the slope over time was calculated using *GraphPad PRISM* software.

3.2.3 Further Methods

3.2.3.1 Chromium-51 release assay

Chromium-51 release assay was performed with K562 or Ba/F3 cells as target cells. 5×10^6 cells were harvested and resuspended in CTL medium (IMDM + 10% FCS + 1% P/S). Cells were labeled with 100 µCi ^{51}Cr (3.7 MBq) for 1 h at 37°C, washed two times with fresh medium and resuspended at a concentration of 5×10^4 cells/ml. NK cells were harvested and plated in a 96-U-well plate in serial dilutions from 10:1 to 1,25:1 (NK:target cell) in triplicate. 5×10^3 target cells were added to the NK cells in a final volume of 200 µl per well and incubated for 4 h at 37°C. For a serial killing Chromium-

51 release assay, the NK cells were diluted from 1:1 to 0,125:1 (NK:target cell) and incubated for 16 h at 37°C with 5×10^3 target cells/well. If mentioned, inhibitors were added during coincubation. The maximum release was determined by lysis of target cells with 1% TritonX-100, spontaneous release was determined by incubating target cells without NK cells and the percentage of specific lysis was calculated as:

$$\% \; specific \; lysis = \frac{\text{experimental release} - spontaneous \; release}{\text{maximum release} - spontaneous \; release} \; x \; 100$$

3.2.3.2 ELISA assay

NK cells were either preincubated with concanamycin A (50 nM) or DMSO for 2h and coincubated with K562 wildtype or K562-Sb9-CD107a for 16 h. The cell mix was centrifuged and the supernatant frozen at -80°C. The supernatants of three independent experiments were thawed and analyzed using IFN-γ or TNF-α ELISA Kit according to the manufacturer's instructions. The samples were analyzed by a tecan M200 pro microplate reader and the data was analyzed with *GraphPad PRISM* software.

3.2.3.3 Preparation of dead cell supernatant

Dead cell supernatant (DCS) was prepared by slowly killing K562 or HEK293T cells by heat shock. $2\text{-}3 \times 10^6$ cells/ml K562 or HEK293T cells were resuspended in fresh medium and incubated for 7 minutes (K562) or 10 minutes (HEK293T) at 56°C. Cells were then incubated for 2 days at 37°C to allow cells to die slowly. At the day of the experiment, cells were centrifuged and supernatant was used in Chromium-51 release assays (see 3.2.3.1) or detachment assays (see 3.2.2.6). As controls, 0.6×10^6 cells/ml were incubated without heat shock for 2 days (Living cells supernatant – LCS) or fresh medium was used.

3.2.3.4 Statistical analysis

Statistical analyses were performed using *GraphPad Prism v.5.0f* (GraphPad Software, Inc., San Diego, CA, USA). Two data groups were analyzed using paired, two-tailed student's t-test. Other statistics methods are indicated. P-values < 0.05 were considered significant differences and are marked: * < 0.05; ** < 0.01; *** < 0.001; ns = not significant.

4 Results

4.1 A flow cytometry-based assay system to determine the NK:target cell conjugate half-life

The process of NK cell killing can be divided in multiple steps. After a firm adhesion and activation of the NK cell, the delivery of cytotoxic proteins induces target cell apoptosis. The last step is the termination of the immunological synapse and detachment of the NK cell. Although much is known about NK cell-mediated killing of transformed or infected cells, the process of NK cell detachment remains relatively unclear.
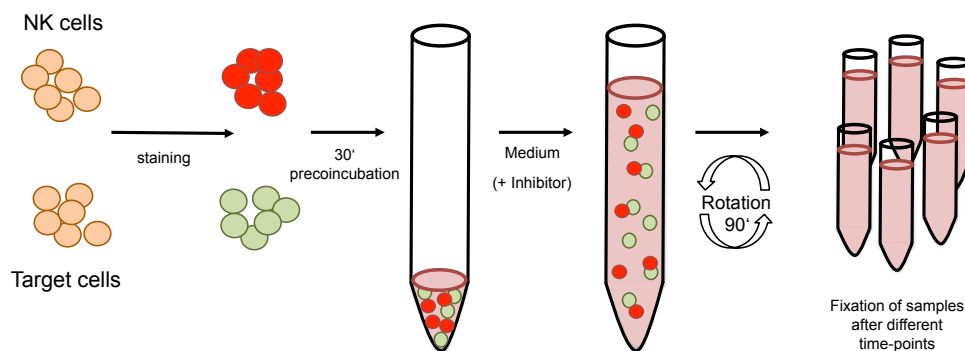

Figure 3. The procedure of a detachment assay as a schematic illustration. Primary NK and target cells were stained with membrane dyes in different colors and coincubated in a small volume to form conjugates (E:T=1:2). After 30 minutes pre-coincubation, cells were diluted with medium and inhibitor was added if indicated. Cells were rotated for 90 minutes and samples fixed after 0, 10, 20, 40, 60 and 90 minutes with 4% PFA.

In order to further analyze the process of NK cell detachment this study focused on the question how NK cells recognize the target cell death and how NK cell detachment is initiated and regulated. To investigate the NK cell detachment, a modified conjugate assay described by Culley et al. and modified by Netter (Culley et al., 2009; Netter, 2014) was used. The detachment assay can be differentiated into two phases. If not mentioned otherwise, primary NK cells were used after 2-4 weeks cultivation in IL-2/IL-21/IL-15 and feeder cells containing medium, when they show the highest activation (Granzin et al., 2016). To distinguish between single cells and conjugates, NK and target cells were stained with intracellular fluorescence dyes in different colors and afterwards coincubated for 30 minutes in a small volume (Figure 3). In this precoincubation phase, NK cells form a significant amount of conjugates but remain in the exponential phase of conjugate formation, when more conjugates are formed than decayed. Importantly, minor changes in the effector to target ratio (E:T) or pre-coincubation duration had no significant effect on the conjugate half-life (Netter, 2014). To stop the continuing formation of conjugates, the cells were diluted 25-fold and subdivided into

multiple samples. Each sample was rotated for different time-periods from 0 to 90 minutes. In the end, the samples were vortexed briefly to separate loosely adherent cells, which did not form a firm conjugate and were fixed with paraformaldehyde (Figure 3). NK and target cells, which have been incubated separately and were mixed at the point of dilution, did not form new conjugates (Figure 4C).
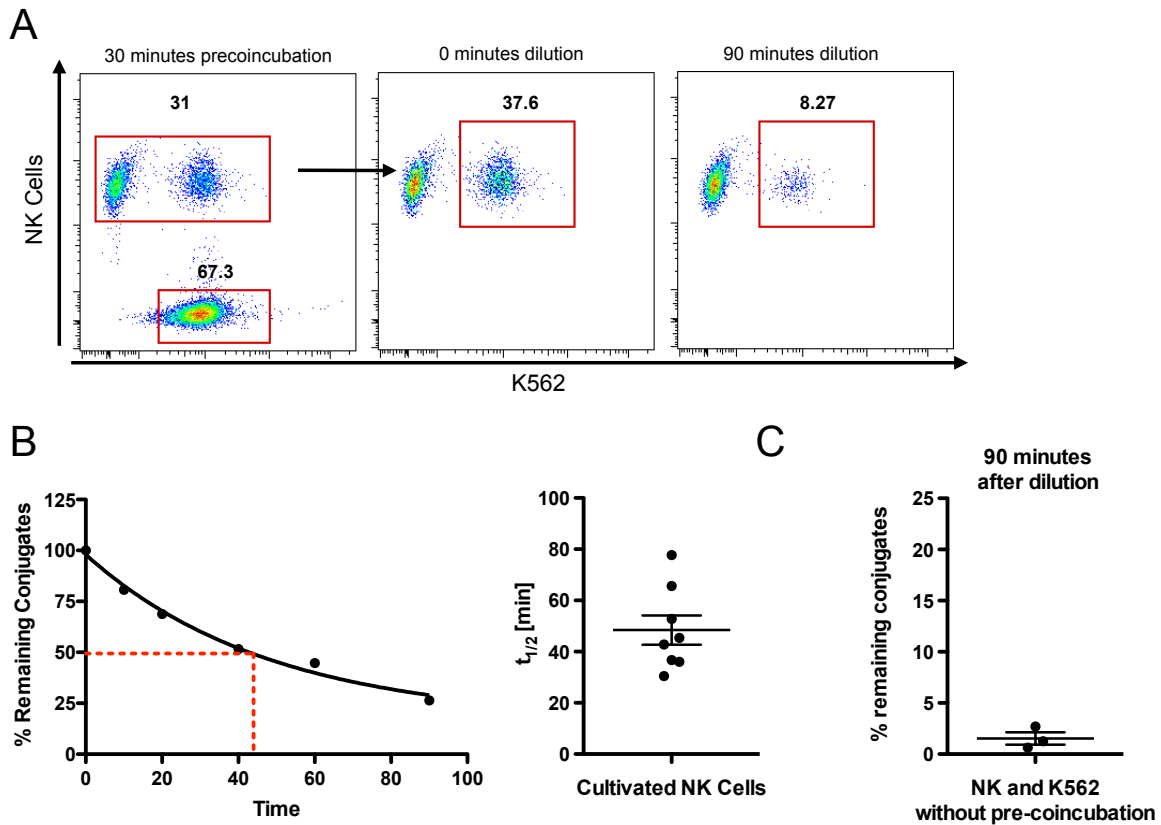

Figure 4. Cultured NK cells detach after approximately 50 minutes. Gating strategy of the detachment assay: NK cells are plotted on the Y-axis and target cells (here K562) are plotted on the X-axis. First a gate including all NK cells was drawn and within these cells on the double-positive events, which display the NK cells in conjugates, was gated. Plots are shown exemplarily for 0 and 90 minutes after dilution **(A)**. The percentage of NK cells in conjugates was determined for all time-points (0, 10, 20, 40, 60 and 90 minutes), normalized to time point 0 minutes after pre-coincubation and plotted against the time. Using the formula for one-phase exponential decay the half-life for the NK cell detachment was calculated **(B, left panel)** and is quantified for NK cells from 8 different healthy donors **(B, right panel)**. The percentage of remaining conjugates after 90 minutes without pre-coincubation of NK and K562 cells **(C)**.

Directly after fixation, the samples were analyzed by flow cytometry. The staining with two different membrane dyes makes it possible to separate three cell populations. Free NK cells and free target cells appear as single positive events, conjugates between an NK and a target cell appear as double positive events. To determine the percentage of NK cells in conjugates after each time-point, the fraction of NK cells engaged in conjugates of all NK cells was calculated (Figure 4A). The number of NK cells in conjugates after 0 minutes of dilution was set to 100% and the following time points were normalized to this sample and plotted against the time. To calculate the half-life of the conjugates, a non-linear regression curve for one-phase decay was used to analyze the data. The half-life

determines the time-point when 50% of the initial conjugates have been decayed (Figure 4B). Cultured NK cells detach after 48.43 ± 16.16 minutes. To exclude the possibility, that NK cells and target cells form new conjugates after dilution, they were mixed in a large volume of medium without prior pre-coincubation. After the following 90 minutes coincubation, only 2-3 % of these NK and target cells were in conjugates (Figure 4C). The data show, that this assay is capable to study NK cell detachment.

4.2 Influence of cytokine-induced pre-activation on NK cell effector functions

The ability of NK cells to kill a target cell is independent of a pre-activation. Nevertheless, the activity of NK cells can be increased by a broad range of cytokines secreted by other cells of the immune system. The main cytokines with activating properties are IL-2, IL-15 and IL-21.

To analyze the effects of NK cell activation on the cytotoxicity, degranulation and formation of conjugates as well as detachment, differentially activated NK cells were compared. After isolation, NK cells were subdivided in three groups. The first group was rested over night in medium without the addition of any cytokines. The second group was treated for 16 h with 200 IU/ml IL-2 to initiate NK cell activation. The third group was cultured for 2-3 weeks in *vericyte* NK cell medium, which contains high levels of IL-2 (1000 IU/ml), IL-15 (10 ng/ml) and a not otherwise specified *factor 115*. This group was supposed to have the maximum activity. The fresh and IL-2 activated NK cells were tested in functional assays one day after isolation, the cultured NK cells after 2-3 weeks.
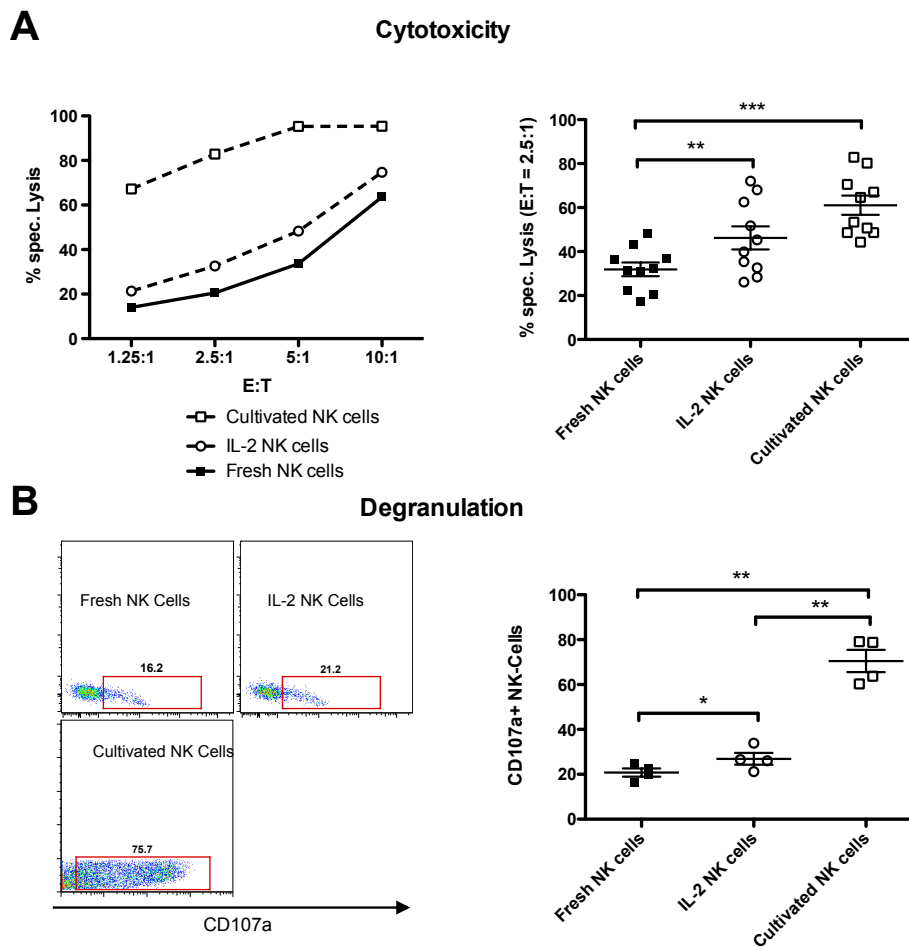
A

Cytotoxicity

B

Degranulation

Figure 5. Degranulation and killing of K562 target cells is dependent on NK cell pre-activation. After isolation NK cells were rested over night in CTL medium, incubated for 16 h with IL-2 (200 IU/ml) or cultured for 2-3 weeks in *vericyte* NK cell medium. Cytotoxicity was tested in a standard Chromium-51 release assay against K562 cells. Left panel shows one representative donor, right panel quantification of 10 different donors. **(A)**. Degranulation was analyzed after 2 h coincubation with K562 cells (E:T=1:2) and staining for CD107a. Left panel shows one representative donor, right panel quantification of four different donors **(B)**. Values are shown as mean ± SD (*p< 0.05; **p< 0.01; ***p< 0.001).

In a standard Chromium-51 release assay the ability of NK cells to lyse K562 target cells in relation to the ratio between both cell types was analyzed. The fresh, rested NK cells were able to kill K562 in a dose dependent manner. The average percentage of lysed cells at a ratio of 2.5:1 (NK cells:K562 cells) was 31.91% ± 9.8. The cytotoxicity increased after activation of NK cells with IL-2 over night and the percentage of lysed cells was significantly higher compared to the resting cells (46.19% ± 16.67 %). The cultivation in *vericyte* medium for 2-3 weeks boosted this effect even more. The killing was significantly increased (61.08% ± 13.90%) and reached a plateau at a ratio of 5:1 (Figure 5A).

