Influence of NK Cells on the Anti-influenza Immune Response

By

David L. Rose

(Under the direction of Kimberly Klonowski and S. Mark Tompkins)

Abstract

Natural Killer (NK) cells act as early defenders against influenza infection by directly recognizing and responding to the presence of viral hemagglutinin, but are also capable of modulating the subsequent anti-viral CD8 T cell response. In these studies, we utilized an NK cell depletion model to investigate the contribution of NK cells to the generation and development of anti-influenza specific memory CD8 T cells. We found that the absence of NK cells during primary influenza infection resulted in increased numbers of influenza-specific memory CD8 T cells present in the lung and lung draining lymph node. Furthermore, this enlarged anti-influenza memory CD8 T cell pool was capable of mediating heterosubtypic protection against lethal viral challenge with fewer reactivated CD8 T cells infiltrating the respiratory tract, likely through increased early production of the anti-viral cytokines IFN-γ and TNFα. Additionally, we utilized *in vitro* influenza infection and protein expression models to examine how hemagglutinin intrinsic features, such as receptor binding specificity and glycosylation levels, impact NK cell activation. We confirmed that influenza hemagglutinin can functionally activate NK cells in an NKp46 dependent manner. We demonstrated that human NKp46 preferentially binds to avian origin hemagglutinins when compared to those of human origin suggesting hemagglutinin sialic acid binding preference may contribute to NK cell activation. However, we also found evidence of previously undescribed sialic acid independent interactions mediating binding of NKp46 to influenza hemagglutinin. Lastly, we found that increasing the glycosylation level of influenza hemagglutinin led to decreased NKp46 binding suggesting that the natural accumulation

of glycosylation sites by circulating influenza strains may act as a viral mechanism to evade NK cell activation. Together these data suggest that modulation of NK cell activation represents a novel method to improve anti-influenza memory CD8 T cell generation during vaccination and that mechanisms of NK cell activation should be considered in rational vaccine design.

INDEX WORDS: NK cells, CD8 T cells, memory, Influenza, respiratory

Influence of NK Cells on the Anti-influenza Immune Response

by

David L. Rose

B.S., Clarion University of Pennsylvania, 2010

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GA

2016

Influence of NK Cells on the Anti-influenza Immune Response

by

David L. Rose

Major Professors: Kimberly Klonowski
S. Mark Tompkins
Committee: Wendy Watford
Balazs Rada
Ralph Tripp

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2016

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

Introduction and literature review

Introduction

Influenza infections are the cause of significant morbidity and mortality which result in an estimated 3-5 million cases of severe respiratory infection and 250,000-500,000 deaths annually (1). The causative agent, generally Influenza A in humans, is an enveloped member of the Orthomyxoviridae family of RNA viruses and can be categorized by the expression of the hemagglutinin (HA) and neuraminidase (NA) proteins expressed on the viral surface (2). These two surface proteins are also the major antigenic targets of the humoral immune response to influenza A, and as such they represent the major targets for current anti-influenza vaccines (3). To date vaccines against influenza A have shown mixed results, owing primarily to the high rate of mutation within the HA and NA segments of the genome as well as the ability of this virus to undergo genetic reassortment resulting in poor protection against disparate viral strains (3). In an effort to develop a better, broadly protective, and consistently effective vaccine, the generation of anti-influenza CD8 T cells has become a major focus of recent design strategies.

CD8 T cells, like the humoral response, are part of the adaptive immune system. However unlike B cells which primarily target the externally expressed HA and NA proteins, CD8 T cells target highly conserved epitopes contained within internal viral proteins which show little propensity for mutation and similarity across viral strains (3). Additionally, CD8 T cells are known to be important for proper viral clearance following infection and protection against subsequent viral infections (4, 5). Thus the generation of anti-influenza memory CD8 T (T_{MEM}) cells represent an ideal supplement to and possible alternative for current vaccine strategies. Unfortunately, the generation of T_{MEM} is a complex and multifaceted process which has only been partially elucidated.

The generation of T_{MEM} first requires activation of the T cell through recognition of cognate antigen presented by a professional antigen presenting cells (APC), usually a dendritic cell (DC). This process of

activation requires peptide antigen be presented to the CD8 T cell by a DC in the context of a MHC-I molecule and in the presence of proper costimulatory molecules and cytokines which allow the T cell to undergo clonal expansion (6). Cytokines also play an important role in determining the eventual fate of individual T cells and whether or not they will survive to become memory cells (7). For example, pro-inflammatory cytokines such as type I interferons as well as IL-12 promote the activation and expansion of effector CD8 T cells (8). In addition to DCs and intracellular cytokine signaling, which are crucial for T cell development, other often neglected accessory cells can also play an important role in shaping the T cell response. One such accessory cell type, Natural Killer (NK) cells, have been shown to modulate the CD8 T cell response in a variety of ways including direct and indirect inhibition of T cell proliferation and expansion (9). Importantly in the context of influenza infection, NK cells are abundant in the lung and lung airways (the site of influenza infection), and are able to both recognize and be directly activated by influenza infected cells (10, 11). Thus, NK cells present a promising and novel mechanism which may be utilized to modulate the CD8 T cell response and promote the generation of long-lived, protective memory CD8 T cells.

Natural Killer Cells

Natural Killer (NK) cells were first described in 1975 based on their characteristic ability to recognize and lyse aberrant tumor cells without the need for prior stimulation or the presence of professional APCs (12, 13). Since their discovery, much more has been elucidated about their development, maturation, and functions. NK cells develop from common lymphoid progenitor (CLP) stem cells which also give rise to T cells, B cells and the recently described innate lymphoid cells (ILC) (14, 15). While T and B cells utilize RAG enzymes for recombination of highly specialized antigen-specific immune receptors, NK cells do not require RAGs and instead utilize a plethora of non-specific, non-rearranged immune receptors to survey and protect their host suggesting that NK cell development diverges early from that of T and B cells (14, 16-18). Interestingly the newly characterized ILCs and NK cells share similar developmental pathways, and it has been suggested that NK cells could be re-categorized as ILC type 1 cells (19). Early NK cell development and lineage commitment appears to take place primarily in the bone marrow as targeted bone

marrow irradiation depletes mice of NK cell activity, and this development has been well characterized in murine models (20). Alternatively, NK cells can arise from hematopoietic stem cells (HSC) and early NK cell precursors (NKP) found in circulation, lymphoid tissues and non-lymphoid tissues (21). It is possible that these HSCs and NKPs in peripheral tissues allow for the development of tissue specific NK cells in a number of organs including the lungs, however more thorough investigations are needed to confirm this hypothesis (22).

NK cells require the expression of CD122 (IL-2Rβ) as signaling through IL-2 and IL-15 are crucial for development and survival *in vivo*, although a combination of IL-7, SCF, and Flt3-L are able to compensate for lack of IL-15 *in vitro* (15, 23). IL-15 signaling is known to promote expression of survival factor Bcl-2 in NK cells as well as the transcription factor Nilf3 (24, 25). Nilf3 in turn promotes expression of the transcription factor Id2, a key transcription factor in development of ILCs and NK cells which prevents development into myeloid cells or B cells by blocking binding of Tcf3 (26). A number of other transcription factors including Ets-1, GATA-3, and Blimp-1 also have distinct roles in delineating the stages of NK cell development (27-29). The T-box transcription factor T-bet, has recently been found to stabilize NK cells in an early, immature developmental stage. Another T-box transcription factor, EOMES is necessary to push past this stage to fully mature NK cells characterized by expression of the full range of NK cell receptors (30). Additionally, the loss of EOMES expression causes NK cells to regress back to an immature phenotype (30). This suggests a step-wise induction of transcription factors is responsible for complete functional maturation of NK cells and expression of a complete repertoire of surface receptors.

Mature natural killer cells are capable of expressing a large array of germ-line encoded receptors on their surface to aid in identification of threats from both host tissue and foreign pathogens. Broadly speaking, these receptors can be classified into two categories: inhibitory and activating or co-activating receptors. In mice the Ly49 family of receptors acts mainly as inhibitory receptors while in humans the killer immunoglobulin-like receptor (KIR) family takes on this role (31). There are some exceptions, however, as both families contain a number of activating receptors as well (32). Activating receptors do not fall into a single broad family of receptors, but all share common signaling pathways by associating with

immunoreceptor tyrosine-based activation motif (ITAM) bearing transmembrane proteins (18). These accessory proteins include DAP-10, DAP-12, FcεRI, and CD3-ζ which upon stimulation leads to the phosphorylation by Src family and ZAP-70 kinases beginning a signaling cascade culminating in cytoskeletal reorganization and degranulation as well as transcriptional activation of various cytokines and chemokines (18, 32). On the other hand, the inhibitory receptors all possess cytosolic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which upon stimulation recruit lipid and tyrosine phosphatases to the cell surface, essentially blocking ITAM signaling via dephosphorylation (18). It is thought that proper NK cell function and regulation requires a balancing act between activating and inhibiting signals and relies on an early "education" of NK cells.

As our understanding of NK cell biology has increased there has been a shift from an early "missing-self" hypothesis, in which NK cells recognize and kill target cells on the basis of missing or downregulated MHC-I expression, to a more encompassing hypothesis on NK cell education. One of the main reasons for this thought shift is the discovery that nearly 15% of murine splenic NK cells do not express an inhibitory receptor that recognizes MHC-I or non-classical MHC-I like molecules and that these cells are still able to fully mature, despite functional hyporesponsiveness to MHC-I deficient and tumor cell lines (33). Additionally, it has been found that NK cells from C57BL/6 mice can express anywhere from 0-3 MHC-I recognizing inhibitory receptors and that the number of these receptors expressed correlates with the level of NK cell responsiveness (34). Current literature points to a "tuning model" of NK cell education in which individual NK cells receive varying amounts of activating and inhibiting signals based on their environment and which receptors are expressed (35). In this theory, all signals are taken into account and the NK cell responds proportionally to the net amount of activating signal received. During the education stage of development, NK cells receiving higher levels of inhibitory signaling will have a high responsive state to stimulation while NK cells not receiving these inhibitory signals (through MHC-I binding receptors) will become hyporesponsive (35). Due to the high levels of heterogeneity in NK cell receptor expression within any given animal a wide range of responsiveness from individual NK cells provides a gradient

consisting mostly of an intermediate and protective level of responsiveness from the population as opposed to deleterious hyper or hypo-responsive extremes.

