Identification and Characterization of serial killing Natural Killer Cells

Dissertation

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Thesis by	Jens Alexander Niemann (born in Osnabrück)
Examiners	Prof. Dr. Carsten Watzl Prof. Dr. Dr. Philipp Zimmer

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Abbreviations

Abbreviation	Abbreviated
ADCC	Antibody Dependent Cellular Cytotoxicity
AI	Artificial Intelligence
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
gMFI	geometric Mean Fluorescence Intensity
GSEA	Gene Set Enrichment Analysis
HLA	Human Leukocyte Antigen
IFN-γ	Interferon γ
IS	Immunological Synapse
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KIR	Killer cell Immunoglobulin-like Receptor
KLRB1	Killer cell lectin-like receptor B1
KLRG1	Killer cell lectin-like receptor G1
MHC	Major Histocompatibility Complex
MTOC	Microtubule Organizing Center
NCR	Natural Cytotoxicity Receptor
NK cell	Natural Killer cell
PBMC	Peripheral Blood Mononuclear Cell
PLZF	Promyelocytic Leukemia Zinc Finger
qPCR	quantitative Polymerase Chain Reaction
SD	Standard Deviation
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
w/o	without

Abstract

Human natural killer (NK) cells have been shown to be heterogeneous and differ in their ability to kill target cells. NK cells with the ability to sequentially kill multiple targets are called serial killers. The serial killing activity of NK cells is essential for their cytotoxic function since the majority of kills can be performed by a minority of cells. Consequently, serial killing NK cells are of particular interest for therapeutic applications. However, while it is known that only a fraction of NK cells perform serial killing, it is currently not possible to predict which NK cells will engage in serial killing. Additionally, current methods to identify serial killing NK cells rely mostly on low throughput, time consuming microscopy or micro-fluidic setups which makes it hard to integrate them into a normal workflow.

To investigate serial killing NK cells, we established a staining protocol that can differentiate between the timing and the count of multiple NK cell degranulation events occurring during target cell co-culture. NK cells are analyzed via flow cytometry which enables us to combine degranulation data with other phenotypic readouts. Our method can reproducibly identify donors with high proportions of serial degranulating NK cells. Multiple degranulation events were paralleled by a stepwise loss of granzyme B and perforin. Loss of CD16 was associated with the number of degranulation events, whereas the upregulation of CD69 correlated with the timing of degranulation. Isolating multiple, single and not degranulated NK cells and rechallenging them resulted in the same pattern emerging. Education influenced the performance of NK cells in the serial degranulation assay with educated NK cells outperforming uneducated ones. RNAseq analysis of sorted NK cells that degranulated multiple times compared to ones that did not degranulate identified several differentially expressed genes. However, most of these genes were activation induced and therefore they cannot be used to identify serial killers beforehand. Investigation of a selection of NK cell receptors did also not result in a clear marker for serial killing NK cells. Still, enrichment could be seen for the receptor NKG2A which is involved in education. When sorting NK cells for expression of that receptor the sorted cells reflected the prediction. Overall, our data support a stochastic model of serial killing compared to a predetermined one and offers a method that can be easily picked up and adapted by other research groups.

Zusammenfassung

Humane natürliche Killerzellen (NK-Zellen) sind heterogen und unterscheiden sich in ihrer Fähigkeit Ziel-Zellen abzutöten. NK-Zellen die in der Lage sind sequenziell mehrere Ziel-Zellen zu töten werden als „serial killer“ bezeichnet. Dieses serielle Töten ist ausschlaggebend für die Zytotoxizität der NK-Zellen da eine Minderheit der Zellen für den Großteil des Effektes sorgen kann. Daher sind „serial kiling“ NK-Zellen von besonderem Interesse für therapeutische Anwendungen. Es ist zwar bekannt, dass nur ein Teil der NK-Zellen dieses Verhalten aufweist, aber es ist nicht möglich vorherzusagen welche. Zusätzlich lässt sich die Analyse von „serial killing“ nur schwer in einen normalen Arbeitsablauf einarbeiten, da sie meist auf zeitraubender Mikroskopie mit geringem Durchsatz oder spezialisierter Mikrofluidik beruht.
Um seriell tötende NK-Zellen zu analysieren haben wir eine Färbemethode entwickelt, die es ermöglicht die zeitliche Verteilung und Anzahl mehrerer Degranulations-Events von NK-Zellen in Co-Kultur mit Zielzellen zu messen. Die Durchflusszytometrie-basierte Methode ermöglicht es Degranulations-Messungen mit Phänotyp-Messungen zu verbinden. Unsere Methode kann reproduzierbar Spender mit einem hohen Anteil an „serial killing“ NK-Zellen identifizieren. Vermehrte Degranulation geht einher mit einem schrittweisen Verlust von Granzym B und Perforin. Der Verlust von Oberflächen-CD16 steht in Verbindung mit der Anzahl der Degranulationen und die Hochregulierung von CD69 korreliert mit dem Zeitpunkt der Degranulation. Isolierte mehrfach-, einfach- oder nicht-degranulierte NK-Zellen zeigen ein entsprechendes Verhalten bei erneutem Zielzellenkontakt. NK-Zell „education“ beeinflusst, wie sich die NK-Zellen im Versuch verhalten, „educated“ NK-Zellen sind aktiver als nicht „educated“ NK-Zellen. RNA-Sequenz-Analyse von mehrfach degranulierten und nicht degranulierten NK-Zellen zeigt mehrere differenziell exprimierte Gene. Die meisten von ihnen sind allerdings durch die Aktivierung induziert worden und daher nicht nutzbar um „serial killing“ NK-Zellen im Vorhinein zu identifizieren. Auch die Analyse von ausgewählten NK-Zell-Rezeptoren brachte kein Erkennungsmerkmal hervor. Dennoch ist für Rezeptoren wie NKG2A, welcher in der NK-Zell „education“ eine Rolle spielt, eine Anreicherung zu beobachten. NK-Zellen die entsprechend der Expression dieses Rezeptors sortiert wurden spiegeln das erwartete Degranulations-Muster wider. Insgesamt sprechen unsere Daten eher für ein stochastisches Modell des „serial killing“ als für ein vorherbestimmendes. Die verwendete Methode kann leicht von anderen Forschungsgruppen genutzt und angepasst werden.

1. Introduction

1.1 Natural Killer cells, an overview

Natural killer (NK) cells belong to the innate arm of the human immune system. They act, as the name implies, as cytotoxic effector cells able to kill virally infected and transformed cells. Additionally, they produce cytokines that further the immune response and activate other parts of the immune system.[1, 2] NK cells are large granular lymphocytes and as such derived from common lymphoid progenitor cells in the bone marrow. 5 – 15 % of the peripheral blood mononuclear cells (PBMCs) are NK cells.[3] They are also found in tissues, lymphoid as well as non-lymphoid and play an important role during pregnancy with umbilical cord blood having increased numbers of NK cells.[4, 5] The activity of NK cells is tightly regulated by a mix of germline encoded activating and inhibitory receptors.[6]

When NK cells mature, they go through distinct stages of maturation. The classical and most common way is to distinguish between $CD56^{bright}$ and $CD56^{dim}$ NK cells.[7, 8] $CD56^{bright}$ NK cells are involved in cytokine production and less cytotoxic. They are highly responsive to cytokine stimulation. When maturing into $CD56^{dim}$ NK cells, they start to express the Fc receptor CD16, become more cytotoxic and less cytokine producing. However, upon activation by target cells they possess a higher overall cytokine production capability.[9] Among $CD56^{dim}$ NK cells those ones expressing CD57 are seen as terminally differentiated. They are less responsive to cytokine stimulation but are very sensitive to stimulation through the Fc receptor CD16 and are highly cytotoxic.[10]

1.2 NK cell regulation

NK cells express a variety of germline encoded activating and inhibitory receptors. When NK cells interact with a healthy cell the inhibitory signaling overweighs activating signals and the NK cells are not activated. Two paths, leading to NK cell activation have been described. As part of immune evasion directed against the T cells of the adaptive immune system virus infected and transformed cells downregulate MHC-I molecules to bypass detection. For NK cells however MHC-I acts as an inhibitory signal. This activation through loss of inhibitory signaling was termed ‘missing self’. Virus infection or transformation can also lead to the stress-induced upregulation of ligands for activating NK cell receptors. These higher activating signals can overpower inhibitory signaling, activating NK cells through a process termed ‘induced self’. In the following a selection of NK cell inhibitory and activating receptors will be explored.

1.2.1 Inhibition

Most inhibitory receptors convey their signaling through an immunoreceptor tyrosine-based inhibitory motif (ITIM). Once phosphorylated it recruits and activates the tyrosine phosphatases SHP-1 and SHP-2 and the inositol phosphatases SHIP-1 and SHIP-2 by binding of the SH2 domain. They in turn target various compounds of the activating signaling cascade, dephosphorylating and deactivating them.[11]

Killer-cell immunoglobulin-like receptors (KIRs) display high allelic polymorphism. They interact with the also polymorphic HLA class I molecules. The inhibiting members of the KIR family possess a long ITIM containing cytoplasmic tail.[12] They are denoted with an `L` in their naming (e.g. KIR2DL1). There are also activating KIRs. They have a short cytoplasmic tail lacking the ITIM. In naming they are denoted with an `S`. To induce activation, they associate with the immunoreceptor tyrosine-based activation motif (ITAM) containing adapter protein KARAP/DAP12.

The inhibitory receptor NKG2A (CD159a) is expressed early on during NK cell maturation, bevor they start expressing KIRs.[12] It competes with the activating receptor NKG2C for CD94. Dimerization with CD94 is needed for proper surface expression. NKG2A and C also share their ligand HLA-E. Like for the KIRs signaling of NKG2A is conveyed through an ITIM domain.[13]

Killer cell lectin-like receptor B1 (KLRB1, CD161) signals through an ITIM domain upon engagement of its ligand lectin-like transcript 1 (LLT1) and thereby inhibits cytotoxicity.[14] Another member of the KLR family, KLRG1 also possesses an ITIM domain. It is described to be expressed on $CD56^{dim}$ NK cells with little proliferative capacity.[15] Inhibition has been shown upon interaction with its ligand E-cadherin.[16]

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) shares its ligands CD80 (B7.1) and CD86 (B7.2) with CD28 which is a costimulatory receptor prominently involved in T cell activation.[17] Since it lacks an ITIM domain its inhibitory function was proposed to result from ligand competition.[18] NK cells do not express CD28 but blocking of CTLA-4 was shown to increase their cytotoxicity.[19] While its inhibitory effect on NK cells was shown it is most notably known as the first target of immune checkpoint inhibition (ICI) therapy.[20, 21]

T cell receptor with immunoglobulin and ITIM domain (TIGIT) is also expressed on NK cells. In addition to its ITIM domain it also possesses an immunoglobulin tail tyrosine (ITT) motive. The ITT can also signal through SHIP-1 to downmodulate NK cell effector function.[22] Additionally it can reduce activation through DNAM-1 by competing for their shared ligands poliovirus receptor (PVR) and nectin-2 (CD112).[23]

1.2.2 Activation

While NK cell activation signaling is diverse and employs multiple signaling motives, an important one is the immunoreceptor tyrosine-based activation motif (ITAM). Upon receptor engagement Src-family kinases phosphorylate its tyrosine residues. The kinases Syk and ZAP70 are recruited and bind the phospho-tyrosines via SH2

domains.[24] Further downstream Ca^{2+}-influx and MAP kinase signaling are triggered.[25] Most activating NK cell receptor types do not provide sufficient signaling but rely on costimulation of multiple receptors to induce activation.[26]

NKG2D is a lectin-like receptor and one of the most important NK cell activating receptors.[27] Its ligands are the stress induced proteins MICA, MICB and members of the ULBP family. For signaling it associates with the signaling adapter molecule DAP10. For NKG2D costimulation often happens through SLAM protein family member 2B4 whose ligand CD48 is expressed on hematopoietic cells.[28]

A prominent group of receptors in NK cell activation is the Ig-like family of natural cytotoxicity receptors (NCRs). They encompass the members NKp30, NKp44 and NKp46.[29] NCRs don't possess an ITAM themselves but associate with ITAM containing DAP12, CD3ζ and FcRγ adapter proteins to convey their activating signaling. Ligands differ between the NCRs and some are still not identified.[30] One of the ligands for NKp30 is B7-H6 which is prominently expressed on multiple tumor cells, making cell lines of these cancers often good targets for NK cell research.[31]

CD16 (FcγRIII) is a low affinity Fc receptor and as such able to bind the constant part of antibodies. When bound to IgG it directs the NK cell against the antibody target.[32] This process is called antibody dependent cellular cytotoxicity (ADCC).[33] Activation through CD16 works without the addition of costimulatory signaling and happens through an ITAM domain.[26, 34]

NKG2C as discussed before is the activating counterpart to NKG2A. For its signaling it pairs up with the ITAM containing DAP12.[13]

4-1BB (CD137) is a member of the tumor necrosis factor receptor superfamily. It acts costimulatory on NK cells and was shown to enhance ADCC.[35]

CD38 is involved in a multitude of functions.[36] It metabolizes NAD^+ and does that also as an ecto-enzyme. It was shown to also work as an activating receptor upon antibody stimulation but needs to associate with CD16 to facilitate the signaling.[37]

The member of the tumor necrosis factor (TNF) family CD27 is a costimulatory receptor linked to memory and stem like features of NK cells.[38] Its ligand CD70 has recently come to light as a marker for low cytotoxicity on long-term ex vivo cultured NK cells.[39]

Programmed cell death ligand 1 (PD-L1) gained traction originally as the ligand for programmed cell death protein 1 (PD-1). PD-1 is an inhibitory receptor present both on NK cells and T cells. PD-1 blockade was the second approved ICI therapy.[21] Besides being expressed on tumor cells PD-L1 is also upregulated on activated NK cells. Recently ligation of PD-L1 on the NK cell was shown to induce a metabolic shift and prime the cytoskeleton for migration resulting in improved tumor control.[40] PD-L1 expression on NK cells was also linked to favorable outcomes of PD-1/PD-L1 blockade in neuroblastoma.[41]

1.2.3 Other receptors

CD69 is a protein with a C-type lectin domain.[42] Since it is upregulated upon NK cell activation it is used as an activation marker.[43]

HLA-DR expression is mostly found on less mature NK cells. Through activation its expression can be induced and marks highly proliferative and active NK cells.[44] When expressing this class II HLA NK cells are able to fulfill a weak antigen presenting role.[45]

CD8 is classically known to distinguish cytotoxic from non-cytotoxic T cells. It is however also expressed on some NK cells.[46] CD8α was found as a marker for highly cytotoxic NK cells following long-term culture.[39]

To exert some of their effector function NK cells not only need to get activated, they also need to make contact to the target and stay there.[47, 48] Contact formation is done in part with the help of integrins. They also play a role in cytoskeletal reorientation and polarization needed for effector functions. CD18 builds the β subunit for multiple different integrins. In combination with CD11a it forms LFA-1, which when engaging its ligands ICAM-1/2 can already provide early activating signals for NK cells.[49]

CD56 is commonly used as marker for NK cells. However, it is also expressed on NK-T cells. To distinguish this two cell types CD3 which is expressed on NK-T cells but not NK cells is also needed.[50] Besides its use to identify NK cells CD56 was also shown to be able to activate already preactivated NK cells.[51]

1.3 Educated and adaptive NK cells

An important aspect of NK cell functionality is their education. NK cells that receive constant inhibitory signals from human leukocyte antigens (HLA) become ‘educated’ which means that they are more cytotoxic when they do get activated.[52] This can also be seen in a higher load of lytic granules in educated NK cells.[53] This inhibitory signaling works through the KIR family of receptors and some others like NKG2A on the NK cells.[54] KIR expression differs between NK cells of an individual and which KIR is educating is dependent on the HLAs. For example, the ligand of KIR2DL1 is HLA-C2, for KIR2DL2 and 3 it is HLA-C1 and for KIR3DL1 it is the Bw4 epitope of HLA-A and B. Genes for KIRs and HLAs lie on different chromosomes and thereby it is possible for an NK cell to express a KIR whose ligand will not be expressed. In addition to the inhibitory KIRs NK cells can also express activating ones. If NK cells express inhibitory as well as activating KIRs with the same ligand the educating effect of the inhibitory KIR has been shown to be tuned down.[55]

The NKG2A ligand, HLA-E presents the leading peptides of HLA class I. Depending on the amino acid of the HLA class I proteins HLA-A and B at position 21 being methionine or threonine the presentation of their leading peptide on HLA-E is higher or lower respectively. Higher presentation is linked to stronger education through NKG2A on the NK cells.[56]

Even though NK cells belong to the innate arm of the immune system some memory like features have been described for them.[57] Upon human cytomegalovirus infection

a distinct subset of NK cells emerges. They were first described as being CD57 positive and high in expression of the activating receptor NKG2C.[58] These so called adaptive NK cells are clonally expanded and possess DNA methylation patterns that are similar to those of effector T cells.[59] Adaptive NK cells are strongly activated through engagement of CD16 and have characteristically low expression of the transcription factor Promyelocytic leukemia zinc finger (PLZF).

1.4 NK cell effector functions

1.4.1 Cytokine production

One feature of NK cell effector function is the production of cytokines and chemokines. Thereby NK cells influence other parts of the innate as well as the adaptive immune response. Key cytokines produced by NK cells are interferon γ (IFN-γ) and tumor necrosis factor (TNF).[9] IFN-γ is a type II interferon affecting transcriptional activators involved in cell cycle regulation and inflammation signaling.[60] Its production can be induced by target cell contact but also through stimulation with IL-2 and IL-12.[61] In turn IFN-γ can aid in the induction of IL-12 production by dendritic cells, creating a positive feedback loop.[62] TNF is also proinflammatory in its nature. It has immunostimulatory effects but can also induce direct apoptosis through binding of its receptors TNFR-1 and -2.[63] Pre-stimulated NK cells are also able to produce granulocyte-macrophage colony-stimulating factor (GM-CSF).[64] It is involved as the name suggests in stimulating cells of the myeloid lineage.[65]

NK cells are also able to produce chemokines, especially after being activated. Chemokines they produce encompass CCL2, CCL3, CCL4, CCL5, CXCL8 (IL8) and CXCL10 (IP-10). NK cells are able to shape the overall immune response and orchestrate their interplay with other immune cells.[9]

1.4.2 Cytotoxicity

The other side of NK cell effector function is the killing of virally infected and transformed cells. Killing has also been observed to fulfill a regulatory role by eliminating T cells to limit autoimmunity.[66] For an NK cell to get activated by a target cell it needs to form an immunological synapse (IS). The IS is characterized by a close cell-cell interaction that is enabled by adhesion molecules and activating receptors engaging their ligands. It is formed through remodeling of the actin cytoskeleton, polarizing the NK cell to the target cell and includes the assembly of the microtubule organizing center (MTOC).[67] Maintenance of the IS is a regulated and energy-dependent active process.[68] If the NK cell gets activated it can kill the target cell in two distinct ways. The first way is the engagement of the death receptors. NK cells express the death receptor ligands FasL (CD178) and TRAIL (CD253). They are stored in vesicles and are brought to the NK cell surface upon activation.[69] Engagement of the

death receptors Fas (CD95) and TRAIL-R1/2 on the target cell triggers a caspase cascade inside the target cell ending in apoptosis.[70, 71]

The other process is called granule-mediated killing. NK cells contain lytic granules, upon activation they are recruited to the MTOC. From there they are brought to the IS with motor proteins and their content is released onto the target cell, in a process called degranulation.[67] This release is directed only against the target cell, leaving bystander cells unharmed. Contained in the granules are perforin, granulysin and granzymes. Perforin oligomerizes and then can insert itself into cell membranes and form pores, causing membrane leakage and allowing granulysin and granzymes to enter.[72] High amounts of perforin can also directly lead to membrane damage-induced necrosis.[73] Granulysin belongs to the saposin-like family of proteins and is antimicrobial in its nature.[74] Human NK cells produce five different proteins of the granzyme family, granzyme A, B, H, K and M.[75] Granzyme A and B are the best researched with granzyme B being the most prominently used.[76] Once inside the target cell, granzyme B can either directly start a caspase cascade by directly cleaving caspase 3, or through a mitochondria-dependent pathway starting with the cleavage of Bid.[77] Both paths lead to apoptosis. NK cells store granzymes and perforin in an inactive state.[78] Additionally, more mechanisms are in place to keep the NK cell from damage by its own cytotoxic cargo. SerpinB9 is expressed by NK cells in their cytoplasm and inhibits granzyme B by blocking its catalytic domain.[79] The membrane lipids inside the IS are tightly packed which inhibits perforin insertion.[80] CD107a (LAMP1) is present in the membrane of lytic granules and also inhibits perforin from binding.[81] After lytic granules fuse with the NK cell membrane to release their content, CD107a is expressed on the NK cell surface, continuing to protect the NK cell. Since degranulation is the usual way for CD107a to get to the NK cell surface it is commonly used as a marker for NK cell degranulation and activity.[82] NK cells contain about 200 lytic granules and release around 10 percent of them during a single degranulation event, with as few as 2-4 granules being sufficient to induce target death.[83] After target cell death the IS can be dissembled and the NK cell detach from the target cell.[84]

Since NK cells survive their cytotoxic activity and retain most of their cytotoxic payload after being activated they are able to kill multiple target cells. This happens in a sequential manner in a process termed serial killing. It has been observed for 6 and more subsequent kills.[85] Release of lytic granules was shown to be the primary way for serial killing with death receptor engagement only being the cause for the last kill of a series.[86] The first kill can prime an NK cell for the following one, making subsequent activation faster, leading to what is described as burst kinetics.[85] The next part will focus more on serial killing and how it can be analyzed.