The degranulation of the differentially activated NK cells showed a similar pattern. During 2 h coincubation with K562, NK cells were stained with anti-CD107a as a marker for degranulation. Fresh,

rested NK cells were able to degranulate after coincubation with K562 (20.78% ± 3.65). The degranulation was slightly but significantly increased if NK cells were pre-activated with IL-2 over night (26.90% ± 5.24). This was considerably and significantly increased, if the NK cells were cultured in *vericyte* medium for 2-3 weeks (70.48% ± 9.89) (Figure 5B).
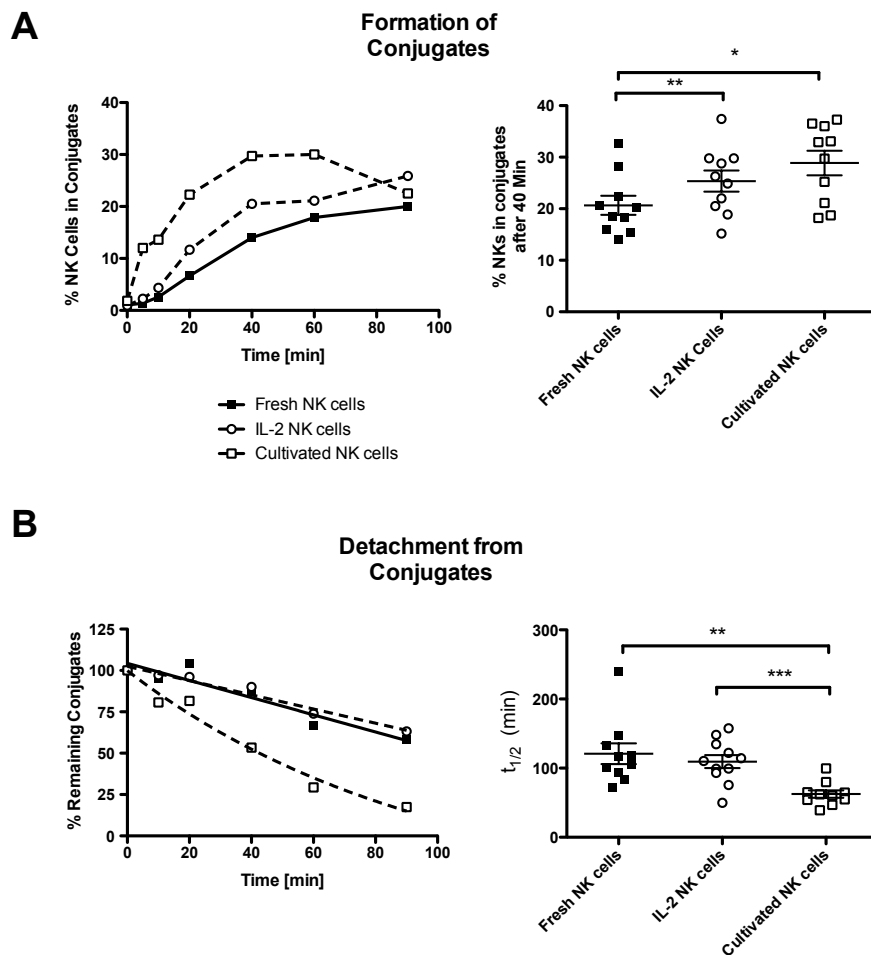

Figure 6. Formation of conjugates and detachment from a target cell is dependent on NK cell pre-activation. After isolation, NK cells were rested over night in CTL medium, incubated for 12 h with IL-2 (200 IU/mL) or cultured for 2-3 weeks in *vericyte* NK cell medium. Formation of conjugates with K562 target cells was determined in a conjugate assay **(A)**. Detachment was tested in a detachment assay against K562 cells **(B)**. Left panels show one representative donor, right panels the quantification of 10 different donors. Values are shown as mean ± SD (*p<0.05; **p<0.01; ***p<0.001).

To further investigate the effect of NK cell activation, the ability of NK cells to form conjugates with K562 target cells was assessed. Again, fresh NK cells were able to bind a target cell and form a stable immunological synapse. But the number of NK cells in conjugates after 40 minutes coincubation was the lowest (20.66% ± 5.87) and could be significantly increased by activation with IL-2 over night (25.36% ± 6.47) or even more after cultivation in *vericyte* medium (28.87% ± 7.50) (Figure 6A). The

relationship between NK cell activation and detachment has not been assessed so far. Interestingly, the results differ somewhat from the other functional assays. The half-life of NK:K562 conjugates was very similar for fresh and IL-2 activated NK cells (120.9 ± 47.27 min and 109.5 ± 31.31 min). Only the cultivation in *vericyte* medium led to an acceleration of the NK cell detachment and significant decrease of the conjugate half-life (62.60 ± 17.08 min).

These results show, that NK cell effector functions are highly dependent on the activation status of the NK cell. Not pre-activated NK cells formed the fewest number of conjugates, degranulate less and killed the least amount of target cells. The pre-activation with cytokines increases all of these effector functions. Here, the long-term activation with high concentrations of cytokines IL-2 and IL-15 (*vericyte* medium) boosts these effects more than short-term activation with IL-2 over night. Surprisingly, even though IL-2 activated NK cells lysed target cells more effectively than fresh NK cells the detachment kinetic was similar and was only accelerated, when NK cells were activated in *vericyte* medium (Figure 6B).

In addition to the activation of NK cells by cytokines, it was investigated how the availability of ligands for activating NK cell receptors influences the effector functions of NK cells. For this, mouse Ba/F3 cells, transfected with different human NK cell ligands were used. Cultured primary NK cells were coincubated with these target cells and the formation of conjugates, detachment, cytotoxicity and degranulation was analyzed with the respective functional assays.
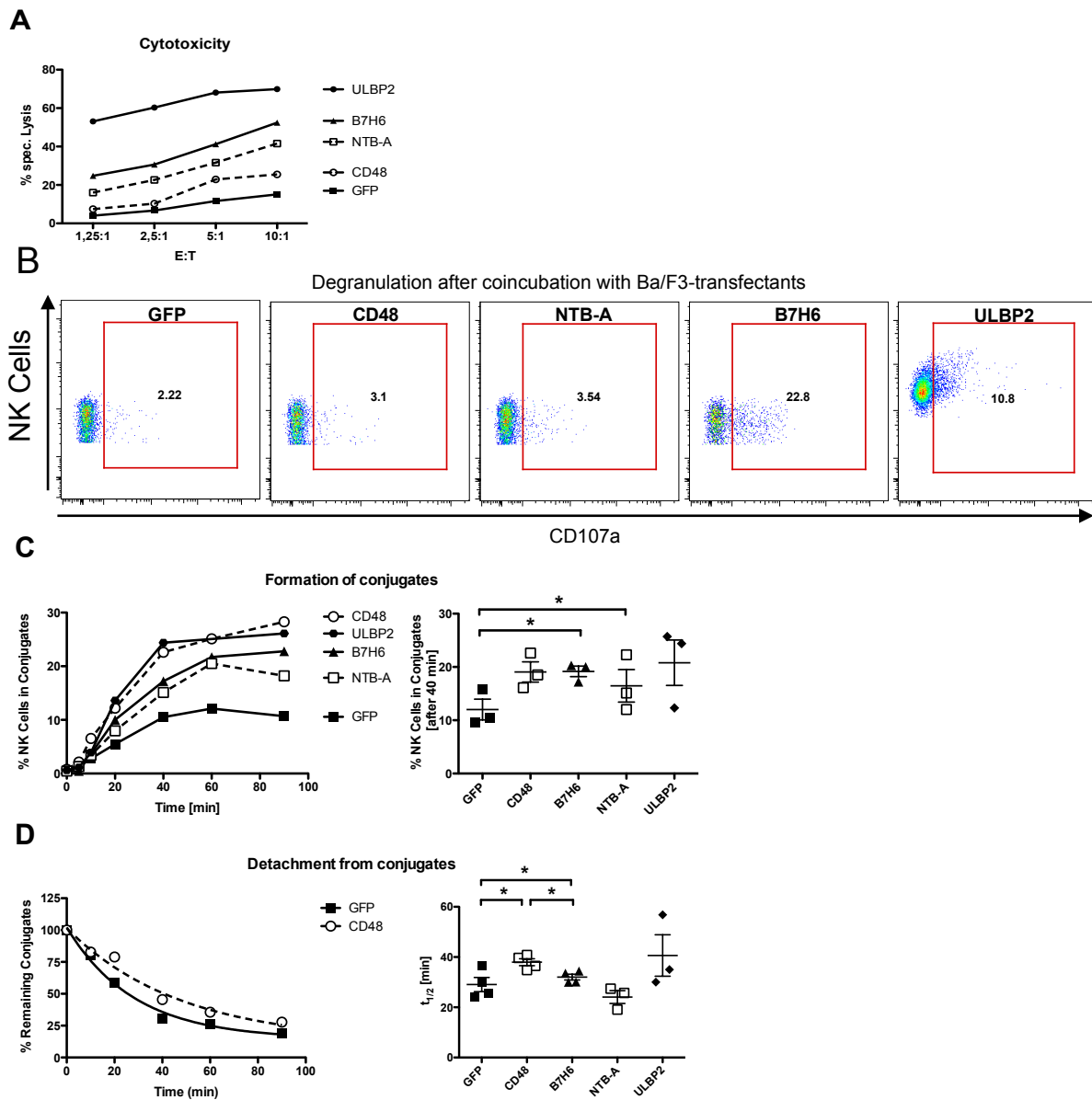
Figure 7. Different receptor-ligand interactions change the ability of NK cells to form conjugates and the detachment kinetic from a target cell. Cultured primary NK cells were coincubated with Ba/F3 cells, which have been stably transfected with NK cell ligands CD48, ULBP2, B7H6, NTB-A or GFP as a control. The cytotoxicity was analyzed using a standard Chromium-51 release assay **(A)**. Degranulation was analyzed after 2 h coincubation (E:T=1:2) and staining for CD107a on NK cells **(B).** Formation of conjugates was determined in a conjugate assay. Left panel shows one representative experiment, right panel shows the quantification of 3 different NK cell donors **(C)**. The detachment was analyzed in a detachment assay. Left panel shows the detachment of one representative experiment between GFP and CD48 transfected Ba/F3 cells. Right panel shows the quantification of 3-4 NK cell donors **(D)**. Values are shown as mean ± SD (*p<0.05).

The availability of different NK cell ligands on the target cell surface had a large impact on the ability to kill the target cell. Cultured primary NK cells lysed only few Ba/F3 cells transfected with a GFP-containing control vector. The expression of CD48, the natural ligand of SLAM-related receptor 2B4, slightly increased the number of killed target cells. This effect was enhanced, if the cells were transfected with NTB-A, a homophilic NK cell receptor. The NCR NKp30 is known to deliver a strong activating signal. The availability of its ligand B7H6 on the target cells facilitated the NK cell to kill

even more target cells. Expression of ULBP2, the ligand for C-type lectin-like receptor NKG2D, had the biggest effect on the pre-activated NK cells and led to the lysis of most of the target cells (Figure 7A). Next, I wanted to examine if NK cells degranulate differently after contact with the different ligands. I coincubated the Ba/F3-transfectants with cultured primary NK cells and determined the degranulation by staining with anti-CD107a antibody. The results show, that degranulation and the ability to kill the target cells are strongly associated. Only very few NK cells degranulate after contact to GFP control transfected Ba/F3 cells. Ba/F3-CD48 and Ba/F3-NTB-A cells, which have been killed only to a small extent, also stimulated the NK cells the least. In contrast, triggering of NKp30 and NKG2D with their ligands B7H6 and ULBP2 clearly enhanced the NK cell degranulation (Figure 7B). To figure out, which ligands are necessary for the formation of conjugates, I performed conjugate assays and quantified the number of conjugates after 40 minutes. Although Ba/F3 cells are mouse cells, its ICAM-1 is recognized by human LFA-1 and enables adhesion of human NK cells. Cultured primary NK cells were able to form conjugates with GFP control transfected Ba/F3 cells to a small but measureable extent. After 40 minutes, 12.02 ± 3.39 % of the NK cells were in a conjugate. Again, the presence of NTB-A has the smallest effect on NK cell effector functions. Only 16.46 ± 5.28 % of the NK cells formed conjugates with Ba/F3-NTB-A cells. The number of conjugates was clearly increased, if NKp30 was triggered by Ba/F3 cells that were transfected with B7H6 (19.17 ± 1.71 %). Interestingly, NK cells form conjugates to the same extent (19.07 ± 3.29 %) with Ba/F3-CD48 cells, which showed only a slight effect in the previously shown cytotoxicity assays. Again, the triggering of NKG2D by ULBP2 had the strongest effect on NK cells and led to the highest percentage of NK cells in conjugates after 40 minutes (20.80 ± 7.39 %) (Figure 7C). Finally I was interested in the influence these ligands have on NK cell detachment. Importantly, as shown in Figure 5C, the number of preformed conjugates differs between the Ba/F3-transfectants after 30 minutes pre-coincubation. Nevertheless, I could perform detachment assays and saw differences in the conjugate decay between NK cells and the differentially transfected target cells (Figure 7D, left panel). The quantification shows, that similar to the results of conjugate formation, CD48 and ULBP2 had the biggest effect on NK cell detachment. Transfection with these two ligands increases the conjugate half-life. The effect of B7H6 was clearly smaller and NTB-A showed no differences compared to the GFP control cells. These results show, that the interaction of different NK cell receptors and their ligands has an effect on NK cell effector functions. While the effect of NTB-A was small, the NKG2D:ULBP2 interaction showed a strong effect on all NK cell functions. In contrast, the interaction of 2B4 with CD48 did hardly increase the cytotoxicity but influenced the adhesion of NK cells.

4.3 Reduction of NK cell cytotoxicity leads to slower detachment

NK cell killing is largely mediated by the exocytosis of lytic granules. In a former study in our lab, we wanted to investigate if NK cell detachment is depending on intrinsic or extrinsic signals. Therefore we inhibited the target cell killing by depleting mature perforin. The NK cells were treated with the drug concanamycin A, an inhibitor for vacuolar H^+-ATPases. Concanamycin A inhibits the vacuolar type H^+-ATPase and thereby acidifies the lytic granules. The acidification inhibits cathepsin L, which is needed for cleavage of immature perforin into its active form (Kataoka et al., 1996; Kataoka et al., 1994). Thus, treatment with concanamycin A prevents the maturation of perforin. We could show that concanamycin A effectively decreases the amount of mature perforin in NK cells, which completely abolished the killing of K562 cells. This had no effect on the formation of conjugates but strongly decelerated the NK cell detachment (Netter, 2014).
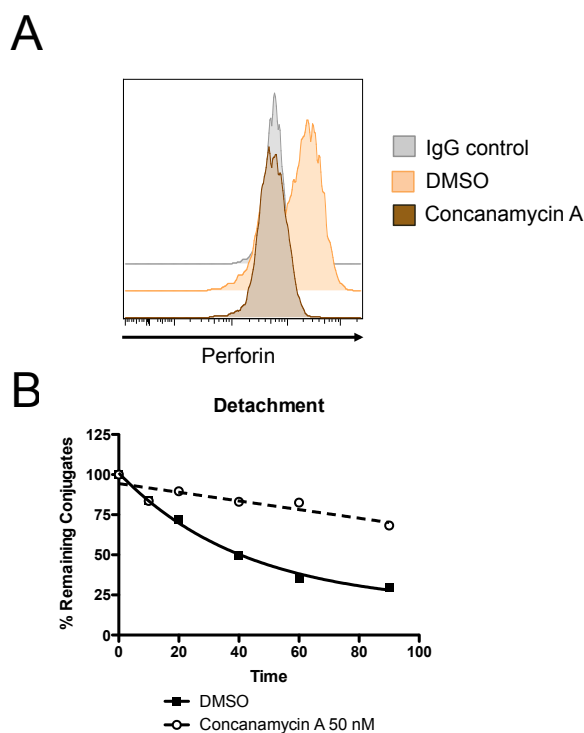

Figure 8. Concanamycin A treated primary NK cells express less perforin and have a prolonged contact time with K562 target cells. Cultured primary NK cells were treated with 50 nM concanamycin A or DMSO as a control for 2 h. Cells were fixed with 4 % PFA, permeabilized and DMSO (yellow histogram) and concanamycin A (brown histogram) treated cells were stained with α-perforin antibody or IgG-control (grey histogram) **(A)**. Concanamycin A (open circles) and DMSO (closed squares) treated NK cells were coincubated in a detachment assay with K562 target cells **(B)**.

In a first step, I wanted to replicate and confirm these results. I cultured primary NK cells with 50 nM concanamycin A for 2 h or DMSO as a control to prevent the maturation of functional perforin. As expected, intracellular staining with α-perforin antibody shows, that the treatment with concanamycin A depleted mature perforin nearly completely (Figure 8A, brown histogram). In contrast, NK cells treated with DMSO as a control were able to normally maturate perforin (Figure 8A, yellow histogram). Next, I could confirm that the absence of mature perforin due to concanamycin A treatment clearly decelerates the NK cell detachment from K562 target cells

compared to cells treated with DMSO (Figure 8B). The half-life in a detachment assay increased from ~ 29 minutes to ~ 550 minutes. These findings confirm the results from Netter and allow me to use concanamycin A in my experiments to further investigate the effect of perforin depleted NK cells with a decelerated detachment.