As previously stated, once mature NK cells reach their activation threshold they are capable of lysing target cells (12, 13). In addition to their cytolytic capabilities, activated NK cells are potent producers of pro-inflammatory cytokines and chemokines, and can act as both an early warning system and first line of defense against invading pathogens (36, 37). For example, active NK cells can produce CCL3, CCL4, and RANTES which act to recruit leukocytes to the site of infection (38, 39). Additonally, NK cells are known to be potent producers of the pro-inflammatory cytokines IFN-γ and TNFα (38). IFN-γ is a pleotropic cytokine which stimulates the production of a large number of interferon stimulated genes (ISGs), and generally promotes inflammation, the induction of a host anti-viral state, and the recruitment of immune cells (40, 41). TNFα similarly acts to promote inflammation and the anti-viral state via signaling through a number of pathways including NFκB and MAPK/ERK (42, 43). Activation of NK cells, and thus their downstream effects, is governed by a delicate balance of both activating and inhibitory receptors expressed on the NK cell surface (44). Importantly, as these receptors primarily recognize MHC-I and MHC-I like receptors they are not specific to a single antigen, but have the potential to become activated by multiple different pathogens (36).

NK cells, though morphologically and functionally similar, compose an extremely heterogenic population when characterized by expression of surface receptors. Almost all human NK cells express CD56 and CD16, and human NK cells have been defined as $CD3^{-}CD56^{+}$ lymphocytes (26). More recently, the natural cytotoxicity receptor NKp46 (NCR1 in mice), which is conserved across many mammalian species, has been shown to be present on almost all human and murine NK cells (45) and has since been used as an NK cell specific identification marker. Although NK cells do share these common receptors their differences are abundant. Recent studies have predicted that there could be as many as 30,000 different NK cell phenotypes within a single human based on NK cell activator and inhibitory receptor expression and that no phenotype constituted more than 7% of the total NK cell population (46). Additionally, using adult twins and unrelated donors it was found that NK cell inhibitory receptors are controlled more strictly by

genetics, while expression of activating receptors are dependent on the environment (46) suggesting a role for pathogens in shaping NK cell repertoires.

NK cells contribute to, but are not sufficient for protection against a number of human pathogens including systemic infections such as malaria and Ebola virus as well as respiratory infections like tuberculosis and influenza (47). This is due to the fact that after development in the bone marrow, NK cells continually migrate through peripheral and lymphatic tissues, utilizing the circulatory and lymphatics systems, in order to mediate immune surveillance for aberrant cells and infectious pathogens (48). NK cells are generally short lived and constitute only a small fraction of lymphocytes in lymphoid and peripheral tissues and constitute only 5%-15% of lymphocytes in the human peripheral blood (26). Similarly, in mice NK cells constitute only around 2% of peripheral blood lymphocytes (36). However, natural killer cells are especially important in the respiratory tract, as they constitute a large proportion of lymphocytes in the lung tissue during the steady state, are functionally mature, and quickly accumulate following respiratory infection (10, 48). Once activated, an NK cell's primary function is elimination of aberrant host cells expressing ligands for NK cell activating receptors, however they also have secondary functions which can affect the downstream adaptive immune response (9, 47, 48).

The ability of NK cells to modulate the adaptive immune response is not a new concept, as NK cells have long been appreciated for their ability to secrete pro-inflammatory cytokines and contribute to DC maturation via NK-DC cross talk (36, 49). More recently, however, it has become apparent that NK cells often act to limit the magnitude of the adaptive immune response through a number of mechanisms (9). For instance, NK derived IL-10 and TGF-β can act as negative regulators of T cell activation (50, 51). Similarly, NK cells can indirectly impact T cell activation by limiting antigen availability via lysis of target cells and lysis of DCs (9). Finally, NK cells are also able to directly lyse activated T cells which upregulates expression of NK activing receptor ligands and downregulates ligands for NK inhibitory receptors (52, 53). Together, these NK cell functions can act as an immunological rheostat which acts to stimulate T cells early after infection and dampen the response over time.

CD8 T cells

CD8 T cells are members of the adaptive immune response which share a number of commonalities with NK cells. Like NK cells, CD8 T cells are known to be potent producers of pro-inflammatory cytokines such as IFN-γ and TNFα and their primary function is the lysis of aberrant and infected target cells (43, 54, 55). However, while NK cells express a multitude of activating receptors which recognize a broad span of antigens, each CD8 T cell expresses only a single T cell receptor (TCR) which is highly specific for a single peptide epitope (6). Similarly, while NK cells undergo an "education" process to limit unwanted activation by healthy host tissues, CD8 T cells undergo a "selection" process in the thymus to protect the host against autoimmunity (44, 56). In contrast to NK cells however, once this selection process is over naïve T cells preferentially migrate to secondary lymphoid tissues rather than peripheral tissues (6). From here, naïve CD8 T cells survey the lymphoid tissues until they come into contact with an activated DC which is expressing the T cell's cognate antigen in the context of MHC-I (6).

The activation and differentiation of CD8 T cells is a complex and multifactorial process. The initial priming of CD8 T cells requires three signals. First the T cell receives stimulation through its TCR when it complexes with its cognate antigen presented on an MHC-I molecule by an APC, generally a DC. Additionally, the DC must be expressing costimulatory molecules (such as CD80 and CD86). Finally, CD8 T cell activation requires the presence of pro-inflammatory cytokines in the priming microenvironment (6). Once all three have been received the T cells undergo clonal expansion and differentiation with some destined to become short lived effector cells (SLECs) while other memory precursor cells (MPECs) survive as long-term memory cells protecting against future infection due in part to their expression of the IL-7Rα subunit (CD127) (57). The factors determining T cell differentiation and fate determination are still unclear, however some factors have been elucidated. For example, the strength and length of TCR signaling have both been implicated in memory development, with strong TCR signaling associated with terminal differentiation while weaker and shorter signaling is associated with memory formation (58). Similarly, antigen availability can alter CD8 T cell differentiation with memory formation showing an inverse correlation with antigen availability (58). Additionally, the priming environment can greatly impact CD8

T cell fate determination with the pro-inflammatory cytokine IL-12 driving terminal effector differentiation by stimulating the production of the transcription factor T-bet, while the anti-inflammatory IL-10 promotes memory formation via as yet undetermined mechanisms (59, 60). The interplay of these and likely other factors on individual T cells culminates in programming them as either SLECs, which express high levels of the marker Killer Cell Lectin Like Receptor G1 (KLRG1) and low levels of CD127, or MPECs, which express high levels of CD127 and do not express KLRG1 (57).

Throughout the contraction phase of the response MPECs are protected from apoptosis via IL-7 signaling through CD127 and many survive as long term memory cells (61, 62). These surviving memory cells can be broadly categorized into central (T_{CM}) and effector (T_{EM}) memory subsets based on surface expression of unique markers which mediate their localization to distinct tissues – secondary lymphoid tissues and peripheral tissues respectively (63). Futhermore, within the T_{EM} population a distinct subset of tissue resident memory cells (T_{RM}) has recently been characterized by their localization to specific peripheral tissues and limited mobility thereafter (63). T_{RM} cells recovered from many tissues also tend to express a combination of the activation marker CD69 as well as the integrin CD103, however expression is variable based on the tissue microenvironment as exemplified by low expression of both CD69 and CD103 on T_{RM} recovered from respiratory tissues (63). This particular subset of CD8 T cell memory, T_{RM}, is especially important from mediating long-term protection against respiratory influenza infection as these cells have been shown to mediate heterosubtypic immunity and loss of T_{RM} CD8 T cells correlates with loss of protection against the virus (5, 64, 65).

Recognition of Influenza A by CD8 T cells and NK cells

Influenza A viruses are members of the Orthomyoviridae family which possess a single stranded, segmented RNA genome which encodes a number of internal and external viral proteins (2). In humans, influenza infection occurs primarily in the respiratory tract which possesses a unique immune environment (2, 63). As previously mentioned, CD8 T cells require priming by DCs to become activated and execute their functions. Respiratory DCs (RDC) can be divided into two major subsets based on external expression

of expression of the markers CD103 and CD11b as well as localization of these subsets to the respiratory epithelium and lung parenchymal tissues respectively (66, 67). Once activated, $CD103^+$ DCs migrate to the draining lymph node and are responsible for priming the CD8 T cell response (68, 69). The majority of this DC migration from the infected lung to the draining lymph node occurs within the first 24-48 hours following infection, with different strains of influenza virus exhibiting differential kinetics of DC migration (70, 71). While these $CD103^+$ DCs have been shown to be crucial for the development of the CD8 T cell response to influenza, lymph node resident $CD8^+$ DCs have also been shown to be capable of presenting antigen suggesting that multiple DC subsets may likely contribute to CD8 T cell priming (72-74). Additionally, TNF/iNOS producing DCs (tip-DCs) are thought to be important for migration of activated CD8 T cells to the lung and or maintenance of the CD8 T cell response within the lung tissue as loss of these cells results in decreased numbers of antigen-specific CD8 T cells in the lung (75). CD8 T cells primarily recognize epitopes derived from the internal nucleoprotein (NP) and components of the viral RNA polymerase (PA and PB1) which are highly conserved across a broad range of divergent viral strains (3, 76). This feature allows CD8 T cells to both contribute to the clearance of a primary influenza infection as well as mediate heterosubtypic immunity against disparate influenza A viruses via early activation of CD8 T_{RM} cells (3, 64). Once activated, the influenza-specific CD8 T cells must undergo clonal expansion to acquire cytolytic functions, as these functions appear to be tied to cellular division, with increased function correlated to increased number of divisions (77). The accumulation of effector CD8 T cells in the lung and lung airways as well as the acquisition of their effector functions peaks around 10 days post infection in mice, corresponding to viral clearance (77, 78). The timing of the CD8 T cell response and viral clearance in mice as well as studies utilizing CD8 T cell depletion have demonstrated the importance of effector CD8 T cells to the control and clearance of influenza A infection (78). Furthermore, human studies have shown that the presence of influenza-reactive CD8 T cells prior to infection correlates with decreased disease severity (79). Similarly, murine studies have demonstrated that memory CD8 T cells are long lived and capable of mediating heterosubtypic immunity (3, 5). Importantly, these memory cells are poised for quick anti-viral response upon secondary challenge with their cognate antigen, allowing them to limit early

viral replication (80, 81). Together, these studies demonstrate the importance of CD8 T cells in clearance of primary influenza infections as well as the control of and protection against secondary infections with disparate influenza viral strains.