1.4.3 Serial killing and its analysis

Serial killing was first described for cytotoxic T cells. This was already in 1972 when it was recognized, that in an assay more target cells died then effector cells were present.[87] It was shown that this killing was not bystander killing but killing in a sequential manner and therefore termed serial killing.[88, 89] For NK cells it has been first reported in 2007.[90] Since then, multiple different methods have been developed to investigate serial killing. Serial killing is a multi-step process.[91] First, contact is made to the target cell, then it is recognized as a target and the NK cell is activated. After the target cell dies, the NK cell needs to detach and find a new target, that also activates its cytotoxic function. Some literature reports about serial killing in a sense of rechallenge with new target cells in assays spanning multiple days.[92, 93] For our purposes we define serial killing as back-to-back NK cell activity without prolonged recovery time happening in under 16 hours.[94] Early serial killing analysis was done in bulk killing assays with an excess of target cells.[90] When more targets die than effectors are present, serial or bystander killing was the reason. The information gained however is limited, since it is not clear, if it is uniform low serial killing or only some cells that highly perform. Due to its complex nature, analysis of serial killing is challenging. Most approaches are based on time lapse microscopy, following individual NK cells throughout the whole duration of the experiment.[90, 95] To help with that, specialized microscopy dishes have been designed, termed micro-wells.[95] These micro-wells can be observed in one field of view and hinder NK cells from leaving the field of view. Analysis of these videos was and still is mostly performed manually, bringing about biases and costing time. Other setups with shallow wells hosting only an individual target cell and letting NK cells reach them from the top were developed to ensure equal distances between the target cells.[96] To facilitate the readout to an endpoint analysis, even smaller setups were developed housing only a single NK cell and multiple target cells.[97, 98, 99] With only a single NK cell present all dead target cells can be attributed to the NK cell. Thereby it is sufficient to only check at the end and not monitor continuously. Similar effects were reached with microfluidic setups encasing a single NK cell in a droplet with multiple target cells. The microfluidics-based setup also enables easy retrieval of the NK cells for downstream analysis with other methods.[100, 101] In the more standard setups this would only be possible with cell picking. In addition to the droplet-based method, microfluidics was used to put individual NK cells into small chambers and sandwiching them between two target cells, thereby ensuring contact.[102] Even though microfluidics eases handling and retrieval of the NK cell the resulting cell numbers for downstream analysis are low. The specialized equipment needed for many of those approaches also limits their use to a select number of laboratories.

While a signature that identifies a serial killing NK cell has not been found, many insights could be gained. For an NK cell to kill a new target it first needs to detach from the original one. If it fails to do so serial killing is impaired.[103] Death of the target cell helps the NK cells to detach.[84] Target cells that do not die result in longer contact times

and drive NK cells to produce more cytokines.[104] For T cells it was shown that the physical contraction of the target cell facilitates detachment.[105] Contact to a new target and formation of a new IS also help NK cells detach from the previous one.[68]
One aspect that leads to the termination of an IS is the loss receptor ligand interaction with the target cell.[68] The activating receptor NKG2D is endocytosed upon activation, which allows for continued activating signaling, while removing its physical interaction with the target cell.[106, 107] On the other hand, CD16 is shed from the NK cell surface by metalloproteases like ADAM17 regardless of its involvement in the NK cell activation.[108] Regarding ADCC, CD16 shedding is necessary for serial killing but also makes subsequent activations harder, since less of the activating receptor is present.[109] Strong activating signaling increases serial killing. Afucosylation of antibodies increases their affinity for CD16 leading to increased serial ADCC.[110] Expression of CD2 on $CD16^+$ NK cells correlates with high serial killing. A potential mechanism is costimulatory signaling by CD2 when it associates with CD16.[33]
Priming by cytokines can also boost serial killing, as treatment with an IL-15 based super-agonist was shown to lead to higher serial killing levels in hematopoietic progenitor cell derived NK cells.[111]
Serial killing NK cells mainly rely on their lytic granules. Denovo synthesis of the effector molecules is an energy consuming process.[112] NK cells have been observed to endocytose granzyme B that has not yet entered the target cell during IS termination.[113] The granzyme B is recycled via endosomes to form new lytic granules, saving resources and promoting longer sustain of activity. This recycling also happens with membrane proteins CD107a and the granule priming factor Munc13-4.[114, 115] When glucose uptake is limited but not completely blocked, NK cells were shown to become better serial killers, further linking serial killing activity to metabolism.[116, 117]
Despite all the methods developed and insights gained, the question remains unanswered, if there is a subset of serial killing NK cells or if serial killing is just a process that emerges stochastically.

1.5 NK cells in the clinic

NK cells have gained traction as an addition to the roster of tumor-immune-therapies. Compared to T cells which are already in use they bring benefits regarding safety and versatility. Less proinflammatory cytokines are released by NK cells reducing the risk of cytokine release syndrome.[9, 118] Additionally, allogeneic NK cells from healthy donors can be given without a risk of graft versus host disease.[119] This opens up possibilities for an off the shelf product, reducing waiting and manufacturing times and cost drastically. The use of chimeric-antigen-receptors for NK cells has already been demonstrated and until the start of 2025 at least 120 clinical-trials using this technology were run.[120] Dosage, stimulation protocols and source of NK cell differ between the trials and no universal approach was found yet. Another way to harness the activity of NK cells clinically is through ADCC by giving antibodies.[90, 110] These can be modified to increase CD16 affinity, also crosslink other NK cell receptors or be directly linked to potent NK cell stimulators like IL-15 in so called BiKEs and TriKEs which are bi- and trispecific NK cell engagers.[121, 122] Since all NK cell therapies rely on their cytotoxicity they could be boosted by serial killing. Insights into a subset of serial killing NK cells or ways to stimulate and trigger NK cells into serial killing would translate into potentially better therapies.

2. Aim

NK cells are a prime candidate for developing new cell-based cancer immunotherapies. This project aims to deepen our knowledge of these cells and their behavior. The focus is on the ability of NK cells to sequentially kill multiple target cells. To that end we tried improving existing tools and to develop a new tool for serial killing analysis. Classical microscopy-based methods to identify serial killing NK cells have a low thruput and are not easily combined with further analysis of cells of interest. The here shown serial degranulation assay can identify NK cells that are able to get sequentially activated. Since it is based on flow cytometry it easily adapts to phenotyping or sorting of cells of interest.

In a second step we started utilizing this tool to seek out what drives serial killing behavior and to find a marker able to identify serial killing NK cells beforehand. The goal is to deepen the knowledge about serial killing NK cells and what differentiates them from non-serial killing NK cells, potentially opening ways to influence this behavior.

3. Material

3.1 Antibodies

Flow cytometer / cell sorter used to measure the cells is indicated in brackets as Cytek® Aurora (C), BD LSRFortessa™ (F), BD FACSAria™ Fusion (A), BD FACSmphony™ A5 (S).

Table 3.1.1: Antibodies for CD107a surface retainment (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	FITC	200	BioLegend (San Diego, CD, USA)
CD56 (MEM-188)	PE	200	BioLegend

700-fold diluted Zombie NIR, BioLegend

Table 3.1.2: Antibodies for CD107a maximal effect blocking (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD56 (MEM-188)	PE	200	BioLegend

Table 3.1.3: Antibodies for in assay CD107a blocking (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD3 (UCHT1)	BUV563	200	BD biosciences (San Jose, CA, USA)
CD56 (B159)	BUV805	500	BD biosciences

700-fold diluted Zombie NIR, BioLegend

Table 3.1.4: Antibodies for serial degranulation assay with multiple incubation times (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD3 (UCHT1)	BUV563	200	BD biosciences
CD56 (B159)	BUV805	500	BD biosciences
TIGIT (741182)	BV786	100	BD biosciences

NKG2A (Z199)	PE	100	Beckman Coulter (Brea, CA, USA)
CD16 (3G8)	BUV615	400	BD biosciences
CD161 (HP-3G10)	BUV661	100	BD biosciences
HLA-DR (L243)	BV605	100	BD biosciences
CD38 (HB7)	BUV395	100	BD biosciences
NKG2C (134591)	BUV496	50	BD biosciences

700-fold diluted Zombie NIR, BioLegend

Table 3.1.5: Antibodies for serial degranulation assay with different target cells lines and different effectors (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD3 (UCHT1)	BUV563	200	BD biosciences
CD56 (B159)	BUV805	500	BD biosciences

700-fold diluted Zombie NIR, BioLegend

Table 3.1.6: Antibodies for serial degranulation assay validation experiment (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD3 (UCHT1)	BUV563	200	BD biosciences
CD56 (B159)	BUV805	500	BD biosciences
CD16 (3G8)	BUV615	400	BD biosciences
CD69 (FN50)	BV750	400	BioLegend

700-fold diluted Zombie NIR, BioLegend

Table 3.1.7: Antibodies for sorting degranulation status (A)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD56(MEM-188)	PE	200	BioLegend

Table 3.1.8: Antibodies for serial degranulation assay with degranulation status sorted cells (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	PerCP	100	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD56(MEM-188)	PE	200	BioLegend

700-fold diluted Zombie NIR, BioLegend

Table 3.1.9: Antibodies for serial degranulation assay with education readout (S)

Antibody (clone)	Conjugate	Dilution	Source
CD56 (N901)	ECD	25	Beckman Coulter
CD57 (HCD57)	FITC	50	BioLegend
CD38 (HB-7)	BV650	50	BioLegend
CD3 (UCHT1)	V500	50	BD biosciences
CD14 (M5E2)	V500	50	BD biosciences
CD19 (HIB19)	V500	50	BD biosciences
KIR3DL1 (DX9)	BV421	50	BioLegend
KIR2DL1 (REA284)	APC-Cy7	50	Miltenyi Biotec (Bergisch Gladbach, Germany)
KIR2DL1/S1 (11PB6)	PE-Cy7	50	Miltenyi Biotec
KIR2DL3/L2/S2 (GL183)	PE-Cy5.5	50	Beckman Coulter
NKG2A (Z199)	APC	50	Beckman Coulter
NKG2C (REAA205)	PE	50	Miltenyi Biotec
Granzyme B (GB11)	AlexaFluor 700	100	BD biosciences

Table 3.1.10: Antibodies for serial degranulation assay with education readout (S)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	PerCP	100	BioLegend
CD107a (H4A3)	PE	400	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
KIR2DL2/L3/S3 (DX27)	RB705	100	BD biosciences
CD57 (QA17A04)	BV605	200	BioLegend
KIR3DL1 (DX9)	BV711	50	BD biosciences
NKG2A (S19004C)	BV785	200	BioLegend

CD56	ECD	100	Beckman Coulter
NKG2C (S19005E)	FITC	50	BioLegend
KIR2DL1/S1/S3/S5 (HP-MA4)	PE-Cy7	25	BioLegend
CD38 (HIT2)	BUV395	100	BioLegend

4000-fold diluted LIVE/DEAD™ Fixable Near IR (780) Viability Kit, Thermo Fisher Scientific (Waltham, MA, USA)

Table 3.1.11: Antibodies for serial degranulation assay with effector molecule readout (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD3 (UCHT1)	BUV563	200	BD biosciences
CD56 (B159)	BUV805	500	BD biosciences
Granzyme B (QA18A28)	PE-Cy7	100	BioLegend
Perforin (dG9)	PerCP-Cy5.5	50	BioLegend

700-fold diluted Zombie NIR, BioLegend

Table 3.1.12: Antibodies for serial degranulation assay with education and effector molecule combined readout (S)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	PerCP	100	BioLegend
CD107a (H4A3)	PE	400	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
KIR2DL2/L3/S3 (DX27)	RB705	100	BD biosciences
CD57 (QA17A04)	BV605	200	BioLegend
KIR3DL1 (DX9)	BV711	50	BD biosciences
NKG2A (S19004C)	BV785	200	BioLegend
CD56	ECD	100	Beckman Coulter
NKG2C (S19005E)	FITC	50	BioLegend
KIR2DL1/S1/S3/S5 (HP-MA4)	PE-Cy7	25	BioLegend
CD38 (HIT2)	BUV395	100	BioLegend
KIR2DL2/L3/S3 (DX27)	RB705	100	BD biosciences

Granzyme B (GB11)	AlexaFluor 700	100	BD biosciences

4000-fold diluted LIVE/DEAD™ Fixable Near IR (780) Viability Kit, Thermo Fisher Scientific

Table 3.1.13: Antibodies for serial degranulation assay with phenotyping readout, RNA sequencing hits (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD3 (UCHT1)	BUV563	400	BD biosciences
CD56 (B159)	BUV805	1000	BD biosciences
CD184/CXCR4 (12G5)	BV786	400	BD biosciences
CD72 (J4-117)	RB545	400	BD biosciences
CD108 (KS-2)	BB700	800	BD biosciences
LDLR (C7)	RB780	400	BD biosciences
Pref-1 (211309)	DyLight 550	200	R&D Systems (Minneapolis, MN, USA)
CD355 (REA1225)	PE-Vio 615	200	Miltenyi Biotec
PCDH1 (648127)	AF700	50	R&D Systems

700-fold diluted Zombie NIR, BioLegend

Table 3.1.14: Antibodies for serial degranulation assay with phenotyping readout, general NK cell receptors (C)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD3 (UCHT1)	BUV563	400	BD biosciences
CD56 (B159)	BUV805	1000	BD biosciences
CD38 (HB7)	BUV395	100	BD biosciences
NKG2C (134591)	BUV496	100	BD biosciences
CD161 (HP-3G10)	BUV661	100	BD biosciences
KLRG1 (2F1)	BV510	50	BioLegend
HLA-DR (L243)	BV605	50	BD biosciences
TIGIT (741182)	BV786	100	BD biosciences

CD8a (RPA-/8)	AF532	200	Thermo Fisher Scientific
CD27 (M-T271)	PerCP-Cy5.5	50	BD biosciences
NKG2A (Z199)	PE	100	Beckman Coulter
CTLA-4 (BNI3)	PE-Dazzle	100	BioLegend
4-1BB (4B4-1)	PE-Cy7	100	BioLegend
PD-L1 (MIH3)	PerCP-Fire806	200	BioLegend
CD18 (TS1/18)	AF700	800	BioLegend
CD70 (REA292)	APC-Vio770	200	Miltenyi Biotec

700-fold diluted Zombie NIR, BioLegend

Table 3.1.15: Antibodies for sorting on CD38 and NKG2A expression (A)

Antibody (clone)	Conjugate	Dilution	Source
CD56 (B159)	PerCp-Cy5.5	50	BD biosciences
CD38 (HIT2)	PE-Cy7	200	BioLegend
NKG2A (Z199)	PE	100	Beckman Coulter

Table 3.1.16: Antibodies for serial degranulation assay with CD38, NKG2A sorted cells (F)

Antibody (clone)	Conjugate	Dilution	Source
CD107a (H4A3)	BV421	50	BioLegend
CD107a (H4A3)	FITC	200	BioLegend
CD107a (H4A3)	APC	200	BioLegend
CD107a (H4A3)	unconjugated	50	BioLegend
CD56 (B159)	PerCp-Cy5.5	50	BD biosciences

500-fold diluted Zombie Yellow, BioLegend

3.2 Reagents

Table 3.2.1: Reagents

Name	Source
BD FACS Flow™	Thermo Fisher Scientific
BD FACS™ Permeabilizing Solution 2	BD biosciences
BD Perm/Wash™ Perm/Wash Buffer	BD biosciences
Brilliant Stain Buffer	BD biosciences
CASYton	OLS® OMNI Life Science (Bremen, Germany)
CellTracker™ Green CMFDA	Thermo Fisher Scientific
CellTracker™ Red CMTPX	Thermo Fisher Scientific
DMEM	Thermo Fisher Scientific
DPBS	Thermo Fisher Scientific
Fetal calf serum (FCS)	Thermo Fisher Scientific
HEPES (1M)	Thermo Fisher Scientific
Human TruStain FcX (Fc Receptor Blocking Solution)	BioLegend
IMDM	Thermo Fisher Scientific
IMDM GlutaMAX™	Thermo Fisher Scientific
LIVE/DEAD™ Fixable Near IR (780) Viability Kit	Thermo Fisher Scientific
Lymphoprep™	STEMCELL Technologies (Vancouver)
NK MACS® Medium, human	Miltenyi Biotec
Pancoll human	PAN-Biotech (Aidenbach, Germany)
Paraformaldehyde (PFA)	Sigma-Aldrich
Penicillin-Streptomycin (P/S)	Thermo Fisher Scientific
Poly-L-Lysin Solution	Sigma-Aldrich (St. Louis, MO, USA)
Puromycin	Calbiochem/Merck Millipore (Billerica)
Recombinant human IL-15	PAN-Biotech
Recombinant human IL-2	NIH Cytokine Repository (Frederick MD, USA)
RPMI 1640	Thermo Fisher Scientific
SYTOX™ Deep Red	Thermo Fisher Scientific
TaqMan™ Fast Advanced Master Mix for qPCR	Thermo Fisher Scientific
TrypLE™ Express™ (Trypsin)	Thermo Fisher Scientific
Zombie NIR	BioLegend
Zombie Yellow	BioLegend

3.3 Media / Buffer

Table 3.3.1: Media / Buffer

Name	Components
NK cell medium	IMDM GlutaMAX™, 10% FCS, 1% P/S
HeLa medium	DMEM, 10% FCS, 1% P/S
K562 medium (CTL)	IMDM, 10% FCS, 1% P/S
FACS buffer	DPBS, 2% FCS
Dynal buffer	DPBS, 0.1% BSA, 2 mM EDTA
CITEseq staining solution	DPBS, 1% BSA, 2 mM EDTA

3.4 Kits

Table 4.4.1: Kits

Name	Source
Dynabeads™ Untouched™ Human NK Cells Kit	Invitrogen™ (Waltham, MA, USA)
NK Cell Isolation Kit, human	Miltenyi Biotec
RNeasy Mini Kit	Qiagen (Venlo, Netherlands)
QIAshredder	Qiagen
First Strand cDNA Synthesis Kit for RT-PCR (AMV)	Roche (Base, Switzerlandl)

3.5 Cells

Table 3.5.1: Cells

Name	Transfected with	Origin
Primary NK cells	untransfected	Whole blood from healthy humans
K562	untransfected	CML
K562 (feeder)	mbIL-15-mbIL-21-41BBL	CML
Hela	CD48	Cervical cancer cell line
Jurkat	untransfected	T cell leukemia
721.221	untransfected	B-cell lymphoma

3.6 Devices

Table 3.6.1: Devices

Device	Name	Source
Cell counter	CASY Cell Counter & Analyzer	OLS® OMNI Life Science
Incubator	HERAcell240i CO_2 Incubator	Thermo Fisher Scientific
Sterile workbench	HERA Safe 2020	Thermo Fisher Scientific
Centrifuge	Heraeus Multifuge 3 S-R Heraeus Megafuge 40R Heraeus Fresco 21 centrifuge	Thermo Fisher Scientific
Flow cytometer	Cytek® Aurora	Cytek Bioscience Inc. (Fremont, CA, USA)
Flow cytometer	BD LSRFortessa™	BD biosciences
Flow cytometer	FACSmphony™ A5	BD biosciences
Cell sorter	BD FACSAria™ Fusion	BD biosciences
Microscope - Objective	Mica WideFocal Live Cell HC PL FLUOTAR 10x/0.32	Leica Microsystems (Wetzlar, Germany)
UV/VIS-Spectralphotometer	NanoDrop™ One^C	Thermo Fisher Scientific
Thermal Cycler	C1000 Touch	Bio-Rad (Hercules, USA)
Real-Time PCR Detection System	CFX96 Touch	Bio-Rad

3.7 Consumables

Table 3.7.1: Consumables

Type	Name	Source
Chambered coverslip	µ-Slide 8 Well high (ibidi Polymer)	ibidi (Gräfelfing, Germany)
96-well U-bottom plate	Nuclon™ Delta Surface	Thermo Fisher Scientific
96-well V-bottom plate	Microtest Plate 96 Well	Sarstedt (Nümbrecht, Germany)

4. Methods

4.1 Cell culture

4.1.1 Cell lines

K562 cells were cultured in IMDM (10% FCS, 1% penicillin/streptomycin (P/S)) in T75 culturing flasks at 37 °C and 5% CO_2. Cell culture was split three times per week. For experiments K562 cell culture was split the day before to a concentration of 3 x 10^5 cells/mL.

Jurkat cells were cultured in RPMI (10% FCS, 1% P/S) in T75 culturing flasks at 37 °C and 5% CO_2. Cell culture was split three times per week. For experiments Jurkat cell culture was split the day before to a concentration of 3 x 10^5 cells/mL.

721.221 cells were cultured in IMDM (10% FCS, 1% P/S) in T75 culturing flasks at 37 °C and 5% CO_2. Cell culture was split three times per week. For experiments 721.221 cell culture was split the day before to a concentration of 3 x 10^5 cells/mL.

