UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Heinrich-Pette Institut
Leibniz-Institut für Experimentelle Virologie

Prof. Dr. Marcus Altfeld

Antigen-Specific Immune Responses Mediated by NK Cells in HIV and Influenza

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Jana Regina Leonhards
aus Zwiesel

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GLOSSARY OF TERMS

ADCC .....antibody dependent cellular cytotoxicity

AML.....acute myeloid leukemia

ART .....antiretroviral therapy

BCG .....Bacille Calmette-Guerin (former vaccine used against tuberculosis)

BL.....time point before vaccination

B-LCL.....EBV-transformed B-lymphoblastoid cell line

BLT .....bone marrow, liver, thymus (humanized mice generated by surgical transplantation of human fetal liver, thymus tissue fragments and pluripotent hematopoietic stem cells)

CD .....cluster of differentiation

CEF .....peptide pool containing peptides from CMV, EBV and influenza virus

CHS.....contact hypersensitivity

CIML.....cytokine induced memory like

EBV .....Epstein-Barr-Virus

FluMist.....live attenuated influenza vaccination, nasal application

HA .....hemagglutinin

HCMV.....human cytomegalovirus

HCV.....hepatitis C virus

HESN .....HIV-exposed seronegative

HLA .....human leukocyte antigen

IFN-γ .....interferon-γ

IL .....interleukin

ITAM.....immune tyrosine-activating motif

KIR .....killer cell immunoglobulin-like receptor

MCMV .....murine cytomegalovirus

MFI .....mean fluorescence intensity

MHC .....major histocompatibility complex

MICA/B.....MHC class I chain-related protein A/B

NA .....neuraminidase

NCR.....natural cytotoxicity receptor

NHP.....nonhuman primate

NK cell .....natural killer cell

PBMC.....peripheral blood mononuclear cells

PV.....2 months post second vaccination

PVe.....5 days post vaccination

PVIa.....30 days post vaccination

RAG .....recombinant activating gene

SAP .....SLAM-associated protein

SEMA7A .....semaphorin 7A

SHIV .....simian-human immunodeficiency virus

SIV.....simian immunodeficiency virus

SLAM .....signaling lymphocytic activation molecule

TNF-α .....tumor necrosis factor α

TRAIL.....TNF-related apoptosis-inducing ligand

ULBP.....UL-16 binding protein

VZV .....varizella zoster virus

1 Introduction

1.1 NATURAL KILLER CELLS AND INNATE IMMUNITY

The human immune system is divided into innate and adaptive. An enormous variety of clonally expressed molecules resulting from somatic recombination defines cells part of the adaptive immune system (e.g. B and T cells). The highly diverse repertoire of B and T cell receptors generated through this process allows the cells of the adaptive immune system to respond to pathogens in an antigen-specific manner(1). In addition, adaptive immune responses are long lasting. Natural killer (NK) cells have been traditionally considered a part of the innate immune system and have been initially identified through their ability to lyse tumor cells via cytotoxicity(2). They are large granular lymphocytes that can not only efficiently eliminate tumor cells but also virus-infected cells without prior sensitization(3). NK cells represent a first cell line defense to minimize pathogenic threat entering the human body while also sending signals to the adaptive immune system through cytokines and chemokines, thus playing an important role in the development and regulation of the adaptive immunity(4). Although NK cells are considered a part of the innate immune system, studies from the past 14 years have uncovered data about adaptive immune responses by NK cells and even demonstrated antigen-specific NK cell memory responses to a variety of different haptens and viral pathogens in mice, nonhuman primates and humans(5-16). Based on these studies, we aimed to pursue further experiments to confirm former results and deepen knowledge about antigen-specific NK cell responses in humans. In particular, whether human NK cells develop memory of influenza is not clear.

1.2 NATURAL KILLER CELL RECEPTORS AND THEIR FUNCTIONS

To fulfill their duty, natural killer cells are equipped with an extensive set of activating and inhibitory receptors, so called germ-line encoded receptors which do not have the ability of somatic recombination but are established within the sequences of the NK cell genome(17, 18). NK cells are defined as CD3-negative lymphocytes expressing the cell adhesion molecule CD56 and the Fc Receptor FcγRIII (CD16). NK cells are subdivided based on CD56 and CD16 cell surface expression levels into $CD56^{dim}$ (CD3-CD56+CD16+), $CD56^{bright}$ (CD3-CD56+CD16-) and $CD56^{neg}$ (CD3- CD56-CD16+) NK cells, each subset displaying different functions(19, 20). $CD56^{bright}$ NK cells exhibit potent immunoregulatory abilities based on the combination of abundant cytokine

production and high proliferation potential(20-22). The $CD56^{dim}$ NK cell subset is endowed with potent cytotoxic functions(2, 23) whereas $CD56^{neg}$ NK cells expand in chronic viral infections such as HIV and hepatitis C virus (HCV) and might represent an anergic subset(24, 25). CD16 is a low affinity IgG Fc receptor that is primarily expressed on NK cells and granulocytes. The receptor is able to bind to the Fc portion of antibodies and triggers cellular cytotoxicity due to the target bound activation, which is also known as antibody dependent cellular cytotoxicity (ADCC)(26).

Inhibitory receptors binding to human leukocyte antigen (HLA) class I predominate with their ability to recognize 'human self', a trait generated during development. A fail of self-recognition stops NK cell development and results in hyporesponsiveness to avoid autoimmunity(27). Target cells deficient in expression of HLA class I proteins are eliminated by NK cells since inhibitory signals are revoked, a phenomenon also known as "missing self"(27). NK cell interactions are dominated by inhibitory signals that are conducted by the killer-cell immunoglobulin-like receptor (KIR) family containing the long cytoplasmic tail and by CD94-NKG2A, a C-type lectin heterodimer. CD94-NKG2A carries importance in sensing the overall expression of HLAs on the surface of other cells, through HLA-derived peptides that are loaded on HLA-E(28).

NK cell activation only occurs when enough inhibiting signals are silenced and activating receptors engaged, a homeostatic interplay between both receptor groups. C-type lectin receptors like NKG2D and NKG2C or natural cytotoxicity receptors (NCR) represented by NKp30, NKp44 and NKp46 play a crucial role within the family of activating receptors. NCRs interact with molecules derived from tumors and viruses (Table 1) (29, 30). Interestingly, influenza hemagglutinin (HA) binds to NKp46 and NKp44 and therefore triggers the cytolysis of influenza-infected cells (31, 32). The binding between NKp46 as well as NKp44 and their ligand hemagglutinin is only possible due to the sialylation of the receptors(33). The research group around Mandelboim even found a highly conserved sugar carrying residue to be crucial for the outstanding binding abilities of NKp46 to HA and other tumor ligands(33).

In addition to the natural cytotoxicity receptors, KIRs with short cytoplasmic tails constitute activating receptors that are triggered by molecules connected to the immune tyrosine-activating motif (ITAM). KIRs are found to bind to the peptide- binding region of the different allotypes of HLA molecules when a peptide is presented(34-38). While the interaction of inhibitory KIRs with HLA class I ligands has been studied in detail,

ligands for most activating KIRs remain elusive. However, in the past few years, substantial progress has been made and open conformers of the non-classical HLA class I molecule HLA-F have been demonstrated as ligands for KIR3DS1, whereas KIR2DS1, -2DS2 and -2DS4 have been proposed to bind specific HLA class I molecules presenting synthetic peptides (KIR2DS1) or peptides derived from CMV (KIR2DS1), flaviviruses (KIR2DS2) or bacteria (KIR2DS4)(39-43). Furthermore, the C-type lectin family presents a set of receptors including the CD94- NKG2C heterodimer and the NKG2D homodimer that both deliver activation signals to the NK cell(44-47). Interestingly, NG2D is expressed on all NK cells and binds to stress-induced ligands such as MICA, MICB and UL16-binding protein (ULBP) 1-5. These ligands are glycoproteins that are distantly related to major histocompatibility complex (MHC) class I molecules, hence termed 'MHC class I chain related protein A (MICA) or B (MICB)'. Since NKG2D represents one of the most potent activating NK cell receptors, viruses have gained the ability to evade the recognition by NKG2D+ NK cells. NKG2C made its way into the spotlight lately since several studies have reported significant expansion of NKG2C+ NK cells endowed with adaptive features in individuals seropositive for CMV (48-52). NKG2C as well as NKG2A bind to the ligand HLA-E. Interestingly, the binding affinity of HLA-E to NKG2A is much higher than to NKG2C. NKG2A signaling leads to the inactivation of the NK cell (53) whereas NKG2C activates the NK cell when bound to HLA-E(54). Hence, it's not the ligand that directs an NK cell response but rather the peptide that is loaded on HLA-E that defines which receptor is being bound and which NK cell response will follow(55). A great variety of different HLA-I derived leader peptides leads to the successful inhibition of NKG2A+ NK cell whereas the activation of NKG2C+ NK cells is limited to selected peptides(56). For instance, the HLA-E complex with HLA-G leader peptide leads to a 3.2-fold enhanced NKG2C+ NK cell response compared to control conditions like HLA class I leader peptides, CMV
derived peptides or Hsp60 peptide(56). These properties allow the NKG- NK cell family to detect HLA-1 changes within the immune system without triggering an immune reaction through NKG2A but also enable controlled NK cell triggering by NKG2C activation(57).

CD94-NKG2E is often cited to be an activating receptor since it has shown activating properties in response to Qa-1(58), the murine ortholog of HLA-E, and also exhibited activating functions in defense against viral pathogens in mice(59). However, there is a

lack in data proving the surface expression of NKG2E in humans owed to the fact that there is no specific antibody that can bind NKG2E. In addition, the association between the activating motif DAP12 for NKG receptors and NKG2E has not been confirmed yet. Studies suggest that NKG2E forms an intracytoplasmic complex with CD94 and DAP12, however its function still has to be further evaluated(47).

Generally, activating signals trigger the release of cytoplasmic granules like perforin and granzymes for lysis of the target cell, which represents the dominant NK cell killing mechanism. This process takes place within the first hours of infection so that the adaptive immune system is given enough time developing in presence of the virus(60). Another killing mechanism enables NK cells to immediately induce apoptosis via a ligand called FAS or through special signaling known as TRAIL, TNF- related apoptosis-inducing ligand. However, while all NK cells display the variety of these different killing functions, not all NK cells share the same receptor repertoire. For instance, the expression of KIRs on different NK cells within one individual can vary, which is based on NK cell development regulating different expression patterns(61). In contrast, 2B4, an activating co-receptor of the signaling lymphocytic activation molecule (SLAM)-related receptor family displaying important immune- modulating functions is expressed on the surface of all NK cells. In primary NK cells, the receptor is able to enhance natural cytotoxicity in synergy with NKp46 and also directly recognizes influenza viral hemagglutinin(62). In contrast, NK cells activated by IL-2 exhibit cytotoxic functions based on 2B4 stimulation alone without co- stimulation of other activating receptors which is based on a higher expression of the SLAM-associated adapter protein (SAP) important for signal transduction(63). There are many more receptors involved in regulating NK cell function that do not belong to the three major receptor families of NCR, C-type lectin or KIR. Activating receptors like NTB-A, CRACC or DNAM-1 that are part of the CD2-family or the inhibitory receptor LILRB1 as one of three receptors belonging to the LILR receptor lineage show the extent of receptors that influence NK cell interactions(45). In addition, it might be possible that we haven't identified all NK cell receptors yet, which becomes transparent when trying to solve the mystery behind the new NK cell trait "memory". NK cells with adaptive immune responses have been described in different experimental settings (Table 2), also including true antigen-specific memory. However, receptors mediating antigen-specific recognition by NK cells have not been identified yet, and markers allowing to specifically discriminate antigen-specific memory NK cells in nonhuman primates and humans are still missing.

Table 1. Major activating and inhibitory NK cell receptors and their ligands

Receptor Family	*Receptor*	*Ligand*	*Function*
KIR	KIR2DL1	HLA-C2	*Inhibitory*
	KIR2DL2/3	HLA-C1, HLA-C2, HLA-B*46:01, HLA-B*73:01 C1 epitope	
	KIR2DL4	HLA-G	
	KIR2DL5	Unknown	
	KIR3DL1	HLA-A with Bw4 motif,	
	KIR3DL2	HLA-A3/11, HLA-F, HLA-A*11:01	
	KIR3DL3	Unknown	
	KIR2DL3	HLA-G	*Activating*
	KIR2DS1	HLA-C2	
	KIR2DS2	HLA-C1, HLA-A*11:01, HLA-C*0102	
	KIR2DS3	Unknown	
	KIR2DS4	HLA-C*05:01, A*11:01, C*16:01	
	KIR2DS5	Unknown	
	KIR3DS1	HLA-F open conformers	
C-type lectin	CD94-NKG2A	HLA-E	*Inhibitory*
	CD94-NKG2C	HLA-E	*Activating*
	NKG2D	MICA/B, ULBP 1-5	
	CD94-NKG2E	HLA-E	*Function unclear*
NCR	NKp30	HS GAGs, main tegument of protein of human CMV, (DBL)-1 α domain of plasmodium falciparum erythrocyte membrane protein (PfEMP1), BAT3/BAG6, B7-H6, Galectin-3, β-1,3 glucan	*Activating*
	NKp44	HS GAGs, HA of influenza virus, HN of avian Newcastle disease virus, Sendai virus human parainfluenza virus, PCNA, NKp44L expressed on tumor cells	
	NKp46	Heparan sulfate glycosaminoglycan, HA of influenza virus, HA of human vaccinia virus, HN of avian Newcastle disease virus, Sendai virus, human parainfluenza virus, (DBL)-1 α domain of plasmodium falciparum erythrocyte membrane protein (PfEMP1), Vimentin expressed on cell infected with myco-bacterium tuberculosis, unidentified ligand expressed by fusobacterium nucleatum, unidentified ligand expressed by pancreatic β cells, C. glabrata epa proteins, Complement factor P (popderin)	

1.3 ANTIGEN-SPECIFICITY AND MEMORY

In 1975, natural killer cells appeared on scientific screens exposing exquisite abilities in lysing tumor cells (64). The extent of cytotoxicity and the fact that they could act without prior antigen exposure led scientists to name those cells “natural killer”(65). Since then, NK cells have been strictly classified as part of the innate immune system, expressing germ-line encoded receptors without the ability to mediate recall responses against previously encountered antigens(17, 18). Yet, about 14 years ago, NK cell experiments conducted in mice broke the status quo and suggested adaptive NK cell immune features. Eventually, these features could be further classified into three main types of memory discussed in detail below: Memory-like NK cells, cytokine-induced memory-like NK cells and antigen-specific memory NK cells.

Memory-Like NK Cells

Memory-like NK cells can be subdivided into “evolved” memory NK cells and “Delta G” NK cells.

Evolved memory NK cells were discovered in human cytomegalovirus (HCMV)- positive subjects expressing high levels of NKG2C+ NK cells (49, 52, 66-68) as well as in murine NK cells expressing Ly49H(11, 69). Generally, NKG2C+ NK cells persist long term in the host and also increase in numbers after reactivation(49, 52). Additional to the expression of NKG2C, evolved memory NK cells display remodeled epigenetic modifications compared to conventional NK cells(70-73). A recent study confirmed HCMV strains encoding variable UL40 peptides presented by HLA-E, the ligand for NKG2C, to be responsible for the expansion and differentiation of this CD94-NKG2C+ NK cell subset(51).

Delta G NK cells express high levels of Fc R but lack the intracellular γ-signaling chain that is a component of the Fc R as well as the Syk adaptor protein, a spleen tyrosine kinase(68, 70, 73-75). This special phenotype is referred to as FcγRΔg NK cell. Interestingly, FcγRΔg NK cells are found in all individuals but expand substantially in HCMV seropositive subjects and have been shown to partly overlap with the NKG2C+ NK cell population(75). However, FcγRΔg NK cells contain both, NKG2C- and NKG2+ NK cells and show especially strong effector functions by CD16-engagement(76, 77). After exposure to CMV, epigenetic modification modulates FcγRΔg NK cells so that they become long-lived and are able to perform more potent antibody-dependent functions compared to other NK cell subsets.

Cytokine-Induced Memory-Like NK Cells

Cytokine-induced memory-like (CIML) NK cells were initially described by the team of Yokoyama, showing that adaptive NK cells in mice can be generated by short-term stimulation with a combination of IL-12, IL-15 and IL-18(78). Eventually, similar properties were described for human NK cells(79, 80). The preactivation with cytokines leads to the expression of a functional high-affinity IL-2 receptor and demethylation of the conserved upstream noncoding enhancer region of the interferon (IFN)-γ gene. In addition, anergic unlicensed NK cells with enhanced antibody-mediated functions are recruited. Cytokine stimulation also releases these memory-like NK cells from KIR-mediated inhibition(72, 81-83). Cytokine-induced NK cells retain enhanced functionality for weeks after the initial cytokine stimulation. The lab around Jost just recently highlighted semaphorin 7A (SEMA7A), a transmembrane protein also known as CD108, as part of a new mechanism underlying the formation of CIML NK cells(84). The cytokine-enhanced interaction between SEMA7A and its ligand integrin-β1 leads to CIML NK cell differentiation and in addition to a more potent IFN-γ release.

Importantly, CIML NK cells have tremendous potential for cancer immunotherapy as a first phase I clinical trial in refractory/relapsing acute myeloid leukemia (AML) patients has shown to adoptively transferred CIML NK cells expanded in vivo efficiently eliminated tumor cells ex vivo, and led to complete remission in 6 out of 11 patients who could be evaluated(85).

Antigen-Specific Memory NK Cells

Antigen-specific memory NK cells are not only able to expand and respond with higher cytotoxicity but in addition are able to recognize a single antigen that it was previously exposed to, a trait formerly reserved for B and T cells. First demonstrations of antigen-specific memory NK cells were shown by the team around Von Andrian that revealed data on hapten-induced antigen-specific recall responses with the receptor CXCR6 playing an important role(5, 6). However, so far there is only one clear demonstration of recall responses mediated by NK cells in nonhuman primates(14) and one in humans(15).