While CD8 T cells recognize highly conserved internal viral proteins, NK cells recognize the ectopically expressed viral hemagglutinin (HA) protein and mediate early viral control during primary influenza infection (11, 82). The viral HA protein is expressed in a trimeric form on the surface of infected cells and virions (2). The expression of this protein on the surface requires co-translation of the monomers at the host cell's ER for proper folding and translocation to occur (83). The resulting trimeric HA protein is then glycosylated, shipped through secretory pathway, and arrives on the cell surface in an inactive (HA0) form (84). Once on the cell surface, the inactive HA0 form of the protein is cleaved by host proteases at a conserved basic amino acid or in some cases at a polybasic cleavage site to produce the active form of the viral HA consisting of linked HA1 and HA2 subunits (85). Thus, the resulting active trimeric HA protein consists of 3 HA monomers, each composed of two linked subunits (HA1 and HA2) which form globular head (HA1) and stem (mostly HA2) regions of the protein respectively (85). At the distal end of the globular head is a highly conserved pocket which mediates binding to sialic acid (86). The tertiary structure of the HA binding pocket confers a preference for HA binding to either α2,3 (mainly recognized by avian origin viruses) or α 2,6 (mainly recognized by human origin viruses) linked sialic acids (87-89). Although the receptor binding pocket itself is highly conserved, the overall hemagglutinin protein is prone to mutation (86, 90). Furthermore, these mutations can be guided by immune selection pressures such as the presence of host neutralizing antibodies (90, 91). Although these mutations can alter and/or mask antigenic sites in order to evade humoral immunity, they cannot greatly alter the ability of the HA to bind to sialic acids without incurring a significant reproductive cost (91). Thus, immune recognition mechanisms which are mediated through the highly conserved sialic acid binding function of the influenza HA would be less susceptible to immune evasion via HA mutation.

Recognition of viral HA by NK cells is mediated primarily through interactions with the NK cell activating receptor NKp46 in mice and humans and to a lesser extent the activating receptor NKp44 in

humans – which is not expressed by mice (11, 92). NKp46 is a transmembrane member of the Immunoglobulin superfamily which has been noted for its activation of NK cell cytolytic activities against a broad range of target cells and has been shown to directly bind to a number of hemagglutinin proteins expressed by influenza A viruses (11, 93, 94). This binding interaction is mediated by the viral HA, which binds to specific sialic acid residues expressed on the NKp46 receptor (95). Point mutations of the NKp46 receptor have revealed that the sialic acid residues which mediate the HA-NKp46 interaction are attached to the T225 (recognized by H1 and H5 hemagglutinins) and N216 (recognized by H5 hemagglutinins) amino acids of human NKp46 via O-linked glycosylation (93). *In vitro* this HA-NKp46 interaction has been shown to be sufficient for activation of both cytokine production as well as cytolysis (11, 96). Furthermore, studies utilizing *in vitro* infection of target cells with human origin (preferring α2,6 sialic acid linkages) and avian origin viruses (preferring α2,3 sialic acid linkage) found that cells infected with avian origin influenza viruses demonstrated an increased ability to activate NK cells as demonstrated by increased cytotoxicity (93). Additionally, co-culture of human NK cells with virus like particles derived from disparate influenza viruses found that HAs from different viral strains differentially activated the NK cells as demonstrated by increased cytokine production and relative levels of the NK cell activation marker CD69 (97). It is unclear however why disparate influenza HAs differentially activated NK cells as neither of these studies looked for a mechanism of action. A combination of murine and porcine models as well as human studies have further demonstrated that this interaction of NKp46 and HA proteins is essential for optimal NK cell activity against influenza viruses (98-100). Early experiments found that *in vitro* recognition and cytolysis of influenza infected cells by human NK cell lines was mediated in large part by NKp46 (11), and these experiments were expanded upon using a number of disparate strains of influenza (92, 93, 101, 102) finding that NKp46 can recognize and kill cells expressing H1, H3, and H5 subtypes of influenza HA. A study by Gazit *et al.* additionally found that transgenic mice in which the gene for NKp46 has been replaced by a GFP cassette were more susceptible to influenza but not vaccinia infection and that increased susceptibility was not due to deficiency in NK cell trafficking to the site of infection (103).

A recent study using swine found that porcine NKp46 is able to directly bind to influenza H1, similar to its murine and human homologues. Furthermore, this study found that NKp46^{+} but not NKp46- NK cells were recruited from the blood into the lung tissue of influenza infected animals over the first 3 days of infection and that these cells localized to areas of the lung where influenza NP was found (98). This specific recruitment of NKp46^{+} NK cells from the blood suggests that this subset of NK cells is selectively activated and recruited. Indeed, a phenomena in which NKp46^{+}NKp44^{+} subsets of NK cells are significantly increased in the peripheral blood has also been described in human patients with moderate and severe cases of influenza A infection (99). Interestingly, patients with severe influenza infection had significantly fewer NKp46+NKp44- NK cells as well as increased expression of the inhibitory receptors KIR3DL1 and KIR2DL1/DS1 when compared to healthy and moderately infected individuals, suggesting that more severe infections may lead to or be the result of increased inhibition of NK cells. It has also been reported that NKp46 expression is transiently decreased in human PBMCs for 24-48 hours following intramuscular vaccination (99, 100). This could correspond to specific recruitment of this subset of NK cells to the site of vaccination, similar to the recruitment seen in swine following infection. Taken together this data suggests that NK cells are recruited to the site of influenza infection or vaccination, activated by the direct interactions between viral HA and NKp46, and carry out cytolysis and cytokine production. However, it remains unclear if NK cells act as modulators of the CD8 T cell response following influenza infection, as previously described in other infection models, and how differential activation of NK cells by disparate influenza HAs can impact NK cell function.

CHAPTER 2

Enhanced generation of influenza-specific tissue resident memory CD8 T cells in NK-depleted mice[1]

[1]David L. Rose, S. Mark Tompkins and Kimberly D. Klonowski. To be submitted to *Frontiers in Immunology*.

Abstract

Natural Killer (NK) cells are among the first effectors to directly contact influenza and influenza infected cells and their activation affects not only their intrinsic functions, but also subsequent CD8 T cell responses. In this study we utilized a NK cell depletion model to interrogate the contribution of NK cells to the development of anti-influenza CD8 T cell memory. We found that ablation of NK cells during infection leads to increased numbers of influenza-specific memory CD8 T cells in the respiratory tract and lung draining lymph node, possibly through loss of early IFN-γ expression and dysregulation of CD127 on respondent T cells. Interestingly, animals depleted of NK cells during primary influenza infection were protected as well as their NK intact counterparts despite having significantly fewer reactivated CD8 T cells infiltrating the respiratory tract after a lethal, heterosubtypic challenge. Instead, protection in NK deficient animals was conferred by an enlarged pool of lung tissue-resident (T_{RM}) memory cells reactivated in situ to produce the anti-viral cytokines IFN-γ and TNFα the first two days post challenge. Therefore, reduction of NK cell activation after vaccination with live, non-lethal influenza virus increases compartmentalized, broadly protective memory CD8 T cell generation and decreases the risk of CD8 T cell-mediated pathology following subsequent influenza infections.

Introduction

Seasonal influenza A viruses cause significant morbidity worldwide resulting in high rates of influenza-associated hospitalization (104, 105). Additionally, influenza A infected hosts are more susceptible to secondary bacterial infection in part due to immunopathology within the lung (106). Current influenza vaccines target humoral responses to strain-specific hemagglutinin (HA) but weakly elicit cross-protective immunity against divergent and mutated viruses (3). In contrast, anti-influenza CD8 T cells recognize conserved, internal viral proteins, and clear virus infection. Therefore, targeting the generation of anti-viral memory CD8 T cells may provide a better alternative to combat evolving influenza strains (107).

The generation of CD8 T cell memory is a multifactorial and not fully understood process. Nonetheless, some factors influencing the generation of CD8 T cell memory have been elucidated. For example, the strength of TCR signaling, duration of T cell-APC interactions and in some cases CD4 help have all been linked to CD8 T cell fate determination (57). Moreover, the integration of TCR and co-stimulatory signaling as well as local cytokines in the priming environment can tune the PI3K and mTOR signaling pathways towards either an effector or memory cell differentiation program (57). Adding to the complexity of this developmental process, the circumstances surrounding the activation of an individual T cell can be unique, with different clones of a single specificity and progeny of a single naïve T cell capable of generating diverse lineages (108). The transcription factors T-bet, Eomesodermin, and STAT3 direct specific CD8 T cell fates (59, 109) via transcriptional programs that are linked to expression of cell surface markers which can identify specific fate lineages early after priming. For example, cells destined to become memory CD8 T cells preferentially express the IL-7 receptor alpha subunit (CD127) and signaling via this receptor promotes the survival of memory cells through the contraction phase of the immune response (61, 62). In contrast, Killer Cell Lectin-Like Receptor Subfamily G Member 1 (KLRG-1) expression is correlated with terminal differentiation and cell death in the absence of CD127 expression (57). All of the factors driving this differentiation process are contextual, with the pathogen and specific priming microenvironment dictating memory CD8 T cell potential. Importantly, and relevant to this work, strong inflammation promotes terminal differentiation at the expense of memory formation in part via the cytokines IL-12 and IFN-γ (59, 60, 110). The knowledge of how individual immune cells supporting the inflammatory response contribute to the CD8 T cell priming environment, either positively or negatively, and affect resultant memory cell development and function could be used to improve influenza vaccine design.

Natural Killer (NK) cells precede CD8 T cells in the respiratory tract after influenza infection, suggesting these cells and their anti-viral cytokines could influence regional adaptive immunity. Indeed, NK cells can directly suppress recently activated CD8 T cell proliferation (111, 112) and affect the magnitude of CD8 T cell priming through direct elimination of virus (113). Moreover, NK cells can

recognize and lyse activated $CD4^+$ helper T cells, subsequently limiting the CD8 T cell response to viral infection (114). It is therefore likely that NK cells are either directly or indirectly influencing the formation and long-term establishment of memory CD8 T cells thus making them an important variable to consider for elicitation of cross-protective CD8 T cell immunity.