HeLa-CD48 cells were cultivated in DMEM (10% FCS, 1% P/S) supplemented with 1 µg/mL Puromycin for CD48 selection in T75 culturing flasks at 37 °C and 5% CO_2. TrypLE™ Express™ (Thermo Fisher Scientific) was used to detach the cells for splitting. Cell culture was split three times per week according to their confluence.

4.1.2 Primary cells

PBMC isolation

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood of healthy donors by density centrifugation. Pancoll human (PAN biotech) was covered by a layer of blood and centrifuged (1025 x g for 25 min). The white layer containing the PBMCs was harvested and washed multiple times with PBS. PBMC were used directly for NK cell isolation or cryopreserved in gas phase of liquid nitrogen in FCS with 10 % DMSO for later use.

NK cell culture

Fresh NK cells were isolated from PBMCs using the Dynabeads® Untouched™ Human NK Cells Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. NK cells were rested over night as detailed in the experiment or preactivated for long term culture. For preactivation NK cells were stimulated with K562-mbIL-15-mbIL-21-41BBL and 200 U/mL IL-2. They were cultured for at least 3 weeks before experiments in NK cell medium with 100 U/mL IL-2 in 96-well-U-bottom plates at 37 °C and 5% CO_2. Cell concentration was kept between 1 x 10^6 and 3 x 10^6

cell/mL and cells split accordingly or medium exchanged every two to three days. Naming of donors is done one a per experiment basis.

4.1.3 NK cell culture for education experiments

PBMC isolation

Experiments regarding NK cell education were carried out in collaboration with the Malmberg group at Oslo University Hospital under help from Giovanna Perinetti Casoni. PBMC were isolated from buffy coats of healthy donors provided by the hospital. Buffy coats were diluted 2:1 with PBS, Lymphoprep™ (STEMCELL Technologies) was layered below and together centrifuged (800 x g for 20 min). The white layer containing the PBMCs was harvested and washed multiple times with PBS.

NK cell culture

Fresh NK cells were isolated from PBMCs using the NK cell Isolation Kit, human (Miltenyi Biotec) according to the manufacturer's instructions. They were rested overnight in RPMI 1640 (VWR) + 10% FCS + 10 mM HEPES at 37 °C and 5% CO_2.

4.2 Microscopy

Polymer µ-Slide 8 Well coverslips (ibidi) were coated with Poly-L-Lysin Solution (Sigma Aldrich) according to the manufacturer's instructions. Cells were stained as 1 million cells / mL in serum free CTL for 30 minutes at 37 °C and 5% CO_2. NK cells were stained with 1 µM CellTracker™ Green CMFDA (Thermo Fisher Scientific) and K562 with 5 µM CellTracker™ Red CMTPX (Thermo Fisher Scientific). Microscopy was performed in sterile filtered CTL without phenol red in the presence of 0.5 µM SYTOX™ Deep Red (Thermo Fisher Scientific) and 10 mM HEPES (Thermo Fisher Scientific). 100 µL medium were added into each well and the slide pre-warmed. The Slide was placed into the Mica WideFocal Live Cell (Leica Microsystems) and 60.000 K562 cell in a volume of 150 µL added. Microscopy was performed with a 10X objective at 37 °C with humidity control. When K562 cells have sunken down an area near the center of the well was chosen to image as a 2 by 2 with 10 % overlap for stitching. 5000 NK cells were added in 25 µL into the center of the well and imaging was started immediately. In addition to brightfield, images for each fluorophore were acquired every 3 minutes for 12 hours.

Video analysis was performed using napari version 0.4.19 and the plugin MMV_H4Tracks in cooperation with Justin Sonneck and Jianxu Chen of the AMBIOM group from the Leibniz-Institut für Analytische Wissenschaften – ISAS.

4.3 CD107a surface retainment

As previously described.[123] Hela cells were seeded in 6 well plates and allowed to adhere overnight. The next day NK cells were added, and 60 min later NK containing supernatant was removed, and remaining NK cells washed off with PBS twice. Supernatant and washed off NK cells were combined. Cells were spun down, supernatant discarded, and NK cells resuspended in fresh media. Cells were gently rotated in the incubator to inhibit reattachment in case HeLa cells were carried over. Anti-CD107a-FITC antibody was added at indicated timepoints and 5 minutes post antibody addition cells were washed and stained with live dead marker for 10 minutes. Subsequently cells were washed again and then fixed with 2% PFA for 10 minutes. Lastly cells were stained with anti-CD56-PE antibody for 10 minutes. Panel used can be found in table 3.1.1.

4.4 CD107a blocking

As previously described.[123] K562 cells and NK cells were co-incubated at an E:T of 2:1 for 40 minutes. Either BV421 conjugated anti-CD107a antibody, 10-fold unconjugated anti-CD107a antibody or nothing was added for 5 min. Cells were washed and directly treated with FITC conjugated anti-CD107a antibody for 5 minutes. Afterwards cells were washed again, fixed with 2% PFA for 10 minutes, washed and stained with anti-CD56-PE antibody for 15 minutes. Lastly cells were washed and analyzed by flow cytometry. Panel used can be found in table 3.1.2.

4.5 CD107a blocking tested in experimental setup

Serial degranulation assay was performed as described below in **4.8** with PBMCs and K562 as target cells, up to the second staining. This was done with or without the addition of 10x unconjugated anti-CD107a-antibody during the first blocking step. The second blocking and third staining were skipped and directly continued with the dead cell stain. Panel used can be found in table 3.1.3.

4.6 Serial degranulation assay with overlapping differing incubation times

Serial degranulation assay was performed similar to the described version below in **4.8** with PBMCs and K562 as target cells. Labeled anti-CD107a-antibodies however were present during the coincubation time. The experiment was performed with 3 different incubation times. Incubation times were 10, 30 and 50 minutes. All 3 incubation periods were started in parallel (Figure 5A). Panel used can be found in table 3.1.4 and was prepared with 50% Brilliant Stain Buffer (BD biosciences). The panel also included several surface markers that were not of interest for the analysis shown here.

4.7 Serial degranulation assay comparing target cell lines

Done by Vanna Imširović.
Serial degranulation assay was performed as described below in **4.8**. Different target cell lines K562, 721.221 and Jurkat (1 million cells) against 1 million cells PBMCs. Panel used can be found in table 3.1.5.

4.8 Serial degranulation assay

As previously described.[123] PBMCs were thawed the day before the experiment and cultured overnight in NK cell medium with the addition of 0.5 ng/mL IL-15 at 37°C and 5% CO_2. Freshly isolated NK cells were rested overnight after isolation in the same manner. The assay was performed in 96-well-v-bottom-plates, this allows manipulation of 12 samples in parallel in one row. 1 million K562 cells were used as target per well. 1 million PBMCs were used as effector cells to gain an E:T ratio of roughly 1:10. To keep the pH constant even while handling the cells, the assay was performed in IMDM (10% FCS, 1% P/S) + 10 mM HEPES (assay medium). 10 x predilutions of the anti-CD107a-antibodies (see table 3.1.6) were prepared beforehand in assay medium and pre-placed in individual rows of another 96-well-v-bottom-plate. 2 x predilution (2 x master mix) of phenotyping antibodies (CD3, CD56, CD16, CD69; see table 3.1.6) is prepared in PBS + 2% FCS. This plate is kept in the dark and on ice. Multi-channel pipettes were used to handle the samples in parallel.

Procedure:
-Add effector cells to V-bottom plate, spin down (5 minutes at 500 x g) and remove supernatant.
-Add 50 µL assay medium with target cells or without (no target control) to the effector cell pellet.
-Resuspend and mix cells by pipetting
-Spin down for 10 seconds to enable direct contact formation
-Incubate the plate for 10 minutes at 37 °C and 5% CO_2
-Add 5 µL 10x anti-CD107a-BV421 and resuspend the cells by pipetting, separating contacts
-Incubate the plate for 5 minutes at 37 °C and 5% CO_2
-Add 5 µL 10x anti-CD107a-blocking antibody and resuspend the cells by pipetting
-Incubate the plate for 5 minutes at 37 °C and 5% CO_2
-Wash by adding 100 µL of assay medium and centrifuge for 5 minutes at 500 x g
-Remove supernatant
-Resuspend in 50 µL assay medium and mix cells by pipetting
-Spin down for 10 seconds to enable direct contact formation
-Incubate the plate for 10 minutes at 37 °C and 5% CO_2
-Add 5 µL 10x anti-CD107a-FITC and resuspend the cells by pipetting, breaking up contacts

-Incubate the plate for 5 minutes at 37 °C and 5% CO_2
-Add 5 µL 10x anti-CD107a-blocking antibody and resuspend the cells by pipetting
-Incubate the plate for 5 minutes at 37 °C and 5% CO_2
-Wash by adding 100 µL of assay medium and centrifuge for 5 minutes at 500 x g
-Remove supernatant
-Resuspend in 50 µL assay medium and mix cells by pipetting
-Spin down for 10 seconds to enable direct contact formation
-Incubate the plate for 10 minutes at 37 °C and 5% CO_2
-Add 5 µL 10x anti-CD107a-APC and resuspend the cells by pipetting, separating contacts
-Incubate the plate for 5 minutes at 37 °C and 5% CO_2
-Wash by adding 100 µL of assay medium and centrifuge for 5 minutes at 500 x g
-Remove supernatant
-Add 25 µL PBS + Zombie NIR 1:700 and resuspend the cells by pipetting
-Incubate the plate on ice for 10 minutes
-Add 25µL 2X master mix and resuspend the cells by pipetting
-Incubate the plate on ice for 15 minutes
-Wash by adding 100 µL of PBS + 2 % FCS and centrifuge for 5 minutes at 500 x g
-Remove supernatant
-Resuspend cells vigorously in 25 µL Enzyme Free Cell Dissociation Solution
-Incubate the plate on ice for 10 minutes
-Pipet again to separate cell-cell contacts
-To fix the cells add 25 µL 4 % PFA and mix by pipetting
-Incubate the plate on ice for 10 minutes
-Wash by adding 100 µL of PBS + 2 % FCS and centrifuge for 5 minutes at 500 x g
-Resuspend cells in desired volume for flow cytometry

4.9 Serial degranulation assay comparing effector cells

Serial degranulation assay was performed as described below in **4.8**. Different effector cells were used. 1 million PBMCs, 0.1 million fresh or preactivated NK cells against 1 million K562 cells. Panel used can be found in table 3.1.5.

4.10 Stability test of degranulation phenotype

Done together with Maren Claus.
Freshly isolated NK cells were cultured overnight in IMDM GlutaMAX™ (10% FCS, 1% P/S) with the addition of 0.5 ng/mL IL-15 at 37 °C and 5% CO_2. 7-13 million NK cells were used to achieve sufficient cell numbers after sorting with K562 cells as targets cells at an E:T of at least 1:2. The serial degranulation assay was performed similar to the description in **4.8**. To handle high cell numbers, it was performed in 15 mL centrifuge tubes in a volume of 1.2 mL. After the third CD107a staining cells were

washed and then stained for 15 minutes with anti-CD56-PE-antibody. After washing the cells, they were resuspended in Dynal buffer, put through a 35 µm cell strainer and kept on ice until sorting. Cells were sorted using a BD FACSAria™ Fusion as triple degranulated, degranulated in the beginning, degranulated at the end and not degranulated into FCS. Panel used can be found in table 3.1.7.

Sorted NK cells were cultivated in 96-well-round-bottom-plates at 37 °C and 5% CO_2 for 2 days. They were plated in NK MACS complete medium prepared with FCS instead of human AB serum and adding 1% P/S, 100 U/mL IL-2 and 1 ng/mL IL-15. The plating concentration was 1 million cells/mL.

On day 2 a follow-up serial degranulation assay was performed as described in **4.8** without fixation. Anti-CD107a-BV421-antibody remained on the cells after 2 days, so it was exchanged against anti-CD107a-PerCP-antibody for the second readout. Panel used can be found in table 3.1.8.

4.11 Determining education status of NK cells

Done together with Giovanna Perinetti Casoni.
PBMCs were stained extracellularly after isolation for 15 minutes and washed. BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD biosciences) was used to fix and permeabilize the cells for 10 minutes at 4 °C. Cells were washed twice and granzyme B stained intracellularly for 30 minutes followed by two last washing steps, all done in BD Perm/Wash™ Buffer. Panel used can be found in table 3.1.9.
As described by Goodridge et al. educated NK cells have higher amounts of granzyme B as uneducated ones.[53] HLA-C type education was inferred on $NKG2A^-$, $CD57^-$, $KIR3DL1^-$ NK cells by comparing median granzyme B fluorescence intensity of $KIR2DL1^+$, $KIR2DL3^-$ NK cells with $KIR2DL1^-$, $KIR2DL3^+$ NK cells. A ratio greater than 1.3 was deemed as educated by that KIR. For gating and example see supplementary Figure 4A.

4.12 Serial degranulation assay with education readout

Done together with Giovanna Perinetti Casoni.
Assays regarding education were carried out in collaboration with the Malmberg group at Oslo University Hospital, this led to some changes to the protocol in **4.8**. NK cells were isolated the day before and rested over night without addition of IL-15. The serial degranulation assay was carried out in RPMI medium. After the third CD107a staining, cells were washed, then stained with a live/dead stain for 5 minutes, FC-blocking-solution Human TruStain FcX (BioLegend) for 10 minutes and the phenotyping master mix for 30 minutes. Afterwards cells were washed twice, fixed for 20 minutes with 2% PFA and washed again. Panel used can be found in table 3.1.10.

4.13 Serial degranulation with effector molecule readout

Serial degranulation assay was performed as described in **4.8**. After fixation cells were washed and permeabilized at room temperature for 10 minutes using BD FACS™ Permeabilizing Solution 2 (BD biosciences). Cells were washed again and stained for granzyme B and perforin content for 20 minutes. Panel used can be found in table 3.1.11.

4.14 Serial degranulation with education and effector molecule readout

Done together with Giovanna Perinetti Casoni.
Serial degranulation assay was performed as described in **4.12**. The last washing step is carried out using BD Perm/Wash™ Perm/Wash Buffer (BD biosciences) afterwards the cells are resuspended in fresh BD Perm/Wash™ Perm/Wash Buffer. Cells were incubated for 15 minutes, then washed and stained intracellularly for granzyme B for 30 minutes in BD Perm/Wash™ Buffer. Lastly cells are washed again. Panel used can be found in table 3.1.12.

4.15 Bulk RNA sequencing

4.15.1 Sorting degranulated NK cells for RNA sequencing

Freshly isolated NK cells were cultured overnight in IMDM GlutaMAX™ (10% FCS, 1% P/S) with the addition of 0.5 ng/mL IL-15 at 37 °C and 5% CO_2. 12 million NK cells were used to achieve sufficient cell numbers after sorting. The serial degranulation assay was performed similar to the description in **4.8**. Cells were split into 24 wells, with 0.5 million NK cells and 1.5 million K562 target cells per well, for an E:T of 1:3. After the third CD107a staining cells were washed and then stained for 15 minutes with anti-CD56-PE-antibody. After washing the cells, they were resuspended in Dynal buffer, put through a 35 µm cell strainer and kept on ice until sorting. Cells were sorted using a BD FACSAria™ Fusion as triple degranulated or not degranulated into FCS. Panel used can be found in table 3.1.7. Sorted cells were pelleted and stored at -80 °C as dry pellet.

4.15.2 RNA isolation for RNA sequencing

0.12 - 0.5 million cells in case of triple degranulated or 1 million cells in case of non-degranulated NK cells were used for RNA isolation. Cells were thawed and the RNeasy Mini Kit (Qiagen) with the QIAshredder (Qiagen) was used for RNA isolation according to the manufacturer's instructions with the additional optional on-column

DNase digestion step. RNA was stored in water at -80 °C until all samples were collected.

4.15.3 Bulk RNA sequencing

Bulk RNA sequencing was performed by the Genomics & Transcriptomics Facility at Hospital University Essen in cooperation with Bettina Budeus. It was prepared using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen) and sequenced on a NextSeq2000 (Illumina) with a P1 flow cell and 100 cycles.

4.15.4 Flow cytometry-based validation of sequencing results

Serial degranulation assay was performed as described in **4.8**. 2 million PBMC and K562 target cells were co-incubated per well and split in two after the live / dead staining. The other half was used for the phenotyping described in **4.16**. After master-mix staining cells were washed and directly measured. Panel used can be found in table 3.1.13.

4.15.5 qPCR based validation of sequencing results

Done by Mina Sandusky.
RNA was prepared as described in **4.15.1** and **4.15.2**. Two more groups were sorted, bulk NK cells from the normal assay conditions and bulk NK cells, where the assay was performed in the absence of target cells. RNA was eluted in 10 mM Tris, 0.1 mM EDTA and quantified with a NanoDrop™ One^{C} (Thermo Fischer). Reverse transcription was performed using 10 ng of RNA from each condition and a Transcriptor First Strand cDNA Synthesis kit (Roche). Gene expression was analyzed by real-time PCR using an Applied Biosystems TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) and Applied Biosystems TaqMan Gene Expression Assays (PN 4453320). Analyzed genes are listed below with their primers. Housekeeping genes notated with an asterisk.
RPLPO* FAM Hs 00420895_gH
B2M* FAM Hs 00187842_m1
RPS18* FAM Hs 01375212_g1
EGR3 FAM Hs 00231780_m1
XCL1 FAM Hs 00751481_s1
NFKBIZ FAM Hs 00230071_m1

Real-time PCR was performed using the following cycling protocol on a Bio-Rad CFX96 Real-Time System / C1000 Touch Thermal Cycler.
Step 1: 50 °C, 2 minutes
Step 2: 95 °C, 1 minute
Step 3: 95 °C, 15 seconds
Step 4: 60 °C, 35 seconds
Step 5: Go To step 3 x 39 cycles

ΔCq was calculated by subtracting Cq of gene of interest from average Cq of the three housekeeping genes. RT-PCR was done twice and ΔCq shown is the average of the two technical replicates.

4.16 Serial degranulation with deep NK cell phenotyping

Serial degranulation assay was performed as described in **4.8**. 2 million PBMC and K562 target cells were co-incubated per well and split in two after the live / dead staining. The other half was used for the phenotyping described in **4.15.4**. After master-mix staining cells were washed and directly measured. Panel used can be found in table 3.1.14.

4.17 Sorting NK cells based on CD38 and NKG2A expression

Done together with Maren Claus.
Freshly isolated NK cells were cultured overnight in IMDM GlutaMAX™ (10% FCS, 1% P/S) with the addition of 0.5 ng/mL IL-15 at 37 °C and 5% CO_2. 20 million NK cells were used to achieve sufficient cell numbers after sorting. NK cells were stained in 1 mL FACS buffer for 30 minutes at 37 °C and 5% CO_2. Panel used can be found in table 3.1.15. After washing the cells, they were resuspended in Dynal buffer, put through a 35 µm cell strainer and kept on ice until sorting. Cells were sorted using a BD FACSAria™ Fusion. During sorting lights were turned of and the loading chamber cooled to ensure fluorophore stability. Cells were sorted as bulk CD56dim and from the CD56dim NK cells further divided into $CD38^+$ $NKG2A^-$, $CD38^+$ $NKG2A^+$, $CD38^-$ $NKG2A^+$ and $CD38^-$ $NKG2A^-$ into FCS. The cells were rested overnight in IMDM GlutaMAX™ (10% FCS, 1% P/S) with the addition of 0.5 ng/mL IL-15 at 37 °C and 5% CO_2 at 2 million cells / mL.

4.18 Serial degranulation assay with CD38 / NKG2A sorted NK cells

Serial degranulation assay was performed as described in **4.8**. Up to 0.1 million NK cells with 1 million K562 cells were used. After master-mix staining (CD56) cells were washed and directly measured. Panel used can be found in table 3.1.16.

4.19 Flow cytometry analysis

Flow cytometry was performed adhering to “Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition)”.[124] Analysis was done with FlowJo version 10.10. General gating is shown in the supplement. Data cleanup with FlowAI_v2.3.2 was tested on the dataset generated in 4.8 and did not change the downstream result, it was not performed for the rest of the experiments.

4.20 Data analysis

Data analysis, statistics and visualization were performed with GraphPad Prism version 10.3.1.