Another approach to reduce the cytotoxicity of NK cells was the treatment with the lactone antibiotic Brefeldin A. Brefeldin A interrupts the vesicle transport from ER to Golgi and therefore prevents exocytosis of vesicles (Fujiwara et al., 1988; Klausner et al., 1992). Exocytosis of cytotoxic granules is the main strategy to kill a target cell and I tried to block this process and analyze the effects on NK cell detachment. To analyze the effect of Brefeldin A on NK cell effector functions, I treated NK cells with 5 µg/ml Brefeldin A or ethanol as solvent control and coincubated them with K562 target cells. Performing the respective functional assays, I analyzed the formation of conjugates, cytotoxicity, degranulation and detachment.
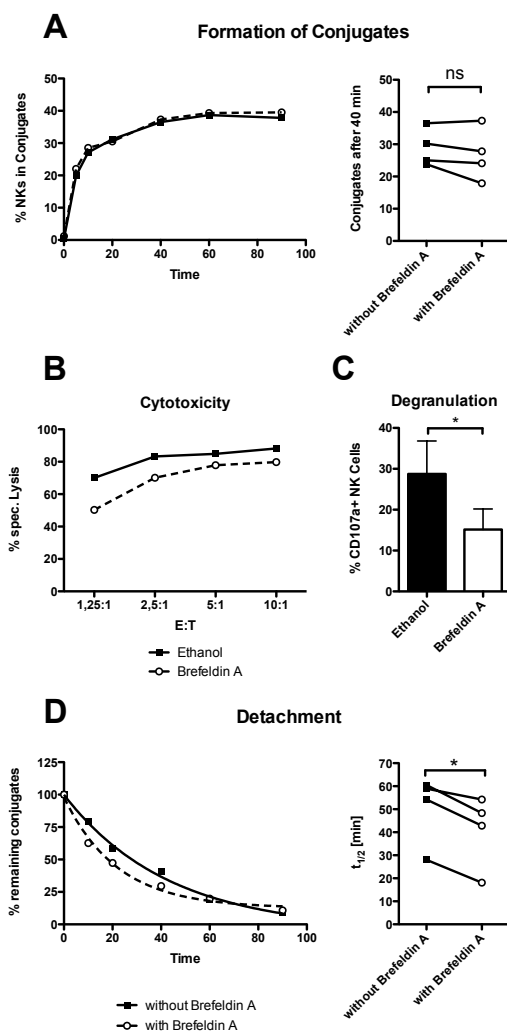
Figure 9. Treatment of NK cells with Brefeldin A has no influence on the formation of conjugates but accelerates the detachment. NK cells were treated with 5 µg/ml Brefeldin A directly before coincubation with K562 target cells. The formation of conjugates was analyzed in a conjugate assay. Left panel shows one representative experiment, right panel shows the number of conjugates after 40 min. from four independent experiment **(A).** The cytotoxicity was assessed in a standard Chromium-51 release assay **(B).** The degranulation was measured using an α-CD107a antibody after 2 h coincubation of Brefeldin A treated NK and K562 cells **(C).** The detachment of preformed NK:K562 conjugates was assessed in a detachment assay. Left panel shows one representative experiment and the right panel the quantification of NK cells from four different donors **(D).** Values are shown as mean ± SD (*p<0.05).

The treatment of cultured primary NK cells with 5 µg/ml Brefeldin A had no significant effect on the formation of conjugates (Figure 9A). After 40 minutes, 28.88 ± 5.79 % of the Brefeldin A treated and 26.78 ± 8.12 % of the ethanol treated NK cells were in conjugates. Analyzing the cytotoxicity I could show, that Brefeldin A reduces the specific lysis of K562 in a standard Chromium-51 release assay (Figure 9B). This was probably due to a decreased ability to degranulate, as I could detect less CD107a on the cell surface of Brefeldin A treated NK cells after coincubation with K562 cells compared to ethanol treated control cells (Figure 9C). Surprisingly, Brefeldin A treatment accelerated the detachment of NK cells. The average half-life for Brefeldin A treated cells was 40.89 ± 15.88 minutes and therefore slightly but significantly shorter then for the control treated cells (50.44 ± 15.20 minutes). These results show, that Brefeldin A treatment can decrease the NK cell degranulation and killing of K562 cells. But in contrast to concanamycin A, the Brefeldin A treatment did not decelerate but accelerate the conjugate decay.

In the next step I wanted to exclude the possibility, that the decelerated detachment of concanamycin A treated NK cells was a side effect of the drug. I genetically modified the target cell line K562 to make them more resistant against NK cell-mediated killing. For this, I overexpressed Serine protease inhibitor 9 (SerpinB9) and CD107a. This gave me the opportunity to leave the NK cells completely untouched.

Cancer cells have evolved multiple ways to escape from NK cell-mediated immunosurveillance. One strategy is the overexpression of SerpinB9 (Bladergroen et al., 2002), which was shown to inhibit Granzyme B and therefore prevent the killing by cytotoxic immune cells (Bird et al., 1998). The lysosome-associated membrane protein CD107a/LAMP-1 was recently shown to prevent the insertion of perforin and the formation of pores in the cell membrane, which hinders Granzyme B to enter the cell to induce apoptosis. Cytotoxic immune cells express CD107a to protect themselves against their own perforin (Cohnen et al., 2013).
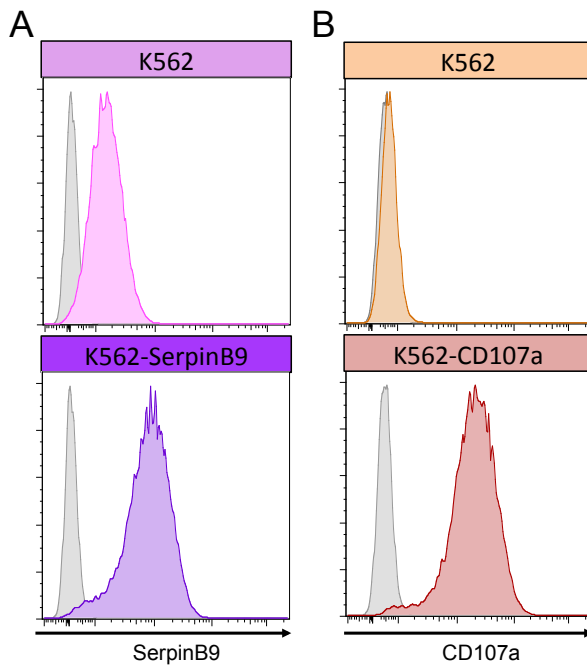
Figure 10. K562 cells could be stably transfected with SerpinB9 and CD107a. K562 cells were retrovirally transfected with SerpinB9 and CD107a. K562 wildtype cells (upper panels) and stably transfected cells (lower panels) were stained with α-SerpinB9 **(A)** or α-CD107a antibody **(B)**. Grey histograms show staining of the respective cells with IgG antibody as a control.

I used a retroviral transfection system to transfect K562 cells with SerpinB9 and CD107a. Two days after transduction with the viruses containing the constructs, the transfected cells were treated with Puromycin for selection. Resistant cells were checked for the overexpression of the transfected protein after 1-2 weeks. After intracellular staining with α-SerpinB9 antibody, endogenous SerpinB9 could be detected in the K562 wildtype cells. However, the K562-SerpinB9 cells showed a clearly increased SerpinB9 expression (Figure 10A). In contrast, the K562 wildtype cells showed no cell surface expression of CD107a. Only the K562-CD107a cells could be stained by α-CD107a antibody. (Figure 10B)

In the next step I wanted to check, if the stable overexpression of SerpinB9 and CD107a in K562 cells reduces the NK cell-mediated killing. In a standard Chromium-51 release assay cultured primary NK cells from the same donor killed less K562-SerpinB9 and less K562-CD107a cells compared to K562 wildtype cells (Figure 11A). Of note, the overexpression of CD107a on the cell surface of K562-CD107a cells did not influence the ability of NK cells to form conjugates (Figure 11B). Remarkably, the detachment of NK cells from K562-SerpinB9 or K562-CD107a conjugates was clearly decelerated compared to K562 wildtype conjugates. NK cells detached after 60.07 ± 18.44 minutes from K562 wildtype cells. The overexpression of SerpinB9 significantly increased the half-life to 115.8 ± 22.59 minutes and the overexpression of CD107a to 85.03 ± 22.43 minutes. These results confirmed the experiments with concanamycin A treated NK cells. Reduction of the NK cell cytotoxicity or increasing of the target cell resistance both clearly decelerates the NK cell detachment.
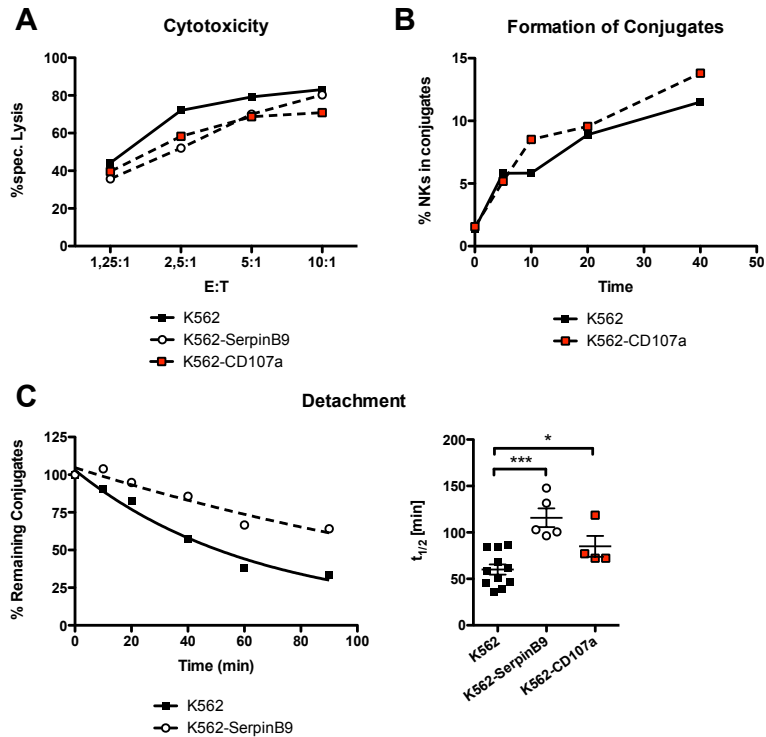
Figure 11. The overexpression of SerpinB9 and CD107a reduces NK cell-mediated killing and decelerates the detachment from target cells. NK cells were coincubated with K562-SerpinB9 and K562-CD107a. The cytotoxicity of NK cells against the transfectants was assessed in a standard Chromium-51 release assay **(A)** and the ability to form conjugates in a conjugate assay **(B)**. One representative experiment of 3 is shown. The detachment from target cells was analyzed in a detachment assay **(C)**. Left panel shows one representative experiment, right panel shows the quantification of the half-life (n = 4-11). Values are shown as mean ± SD (*p<0.05; ***p<0.001).

After I could show, that NK cells detach later if their ability for killing the target cell is decreased, I wanted to investigate the opposite situation. I induced apoptosis in target cells while they were in a conjugate with an NK cell. So I could study if NK cells recognize the target cell death and initiate the detachment, even if the target cell dies for an NK cell independent reason. For this I induced apoptosis in K562 cells by the treatment with H_2O_2, coincubated them with cultured primary NK cells and studied the ability to form conjugates and detach with the respective assays.
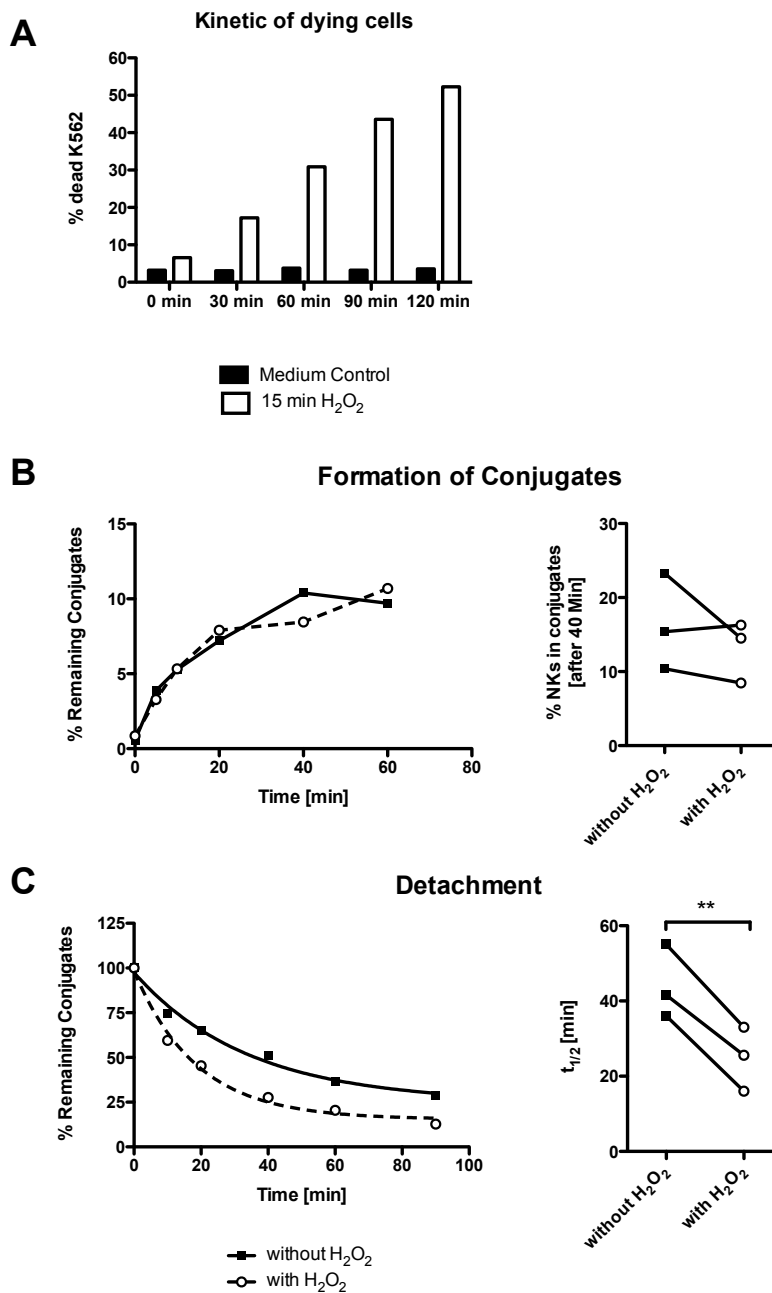
A

Kinetic of dying cells

B

Formation of Conjugates

C

Detachment

Figure 12. NK cell detachment accelerates when the target cell is apoptotic. K562 cells were treated with 25 mM H_2O_2 for 15 minutes and then washed. Samples were taken all 30 minutes and stained with life/dead-dye zombie-aqua. Percentage of dead cells is shown for H_2O_2 treated cells (white bars) and untreated cells (black bars) **(A)**. The formation of conjugates was assessed in a conjugate assay **(B)** and the detachment from K562 target cells was analyzed in a detachment assay **(C)**. Left panels show one representative experiment, right panels show the quantification of three independent experiments with NK cells from three different donors (**p<0.01).

K562 cells were treated with 25 mM H_2O_2 for 15 minutes, then washed thoroughly and used in conjugate and detachment assays. Staining with a live/dead marker allowed me to illustrate the kinetics of apoptosis in the H_2O_2 treated cells. About 50 % of the K562 cells died in the first 120 minutes, which represents the duration of a detachment assay. During the first 30 minutes, the time-period I use to let the NK cells form conjugates, only ~ 17 % of the K562 cells died (Figure 12A). I did not find differences in the formation of conjugates when I coincubated cultured primary NK cells with H_2O_2 treated or untreated K562 target cells (Figure 12B, left panel). After 40 minutes, the numbers of conjugates were comparable (Figure 12B, right panel). This gave me the opportunity, to use the cells in our detachment assay and analyze the effect of NK cell independent apoptosis on the decay of

NK:K562 conjugates. NK cells detached clearly faster if coincubated with H_2O_2 treated K562 (Figure 12C, left panel). The half-life was significantly decreased and dropped from 44.19 ± 9.77 minutes without H_2O_2 to 24.86 ± 8.52 minutes with H_2O_2 (Figure 12C, right panel). These results show that NK cell detachment is dependent on extrinsic signals from the target cell. NK cell independent apoptosis of the target cell leads to alterations of the target cell, which are recognized by the NK cell and induce detachment.

The immune disorder familial hemophagocytic lymphohistiocytosis 2 (FHL-2) is characterized by the absence of perforin in cytotoxic lymphocytes (Janka, 2012; Stepp et al., 1999a). This results in the overproduction of inflammatory cytokines TNF-α and IFN-γ and a prolonged conjugation time of the NK cells (Jenkins et al., 2015). I wanted to investigate, if NK cells show the same cytokine overproduction if they are treated with concanamycin A or coincubated with NK cell resistant K562 transfectants. To boost the protective effect of SerpinB9 and CD107a, I cotransfected K562 with vectors for both proteins and sorted the cells to increase the amount of cells coexpressing both proteins. The newly transfected K562-SerpinB9-CD107a cells expressed clearly more SerpinB9 and CD107a compared to K562 wildtype cells (Figure 13A).
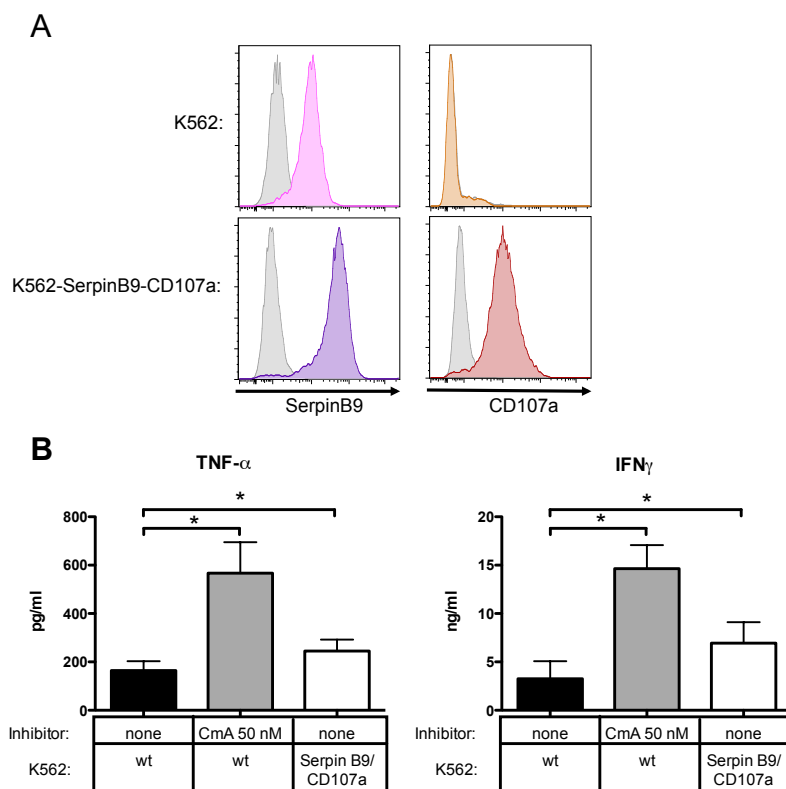
Figure 13. NK cells produce more of the cytokines TNF-α and IFN-γ if treated with concanamycin A or coincubated with K562-SerpinB9-CD107a. K562 cells were retrovirally transfected with SerpinB9 and CD107a. K562 wildtype cells (upper panels) and stably transfected cells (lower panels) were stained with α-SerpinB9 or α-CD107a antibody. Grey histograms show staining of the respective cells with IgG antibody as a control (A). Cultured primary NK cells were coincubated with K562 cells over night and production of TNF-α (left panel) or IFN-γ (right panel) was assessed by ELISA. NK cells were either treated with concanamycin A (50 nM) for 2 h and coincubated with K562 wildtype (wt, grey bar). Or untreated cultured primary NK cells were coincubated with K562-SerpinB9-CD107a cells (white bar). As a control, cultured primary NK cells were coincubated with K562 wildtype cells (black bar) (B). Values are shown as mean ± SD (*p<0.05).