Here, we demonstrate that NK cells are an early source of IFN-γ after influenza infection; and loss of IFN-γ is inversely correlated with higher CD127 expression on memory CD8 T cells. In addition, systemic depletion of NK cells throughout a primary influenza A infection reduces *IFNg* mRNA detected in the lung and lung-draining lymph node and results in a numerically larger pool of influenza-specific memory CD8 T cells in these sites. Furthermore, the CD8 memory T cells generated after acute depletion of NK cells are enriched for tissue-resident memory cells (RM) which are positioned within the lung parenchyma and outside of the associated vasculature. Under the NK cell depletion conditions, animals are as protected as their NK intact counterparts from a heterosubtypic viral challenge, however significantly fewer peripheral, reactivated memory CD8 T cells are recruited to the lungs. Instead, viral titers are kept under control by numerically enriched T_{RM} pre-positioned in the lungs of NK depleted animals which produce significantly more anti-viral cytokines early after challenge. Together, these studies demonstrate that ablation of NK cells increases the generation of anti-influenza CD8 T cell memory and allows for protection from subsequent infections with substantially less cellular infiltrate by positioning a greater number of T_{RM} in the respiratory tract. Further, these studies suggest that modulation of NK cell activation and function may present a novel method to elicit optimal CD8 T_{RM} during vaccination.

Materials and Methods

Mice, viruses, infections and NK cell depletion

Age and sex matched C57BL/6 mice (Charles River (Wilmington, MA)) were anesthetized using Avertin (2, 2, 2-Tribromoethanol) and infected intranasally (i.n.) with 10^3 pfu A/Hong Kong/1/68 x A/Puerto Rico/8/34 2:6 reassortant (x31, H3N2), 500 pfu A/Puerto Rico/8/34 (PR8, H1N1) or 10^5 pfu PR8 in 50µl PBS. Where noted, NK cells were depleted by injecting mice intravenously (i.v.) with 200µg of the anti-NK1.1 antibody (clone PK136) (BioXcell, West Lebanon, NH) in 200µL sterile PBS every other day for 5 days starting 1 day prior to infection. All animal studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Georgia.

Quantitative RT-PCR

Whole lung tissues were collected and RNA purified from the samples using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Reverse transcriptions were performed using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA). Quantitative PCR assays were prepared using the ABI TaqMan Gene Expression Master Mix from ABI 7500 Real Time PCR System (Applied Biosystems, Grand Island, NY). Quantitative real-time RT-PCR was performed using TaqMan technology using Ifng-FAM (Mm01168134_m1), IDO1-FAM (Mm00492586_m1) and 18s-VIC (#4319413E) assays from Life Technologies (Grand Island, NY) in single-plex reactions and assessed on a 7500 Real Time PCR System (Applied Biosystems, Grand Island, NY). Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, and 45 cycles of denaturation (95°C for 15 s) and annealing (60°C for 60 s).

Plaque Assays

Plaque assays were performed as previously described (115). Briefly, whole lungs isolated from infected mice were isolated and homogenized using a Tissue Lyser (Qiagen, Hilden, Germany). Serial dilutions of 10% homogenate were made in dilution media and incubated for 1 hr atop confluent monolayers of Madin-Darby kidney cells grown in 12 well plates for 1 hr at 37°C. Following infection, cell layers were washed and overlaid with MEM containing 1.2% Avicel microcrystalline cellulose (FMC BioPolymer,

Philadelphia, PA). After 72 hrs at 37°C, the overlay was removed and the cells were washed, fixed with cold methanol/acetone (60:40%) and stained with crystal violet. Plaques were counted and plaque-forming units per mL of lung homogenate determined.

Tissue Preparation and Flow Cytometry

Single cell suspensions from tissues were obtained as previously described (116). Cells from the lung airways were obtained by means of brocheoaveolar lavage (BAL) in which the trachea was intubated and 1 ml of PBS introduced and recovered from the lung airway 3x. Following BAL, perfused lungs were excised, minced and incubated with 1.25 mM EDTA at 37°C for 30 min followed by a 1 hr incubation with 150 units/mL collagenase (Life Technologies, Grand Island, NY). After passage through cell strainers, lymphocytes were resuspended in 44% Percoll underlaid with 67% Percoll, centrifuged and the cellular interface collected. Lymph nodes and spleen were mechanically disrupted then passed through a cell strainer. Erythrocytes were depleted from the spleens using Tris-buffered ammonium chloride and cells enumerated using a Z2 Coulter Particle Counter (Beckman Coulter, Indianapolis, IN). Intravascular staining was performed by injecting mice with 3µg FITC-conjugated anti-CD45.2 i.v. three minutes prior to euthanasia.

The influenza nucleoprotein (NP) MHC class I [H-2D(b)/ASNENMETM] tetramers were generated at the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University, Atlanta, GA). Staining was carried out at RT for 1 hr with a combination of other mAbs: PerCP-Cy5.5-conjugated anti-CD8α, anti-NKp46, anti-CD69, FITC-conjugated anti-CD44, anti-NKp46, PE-conjugated anti-CD127, anti-NKp46, anti-NK1.1, anti-CD103, APC/Cy7-conjugated anti-CD62L, anti-CD8α, anti-CD127, PE/Cy7-conjugated anti-KLRG1, anti-CD8α APC-conjugated anti-CD3 or for 20 min at 4°C without tetramer. All antibodies were purchased from eBioscience or Tonbo Biosciences (both San Diego, CA). Data was acquired using an LSR II with FacsDiva software (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star INC, Ashland, OR).

CD8 T cell restimulation assay

Following isolation, lymphocytes were incubated at 37°C for 5 hrs in the presence of influenza $NP_{366-374}$ (ASNENMETM) or the irrelevant ovalbumin $(OVA)_{257-264}$ (SIINFEKL) peptide in the presence of GolgiStop (BD Pharmigen, San Diego, CA). Subsequently cells were stained with anti-CD8 and anti-CD44 Abs for 20 min at 4°C and fixed in 2% paraformaldehyde overnight. The next day, samples were permeabilized with Perm/Wash (BD Biosciences, San Diego, CA), stained with FITC-conjugated anti-IFNγ (BD Pharmingen, San Diego, CA) and PerCP5.5-conjugated anti-TNFα (eBioscience, San Diego, CA) for 30 min at 4°C and analyzed by flow cytometry.

Statistics

Statistical analysis was carried out using Prism 5 software (GraphPad Software, La Jolla, CA). Significance was determined using Student's two sided t-test (and Holm-Sidak multiple comparisons correction when appropriate) where the p-value was $p<0.05$.

Results

Systemic depletion of NK cells results in numerically increased memory CD8 T cells

Innate immune cells act as first responders, decreasing Ag load and producing cytokines at the infection site, and, as such, can modulate the subsequent adaptive immune response (10, 113). Indeed, in some situations NK cells have been shown to impact the magnitude and longevity of subsequent anti-viral T cell responses (113, 114, 117, 118). However, it is unclear how NK cells modulate anti-influenza CD8 T cell responses over the complete course of activation and differentiation into memory subsets and how this modulation impacts recall responses *in vivo*. To examine how the activation of NK cells during influenza infection affects the development of CD8 T cell immunity, we administered the monoclonal antibody PK136 (anti-NK1.1) to deplete NK cells starting 1 day prior to and ending 3 days post sub-lethal infection with a low pathogenicity influenza virus x31. This protocol results in greater than 90% NK cell depletion through 5 days post infection (dpi), the time period over which NK cells accumulate and become activated within the lung (Supplemental Figure 2.1A, B). While the anti-NK1.1 mAb can deplete $NK1.1^+$ NKT cells, we found that NKT cells neither expanded nor significantly up-regulated CD69 after influenza infection suggesting that they do not contribute to the anti-influenza response to the same extent as NK cells in our model (Supplemental Figure 1). Therefore, our depletion protocol preferentially affected NK cells responding to influenza infection, allowing us to examine their specific contribution to the CD8 T cell response.

To determine whether depletion of NK cells affects the magnitude of the anti-influenza CD8 T cells response, we monitored the numerical accumulation of the immunodominant anti-influenza nucleoprotein (NP) specific CD8 T cells recovered from the lung, lung airways (via bronchial alveolar lavage (BAL)), and lung-draining mediastinal lymph node (MdLN) after infection with influenza in the presence or absence of NK cells. We found that depletion of NK cells increased the numbers of influenza NP-specific CD8 T cells recovered from all respiratory associated tissues at 10 dpi, the peak of the proliferative CD8 T cell response (Fig 2.1A). Interestingly, this disparity increased over time in the lung where more than double the number of NP-specific CD8 T cells were isolated from the respiratory tract of NK depleted vs. sufficient

mice 45 dpi. In the MdLN we observed a similar trend, albeit slightly delayed, with nearly double the number of anti-NP CD8 T cells recovered from NK depleted vs. sufficient mice 90 dpi (Fig 2.1A). While the number of Ag-specific CD8 T cells isolated from the BAL of NK depleted mice was elevated over NK sufficient mice at all time points examined, the difference between the two groups did not reach statistical significance, perhaps due to the increased variability inherent with BAL isolation. Nonetheless, these data demonstrate that depletion of NK cells numerically increased the pool of anti-influenza memory CD8 T cells in both the lung parenchyma and lung draining MdLN after influenza infection. As demonstrated previously (113), this suggests that NK cells limit the numerical accumulation of memory CD8 T cells; however, it remains unclear whether ablation of NK cells affects the development (and/or survival) and quality of the resultant memory cells.

NK cell depletion increases the pool of CD127 expressing memory cells following influenza infection

The cell surface markers KLRG-1 and CD127 (IL-7Rα) have been established in other models systems to track the formation of effector and memory CD8 T cells following infection (57). KLRG-1^{hi}CD127^{lo} cells represent terminally differentiated short lived effector cells (SLECs) and KLRG-1^{lo}CD127^{hi} are considered memory precursor cells (MPECs). Indeed, it has been reported that CD127^{hi} CD8 T cells contract at a slower rate than CD127^{lo} cells (119). Cells that are KLRG-1^{lo}CD127^{lo} represent a multipotent precursor population of early effector cells (EECs) which could in a brief interim convert to SLECs or MPECs (59). Interestingly, in respiratory infections the EEC population persists longer and at a higher proportion compared to other infections, possibly owing to the unique environment of the respiratory tract (63).