5. Results

5.1 Microscopy based analysis of serial killing NK cells

Live cell video microscopy is the gold standard for serial killing analysis of NK cells as well as T cells. In that, micro-well-dishes can be utilized to keep NK cells in the field of view and enable long term observation of individual cells. This can introduce some novel problems of NK cells not moving freely but sticking to the wall of the dish and cells clumping together at the edges. Also, only a low number of cells can be analyzed in a given well. These limitations can be overcome with faster microscopy setups by using larger microscopy dishes and imaging a large area, stitching them together.[85] Thereby, even if some NK cells leave the field of view on the edge and cannot be analyzed there are enough other NK cells that stay inside. For high numbers of NK cells, the next bottleneck becomes the downstream analysis. Traditionally each NK cell is analyzed manually. For hundreds of cells this becomes very time consuming and can introduce bias. To overcome this, we started a collaboration with the AMBIOM group of the Leibniz Institute for Analytical Sciences, who have a background in automated video analysis and cell tracking. To track each cell type individually effector cells and target cells were fluorescently labeled with different dyes. Effector NK cells were labeled with CellTracker™ Green CMFDA and target K562 cells with CellTracker™ Red CMTPX. In this way each cell type can be tracked in the corresponding fluorescence channel (Figure 1 B). To identify dying cells a dead cell stain in another color was added. Cells were coincubated for up to 12 hours and images of all three fluorescence channels and brightfield were taken every 3 minutes. About 3 minutes are optimal to enable cell tracking. Longer times would enable the cells to move further between images making them harder to track, while shorter times unnecessarily stress the cell by increasing light exposure. A stitched image of 2 by 2 with a 10X objective covered an area large enough to get sufficient numbers of NK cells which do not leave the field of view. We provided the AMBIOM group with manually segmented frames they used to train AI models to identify individual cells. For segmentation the fluorescent images were used in conjunction with the brightfield image to get the actual shape of the cells. The models were then trained only on the fluorescent images. Each fluorescence channel (effector, target, dead) got their own predictor. The predictors were used to calculate segmentations for the complete videos (Figure 1 C). Individual frames of these predictions were manually corrected and then again used to train the models in an iterative process.

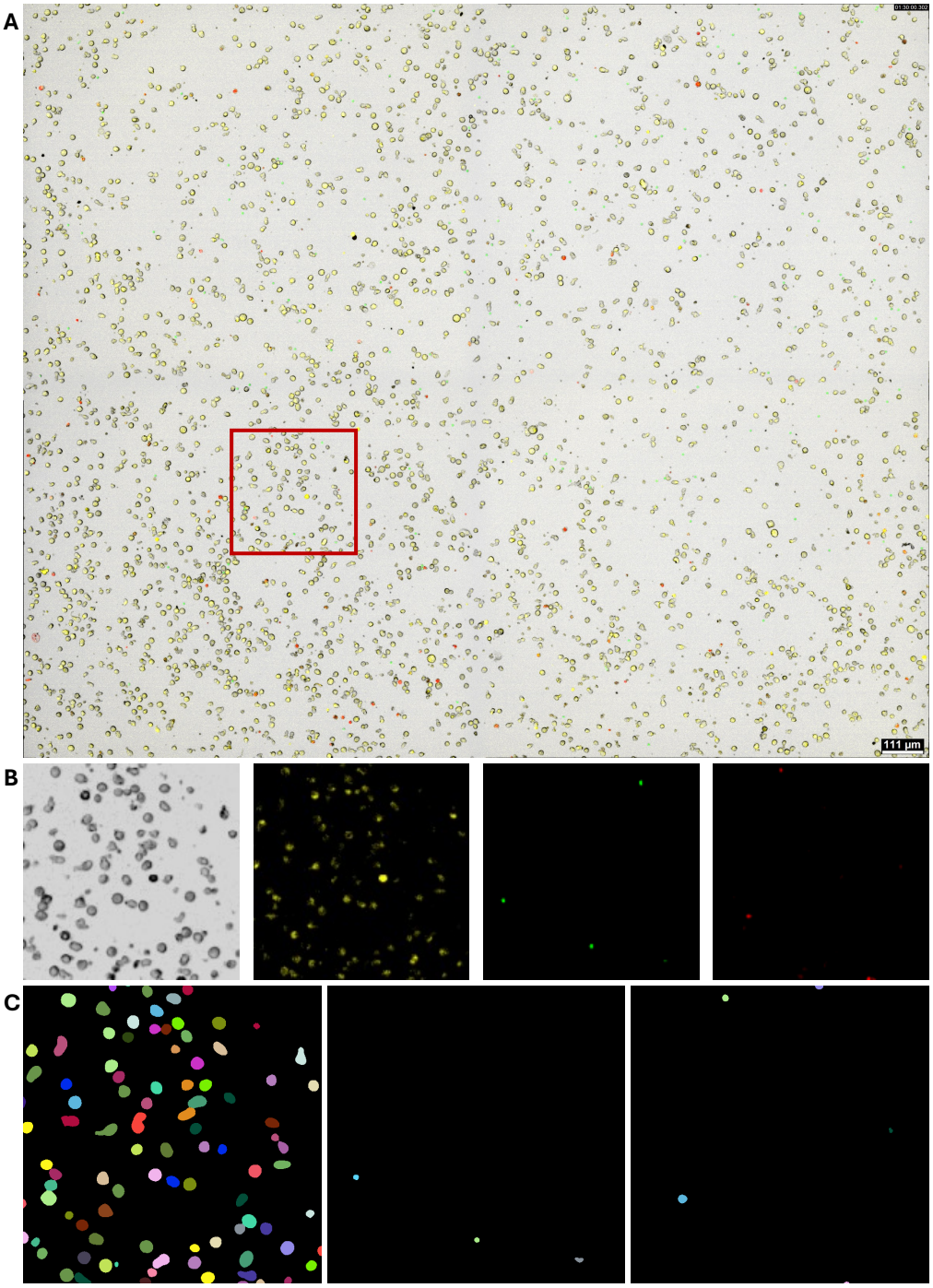

Figure 1: Machine-learning assisted cell segmentation
A) Composite image of brightfield and 3 fluorescence images merged from 4 fields of view, showing K562 cells in co-culture with NK cells after 90 minutes. K562 cells are shown in yellow, NK cells in green and dead cells in red. The red box indicates the area depicted in B and C. B) Individual channels showing bright field, CellTracker™ Red (K562), CellTracker™ Green (NK cells) and Sytox™ Deep Red (dead cells) in that order. C) Automatic segmentation of the fluorescent channels by the MMV_H4Tracks napari plugin, trained on manually segmented data. Done in cooperation with Justin Sonneck.

At the moment the automated segmentation still needs more training data and iteration to improve performance. Shifts in signal strength need a more robust model to still enable good predictions. Bleaching or leaking of fluorophore reduces signal strength

during the videos. Slight differences in staining efficiency leads to intensity differences between assays.
When the segmentation works to a satisfying degree the next step is tracking. When tracking works, the tracks can be analyzed. When an NK cell track overlaps with a target cell track this can be counted as a contact. Next the contacts need to be filtered for duration, if the overlap is only for 1 frame it could be an NK cell just passing. Passing by would mean no real contact with formation of an IS and probing of the target cell. For each contact the dead cell track needs to be checked if a new dead cell track starts during the contact at that position. If the dead cell track was persistent before the contact was formed, the NK cell made contact to an already dead target cell. The end result will be for each tracked NK cell a list of contacts, their duration and if it led to the death of a target cell. This finally needs to be filtered for multiple NK cells being present for the same death. In that case it is unclear if one or more NK cells were involved in the kill, so all involved NK cells need to be removed from the analysis. Advances in machine learning and image recognition software further help the development of such automated serial killing analysis. Potentially also allowing label free identification and separation of effector and target cells, further reducing stress on the cells and making the setup more physiological.
One not so easily overcome problem is the limited ability to phenotype the NK cells in a microscopy setup. NK cells could be sorted beforehand and then their killing analyzed but this only allows for a limited number of conditions in an experiment and analyzing NK cells after the serial killing would require specialized equipment for cell picking. These shortcomings in phenotyping could be easily overcome when the serial killing readout could be done with flow cytometry. High dimensional flow cytometry can be used for deep phenotyping of cells and can easily be adapted for cell sorting to isolate cell populations of interest. To gains these benefits, we tried to establish a flow cytometry-based assay to assess serial killing ability of NK cells.

5.2 CD107a surface retainment and antibody binding

When NK cells kill a target cell, they can do this in two different ways. Via death cell receptor mediated signaling or the release of granzymes and perforin from lytic granules, called degranulation. Earlier microscopy based studies have shown that serial killing is performed prominently via the second pathway.[86] During degranulation the lytic granules fuse to the NK cell membrane leaving proteins from the vesicle membrane exposed at the cell surface. One of these proteins is CD107a. It is commonly used as marker for NK cell degranulation and thereby a proxy for activation and the attempt to kill.[82] Most current protocols only measure if degranulation occurred at all, some also inhibit endocytosis not to lose surface expression again. To establish a flow cytometry-based assay able to detect serial killing NK cells we want to measure serial degranulation of NK cells. This will be done by sequentially staining for CD107a in different colors to differentiate between the stainings. The idea to use multiple CD107a stainings is not new, and a protocol utilizing 2 differently labeled anti-CD107a-antibodies and incubation times of 2 hours and 4 hours has been published.[125] We want to increase the differentiation to 3 staining instances and optimize the timeframe to fit better to serial killing timelines of under 1 hour as they are observed in microscopy.[86] Important for the setup is how long CD107a stays on the NK cell surface after degranulation.

To test this, we cocultured NK cells with adherent HeLa target cells and then removed the NK cells from the target cells. NK cells were stained for CD107a at different times after target cell removal (Figure 2 A).

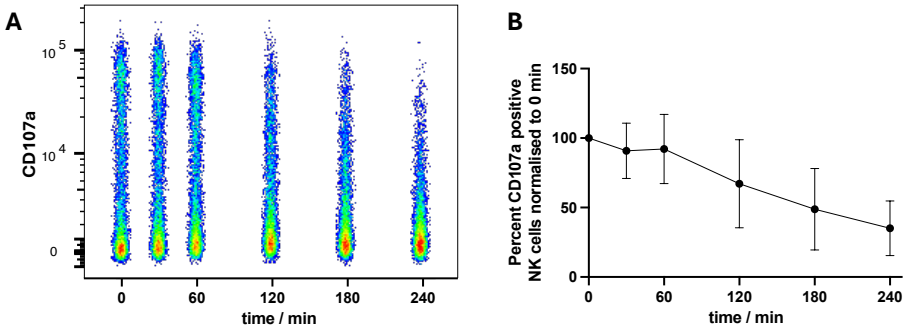

Figure 2: CD107a surface retainment

A)[123] Representative pseudo-color-dot-plot showing CD107a expression on NK cells at different time points after removal from stimulus (HeLa cells). B)[123] Percent of NK cells positive for CD107a at different time points after removal of stimulus normalized to time point 0. Shown is mean with standard deviation (SD); n = 3

For the first hour the amount of surface CD107a stayed stable before declining (Figure 2 B). Afterwards surface expression declined but was still detectable on about half the degranulated cells after 4 hours. For stainings of CD107a up to 1 hour after degranulation endocytosis does not need to be inhibited.

The planned assay should detect when during the assay the NK cell degranulated, not only if it did at all. To achieve this CD107a will be stained at multiple time points with

antibodies linked to different fluorophores. Incomplete staining during the first step could leave CD107a from this step exposed to be stained with the antibody used for the second step. To test this carryover, NK cells were co-incubated with K562 target cells and then either left unlabeled in the first step, stained for CD107a with standard staining concentrations of fluorophore-linked antibody or blocked with a tenfold concentration of unlabeled anti-CD107a-antibody of the same clone for 5 minutes. Directly following that the NK cells were stained with the same clone anti-CD107a-antibody labeled with a different fluorophore (Figure 3 A).

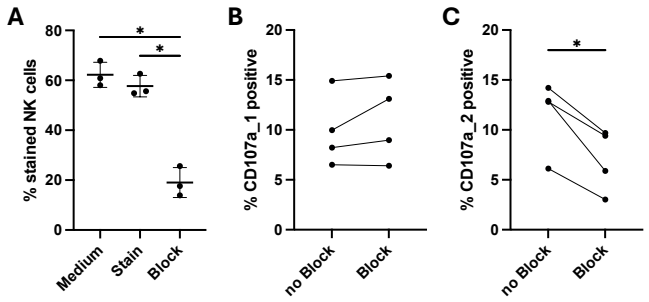

Figure 3: Effect of receptor-blocking on CD107a staining
A)[123] Percent of NK cells co-incubated with K562 cells that can be stained for CD107a after treatment of NK cells with nothing (medium), normal (stain) amounts of anti-CD107a-antibody or high (block) amounts of anti-CD107a antibody. B, C)[123] Percent of NK cells positive for the first CD107a staining (B) and the second CD107a staining (C) with or without the addition of blocking antibody after the first CD107a staining during a serial degranulation assay.
A: Shown is mean with SD. RM 1-way ANOVA with Geisser-Greenhouse correction, Tukey's multiple comparison. n = 3; C: paired t-test. n = 4; *p ≤ 0.05

Staining with standard conditions for 5 minutes before staining again resulted in almost the same level of CD107a positive NK cells in the second staining as just leaving them in the medium. However, the tenfold concentrated blocking condition resulted in a reduction of positive NK cells from around 60 % down to on average 20 % (Figure 3 A). Blocking in this way did not completely eliminate carryover staining but drastically reduced it.

To see if a previous CD107a staining would be affected by a blocking step following it, a blocking step was tested between two CD107a staining steps. The used target cells are suspension cells, they stay with the NK cells throughout all staining steps. NK cells were co-incubated with K562 target cells, stained for CD107a a first time (CD107a_1), treated with blocking antibody or not and after a second incubation period stained for CD107a again (CD107a_2) using differently labeled antibody (Figure 3 B and C). The CD107a staining preceding the blocking step was not affected by it while the second staining was reduced. This indicates the added blocking step reduces false carryover staining and therefore should be included in the final protocol.

5.3 Establishment of serial degranulation assay

Since serial killing has been described for up to 6 and more sequential kills depending on the target, one more staining was added to the serial degranulation assay.[85] 3 degranulation events, better cover the range of NK cell activity. The 3 individual CD107a stainings each represent degranulation in a different time frame, adding temporal resolution (Figure 4 A). 3 stainings give rise to 8 possible combinations of when an NK cell degranulates (Figure 4 B). These 8 outcomes can be condensed again either according to the number of degranulations (count: 3, 2, 1, 0) or when the NK cell first degranulated (timing: early, late, never).

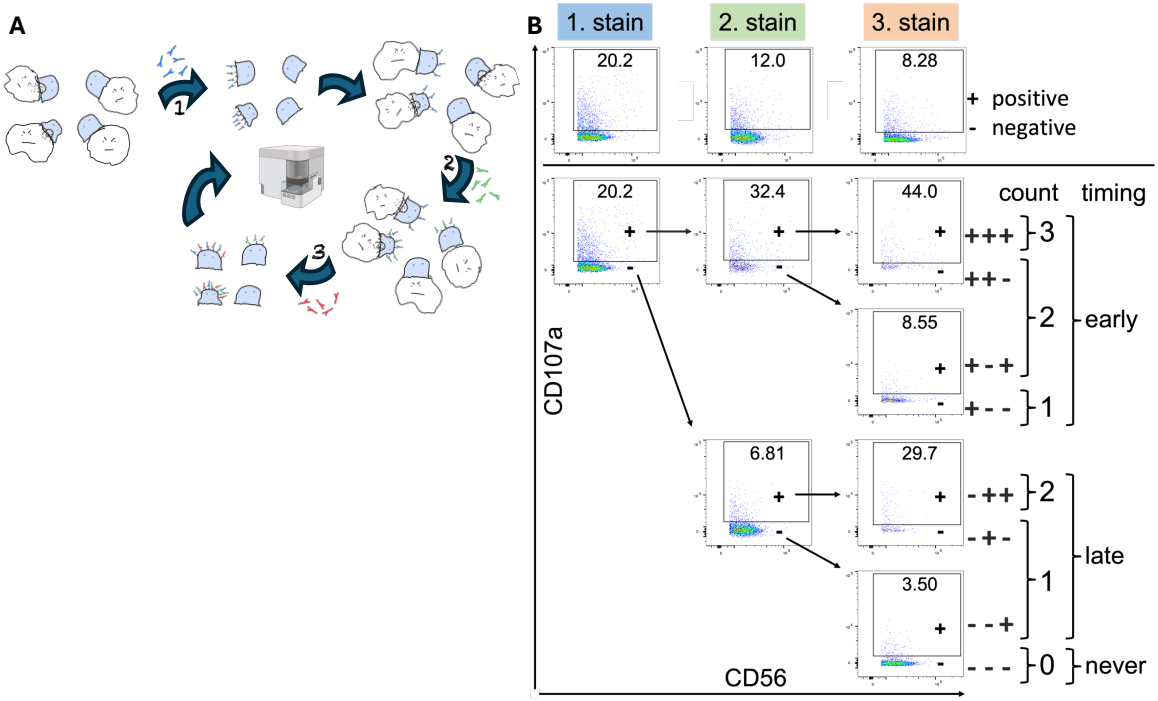

Figure 4: General gating strategy of serial degranulation assay
A)[123] Cartoon depiction of the serial degranulation assay. Flow cytometer from BioRender© B)[123] Gating (general gating on NK cells in Supplementary Figure 1). Top: All NK cells positive for each of the 3 CD107a stains are shown. Bottom: Sequential gating on CD107a positive and negative NK cells for each degranulation time range. Only the NK cells gated on are shown in the following plot indicated by arrows. 3 time points of CD107a staining give rise to 8 different staining outcomes. These can be condensed together according to the count or timing of degranulation.

Serial killing has been shown to happen fast compared to NK cells killing only a single time but the timeline is not strict and varies widely from NK cell to NK cell.[86] Different lengths of coincubation before going to the next staining step were tested to optimes for serial degranulation. CD107a is stable on the surface for about an hour (Figure 2 B). 50, 30 and 10 minutes of incubation per staining step were tested (Figure 5 A). The assay was setup in a way that the end point of the second incubation period from a short incubation time will be at the same time as the end of the first incubation period of a longer incubation time (Figure 5A, dashed lines). The same is true for the end of the third incubation period. This allows to test if the same amount of NK cells degranulate during multiple short and during one long incubation time. In this setup

the anti-CD107a-antibody was present for the whole duration of coincubation with K562 target cells.

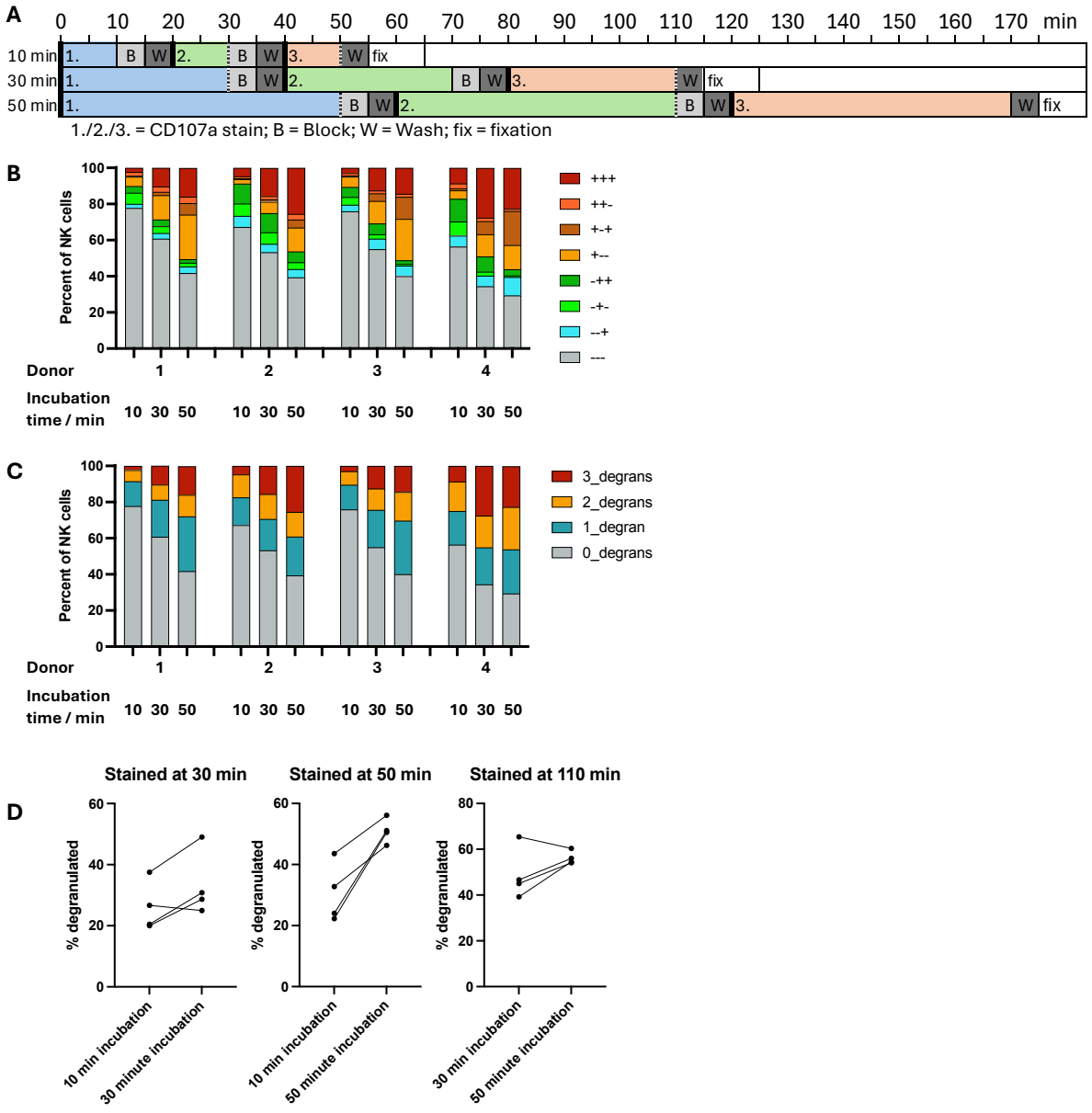

Figure 5: Comparison of incubation times for serial degranulation assay
A) Experimental setup testing multiple incubation times in parallel. Assay was performed with PBMCs and K562 as target cells. B, C) Percent of NK cells in the different degranulation groups (B) or condensed according to the degranulation count (C) shown for the 3 tested incubation times and 4 different doors. D) Percent overall degranulated NK cells at 30, 50 or 110 minutes after the start of the assay. At each of this time points two incubation time setups have concluded a staining step, depicted as dashed line in A.