1.3.1 NK Cell Memory in Mice

1.3.1.1 Antigen-Specific NK Cell Memory

O'Leary et al. conducted contact hypersensitivity (CHS) experiments in mice that were deficient for the recombination activating gene (RAG)-2, a gene required for functional T and B cells(5, 86). That way they investigated whether other cells than T and B cells could mediate hapten-induced contact hypersensitivity(87, 88). Surprisingly, they discovered an NK cell-mediated CHS response that could still be detected at least one month after chemical hapten-specific priming which was quite striking knowing that the half-life span of a mature NK cell in steady-state-condition is thought to count 17 days(89). The scientists discovered that primed NK cells reside solely in the liver and not in the spleen or in lymph nodes. Additionally, when transferring liver NK cells into naïve mice, recall responses could be achieved in the recipient mice. Continuing and based on these first experimental results, Paust et al. released phenomenal data on antigen-specific NK cell responses in mice vaccinated with non-infectious virus-like particles from influenza, ultraviolet-light-inactivated vesicular stomatitis virus or HIV-1 in the year of 2010(6). Adoptive transfer of hepatic NK cells from vaccinated mice conferred enhanced survival of naïve recipient mice lacking functional T or B cells after lethal challenge with the sensitizing virus but did not confer protection after challenge with any of the other viruses. Transfer of splenic or naïve NK cells did not enhance NK cell immune responses. Corroborating memory NK cell protection against influenza, Li et al. could show that the infection of mice with the mouse-adapted PR8 influenza strain leads to the formation of memory NK cells displaying a CD49a+ DX5- NK cell phenotype and homing to the liver, which confer protection upon lethal viral challenge, in contrast to influenza-primed lung NK cells(90). The protective effect of hepatic memory NK cells is explained by the finding that in mice, the hepatic chemokine receptor CXCR6 is crucial for memory NK cell persistence. CXCR6 is likely not directly involved in antigen recognition, yet the finding of CXCR6 as first murine surrogate marker for NK cell memory along with the data proving memory formation in the presence of viral antigens represented a great step forward. Subsequently, several other studies confirmed liver-restricted NK cell memory in mice(7, 8, 90, 91). Overall, the team around Ulrich von Andrian with O'Leary and Paust opened up a new chapter in NK cell history: NK cell memory.

Van den Boorn et al. also confirmed previous antigen-specific CHS studies by using the hapten monobenzone, a therapeutic depigmenting agent for vitiligo universalis in patients(92). The difference between Van den Boorn's paper and O'Leary et al.'s CHS study in 2006 is that O'Leary experimented with chemical haptens, which have no use in human therapies whereas monobenzone is used as a therapeutic mean(5). Van den Boorn conducted his experiments in mice deficient in the RAG-2. The scientist could prove recall CHS responses upon elicitation with monobenzone by NK cells deriving from the liver. When transferring those hepatic memory NK cells to naïve animals, the immunological adaptive response was still retrievable and could be elicited as late as 4 months after the initial sensitization. In accordance with previous studies (5) Van den Boorn also found NKG2D to be relevant for the CHS response in monobenzone-sensitized mice. When trying to dismantle the mystery behind the memory forming process, Van den Boorn found that monobenzone- triggered memory NK cells migrate to lymph nodes for their temporary residence. The following CHS induction relied on the lymph node recruitment of macrophages without the typical engagement of dendritic cells in any of these steps. Nevertheless, the entire CHS response by antigen-specific NK cells and the homing of macrophages to the lymph nodes could only be induced by the engagement of the NLRP3 inflammasome. NLRP3 was not only required for recall responses but also for the memory formation process when engaging with monobenzone for the first time. Interestingly, those memory NK cells originated from hepatic hematopoietic progenitors and stem cells and not from bone marrow like expected, which coincides with Paust's findings in 2010. In 2017, Venkatasubramanian et al. released data on antigen-specific NK cells in mice that underwent Bacille Calmette-Guerin (BCG) vaccination(12). NK cells expressing NKp46, CD27 and KLRG1 produced significant amounts of IFN-γ and also highly expanded after immunization. Adoptive transfer of CD3-NKp46+CD27+KLRG1+ NK cells from the spleen and lymph nodes to naïve mice granted long term protection when challenged with Mycobacterium tuberculosis one and three months later. Interestingly, the expansion of this antigen-specific NK cell phenotype depended on the stimulation with IL-21.

1.3.1.2 Cytokine-Induced NK Cell Memory

In addition to antigen-specific NK cell research, scientists also acquired data on cytkine-induced NK cell memory. Cooper et al. released a significant paper stating that short ex vivo stimulation of murine NK cells with cytokines (IL-12, IL-15 and IL- 18) leads to the formation of adaptive NK cells that produce significantly more IFN-γ upon restimulation in vivo (78). This study displayed a new memory strategy in 2009 carrying out adaptive immune functions through an intrinsic pathway instead of viral or hapten triggers, thus called cytokine-induced NK cell memory. Cooper found that there was no phenotypical difference between cytokine-activated and control NK cells that were adoptively transferred into naïve hosts. However, these NK cells released significantly more IFN-γ when restimulated with cytokines in vitro 1-3 weeks later. Interestingly, the capacity for enhanced IFN-γ production was passed on to next generations of NK cells suggesting that NK cell changes were linked to possible transcriptional or epigenetic modifications. Pre-activation of NK cells did not result in enhanced cytotoxicity. Further studies in mice revealed that cytokine-induced memory-like NK cells posses potent anti-tumoral functions(93), especially against myeloid leukemia which could be reproduced in humans by Romee et al. 2 years later(85). In addition, most recent data presented CIML NK cells with activity against solid tumor(94).

1.3.1.3 Memory-Like NK Cells

At the same time as antigen-specific NK cell recall responses against haptens and viral antigens were described by the team of Ulrich von Andrian, Sun et al. reported that murine cytomegalovirus (MCMV) infection elicits NK cell memory in mice(11). For this venture, the scientists used mice containing a resistance locus in their genome called CMV1, which encodes for the activating NK cell receptor Ly49H in mice(95). After MCMV infection, Ly49H recognized the viral glycoprotein m157 on the surface of virus-infected cells, which on the other hand led to significant expansion of Ly49H+ NK cells in spleen and especially in the liver. Upon re- exposure to MCMV one month later, those MCMV-experienced NK cells rapidly degranulated and produced cytokines more efficiently than NK cells in naïve mice. Sun et al. was within one the first teams to discover a specific viral antigen that generated evolved memory-like NK cells after

exposure. After two months, Ly49H+ NK cells were found in all different organs (e.g. spleen, lymph node, liver, lung) before settling permanently into peripheral and lymphoid tissues. Adoptive transfer of these NK cells into naïve animals, which was followed by a viral challange, also showed protective immunity and a stronger secondary response .

1.3.2 NK Cell Memory in Nonhuman Primates

1.3.2.1 Antigen-Specific NK Cell Memory

In 2015, Reeves et al. published data that demonstrated long-lived antigen-specific NK cell responses in nonhuman primates (NHP) for the first time(14). Two macaque cohorts that were included in the experiments, either infected with simian-human immunodeficiency virus (SHIV) or simian immunodeficiency virus (SIV) developed enhanced NK cells responses upon challenge with antigens that they were primed with, consistent with antigen-specific recall responses. He further tested NK cell memory responses in vaccinated macaques, assessing NK cell responses 5 years following immunization with adenoviral vectors expressing either HIV Gag or HIV Env. For the first time in a primate species, scientists showed strong antigen-specific NK cell memory responses that could still be detected after 5 years in the absence of viral antigens. These elevated adaptive immune functions were shown in NK cells residing in the liver as well as in the spleen, and to a lesser extent in the blood(96). Interestingly, Reeves et al. found significant impact of the NKG2 family on NK cell activity. When blocking NG2A or NKG2C, NK cell killing of antigen-pulsed dendritic cells was markedly reduced by up to 80%. Comparable to the importance of NKG2D in the CHS studies(5, 92), these receptors still have to be further evaluated regarding their full effector functions in antigen-specific responses by NK cells. Taken together, Reeves et al.'s data opened up new dimensions in the NK cell memory chapter showing the development of memory NK cell responses after infection and vaccination in NHPs, strongly suggesting that similar responses exist in humans.

1.3.2.2 Memory-Like NK Cells

The team around Reeves continued experiments in HIV/SIV infected rhesus macaques allowing him deeper insight into memory-like NK cells(97). The data was conform with previous results in that g-chain- Syk- NK cells were only generated in presence of rhesus CMV, however the migration into tissues could be shown to be dependent on SIV infection. The number of adaptive NK cells correlated with the rhesus CMV titers in the blood. Further studies have to clarify in how far SIV/HIV is able to modulate CMV induced adaptive NK cells and whether this NK cell phenotype posseses protective features against other viral infections.

1.3.3 NK Cell Memory in Humans

1.3.3.1 Antigen-Specific NK Cell Memory

When looking at antigen-specific NK cell memory in humans, only a couple studies were released and clear mechanisms behind this type of memory are still missing. Experiments with CMV, Epstein-Barr-Virus (EBV) and BCG suggest expansion of specific NK cell phenotypes in presence of viral targets and long term NK cell persistence, however the provided data is limited to a few studies revealing true antigen-specific NK cell memory in humans(13, 15, 48, 98, 99). The group around Paust just released a significant paper on true antigen-specific NK cell responses covering all three entities of immunological memory, hence vaccination dependency, antigen specificity and longevity(15). They demonstrated that human NK cells isolated from the liver of humanized BLT mice vaccinated with HIV-Env mediated vaccination-dependent and an antigen-specific killing in vitro. NK cells from the spleen did not show similar results. In addition, they tested the longevity of NK cells in humans infected with varizella zoster virus (VZV) showing large numbers of NK cells being recruited to the site of infection decades after the initial exposure when being re-challenged with the virus.
Recent groundbreaking data by Wang et al. revealed mechanisms of NK cell memory establishment in HIV-1 infected subjects similar to the memory formation process of T cells(16). They found CD94+CD56^{hi} NK cells with high-level expression of transcription factor 7 (TCF7) to increase in subjects with HIV-1 infection, a transcription factor known to be responsible for the memory forming process in CD8+ T cells(100).

The WNT signaling pathway and TCF7 could also be confirmed to be crucial for the establishment of this specific memory NK cell subset. Interestingly, the proliferation and enhanced degranulation of CD94+TCF7+CD56^{hi} NK cells was only initiated after HIV-1-specific cytokine stimulation. Additionally, the assessment of transcription factors and the elevated degranulation response to HIV-infected CD4+ T cells suggest close resemblance to memory T cells.

1.3.3.2 Cytokine-Induced NK Cell Memory

First successful observations on cytokine-induced NK cell memory in humans were made by Romee et al. who confirmed cytokine primed NK cell activation in humans based on Cooper et al.'s paper, an important milestone in the NK cell memory chapter since all of the obtained data until 2012 was primarily based on animal or in vitro experiments(80). Romee et al. could not only show cytokine-induced NK cell responses in humans for the first time but also found them to exhibit exquisite cytotoxic abilities in response against AML in mice and humans 4 years later(85). These NK cells produced high levels of IFN-γ and mediated enhanced cytotoxicity against AML cell lines as well as primary human acute myeloid leukemia blasts in vitro. Interestingly, those memory functions were triggered regardless of KIR to KIR- ligand interactions. When transferring human CIML NK cells into mice, AML burden in vivo was substantially reduced and overall survival improved. In addition to these basic AML experiments, Romee et al. were able to test the potential of cytokine-induced memory like NK cells for cancer immunotherapy in a first-in-human phase 1 clinical trial. They could demonstrate robust memory-like NK cell responses against leukemia targets after efficient proliferation and expansion in vivo, leading to remission in a subset of AML patients(85). Based on those promising results, the clinical potential of CIML NK cells is currently being further evaluated in several phase II clinical trials.

Within the same year, Goodier et al. released data on cytokine-induced memory-like NK cells after vaccination with influenza(101). First, he found enhanced IL-2 dependent NK cells responses to influenza, which were still detectable 4 weeks post- vaccination. Interestingly, those responses were higher in individuals tested negative for CMV. In addition, the administered influenza vaccination triggered enhanced NK cell responses to innate cytokines, which however were mostly seen in individuals tested

positive for CMV. These elevated cytokine responses could also be detected in vitro. In 2019, the team around Goodier released another paper on cytokine induced memory in NK cells describing that this type of memory can be induced by influenza vaccination showing heightened responses to cytokine stimulation months after (102). This study opened speculations on the formation of cytokine-induced memory NK cells in response to vaccination. Such NK cell responses could significantly contribute to existing vaccinations representing an additional arm of vaccine-induced immunity. Further studies have to clarify the relevance of this pathway in post-vaccination immunity to influenza and other viruses.

1.3.3.3 Memory-Like NK cells

Following first cytokine-induced NK cell data in humans, scientists were eager to see whether experiments with MCMV-infection in mice could be transferred to human settings with similar results. In 2004, Guma et al. provided first evidence that individuals infected with HCMV carry higher frequencies of CD94-NKG2C+ NK cells compared to individuals negative for HCMV(66). Comparable to MCV studies in mice, Guma et al. suggested that the binding of NKG2C and its ligand on HCMV- infected cells leads to the formation of memory NK cells. Consecutive studies with subjects either infected with HCMV or after reactivation due to immunosuppression after solid organ or hematopoietic stem cell transplantation confirmed former results(49, 52, 103). They found CD94-NKG2C+ NK cells to expand during acute infection and also persist as memory NK cells over time. In some individuals, the CD94-NKG2+ NK cell subset represented up to 70% of the total NK cell population. In addition, Foley et al.'s study on NK cells in hematopoietic stem cell transplantation demonstrated enhanced CD94-NKG2C+ NK cell functions after re-exposure to HCMV, when both the donor and the recipient were positive for HCMV(49), suggesting NK cell memory in response to HCMV infection. In contrast, CD94- NKG2C+ NK cells from HCMV-seronegative donors did not lead to such results. Expansion of CD94-NK2C+ NK cells in populations infected with HCV(67), hantavirus (104) or HIV (105) have been shown however only in individuals that were persistently infected with HCMV.

Guma et al. allowed early insight on the interaction between NKG2C and HLA-E based on in vitro experiments with HCMV-infected fibroblast co-cultured with NK cells either knocked down for HLA-E or treated with NKG2C-specific blocking antibodies(50). The

bond between NKG2C and HLA-E represents the driving force for the expansion of the NKG2C+ NK cell population in HCMV-positive individuals. However, it took a few more years to show specific CMV-peptide recognition by NKG2C via HLA-E(51, 106, 107). Heatley et al. discovered UL40, an immunmodulatory glycoprotein, to be essential in the interaction between NKG2C and HLA-E(106). Individuals that underwent hematopoietic stem cell transplantation and re-infection with HCMV revealed UL40 proteins with polymorphic hotspots in its HLA-E binding sequence. Most of these polymorphisms led to reduced affinity of the interaction between HLA-E and CD94-NKG2C and therefore reduced NK cell activation. However, a few peptides, most notably the HLA-G derived peptide VMAPRTLFL, led to increased lysis of peptide-pulsed HLA-E transfectans by NKG2C+ NK cell clones. Rolle et al. as well as Hammer et al. underlined these results highlighting the role of the HLA-G derived peptide VMAPRTLFL and its ability to induce enhanced effector functions in NKG2C+ NK cells(51, 107).

In parallel, studies showed the expansion of a subset of NK cells lacking the intracellular signaling adapter FcRIy in CMV-infected individuals(68, 74). In addition these NK cells exhibited superior ADCC abilities to antibody-coated virally infected targets compared to FcRIy+ NK cells. The expansion of this subset was strongly associated with the expression of CD57 and NKG2C, but was also seen in NKG2C- NK cells indicating that the expansion of FcRIy- NK cells, now termed FcγRΔg, indeed partly overlap but also have different distinct functionality(74). FcRIy-NKG2C+ NK cells showed memory-like properties like clonal expansion and longevity with strong effector functions by CD-16 engagement but weak responses to K562 exposure(77), whereas FcRIy+NKG2C+ NK cells showed elevated functionality in both settings. Preceding studies focusing on FcγRΔg NK cells in CMV infected subjects concentrated on the epigenetic regulation of this specific NK cell subset(70, 73). Lee et al. found the formation of distinct subsets of memory-like NK cells in presence of HCMV often to be deficient for several transcription factors and signaling proteins, e.g. the tyrosine kinase SYK, which was largely confined to the newly found NK cell subset(73). In the same issue of Immunity, Schlums et al. described $CD56^{dim}$ NK cells devoid of FcRγ, SYK and EAT-2 expression, correlating with promoter DNA methylation in HCMV-seropositive individuals(70). Interestingly, DNA modifications in adaptive NK cells showed similarities to memory CD8+ T cells. Functionally, DNA modifications in NK cells led to heightened resistance to apoptosis during inflammation and superior proliferation in the presence of antibodies.

While antibody-dependent adaptive NK cell properties are not antigen-specific, both studies gave new insight on the formation of memory-like NK cells in humans following HCMV infection. The following years, different study groups confirmed those previous results and also provided insight into deeper mechanisms of memory-like NK cells in challenge with other viruses like HIV or HCV(108, 109).