I treated cultured primary NK cells with 5 nM concanamycin A to prevent the maturation of perforin and reduce the cytotoxicity. I coincubated the NK cells with K562 wildtype cells for 16 h and

measured the concentration of secreted TNF-α and IFN-γ in the supernatant. I found the secretion of TNF-α to be ~ 3.4 fold increased if the NK cells were treated with concanamycin A compared to untreated NK cells. Additionally, the amount of secreted INFγ was more than 4.5 fold higher.

In a second approach I coincubated untouched cultured primary NK cells with K562-SerpinB9-CD107a cells. Notably, the cotransfection with SerpinB9 and CD107a reduced the NK cell-mediated killing and delayed the detachment similar as I saw with K562 cells overexpressing only one of these proteins (data not shown). But this effect was not boosted by the overexpression of both proteins. The coincubation of cultured primary NK cells with K562-SerpinB9-CD107a also resulted in a significantly higher production of TNF-α and IFN-γ. The secretion of TNF-α was increased by 1.5 fold and the secretion of IFN-γ by ~ 2 fold. These results show, that delayed NK cell detachment increases the secretion of proinflammatory cytokines TNF-α and IFN-γ (Figure 13B).

4.4 Calcium and redox signaling are important second messengers for NK cell detachment

Calcium is an important second messenger in all eukaryotic cells. In lymphocytes, calcium is responsible for many cell functions like proliferation and cytotoxicity. NK cells get stimulating signals over the whole period of conjugation. I wanted to analyze, how the calcium signal changes over time.
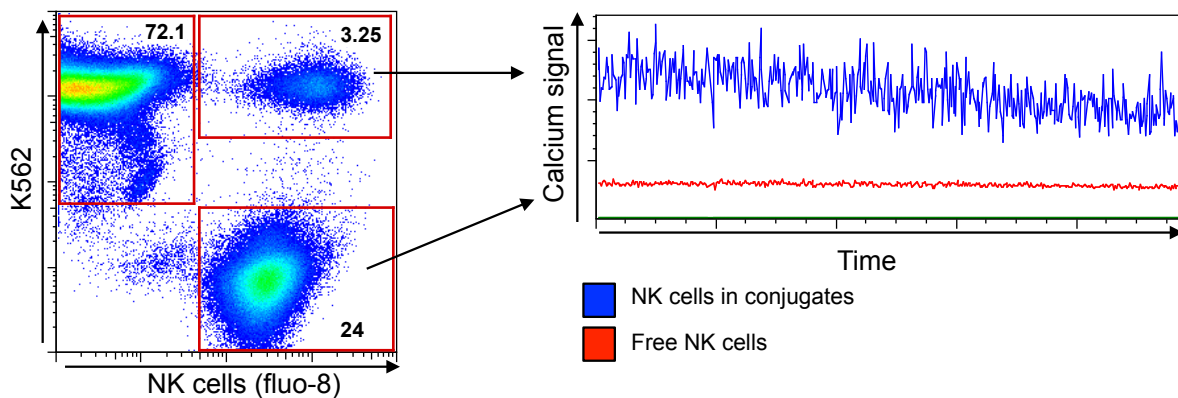
Figure 14. The calcium signal of NK cells in conjugates is increased compared to free NK cells. Cultured primary NK cells were coincubated with K562 target cells for 30 minutes (E:T = 1:2). NK cells were stained with calcium binding dye fluo-8 and K562 cells with cell tracker orange to distinguish between free NK cells and K562 as well as conjugates (left panel). The calcium signal of free NK cells (red line) and NK cells in conjugates (blue line) was analyzed over 10 minutes (right panel).

NK cells were stained with calcium binding dye fluo-8 and K562 with cell tracker orange. This allowed me to distinguish between free NK cells, free K562 cells and conjugates consisting of both cell types (Figure 14, left panel). Similar to a detachment assay, NK and K562 cells were coincubated for 30 minutes to form conjugates and then diluted 1:25. Cells were divided in multiple samples and rotated to allow NK cells to detach. All samples were measured in succession, each for 4 minutes. Using this

approach, I can analyze the calcium signal in NK cells, which have been in conjugates for different time periods and combine these data to one continuous kinetic.

Free NK cells, stained with fluo-8, showed a low calcium signal (Figure 14, red line). This intracellular calcium signal was constant and did not change over time. In contrast, NK cells in conjugates showed a clearly increased calcium signal (Figure 14, blue line). The calcium signal was at any time higher than the calcium signal from free NK cells, but the signal decreased over time.

In the next step I wanted to analyze the effect of calcium signaling on NK cell detachment, which has not been investigated before. In our flow cytometry system it is not possible to measure the calcium signal of a single NK cell over the time of conjugation and detachment. Therefore, I measured the calcium level of the whole population of NK cells in conjugates over time as described for Figure 14.
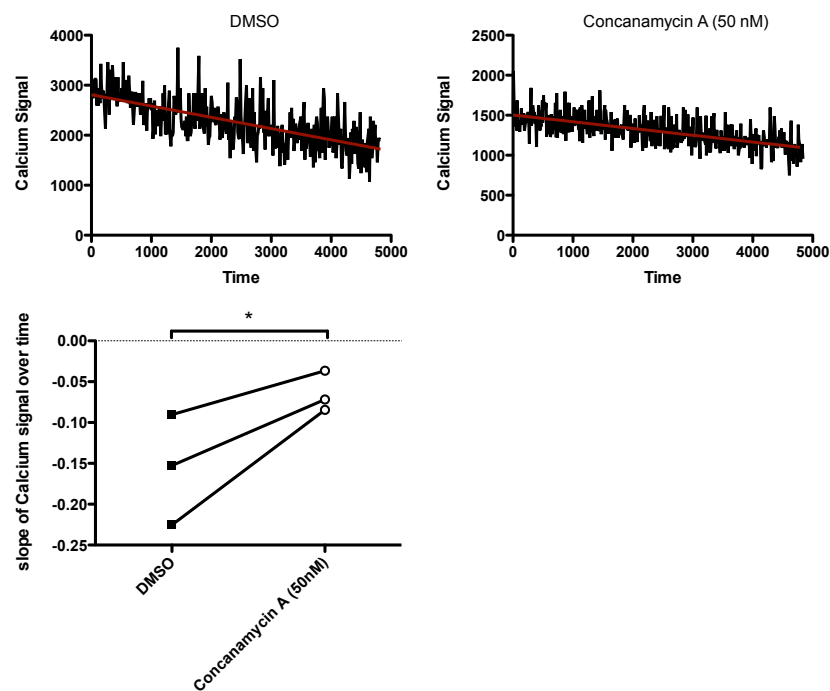
Figure 15. Decrement of the calcium signal of NK cells in conjugates is decelerated if NK cells are pretreated with concanamycin A. NK cells were pretreated for 2 h with 50 nM concanamycin A or DMSO as a control and stained with calcium binding dye fluo-8. After 30 min pre-coincubation, detachment was analyzed over 90 minutes. Calcium signal of NK cells in conjugates was plotted against the time and regression line was calculated (top panels). Slope of calcium signal over time for DMSO and concanamycin A treated NK cells is illustrated for 3 different donors (lower panel) (*p<0.05).

I wanted to investigate how the calcium signal in NK cells changes if the cells were unable to detach from target cells. For this, I treated cultured primary NK cells for 2 h with 50 nM concanamycin A to prevent the maturation of perforin and thereby reduce the detachment (see Figure 8). NK cells treated with DMSO as a control, showed a strong calcium signal in the beginning of the experiment. The NK cells measured later during the detachment assay, showed lower calcium levels. I saw the same effect in NK cells treated with concanamycin A (Figure 15, upper panels). However, the calcium signal decreased less over time. Importantly, the curve shows changes of the mean fluorescence intensity of the calcium signal from the NK cell population in conjugates over time. From these data, I could calculate a regression line and estimate the slope of the calcium signal in NK cells over time. The slope of the calcium signal in DMSO treated NK cells was significantly decreased compared to NK cells treated with concanamcin A (Figure 15, lower panel). These results show, that the calcium signal in NK cells is maintained if the NK cells cannot kill and detach from the target cell.

An important step for the formation of the immunological synapse is the binding of adhesion receptor $\alpha_L\beta_2$ integrin (LFA-1) to its ligands Intercellular Adhesion Molecules (ICAMs). The adhesiveness of LFA-1 depends on its conformation state and clustering and is controlled by inside-out signaling. Activation of the NK cell leads to increased affinity and avidity of LFA-1.

The question has been raised, if NK cells downregulate the activity of LFA-1 after recognizing the target cell death to decrease adhesion and force detachment. I performed a LCAA-Assay to analyze the activity of LFA-1 by staining with antibody labeled ICAM-1-complexes and compared fresh unstimulated NK cells 1) before conjugation 2) in conjugates with a target cell and 3) freshly detached (Figure 16A). ICAM-1 complexes bind to the activated high affinity and high avidity forms of LFA-1. To stimulate the binding, I used HeLa-CD48 as target cells.
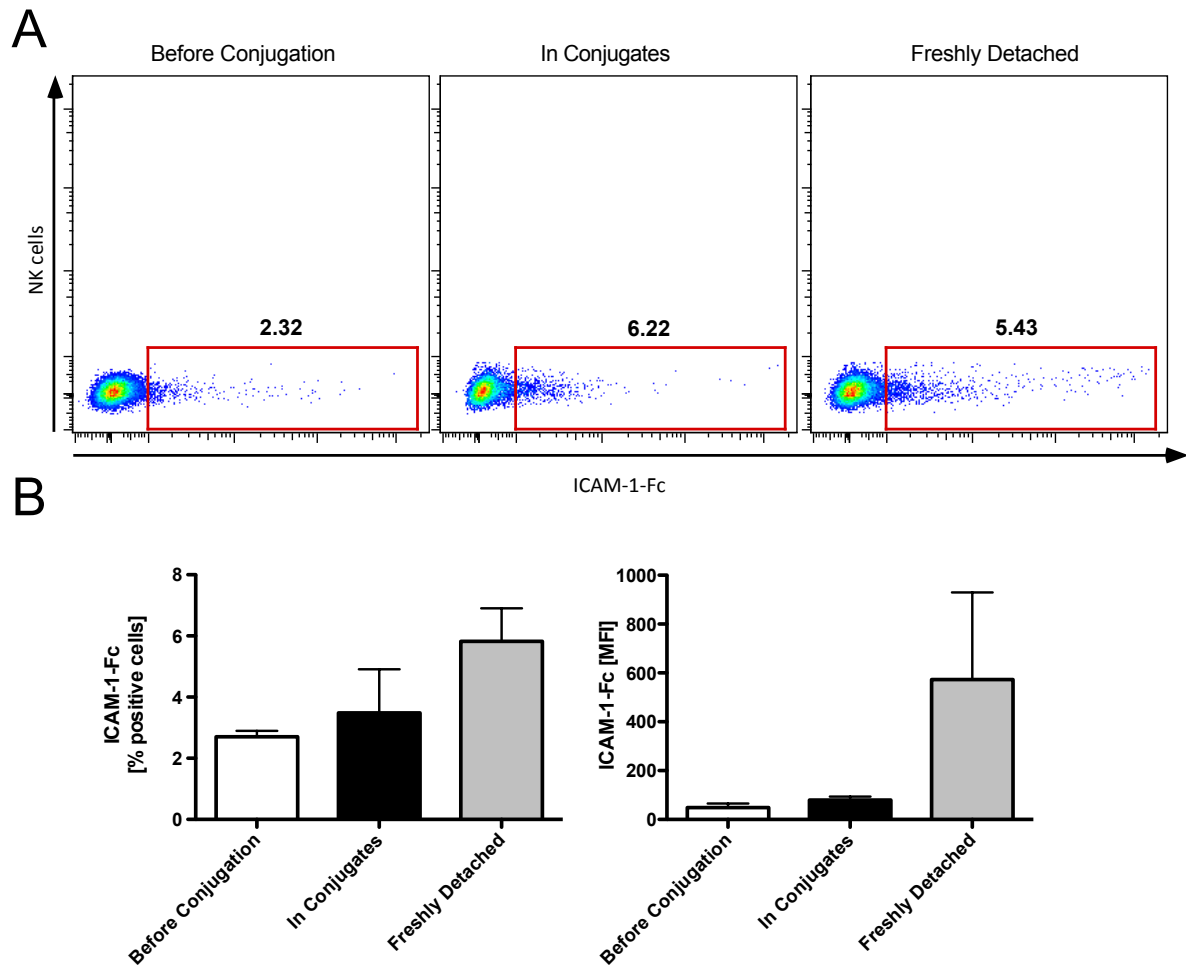
Figure 16. LFA-1 activity is increased in freshly detached NK cells compared to NK cells in conjugates. NK cells were coincubated with adherent HeLa-CD48 target cells for 30 minutes in the presence of ICAM-1-Fc complexes. NK cells, which have not been attached in the next 30 minutes, were fixed and the NK cells in conjugates incubated for additional 30 minutes. In the end, freshly detached NK cells and NK cells in conjugates were fixed **(A)**. The number of ICAM-1-Fc positive NK cells and the MFI is quantified for 2 independent experiments. Values are shown as mean ± SD **(B)**.

NK cells, which had not formed a conjugate in the first 30 minutes of coincubation with HeLa-CD48 cells, had the lowest LFA-1 activity. The ICAM-1 complexes bound only to 2.32 ± 0.34 % of the NK cells. But I can't exclude, that those NK cells have been in contact with a target cell in the 30 minutes before and detached really fast. NK cells, which were in a conjugate showed an increased LFA-1 activity. 3.48 ± 2.47 % of the NK cells bound the ICAM-1 complexes. The mean fluorescence intensity (MFI) can be used to compare the overall amount of ICAM-1 complexes bound to the cells. I measured the MFI of all NK cells in the three samples. This value was moderately increased in NK cells in conjugates (79.57 ± 24.33) compared to free NK cells (48.97 ± 28.36). Interestingly, NK cells which have been in a conjugate after 30 minutes coincubation and detached in the following 30 minutes, showed the highest LFA-1 activity. 5.82 ± 1.88 % of the cells bound ICAM-1 complexes. The MFI was 5-fold higher compared with free NK cells. The results show that NK cells increase the LFA-1

affinity and avidity after activation and binding to a target cell. But differently than expected, the freshly detached NK cells displayed a higher LFA-1 activity than NK cells in conjugates (Figure 16B).