Different incubation times were tested with 4 different donors (Figure 5 B and combined for count Figure 5 C). The overall fraction of degranulating NK cells increases with longer incubation times. The fraction of triple degranulating NK cells increases from 10 to 30 minutes of incubation. This increase continues for the longest incubation time for the first two donors, but the third donor shows little increase while the 4th donor even shows a reduction in triple degranulated cells. The reduction is

probably due to NK cells degranulating multiple times during the first incubation time and then no more during the later ones. This fits to the observation that the fraction of NK cells degranulating only in the beginning increases with increasing incubation time. The assay for all incubation times was performed in parallel and timed in a way that multiple shorter incubation times end at the same time as fewer longer ones (dashed lines Figure 5 A). The idea was that after the same total experimental runtime the same amount of NK cells would degranulate regardless of incubation time length. By that the shorter incubation times could be used to dissect the longer incubation times where NK cells could degranulate multiple times. However, the longer incubation times tend to result in more overall degranulation after the same amount of total experiment time (Figure 5 D). This effect is most prominent at 50 minutes of total experimental runtime.

Since information from different incubation times cannot be directly integrated with each other and longer incubation times reduce the resolution on serial degranulation a single short incubation time was chosen for the assay. To focus in on NK cells that have a high amount of CD107a on the surface the staining time was reduced to 5 minutes instead of the complete time of incubation. However, these 5 minutes of staining time were added to the end of the incubation time, for a total of 15 minutes. At the start of each incubation time the NK and target cells are spun down to directly enable contact formation. The first two staining step are followed by blocking with high concentration of unlabeled antibody. The finalized protocol has been published and is depicted in Figure 6.[123]

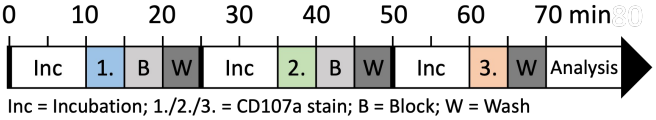

Figure 6: Experimental setup of serial degranulation assay

[123]Timeline of serial degranulation assay. Effector and target cells are put together at time point 0. Bold lines at the start of incubation periods indicate spin down of cells to directly enable contact formation. Cells are resuspended vigorously to break up contacts when staining or blocking antibody is added. Washing step includes spin down and removal of supernatant followed by resuspension.

The protocol uses suspension cells as target cells and is not limited to the use of only K562. The suspension cell lines 721.221 (B cell) and Jurkat (T cell) which are also susceptible to NK cell killing were tested (Figure 7). K562 cells show the highest activation of NK cells as measured by this degranulation assay. Notably there is donor variance in how good a given target cell activates the NK cells. Donor 1 (D1) shows comparable levels of degranulation for 721.221 and Jurkat cells but lower levels for K562 cells compared to the other donors. Donor 4 (D4) shows average degranulation against Jurkat and K562 cells but is less activated by 721.221 cells.

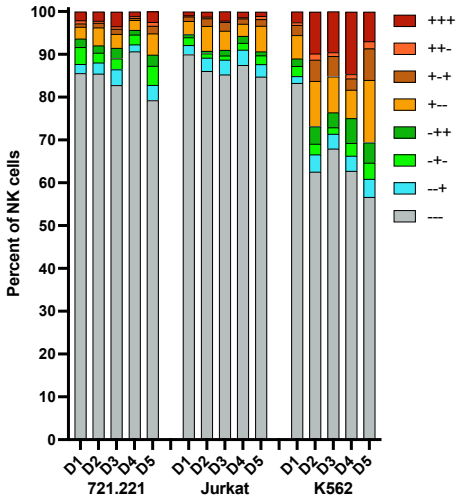

Figure 7: Serial degranulation assay with different target cell lines
[123]Serial degranulation assay was performed with PBMCs of 5 donors and either 721.221 cells, Jurkat cells or K562 cells as target cells. Shown is the percentage of NK cells in the different degranulation groups. Done in cooperation with Vanna Imširović.

K562 cells gave the highest overall degranulation and the most triple degranulating NK cells. The following experiments were all carried out following this optimized protocol against K562 target cells.

5.4 Validation of serial degranulation assay

Having already seen differences between donors a next step was to compare donor variance to interexperimental variance and test the robustness of the assay. PBMCs of 5 donors were aliquoted and cryo-preserved. All 5 donors were tested in a serial degranulation assay in parallel. This was done 3 times at different days using cells from a freshly thawed aliquot each time.

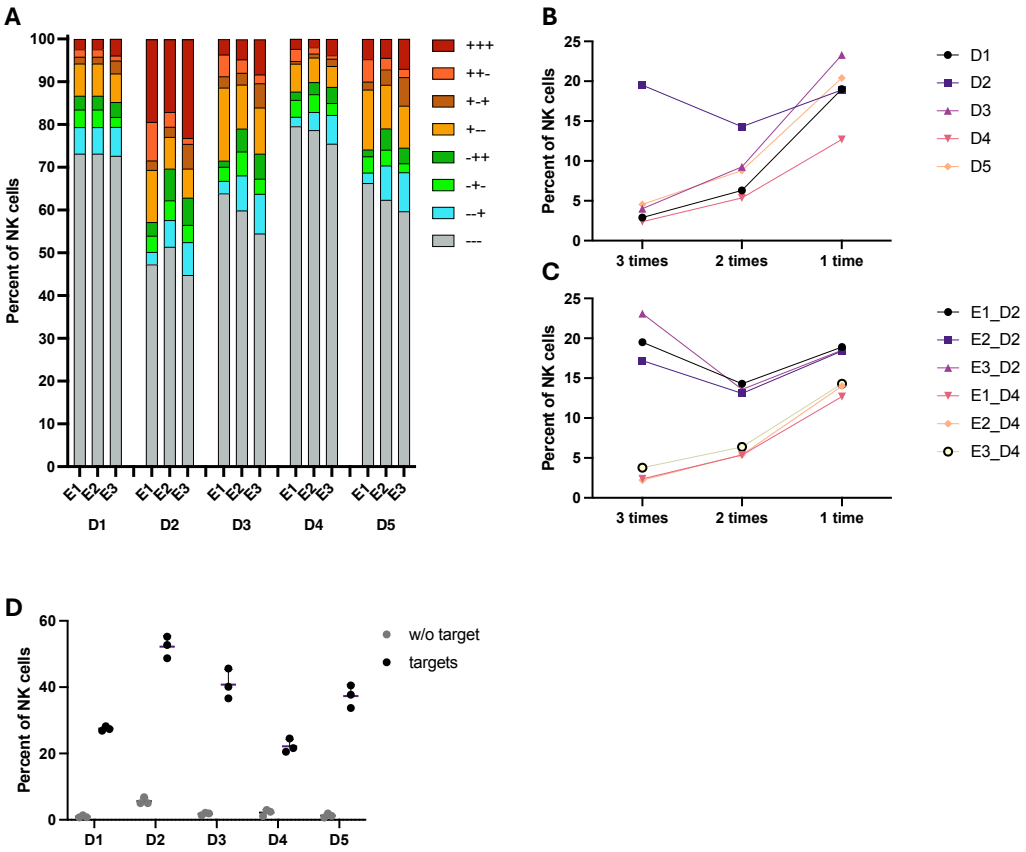

Figure 8: Inter- and intra-experimental variance of serial degranulation assay
PBMCs of 5 donors were frozen in aliquots. The serial degranulation assay was performed 3 times with all donors in parallel using K562 as target cells and PBMCs from a freshly thawed aliquot. A)[123] Percent of NK cells in the different degranulation groups for each donor per experiment. B)[123] Intra-experimental biological variance between 5 donors in one experiment. C)[123] Inter-experimental technical variance between repeats of the experiments for 2 donors. D)[123] Percent of overall degranulating NK cells of all 3 repeats with or without (w/o) the addition of target cell.

As expected, differences between the donors could be observed with donor 2 (D2) being especially active against the K562 cells (Figure 8 A, B and Supplementary Figure 2). Between repeats of the experiment the performance of the donor stayed constant (Figure 8 A, C and Supplementary Figure 2). Activation of the NK cells was induced by the presence of the target cells and not induced through constant pipetting during the assay itself. This was confirmed in the condition without target cells (Figure 8 D). Only donor 2 showed a low level of baseline degranulation further arguing for a donor with very active NK cells. Figure 8 D shows overall degranulation, the positive signal for donor 2 in the no target condition is nearly solely from the first staining (data

not shown). This further indicates that the assay itself does not induce NK cell activation and each staining plus blocking step binds the present CD107a nearly completely.

Bystander cells have been shown to affect the killing behavior of NK cells. This can happen through their physical presence alone but also through released cytokines acting on the NK cells.[126] Isolated NK cells or whole PBMCs of the same donors were used in a serial degranulation assay to test the bystander effect. When cultivating NK cells over long periods or for clinical application they are often cultured in the presence of activating cytokines like IL-2 and IL-15 or feeder cells.[127] NK cells pre-activated through co-culture with K562-mbIL-15-mbIL-21-41BBL and IL-2 were also tested in the assay.

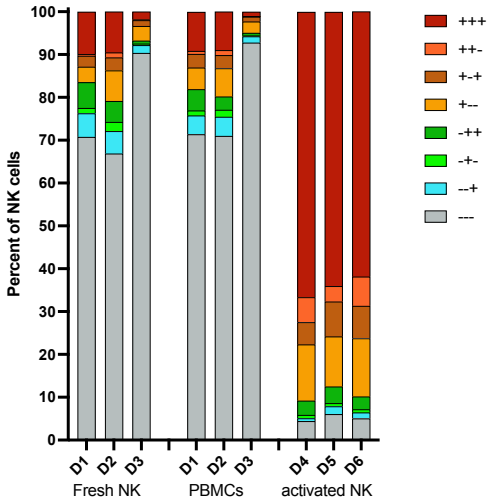

Figure 9: Effect of NK cell activation on serial degranulation assay
[123]Serial degranulation assay was performed with K562 cells as target cells. Freshly isolated NK cells, PBMCs of the same donor or pre-activated NK cells were used in a serial degranulation assay. Shown are percent of NK cells in the different degranulation groups.

Fresh NK cells showed no difference compared to their whole PBMCs and retained their donor differences (Figure 9). Presence of bystander cells seems to have little effect in this assay setup. Therefore, if possible PBMCs were used in the following assays to save time and resources. Activated NK cells showed highly increased levels of overall degranulation and triple degranulation in particular. Since only 3 donors were tested and different donors were tested between the fresh and activated condition, no information if the activation scheme removes donor variance was gained.

NK cells undergo changes in protein surface expression once activated. We used proteins with known changes to further validate our setup. CD69 expression is induced and increases on activated NK cells.[43] CD16 which can itself activate NK cells in the context of ADCC, is cleaved off by metalloproteases like, a disintegrin and metalloprotease-17 (ADAM-17) upon activation.[108] To measure surface levels of these proteins a surface staining was done on washed NK cells directly after the third

CD107a staining of the serial degranulation assay. This was assessed in the three repeats of the 5 donors shown before in figure 8.

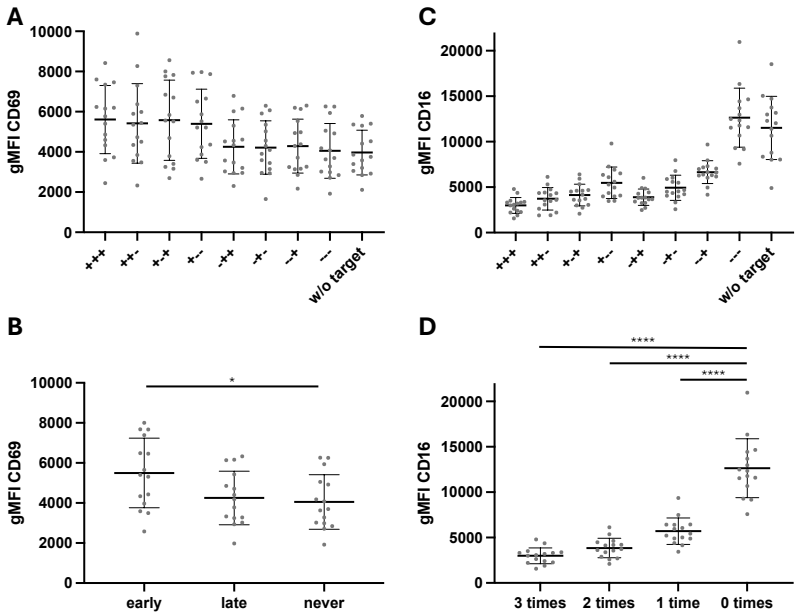

Figure 10: Effect of serial degranulation on CD69 and CD16 expression
Serial degranulation assay was done with PBMCs and K562 target cells. A, B)[123] CD69 expression for all degranulation groups (A) or pooled according to degranulation timing (B). C, D)[123] CD16 expression for all degranulation groups (C) or pooled according to degranulation count (D).
Shown is mean and SD. Statistics were performed for B and D. Nested 1 way ANOVA with multiple comparison; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; Data pooled from the experiments depicted in Figure 8; 5 donors and 3 repeats.

As expected CD69 expression increased on activated NK cells (Figure 10 A). However, only in the NK cells that degranulated early, this increase was significant when comparing them to the non-degranulated ones (Figure 10 B). The total runtime of the assay only enables NK cells activated early to upregulate CD69. For setups with longer incubation time an upregulation of CD69 was also observed in NK cells degranulating at a later time point (data not shown). The cleavage of CD16 on the other hand is a fast process. The levels of CD16 decrease on activated NK cells as expected. However, in contrast to the CD69 upregulation, a pattern based on number of degranulations emerges for CD16 (Figure 10 C). This nicely shows that with increasing numbers of degranulation events CD16 is cleaved off repeatedly, thereby providing additional evidence that multiple degranulation stainings in fact represent multiple activation events of NK cells and not carryover from earlier activations.

5.5 Phenotype stability of serial degranulating NK cells

Serial killing has been observed for a fraction of NK cells, it is however not known if this is a stable phenotype. To address this, a serial degranulation assay was performed and the NK cells subsequently sorted according to their degranulation status. Triple degranulated NK cells, cells that degranulated only in the beginning, those that degranulated only at the end and NK cells that did not degranulate at all were rested for 2 days and then again used in a serial degranulation assay (Figure 11 A and Supplementary Figure 3).

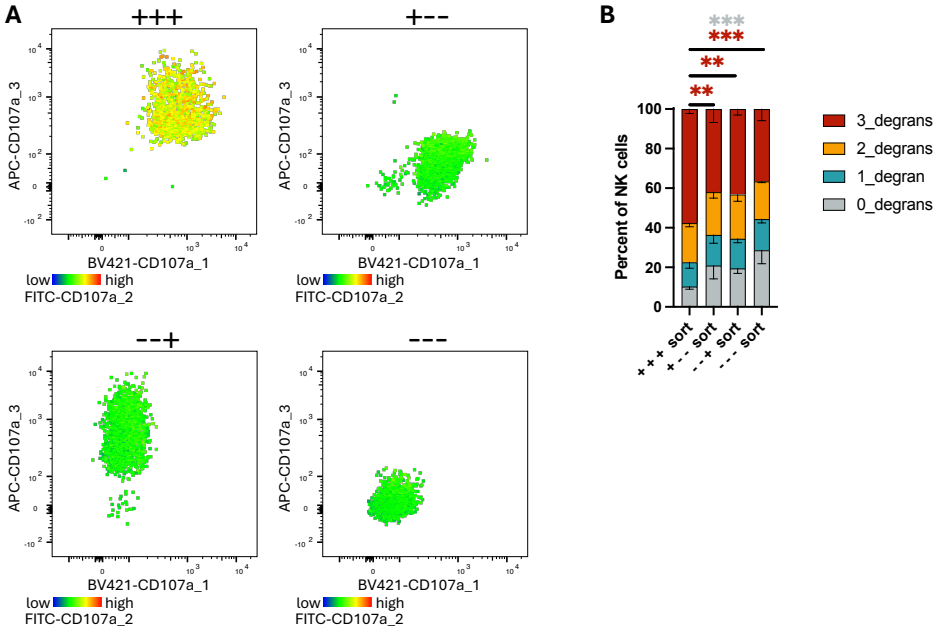

Figure 11: Retainment of degranulation phenotype
A) A Serial degranulation assay was performed and NK cells sorted as triple degranulated (+++), degranulated only in the beginning (+- -), degranulated only at the end (- -+) or not degranulated (- - -). Shown are representative dot plots of NK cells measured again directly after sorting. B) After resting 2 days NK cells were again used in a serial degranulation assay. Shown are percent of NK cells according to groups based on count of degranulations.
A: Done in cooperation with Maren Claus; B: Shown is mean and SD. RM 2way ANOVA with Geisser-Greenhouse correction, Tukey's multiple comparison; n = 3

NK cells that initially degranulated three times showed the highest overall degranulation in the re-challenge (Figure 11 B). They also have the highest amount of triple degranulating cells. Target cell contact can act as a sort of pre-activation, priming NK cells for the next encounter.[128] Since triple degranulated NK cells also performed better than NK cells which degranulated once, one could argue either that they were pre-activated more strongly or that in fact the serial degranulation phenotype is stable to some degree. Of note however not all triple degranulated NK cells degranulated three times in the re-challenge, some not at all. This indicates that some other cellular state could be important for activation, not just the ability to recognize the target cells. This is supported by a noticeable fraction of previously non-degranulated NK cells degranulating in the re-challenge. The overall degranulation in the re-challenge is

increased compared to the pre-sorting assay outcome (data not shown). This can be due to stimulation by the cytokines which are needed to ensure survival during the 2 day resting period.
The assay gives hints that serial degranulation could be a stable phenotype but to validate this further testing will be needed, somehow uncoupling it from stronger pre-activation.

5.6 Impact of NK cell maturity on serial degranulation

During maturation NK cells undergo changes in protein expression and functionality. The most common distinction is between CD56^{bright} and CD56^{dim} NK cells. The CD56^{bright} NK cells reflect an earlier state associated with more cytokine release while the CD56^{dim} NK cells are more mature and are shown to be more involved in the cytotoxic effector function.[7, 8] Our assay was used to asses if these differences are also reflected in the ability of CD56^{bright} and CD56^{dim} NK cells to serially degranulate.

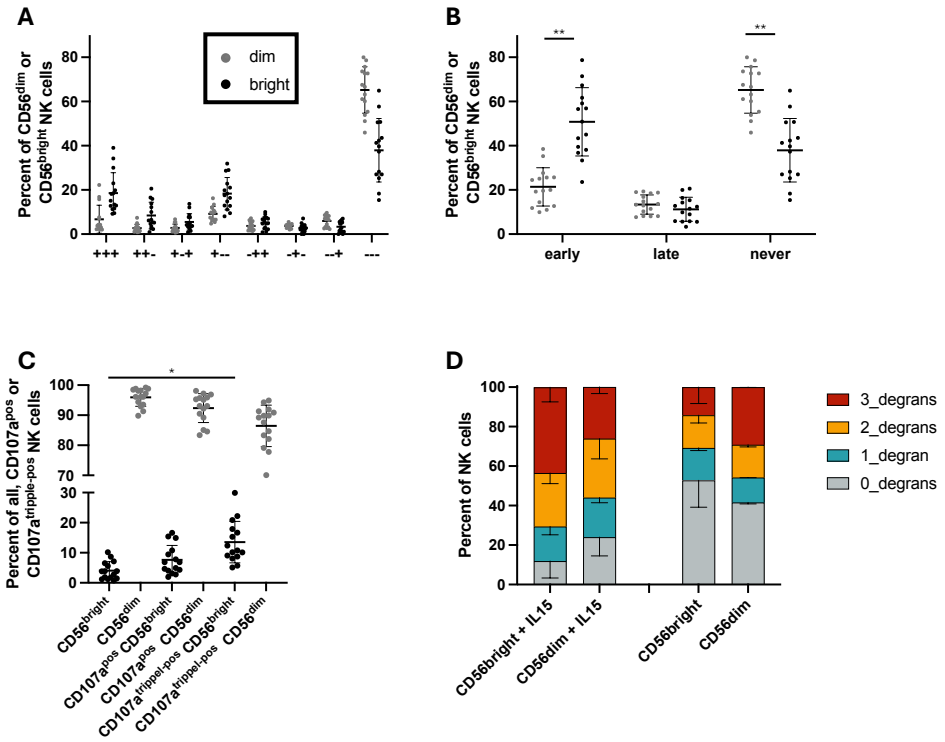

Figure 12: Performance of CD56^{bright} and CD56^{dim} NK cells in serial degranulation assay
A – C)[123] Serial degranulation assay was done with PBMCs and K562 target cells. Shown is the distribution of CD56^{bright} or CD56^{dim} NK cell in the different degranulation groups (A), in groups based on degranulation count (B) or the percentage of CD56^{bright} and CD56^{dim} NK cell in all NK cells, NK cells that did degranulate or triple degranulated NK cells (C). D) Serial degranulation assay was performed with K562 target cells and NK cells cultured overnight with or without added IL-15. Shown is percentage of NK cells in degranulation groups condensed by count for CD56^{bright} and CD56^{dim} NK cells, respectively.
Shown is mean and SD. Statistics were performed for B and C, Nested 1way ANOVA with multiple comparison; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 ****p ≤ 0.0001; A-C: Data pooled from the experiments depicted in Figure 8a. 5 donors and 3 repeats; D: n = 2. Done in cooperation with Giovanna Perinetti Casoni.