When strolling along the memory pathway from 2006 until now, the NK cell field took great steps in discovering more details about the newly uncovered characteristic of memory NK cells. In mice, NK cell memory has been described in three settings with CXCR6+ hepatic NK cells that can mount antigen-specific transferrable recall responses to a vast array of haptens and viral antigens, Ly49H+ NK cells mediating MCMV-specific recall responses that also show enhanced adaptive immune responses upon adaptive transfer and last but not least cytokine-induced adaptive NK cells, which are not antigen-specific nonetheless.
In nonhuman primates, the lab around Reeves showed data on antigen-specific NK cell recall responses induced by vaccination as well as on memory-like NK cells in rhesus macaques infected with CMV. NK cells with memory characteristics in humans have been described and can be triggered by specific combinations of cytokines (78, 80) or cytomegalovirus infection (70, 73). Cytokine-elicited memory-like NK cells impressed by revealing a huge potential in immunotherapies against cancer and are currently tested for antitumoral properties in humans. True antigen-specific NK cells responses were suggested in experiments conducted with CMV, EBV, BCG and HIV(13, 15, 48-52, 99). Moreover, besides the expansion of phenotypically and functionally distinct adaptive NK cell subsets in CMV-seropositive individuals(110), only a limited number of studies in humans have investigated pathogen-induced expansion of specific NK cell subpopulations. Therefore, the recent data release by Paust's study group showing vaccination-dependent and true antigen-specific human NK cell responses represents a great step forward in the new field of NK cell memory(15). The demonstration of antigen-specific NK cells with activity against particular viruses in humans would be highly significant, as it would provide a third arm of the immune system that can be targeted by vaccines to prevent infectious diseases for which there is currently no available or effective enough prophylaxis, such as HIV and influenza.

Table 2. NK cell memory

Memory-like	*Cytokine-induced*	*Antigen-specific*	
• **MCMV** Scalzo et al., 2005 [10] Kielczewska et al. 2009 [9] Sun et al., 2009 [11]	• Cooper et al., 2009[78] • **TUMOR** NI et al., 2012 [93] Uppendahl et al., 2019 [94]	• **Haptens** O'Leary et al., 2006 [5] Paust et al., 2010 [6] Majewska-Szczepanik et al., 2013 [7] Peng et al., 2013 [8] Van den Boorn et al., 2016 [92] • **HIV** Paust et al., 2010 [6] • **Influenza** Paust et al., 2010 [6] Li et al., 2017 (90) • **Vesicular stomatitis** Paust et al., 2010 [6] • **Vaccina virus** Gillard et al., 2011 [91] • **BCG/M. tuberculosis** Venkatasubramanian et al., 2017 [12]	*MOUSE*
• **Rhesus CMV** Manickam et al., 2019[97]	—	• **SHIV/SIV** Reeves et al., 2015 [14]	*NHP*
• **HCMV** Guma et al., 2004/2006[50,66] Lopez-Vergès et al., 2011 [52] Foley et al., 2012 [49] Hwang et al., 2013 [68] Zhang et al., 2013 [74] Lee et al., 2015 [73] Schlums et al., 2015 [70] Hammer et al., 2018 [51] • **HCV** Oh et al., 2016[108] • **HIV** Zhou et al., 2015[109]	• **In vitro** Romee et al., 2012[80] • **AML** Romee et al., 2016[85] • **Influenza** Goodier et al., 2016[101]	• **EBV** Hendricks et al., 2014[48] Tong et al., 2014 [98] Jud et al., 2017 [99] • **BCG** Suliman et al., 2016[13] • **HIV** Nikzad et al., 2019[15] Wang et al., 2020 [16]	*HUMAN*

[a] *divided into memory-like, cytokine-induced and antigen-specific NK cell responses and subject that was used within the study (mouse, non human primate (NHP), human)*

1.4 NK CELLS IN HIV INFECTION

An effective HIV vaccine is urgently needed after failure or marginal efficacy of several HIV vaccine trials conducted since the finding of HIV itself(111). Since the start of the HIV epidemic in 1980, the virus has infected 74.9 million people and 32 million people were killed overall(112). Although antiretroviral therapy (ART) has decreased annual AIDS-related deaths by 56% since the peak in 2004, with 37.9 million people being infected, 1.7 million new infections and 770.000 deaths from AIDS-related illnesses at the end of 2018, HIV still represents one of the greatest medical burdens known(112). It would be highly significant to be able to harness NK cells for future HIV vaccine designs as increasing evidence suggest that NK cells can mediate anti-viral activity against HIV(24, 113-131), and develop memory of HIV(14- 16).

1.4.1 NK Cells and Protection against HIV

Certain receptor arrangements help individuals to undergo slower progression or make them less likely to get infected by HIV. When taking a look at KIR receptors, studies suggested that especially the activating KIR allele KIR3DS1 in the presence of HLA-B alleles that encode an isoleucine at position 80 leads to slower AIDS progression(113). The presence of the ligand alone did not show any protection whereas the presence of KIR3DS1 without its putative binding ligand HLA-B led to more rapid progression to AIDS(113). Individuals expressing HLA-Bw4-80I display a preferential expansion of KIR3DS1+ NK cells, a subset that shows strong functional inhibition of HIV replication(114). Furthermore, individuals carrying specific alleles of the inhibitory KIR3DL1/HLA-Bw4 genotype showed lower levels of HIV expression and slower progression to AIDS(115). Eventually, KIR3DL1 in combination with HLA- B*57 was found to lower the risk of HIV infection in HIV-exposed uninfected individuals(116). These results seem to be contradictive comparing an activating and inhibitory receptor to have the same outcome. Yet, studies suggested looking at both receptors as a state of synergy and also partly as a mean of licensing. Inhibition promoted by KIR3DL1 forces the NK cell into an inhibitory state. Yet, once the activation threshold is reached and inhibition is abrogated, KIR3DL1/HLA-B+ NK cells might show increased functionality. The susceptibility for HIV decreases depending on the number of gene copies for KIR3DL1

with at least one copy existing for KIR3DS1 and one for HLA-Bw4-80I (117). However, a study comparing HIV-infected and highly exposed uninfected individuals showed that not all subtypes of the inhibitory KIR3DL1 are associated with reduced risk of infection, which is linked to distinct allotypes resulting in distinct surface expressions and therefore varying effector functions(118). A study in 2013 released new data on protection against HIV transmission in sexual partners that revealed KIR/HLA incompatibility(132). Recipient seronegative partners did not show efficient killing of HIV-infected target cells whenever the sexual partner expressed the matching KIR ligand. In contrast, a clear increase in CD4+ T cell death was detected whenever an allogeneic HLA/KIR mismatch was present possibly due to alloreactive NK cells triggering a missing-self mechanism(132). Besides the role played by KIRs in HIV infection, Carrington et al. published data on high levels of HLA-A on NK cells which resulted in poorer control of HIV(133). HLA-A expression led to higher expression rates of HLA-E through an HLA-A derived signal peptide. Therefore, high HLA-E expression promoted NKG2A-mediated NK cell inhibition impairing NK cell mediated lysis of HIV-infected targets. These results suggested therapeutic blocking of the interaction between HLA-E and NKG2A for better outcome of HIV infection progression.

Overall, NK cells are able to express certain receptor repertoires that enable the cell to slow down or to suppress HIV infection and progression in a stronger manner. A listed overview on all KIR alleles that influence possible HIV outcomes in regards to HIV resistance, slower and rapid disease progression can be seen in Hens et al.'s paper(134).

1.4.2 HIV Immune Escape from NK Cells

HIV responds to immune pressure with evasions strategies that help the virus to establish a chronic reservoir and dangerously deplete the CD4+ T cell count so that AIDS becomes manifest. Physiologically during acute HIV infection NK cell redistribution from $CD56^{dim}$ towards $CD56^{neg}$ occurs, generating a variety of dysfunctional NK cells(135). The decrease of cytotoxic NK cells and the increase of NK cells that show reduced expression of activation KIR receptors and secretion of cytokines as well as an increase in inhibitory KIR receptors on the surface delivers HIV the chance to progress and replicate within the human host(24, 25, 130). In addition, immune pressure has highly sup-

ported the development of different efficient HIV evasion strategies. Alter et al. showed that HIV is capable of escaping from KIR+ NK cell pressure by selecting for sequence polymorphisms that encode for new amino acids in HLA-I–presented peptides(119). These peptides enhance the binding of inhibitory KIRs to HIV-infected CD4+ T cells so that virus-infected cells are protected from killing. Also controlling the up and down regulation of the surface expression of cellular ligands for NK cell receptors helps the virus to escape immune recognition(120). Different cells of the immune system like CD8+ T cells and NK cells control viral replication. Cytotoxic CD8+ T cells recognize peptides presented on HLA-I molecules. Consequently, HIV forces the down regulation of HLA-I receptors, which in turn activates NK cells due to the lack of HLA-I(121-123). Therefore, HIV developed a self-sustaining strategy by producing proteins (e.g. Nef) that down regulate the major binding sites for cytotoxic T cells but sustain HLA class I molecules that lead to inhibitory signals for NK cells(124-126). Accordingly, HIV treats the up regulation of NKG2D on NK cells the same way mostly by downregulating ligands from the surface(127). In addition, hyperactivation through NKG2D and NCR triggers their chronic downregulation in return(136). Furthermore, the virus is able to modulate the receptor expression levels on NK cells thus increasing the frequency of inhibitory NK cell receptors whereas decreasing natural cytotoxicity receptors(24, 128-130). Based on limited therapeutic interventions against HIV and its immune evasion strategies, a better understanding of potential antiviral immune mechanisms that could help control HIV infection, especially memory NK cells with anti-HIV activity might highly contribute towards the efforts of developing an efficient vaccine against HIV.

1.5 NK CELLS IN INFLUENZA INFECTION

HIV is not the only disease that is in need of an effective vaccine these days. Influenza still represents an annual threat to mankind due to short-term and variable vaccine efficacy that urges annual vaccine reformulations. The need of new vaccine designs is based on rapid flu virus mutations, also known as antigenic drifts, especially in the influenza A hemagglutinin (HA) and neuraminidase (NA) antigens that facilitate the virus to escape from the host immune system. The delay between the start of annual vaccine manufacture and the spread of the epidemic results in vaccines of variable effica-

cy. Poor vaccine-elicited protection can lead to seasonal epidemics, which result in an estimated 250,000-650,000 deaths and 3 to 5 million cases of severe illness per year worldwide(137). Moreover, direct transmission of an avian influenza virus to humans or reassortments of entire viral RNA segments between avian and human influenza viruses can lead to the spread of pandemic strains of influenza viruses. In these cases, the population is exposed to a new virulent virus against which there is little natural immunity, resulting in extremely high rates of morbidity and mortality. In 2009, emergence of a new swine-derived H1N1 influenza strain (A/California/07/2009) set off an international pandemic starting in Mexico/California, which was followed by concerning cases of human infections with the highly pathogenic avian influenza A (H7N9) in 2013 in China(138). For now, influenza vaccines only provide seasonal protection such that the flu can still infect previously vaccinated individuals(139).

1.5.1 NK Cells and Protection against Influenza

Recovery from acute influenza infection relies on the ability of the immune system to produce neutralizing antibodies targeting the HA and NA glycoproteins as well as on the influenza-specific killing of CD8+ T cells(140, 141). However, numerous studies have highlighted the importance of NK cells during immune responses against influenza infections(32, 142-156). Healthy lungs harbor 5-20% of lymphocytes whereof residential $CD56^{dim}$CD16+ NK cells represent a substantial portion(157). Within days of infection or hours after inflammation initiation additional circulating NK cells are recruited to the lungs to execute two major immune functions: secretion of cytokines and killing of virus-infected cells to contain viral replication(45). Nonetheless, also resident lung $CD56^{bright}$CD49a+ NK cells exhibit significant antiviral activity against influenza-infected cells by secreting IFN-γ and granzyme B(158). Enhanced IFN-γ secretion could not only be detected during influenza infection but was also shown in experiments using influenza vaccination(146, 159-161).

Studies around the H1N1 swine-origin pandemic in 2009 associated severe and/or lethal influenza infections with reduced proportions of NK cells in the peripheral blood and the lungs suggesting a great importance of NK cells in controlling the virus load(162, 163). The same pandemic also allowed for gene association studies showing correlation between specific KIR alleles and better control or increased susceptibility during

influenza infection although due to the small size of those study cohorts, these results and their clinical relevance have to be further determined(164, 165). More importantly, studies found NKp46 to be a crucial receptor for activation of NK cells during influenza infection in humans, which could be confirmed in mice by the equivalent receptor NCR1 interacting with hemmagglutinin through sialic acid motifs(32, 143, 166). Although the recognition of HA varies among the different influenza subtypes, NKp46 is essential in controlling the virus titer levels by lysing influenza-infected cells(32, 167). In addition, NKp44, 2B4 and NTB-A also significantly mediate cell lysis by directly binding to the HA proteins present at the surface of influenza-infected cells(62, 142). Additionally, latest studies revealed a CD49a+DX5- NK cell memory phenotype that resides in the liver of Rag-1 deficient mice which is of vital importance in protection against acute secondary influenza infection in mice(90) and Goodier et al. released data on elevated NK cell IFN-γ responses to a specific influenza virus (A/California/7/2009) up to 4 weeks post- vaccination(101). Those NK cell responses could be enhanced in the presence of innate cytokines although they were also dependent on the CD4+ T cell secretion of IL-2.

1.5.2 Influenza Virus Immune Escape from NK Cells

Further corroborating a role for NK cells in controlling influenza replication, the virus shows evasion strategies to avoid NK cell lysis. Influenza targets the recognition of HA by slowly mutating and removing the sialic acid residues on NKp46 so that the viral protein cannot be bound anymore(168). Consequently, NK cells lyse influenza- infected cells less effectively. In comparison, deletion of mutated sites within NKp46 eventually increases the lysing potential of NK cells again(152). Yet, not only mutation of the sialic acid sites helps the virus to escape but also their entire deletion by the influenza derived antigen neuraminidase (168). Besides, influenza is also able to infect and kill NK cells directly. In vitro studies showed the binding of the virus to sialic acid motifs on receptors with subsequent mediate endocytosis, which led to apoptosis of the target cell(169). Furthermore, Manicassamy et al.'s study suggested the infection of NK cells by influenza in vivo via green fluorescent protein (GFP) reporter viruses, which eventually revealed a proportion of GFP positive NK cells supporting their hypothesis(170). Although influenza immunization efforts are ahead of those for HIV with the production of an annual influenza vaccine, influenza is still a disease that causes severe illness

and death, especially to high-risk populations including neonates and elderly(138, 171), and the threat of a pandemic represents a major public health concern. Just like HIV, influenza has found its way to escape from human immune mechanisms, which make it difficult to find a preventive cure. Therefore, NK cells with antiviral activity against influenza represent a potential effector cell population, which might be able to recognize more conserved regions of influenza than those targeted by T and B cells and could significantly contribute to prevent influenza infections. Interestingly, previous studies by Dr. Jost reported that influenza vaccination promotes the expansion of a subset of NK cells expressing high levels of 2B4 ($2B4^{bright}$) and low levels of NKp46 ($NKp46^{low}$) (172). However, whether a subset of long-lived $2B4^{bright}NKp46^{low}$ NK cells endowed with anti-influenza specificity persists in individuals exposed to influenza and is associated with control of influenza replication remains to be determined. Thus, after four influenza pandemics within 100 years and the missing success of providing a vaccine against HIV, it is more important than ever to explore the different arms of the immune system in more detail to work towards an effective vaccine that can protect against a broader spectrum of influenza strains.

For my thesis, I sought to look at antigen-specific NK cell responses in HIV and influenza. Preliminary results obtained in Dr. Jost's laboratory showed enhanced anti- HIV Gag activity mediated by NK cells in HIV-infected subjects, suggesting for the first time antigen-specificity among human peripheral blood NK cells. Building on those preliminary results, we performed experiments in a cohort of HIV-exposed seronegative (HESN) subjects testing the hypothesis that HIV-specific NK cell responses would be stronger in HESN than in healthy individuals, and partly responsible for protection. The results in the HIV-exposed seronegative cohort showed no significant difference in killing of HIV-infected cells compared to those in the negative control group. Both groups similarly eliminated HIV-presenting target cells. In parallel, we assessed the existence of influenza-specific human NK cell memory in healthy subjects receiving serial vaccination with a live attenuated influenza virus (FluMist) as a model to analyze the kinetics of NK cell antigen-specific expansions following repeated exposure to the same viral antigen. NK cell responses mediated by the $2B4^{bright}NKp46^{low}$ subset against a recombinant H1N1 HA protein close to the strain included in the vaccine were enhanced after immunization compared to baseline, consistent with adaptive immune responses mediated by NK cells.

2 Material and Methods

2.1 STUDY SUBJECTS

2.1.1 Influenza Vaccine Cohort

A total of 7 healthy volunteers (three women and four men, median age 24 years, range 24-39 years) immunized with a live attenuated influenza virus vaccine administered intranasally (FluMist, MedImmune) were included in this study and referred to as FLUM001 to FLUM007 hereafter (Table 3). According to self-reported flu history, four of the subjects were never vaccinated before (FLUM001, FLUM003, FLUM006 and FLUM007). One of those unvaccinated subjects reported to have undergone influenza infection (FLUM003). FLUM004 was not entirely sure to have never been vaccinated. Two subjects had received a one-time intramuscular inactivated influenza vaccination in 2007 (FLUM002) and 2008 (FLUM005), so two and one year prior to the first study vaccination with FluMist, respectively. As part of this study, all subjects were immunized intranasally with 0.2mL of the FluMist 2008- 2009 formula containing 106.5-7.5 FFU (fluorescent focus units) of live attenuated influenza virus reassortants of the following strains: A/SouthDakota/6/2007(H1N1)(A/Brisbane/59/2007-like), A/Uruguay/716/2007 (H3N2) (A/Brisbane/10/2007-like) and B/Florida/4/2006.

The subjects were vaccinated two or three times at intervals of one month. Peripheral blood mononuclear cells (PBMC) and plasma were isolated from blood samples taken before vaccination (BL) and then approximately 5 (PVe) and 30 (PVIa) days after each immunization. The study was approved by the MGH Institutional Review Board and each subject gave written informed consent for participation in the study.