In the next step, I wanted to further investigate the effect of calcium signaling on NK cell detachment. I used the drug Thapsigargin to manipulate intracellular calcium levels and analyzed the effect on conjugate formation, cytotoxicity, degranulation and the detachment from K562 target cells. Thapsigargin interrupts calcium homeostasis by inhibition of the Sarco-Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) (Thastrup et al., 1990). This blocks the transport from cytosolic calcium into the ER and therefore quickly increases the cytosolic calcium concentration.
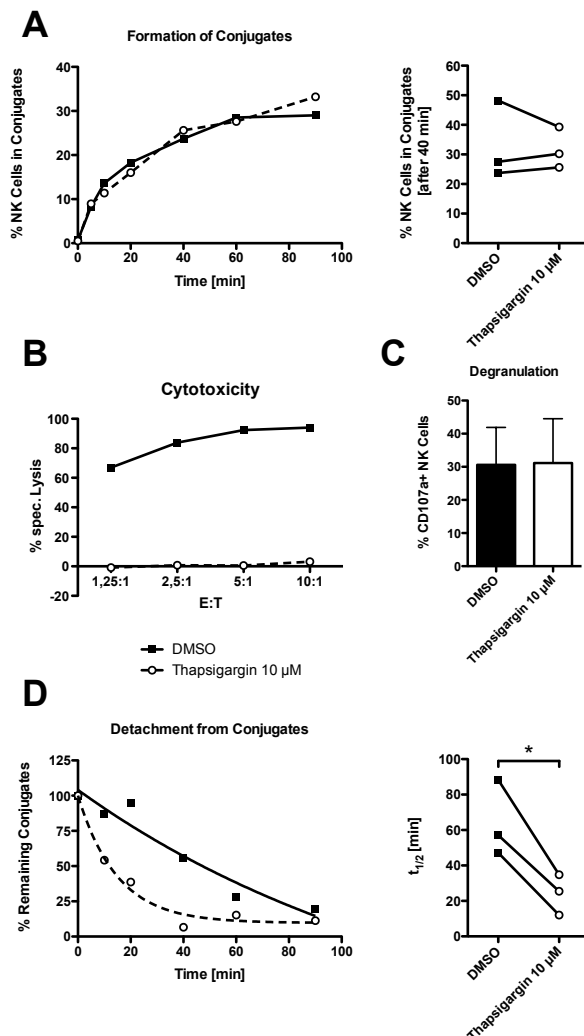
Figure 17. NK cell detachment is accelerated and killing is abolished after treatment with Thapsigargin. NK cells were treated with 10 µM Thapsigargin directly before coincubation with K562 target cells and the formation of conjugates was analyzed in a conjugate assay. Left panel shows one representative experiment and the right panel the quantification of NK cells from three different donors **(A)**. The cytotoxicity was analyzed in a standard Chromium-51 release assay (One representative experiment from three independent experiments) **(B)**. The degranulation was measured in a CD107a-Assay after 2 h coincubation (mean ± SD of three independent experiments) **(C)**. The detachment of preformed NK:K562 conjugates was assessed in a detachment assay. Thapsigargin was added directly after dilution of conjugates. Left panel shows one representative experiment and the right panel the quantification of NK cells from three different donors (*p<0.05) **(D)**.

Examining the effect of artificially induced high cytosolic calcium level, I treated cultured primary NK cells directly before coincubation with K562 target cells with 10 µM Thapsigargin and analyzed the impact on the formation of conjugates. I could not observe any changes in the adhesion of NK cells to K562 cells after Thapsigargin treatment compared to DMSO treated NK cells (Figure 17A). In contrast, the rise of cytosolic calcium levels by Thapsigargin abolished the cytotoxicity completely. After a 4 h

Chromium-51 release assay, hardly any target cells have been lysed (Figure 17B). Notably, this was a long-term effect. I used a 16 h Chromium-51 release assay with very low NK to K562 ratios to monitor serial killing. Even in this 16 h assay, NK cells totally lost the ability to lyse target cells (data not shown). In order to find the reason for this defect in cytotoxic capability, I tested if the cells degranulate after binding a target cell, but I could not find any difference in NK cells treated with Thapsigargin or with DMSO as a control (Figure 17C). Finally, analyzing the NK cell detachment, I found NK cells to detach significantly earlier after treatment with Thapsigargin. The half-life was reduced from 64.24 ± 21.48 minutes to 24.09 ± 11.47 minutes (Figure 17D). These results show, that NK cells need calcium homeostasis for conjugate maintenance. Disruption of this homeostasis leads to decay of the conjugate and abolishes the NK cell cytotoxicity.

Another possibility to raise intracellular calcium concentration is the use of the ionophore Ionomycin. Ionomycin can transport calcium ions freely across the cell membrane, which allows ions to diffuse into the cytosol. I tested the effect of Ionomycin on NK cell effector functions as described for Thapsigargin in Figure 18.

Figure 18. Treatment with calcium ionophore Ionomycin accelerates detachment but reduces cytotoxicity. NK cells were treated with 1.3 mM Ionomycin directly before coincubation with K562 target cells and the formation of conjugates was analyzed in a conjugate assay. Left panels show one representative experiment and right panels the quantification of NK cells from three different donors **(A)**. The cytotoxicity was analyzed in a standard Chromium-51 release assay (One representative experiment out of three) **(B)**. The degranulation was measured in a CD107a-Assay after 2 h coincubation. Three independent experiments are quantified. Values are shown as mean ± SD (*p<0.05) **(C)**. The detachment of preformed NK:K562 conjugates was assessed in a detachment assay. Ionomycin was added directly after dilution of the conjugates. Left panel shows one representative experiment and the right panel the quantification of NK cells from three different donors (*p<0.05) **(D)**.
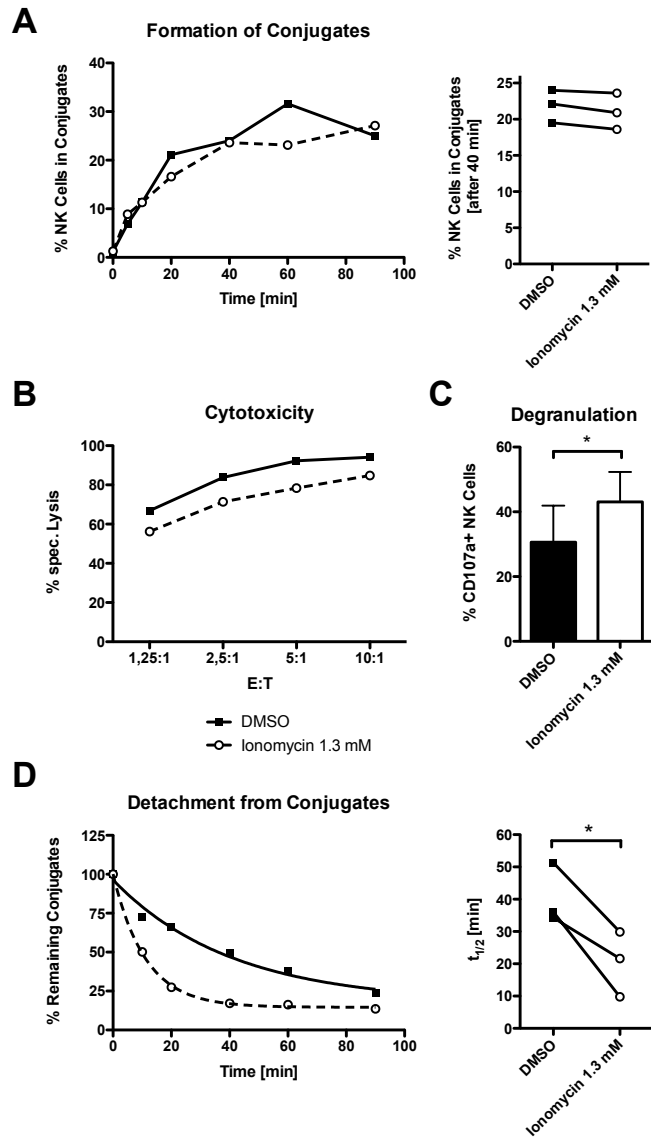
Similar to the treatment with Thapsigargin, the addition of 1.3 mM Ionomycin did not have any effect on the formation of conjugates. After 40 minutes ~ 20 % of both Ionomycin but also the DMSO treated NK cells formed conjugates with K562 target cells (Figure 18A). The specific lysis of K562 cells in a 4 h Chromium-51 release assay was slightly reduced in the presence of Ionomycin (Figure 18B). But this effect was less stable compared with the Thapsigargin treatment. In a 16 h Chromium-51 release assay, I found no difference in the specific lysis of K562 cells between Ionomycin treated and untreated NK cells (data not shown). Romera-Cardenas et al. reported, that after 16 h treatment with Ionomycin, NK cells became hyporesponsive and showed a clear reduction in the ability to degranulate. Nevertheless, after 2 h I found NK cells to degranulate more in response to K562 cells if they had been treated with Ionomycin compared to DMSO control (Figure 18C). It has to be investigated, if the secretion of lytic granules was directed towards the target cell or if Ionomycin just stimulated the undirected secretion of lytic granules. An explanation for the reduced cytotoxicity

could be a strong acceleration in the NK cell detachment. If Ionomycin was added at the point of dilution, NK:K562 conjugates decayed very fast compared to DMSO treated control samples (Figure 18D). Ionomycin significantly reduced the half-life from 40.58 ± 9.37 minutes to 20.44 ± 10.11 minutes. These results demonstrate, that calcium is an important signaling molecule for NK cell effector functions. Manipulating the calcium levels by treatment with calcium ionophore Ionomycin has a large impact on the maintenance of NK conjugates and reduces the stability clearly.

Besides calcium signaling, many intracellular proteins are regulated by redox signaling. Mainly NADPH oxidases produce ROS, which oxidize and thereby activate or inhibit other molecules (Ushio-Fukai, 2006). NK cell effector functions are known to be influenced by redox signaling. For example, the activity of transcription factor NFκB is among others dependent on ROS (Stottmeier and Dick, 2016). I wanted to investigate if redox signaling is also important for NK cell detachment and maintenance of NK:K562 conjugates and NK cell cytotoxicity. Therefore I treated NK cells with the antioxidant N-acetylcysteine, which reduces ROS and thereby manipulates intracellular redox signaling.
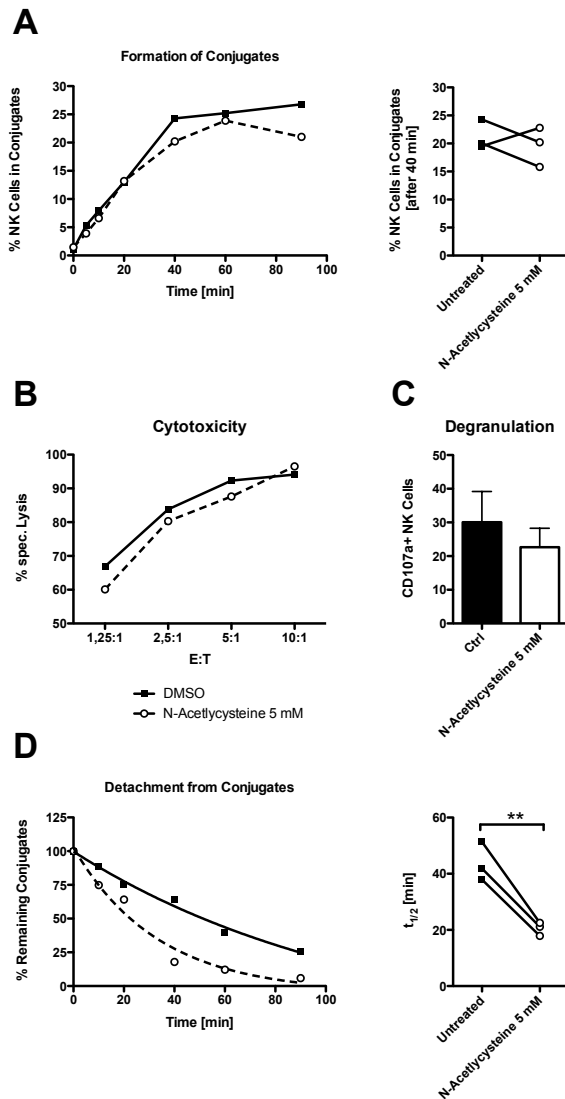
Figure 19. Treatment with antioxidant N-Acetylcysteine has a slight effect on killing and decreases NK:K562 conjugate stability. NK cells were treated with 5 mM N-Acetylcysteine directly before coincubation with K562 target cells and the formation of conjugates was analyzed in a conjugate assay. Left panel shows one representative experiment and the right panel the quantification of NK cells from three different donors **(A)**. The cytotoxicity was analyzed in a standard Chromium-51 release assay (One representative experiment from three independent experiments) **(B)**. The degranulation was measured in a CD107a-Assay after 2 h coincubation. Three independent experiments are quantified **(C)**. The detachment of preformed NK:K562 conjugates was assessed in a detachment assay. N-Acetylcysteine was added directly after dilution of conjugates. Left panel shows one representative experiment and the right panel the quantification of NK cells from three different donors (**p<0.01) **(D)**

The addition of 5 mM N-Acetylcysteine at the point of coincubation did not lead to differences in the formation of conjugates (Figure 19A, left panel). Quantification of the percentage of conjugates after 40 minutes showed, that the treatment with N-Acetylcysteine has no significant influence on the formation of conjugates (Figure 19A, right panel). But I could reveal, that N-Acetylcysteine had a little effect on NK cell cytotoxicity. The killing of K562 target cells was slightly decreased in a 4 h (Figure 19B) and a 16 h Chromium-51 release assay (data not shown). I also saw the trend that N-Acetylcysteine reduces the ability of NK cells to degranulate after contact with target cells (Figure 19C). Applied in a detachment assay, N-Acetylcysteine clearly accelerated the NK cell detachment (Figure 19D). This led to a significantly reduced half-life of NK:K562 conjugates in the presence of N-Acetylcysteine and could explain the decreased cytotoxicity. These results show, that redox signaling is also involved in the maintenance of NK cell conjugates and manipulation of the intracellular redox milieu can decrease NK cell effector functions.

4.5 Influence of the inhibiting receptor CD300a on NK cell detachment

So far, the depicted results show that NK cells are able to detect the target cell death and react by immediate detachment. In order to find the mechanism how NK cells recognize the death of the target cell, several possibilities have to be considered. The existence of a special NK cell receptor that can identify and bind damage associated molecular patterns (DAMPs) and inhibit the NK cell functions is one possible mechanism. Cantoni et al. reported, that the NK cell receptor CD300a is expressed on all NK cells and has inhibitory functions through intracellular ITIM motifs (Cantoni et al., 1999). This receptor can bind to phosphatidylserine (Nakahashi-Oda et al., 2012), a lipid that is only present on the cell surface of apoptotic cells. The ligation of CD300a with PS leads to an inhibitory signal and reduces NK cell cytotoxicity (Lankry et al., 2013).
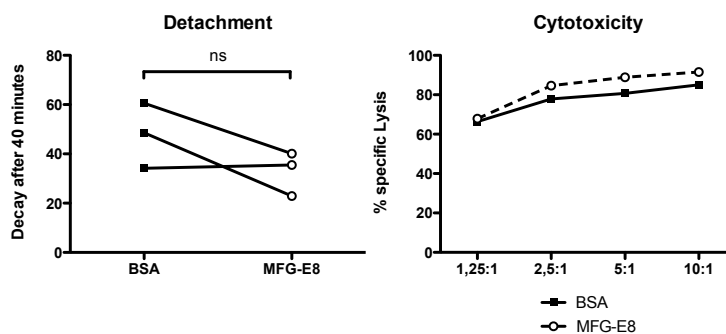
Figure 20. Blocking of CD300a-phosphatidylserine interaction has no effect on the cytotoxicity or detachment of NK cells. Cultured primary NK cells were coincubated with K562 target cells for 30 minutes. Directly after dilution 10 µg/ml MFG-E8 was added to the cells and the detachment was analyzed after 40 minutes (left panel). The cytotoxicity was analyzed after addition of 10 µg/ml MFG-E8 in a standard Chromium-51 release assay (right panel, n=1) ns=not significant.

I tested if the interaction of CD300a and its ligand phosphatidylserine has any effect on NK cell detachment and cytotoxicity by blocking with MFG-E8. MFG-E8 binds to phosphatidylserine and prevents the ligation with CD300a (Lankry et al., 2013). To investigate the effect on NK cell detachment, I diluted preformed NK:K562 conjugates with medium and added 1 µg/ml MFG-E8 or BSA as a control. I analyzed the amount of NK cells in conjugates after 0 and 40 minutes. The result for 0 minutes sample was set to 100 % and the 40 minutes time-point was normalized. I could not find any significant differences in the detachment after treatment with MFG-E8 (Figure 20, left panel). Also I could not find a reduced ability to kill K562 target cells, if 1 µg/ml MFG-E8 was added in a standard 4 h Chromium-51 release assay (Figure 20, right panel). This indicates, that the interaction of inhibitory receptor CD300a and phosphatidylserine has no effect on NK cell detachment.

4.6   The effect of soluble factors on NK cell effector functions

Another possibility I considered was the influence of soluble factors, which are secreted by the dying target cell and influence the NK cell functions. It is well known that cells shed surface proteins and secrete apoptotic vesicles in the process of apoptosis. I wanted to investigate, if NK cells sense these proteins or vesicles and use them as an indicator of target cell death to initiate the process of detachment.
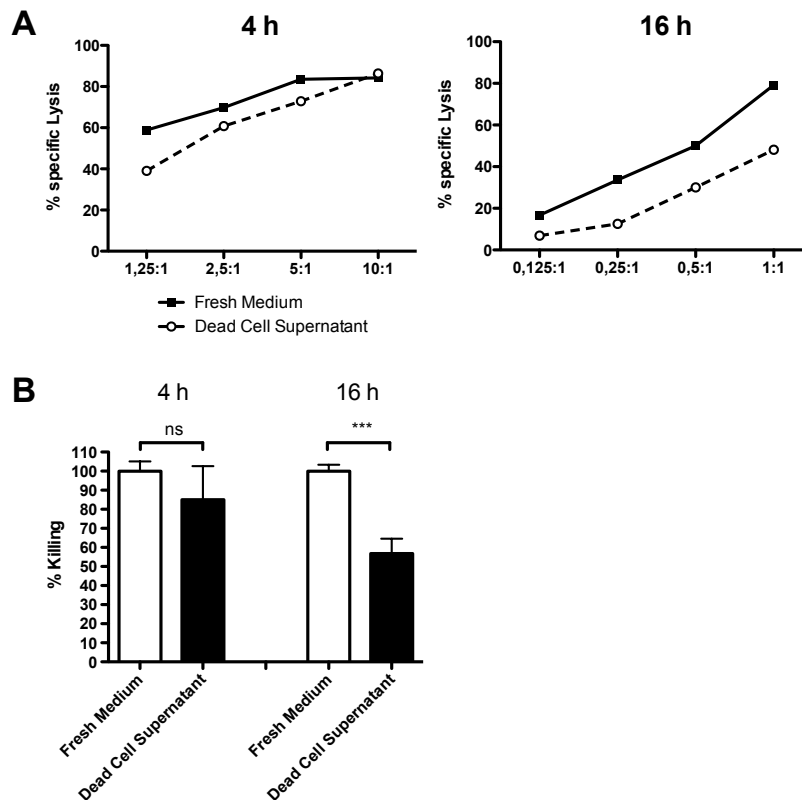
Figure 21. Dead cell supernatant decreases the cytotoxicity of NK cells in a 16 h serial killing assay. Dead cell supernatant was created by killing of $3x10^6$ HEK293T / ml by heat shock over two days and discarding the dead cells by centrifugation. NK cells and K562 target cells were coincubated in the dead cell supernatant in a standard 4 h and a 16 h serial killing Chromium-51 release assay **(A)**. The cytotoxicity at the E:T 1.25:1 (4 h assay) and 1:1 (16 h assay) for four different experiments with different NK cell donors was quantified and normalized to 100 % to control. Values are shown as mean ± SD (ns=not significant; ***p<0.001) **(B)**.