$CD56^{bright}$ NK cells show more serial degranulation (Figure 12 A). This can be attributed to a faster overall response time with more $CD56^{bright}$ cells also being in the +-- group while the --+ group is bigger in the $CD56^{dim}$ fraction. Combining the groups according to timing reflects this, showing significantly more early and less non-degranulating NK cells in the $CD56^{bright}$ NK cells (Figure 12 B). However, since the majority of the NK cells are $CD56^{dim}$, they still make up the bulk of triple degranulating NK cells, even though the $CD56^{bright}$ fraction is significantly enriched in the triple degranulated NK cells (Figure 12 C). This seems to go against the described functions of these NK cell subgroups, but since only degranulation is measured one cannot infer if the degranulation of $CD56^{bright}$ NK cells leads to target cell death in the same capacity as for $CD56^{dim}$ NK cells. $CD56^{bright}$ and $CD56^{dim}$ cells have different responses to cytokine stimulation. During the cultivation of the NK cells overnight IL-15 was present. $CD56^{bright}$ NK cells show a stronger IL-15 response compared to $CD56^{dim}$ NK cells.[129] Cultivating NK cells without IL-15 reduced overall degranulation with a stronger decrease in $CD56^{bright}$ NK cell activity (Figure 12 D), resulting in more activity in $CD56^{dim}$ NK cells compared to $CD56^{bright}$ NK cells.

Differences in the ability of $CD56^{bright}$ and $CD56^{dim}$ NK cells to serially degranulate were found and are linked to the presence of IL-15 which stimulates $CD56^{bright}$ NK cells more strongly.

5.7 Impact of NK cell education on serial degranulation

Education is another mechanism affecting NK cell activity. NK cells that continuously receive inhibitory signals get educated and are in turn more active when they do get activated.[52, 130] We investigated the effect of NK cell education on serial degranulation. The KIRs KIR2DL1 and KIR2DL3 can educate NK cells through interaction with their ligands HLA-C2 and HLA-C1 respectively. Since humans can have two different alleles of HLA-C either only one or both KIR2DL variants are educating. So, it is dependent on the genotype of the donor if a KIR is educating or not. To observe differences in education between KIR2DL1 and KIR2DL3, other sources of education need to be excluded, so the analysis focused in on NK cells negative for: NKG2A (education through HLA-E)[131], CD57 (maturity)[10], KIR3DL1 (education through Bw4)[132] and KIR2DS1 (activating KIR counteracting education through KIR2DL1)[55] (Supplementary Figure 4 A). HLA-C educated NK cells have a higher granzyme B content than uneducated ones (Supplementary Figure 4 B).[53] This was used to infer the HLA-C allotype of the donors.

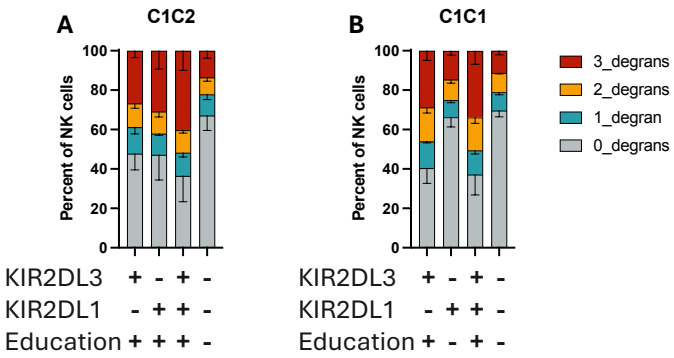

Figure 13: Effect of NK cell education on serial degranulation assay
Serial degranulation assay was performed with fresh NK cells and K562 cells as target cells. NK cells were gated to be only educated by either KIR2DL3 or KIR2DL1 (Supplementary Figure 4 A). Shown are results according to degranulation count. A) Donors that educate NK cells through KIR2DL3 (HLA-C1) and KIR2DL1 (HLA-C2). B) Donors that educate NK cells only through KIR2DL3 (HLA-C1).
Shown is mean and SD. A: n = 4; B: n = 3; A, B: Statistic showing significances is depicted in Supplementary Figure 4 C for clearer visualization. Done in cooperation with Giovanna Perinetti Casoni.

When both KIR2DL1 and KIR2DL3 are educating, more triple and overall degranulated NK cells can be found in subsets expressing either of the two compared to NK cells expressing none (Figure 13 A). NK cells expressing both KIRs seem to perform slightly better than the ones with a single educating KIR. For donors where only KIR2DL3 is educating (C1C1), NK cells expressing this KIR show more activity than the ones which lack KIR2DL3 regardless of KIR2DL1 expression (Figure 13 B).
This demonstrates that education affects NK cells ability to serially degranulate.

5.8 Perforin and Granzyme B loss during serial degranulation

Perforin and granzyme B are some of the most prominent effector molecules being released during NK cell degranulation. Since they are expelled from the NK cell during degranulation their level inside the cell decreases. The serial degranulation was used to assess perforin and granzyme B loss on a per degranulation basis.

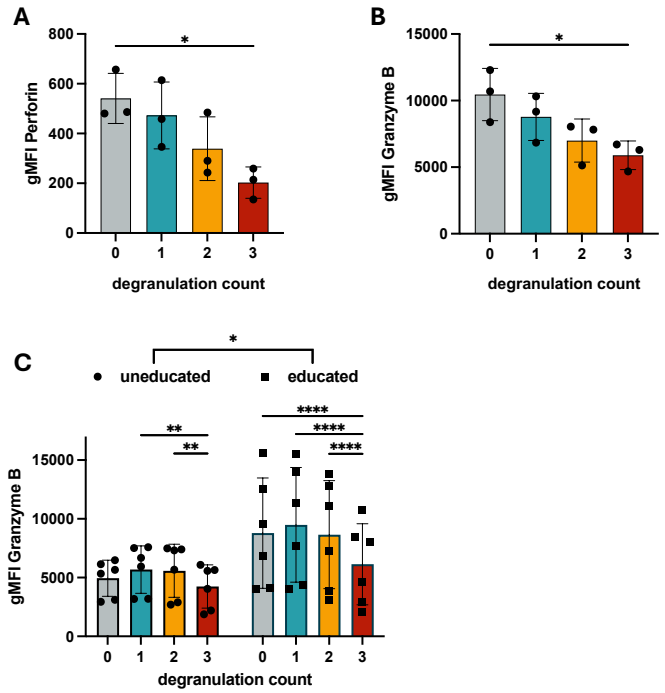

Figure 14: Perforin and Granzyme B loss over subsequent degranulations
A, B) Perforin (A) and Granzyme B (B) geometric-mean fluorescence intensity (gMFI) was measured in NK cells after serial degranulation assay. Assay was performed with PBMCs and K562 as target cells C) Granzyme B gMFI was measured in NK cells of known KIR2DL3 / KIR2DL1 education status, after serial degranulation assay. Assay was performed with fresh NK cells and K562 target cells.
Shown is mean and SD. A, B: Friedman test with Dunn's multiple comparison, n = 3; C: RM 2way-ANOVA with Šídák's multiple comparison n = 6, Done in cooperation with Giovanna Perinetti Casoni; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

Perforin shows a significant decrease from non to triple degranulated cells (Figure 14 A) with 1 and 2 degranulations showing the gradual decrease of perforin levels. This fits the model of repeated release of granules and thereby loss of perforin. The same can be observed for granzyme B (Figure 14 B).
To further assess the effect of education on NK cell serial degranulation the same education setup as before was used, comparing HLA-C educated vs. uneducated NK cells. In the education setup general higher levels of granzyme B can be detected in the educated NK cells. The decrease in granzyme B levels with subsequent degranulations can be observed for both groups (Figure 14 C). Some of the triple degranulated educated NK cells have more granzyme B than the less degranulated uneducated ones. It is plausible that these cells could continue degranulating.

5.9 Bulk RNA sequencing of triple- and non-degranulated NK cells

To get a better overview of what affects serial degranulation of NK cells, an RNA sequencing assay was set up. NK cells were put through a serial degranulation assay and subsequently sorted as either non or triple degranulated (Supplementary Figure 3). RNA was isolated from the sorted cells and used for bulk RNA sequencing. The volcano plot shows differentially expressed genes between the two groups (Figure 15).

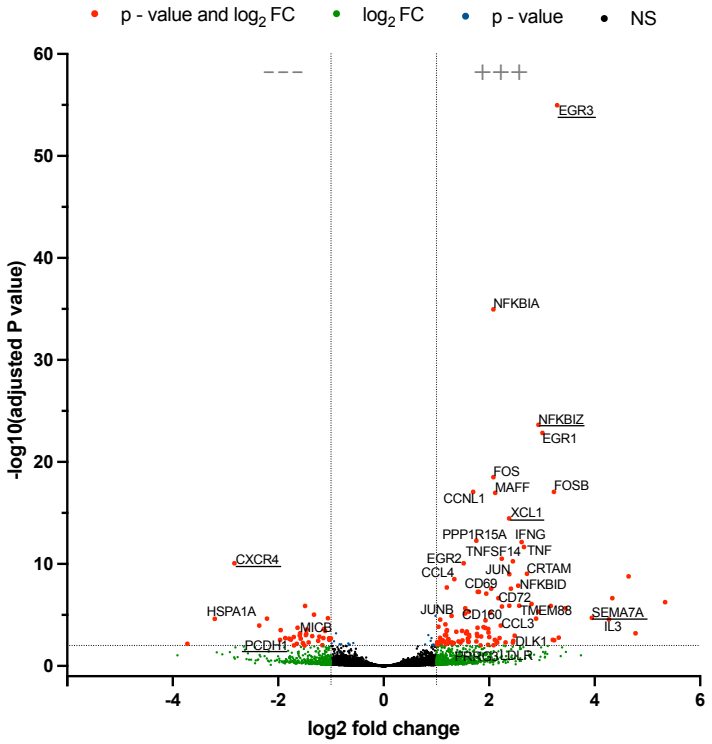

Figure 15: Transcriptional distinction of triple degranulated NK cells
Triple and non-degranulated NK cells were sorted after serial degranulation assay. Bulk-RNA-sequencing results depicted as a volcano plot, triple degranulated vs non degranulated NK cells. Underlined transcripts were further investigated. Done in cooperation with Bettina Budeus.

Comparing transcriptomes of triple to non-degranulated NK cells reveals, that most differentially expressed genes are upregulated and a smaller amount shows downregulation (Figure 15). The best outcome would be the identification of surface markers for serial degranulating NK cells. To this end the follow-up analysis focused on hits that are highly up- or downregulated and whose proteins are described to be present on the cell surface. A serial degranulation assay with subsequent surface staining of PCDH1, SEMA7A (CD108), CXCR4, CD355, CD72 and LDLR was performed. Protocadherin 1 (PCDH1) is involved in cell adhesion in various cell types and tissues.[133] Semaphorin 7A (CD108) has immunomodulatory function linked to inflammation.[134] C-X-C motive chemokine receptor 4 (CXCR4) is involved in chemotaxis.[135] Cytotoxic and regulatory T cell molecule (CRTAM / CD355) is involved

in epithelial adhesion and T cell activity.[136, 137] CD72 is described for its regulatory role in B cell activity.[138] The low density lipoprotein receptor (LDLR) facilitates the uptake of low density lipoproteins into the cell.[139]

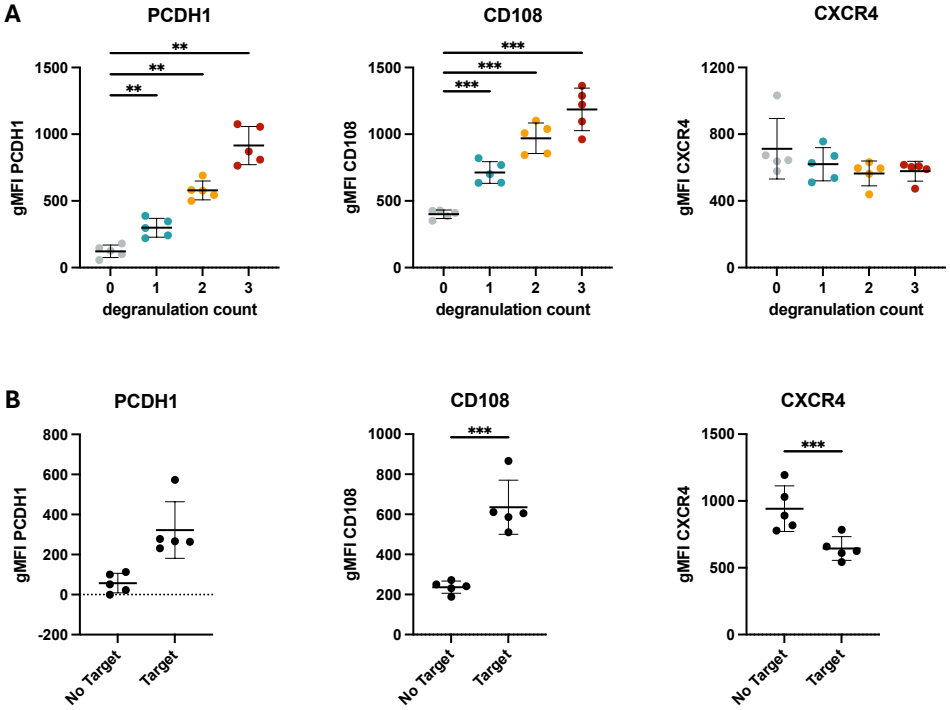

Figure 16: Flow cytometry-based analysis of activation induced phenotypic changes
A, B) Serial degranulation assay was done with PBMCs and K562 target cells and proteins of interest stained. Shown is expression in NK cells that degranulated different amounts of times (A) or comparing between all NK cells with or without target cells present (B).
A: RM one-way ANOVA, with Geisser-Greenhouse correction; B: Ratio paired t-test. For PCDH1: Wilcoxon matched pairs signed rank test; n = 5; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

Surface levels of Pref-1, CD355, CD72 and LDLR were not detectable and were not further investigated (not shown). Surface levels of PCDH1 and CD108 significantly increased on the NK cells with increasing number of degranulations, this nicely reflects the higher levels of mRNA found in triple degranulating NK cells (Figure 16 A, Figure 15). While changes of CXCR4 are not statistically significant a trend to lower expression on NK cells that degranulated more often can be observed. This also fits to the observed lower mRNA levels. If no active process is involved in removing a protein from the surface, a reduction in surface protein level can take a longer time following reduction in mRNA levels compared to an increase. However, when looking at the protein expression difference between bulk NK cells with and ones without target cell contact, an effect in the same direction as the change in mRNA levels can be observed: A trend of upregulation for PCDH1, and significant up- and downregulation for CD108 and CXCR4, respectively (Figure 16 B). This shows that the observed differences in protein and mRNA expression are activation-induced. By that, they are not excluded from being involved in activating the NK cells, but it makes it less likely they are the underlying reason for an NK cell to be a serial degranulator. Also, in order

to be useful to predict serial killing behavior, the marker must be expressed differentially before the NK cell is activated.
Serial degranulation does not necessarily need to be predictable by a single marker. To see if serial degranulating NK cells differentially use certain pathways, we performed gene set enrichment analysis (GSEA). The GSEA did not produce insights into underlying mechanisms explaining serial degranulation (data not shown). Instead, to validate the enriched gene sets the top 3 driver genes of the enriched gene sets were analyzed via qPCR. NK cells were sorted after a serial degranulation assay as before and qPCR performed on the isolated RNA for EGR3, XCL1 and NFKBIZ in addition to housekeeping genes. Early growth response 3 (EGR3) is a transcription factor described to be involved in the functionality of tumor infiltrating lymphocytes.[140] XCL1 (lymphotactin) is a chemokine, its receptor CXR1 is expressed on dendritic cells.[141] NF-ϰB inhibitor zeta is located in the nucleulus where it regulates the activity of NF-ϰB.[142] Besides triple and non-degranulated cells, bulk NK cells with and without target cell presence were analyzed.

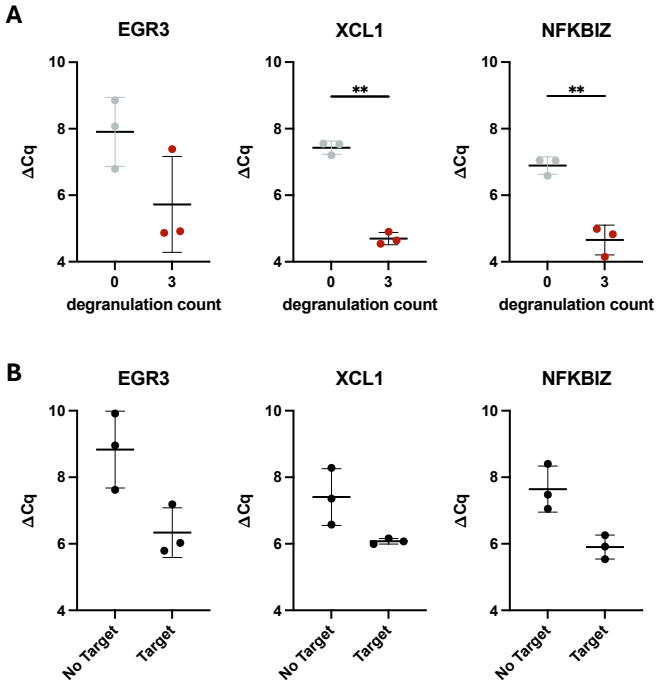

Figure 17: qPCR-based analysis of activation induced phenotypic changes
A, B) Serial degranulation assay was done with PBMCs and K562 target cells and triple and non-degranulated NK cells as well as bulk NK cells sorted. Shown is ΔCq value compared to housekeeping genes determined by qPCR in NK cells that degranulated different amounts of times (A) or comparing between all NK cells with or without target cells present (B).
Paired t-test; n = 3 (average of 2 technical repeats); Done in cooperation with Mina Sandusky; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

Depicted are the delta Cq values, so higher values correspond to more doubling cycles, meaning lower levels of mRNA were present (Figure 17). The main drivers for enriched pathways showed a similar pattern when comparing non- to triple degranulated NK cells and NK cells with and without target cell contact. While only the

differences for XCL1 and NFKBIZ comparing non- to triple degranulated NK cells are significant, EGR3 shows at least a trend (Figure 17 A). Even though the effect is not as potent comparing the with and without target condition, the same trend of an activation induced effect is observable (Figure 17 B). The with target group also includes cells which did not degranulate and thereby did not upregulate the mRNA, therefore reducing the effect strength. Overall, all further investigated differentially expressed genes were shown to be activation-induced.

5.10 Flow cytometry-based phenotyping of serial degranulating NK cells

The unbiased RNA sequencing experiment resulted only in genes that display activation induced changes in expression. Therefore, we switched to check on proteins that are already described to be involved in NK cell activation. A selection of NK cell surface receptors, involved in different steps of NK cell activation, were analyzed to find a possible combination that hints at serial degranulation activity. A serial degranulation assay was performed with PBMCs of 5 donors and surface receptors were stained afterwards (Supplementary Figure 5).