2.1.2 HIV-Exposed Seronegative Cohort

Experiments looking at HIV-specific NK cell responses in HIV-exposed seronegative individuals were performed using samples from 8 subjects enrolled in a cohort of HIV-negative men who have sex with men (MSM) self-identified as ‘high-risk of HIV acquisition’ (173, 174) and from 5 healthy low-risk male control subjects. HIV negative subjects were enrolled on IRB approved protocols at Fenway Health Boston and Massachusetts General Hospital. HIV-negative status was confirmed by OraQuick® Advance HIV1/2 Rapid Antibody Test (OraSure Technologies, Inc. Bethlehem, PA, USA) and Alere DetermineTM HIV-1/2 Ag/Ab Combo (Alere Inc., Waltham, MA, USA). PBMC were isolated by density gradient centrifugation from peripheral blood samples and cryopreserved.

Table 3. Influenza vaccine cohort

	Gender	Age	Self-reported Flu history	Number of FluMist® vaccinations
FLUM001	F	25	*Never vaccinated*	VAX I-III
FLUM002	M	24	*Vaccinated once (2007)*	VAX I-III
FLUM003	F	24	*Never vaccinated - possible past infection*	VAX I-III
FLUM004	F	25	*Possible previous vaccination*	VAX I-III
FLUM005	M	24	*Vaccinated once (2008)*	VAX I-II
FLUM006	M	24	*Never vaccinated*	VAX I-II
FLUM007	M	39	*Never vaccinated*	VAX I-II

2.2 CELL LINES

2.2.1 EBV-Transformed B-Lymphoblastoid Cell Lines (B-LCL)

Fresh whole blood was used to isolate PBMC by Histopaque density gradient centrifugation (Sigma, St. Louis, MO). Autologous B-LCL were generated by incubating lymphocytes from each study subject with the supernatant of B958 cells, a T cell line which constitutively releases high titers of transforming EBV, in the presence of 1ug/mL cyclosporine A and 30% fetal calf serum (FCS) for about 6 weeks in RPMI-1640 medium supplemented with 2mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco) and 100 U/mL streptomycin (Gibco). After 10 days with no medium change, medium was replaced every two days. Once transformed, B-LCL were maintained in RPMI-1640 supplemented with 20% FCS, 2mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin. B-LCL pulsed with peptide pools were used as targets in functional assays to measure antigen-specific NK cell responses.

2.2.2 K562 Cells

K562 cells were the first human immortalized myelogenous leukemia cell line being described in 1973(175). It was found in the blood of a patient with chronic myeloid leukemia in terminal blast crisis. K562 cells are commonly used as target cells for NK

cells in assays in vitro as K562 cells lack cell surface expression of conventional HLA class I molecules that usually inhibit NK cell activation(176). Therefore, this cell line represents an excellent positive control for all the experiments described below. K562 cell lines (American Type Culture Collection, Manassas, VA) were grown in RPMI-1640 supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM L- glutamine (Gibco), 100 U/mL penicillin (Gibco), and 100 U/mL streptomycin (Gibco) at 37°C/5% CO2.

2.3 FUNCTIONAL ASSAYS

2.3.1 Antigen-Specific NK Cell Calcein Acetoxymethyl Ester (CAM) Cytotoxicity Assay

Cryopreserved PBMC were thawed and either used immediately (Influenza) or incubated overnight (HIV) at 1M/mL in RPMI-1640 medium combined with 10% fetal bovine serum (Sigma-Aldrich), 2mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco) and 1ng/mL IL-15 (R&D) prior to NK cell isolation using commercially available negative selection kits (such as NK cell isolation kit and autoMACS® Separator from Miltenyi). These assays typically yield >95% purity of NK cells. NK cell lysis of HLA class I-devoid K562 cells or autologous B-LCL loaded with overlapping peptide pools derived from HIV envelope (HIV-1 Consensus B – NIH AIDS Reagent Program), Influenza HA (A/Brisbane/59/2007(H1N1) (JPT Innovative Peptide Solutions), yellow fever virus NS4B (Negative control) (JPT Innovative Peptide Solutions) or the CEF peptide pool (Positive Control) (Mabtech) was then assessed. The CEF peptide pool consists of HLA class I-restricted peptides from human CMV, EBV and influenza virus and was shown to induce IFN-γ responses by CD8+ T cells in 9 out of 10 Caucasians. To do so, K562 or B-LCL target cells were stained with calcein AM (ThermoFisher Scientific) at a 1:100 dilution for 1h at 37°C/5% CO2 and then pulsed with peptides (2ug/mL) followed by another incubation at 37°C/5% CO2 for 1h. Calcein AM-stained pulsed or unpulsed B-LCL were then plated with purified autologous NK cells at an effector-to-target cell ratio of 5:1 on a 96-well-plate and incubated for 4h at 37°C/5% CO2.

The aim of the assay is to assess the ability of NK cells to kill target cells in an antigen-specific manner, in the absence of signals triggered by infection that commonly activate NK cells (i.e. HLA class I downregulation, cell surface expression of viral or

stress-induced proteins that serve as ligands for the NK cell receptors). Upon recognition and lysis by NK cells, target cells release their calcein AM dye, which then can be measured in the supernatant. The released calcein AM by killed target cells was measured using a Victor Multilabel Plate Reader with an excitation of 485nm and absorption of 530nm. The percent-specific lysis was calculated using the following formula: (test release – spontaneous release) / (maximum release – spontaneous release). Spontaneous release was calculated using target cells only in complete medium, maximum release was calculated using target cells in medium plus 2% Triton X-100.

2.3.2 Antigen-Specific Intracellular Staining (ICS) Assay

This modified ICS is used as surrogate measure for activation of antigen-specific NK cells. The assay evaluates degranulation and cytokine production by NK cells in response to viral peptides and in the absence of IL-2 produced by antigen-specific T cell that significantly induce NK cells to release IFN-γ(160, 177-183). CD3-depletion was performed with thawed cryopreserved PBMC using commercially available negative selection kits (such as CD3 MicroBeads human - lyophilized and autoMACS® Separator from Miltenyi). Activation of NK cells was quantified after stimulation of CD3-depleted PBMC with HLA class I-devoid K562 cells at an effector- to-target cell ratio of 10:1, 3ug/mL of purified influenza virus (A/PR/8/34(H1N1) (Charles River Laboratories, Wilmington, MA), or 2ug/mL recombinant influenza HA protein (A/Brisbane/59/2007(H1N1) (Sino Biological Inc.). To expose CD3-depleted PBMC to inactivated influenza virus, approximately 5.2 x 10^6 non-infectious influenza viral particles were added to 10^6 CD3-depleted PBMC resuspended in 0.1mL RPMI-1640 medium without serum. After 1h of incubation at 37°C/5% CO2, RPMI-1640 supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2mM L- glutamine (Gibco), 100 U/mL penicillin (Gibco) and 100 U/mL streptomycin (Gibco) was added to a final volume of 1mL. Peptide pools and K562 cells were added directly to 10^6 CD-3 depleted PBMC resuspended in 1ml complete medium. Then, CD107a-A647 antibody (Biolegend), 1 mg/mL brefeldin A solution (BD Biosciences) and monensin (GolgiStop; BD Biosciences) at a final concentration of 0.3 ul/mL were added immediately to all the reaction tubes and the total stimulation lasted for 18hr at 37°C/5% CO2. Unstimulated PBMC were similarly treated in parallel

to define the background level of degranulation. NK cells were defined as CD3-CD14-CD19- CD56+CD16+/- cells using following surface antibodies: CD56-BV605 (Biolegend), CD16-allophycocyanin-Cy7 (APC-Cy7) (Biolegend), CD3-BV421 (Biolegend), CD14- BV421 (Biolegend), CD19-BV421 (Biolegend). NK cells were further classified into subpopulations based on their expression levels of CD56 and CD16 as $CD56^{dim}$ (CD56+CD16+), $CD56^{bright}$ (CD56+CD16-) and $CD56^{neg}$ (CD56-CD16+)(19, 20). NK cell function and surface expression of 2B4 and NKp46 were evaluated simultaneously using 2B4-PerCP-Cy5.5 (Biolegend) and NKp46-PEcy7 (BD Biosciences). To monitor production of intracellular cytokine release, PBMC were fixed (BD Cytofix/Cytoperm, BD Biosciences), permeabilized (BD Perm/Wash, BD Biosciences) and finally stained for intracellular IFN-γ-FITC (BD Biosciences) and TNF-α-BV711 (Biolegend). Fixed cells were analyzed on an LSRII instrument using FACSDIVA version 6.1.3 (BD Biosciences). The frequency and phenotypes of NK cells were defined using FLOWJO version 7.5 (Treestar, Ashland, OR) (**Figure 1**).

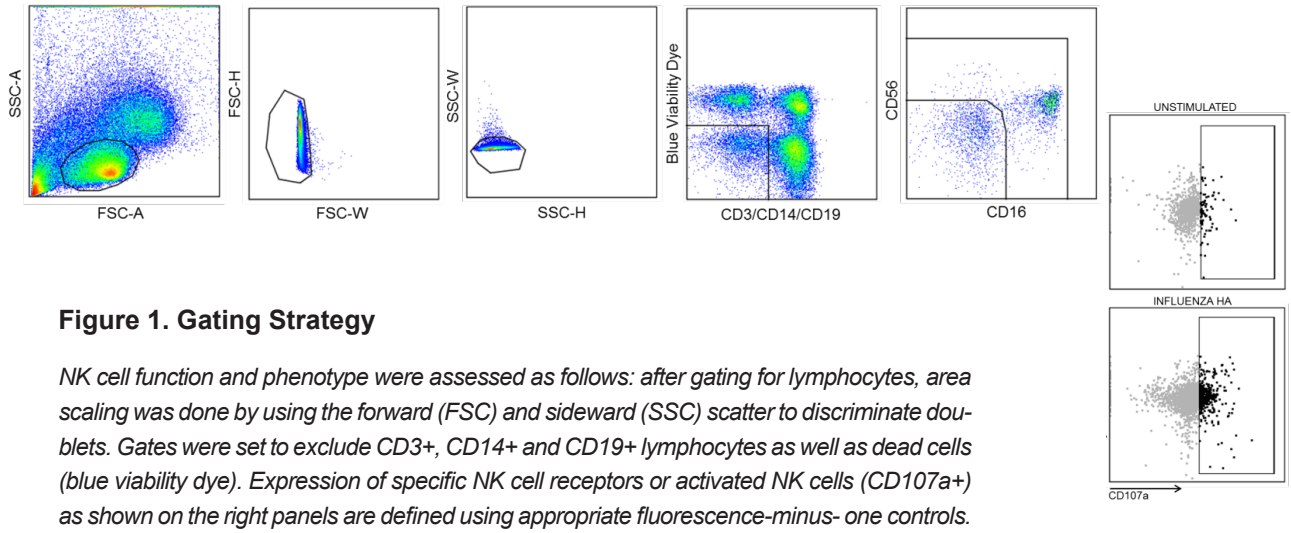


Figure 1. Gating Strategy

NK cell function and phenotype were assessed as follows: after gating for lymphocytes, area scaling was done by using the forward (FSC) and sideward (SSC) scatter to discriminate doublets. Gates were set to exclude CD3+, CD14+ and CD19+ lymphocytes as well as dead cells (blue viability dye). Expression of specific NK cell receptors or activated NK cells (CD107a+) as shown on the right panels are defined using appropriate fluorescence-minus- one controls.

2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism6. The Wilcoxon rank sum test was used to assess differences in phenotype frequencies and functional activities between groups and wilcoxon matched-pairs signed rank tests or paired T tests were used to measure differences pre- and post-vaccination or between different conditions. Values of $p < 0.05$ were considered significant.

3 Results

3.1 PART I: ANTIGEN-SPECIFIC NK CELLS IN HIV

3.1.1 NK Cells Mediate Enhanced Anti-HIV Gag Responses in Chronically HIV-Infected Subjects

Memory cells are long-lived and have the ability to expand more rapidly and display more robust anti-viral responses than naïve cells upon re-exposure to the same antigen. Until recently, only T and B cells were sought to exert such memory functions. However, accumulating evidence suggests that murine NK cells can mediate true antigen-specific recall responses and that immunized rhesus macaques are able to display immunological recall responses for at least a period of 5 years(14- 16, 184). In humans, NK cells with adaptive features have been described and can be triggered by specific combinations of cytokines(78, 80) or CMV infection(68, 70, 73, 74, 185). In addition, some studies have suggested virus-specific recall responses by NK cells in humans (13, 16, 48-52, 99). Especially data on HIV/SIV- specific NK cell responses substantially advanced with experiments in rhesus macaques, humanized mice and humans(14-16). Unpublished data building on preliminary work by the Jost and Reeves laboratories now strongly support the development of memory NK cells induced by HIV infection and HIV vaccination in HIV-infected individuals or healthy volunteers receiving HIV vaccine candidates, respectively(186, 187).

In order to start exploring whether human NK cells can mediate HIV-specific responses, the Jost laboratory originally assessed if NK cells from HIV-infected individuals can mediate stronger HIV-specific NK cell responses than those from healthy controls, similarly to what has been previously demonstrated in nonhuman primates(14). To do so, the Jost laboratory conducted antigen-specific NK cell killing assays using autologous B-LCL pulsed with a peptide pool covering the whole HIV Gag protein as target and purified NK cells from 10 HIV-infected subjects and 4 healthy individuals. HIV-infected subjects included 6 patients on antiretroviral therapy with undetectable viral loads and 4 HIV elite controllers who achieve spontaneous control of viral replication in the absence of treatment (HIV RNA levels <50 copies/mL for at least a year(188)). HLA class I-devoid K562 cells and a CEF peptide pool containing peptides from human CMV, EBV and influenza virus served as positive controls. NK cells from chronically HIV-infected subjects showed higher responses to HIV Gag than NK cells from healthy donors ($p=0.05$) (**Figure 2**). Clear HIV-specific responses were observed in 50% of

HIV-infected subjects and NK cells from elite controllers with detectable HIV Gag-specific cytotoxic activity (2/4) displayed the most potent responses, potentially reflecting higher viral loads in this cohort. Apart from one response, specific killing of HIV Gag-pulsed targets were under 20% likely reflecting low frequencies of antigen-specific NK cells in the peripheral blood compared to liver or spleen as described in animal studies (6, 14, 15). As expected, both cohorts showed similar elevated responses in presence of K562 cells. When I first joined the lab, we performed a set of experiments aimed at identifying epitopes on HIV proteins that are recognized by human NK cells by using subpools of the HIV Gag protein following the assay protocol above. The results were interesting, although they were not reproducible over time using NK cells from the same subject so that studies to optimize the assay including relevant quality controls are ongoing. To conclude, for the first time, HIV Gag-specific NK cells were identified in the peripheral blood of HIV infected individuals serving as first step for further experiments.

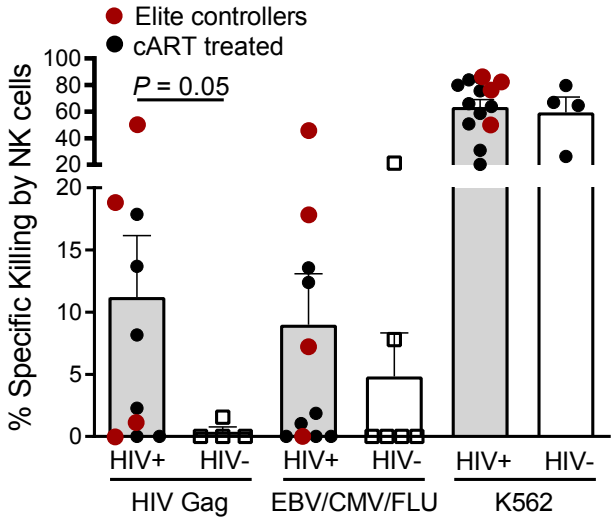


Figure 2. NK cells mediate enhanced anti-HIV Gag activity in HIV-infected subjects
Bar graphs represent mean ± SEM percentages of HIV Gag-specific killing by peripheral blood NK cells isolated from 10 chronically HIV-infected subjects (6 patients on ART, 4 HIV elite controllers) and 4 healthy donors. HLA-deficient K562 cells and CEF (EBV/CMV/FLU) were used as positive control.

3.1.2 NK Cells from HIV-Exposed Seronegative Subjects Do Not Display Enhanced Anti-HIV Env Responses Compared to Controls

To start assessing a potential role for NK cell-mediated HIV-specific responses in the control of HIV infection, we sought to test the hypothesis that HIV-specific NK cell responses would be detectable in HIV-exposed seronegative individuals and important for protection against HIV acquisition. This hypothesis is in line with previous reports demonstrating that NK cells in HIV-exposed seronegative individuals exhibit strong anti-HIV NK cell activity (116, 189-193) and are associated with protection from infection(191, 192, 194-198). To test this hypothesis, we used samples from a previously described cohort of HIV-seronegative men at high risk of HIV acquisition(173, 174). One reported characteristic of this cohort included subclinical endotoxemia that correlates with decreased CD4/CD8 T cell ratio, elevated plasma cytokine levels and markers of T cell exhaustion(173). In this study, we compared the ability to eliminate HIV Env-pulsed B-LCL between NK cells isolated from 8 HIV-exposed seronegative individuals and those from 5 healthy controls. CEF peptide pool and K562 cells served as positive controls. Killing of HIV Env-pulsed B-LCL by NK cells from HESN was not significantly different from that mediated by NK cells isolated from healthy individuals (**Figure 3**). Additionally, both groups similarly eliminated positive control target cells such as K562 cells and B-LCL pulsed with CEF peptide.

Altogether, these results suggest that NK cells in HIV-exposed seronegative individuals do not express elevated responses in exposure to HIV Env than in healthy individuals. Further investigations are warranted to determine the potential role of HIV-specific NK cells in HESN.