To determine whether NK cells affect the memory potential of antigen-specific CD8 T cells we tracked the kinetics of CD127 and KLRG1 expression on influenza-specific CD8 T cells. In the lung parenchyma of NK depleted mice, the proportion of EECs (KLRG1^{hi}CD127^{lo}) was slightly elevated at the expense of MPECs (KLRG1^{lo}CD127^{hi}) 10 dpi, however by 45 dpi this discrepancy was resolved resulting in a net increase in the proportion of MPECs between 10 and 45 dpi (Fig 2.1B). Alternatively, we observed no significant difference between the frequency of EECs and MPECs 10 dpi in the MdLN. However, by 45

dpi NP-specific CD8 T cells from the MdLN of NK depleted animals also showed a clear bias toward cells expressing CD127 (Fig 2.1B). These data demonstrate that between 10 and 45 dpi the proportion of CD127 expressing CD8 T cells is significantly increased in NK depleted vs. intact mice. Thus, the absence of NK cells appears to promote the expression of CD127 following influenza infection. Since $CD127^{+}$ expressing CD8 T cells have been reported to undergo contraction at a slower rate than their $CD127^{-}$ counterparts, NP-tetramer^{+} CD8 T cells isolated from NK depleted mice would have a greater chance of surviving as a memory population (119). Indeed, similar to previous reports $CD127^{hi}$ cells contracted at a slower rate than $CD127^{lo}$ cells in both the lung and the MdLN over the contraction period (data not shown). Together these data show that CD8 T cells activated in the absence of NK cells are more likely to generate $CD127^{hi}$ memory CD8 T cells with a greater survival potential.

IFN-γ antagonizes CD127 expression on CD8 memory T cells

Having established that NK cell depletion increases the number of NP-specific memory CD8 T cells and increases the proportion of memory cells expressing CD127, we sought to determine possible mechanisms by which NK cells could influence anti-viral CD8 T cell memory development. Previous studies demonstrated that NK cells can influence T cells directly by lysing activated T cells, inhibiting CD8 T cell proliferation and controlling viral load (111, 113, 114). However, NK cells can also be potent producers of IFN-γ which can contribute to CD8 T cell activation or alternatively lead to contraction of CD8 T cell responses by increasing pro-apoptotic factors (120-123). Additionally, IFN-γ has been shown to block the development of CD127 expressing memory precursor CD8 T cells if induced during or shortly after T cell priming in a DC immunization model (110). Thus, loss of NK derived IFN-γ could explain both the numeric and phenotypic differences of the anti-viral CD8 T cells observed in NK depleted versus sufficient mice.

To first determine the extent of IFN-γ produced in our influenza model, we measured IFN-γ transcripts in the lungs and MdLN of NK depleted and sufficient mice. As expected, NK depleted mice expressed IFN-γ transcript at lower levels, approximately one half and one quarter of that observed in the lung of NK sufficient mice at 24 and 72 hrs post infection, respectively (Fig 2.2A). Similarly, NK depleted

mice expressed approximately one half and one tenth IFN-γ transcript levels of NK sufficient mice in the MdLN at 24 and 72 hrs post infection, respectively (Fig 2.2A). To explore whether IFN-γ contributes to regulating the number of $CD127^{hi}$ antigen-specific CD8 T cells after influenza infection, we compared the NP-specific CD8 T cell response between wild type and IFN-$\gamma^{-/-}$ mice at 10 and 45 dpi. We did not observe significant differences between the proportion of $CD127^{lo}$ EECs and $CD127^{hi}$ MPECs recovered from either the lung parenchyma or MdLN of NK depleted or sufficient animals 10 dpi (Fig 2.2B). However, by 45 dpi there was a significant increase in the percentage of $CD127^{hi}$ influenza-specific CD8 T cells recovered from both tissues in animals acutely depleted of NK cells during priming. Additionally, the overall CD127 expression on an individual cell was significantly higher on the NP-specific CD8 T cells isolated from both the lung (~1.5x higher) and MdLN (~2x higher) 45 dpi (Fig 2.2C). We did not observe differences in the proportion of SLECs at 10 dpi, and this population does not survive through 45 dpi (data not shown). Interestingly, we did not observe significant differences in the number of Ag-specific CD8 T cells between wild type and IFN-$\gamma^{-/-}$ mice at 10 or 45 dpi (data not shown). Together, these data suggest that IFN-γ participates in suppressing CD127 expression on influenza-specific CD8 T cells but does not influence the number of NP-specific CD8 T cells in the memory pool. Thus NK-derived IFN-γ may be one of multiple mechanisms by which NK cells regulate memory CD8 T cell development.

NK cell ablation during primary influenza infection leads to numerically smaller but equally protective CD8 T cell recall response

To determine whether the increased number of memory CD8 T cells harbored in the lung of NK depleted mice generated a more robust recall response, we generated x31 immune animals in the presence or absence of NK cells and challenged the mice at memory (between 45-50 dpi) with a heterosubtypic strain of influenza virus, PR8. The PR8 strain of influenza shares the CD8-specific NP epitope with x31 but expresses distinct external epitopes and antibody targets. Prior to challenge we were able to recover nearly double the number of NP-specific CD8 T cells from the lung tissue of NK depleted compared to sufficient mice (Fig 2.3A). We hypothesized that having more Ag-specific memory cells positioned in the lung prior

to challenge would result in a larger recall response in the animals depleted of NK cells in the primary response, resulting in enhanced protection and reduced viral titers after challenge.

First, we monitored the accumulation of the NP-specific memory CD8 T cells in the BAL and lung at 8 and 15 days post-challenge (dpc). Interestingly, we recovered fewer NP-specific CD8 T cells from the BAL and lung of NK depleted vs. sufficient mice (nearly 1/3 and 1/2 respectively) at the proliferative peak of the recall response at 8 dpc (Fig 2.4A). Despite a numerically reduced recall response, NK depleted mice were sufficiently and equally protected after challenge as they demonstrated similar weight loss (Fig 2.3B) and survival (no mice succumbed to infection) to NK sufficient animals (data not shown). Viral titers were decreased in NK depleted animals at 2, 4 and 6 dpc, however these data were not statistically significant (Fig 2.4C). Moreover, there was no observed difference between the groups regarding time to viral clearance. Therefore, our data suggest that memory T cells generated in the absence of NK cells may be functionally superior upon recall when compared to their counterparts generated in the presence of NK cells.

To compare the functionality of memory T cells developed in the absence of NK cells, we generated x31 immune mice in the presence or absence of NK cells and challenged with PR8 as previously described. T cells were subsequently recovered from the lung a 2, 4, 6 and 8 dpc, restimulated *ex vivo* with influenza $NP_{366-374}$ peptide, and analyzed for their ability to produce the effector molecules IFN-γ and TNFα, which are known to be important for recall responses to influenza infection (43, 54). Memory CD8 T cells recovered from the lung of NK depleted and sufficient mice had equivalent proportions of IFN-γ and TNFα expressing CD8 T cells following restimulation on all days assayed (Fig 2.4A). Additionally, there were no significant differences in the expression levels of IFN-γ or TNFα in the total activated ($CD44^{hi}$) CD8 T cell population on a per cell basis (data not shown). We also monitored the accumulation of IFN-γ^+ CD8 T cells in the lung following challenge. We observed that NK depleted animals had altered CD8 T cell kinetics with a less overall accumulation and earlier peak (6 vs. 8 dpc) resulting in fewer (~1/2) IFN-γ^+ CD8 T cells accumulating in the lung at 8 dpc compared to NK sufficient animals (Fig 2.4B). Interestingly however, at 2 dpc we recovered nearly 2x more IFN-γ^+ CD8 T cells from the lung of NK depleted vs. sufficient animals.

Studies by Wu *et al*. demonstrated that infiltration of reactivated peripheral memory CD8 T cells does not occur before 3 dpc (64), suggesting that these IFN-γ^+ CD8 T cells were likely tissue resident memory T cells (T_{RM}) reactived rapidly in situ. Together, these data demonstrate that ablation of NK cells during primary immune response to influenza allows for an equally functional, protective recall response with fewer antigen-specific CD8 T cells entering the lung and lung airways after challenge. Furthermore, this data suggests that ablation of NK cells during primary influenza infection leads to increased numbers of functional lung CD8 T_{RM} which are quickly activated and converted to secondary effector cells.

NK Cell depletion increases the number of Ag-specific CD8$^+$ tissue resident (T_{RM}) cells

Memory cells can be broadly categorized as central (T_{CM}) or effector (T_{EM}) memory based on expression of distinct cell surface markers that dictate their localization (63). Whereas T_{EM} patrol peripheral tissues, $T_{RM,}$ a subset of $T_{EM,}$ have limited migratory potential. It has long been appreciated that anti-influenza CD8 T cells positioned at the site of infection are crucial for heterosubtypic immunity and the waning of heterosubtypic immunity correlates with loss of respiratory CD8 T cells over time (5, 65). Recent studies have attributed protective immunity against secondary viral challenge to the presence of T_{RM} whereas infiltrating memory CD8 T cells are associated with immunopathology (64). In this study, we have demonstrated that there are numerically more memory CD8 T cells generated in the absence of NK cells compared to NK intact animals. Furthermore, we have observed increased numbers of memory CD8 T cells responding early after heterosubtypic challenge in NK depleted vs. intact animals. This would suggest that NK depleted animals harbor a greater frequency of T_{RM} poised for reactivation in their respiratory tract.

To examine the contribution of NK cells to the generation of T_{RM} cells, we differentiated circulating from resident memory CD8 T cells in NK depleted and sufficient mice 50 dpi using an established intravenous staining protocol (124). Briefly, 3 minutes prior to sacrifice, animals are injected i.v. with an anti-CD45 mAb. NP-specific T_{RM} will not stain with the i.v. injected mAb within this brief window whereas memory CD8 T cells localized within the vasculature and accessible to the mAb will stain (124). We did not observe any difference in the frequency of circulating T_{EM} versus T_{RM} CD8 T cells between the NK depleted and sufficient groups (Fig 2.5A). However, since overall CD8 T cell numbers in the lungs of NK

depleted animals were increased, the total number of T_{RM} was coordinately increased by 2 fold (Fig 2.5B). T_{RM} cells in the skin, gut and brain can also be identified by the markers CD103 and CD69, whereas the majority of lung T_{RM} are $CD103^{-}CD69^{-}$ (63). Loss of NK cell signals during CD8 T cell priming, however, did not enrich for any additional subset of T_{RM} (Fig 2.5C). In summary, these data demonstrate that depletion of NK cells increases the number of lung T_{RM} cells (Fig 2.5) by nearly two-fold, which could enhance heterosubtypic immunity and improve virus control and clearance.