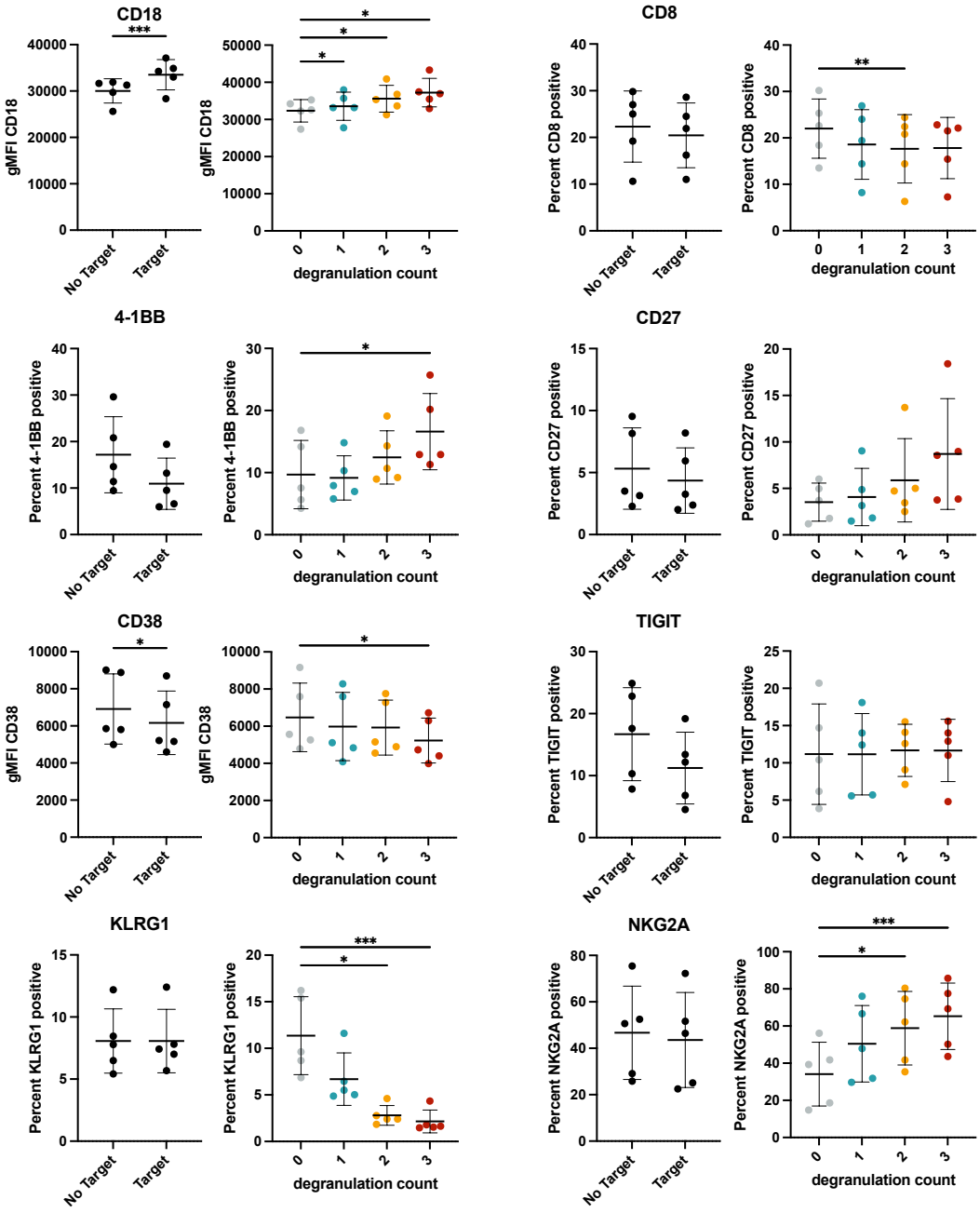

Figure 18: Effect of NK cell phenotype on serial degranulation outcome
Serial degranulation assay was performed with PBMCs and K562 as target cells. Multiple surface proteins have been measured and are shown as gMFI or percent positive dependent on the protein (Supplementary Figure 5).
For gMFI: Ratio paired t-test and RM one-way ANOVA, with Geisser-Greenhouse correction; For percent: Wilcoxon matched-pairs signed rank test and Friedman test with Dunn's multiple comparison. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

CD18 is expressed on all NK cells as part of LFA-1 and as such not usable as a marker to identify a subgroup of NK cells (Supplementary Figure 5). Nonetheless, an activation-induced increase can be observed with higher levels of increase for NK cells that degranulated more often (Figure 18). CD8α, KLRG1 and NKG2A seem unaffected by the presence of target cells while 4-1BB, CD27 and TIGIT might show a trend to decreased levels. For CD38 a reduction can be observed in the gMFI levels.

Even though statistically significant, the difference in expression of CD8α on NK cells that degranulated more often is small. KLRG1 shows the same trend but to a stronger extent with only about 2 % of triple degranulated NK cells expressing it. This is significantly less than the 12 % of not degranulated NK cells. The low overall expression however makes this a bad predictor of serial degranulation since close to 90 % of not degranulated NK cells are also KLRG1 negative. The CD38 gMFI level are lower on triple degranulated compared to non-degranulated NK cells. This small effect was only observable as a trend in the gating for CD38 positive cells (data not shown). TIGIT is uniformly expressed, regardless of degranulation status. A higher expression on NK cells that degranulate more often can be seen for 4-1BB, NKG2A and as a trend for CD27. In all cases no marker is expressed on all NK cells showing high levels of degranulation. The strongest effect can be observed for NKG2A which is involved in NK cell education. Other aspects of education were not investigated in this assay.

To validate findings of the assay, the inverse was tested. $CD56^{dim}$ NK cells were sorted for expression of NKG2A and CD38 and after resting used in a serial degranulation assay. NKG2A was chosen as it showed the strongest effect in the previous assay. While CD38 expression has only a small effect, there is the trend of CD38 negative NKG2A positive NK cells outperforming the double positive ones (Figure 19 A Supplementary Figure 6 A). Additionally, the strong expression of these two markers helped with sorting the cells (Supplementary Figures 5, 6 B and C).

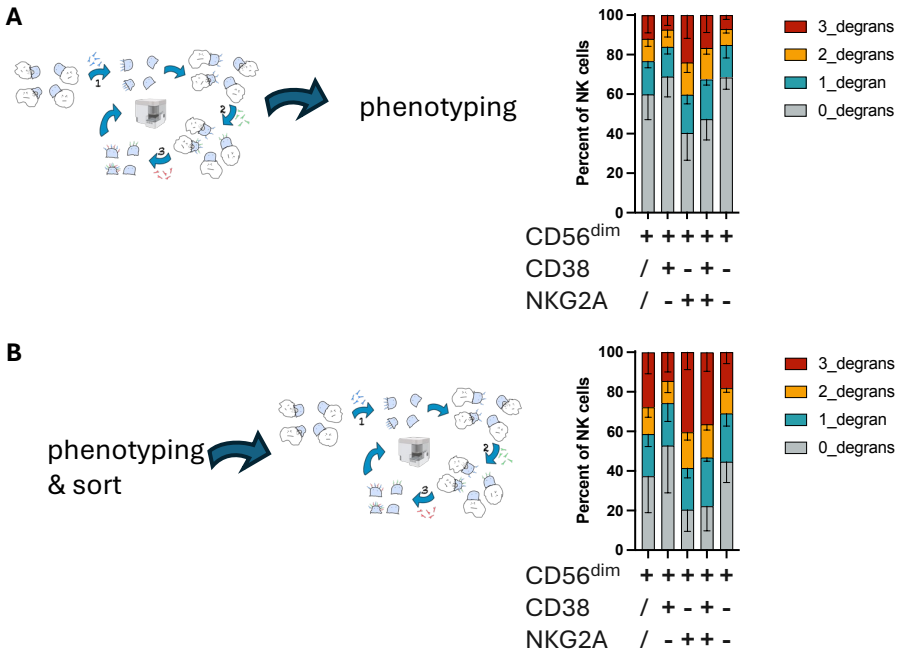

Figure 19: Validating serial degranulation performance of CD38 and NKG2A sorted NK cells
A) Serial degranulation assay was performed with PBMCs and K562 target cells followed by surface receptor phenotyping (Figure 18). Shown is the degranulation group distribution based on degranulation count in dependence on CD38 and NKG2A expression for $CD56^{dim}$ NK cells. B) NK cells were sorted as $CD56^{dim}$ and based on CD38 and NKG2A expression (Supplementary Figure 6 B, C). After resting overnight, serial degranulation assay was done with K562 target cells. Shown is the degranulation group distribution based on degranulation count.
Shown is mean and SD; A: n = 5; B: n = 4-6; statistic showing significances is depicted in Supplementary Figure 6 A (for A) and D (for B) for better clarity. B: Done in cooperation with Maren Claus.

Overall degranulation was higher for sorted NK cells (Figure 19 A and B). Other donors were used, so it could be just a donor effect, but the additional day of culture in the sorting experiment probably preactivated the NK cells, resulting in higher overall degranulation. The sorting experiment validates the previous finding and shows that those markers work independently of previous activation. NKG2A negative NK cells degranulate less than bulk $CD56^{dim}$ NK cells, while among NKG2A positive NK cells the ones lacking CD38 show a trend for more overall and triple degranulation.
Overall, the assay provides the ability to isolate serial degranulating NK cells and identify markers that influence this behavior.

6. Discussion

Serial killing is a key aspect of NK cell cytotoxic behavior. It has been described over 15 years ago and methods to directly investigate it are around for at least a decade. Still, it is not clear what differentiates a serial killing NK cell from one that does not. A major bottleneck in the investigation of serial killing is its complex nature, involving multiple sequential cell-cell interactions. Methods deployed today are limited in at least one area. They have little information gain, are very labor intensive or rely on specialized equipment. This keeps investigation of serial killing to a few labs and holds others from just integrating it into their workflow.

In the context of tumor immune therapies serial killing is a desirable trait for effector cells. It could boost potency by allowing the same number of cells to be more effective or reduce side effects by keeping potency but reducing the number of cells needed. To this day there is no marker to identify serial killer NK cells. A marker would be interesting for application purposes but also to better investigate serial killing in itself. A surface marker could be used to isolate and further characterize this subset of NK cells. This would not be possible for intracellular markers, but they could still enable investigation of underling mechanisms.

Work done here tried to fill this gap in knowledge by providing a new tool in the arsenal of serial killing investigation. It improves upon already published methods by increasing the differentiable events from 2 to 3 and better fits to the timescale of serial killing in the range of multiple minutes not hours.[125] The serial degranulation assay does not measure killing directly but only degranulation. Degranulation, while it correlates with killing, is only a proxy since target cells can survive contact with a degranulating immune cell. This is prominently known for T cells where multiple sublethal hits from different T cells need to add together to kill certain target cells.[143] For NK cells, contacts that do not lead to a kill are mostly observed after they have already killed.[86] NK cells can kill by engaging death receptors on the target cell. In the NK cell line YT it was shown that the death ligand FasL is not present in vesicles together with the degranulation marker CD107a.[144] However, in order to do serial killing, NK cells need to rely on their lytic granules since engagement of death receptors only leads to single kills or the last kill in a sequence.[86] Therefore, serially degranulated NK cells should represent NK cells that at least tried to kill multiple target cells. The clear advantage of the serial degranulation assay compared to microscopy is that it is based on flow cytometry. With the emergence of spectral flow cytometry, 20 and more immune parameters can be easily measured simultaneously, enabling deep phenotyping. Cells of interest can be isolated via fluorescence activated cell sorting. The number of cells analyzed is magnitudes higher than for the manually analyzed microscopy assays, ranging in the low hundreds.[86, 145] This makes it possible to identify rarer events and smaller effect strength. Acquisition and analysis of flow cytometry-based data is also generally faster. To analyze NK cells via microscopy, they often

need to be isolated, for the serial degranulation assay itself this can be reduced to only isolating the PBMCs and working directly with them.

There are other flow cytometry-based assays that read out the activity on the NK cell instead of the target cell side. They utilize fluorescent dyes released by the dying target cell. These dyes are then taken up by the NK cell or stick to the surface of the NK cell that has been equipped with dye specific antibodies.[146] Such approaches however cannot distinguish between individual activations of the NK cells and high fluorescence intensity of an NK cell can only roughly be linked to serial killing. Our approach on the other hand uses multiple instances of staining that can be clearly linked to individual periods of activity.

During the establishment of the serial degranulation assay different incubation times were tested. Longer incubation times lead to higher total degranulating NK cells than multiple shorter incubation times adding up to the same time. Handling of the cells possibly interrupts the formation of IS or destroys already built IS, keeping NK cells from getting activated and degranulating. This would explain the increased degranulation for longer incubation times but is also a desired effect to give each NK cell a fresh start for each new incubation time. By removing existing contacts, NK cells starting to degranulate at the end of one incubation time that could continue in the following are less likely to occur. When testing different target cell lines, K562 cells resulted in the highest degranulation and were accordingly used for subsequent experiments. The differences in NK cell activation between the different target cell lines will result from their unique expression of NK cell ligands. While all of them show expression of ligands for the activating NCR NKp44, they do so to varying extents, with K562 cells having the lowest expression.[147] K562 cells and 721.221 cells both lack the expression of inhibitory MHC class I molecules, but NKp30 ligand and the NKG2D ligand ULBP-2 are only expressed on K562.[148, 149] Jurkat do express MHC class I, but also the NKG2D ligands ULBP-1 and -2.[150] To investigate serial degranulation against 721.221 cells, Jurkat cells or other target cells that trigger NK cell degranulation to a lesser degree than K562 cells, the incubation time could be increased, giving more overall degranulation and a better spread to make differences in degranulation groups visible.

The degranulation phenotype after sorting reflected the performance of the cells in the assay used for sorting. This opens up the possibility to further investigate the link between performance in the serial degranulation assay and actual serial killing. As mentioned before degranulation does not necessarily need to lead to the death of the target cell. Additionally, during the resuspension steps NK cells and target cells can potentially be separated before the target death occurs. NK cells sorted for their performance in serial degranulation can be compared in a microscopy-based setup instead of another serial degranulation assay. This will show if the NK cells which got activated and tried to kill a target cell are actually successful. Differences between the groups will possibly be diminished by the resting period as seen for the repeated serial degranulation assay. Resting with activating cytokines pre-activates NK cells which

did not degranulate in the first go, turning them from inactive to active during the second challenge. On the other side some NK cells from the triple degranulated group did not degranulate at all in the rechallenge. One possibility is that these cells entered into an activation-induced exhausted state.[151] A hallmark of exhaustion would be the upregulation of TIGIT, which was not observed. However, TIGIT levels were measured directly after the target cell contact, so slower changes in expression are not visible. This could be tested by also checking exhaustion markers at the time of degranulation repeat. Other markers for exhaustion like TIM-3, LAG-3 or PD-1 could also be used.[152]
Another hypothesis stems from the proliferation of activated NK cells. NK cells that are actively engaged in cell division might be less cytotoxic. For ex vivo NK cell homeostasis it was shown that fast cycling NK cells exert less effector function than slower cycling ones.[153] There are methods to detect the cell cycle via flow cytometry that could be paired with the serial degranulation assay to address this. In T cells, glycolysis was shown to be necessary for effector function while proliferation can happen without it.[154] By forcing NK cells to adapt their metabolism, their effector function has been shown to change, with fasting improving NK cell anti-tumor immunity and restriction of glycolysis leading to increased serial killing.[116, 155] The metabolic profile can also be addressed via flow cytometry with the SCENITH assay, which could be coupled to the serial degranulation assay.[156]
The investigation of NK cell maturity via CD56 expression revealed higher activity of $CD56^{bright}$ NK cells. This was observed to be rooted in their higher stimulation through IL-15. IL-15 signals through binding with the heterodimer of IL-2Rβ and the common gamma chain. While both $CD56^{bright}$ and $CD56^{dim}$ NK cells have robust expression of the IL-15 receptor subunit IL-2Rβ, $CD56^{bright}$ NK cells were shown to be more responsive to stimulation with IL-15.[129] Differences in c*is* presentation of IL-15 through the high affinity IL-15 receptor IL-15Rα on the same cell could be the reason for the differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells.[157] For IL-2, which signals through the same β/γ-receptor as IL-15, it is known that $CD56^{bright}$ but not $CD56^{dim}$ NK cells express the high affinity IL-2 receptor chain IL-2Rα.[158] The signaling downstream of IL-15 binding also differs between $CD56^{bright}$ and $CD56^{dim}$ NK cells, with phosphorylation of the signaling molecules AKT and ERK only being present in $CD56^{bright}$ NK cells.[129] The difference in NK cell stimulation could be seen as a bias in the assay but also shows that the assay can be used to test different stimulation techniques. In the context of tumor immune therapy IL-15 is used in some protocols for expansion of the NK cells or even later on co-administered to the patient.[120]
NK cell education is essential for their activity. By getting constant inhibitory stimuli through KIRs or NKG2A they in turn are more cytotoxic when they do get activated.[52] This could also be observed with the serial degranulation assay. However, education alone is not the sole determining factor, and it will be interesting to investigate where the differences between triple- and non-degranulating educated NK cells lie and how they compare to differences between triple- and non-degranulating uneducated NK cells. Another prominent group of NK cells are the adaptive ones. They emerge in

response to infection with CMV and differ in their activity compared to “normal” mature NK cells.[57] One hallmark of the adaptive phenotype is the down-regulation of FcεRIγ and with that also NKp30.[159] K562 cells which were mostly used for the assays shown here can activate NK cells through ligation of NKp30.[149] In turn some adaptive NK cells are especially inept at engaging K562 cells. To study them in this context, other target cell lines should be used. NK cells do not need to recognize their target directly but can also do it indirectly via antibodies bound to the target cells through CD16-mediated ADCC. Adaptive NK cells have been shown to be especially well suited for ADCC.[159] Furthermore, since CD16 shedding is higher with increasing numbers of degranulations, it gives rise to a negative feedback loop that could diminish serial killing. To combat CD16 shedding in clinical settings, uncleavable variants have been used.[160] Conversely, inability to shed CD16 can render NK cells unable to detach from their target cell, also disrupting serial killing.[109, 145] NKG2C is another receptor linked to adaptive NK cells. Most people have the NKG2C gene, but about 4 % of the population have a deletion on both alleles.[161] It is not necessary for clonal expansion of adaptive NK cells, but donors who express NKG2C mostly have it on their adaptive NK cells.[161] Cells of the donors measured in the characterization setup had little NKG2C expression so it was excluded from the further analysis. To properly analyze its impact, donors should be screened beforehand and tested for an adaptive population via an alternative route like staining of PLZF. [159]

The goal of the RNA sequencing experiment was the identification of markers able to predict the serial killing ability of NK cells. Most identified genes were upregulated in their expression. Analysis of activation-induced up- or downregulation of gene expression was not planned for in the experiment design. Increasing gene expression will have a faster impact compared to lowering gene expression if no active degradation mechanisms are involved. Thereby the directionality of differential expression could be due to this speed difference, and not a trend for serial degranulating NK cells to have more different genes expressed or express their genes to a stronger extend. As mentioned, the activation-induced difference in expression was not addressed and so all hits that where further tested showed the behavior of being induced by the activation of the NK cell. Induced up- or downregulation of a genes expression or the protein levels does not exclude it from impacting if an NK cell gets activated and the protein can thereby still be a useful biomarker. However, it does add an additional layer of complexity into the system. To focus in on markers not effected by activation a comparison between resting NK cells and NK cells after target cell contact should be made first. All genes that show differentialm RNA expression between those two groups should be excluded for the comparison of differentially degranulating NK cells inside the group of NK cells after target cell contact.

An activation-induced increase of SEMA7 levels through target cell contact as shown here, as well as through cytokine stimulation has also been shown before.[162] The here shown downregulation of the chemokine receptor CXCR4 could also be triggered through activating cytokines and an upregulation was noted for NK cells treated with

the anti-inflammatory cytokine TGFβ.[163] The observed activation-induced reduction or trend thereof for 4-1BB, CD27, CD38 and TIGIT does not fit to previously described readouts. CD27 is down-regulated upon chronic stimulations with its ligand CD70 which was shown in mice.[164] However, the serial degranulation assay does neither reflect chronic stimulation nor are K562 known to express CD70.[165, 166] Additionally, an activation-induced down-regulation would also not fit to the trend of more CD27 expression on multiple degranulated NK cells. An explanation for this trend might be found in the expression pattern of CD27. It is described to be higher expressed on $CD56^{bright}$ NK cells (confirmed for this experiment, not shown).[167] Since $CD56^{bright}$ NK cells were enriched in the triple degranulated fraction - at least in the setup used for the characterization - the enriched CD27 expression could be a reflection of that. Proteins that are commonly co-expressed would potentially both be usable as markers, but with regards to mechanisms need to be closely examined as to who is actually conveying the effect. CD38 expression is lower on adaptive NK cells. It has been linked to resistance of these NK cells to oxidative stress.[168] Some clinical approaches have opted to knock out CD38 to give these adaptive like features to their cell product.[169] The serial degranulation protocol was performed in v-bottom plates in which NK cells with an excess of target cells were spun down. The so formed loose pellet of many cells in close contact could mimic TME-like features like high oxidative stress. Sorted NKG2A positive, CD38 negative NK cells were performing slightly better than CD38 positive ones in the serial degranulation assay. Possibly, the lack of CD38 helped the NK cells in overcoming the conditions in the loose pellet. However, it is not clear that a short time frame as used here would be sufficient to convey such a TME-like environment. Down-regulation of CD38 can be induced by longer ex vivo expansion protocols, but this does not reflect the short assay used here.[170] For TIGIT, down-regulation is not described in the context of activation. As mentioned before, chronic NK cell stimulation would lead to exhaustion-linked up-regulation as seen in HIV patients.[171] Multiple receptors showed increased surface levels on serial degranulated NK cells, while a decrease in surface staining was observed when comparing the without and the with target cell condition. This would imply an activation-induced downregulation on the bulk level, with highly active NK cells keeping their expression. The literature does mostly not support down-regulation under the used conditions. One other explanation could be that the excess of target cells reduces the staining efficacy for some of the used antibodies. To circumvent this, the staining conditions could be adjusted or target cells added to the no-target-group for the staining. The cold staining condition should ensure that the target cells do not induce expression changes in the NK cells in that step, but this would need to be confirmed. Lack of CD70 and expression of CD8α have previously been linked to higher performing NK cells in regards of cytotoxicity.[39] CD70 was not detectable when measured here and for CD8α only a slight but opposite effect was observed. A reason for that could be the treatment of the NK cells. This analysis focused on NK cells that

were nearly unstimulated while the ones in the mentioned publication underwent long-term culture before they were assessed.
Even though no markers directly linked to serial killing could be identified, this does not confirm that no such marker or combination thereof exists. The flow-based characterization approach has the underlying problem that the markers need to be selected beforehand to be tested. The here used bulk RNA sequencing on the other side does not give single cell resolution, so combinatory effects will be lost. To circumvent both these problems CITE-seq could be used to get the best of both worlds. RNA-labeled anti-CD107a-antibodies can be used for the serial degranulation assay and read out with single cell RNA sequencing, giving single cell resolution and the broad spectrum of RNA sequencing.
On the other hand, it cannot be ruled out that an identification marker for serial killing NK cells does not exist. Serial killing is a multi-step process with many factors that need to align. The NK cell needs to find a target cell that induces signaling strong enough to activate it. Next, the target cell needs to die, with fast death being favorable for serial killing. Prolonged contact times can drive NK cells to a more cytokine releasing less cytotoxic state.[172] Complete detachment comes next and needs to happen for the NK cell to move to a new target cell. The way the target cell dies can also impact how fast the effector cell can detach again. [84, 172] Then this chain needs to repeat for following kills without breaking. Previous activations prime NK cells, helping them activate more easily in the future through kinetic priming.[85] Conversely, repeated activations also reduce expression of some activating receptors through shedding or internalization and drain the contents of the lytic granules needed to kill.[106, 107, 109] Taken together, it is possible that the process of serial killing is just stochastic in nature, impacted strongly by random distributions of signaling molecules at the time of contact inside both NK cell and target cell. Nonetheless, conditions can be made favorable for serial killing. Pre-stimulation with cytokines can boost expression of activating receptors and signaling molecules. An adaptive phenotype or culturing conditions could bring NK cells into an more favorable metabolic state.[155, 173] Education can increase the cytotoxic load of the NK cell.[53] Activation can be helped through antibodies engineered for optimal NK cell stimulation like NK cell engagers or with the use of CAR in the clinical context. These can potentially be further improved and tailored since it is unclear if some receptors or co-stimulations trigger NK cells more strongly in the direction of serial killing compared to others. The flip side of NK cells activatability can also be addressed. Chronic stimulation leads to exhaustion. Inhibitory ligands on target cells or signaling molecules like PGE_2 and TNF block NK cell activation. Checkpoint blockade or blockade of those inhibitory interactions can restore NK cell activity and by that should also positively impact serial killing.