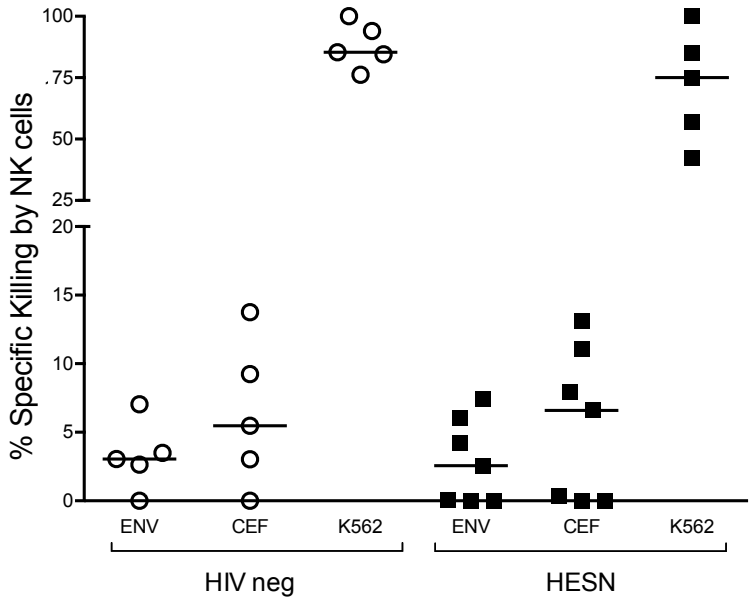


Figure 3. NK cells from HIV-exposed seronegative individuals do not mediate HIV Env-specific responses above background

HIV Env-specific killing by peripheral blood NK cells isolated from 8 highly exposed seronegative (HESN) subjects and 5 healthy donors. Autologous NK cells were co-cultured with Violet dye-labeled B-LCL pulsed with an Env or CEF peptide pool at 5:1 effector-to-target ratio. K562 cells and CEF were used as positive control. y-axis: percent specific killing; x-axis: cohorts with different conditions. Horizontal lines indicate the median percentages. White dots represent individual data for each HIV- negative subject; black squares represent individual data for each HESN participant.

3.2 PART II: ANTIGEN-SPECIFIC NK CELLS IN INFLUENZA

3.2.1 Intranasal Immunization against Influenza Enhances NK Cell Responses to Hemagglutinin

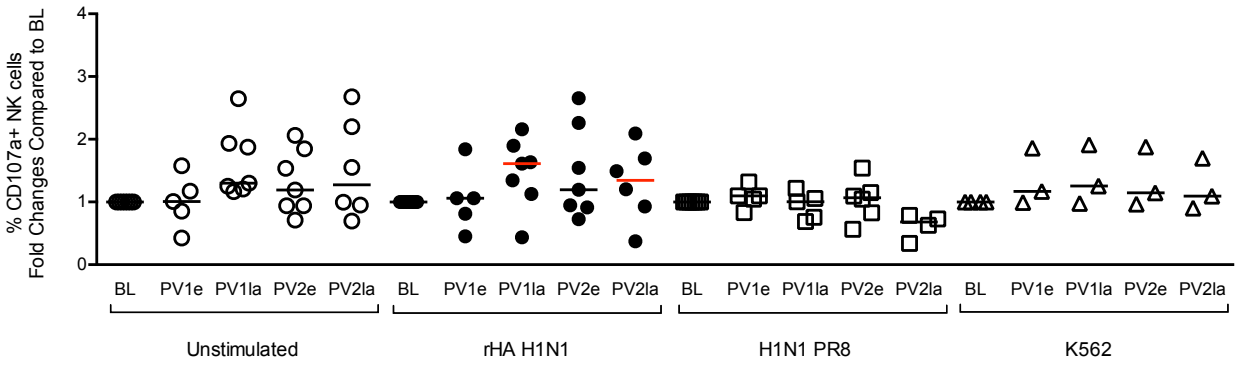
Numerous studies have highlighted the early and pivotal role of NK cells in the control of influenza infection (32, 142-156). It is known that IFN-γ production by NK cells is enhanced following influenza infection or vaccination(146, 159, 160), and increased antigen-specific IFN-γ responses to influenza mediated by NK cells with low NKp46 surface expression up to 6 months post-influenza immunization have been described(161). Previous studies by Dr. Jost et al. reported that influenza vaccination promotes the expansion of a subset of NK cells expressing high levels of 2B4 ($2B4^{bright}$) and low levels of NKp46 ($NKp46^{low}$)(172), two surface receptors that mediate the lysis of influenza-infected cells by directly binding to the HA protein present at the cell surface(32, 62, 143). While it has been demonstrated that influenza-specific memory NK cells can protect mice against lethal influenza challenge(6, 90), the existence of long-lived NK cells with antigen specificity against influenza capable of efficiently eliminating influenza-presenting target cells remains to be demonstrated in humans.

To start addressing NK cell memory of influenza in humans, we employed repeated vaccinations with live attenuated influenza (FluMist), based on the assumption that repeated exposure to live attenuated influenza would result in different kinetics of expansion of influenza-specific NK cells from one vaccination to the next. To do so, 7 healthy volunteers were immunized with three consecutive nasal sprays of FluMist at one-month intervals. We hypothesized that influenza-specific memory NK cells would expand to higher frequencies during the second and third application of the vaccine and display enhanced functionality against influenza compared to non-influenza-specific NK cells in the same individual, consistent with the concept of immunological memory. We assessed upregulation of the surrogate marker of degranulation CD107a as well as IFN-γ and tumor necrosis factor (TNF)-α production in response to the live A/PR/8/34 H1N1 virus (H1N1 PR8), a recombinant A/Brisbane/59/2007 HA protein (rHA H1N1) or HLA class I-deficient K562 cells by NK cells from 7 healthy subjects pre- and post-vaccination. Of note, one of the three influenza strains included in the FluMist formulation is A/South Dakota/6/2007 (H1N1) (A/Brisbane/59/2007-like) and HA from this strain is 99% similar to the rHA H1N1 used in our assays (NCBI BLAST®)(199). Changes in

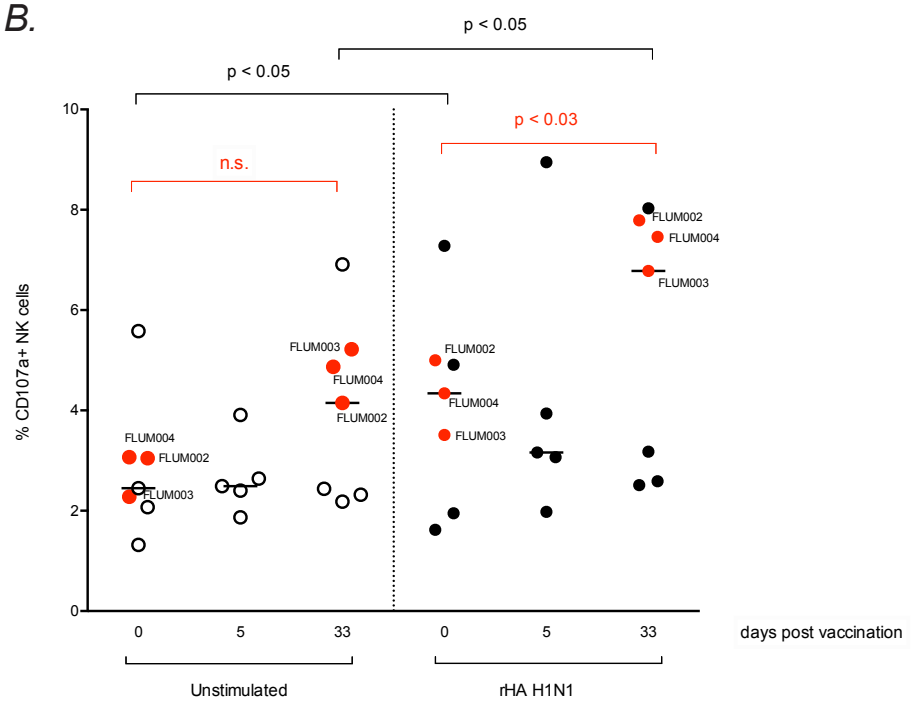
NKp46 and 2B4 expression on NK cells were also monitored over time. Since NK cell IFN-γ responses to influenza after vaccination vastly depend on IL-2 production by influenza-specific T cells(182, 183), we performed our experiments using CD3-depleted PBMC to eliminate any influenza specific-CD4+ T cell-dependent NK cell responses. This intracellular staining flow cytometry-based assay has been optimized in the Jost lab to measure virus-specific NK cell responses.

While CD107a expression remained similar to that at baseline on NK cells left unstimulated or stimulated with H1N1 PR8 or K562 cells, there was a clear trend towards increased NK cell degranulation in response to rHA H1N1 at about one month post-first and second vaccination (PV1la/PV2la)(**Figure 4A and B**). When considering raw data, it appeared clearly that the observed increase in CD107a was driven by 3 out of the 7 subjects analyzed who presented detectable post-vaccination response (**Figure 4B**). For those 3 responders, CD107a is significantly increased in response to the recombinant HA protein 33 days after the first FluMist vaccination. Enhanced CD107a upregulation could not be detected in peripheral blood NK cells 5 days post-first and second vaccination (PV1e/PV2e), possibly reflecting the temporary recruitment of NK cells with activity against influenza at the site of infection (i.e. respiratory tract). IFN-γ or TNF-α production by NK cells did not change significantly over time (**Figure 4C-F**). Thus, NK cells from subjects immunized against influenza revealed elevated CD107a expression levels during re-exposure to antigens derived from influenza strains similar to those included in the vaccine. NK cells co-cultured with live or inactivated influenza strains that were not included in the vaccine did not show augmented responses post-vaccination.

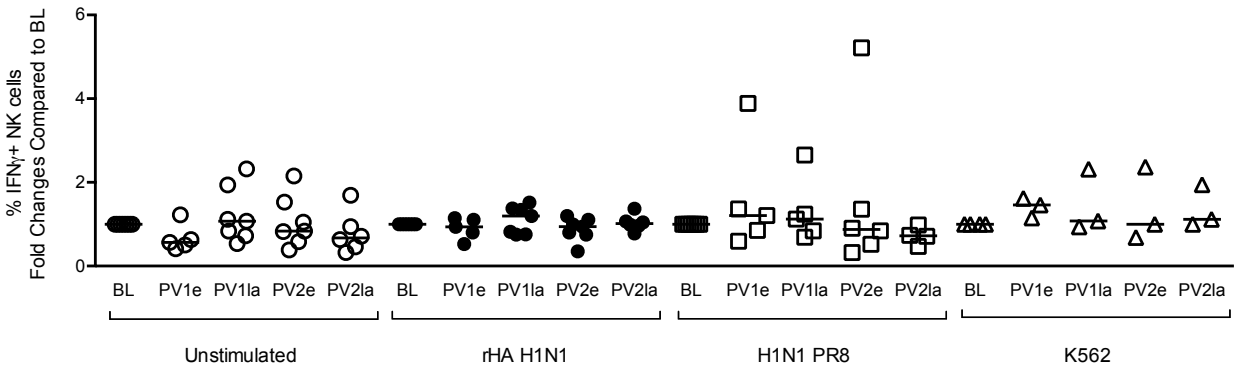
A.


% CD107a+ NK cells
Fold Changes Compared to BL
4
3
2
1
0
BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la
Unstimulated
rHA H1N1
H1N1 PR8
K562

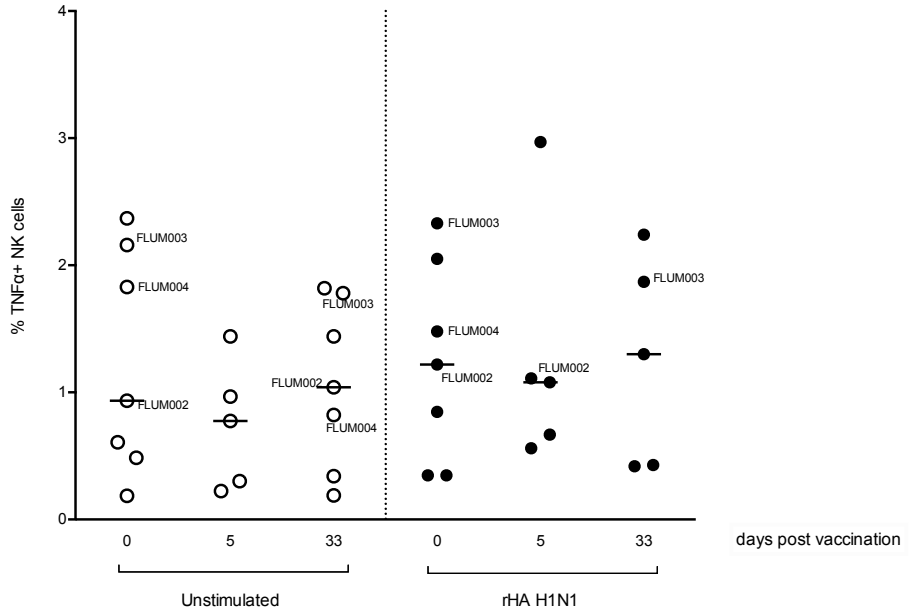
B.


p < 0.05
p < 0.05
p < 0.03
n.s.
% CD107a+ NK cells
10
8
6
4
2
0
FLUM002
FLUM004
FLUM003
FLUM003
FLUM004
FLUM002
FLUM004
FLUM002
FLUM003
FLUM004
FLUM002
FLUM003
0 5 33 0 5 33
days post vaccination
Unstimulated
rHA H1N1

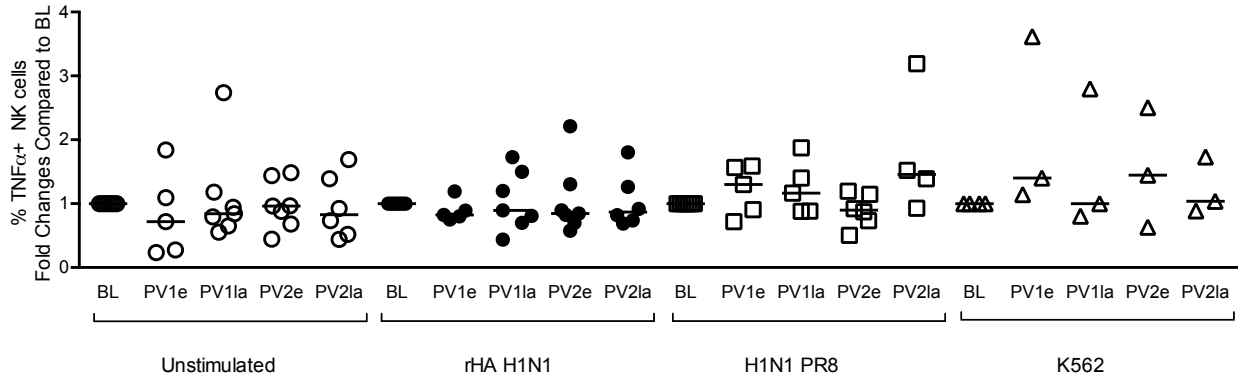
C.


% IFNγ+ NK cells
Fold Changes Compared to BL
6
4
2
0
BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la
Unstimulated
rHA H1N1
H1N1 PR8
K562

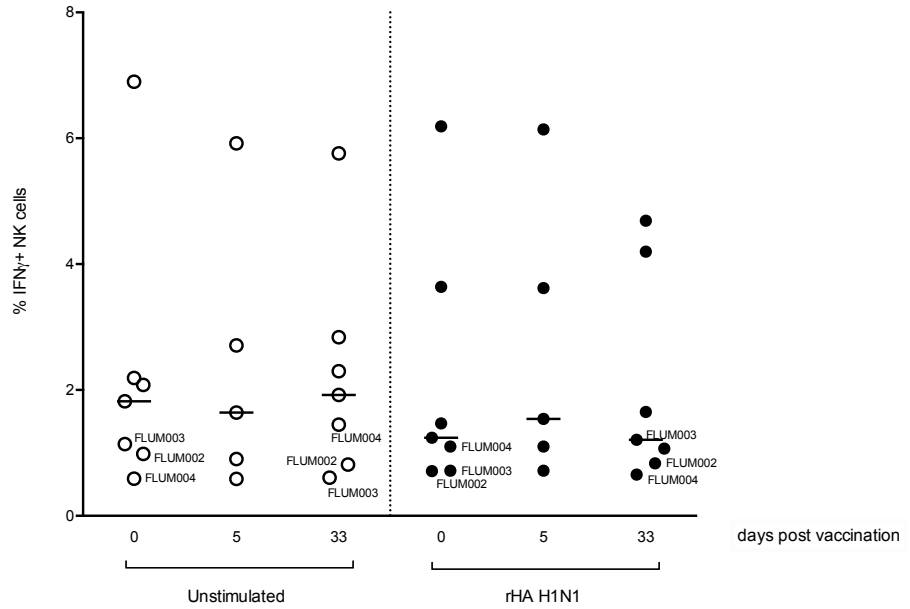
D.


% TNFα+ NK cells
4
3
2
1
0
FLUM003
FLUM004
FLUM002
FLUM003
FLUM002
FLUM004
FLUM003
FLUM004
FLUM002
FLUM002
FLUM003
0 5 33 0 5 33
days post vaccination
Unstimulated
rHA H1N1

E.


% TNFα+ NK cells
Fold Changes Compared to BL
0
1
2
3
4
BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la BL PV1e PV1la PV2e PV2la
Unstimulated
rHA H1N1
H1N1 PR8
K562

F.