To investigate the influence of soluble proteins, vesicles or other molecules on NK cell effector functions, I produced so-called dead cell supernatant (DCS). I induced apoptosis in K562 or HEK293T cells by a short heat shock, let them die slowly over 2 days and collected the supernatant. In this DCS NK cells were coincubated with new K562 target cells and their effector functions were tested in the respective assays.

At first, I performed Chromium-51 release assays to test the cytotoxicity of NK cells in DCS or fresh medium as a control. After 4 h coincubation with K562 cells, cultured primary NK cells killed slightly

less target cells if coincubated in DCS than in fresh medium (Figure 21A, left panel). But these variations were small and did not reach significance (Figure 21B). In contrast, in a 16 h serial killing assay NK cells killed clearly fewer target cells if coincubated in DCS compared to fresh medium (Figure 21A, right panel). The combination of 4 individual experiments showed, that those differences were significant and the cytotoxicity was reduced to 56.77 ± 26.90 % of the fresh medium control (Figure 21B). These data indicate, that cells secrete or lose proteins, molecules or vesicles in the process of dying. They seem to influence NK cells and reduce the ability to kill multiple targets in a row.
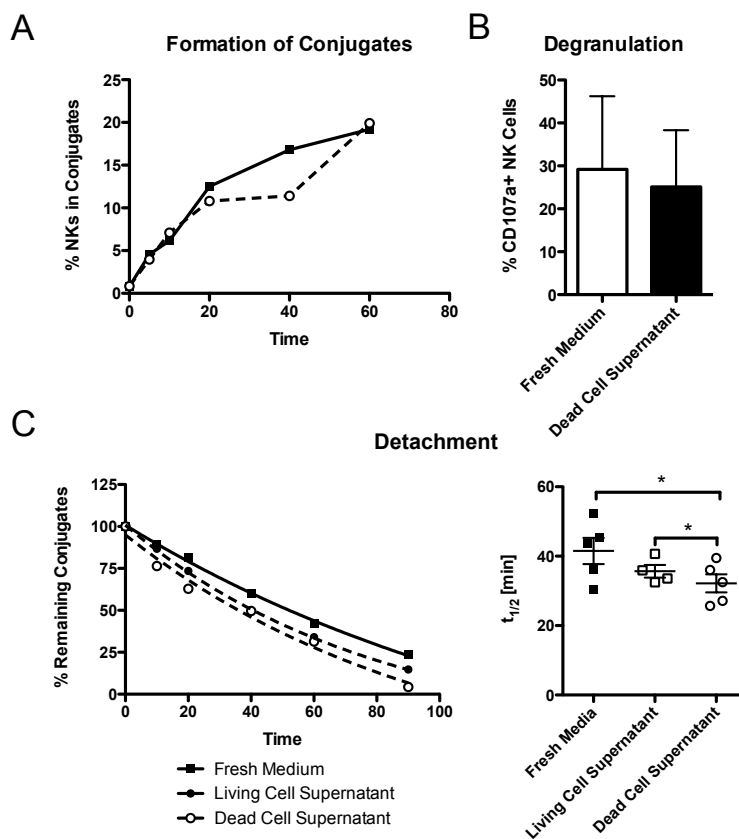
Figure 22. Dead cell supernatant has no effect on the formation of conjugates or degranulation but accelerates the detachment of NK cells. NK cells and K562 target cells were coincubated in dead cell supernatant and the formation of conjugates was analyzed in a conjugate assay. One representative experiment out of three independent experiments is shown (**A**). After 2 h coincubation, the degranulation was assessed by staining with α-CD107a. Results from three independent experiments are quantified. Values are shown as mean ± SD (**B**). Preformed NK:K562 conjugates were diluted in dead cell supernatant in a detachment assay. Left panel shows one representative experiment, right panel the quantification of 5 independent experiments with NK cells from different donors. Values are shown as mean ± SD (*p<0.05) (**C**).

To further analyze the effect of dead cell supernatant on NK cells, I investigated the ability of NK cells to form conjugates, degranulate or detach in the presence of DCS compared to fresh medium. The coincubation of cultured primary NK cells and K562 target cells in DCS had no effect on the formation of conjugates in the first 60 minutes (Figure 22A). The percentage of conjugates was comparable to NK cells, which have been coincubated with K562 in fresh medium. The degranulation of NK cells was also not influenced by DCS. NK cells, which have been coincubated with K562 cells and afterwards stained for the degranulation marker CD107a, showed a similar percentage of CD107a positive cells. The mean for CD107a^{+} NK cells was 29.19 ± 29.53 % for dead cell supernatant and 25.10 ± 22.86 % for fresh medium (Figure 22B). Finally, I investigated if DCS influences the NK cell detachment. As additional control, I collected the supernatant from target cells incubated for 2 days in fresh medium

without a prior heat shock (living cell supernatant = LCS). After 2 days incubation the viability of the K562 cells was below 20% for DCS and above 80% for LCS (data not shown). In a detachment assay I coincubated NK and K562 cells in fresh medium to form conjugates under identical conditions and afterwards used the DCS, LCS or fresh medium to dilute the cells. The detachment of NK cells, which have been diluted in DCS was slightly accelerated compared to LCS or fresh medium (Figure 22C, left panel). Also the half-life was significantly reduced. The coincubation in fresh medium led to a conjugate half-life of 41.51 ± 8.43 minutes and was reduced to 35.65 ± 3.65 minutes if the cells were diluted in LCS. The use of DCS significantly decreased the half-life even more to 32.17 ± 5.83 minutes. These data indicate, that proteins, molecules or vesicles, which have been secreted by dying cells also influence the detachment of NK cells.

I tried to identify the proteins, molecules or vesicles that are responsible for this effect. To analyze if apoptotic bodies caused the observed accelerated detachment, I isolated all vesicles of the DCS by centrifugation (16,000 xg, 30 minutes). NK cells were treated with isolated apoptotic bodies in fresh media or with the vesicle free supernatant. But I could not find any differences in the NK cell detachment if coincubated with or without apoptotic bodies (data not shown). Another theory was that NK cells take up DNA fragments, which have been released by the target cell in the process of dying. Intracellular NK cell receptors could bind the target cell DNA and sense an inhibitory signal, which reduces the NK cell activity and forces the detachment. To investigate this possibility, I treated DCS with 1 mg/ml DNase I to remove any free DNA. The coincubation in medium with or without free DNA had also no effect on the NK cell detachment (data not shown). These data indicate, that neither apoptotic bodies nor free DNA are responsible for the observed reduced cytotoxicity and accelerated detachment of NK cells in DCS.

4.7 NK cell-mediated loss of target cell adhesion molecules and ligands for activating NK cell receptors during target cell death

An important factor to maintain the NK:target cell conjugate is the binding of NK cell receptors to their ligands on the target cell. The constant delivery of an activating signal leads to the ongoing secretion of cytotoxic granules, which in the end induces apoptosis in the target cell. I examined the possibility that apoptosis of the target cell leads to changes in the protein compisition of the target cell surface. If ligands for activating NK cell receptors are lost, this could decrease the NK cell activity and accelerate the conjugate decay.
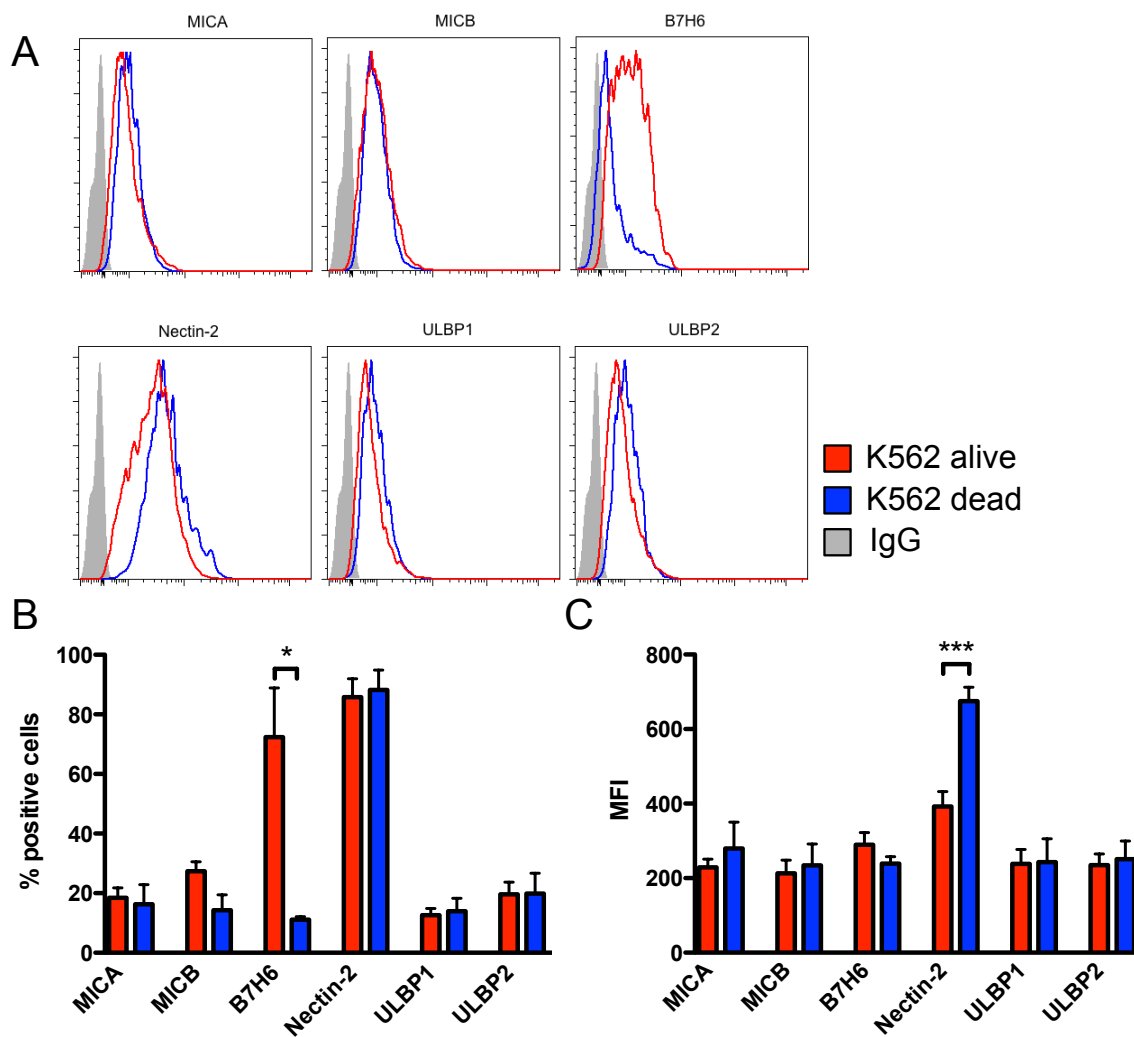
Figure 23. K562 target cells lose activating NK cell ligand B7H6 after NK cell-mediated killing. NK cells and K562 target cells were coincubated for 2 h and stained with life/dead marker Zombie-aqua. Afterwards, cells were stained with antibodies against ligands for activating NK cell receptors MICA, MICB, B7H6, Nectin-2, ULBP1 and ULBP2. Expression of the indicated proteins on living and dead K562 cells was analyzed. Histograms from one representative experiment are shown **(A)**. Percent positive cells **(B)** and the Mean fluorescence intensity (MFI) **(C)** is quantified for three independent experiments. Values are shown as mean ± SD (*p<0.05; ***p<0.001).

To investigate the expression profile of ligands for activating NK cell receptors, I coincubated NK and K562 cells. After 2 h I stained the cells with a live/dead marker and antibodies against ligands for the

activating NK cell receptors NKG2D (MICA, MICB, ULBP1, ULBP2), NKp30 (B7H6) and DNAM-1 (Nectin-2), which are known to be expressed by K562 cells. Using this staining strategy, I could distinguish between K562 cells that were alive or have been killed by NK cells and analyze the expression of the indicated proteins. I evaluated the percentage of positive cells (Figure 23B) and the mean fluorescence intensity (MFI) (Figure 23C), which gives a relative value for the expression level of the antigens on the cell.

The NKG2D ligands MICA, MICB, ULBP1 and ULBP2 were all expressed by the K562 cells (Figure 23A). After 2 h coincubation, the NKG2D ligands were expressed in average by 19.51 ± 5.58 % of the living K562 cells (Figure 23B). The killed K562 cells showed no significant differences in the expression MICA, MICB, ULBP1 and ULBP2 (in average 16.08 ± 9.91 %). The MFI of the NKG2D ligands was also unaltered, which showed that K562 did not lose or gain NKG2D ligands after cell death (Figure 23C). In contrast, Nectin-2 was expressed by the majority of living K562 cells and this number did not change if they have been killed by NK cells. Interestingly, the MFI of Nectin-2 increases after cell death (Figure 23C). The NKp30 ligand B7H6 was also expressed by a majority of the living K562 cells. After the cells have been killed, most of them lost B7H6. The results indicate, that target cells can lose ligands for activating NK cell receptors in the process of NK cell-mediated apoptosis.

Besides ligands for activating and inhibitory receptors, NK cells interact with adhesion molecules on the target cell. Adhesion receptors on the NK cell surface bind to their ligands on the target cell membrane, which results in a firm conjugate. This ligation is maintained during the whole time of conjugation. In order to investigate, if target cells lose adhesion molecules after NK cell induced apoptosis, I compared the adhesion molecules on living and dead K562 cells. I used the same staining strategy like in Figure 23. NK and K562 cells were coincubated and afterwards stained with a live/dead marker and antibodies against several adhesion molecules: CD15 (Lewis X.), CD54 (Intercellular Adhesion Molecule 1, ICAM-1), CD58 (Lymphocyte function-associated antigen 3, LFA-3), CD59 (MAC-inhibitory protein), CD62E (E-Selectin) and CD102 (ICAM-2).
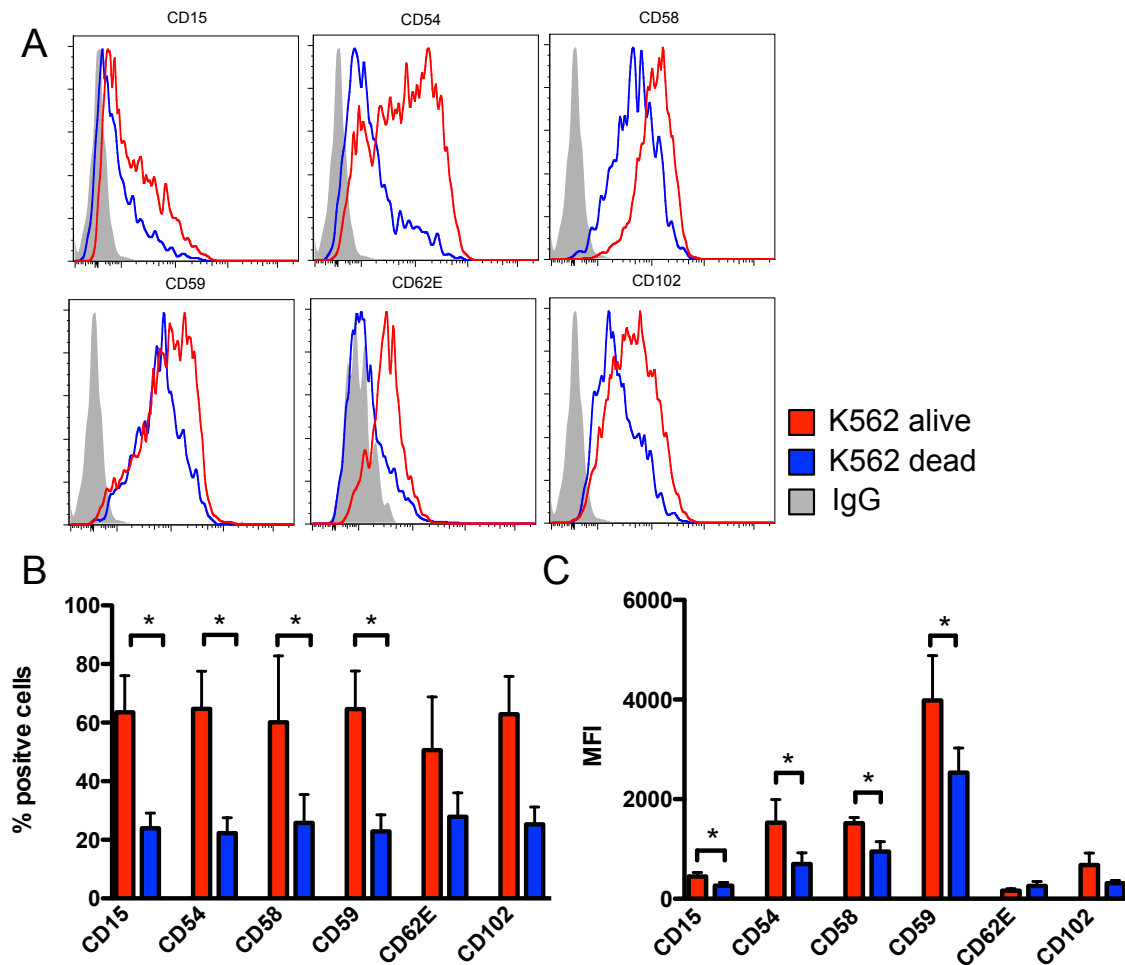
Figure 24. After killing by NK cells, K562 target cells lose a broad range of adhesion molecules. NK cells and K562 target cells were coincubated for 2 h and stained with life/dead marker Zombie-aqua. Afterwards, cells were stained with antibodies against adhesion molecules CD15, CD54, CD58, CD59, CD62E and CD102. Expression of the indicated proteins on living and dead K562 cells was analyzed. Histograms from one representative experiment are shown (**A**). Percent positive cells (**B**) and the MFI (**C**) are quantified for three independent experiments. Values are shown as mean ± SD (*p<0.05).