Discussion

CD8 T cells are instrumental in the control and clearance of a number of highly mutagenic, intracellular human pathogens such as HIV, malaria, and influenza A (3, 125). However, eliciting effective CD8 T cell memory through vaccination has proven difficult due to our incomplete understanding of the conditions necessary to elicit long-lived, functional memory populations. Many studies have prioritized the examination of how inherent T cell-specific factors, the DC-T cell interaction and immunization with different adjuvants and antigens affect the generation of memory CD8 T cells, but the role of accessory or supporting cells in this process is often overlooked.

Natural Killer cells account for a large proportion of the lymphocytes recovered from the lung in the steady state and significant numbers are recruited to the lung after respiratory infection (10), as early as 3 dpi with influenza virus (Supplemental Figure 1). While NK cells are primarily known for cytolysis of aberrant host cells and production of the inflammatory cytokine IFN-γ, they can impact the broader immune response as well. Through the elimination of infected cells, NK cells can limit viral replication and alter antigen availability (113). Activated NK cells also engage in cross-talk with DCs through both contact dependent and independent mechanisms leading to increased DC activation and culling of immature DCs (126, 127). Additionally, NK cells can directly interact with T cells and lyse activated CD4 helper T cells or suppress proliferation of recently activated CD8 T cells (111, 114). Recently, NK cells were found to suppress the development CD8 T cell memory following systemic viral infection (114). This observation is particularly noteworthy when one considers that after influenza infection there is a local concentration of NK cells in the lung (Supplemental 1C?) and lung-draining lymph node (data not shown); and the

development of long-term CD8 T cell memory to respiratory infection is poor (10, 63). Therefore, we hypothesized that regional NK cell activation after influenza infection may affect the anti-influenza CD8 T cell response by limiting memory CD8 T cell development.

In this study we depleted NK cells in order to examine the net contribution of these anti-viral effectors to the subsequent generation of CD8 T cell memory after influenza A infection. We found that the depletion of NK cells during influenza A infection results in numerically increased memory CD8 T cells (Fig 2.1A) akin to what has been reported after systemic LCMV infection in the absence of NK cells (114). In the later study it was determined NK cell mediated culling of activated CD4 helper T cells limited effector CD8 T cell proliferation, resulting in a reduced clonal burst and numerically reduced memory (114). However, we did not observe significantly more CD8 T cells in NK depleted vs. sufficient mice at the proliferative peak following influenza A infection (Fig 2.1A), suggesting an alternate NK cell mediated mechanism is responsible for the increased number of memory CD8 T cells after respiratory infection. In addition, NK-DC crosstalk (126, 127) is not likely affected in our respiratory model as the number of DCs and the level of their surface expression of MHC-I, MHC-II and CD80 at 1-5 dpi was equivalent in NK depleted and sufficient mice (data not shown). Thus, the increased number of memory CD8 T cells we observed in NK depleted mice cannot be ascribed to bystander regulation of other cell types known to impact CD8 T cell memory and suggests that NK cells directly regulate memory CD8 T cell development.

The cytokine IL-7 participates in memory CD8 T cell formation and its receptors are dynamically regulated to confer responsiveness to this growth/ survival factor (128). Moreover, whereas specific inflammatory cytokines have been associated with supporting or inhibiting CD8 memory T cell formation, how these inflammatory signals regulate this developmental pathway and/or specifically IL-7 responsiveness is not well understood. Activated NK cells are potent producers of IFN-γ, a cytokine antagonistic to memory CD8 T cell development when present during or shortly after priming (110, 128). Not surprisingly we observed that depletion of NK cells resulted in significantly decreased IFN-γ transcripts detected in the lung tissue 24 and 72 hrs p.i. (~0.5x and ~0.75x decrease, respectively; Fig 2A) and MdLN (~0.5x and ~0.9x decrease, respectively; Fig2A). IFN-γ is a pleotropic cytokine and could influence the

generation of CD8 T cell memory in a number of ways. For example, the phosphorylation of the transcription factor STAT3 promotes CD8 T cell memory and expression of the IL-7Rα subunit (CD127) via signaling through the mTOR pathway in an IL-10 dependent manner (109). IFN-γ is a known suppressor of IL-10 and can act directly on T cells to dephosphorylate STAT3 (129). Indeed, we observed a higher frequency of $CD127^{hi}$ NP-specific CD8 T cells in IFN-γ KO mice and NK depleted mice 45 dpi compared to wild type and PBS controls, respectively (Fig 2.1B; Fig 2.2B). Interestingly, NK depleted and IFN-γ KO mice did not harbor significantly different frequencies of $CD127^{hi}$ NP-specific CD8 T cells at 10 dpi suggesting that that the effects of IFN-γ do not manifest until after the proliferative peak. Since $CD127^{hi}$ NP-specific CD8 T cells undergo contraction at a slower rate than their $CD127^{lo}$ counterparts, it is possible that the reduction of IFN-γ increased CD127 expression on a per cell basis, reducing their rate of contraction. However, we did not observe any difference in the MFI of CD127 on NP-specific CD8 T cells isolated from either NK depleted or IFN-γ KO mice 10 dpi nor did we observe a slower rate of contraction for NK depleted vs. sufficient mice (data not shown). While IL-10 transcript levels were not significantly increased in the lungs of NK depleted mice 10 dpi (data not shown), loss of early NK cell derived IFN-γ could increase STAT3 phosphorylation within respondent CD8 T cells, promoting CD127 expression. Alternatively, loss of early IFN-γ signaling could promote memory CD8 T cell formation via alteration of mTOR activity (130). Finally, it is possible that other interferon stimulated genes (ISGs) or combinations of ISGs play a role in CD8 T cell fate determination. Overall, our data demonstrate that early IFN-γ signaling alters CD8 T cell programing during the effector phase of the response to promote memory formation and survival after the proliferative peak resulting in increased numbers of antigen-specific CD8 memory T cells.

Although we observed that NK depleted mice harbor a greater number of Ag-specific memory CD8 T cells compared to NK intact mice, it was unclear whether these cells were either functionally distinct or simply numerically sufficient to confer superior protection after challenge. Interestingly, the memory CD8 T cell reservoir in the respiratory tract of animals depleted of NK cells in the primary (and not secondary) infection were equally protective against heterosubtypic challenge compared to control animals, albeit with

reduced CD8 T cell infiltration into the respiratory tract (Fig 2.3). Furthermore, equivalent proportions of reactivated memory CD8 T cells recovered from the lung of NK depleted or sufficient animals produced IFN-γ and TNFα on days 2, 4, 6 and 8 post challenge(Fig 2.4), demonstrating that the recall response was functionally unaltered in NK depleted animals. Given the contribution of CD8 T_{RM} cells in protection against heterosubtypic influenza infection (64), it is possible that positioning more CD8 T_{RM} cells at the site of infection can control viral titers without the need for additional proliferation. Indeed, previous studies have shown that increasing the initial number of memory CD8 T cells present at the time of challenge leads to decreased proliferation of both naïve and memory CD 8 T cell responses as well as decreased pathogen burden (131, 132). Therefore, it is unsurprising that NK depleted animals, which harbored nearly 2 fold more Ag-specific T_{RM} CD8 T cells than NK intact animals at the time of challenge (Fig 2.5A), generated a significantly smaller CD8 T cell recall response (Fig 2.3A) and tended to have lower viral titers at all time points analyzed (2, 4, and 6 dpc) post challenge when compared to NK intact animals (Fig 2.3C). Thus, our data show that depletion of NK cells during a primary influenza infection alters early CD8 T cell programing, possibly via differential inflammatory milieu during T cell priming, leading to increased T_{RM} generation. The resulting increased number of T_{RM} and the early antiviral cytokines they produce allow for equal protection against heterosubtypic challenge with a smaller contribution from, and accumulation of, peripheral memory CD8 T cells.

CD8 T cells are thought to be major contributors to tissue damage during clearance of influenza infection which contributes to secondary bacterial infection and increased mortality. Therefore, limiting T cell proliferation after reactivation could ameliorate these undesirable consequences. Our data would suggest that curtailing NK activation during the initial infection or vaccination could generate sufficient, cross-protective memory CD8 T cells with limited need for proliferation after reinfection. While ablation of NK cells during vaccination is impractical, Influenza A viruses can directly stimulate NK cells via interaction between the viral hemagglutinin and the NK cell activating receptor NKp46 (11). As such, it may be possible to limit or differentially modulate NKp46 receptor ligation and resultant NK cell activation

during vaccination with live attenuated viruses in order to elicit a greater number of protective CD8 T_{RM} while simultaneously decreasing the risks of secondary bacterial infection.

Acknowledgements

We would like to thank Dr. Hillary Shane for critical reading of the manuscript and Jamie Barber for technical assistance with the flow cytometry.