% IFNγ+ NK cells
0
2
4
6
8
FLUM003
FLUM002
FLUM004
FLUM002
FLUM004
FLUM003
FLUM004
FLUM003
FLUM002
FLUM003
FLUM002
FLUM004
0 5 33 0 5 33
days post vaccination
Unstimulated
rHA H1N1

3.2.2 Higher Proportions of 2B4+ NK Cells Following Immunization with FluMist

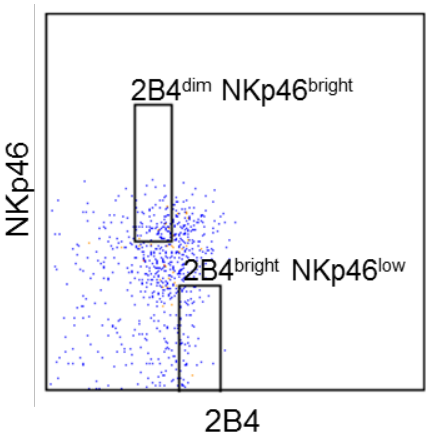
Based on previous results by Dr. Jost's laboratory showing expansion of a subset of NK cells expressing high levels of 2B4 ($2B4^{bright}$) and low levels of NKp46 ($NKp46^{low}$) following intramuscular vaccination with inactivated trivalent influenza vaccines(172), we were interested to see whether the expansion of a subpopulation of NK cells with similar features is associated with enhanced NK cell degranulation against recombinant HA in volunteers receiving FluMist vaccine (**Figure 4A and B**). We first quantified proportions of 2B4+ NK cells as well as percentages of $2B4^{bright}NKp46^{low}$ and $2B4^{dim}$ $NKp46^{bright}$ NK cells in unstimulated samples from each of the 7 individuals, comparing pre- and post-vaccination (**Figure 5A**). We observed a significant increase in proportions of 2B4+ NK cells one month post-first vaccination (PV1la) compared to baseline (BL) ($p = 0.03$), whereas 2B4+ NK cells did not show any sign of expansion 5 days post-first and second vaccination, possibly reflecting early recruitment of NK cells at the site of infection (**Figure 5B**).

Figure 4. Serial immunization with FluMist specifically enhances NK cell responses to a recombinant H1N1 HA that closely matches one of the vaccine strains

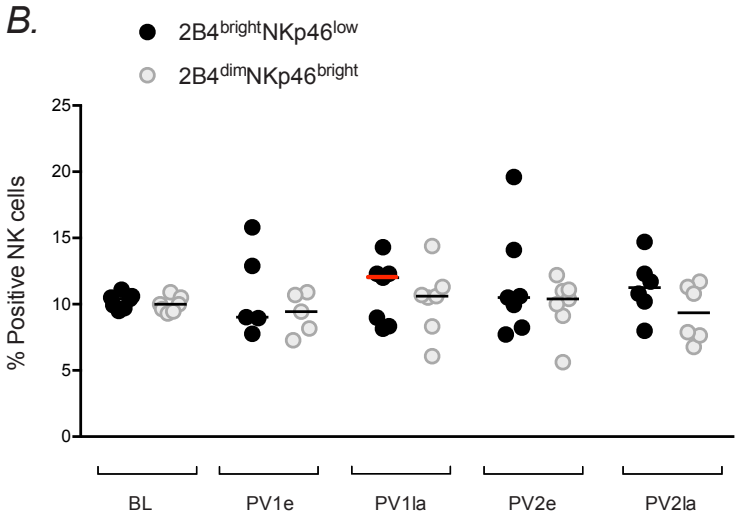
Percentages of NK cells subsets stimulated with recombinant HA H1N1 or live H1N1 PR8 virus in 7 individuals immunized with FluMist. Unstimulated PBMC and K562 cells served as negative and positive control, respectively. Horizontal lines indicate the median percentages. Dots, squares and triangles show single individual results for the respective conditions. Samples from all 7 subjects were not available for all time points. ***A.*** *Percentages of CD107a expression on NK cells stimulated with either rHA H1N1 or live H1N1 PR8 virus. CD107a upregulation was examined using fold changes comparing PBMC approximately 5 days (PV1e/PV2e) and 33 days (PV1la/PV2la) after each vaccination to baseline (BL).* ***B.*** *Percentages of CD107a expression on NK cells stimulated with rHA H1N1 and unstimulated NK cells compared to baseline using raw data approximately 5 days and 33 days after the first vaccination. 3 subjects marked as FLUM002, FLUM003 and FLUM004 showed significant results.* ***C.*** *Percentages of IFNγ expression on NK cells stimulated with either rHA H1N1 or live H1N1 PR8 virus. IFNγ expression was analysed using fold changes comparing PBMC approximately 5 days (PV1e/PV2e) and 33 days (PV1la/PV2la) after each vaccination to baseline (BL).* ***D.*** *Percentages of IFNγ expression on NK cells stimulated with rHA H1N1 and unstimulated NK cells compared to baseline using raw data approximately 5 days and 33 days after the first vaccination.* ***E.*** *Percentages of TNFα expression on NK cells stimulated with either rHA H1N1 or live H1N1 PR8 virus. IFNγ expression was analysed using fold changes comparing PBMC approximately 5 days (PV1e/PV2e) and 33 days (PV1la/PV2la) after each vaccination to baseline (BL).* ***F.*** *Percentages of TNFα expression on NK cells stimulated with rHA H1N1 and unstimulated NK cells compared to baseline using raw data approximately 5 days and 33 days after the first vaccination.*

Furthermore, we could not detect significant longitudinal changes in proportions of 2B4^{bright}NKp46^{low} or 2B4^{dim}NKp46^{bright} NK cell subsets after vaccinations (PV1e/PV-1la and PV2e/PV2la) compared to baseline (**Figure 5C**). However, percentages of 2B4^{bright}NKp46^{low} NK cells tended to be higher one month post-first and second vaccination (PV1la/PV2la) compared to NK cells at baseline or 5 days post- vaccination (PV1e/PV2e), particularly in 4 out of 7 vaccinees. The mean fluorescence intensity of 2B4 or NKp46 on NK cells did not significantly change for any of the time points (**Figure 5D**).

A.



B.



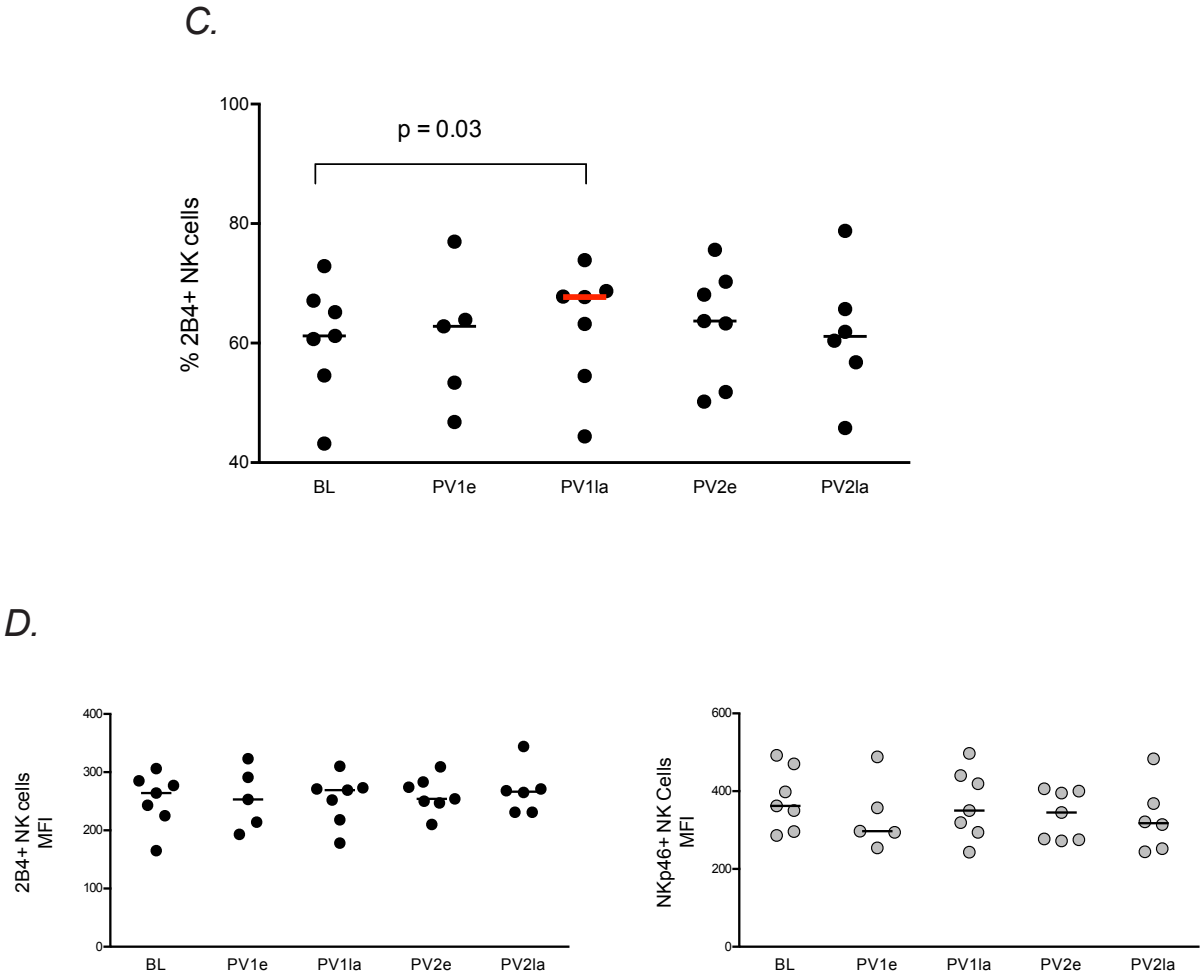
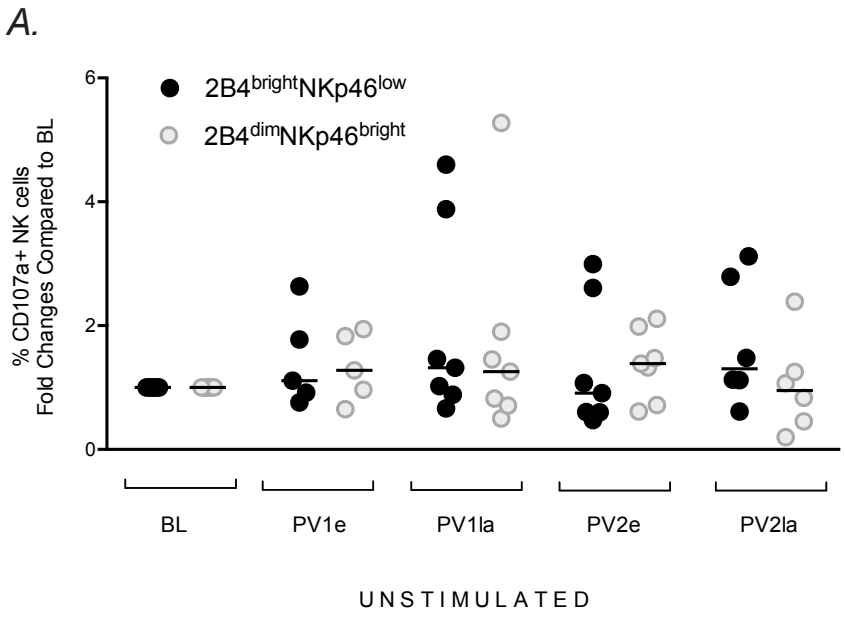


Figure 5. Expansion of 2B4+ NK cells post-vaccination

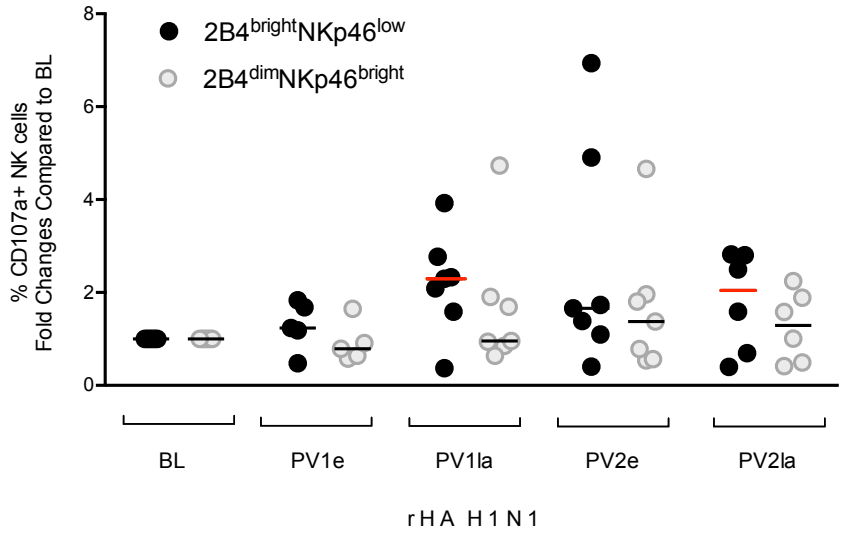
Percentages of NK cell subsets expressing either high levels of 2B4 (2B4^{bright}) and low levels of NKp46 (NKp46^{low}) or low levels of 2B4 (2B4^{dim}) and high levels of NKp46 (NKp46^{bright}) in the peripheral blood of 7 healthy subjects immnunized with FluMist. Unstimulated PBMC were evaluated at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination and compared to baseline (BL). Percentages show the portion of the respective subsets compared to the total of all NK cells (y-axis). Horizontal lines indicate the median percentages. Dots represent each individual result for the respective subset and time point (x-axis) ***A****. NK cell phenotype was assessed using FlowJo software gating for the specific subsets of 2B4^{bright} NKp46^{low} and 2B4^{dim}NKp46^{bright}* ***B****. Percentages of 2B4+ NK cells from unstimulated PBMC at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination* ***C****. Percentages of 2B4^{bright}NKp46^{low} and 2B4^{dim}NKp46^{bright} NK cells from unstimulated PBMC at 5 days (PV1e/PV2e) and one month (PV1la/PV-2la) post-first and second vaccination compared to baseline* ***D****. Mean fluorescence intensity (MFI) of 2B4+ and NKp46+ NK cells for the different time points.*

3.2.3 2B4^{bright}NKp46^{low} NK Cells Mediate Enhanced Responses against rHA H1N1 Following FluMist Immunization

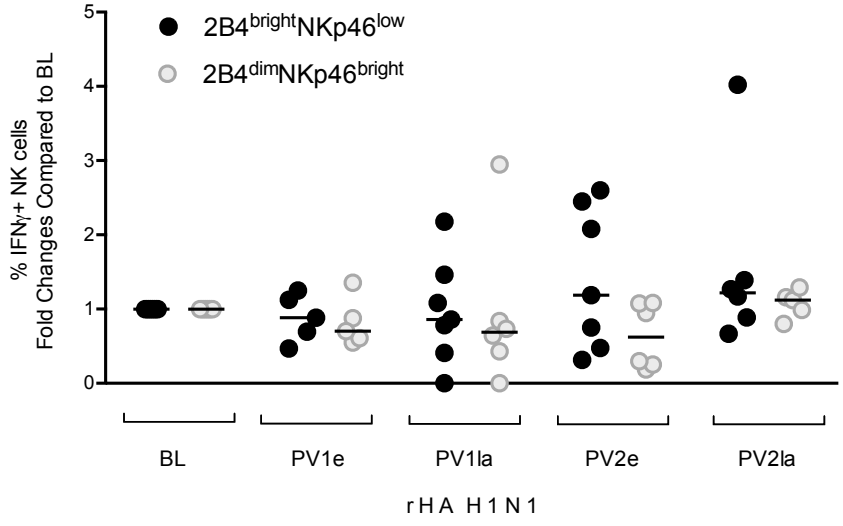
In order to compare the function of the 2B4^{bright}NKp46^{low} and the 2B4^{dim}NKp46^{bright} NK cells, we assessed upregulation of CD107a, IFN-γ and TNF-α on those subsets in response to H1N1 PR8, rHA H1N1 and K562. Although we could not detect a significant expansion of the 2B4^{bright}NKp46^{low} NK cell subsets in the peripheral blood, CD107a upregulation by 2B4^{bright}NKp46^{low} NK cells was increased compared to that of 2B4^{dim} NKp46^{bright} NK cells at 33 days post-first and second vaccination (PV1la/PV2a) in response to rHA H1N1 (**Figure 6A and B**). Proportions of CD107a+ 2B4^{bright}NKp46^{low} NK cells were approximately 2-times higher in response to the rHA H1N1 protein 33 days post-first vaccination compared to unstimulated NK cells. Fold changes for CD107a expression in 2B4^{bright}NKp46^{low} NK cells 33 days post-second vaccination were elevated and only slightly lower compared to the results for one month post-first vaccination (**Figure 6B**). Responses from 2B4^{dim}NKp46^{bright} NK cells or either subset to H1N1 PR8 or K562 did not differ from baseline over time (**Figure S6.1 - S6.3**, **appendix**). NK cells stimulated with rHA H1N1 from either subset did not show enhanced IFN-γ expression at any of the time points. Thus, the 2B4^{bright}NKp46^{low} NK cell subset expressed higher CD107a levels upon restimulation with recombinant influenza HA 33 days after the first and second immunization (PV1la/PV2la) compared to baseline.



B.


2B4brightNKp46low
2B4dimNKp46bright
% CD107a+ NK cells
Fold Changes Compared to BL
8
6
4
2
0
BL
PV1e
PV1la
PV2e
PV2la
rHA H1N1

C.


2B4brightNKp46low
2B4dimNKp46bright
% IFNγ+ NK cells
Fold Changes Compared to BL
5
4
3
2
1
0
BL
PV1e
PV1la
PV2e
PV2la
rHA H1N1

3.2.4 Immunization with FluMist Enhances Influenza-Specific Killing by NK Cells in Subjects Who Have Been Previously Exposed to Influenza

In order to confirm that intranasal influenza vaccination with live attenuated viruses enhances the ability of NK cells to specifically recognize and eliminate influenza- presenting target cells, we used an alternative assay developed in the Jost laboratory to measure virus-specific NK cell cytotoxic activity. To do so, we compared NK cell-mediated killing of autologous immortalized B cells pulsed with an influenza peptide pool prior to and 2 months following the second FluMist immunization. We could show an overall 5-fold-increase in influenza-specific killing by NK cells from individuals who reported previous influenza vaccination (less than 2 years prior to FluMist immunization) or infection, with one subject displaying similar responses pre- and post-FluMist vaccination (**Figure 7**). In contrast, FluMist vaccination did not boost NK cell-mediated killing of target cells presenting influenza in individuals who had never been vaccinated, nor that of K562 cells or target cells loaded with yellow fever peptide pool, which we used as positive and negative controls, respectively. To conclude, these data suggest that influenza exposure might lead to the development of influenza-specific NK cells that can persist for at least a year and display recall responses upon re-exposure to influenza. Alternatively, it might be that, independently of their influenza history, only half of the vaccinated subjects mounted influenza-specific NK cell responses that are detectable in the blood.