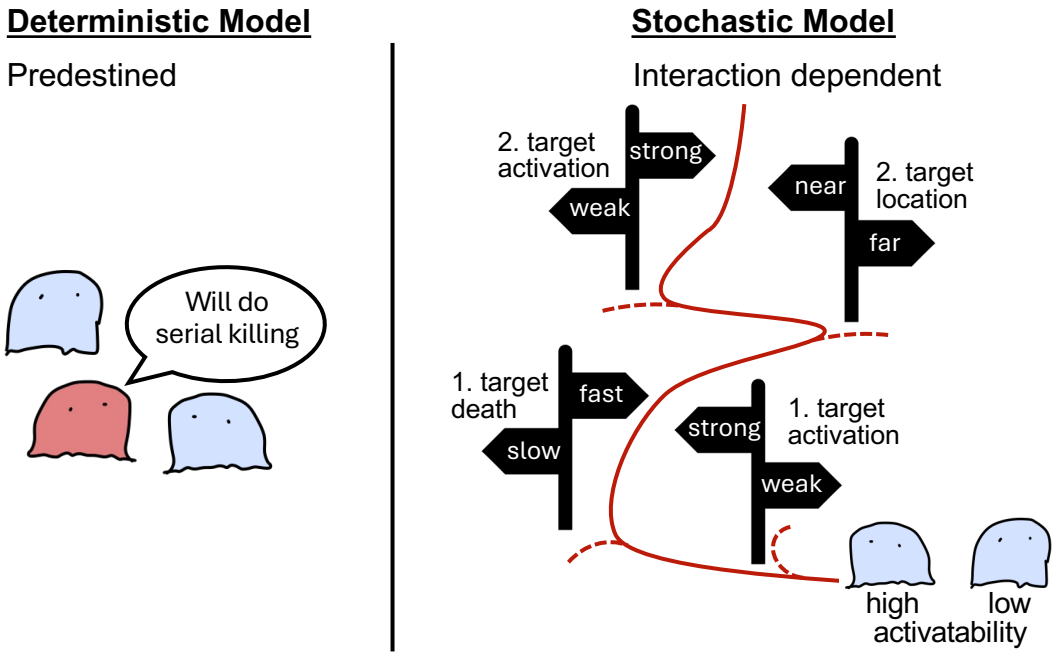

Figure 20: Deterministic and stochastic model of serial killing NK cells
Left: Deterministic model. Only a subset of NK cells is able to do serial killing and this subset is identifiable by some predefined markers. Right: Stochastic model. NK cells vary in their ability to get activated and steps during killing influence if the NK cells will continue to kill afterwards, making it impossible to predict if an NK cell will be a serial killer.

The here provided and utilized method, even though not yet shown to identify actual serial killing behavior, can find highly activatable NK cells. The readout through CD107a could also be used to investigate cytotoxic T cells. With its use of flow-cytometry it is highly adaptable and also easy to take up into the repertoire of any research group. No direct links between expression patterns and serial degranulation could be obtained in this work, which pushes into the direction of a more stochastic model for serial killing (Figure 20). However, there are more aspects of NK cell biology that need to be investigated thoroughly before the “predestined serial killer” can be ruled out. Since most therapeutic NK cell approaches rely on them killing other cells, a deeper understanding of their killing and serial killing behavior will certainly be needed to bring these therapies to their full potential.

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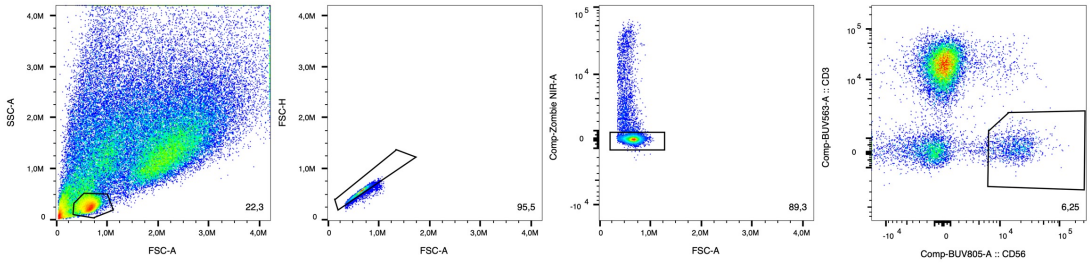
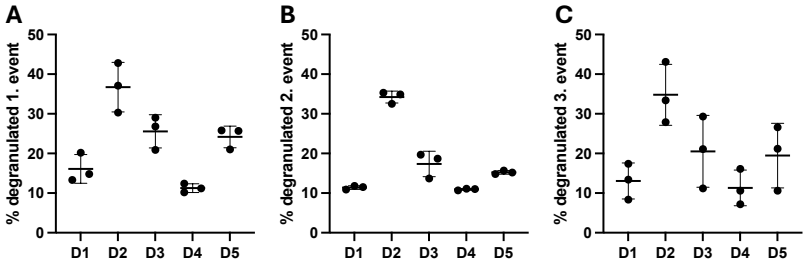
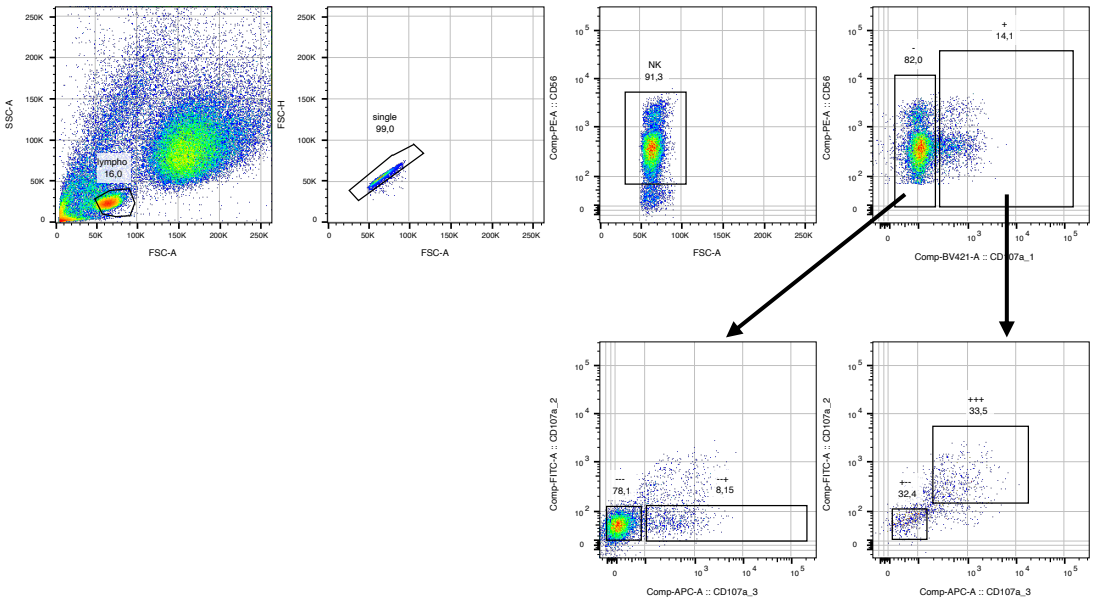
8. Supplement



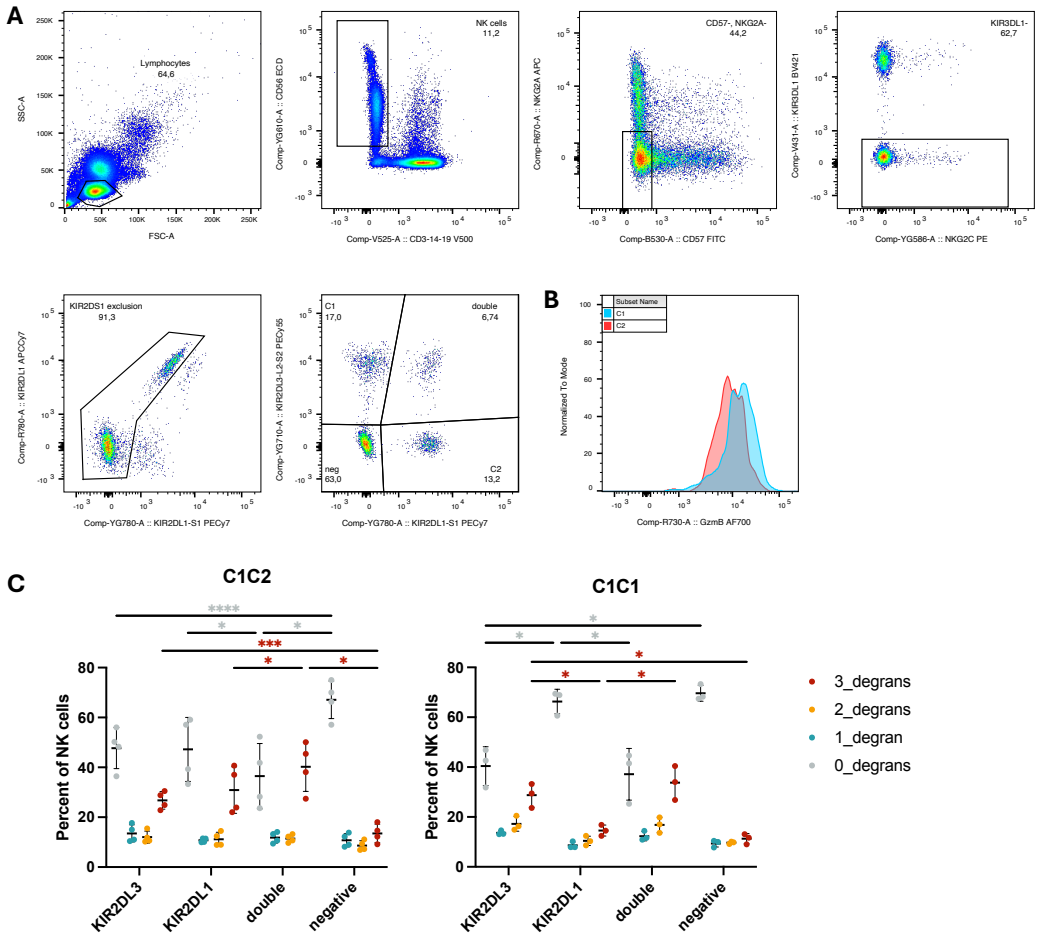
Supplementary Figure 1: General NK cell gating strategy
[123]Gating NK cells from a mix of PBMCs and target cells. Lymphocytes are gated according to size and granularity as single cells negative for dead cell marker. Cells positive for CD56 and negative for CD3 are seen as NK cells.



Supplementary Figure 2: Overall degranulation
Percentage of NK cells positive for the first CD107a stain (A)[123], the second CD107a stain (B)[123] or the third CD107a stain (C)[123] using PBMCs from five different donors (D1-D5) repeated three independent times. Data from the experiment depicted in Figure 8.



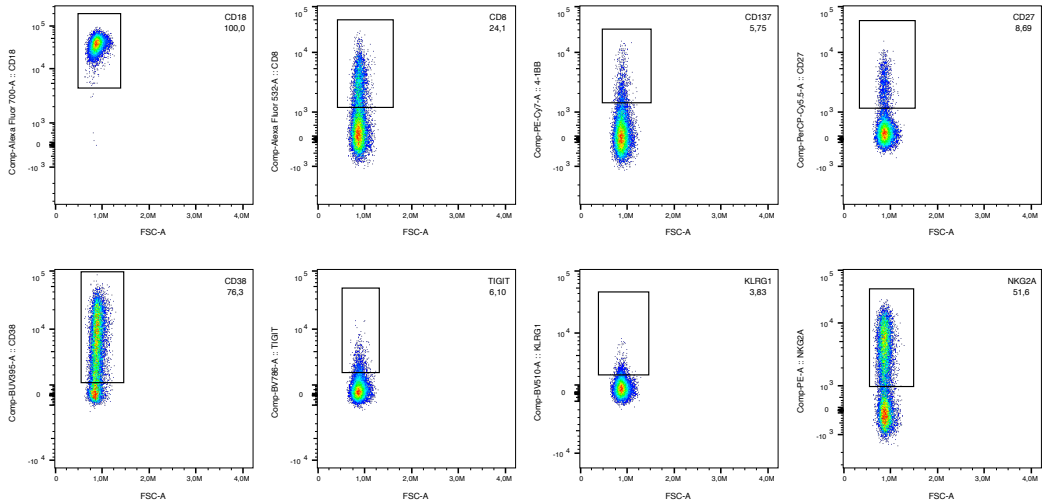
Supplementary Figure 3: Gating of NK cells dependent on degranulation status
NK cells are gated from a mix with target cells dependent on granularity and size as CD56 positive single cells. From those according to expression of CD107a_1, CD107a_2 and CD107a_3 as triple negative (- - -), triple positive (+++), only positive for CD107a_1 (+ - -) or only positive for CD107a_3 (- - +).



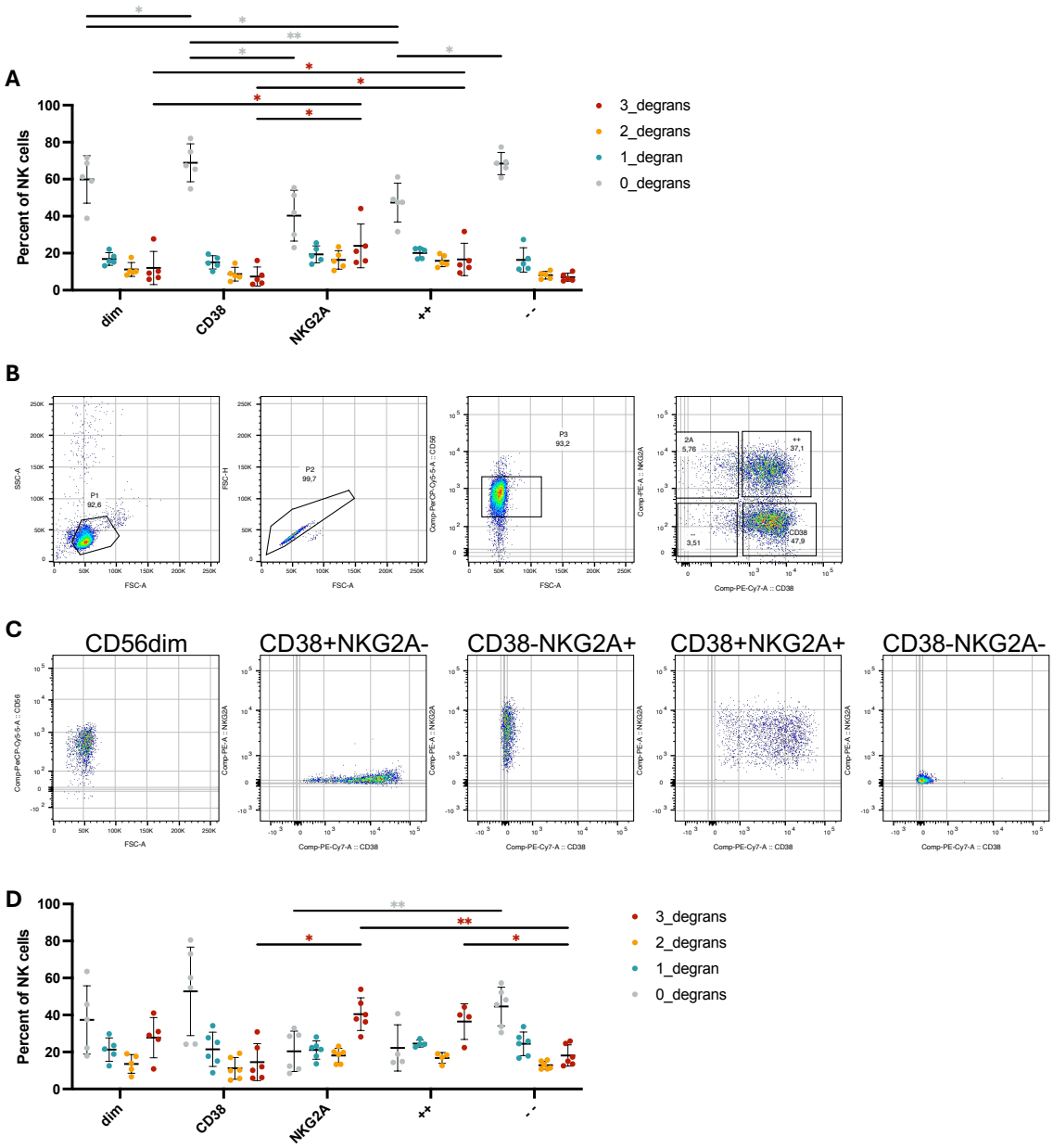
Supplementary Figure 4: NK cell education

A) To compare median granzyme B levels of KIR2DL3 and KIR2DL1 educated NK cell subsets, NK cells were gated from PBMCs according to size and granularity, Positive for CD56 and negative for CD3, CD14 and CD19. NK cells positive for NKG2A, CD57, KIR3DL1 and KIR2DS1 are gated out. B) Representative histograms of NK cells from an HLA-C1 educating donor showing granzyme B expression in HLA-C1 educated (KIR2DL3+ KIR2DL1-; blue) and HLA-C2 educated (KIR2DL3- KIR2DL1+, red) NK cells. C) Distribution of degranulation groups based on degranulation count for NK cells from HLA-C1 and HLA-C2 education donors (left) and HLA-C1 single educating donors (right). Serial degranulation assay was performed with NK cells and K562 target cells. NK cells gated as NKG2A, CD57, KIR3DL1 negative.

C: RM 2way ANOVA with Geisser-Greenhouse correction, Tukey's multiple comparison, for clarity not shown in figure: C1C2: 2_degrans: KIR2DL3 vs negative**, double vs negative**, 1_degran: KIR2DL3 vs negative*, n = 4; C1C1: 2_degrans: KIR2DL3 vs KIR2DL1*, KIR2DL1 vs double*, 1_degran: KIR2DL3 vs KIR2DL1**, KIR2DL1 vs double*, n = 3; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001



Supplementary Figure 5: Gating of Marker from Figure 17
NK cells were gated as shown in Supplementary Figure 1. Expression too low for follow-up analysis and data not shown: CD161, HLA-DR, CD70, CLTA-4, PD-L1 and NKG2C.



Supplementary Figure 6: Serial degranulation performance of CD38 and NKG2A sorted NK cells
A) Other visualization of the data in Figure 18 A. B) Exemplary gating strategy to sort CD56^{dim} NK cells and these further dependent on CD38 and NKG2A expression. C) Exemplary plots of sorted NK cells. D) Other visualization of the data in Figure 18 B.
A: RM 2way ANOVA with Geisser-Greenhouse correction, Tukey's multiple comparison, for clarity not shown in figure: 2_degrans: dim vs CD38*, dim vs ++*, CD38 vs ++*, NKG2A vs --*, ++ vs --*, 1_degran: CD38 vs ++*, n = 5; D: Mixed effect model with Geisser-Greenhouse correction, Tukey's multiple comparison, for clarity not shown in figure: 1_degran: dim vs --***, n = 4 - 6; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

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(Enrolment number)

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