Figures

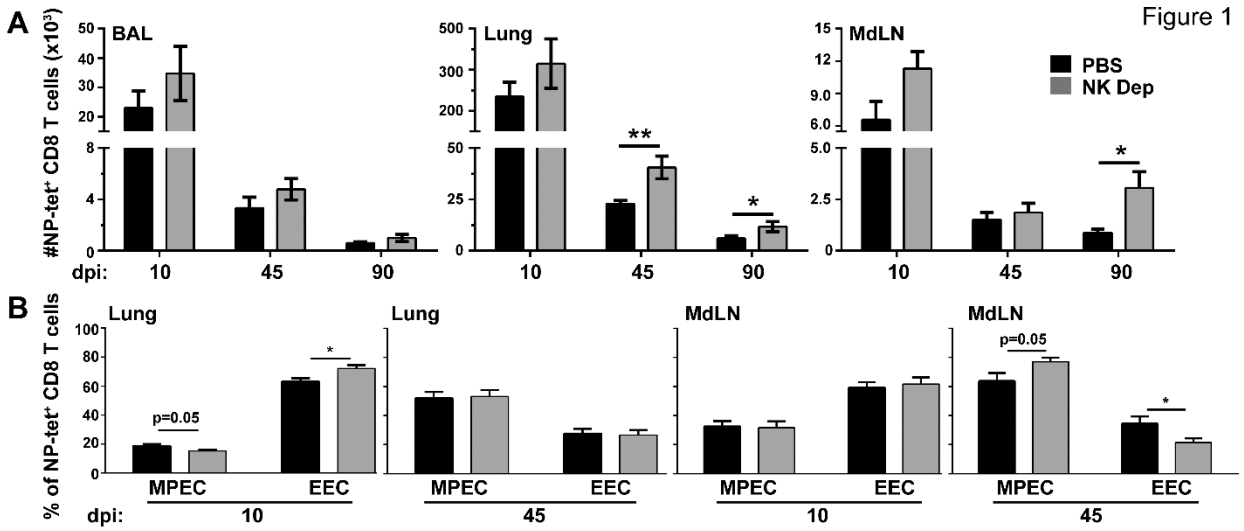


Figure 1
A
BAL
Lung
MdLN
#NP-tet+ CD8 T cells (x10³)
PBS
NK Dep
dpi:
10
45
90
**
*
B
% of NP-tet+ CD8 T cells
p=0.05
MPEC
EEC

Figure 2.1 Anti-influenza CD8 T cells are numerically increased following NK cell depletion. (A) C57BL/6 mice were infected with 10^3 PFU x31 and administered anti-NK depleting antibody at -1, 1, 3 dpi. The kinetics of the CD8 T cell response based on the total number ±SEM of activated ($CD44^{hi}$), NP-specific CD8 T cells recovered from the BAL, lung, and MdLN of animals that were treated with PBS (black) or depleting antibody (grey) is depicted. (B) The proportion ±SEM of activated ($CD44^{hi}$), NP-specific CD8 T cells expressing the indicated phenotype recovered from the lung (left) or MdLN (right) of NK sufficient (black) or NK depleted (grey) mice at the indicated dpi. Graphs represent pooled data from 3 or more independent experiments using $n \geq 3$ mice/group/time point. *$p<0.05$, **$p<0.01$; unpaired Student's t-test with Holm-Sidak multiple comparisons correction.

Figure 2

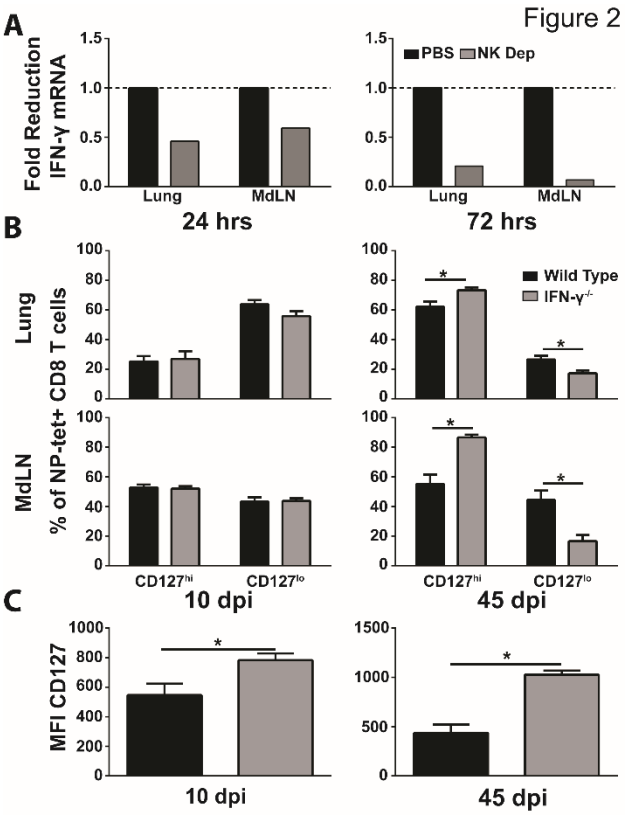

A
Fold Reduction IFN-γ mRNA
1.5
1.0
0.5
0.0
Lung
MdLN
24 hrs
PBS
NK Dep
72 hrs
B
Lung
MdLN
% of NP-tet+ CD8 T cells
100
80
60
40
20
0
Wild Type
IFN-γ-/-
CD127hi
CD127lo
10 dpi
45 dpi
C
MFI CD127
1000
800
600
400
200
1500
500

Figure 2.2. Loss of IFN-γ leads to increased expression of CD127 on memory CD8 T cells. C57BL/6 mice were infected with 10^3 PFU x31 and administered anti-NK depleting antibody on -1, 1, 3 dpi. (A) The relative expression of IFN-γ transcript recovered from bulk lung tissue and MdLN of NK sufficient (black) and depleted (grey) mice 24 and 72 hrs post infection was determined. (B) The proportion of $CD44^{hi}$, NP-specific CD8 T cells expressing CD127 ±SEM recovered from the lung (top) and MdLN (bottom) of wild type (black) and IFN-$\gamma^{-/-}$ (grey) mice at the indicated dpi was determined. (C) Median fluorescent intensity ±SEM of CD127 expression on NP-specific CD8 T cells recovered from the lung (left) and MdLN (right) of wild type (black) and IFN$\gamma^{-/-}$ (grey) mice 45 dpi was determined. Graphs are representative of 3 independent experiments using $n \geq 3$ mice/group/time point. *$p<0.05$; unpaired Student's t-test.

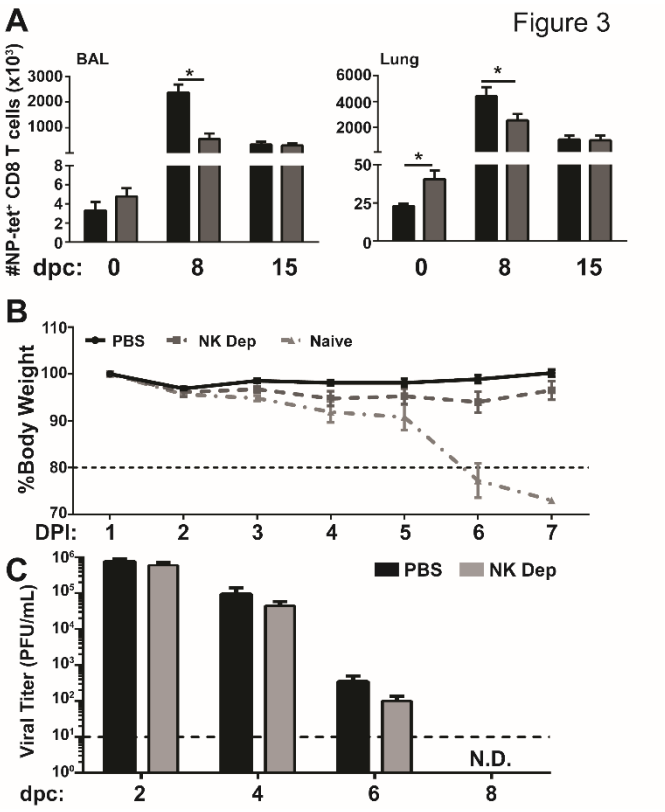

A
Figure 3
BAL
Lung
#NP-tet+ CD8 T cells (x10³)
3000
2000
1000
8
6
4
2
0
6000
4000
2000
50
25
0
*
dpc: 0 8 15
0 8 15
B
PBS
NK Dep
Naive
%Body Weight
110
100
90
80
70
DPI: 1 2 3 4 5 6 7
C
PBS
NK Dep
Viral Titer (PFU/mL)
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
N.D.
dpc: 2 4 6 8

Figure 2.3. NK depleted animals are equally protected from heterosubtypic challenge by a numerically smaller CD8 recall response compared to NK sufficient animals. C57BL/6 mice were infected with 10^3 PFU x31 and administered anti-NK depleting antibody on -1, 1, 3 dpi to develop x31 immune mice. (A) The x31 immune mice were subsequently challenged with 10^5 PFU PR8 on 50 dpi. The total number of antigen experienced ($CD44^{hi}$), NP-specific CD8 T cells ±SEM recovered from the BAL (left) and lung (right) of NK sufficient (black) and NK depleted (grey) mice was determined at 0 (45 dpi), 8, and 15 dpc. (B) The percent of body weight lost ±SEM by x31 immune mice generated in the presence (solid black) or absence (dashed, dark grey) of NK cells or by naïve mice (dash-dot, light grey) after a 500 PFU PR8 challenge was determined. (C) The viral titer of lung homogenate recovered from NK sufficient (black) or depleted (grey) immune animals was measured 2, 4, 6 and 8 dpc (N.D. = not detectable). Graphs represent pooled data from 2 independent experiments using n=5 mice/group/time point. *$p<0.05$; unpaired Student's t-test with Holm-Sidak multiple comparisons correction.