All adhesion molecules were expressed to a similar level on the cell surface of living K562 cells (**Figure** 24A). But after 2 h coincubation the tested adhesion molecules have been clearly reduced on the cell surface of killed K562 cells. On average, ~ 60 % of the living K562 cells were positive for each of the adhesion molecules. After the NK cells have killed the cells, about two-third of the cells lost expression of the tested adhesion proteins (**Figure** 24B). Analyzing the MFI of the adhesion proteins before and after cell death, it became clear that also the average expression level of these molecules is reduced on dead K562 (**Figure** 24C). These results show, that K562 cells lose many of their adhesion molecules during NK cell-mediated killing. Thereby the adherence between NK and target cell is reduced, the detachment is accelerated.

5   Discussion

5.1   Influence of cytokine-induced pre-activation on NK cell effector functions

NK cells deliver cytotoxic proteins through direct contact with the target cells and the formation of an immunological synapse. The efficiency of NK cell killing depends on numerous factors. It is well-understood how NK cells are activated by diseased cells and how the NK:target cell conjugate is formed (Gasser and Raulet, 2006; Mace et al., 2014; Watzl and Long, 2010). Also the mechanism NK cells use to release cytotoxic content and how apoptosis is induced in the target cells was investigated in-depth during the last four decades (Caligiuri, 2008). NK cell killing is most effective if one single NK cell kills multiple target cells in a process called serial killing. Since NK cells can only form one IS at once they first have to break the contact with the first target before they can bind and kill another one. The termination of the NK:target cell conjugate is an active and regulated process (Netter, 2014). Otherwise the NK cells would detach too early and the target cell could survive or the NK cell detaches too late and is ineffective. In this thesis I investigated the regulation of NK cell detachment and adressed the question how NK cells recognize the target cell death.

To investigate the regulation of NK cell detachment I used a modified flow cytometry-based approach after Culley et al.. I could confirm results from former studies in our lab that cultivated human primary NK cells detach after approximately 48 minutes from K562 target cells (Netter, 2014). However, I could observe variations in the conjugate half-life between NK cells from different donors. I assume that besides genetic factors, the pre-activation of NK cells by pro-inflammatory cytokines influences the NK cell detachment. Affected by stress, age, diseases or infections, cells of the immune system from different donors produce variable amounts of pro-inflammatory cytokines (Bernstein and Murasko, 1998; Claus et al., 2016a; Elenkov and Chrousos, 2002; Kleiner et al., 2013). I could show that the pre-activation of human NK cells has a large effect on NK cell effector functions. As described in the introduction in detail, the process of NK cell killing can be discriminated into different phases. Here I compared the ability of differently pre-activated NK cells to adhere, degranulate, kill and detach from K562 target cells for the first time. Freshly isolated NK cells, which have not been pre-activated *in vitro*, are able to bind, degranulate and kill target cells. This is consistent with early findings that NK cells are cytotoxic even without prior sensitization (Herberman et al., 1975; Kiessling et al., 1975). IL-2 is a key activator of NK cells. I could show that short time IL-2 activated NK cells form more conjugates with K562 cells in less time. IL-2 stimulates the high affinity and high avidity conformation of LFA-1, which results in an increased binding to ICAM expressing target cells (Barber and Long, 2003; Suck et al., 2011). The activation of transcription factor NFκB downstream of the IL-2 receptor induces the production of perforin and Granzymes (Huang et al.,

2006; Zhou et al., 2002). Thus, the IL-2 activated NK cells are able to secrete more cytotoxic granules, which increases the lysis of target cells. Cultured NK cells are able to produce even larger amounts of cytotoxic granules. This results in a strongly increased degranulation and killing of the target cells. I show that target cell death is sensed by the NK cell and initiates the IS termination, which explains why fast killing cultured NK cells detach clearly earlier. Surprisingly, the pre-activation with IL-2 was not sufficient to accelerate NK cell detachment even though more target cells were killed. Probably, the cytotoxicity of fresh and IL-2 activated NK cells was too similar and the detachment assay not sensitive enough to display differences in the half-life. Hence, the recognition of target cell death or the initiation of NK cell detachment can be sped up by long-term pre-activation with high amounts of cytokines.

5.2 Triggering of single receptors differently stimulates NK cell detachment

In the next step I investigated the influence of single NK cell receptors on the adhesion, degranulation, killing and detachment by coincubation of NK cells with Ba/F3 cells, transfected with the associated ligand. Importantly, I used cultured NK cells, which have been pre-activated for 2-3 weeks. It is known, that degranulation is positively correlated with killing of target cells (Alter et al., 2004) and I could show that triggering of NKp30 and NKG2D led to the strongest degranulation and thereby highest number of lysed cells. I conclude that in pre-activated NK cells ligation of NKp30 or NKG2D alone is sufficient to induce target cell lysis. In contrast, co-stimulatory receptors NTB-A and 2B4 are less sufficient to induce degranulation, and NK cells stimulated via these receptors thereby lyse clearly less target cells. Human cultivated NK cells are able to bind untransfected mouse Ba/F3 cells through LFA-1:ICAM-1 interaction (Johnston et al., 1990). I detected small differences in the adhesion and detachment of NK cells if only one activating receptor was triggered by the target cell. However, the presence of each of the four different ligands on the target cells clearly increased the adhesion compared to untransfected target cells. This effect was most likely induced through inside-out signaling, which increases the affinity and avidity of LFA-1 (Beals et al., 2001). Although binding to CD48-transfected target cells only slightly increases the cytotoxicity of NK cells, it has a large influence on the formation of conjugates. This has two reasons: On the one hand ligation of NK cell receptor 2B4 to CD48 increases LFA-1 affinity to ICAM molecules through inside-out signaling (Hoffmann et al., 2011; Urlaub et al., 2017). On the other hand CD48 can additionally bind to NK cell adhesion receptor CD2, which further increases the adhesion (Arulanandam et al., 1993). The binding of NKG2D to ULBP2 or NKp30 to B7H6 results in a strong adhesion mainly through activation of LFA-1 (Raemer et al., 2009; Segovis et al., 2009). NTB-A only slightly activates LFA-1 (Urlaub et al., 2017) and showed the smallest effect on NK cell adhesion in my experiments. However, the homophilic interaction with NTB-A molecules on target cells increases the adhesion compared to control cells.

Finally I could show that the dynamics of NK cell detachment are affected by the triggered activating receptor. NK cells detach quickly from control or NTB-A transfected cells, which promote only weak adhesion. In contrast, triggering of the receptors 2B4/CD2, NKG2D or NKp30, which promote strong adhesion, decelerated the NK cell detachment. This shows that termination of the NK:target cell contact needs loosing of NK cell adhesion molecules from their ligands on the target cell. The stronger an NK cell adheres to a target cell, the more effort it needs to release the cell-cell connection. However, this conclusion stands in contrast to the observation that NK cell detachment is accelerated if the killing is increased. It would be possible that NK cells cannot form functional contacts with the GFP-transfected Ba/F3 cells, which causes the fast detachment and short half-life and makes them inappropriate as a control.

5.3 Calcium and ROS are important second messengers for NK cell detachment

Calcium and ROS are important for NK cell effector functions. It is well investigated that especially PLCγ-dependent calcium mobilization is needed for granule exocytosis and killing of the target cell (Patterson et al., 2005). Importantly, formation of a NK:target cell conjugate is independent of raising cytosolic calcium level as I did not find any differences in the formation of conjugates after treatment with Thapsigargin or Ionomycin. The opening of calcium channels is a very fast event. The calcium-influx occurs directly after formation of the IS and triggering of activating receptors (Mace et al., 2014; Oh-hora and Rao, 2008). I could show that the high calcium level in conjugated NK cells was maintained during the whole synapse time, probably due to ongoing activating signaling, which is needed for the IS maintenance (Huppa et al., 2003; Netter, 2014). Reduction of the target cell death with concanamycin A decelerates the detachment and maintains the activating signaling and thereby calcium levels. However, the correct calcium level seems to be important for IS stability as NK cells detach immediately after treatment of pre-formed conjugates with Thapsigargin or Ionomycin, which both increase the cytosolic calcium levels. Calcium-influx is a local event and CRAC channels are recruited to the IS where they increase the calcium level directly at the contact site with the target cell (Hartzell et al., 2016; Lioudyno et al., 2008). Calcium is important for the reorganization of the actin cytoskeleton at the IS (Hartzell et al., 2016) and in T cells the level has to be maintained during the contact time to prevent detachment (Bunnell et al., 2001). Treatment with Thapsigargin or Ionomycin increases the calcium level in the whole cell and not only in the region adjacent to the target cell, which could mimic activation in distant membrane regions. Activating signals could be withdrawn from the IS, which then destabilizes the synapse and forces the detachment. This would be consistent with the observations of Choi and colleagues that activating signals from a new target can accelerate detachment from bound targets (Choi and Mitchison, 2013). After treatment with Thapsigargin or Ionomycin the degranulation of NK cells was not, or only slightly affected. The

decreased lysis of target cells can be explained by the rapid detachment. It is also possible, that the calcium-induced activation of distant membrane regions leads to undirected degranulation.

I could also show that redox signaling influences NK cell effector functions. The maintenance of the IS needs energy in form of ATP (Netter, 2014). The energy is provided by mitochondria, which are recruited to the contact site after formation of the IS (Abarca-Rojano et al., 2009) and are responsible for the production of ROS. Elimination of ROS with antioxidant N-Acetylcysteine accelerated the detachment of NK cells and slightly decreased degranulation and lysis of the target cells. Given that ROS are associated with F-actin dynamics in several cell types (Munnamalai and Suter, 2009; Sakai et al., 2012; Taulet et al., 2012), the mitochondrial ROS production at the IS participates most likely directly in the stabilization of the actin-network and tightens the contact with the target cell. The influence of ROS on F-actin could also increase the LFA-1 dependent adhesion of the NK cell to the target. Treatment with the pro-oxidative compound WF-10 was shown to enrich LFA-1 at the IS. This increases the adhesion of CTLs to their targets and prevents detachment and serial killing (Wabnitz et al., 2016). The clustering of high affinity LFA-1 at the IS is dependent on F-actin flow (Comrie et al., 2015). Thus, it is mandatory for the termination of the IS that ROS production is reduced after target cell death.

5.4 Reduction of NK cell cytotoxicity leads to decelerated detachment

Former studies showed that NK cells detach clearly later from target cells if NK cells were unable to kill due to perforin absence (Jenkins et al., 2015; Netter, 2014). To prevent maturation of perforin I treated NK cells with concanamycin A, which is not a specific perforin inhibitor. Concanamycin A inhibits the vacuolar type H^+-ATPase and thereby raises the pH of lytic granules. The acidification inhibits cathepsin L, which is needed for cleavage of immature perforin into its active form (Kataoka et al., 1996; Kataoka et al., 1994). This raise the question if side effects of the concanamycin A treatment prevents termination of the IS directly. The delayed detachment would be caused by these side effects and not by the decreased NK cell cytotoxicity due to the loss of mature perforin. Therefore, I treated NK cells with Brefeldin A to confirm the concanamycin A results. The lactone antibiotic Brefeldin A interrupts the vesicle transport from ER to Golgi, which leads to an accumulation of proteins in the ER and prevents the exocytosis of vesicles (Fujiwara et al., 1988; Klausner et al., 1992). I used Brefeldin A in order to abolish the secretion of cytotoxic proteins and could detect a decrease in degranulation, which led to a reduced lysis of target cells although these effects were less dramatic compared to concanamycin A. Surprisingly, the detachment of Brefeldin A treated NK cells was slightly accelerated, which was the opposite effect to concanamycin A treatment. A side effect of Brefeldin A is the partial interruption of the microtubule and actin

cytoskeleton (Alvarez and Sztul, 1999). Both are fundamental for the maintenance of the IS (Netter, 2014), which explains the accelerated detachment.

I could show that the NK cell detachment can be decelerated by coincubation with resistant target cells. Importantly, the overexpression of SerpinB9 or CD107a decreases the killing of target cells only mildly compared to the treatment with concanamycin A. It can be assumed that the translocation of CD107a onto the outer cell membrane is not the only mechanism to prevent the insertion of perforin (Cohnen et al., 2013) and is not sufficient to protect a cell completely from perforin-induced killing. Additionally, Granzyme B uses further mechanisms to induce apoptosis beside activation of caspases (Rousalova and Krepela, 2010), which explains why overexpression of SerpinB9 did not abolish NK cell killing completely. Nevertheless, the delayed detachment from resistant target cells compared to untransfected controls shows that target cell death is one important step to initiate NK cell detachment. I hypothesize that apoptosis-induced alterations in the target cell membrane are recognized by the NK cell and induce the termination of the IS.

In the next step I investigated if these apoptosis-induced alterations are necessarily caused by the NK cells. It is known that during the contact time, membrane fragments and proteins are exchanged between both cells, which can reduce the NK cell cytotoxicity (Roda-Navarro et al., 2006). Additionally, Granzymes cleave specific pro-caspases and further proteins in the target cell (Voskoboinik et al., 2015; Waterhouse et al., 2005). I induced NK cell independent target cell death by a hydrogen peroxide pulse and determined the NK cell detachment. Hydrogen peroxide induces apoptosis mainly through activation of caspase-3 and -9 via the mitochondrial pathway (Saito et al., 2006; Singh et al., 2007). In contrast, Granzyme B activates caspase-3 and -7 (Rousalova and Krepela, 2010). I observed a clearly accelerated NK cell detachment from target cells, that died for a NK cell unrelated reason. This demonstrates that termination of the NK cell IS is initiated by apoptosis of the target cell, which has not to be induced by the NK cell. *In vivo*, different immune cells fight together against diseased and infected cells and induce apoptosis by different mechanisms. The ability to recognize NK cell independent apoptosis enables NK cells to detach as early as possible from target cells. This can increase serial killing and thereby the efficiency of NK cells.

5.5 Loss of target cell adhesion molecules and ligands for activating NK cell receptors during target cell death

In order to detect the apoptosis-induced alterations after NK cell-mediated target cell death, I investigated the protein composition of the target cell membrane before and after cell death.

Upon ligation some activating NK cell receptors are internalized. Freshly detached NK cells show a decreased expression of 2B4, DNAM-1 and NKG2D (Netter, 2014). This diminishes activating signaling

over the period of conjugation. However, this down-regulation is not dependent on the target cell death and will probably just down-modulate the NK cell activation, which might facilitate the detachment and formation of a new IS with another target cell. I could show that another mechanism to diminish the NK cell activation is the down-regulation of specific ligands on the target cell membrane. After NK cell-mediated killing, K562 target cells lose the NKp30 ligand B7H6. Since NKp30 is not internalized after ligation with its ligand, this could be a mechanism to reduce NKp30 signaling after target cell death. B7H6 was shown to be shed by metalloproteases "a disintegrin and metalloproteinase" (ADAM)-10 and ADAM-17 from tumor cells (Schlecker et al., 2014). The expression of both metalloproteases is induced after apoptosis and ADAM-10 was shown to colocalize with caspase-3, a main target of Granzyme B (Lizama et al., 2011; Musumeci et al., 2014; Rousalova and Krepela, 2010). In contrast, ligands for NKG2D and DNAM-1, which are both internalized after ligation, are not down-regulated on apoptotic cells.