Figure 6. $2B4^{bright}NKp46^{low}$ NK cell subset mediates enhanced responses to rHA H1N1 following serial immunization with FluMist

Percentages of CD107a and IFN-γ expression on NK cells expressing high levels of 2B4 ($2B4^{bright}$) and low levels of NKp46 ($NKp46^{low}$) or low levels of 2B4 ($2B4^{dim}$) and high levels of NKp46 ($NKp46^{bright}$) stimulated with recombinant HA H1N1 in 7 individuals immunized with FluMist. Bars represent median percentages. PBMC were evaluated at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination and compared to baseline (BL). Percentages show the portion of the respective subsets compared to the total of all NK cells (y- axis). Horizontal lines indicate the median percentages. Dots represent each individual result for the respective subset and time point (x-axis). Samples from all 7 subjects were not available for all time points. ***A****. Percentages of CD107a expression on NK cells from unstimulated PBMC at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.* ***B****. Percentages of CD107a expression on NK cells from PBMC stimulated with rHA H1N1 at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.* ***C.*** *Percentages of IFN-γ expression on NK cells from PBMC stimulated with rHA H1N1 at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.*

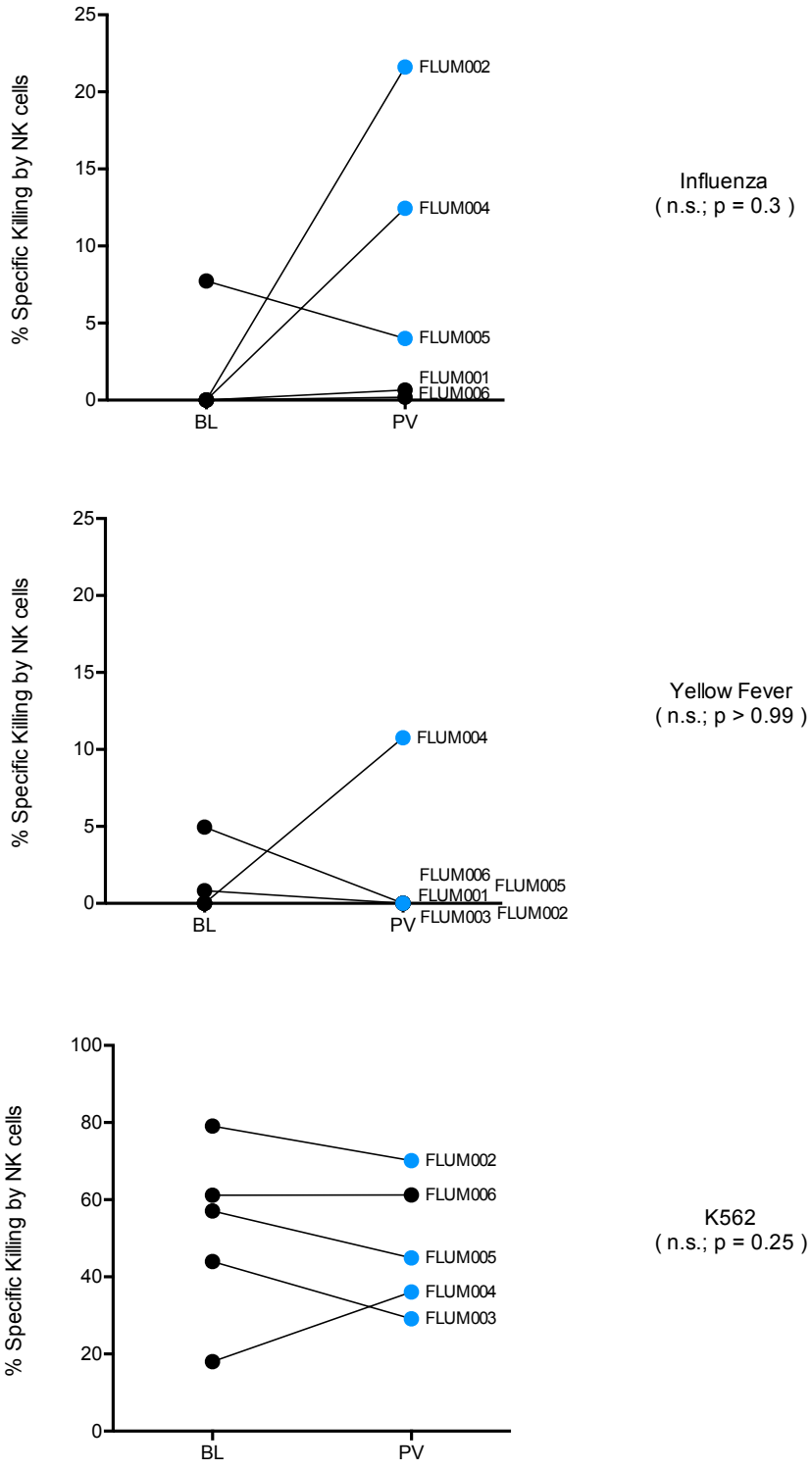


Figure 7. Enhanced NK cell-mediated influenza-specific killing in vaccinees with previous exposure to influenza

Influenza-, and yellow fever-specific lysis as well as lysis of K562 target cells by bulk NK cells isolated from 5 subjects immunized with FluMist. Cytotoxic function of NK cells was assessed using a CAM killing assay before (BL) and 2 months post-second vaccination (PV). Yellow fever vaccination history is not known. The blue dots highlight the four subjects (FLUM002, FLUM003, FLUM004, FLUM005) that were exposed to influenza prior to this study.

4 Discussion

Prior studies presented accumulating evidence on murine NK cells that mediate true antigen-specific recall responses and on immunized rhesus macaques that are able to display immunological recall responses for at least a period of 5 years(14, 184). Subsets of human NK cells exhibit adaptive features following CMV infection (68, 70, 73, 74, 185) and exposure to specific combinations of cytokines(78, 80). In addition, accumulating data suggest human NK cells can mediate virus-specific recall responses(13, 15, 48-52, 99, 186). Notably, the team led by Paust demonstrated VZV-specific memory NK cell responses in VZV-experienced human volunteers as well as vaccination-dependent, antigen-specific human NK cell recall responses to HIV-encoded envelope protein in humanized mice(15). Furthermore, Wang et al. just now revealed the importance of the WNT transcription factor TCF7 in the forming of a specific NK cell memory subtype in humans, which is also found to be crucial in the formation of memory T cells(16). Interestingly, HIV-1-specific cytokines were able to reproduce this NK cell memory subtype. Corroborating these findings, work by Jost and Reeves recently presented at several international HIV conferences showed that human memory NK cells can be induced by HIV and HIV vaccines(186, 187).

Our data build on these prior studies and while we could not find any evidence of HIV-specific NK cells in HESN individuals, our results strongly suggest influenza- specific NK cell responses in human volunteers that received FluMist, an intranasal live attenuated influenza vaccine. After immunization with FluMist, we detected degranulation responses by peripheral blood NK cells, and more particular by the $2B4^{bright}$ $NKp46^{low}$ NK cell subset, against a recombinant HA H1N1 influenza protein that closely matches the H1N1 vaccine strain (**Figure 4A and B**). In addition, influenza-specific killing by NK cells was increased post-FluMist vaccination in individuals that had been previously exposed to influenza (**Figure 7**).

4.1 Antigen-Specific NK Cells in HIV

Based on preliminary results obtained in the Jost lab supporting the existence of antigen-specific NK cells against the HIV protein Gag (**Figure 2**), we started exploring if HIV-specific responses might be associated with the control from HIV acquisition in a cohort of HESN individuals. We tested the hypothesis that HIV- specific NK cell responses would be stronger in HESN than in healthy individuals, and partly responsible for protection against infection with HIV. Former studies on highly exposed seronegative subjects primarily presented data on the expansion of specific NK cell phenotypes and augmented NK cell cytotoxicity as well as natural resistance to HIV (116, 189-193), whereas there are to our knowledge no published studies evaluating HIV-specific NK cell responses in HESN. Using an antigen- specific NK cell killing assay, we detected background levels of NK cell-mediated killing of Env-pulsed target cells (<10%) by NK cells from HESN subjects, similar to Env-specific killing by NK cells from low-risk HIV-negative controls.

One limitation of these experiments is that while non-specific lysis was assessed by measuring killing of unpulsed target cells, we did not test NK cell killing of target cells pulsed with an irrelevant peptide pool as negative control. Virus-specific NK cell killing assays currently performed in the Jost lab include killing of control target cells pulsed with peptides derived from a human protein (self-peptides). NK cells are considered reactive against a specific viral antigen when they mediate specific killing above zero after subtracting non-specific killing and at least twice above specific killing of self-peptides-pulsed control target. Interestingly, killing activity against self- peptides typically ranges between 0% and 10%, suggesting that any response under 10%, as the ones we observed against HIV Env in our HESN and low-risk HIV- negative cohorts is not specific and reflect background responses.

Another limitation of this study is that we only had access to peripheral blood NK cells. While we can detect HIV Gag-specific peripheral blood NK cell responses in a cohort of chronically HIV-infected subjects (**Figure 2**), this study focused on subjects that were highly exposed to the virus but not chronically infected, and it is therefore possible that exposure to HIV antigens is restricted in magnitude and location in HESN, with only few viruses reaching mucosal barriers of the gastrointestinal tract and being available to activate potential tissue resident NK cells. One can speculate that a restricted

number of HIV-specific memory NK cells might reside in the GI tract and only expand and circulate in the peripheral blood upon productive HIV infection and high virus titers. Alternatively, the liver and potentially the spleen have been proposed as homing sites for memory NK cells in mice and NHP. Thus, it would be interesting to see whether assays using NK cells isolated from those tissues can detect HIV-specific memory NK cell responses in HESN. However, such experiments would be challenging, as access to such tissues in healthy individuals is restricted for obvious ethical reasons.

Finally, it has been evaluated that 30%-60% of HESN subjects lack detectable T cell responses to HIV(200, 201), and that in those individuals displaying HIV-specific T cell responses, the breadth and magnitude are significantly lower than in people living with HIV(202, 203). We can speculate that the proportion of individuals displaying HIV-specific NK cell responses, as well as the frequency of their circulating HIV-specific NK cells, which are already low among HIV-infected patients, are even lower among HESN. Therefore, it is likely that a larger cohort might be required to detect HIV-specific NK cell responses in HESN.

Further investigations are warranted to determine the potential role of HIV-specific NK cells in HESN. In consideration of the current literature, the goal is to include more study subjects and test NK cell activity against additional peptide pools (i.e. peptide pools derived from human proteins or other HIV antigens), which might provide us with data showing representative differences between HESN and healthy controls in either peripheral blood or tissues.

4.2 Antigen-Specific NK Cells in Influenza

In parallel to the HESN cohort, I had access to samples from a unique cohort of individuals who received consecutive intranasal immunizations with the FluMist vaccine to explore NK cell recall responses to influenza. We found that serial immunization specifically enhanced degranulation responses to the rHA H1N1 protein approximately 30 days after first and second vaccination (PV1Ia/PV2Ia). Furthermore, in accordance with previous reports by the Jost lab(172), we detected higher proportions of 2B4+ NK cells in the blood following immunization with FluMist. Interestingly, it was the $2B4^{bright}NKp46^{low}$ NK cell subset that seem to mediate these enhanced degranulation responses against rHA H1N1 following vaccination. However, although the $2B4^{bright}$

NKp46^{low} NK cell subset mediated enhanced responses against rHA H1N1 following vaccination it did not significantly expand in the peripheral blood over time. Finally, we were interested to see whether the overall NK cell response against influenza was specifically directed against the virus in this cohort. Our results revealed an increase in NK cell-mediated cytolysis of influenza- pulsed B-LCL two months post-second vaccination in a subset of vaccinees showing influenza-specific NK cell responses.

Our analysis revealed vaccination-associated changes in NK cell surface expression with the upregulation of 2B4 and downregulation of NKp46 (**Figure 5B**), findings that are in line with previous reports by Dr. Jost's lab (172). The activating NKp46 receptor has the ability to interact directly with the influenza antigen HA and is involved in cytolysis of influenza-infected cells(32, 143, 147). The co-activating receptor 2B4 can also bind influenza HA and co-stimulates NKp46-mediated cytotoxicity against influenza(204-206). Therefore, enhanced CD107a upregulation against rHA post-vaccination might mainly reflect the ability of those two NK cell receptors to bind HA and activate NK cells. Experiments to compare the function of 2B4^{bright}NKp46^{low} NK cells against other influenza antigens as well as against live A/Brisbane/59/2007(H1N1) virus pre- and post-vaccination are required to evaluate the full potential of this NK cell subset.

We performed all our ICS assays on CD3-depleted PBMC to prevent NK cell stimulation by IL-2 produced by influenza-specific T cells and to have a better sense of NK cell responses that are mediated by a potential direct recognition of influenza epitopes. Thus, it is unlikely that the observed function of 2B4^{bright}NKp46^{low} NK cells is mediated by IL-2. Further investigations to assess whether the observed T cell help-independent responses by NK cells could be triggered by cytokines like previously suggested (101) or depend on innate cytokines or on influenza-specific antibodies, which were not excluded from our assay, are warranted.

Generally, NK cell activation leads to a downregulation of activating NK cell receptors to prevent cell death. In consequence, enhanced post-vaccination activation of 2B4^{bright}NKp46^{low} NK cells against rHA likely reflects internalization of the receptor resuting from successful recognition of rHA and triggering of NK cell cytotoxicity by NKp46(161). Internalization of NKp46 upon ligation of HA might also reflect a mechanism of the influenza virus to infect the cell itself(148, 169). 2B4 did not fade from the NK cell surface, which might be owed to the fact that it mostly served as a co-signaling

receptor for NKp46 rather than triggering cytotoxicity itself. 2B4^{bright}NKp46^{low} peripheral blood NK cells did not show elevated responses right after the immunization (5 days post-vaccination), which might reflect a temporary recruitment of the NK cell subset at the site of infection (i.e. the respiratory tract) or to lymphoid organs (i.e. lymph nodes, spleen) and therefore can't be seen in the human blood 5 days post-vaccination. Furthermore, it would be interesting to see whether bulk hepatic or splenic as well as 2B4^{bright}NKp46^{low} NK cells in these organs would show significant influenza-specific responses, since it has been shown in animals that memory NK cells are mostly residing in these tissues(6, 14). However, like mentioned before, such experiments are challenging due to limited access to those tissues in healthy individuals. On top, different organs within the human body home different NK cell subpopulations with distinct phenotypes and functions, which might skew experimental results.

Interestingly, enhanced antigen-specific NK cell killing was observed in subjects previously exposed to influenza (FLUM002, FLUM003, FLUM004), either by vaccination or infection, but not in influenza-naïve subjects vaccinated twice with FluMist suggesting that FluMist might not allow efficient generation of influenza- specific NK cell memory. It is possible that the prior NK cell exposure to influenza might have already led to an alteration of NK cells. Since the antigen-specific response was directed against the recombinant HA H1N1, it would be interesting to know whether the influenza vaccine that FLUM002 and possibly also FLUM004 received contained a similar strain to the one in FluMist or the recombinant influenza HA protein (A/Brisbane/59/2007(H1N1)) used in the assay. Subject FLUM004 could not reproduce her vaccination record, subject FLUM002 reported to have been vaccinated once in 2007. According to the World Health Organization, the influenza H1N1 strain used in the trivalent vaccine in 2006-2007 was the A/New Caledonia/20/99 (H1N1)-like virus(207). In 2007-2008, it was the A/Solomon Islands/3/2006 (H1N1)-like virus that was used for the vaccine formulation(207). The A/New Caledonia/20/99 (H1N1) strain showed a 98%-similarity and the A/Solomon Islands/3/2006 (H1N1) a 99%-similarity to the A/Brisbane/59/2007 (H1N1) strain, that was used in the assay which itself is 99% similar to the H1N1 strain used in FluMist (NCBI BLAST®). Given the similarity of these strains, it is possible that the prior exposure led to an alteration of NK cells to respond in an antigen-specific manner to the rHA H1N1 strain that is similar to the strain contained in FluMist. In this context,

it would be interesting to see whether NK cells from responders are capable of killing target cells pulsed with antigens from heterosubtypic strains like H3N2, including the ones that are not part of influenza vaccines (i.e. H5N1, H7N9).

Similarly, it is also possible that the timing we used was not optimal. The subjects were vaccinated two or three times at intervals of one month, expecting an increased NK cell response after each vaccination. NK cell responses 5 days post-vaccination did not show any augmented NK cell activation, assuming that NK cells were still homed at the site of infection. True antigen-specific responses were detected 30 days post-second vaccination, indicating that either the process around the formation of antigen-specificity might take longer than previously assumed or that antigen- specific NK cells can only be seen in the blood by that time. Since only the subjects previously exposed to influenza showed enhanced antigen-specific immune responses, it would be interesting to see whether an expanded vaccination panel (i.e. 3-5 vaccinations) with broader time intervals would lead to increasing NK cell responses over time. Studies on T- and B-cell maturation after vaccination have shown that the optimal interval between the first immunization and the following booster shot should take at least 2-3 months(208, 209). Additionally, later blood draws (>30 days) might also reveal enhanced NK cell responses in subjects that have never been exposed before.

A confounding factor to any NK cell vaccination study could be the high rates of CMV infections in humans skewing the NK cell repertoire. It has been shown that subjects being infected with CMV showed lower NK cell IFN-γ production post-vaccination compared to control group(210). These dampened NK cell responses were primarily seen in the $CD56^{dim}$CD57+NKG2C+ NK cell subset. Interestingly, NK cell IFN-γ responses in HCMV+ subjects were enhanced after vaccination when being stimulated with cytokines like IL-12 and IL-18(211). Since the studied population is ought to have a HCMV seroprevalence of about 60%, enhanced responses by the $2B4^{bright}NKp46^{low}$ NK cell subset might be linked to cytokine stimulation or to co- expression of other key receptors, including CD2 and NKG2C with the latter being associated with individuals seropositive for CMV(48-50, 52). The role of this NK cell subset has to be further investigated, especially 30 days post-first and second vaccination and if other receptors or factors (i.e. CMV, cytokines) reshaping the NK cell repertoire play a bigger role in the grand picture.