I hypothesize that ongoing activating signaling is important to keep LFA-1 in a high affinity and high avidity state during conjugation with a target cell. The loss of activating signals could cause the return of LFA-1 to its low affinity/avidity state and promote the detachment of the NK cell. I could show that after detachment NK cells have more LFA-1 in a higher adhesiveness state than before conjugation. Unfortunately I could not detect high affinity/avidity LFA-1 on NK cells in conjugates. This may have technical reasons because after target cell contact LFA-1 is recruited to the IS (Zheng et al., 2009) and it is not clear if ICAM-1-complexes can enter the IS. Hence I was not able to declare if the conformation state of LFA-1 is changed to its less affinity/avidity state prior to detachment. However, the LFA-1 ligands ICAM-1 (CD54) and ICAM-2 (CD102) were clearly reduced on the cell surface of apoptotic target cells. ICAM-3 (CD50) was earlier shown to be down-regulated on apoptotic cells (Hart et al., 2000). This could be a mechanism to decrease the LFA-1-mediated adhesion of NK cells to dying target cells. At the same time LFA-1 could be retained in an activation state, which is higher compared to LFA-1 on resting NK cells. Thereby the freshly detached NK cell needs less time to bind and kill a new target cell, which was already observed by Choi et al. (Choi and Mitchison, 2013).

Beside ICAM-1 and ICAM-2 I found several other adhesion molecules to be removed from the target cell membrane after induction of apoptosis. CD15 was earlier shown to be removed from the cell surface of neutrophils after induction of apoptosis (Hart et al., 2000). It can bind to CD62E, CD62L and CD62P (E-, L- and P-selectins) on the NK cell surface and is therefore important for the adhesion between both cells (Varki, 1994). Blocking of CD15 on cancer cells with antibodies was shown to decrease the adhesion to CD62E expressing cells (Jassam et al., 2016). After activation, shedding of CD62E and release of soluble sCD62E is a well characterized phenomenon (Pigott et al., 1992). This can explain the observed reduction of CD62E on the cell surface of the target cell after NK cell

conjugation. The reduction of CD15 and CD62E on the target cell after NK cell-mediated apoptosis will therefore decrease the adhesion of NK cells and contribute to the detachment.

CD58 and CD59 are both ligands for the NK cell adhesion receptor CD2, which not only provides adhesion to the target cell but also mediates ERK1/2 phosphorylation (Zheng et al., 2009). The reduction of CD58 and CD59 on target cells after NK cell-mediated apoptosis decreases both, the CD2-mediated adhesion towards the target cell and activating signaling via ERK1/2. The latter is important for the NK cell activity as blocking of CD2 with antibodies clearly decreases NK cell effector functions (Zheng et al., 2009).

Beta-catenin containing cell-cell contacts are cleaved by caspase-3 after induction of apoptosis and decrease adhesiveness to neighboring cells (Brancolini et al., 1997). The reduction of CD62E which I saw after NK cell-mediated apoptosis is most likely mediated by caspases, as the treatment with caspase inhibitor zVAD reduces the shedding of CD62E in the mouse (Harrington et al., 2006). During the execution phase of apoptosis, an apoptotic microtubule network (AMN) is formed beneath the cell membrane and forms a caspases free area. The cleavage and removal of adhesion molecules by proteases is only possible after caspases have depolymerized the AMN (Oropesa-Avila et al., 2013; Sanchez-Alcazar et al., 2007). This could be a mechanism to prevent the premature detachment from the NK cell before the target cell has been successfully killed. Additionally, activated Granzyme B was shown to cleave adhesion molecules like fibronectin on the target cell membrane before entering the cell (Hendel and Granville, 2013). However, this Granzyme B-mediated removal of adhesion proteins is not related to apoptosis and could only serve as a mechanism to reduce the overall adhesion and facilitate the detachment after target cell death. It could also explain the detachment of NK cells, which were unable to kill the target cell after some time.

5.6 Cytokine hypersecretion after decelerated NK cell detachment

The disease familial hemophagocytic lymphohistiocytosis 2 (FHL-2) is characterized by cytokine storms with high serum concentrations of TNF-α and IFN-γ (Henter et al., 1991). Jenkins and colleagues could show, that the absence of functional perforin in NK cells and CTLs in FHL-2 prolongs the synapse time since the effector cells were unable to kill the target cell (Jenkins et al., 2015). This is consistent with my data. I analyzed the secretion of TNF-α and IFN-γ by NK cells *in vitro* after deleting mature perforin or during co-incubation with resistant target cells. Both approaches led to the hypersecretion of TNF-α and IFN-γ. This reveals that cytokine hypersecretion is caused by prolonged contact time between NK cells and their targets and is not a side effect of perforin absence. Ongoing signaling is important for IS maintenance (Huppa et al., 2003; Netter, 2014). The inability to kill the target cell prevents the loss of ligands for activating and adhesion receptors on the target cell membrane and the prolonged activating signaling maintains the high calcium levels. The

persistent calcium influx can lead to the continuous production of pro-inflammatory cytokines (Maul-Pavicic et al., 2011).

5.7 Influence of the inhibitory receptor CD300a on NK cell detachment

Another possible mechanism for NK cell detachment is the recognition of DAMPs by specialized inhibitory receptors. They could reduce the NK cell activity and force the decay of the conjugate after target cell death. Mace et al. propose that the interaction of CD300a with PS on the surface of apoptotic cells could induce the NK cell detachment (Mace et al., 2014). In fact, NK cells have an increased ability to lyse target cells if the CD300a-PS interaction is blocked with MFG-E8 (Lankry et al., 2013). However, blocking CD300a with MFG-E8 in our detachment assay did not lead to an alteration in NK cell detachment. MFG-E8 only blocks the interaction of CD300a with PS and perhaps the interaction with its other ligand PE is more important for NK cell detachment or CD300a simply has no role in the induction of the NK cell detachment. It is conceivable that CD300a functions more as a detector for apoptotic cells and initiator of an inhibitory synapse. The triggering of CD300a could prevent the firm adhesion to dead cells. Notably, I did not see differences in the lysis of K562 cells after blocking CD300a with MFG-E8. It is possible that MFG-E8 can't enter the sealed IS, whereby an investigation in our assay system would not be possible as I added MFG-E8 after formation of conjugates.

5.8 The effect of soluble factors on NK cell effector functions

Shedding of membrane proteins and release of apoptotic vesicles after induction of apoptosis is a well-known phenomenon (Ilan et al., 2001; Kern et al., 2000). After activation of caspases, cleavage of Rho associated kinase ROCK1 promotes membrane blebbing (Coleman et al., 2001; Sebbagh et al., 2001). These apoptotic vesicles seem to have an inhibitory effect on NK cells in my experiments.

The effect of dead cell supernatant on NK cell functions remains ambiguous. It could be mediated by secreted or shed molecules or by apoptotic vesicles. I detected a long-lasting inhibitory effect of dead cell supernatant on NK cells. This indicates a systemic inhibition by the released soluble factors. Yufeng Xie et al. report that CTLs are inhibited by membrane bound transforming growth factor-ß1 (TGF-ß1) on apoptotic bodies, which are released by tumors after radiation-induced apoptosis in the mouse (Xie et al., 2009). TGF-ß1 can decrease NK cell function by down-regulation of receptors like NKG2D and decrease the production of pro-inflammatory cytokines probably through repression of the mTOR pathway (Bellone et al., 1995; Dasgupta et al., 2005; Viel et al., 2016). Future studies have to examine, if K562/HEK293T-derived apoptotic bodies have membrane bound TGF-ß1 and if they interact with and inhibit NK cells. Notably, I also saw a similar inhibitory effect of dead cell supernatant, which was centrifuged to remove apoptotic bodies. However, apoptotic bodies are very

heterogeneous and vary in size from 50-5000 nm (Akers et al., 2013). It is most likely, that not all vesicles have been removed and I cannot clearly link the inhibitory effect to other soluble molecules.

In addition to the inhibition of NK cell cytotoxicity, I saw a direct effect of dead cell supernatant on the NK:target cell conjugate stability. As mentioned before, Choi et al. postulate a mechanism called "kinetic priming" for the detachment of NK cells. Thus, NK cells use signals from new targets in close proximity to force the detachment from the old target (Choi and Mitchison, 2013). The addition of new targets in a detachment assay at the time-point of dilution clearly accelerates the NK cell detachment (Netter, 2014). Apoptotic vesicles or shed ligands could bind receptors distant to the target cell contact site. This could subtract activating signals from the IS, decrease the conjugate stability and accelerate the NK cell detachment.

5.9   Model for NK cell detachment

On basis of my results, I propose a model for the regulation of NK cell detachment. This depends on the recognition of target cell death by NK cells, which initiates the termination of the IS by reduction of ongoing activating signaling.
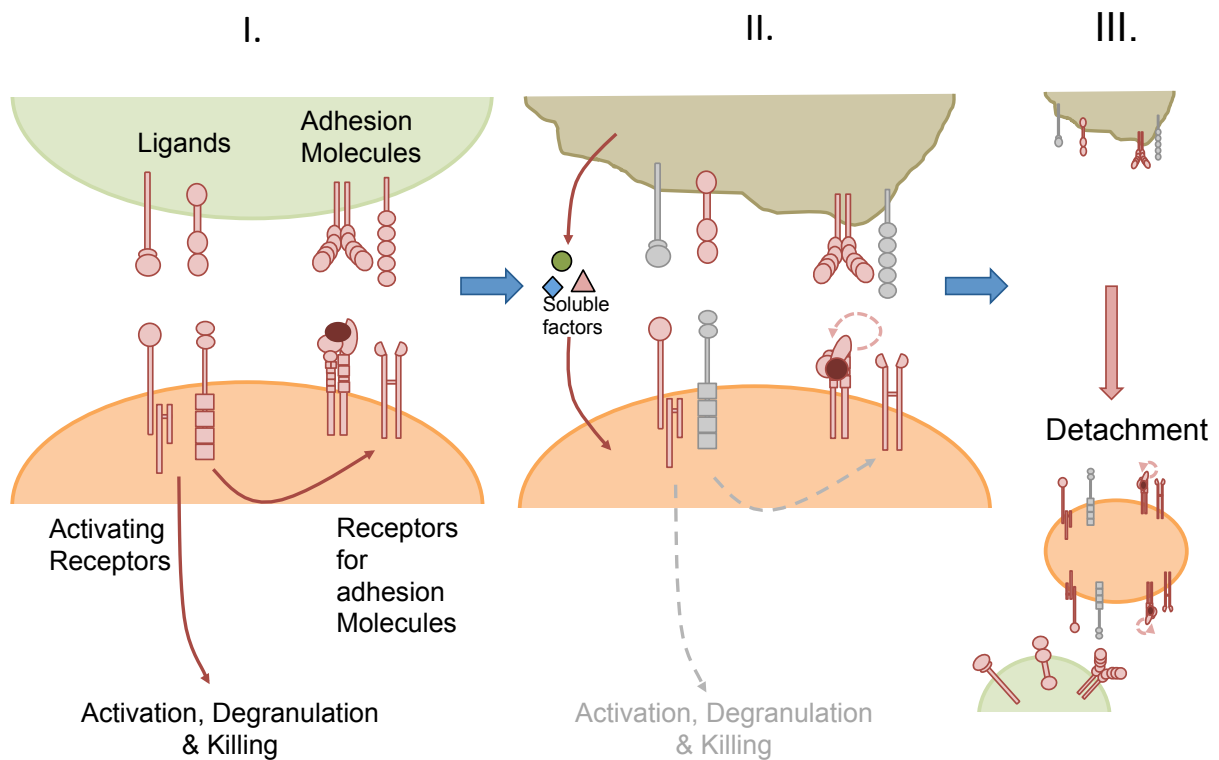
Figure 25. Schematic Model of NK cell adhesion (I) and detachment (II and III).

I. The immunological synapse consists of activating receptors, which bind their ligands on the target cell. Via inside-out signaling, adhesion receptors are clustered at the target cell contact site and the adhesiveness of LFA-1 increases by conformational changes. This leads to a firm adhesion and formation as well as maintenance of the IS. The emerging activating signal initiates degranulation and killing of the target cell.

II. The secreted cytotoxic proteins enter the target cell and induce apoptosis. The dying target cell loses ligands for activating NK cell receptors. Additionally, activating receptors are internalized after ligation, which diminishes the activating signal. Soluble molecules, which are secreted by the apoptotic target cell may also inhibit the NK cell. The decreased inside-out signaling lowers the adhesiveness of adhesion receptors and together with the loss of adhesion molecules on the target cell, this reduces the adhesion towards the target cell.

III. The NK cell detaches from the target cell, and is able to bind and kill another cell.

This model explains the experimental findings that detachment is an important factor for NK cell effector functions. The regulation is highly dependent on extrinsic signals from the target cell after induction of cell death. Loss of NK cell ligands and adhesion molecules on the target cell during apoptosis are mainly responsible for the detachment of NK cells. A reduced ability to kill, due to

resistant target cells or absence of cytotoxic proteins, decelerates the NK cell detachment. This prolonged conjugation time has functional consequences, as ongoing activating signaling and sustained Ca^{2+} flux leads to the production of more pro-inflammatory cytokines. These findings facilitate an important insight in NK cell regulation after degranulation. It could help in understanding diseases like FHL-2 with impaired NK cell functions or fighting cancer diseases with NK cell resistant tumors.

In the end some questions remain. The proposed model of NK cell detachment can only apply to lytic NK cell contacts, which are important for the clearance of diseased cells. However, the stimulation of NK cells by DCs or macrophages requires the formation of a non-lytic, regulatory IS (Borg et al., 2004; Lapaque et al., 2009). This regulatory IS is terminated without lysis of the target cell. Further investigations have to reveal how NK cells detach from contacts without induction of apoptosis.

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7 Abbreviations

ADAM	A disintegrin and metalloproteinase
AKT	Protein kinase B
AMN	Apoptotic microtubule network
Arl8b	ADP-ribosylation factor-like protein 8B
BAT-3	HLA-B-associated transcript 3
CAR	Chimeric antigen receptor
CCL5	CC-chemokine ligand 5
CD	Cluster of differentiation
CDC	Cholesterol-dependent cytolysin
Cdc42	Cell division control protein 42 homolog
CLP	Common lymphoid progenitor
CRAC	Calcium-release-activated calcium
CREB	cAMP response element-binding protein
CTL	Cytotoxic T-Lymphocyte
DAG	Diacylglycerol
DAP10	DNAX-activating protein of 10 kDa
DAP12	DNAX-activating protein of 12 kDa
DC	Dendritic cell
DCS	Dead cell supernatant
DMSO	Dimethylsulfoxide
DNAM-1	DNAX accessory molecule
EAT2	Ewing's sarcoma-associated transcript 2
ER	Endoplasmatic reticulum
ERK	extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FasL	FAS Ligand
Fc	Fragment crystallizable
FCS	Fetal calf serum
FHL-2	Familial hemophagocytic lymphohistiocytosis 2
GADS	GRB2-related adaptor downstream of Shc
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Grb	Growth factor receptor-bound protein
GTP	Guanosintriphosphat
HA	Hemagglutinin
IFN	Interferon
IL	Interleukin
InsP$_3$	Inositol-1,4,5-trisphosphate
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITK	Interleukin-2-inducible T-cell kinase
ITSM	Immunoreceptor tyrosine-based switch motif
JAK	Janus kinase
kDa	Kilo dalton
LAT	Linker of activated T cells
LCAA	Ligand complex–based adhesion assay
LCS	Live cell supernatant
LFA	Lymphocyte function-associated antigen
LSM	Lymphocyte Separation Medium
MACPF	The membrane attack complex/perforin

MAPK	Mitogen-activated protein kinase
MFG-E8	Milk fat globule-EGF factor 8 protein
MHC	Major histocompatibility complex
MICA/B	MHC class I polypeptide-related sequence A/B
MIP	Macrophage inflammatory protein
MTOC	Microtubule-organizing center
mTOR	Mechanistic target of rapamycin
Munc13	Mammalian uncoordinated-13
NCR	Natural cytotoxicity receptor
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK cell	Natural killer cell
NKG2D	Natural killer group 2D
NKT cells	Natural killer T cells
NOX	NADPH oxidase
ORAI1	Calcium-release-activated calcium molecule 1
P/S	Penicillin-streptomycin
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PCNA	Proliferating cell nuclear antigen
PDGF-D	Platelet derived growth factor D
PE	Phosphatidylethanolamine
PI3K	P85α subunit of phosphatidylinositol-3 kinase
PIPKIγ	PtdIns(4)P 5-kinase
PLCγ1	Phospholipase C γ1
PS	Phosphatidylserine
PtdIns(4,5)P$_2$	Phosphatidylinositol-4,5-bisphosphate
PtdIns(4)P) 5-kinase	Type Iγ phosphatidylinositol 4-phosphate
PTP	Protein tyrosine phosphatase
PVR	Poliovirus receptor
ROCK	Rho-kinase
ROS	Reactive oxygen species
SAP	SLAM-associated protein
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
SerpinB9	Serine protease inhibitor 9
SHIP	SH2-containing inositol polyphosphate 5-phosphatase
SHP	Src homology region 2 domain-containing phosphatase
SLAM	Signaling lymphocytic activating molecule
SLP76	SH2 domain containing leukocyte protein of 76kDa
SNARE	Soluble NSF attachment protein receptor
STAT	Signal transducers and activators of transcription
STIM1/2	Stromal interaction molecule 1/2
SYK	Spleen tyrosine kinase
TGF	Transforming growth factor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TNF	Tumor necrosis factor
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
ULBP	UL16 binding proteins
VAMP	Vesicle-associated membrane protein
WASp	Wiskott–Aldrich Syndrome protein
ZAP	Zeta-chain-associated protein kinase
zVAD	zVAD-fmk