While NK cells showed responses against the recombinant HA H1N1 there was no response in presence of live H1N1 influenza virus. It is very possible, that the specificity of NK cells is limited to a certain number of influenza strains. We used a live virus lab strain adapted to mice, which all subjects of the study cohort have never been exposed to. If influenza-specific NK cells mediate the observed NK cell responses against rHA H1N1, it suggests that these cells might have limited cross- reactivity to other influenza strains.

Although we obtained data on NK cells that showed elevated responses towards rHA H1N1 after immunization with FluMist and influenza-specific killing by NK cells two months post-second vaccination, none of the data reached statistical significance. This is likely a consequence of the small size of our cohort. Future experiments based on the current data with bigger subject pools and a greater assortment of blood samples are warranted to confirm those results. Also, a thorough characterization of isolated and clonally expanded $2B4^{bright}NKp46^{low}$ NK cells will give precious insights into the role of this subpopulation in influenza vaccination.

The Jost laboratory has now pursued those investigations and could isolate and expand single influenza-specific NK cells displaying heterosubtypic cytotoxic activity against influenza nucleoprotein. These new data suggest that influenza-specific NK cells that recognize conserved influenza antigens from serologically distinct strains exist in humans and have the potential to be harnessed in future vaccine strategies.

5 Conclusion

Overall, our findings show enhanced NK cell responses against a recombinant HA H1N1 protein by the 2B4^{bright}NKp46^{low} NK cell subset after serial immunization with FluMist as well as influenza-specific NK cell responses two months post-vaccination in humans. Long-lived NK cells with strong antiviral activity against influenza might represent a potential effector cell population that is able to recognize broader and more conserved regions of influenza than those targeted by T and B cells. Previous studies on NK cells in humans have revealed adaptive characteristics in presence of CMV and different cytokines(68, 70, 73, 74, 78, 80, 185) and even antigen-specific NK cells have been described(13, 48-52, 99). Just recently, two reports have been released presenting data on antigen-specific human NK cells after vaccination with HIV(15, 186), however vaccination-induced influenza-specific NK cells responses have not been explored so far.

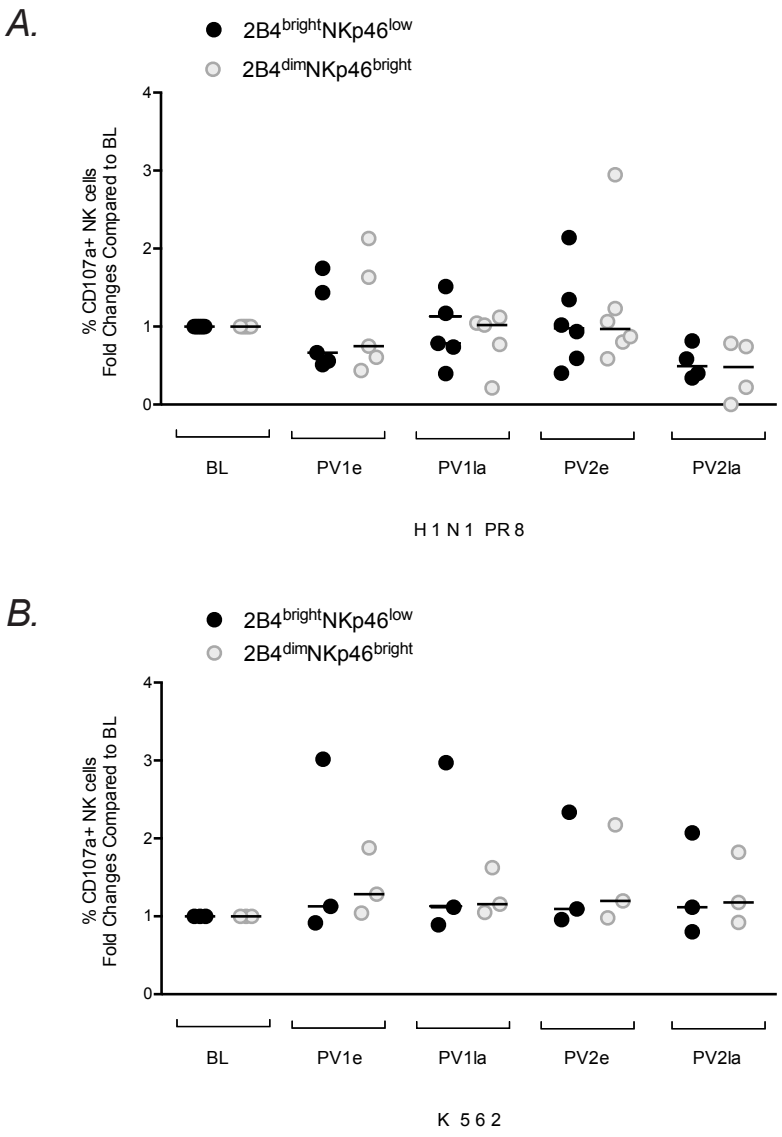
Despite the limitations of our study, our results suggest antigen-specific NK cell responses triggered by influenza vaccination. Although seasonal influenza vaccinations already exist, influenza is still a disease that causes severe illness especially to elderly and neonates due to limited vaccine efficacy against influenza strains that do not match the vaccine(138, 171). Being able to elicit antigen-specific memory NK cells by vaccination could significantly contribute to increasing the efficacy of future universal influenza vaccine harnessing the full force of the immune system.

6 Zusammenfassung

Unsere Ergebnisse zeigen gesteigerte NK Zell Antworten auf ein rekombinantes HA H1N1 Protein durch die spezialisierte 2B4^{bright}NKp46^{low} NK Zelle nach Mehrfachimpfung mit FluMist. Ebenso zeigten 2B4^{bright}NKp46^{low} NK Zellen Influenza-spezifische Immunantworten im Menschen 2 Monate post-Immunisierung. Langlebige NK Zellen mit starker antiviraler Aktivität gegen Influenza stellen eine mögliche effektive Zelllinie dar, welche fähig ist, im Vergleich zu T und B Zellen Influenza-spezifische Regionen in einem breiteren Spektrum zu erkennen und eliminieren. Vorangegangene NK Zell Studien im Menschen zeigten adaptive Charakteristika in Gegenwart von CMV und unterschiedlichen Zytokinen (68, 70, 73, 74, 78, 80, 185) und sogar Antigen-spezifische NK Zellen wurden beschrieben (13, 48-52, 99). Vor Kurzem wurden in zwei Berichten Daten über Antigen-spezifische NK Zellen in Menschen nach Impfung mit HIV veröffentlicht (15, 186), jedoch fehlen noch jegliche Berichte über Influenza-spezifische Zellen durch Vakzination.

Trotz der Limitationen in unserer Studie zeigen wir Antigen-spezifische NK Zell-Antworten, die durch Mehrfachimpfungen mit Influenza induziert wurden. Obwohl die saisonale Influenza-Impfung schon existiert, bereitet das Influenza Virus bei Älteren sowie Neugeborenen aufgrund fehlender Impfstoffeffizienz weiterhin besorgniserregende Krankheitsverläufe (138, 171), die oft auf einem Mismatch der Influenzastränge in der Impfung basiert. Antigen-spezifische Gedächtnis NK Zellen, die durch Vakzination induziert werden, könnten die Effizienz einer Influenza-Impfung signifikant steigern und somit die volle Kraft des Immunsystems nutzbar machen.

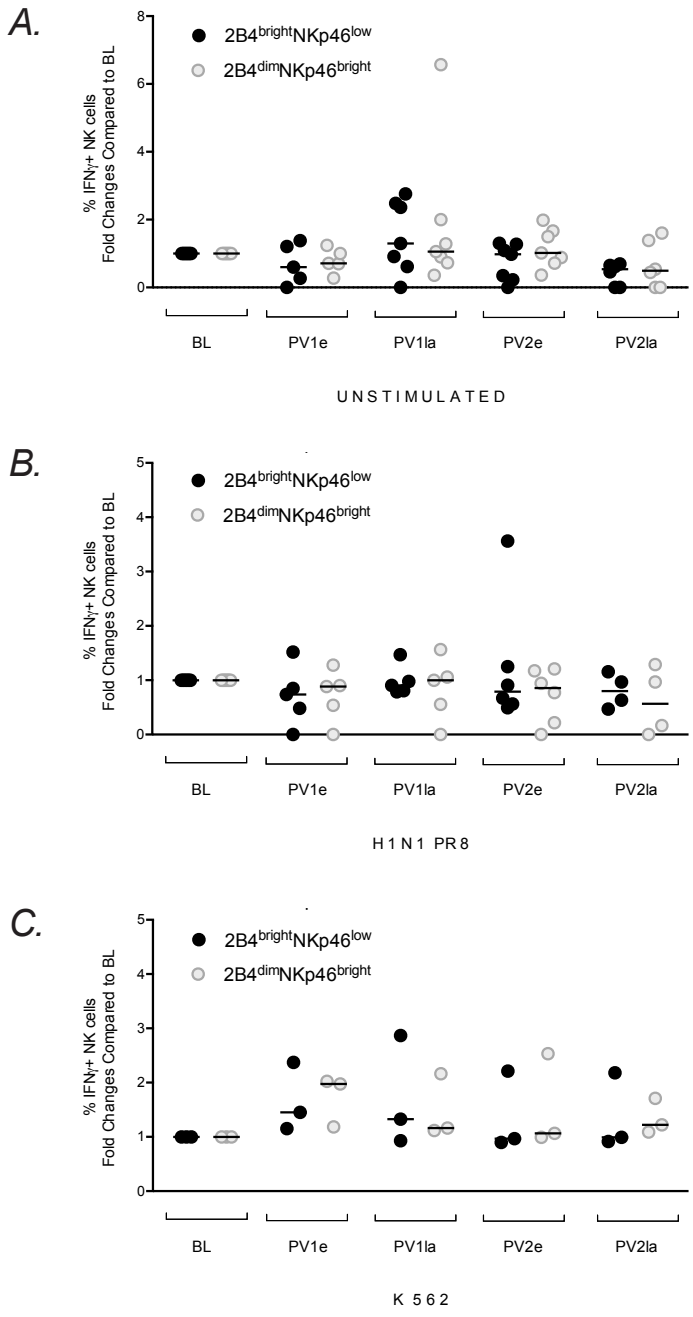
APPENDIX



Supplementary Figure S6.1. Percentages of CD107a expression on NK cells expressing 2B4 and NKp46 stimulated with H1N1 PR8 and K562

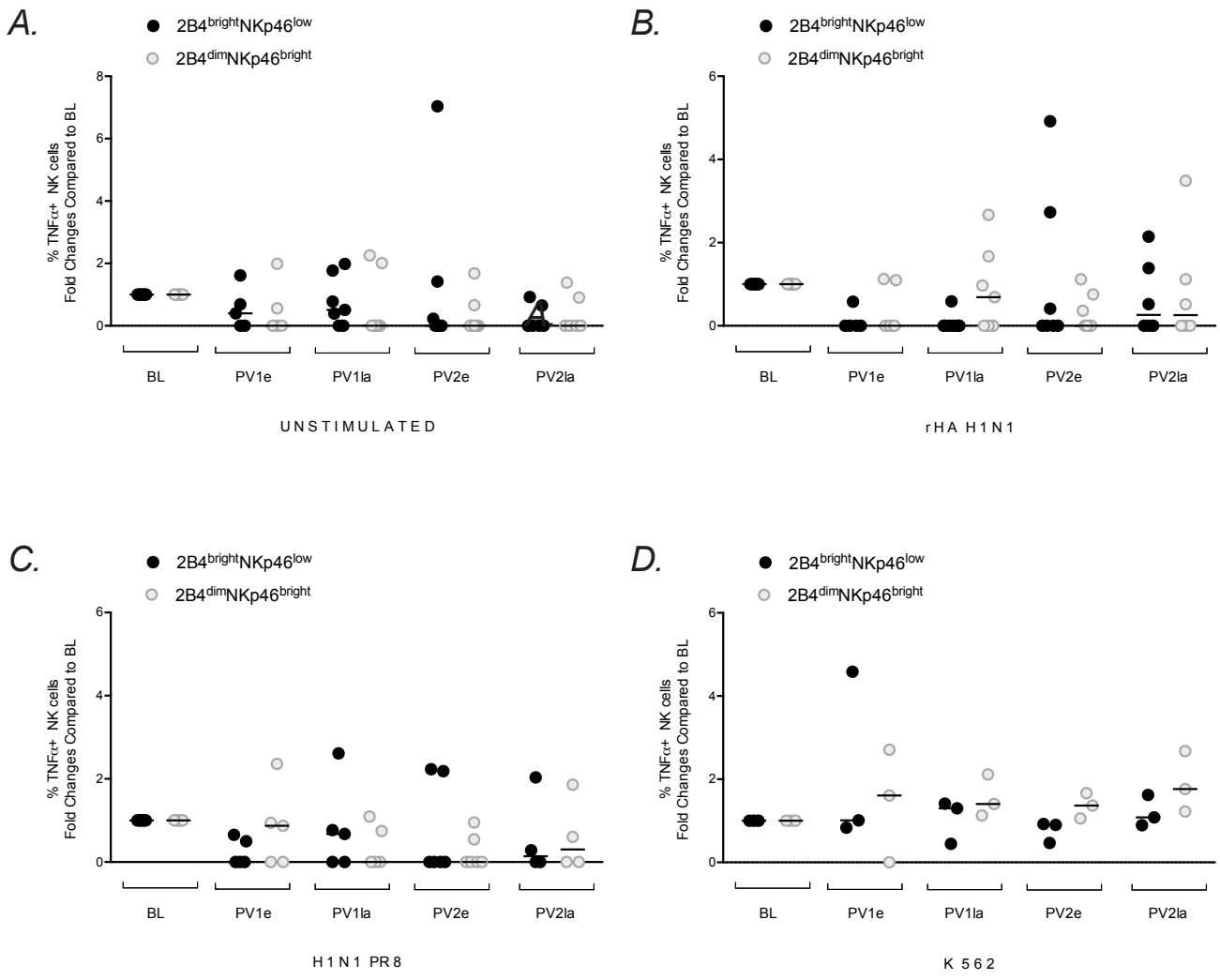
Percentages of CD107 expression on NK cells expressing high levels of 2B4 ($2B4^{bright}$) and low levels of NKp46 ($NKp46^{low}$) or low levels of 2B4 ($2B4^{dim}$) and high levels of NKp46 ($NKp46^{bright}$) stimulated with H1N1 PR8 and K562 in 7 individuals immunized with FluMist. Bars represent median percentages. PBMC were evaluated at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination and compared to baseline (BL). Percentages show the portion of the respective subsets compared to the total of all NK cells (y-axis). Horizontal lines indicate the median percentages. Dots represent each individual result for the respective subset and time point (x- axis). Samples from all 7 subjects were not available for all time points. ***A.*** *Percentages of CD107a expression on NK cells from PBMC stimulated with H1N1 PR8 at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.*

B. *Percentages of CD107a expression on NK cells from PBMC stimulated with K562 cells serving as positive control at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.*



Supplementary Figure S6.2. Percentages of IFNγ expression on NK cells expressing 2B4 and NKp46 unstimulated and stimulated with H1N1 PR8 and K562

Percentages of IFNγ expression on NK cells expressing high levels of 2B4 (2B4^{bright}) and low levels of NKp46 (NKp46^{low}) or low levels of 2B4 (2B4^{dim}) and high levels of NKp46 (NKp46^{bright}) unstimulated and stimulated with H1N1 PR8, K562 in 7 individuals immunized with FluMist. Bars represent median percentages. PBMC were evaluated at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination and compared to baseline (BL). Percentages show the portion of the respective subsets compared to the total of all NK cells (y-axis). Horizontal lines indicate the median percentages. Dots represent each individual result for the respective subset and time point (x- axis). Samples from all 7 subjects were not available for all time points. ***A.*** *Percentages of IFNγ expression on NK cells from unstimulated PBMC at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.* ***B.*** *Percentages of IFNγ expression on NK cells from PBMC stimulated with H1N1 PR8 at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.* ***C.*** *Percentages of IFNγ expression on NK cells from PBMC stimulated with K562 cells serving as positive control at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.*



Supplementary Figure S6.3. Percentages of TNFα expression on NK cells expressing 2B4 and NKp46 unstimulated and stimulated with rHA H1N1, H1N1 PR8 and K562

Percentages of TNFα expression on NK cells expressing high levels of 2B4 (2B4^{bright}) and low levels of NKp46 (NKp46^{low}) or low levels of 2B4 (2B4^{dim}) and high levels of NKp46 (NKp46^{bright}) unstimulated and stimulated with rHA H1N1, H1N1 PR8 and K562 in 7 individuals immunized with FluMist. Bars represent median percentages. PBMC were evaluated at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination and compared to baseline (BL). Percentages show the portion of the respective subsets compared to the total of all NK cells (y-axis). Horizontal lines indicate the median percentages. Dots represent each individual result for the respective subset and time point (x-axis). Samples from all 7 subjects were not available for all time points. ***A.*** *Percentages of TNFα expression on NK cells from unstimulated PBMC at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.* ***B.*** *Percentages of TNFα expression on NK cells from PBMC stimulated with rHA H1N1 at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.* **C.** *Percentages of TNFα expression on NK cells from PBMC stimulated with H1N1 PR8 at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.* ***D.*** *Percentages of TNFα expression on NK cells from PBMC stimulated with K562 cells serving as positive control at 5 days (PV1e/PV2e) and one month (PV1la/PV2la) post-first and second vaccination compared to baseline.*

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