Figure 4

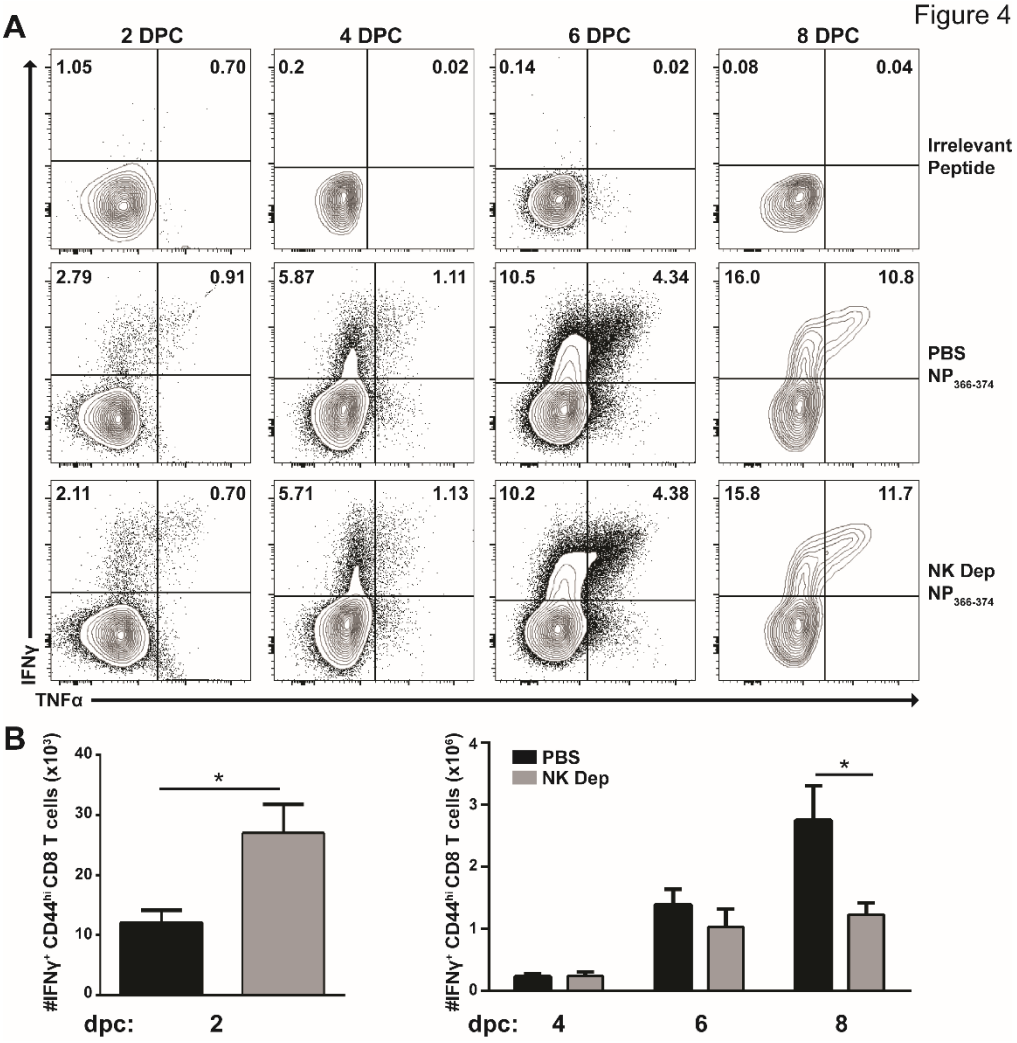

A
2 DPC
4 DPC
6 DPC
8 DPC
1.05
0.70
0.2
0.02
0.14
0.02
0.08
0.04
Irrelevant Peptide
2.79
0.91
5.87
1.11
10.5
4.34
16.0
10.8
PBS NP366-374
2.11
0.70
5.71
1.13
10.2
4.38
15.8
11.7
NK Dep NP366-374
IFNγ
TNFα
B
#IFNγ+ CD44hi CD8 T cells (x10³)
40
30
20
10
0
*
dpc: 2
#IFNγ+ CD44hi CD8 T cells (x10⁶)
4
3
2
1
0
PBS
NK Dep
*
dpc: 4 6 8

Figure 2.4. Memory CD8 T cells generated in the presence and absence of NK cells produce equivalent amounts of IFN-γ and TNFα. x31 immune mice generated in NK sufficient or deficient conditions were challenged with PR8 and lymphocytes were recovered from the lung at the indicated dpc. (A) Following restimulation with the indicated peptide, TNFα and IFN-γ was detected by intracellular staining. (B) Absolute number of IFN-γ^+ CD8 T cells recovered from lung of NK deficient (grey) or sufficient (black) mice on indicated dpc. Graphs represent pooled data from 2 independent experiments using n=5 mice/group/time point. *$p<0.05$; unpaired Student's t-test with Holm-Sidak multiple comparisons correction.

Figure 5

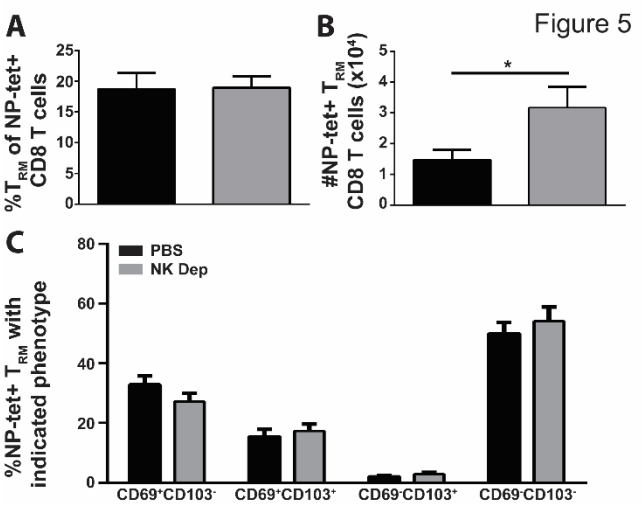
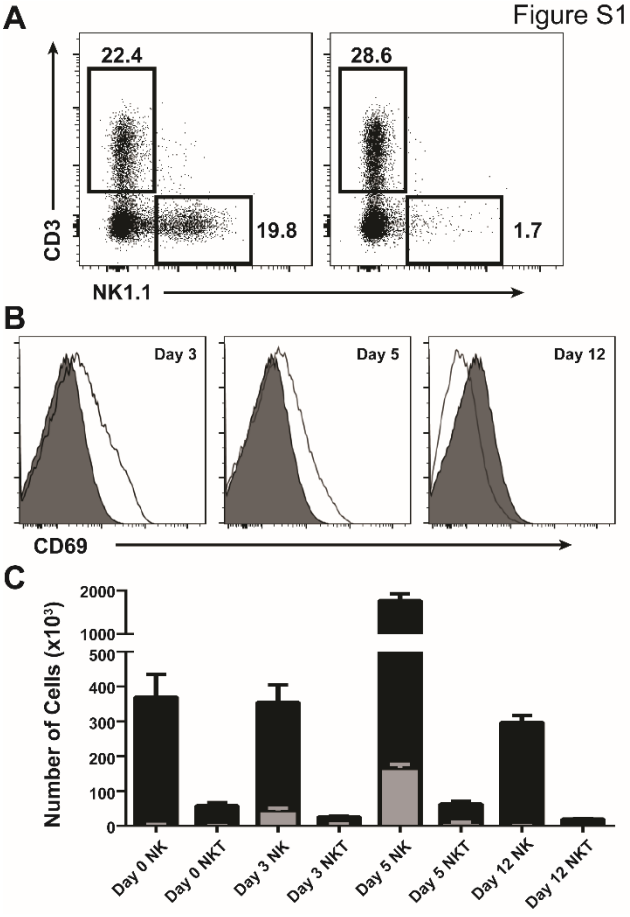

A
%T_RM of NP-tet+ CD8 T cells
25
20
15
10
5
0
B
#NP-tet+ T_RM CD8 T cells (x10^4)
5
4
3
2
1
0
*
C
%NP-tet+ T_RM with indicated phenotype
80
60
40
20
0
PBS
NK Dep
CD69+CD103-
CD69+CD103+
CD69-CD103+
CD69-CD103-

Figure 2.5. Removal of NK cell during CD8 T cell priming enhances the development of lung T_{RM}. C57BL/6 mice were infected with 10^3 PFU x31, administered anti-NK depleting antibody at -1, 1, 3 dpi, and rested for 45 days. T_{RM} were identified by lack of staining with an anti-CD45 administered 3 minutes prior to euthanasia. (A) The frequency, (B) number and (C) phenotype of NP-tet+ T_{RM} CD8 T cells ±SEM recovered from the lung of NK depleted (grey) and sufficient (black) mice are depicted. All graphs represent pooled data from 2 independent experiments with n=5 mice/group/time point. *$p<0.05$; unpaired Student's t-test with Holm-Sidak multiple comparisons correction.

Figure S1


A
22.4
19.8
28.6
1.7
CD3
NK1.1
B
Day 3
Day 5
Day 12
CD69
C
Number of Cells (x10³)
2000
1000
500
400
300
200
100
0
Day 0 NK
Day 0 NKT
Day 3 NK
Day 3 NKT
Day 5 NK
Day 5 NKT
Day 12 NK
Day 12 NKT

Supplemental Figure 2.1. Activation and expansion of NK cells following influenza infection. C57BL/6 mice were infected with 10^3 PFU x31 and administered anti-NK depleting antibody on -1, 1 and 3 dpi. (A) To assess depletion efficiency, the frequency of NK cells was determined in the lung of NK sufficient (left) and NK depleted (right) mice 5 dpi. (B) NK cells recovered from lung tissue of naive (solid histogram) and x31 infected (open histogram) mice on days 3, 5, and 12 post infection were analyzed for expression of the activation marker CD69. (C) To examine the kinetics of the NK and NKT cell expansion and activation, NK and NKT cells were recovered from the lung of x31 infected mice at 0, 3, 5, and 12 dpi and their total number (black) and the number of those cells expressing CD69 (grey) ±SEM were determined. Data are representative of 3 independent experiments using n = 3 mice/group.

Chapter 3

Differential binding of human NKp46 receptor by disparate influenza hemagglutinin proteins

Introduction

Natural Killer cells are innate lymphoid cells which are uniquely poised to serve both as a first line of defense against a host of respiratory pathogens as well as a bridge between the innate and adaptive immune response. NK cells account for a large proportion of the lymphocyte compartment within the respiratory tract and lung parenchyma during steady state and quickly become activated within and recruited to these tissues following respiratory infection (10). Once activated, NK cells carry out effector functions by eliminating infected host cells, but also shape the local environment and subsequent adaptive immune response via secretion of cytokines, NK-DC cross talk, and direct elimination of activated T and B cells (9, 48, 112). We have previously shown (Chapter 2) that depletion of NK cells prior to influenza A infection results in increased numbers of influenza-specific respiratory CD8 T_{RM} cells. Furthermore, we have demonstrated that mice depleted of NK cells before primary infection are equally able to protect against heterosubtypic viral challenge with less CD8 T cell infiltrate into the lung, likely owing to the increased influenza-specific CD8 T_{RM} prior to challenge. These findings point toward the modulation of the NK cell response as possible mechanism to improve current influenza A vaccines through the generation of long lived, broadly cross-reactive memory CD8 T cells, however direct depletion of NK cells would be impractical and potentially dangerous. Therefore, in an effort to identify alternative methods to dampen NK cell activation following influenza infection wanted to examine the mechanisms through which influenza A viruses activate NK cells.

NK cells are known to contribute to the early anti-influenza immune response through the production of pro-inflammatory cytokines and as early cytotoxic cells with the potential to limit viral replication (120, 133, 134). Additionally, NK cells have been reported as producers of the cytokine IL-22 after viral clearance suggesting a role for NK cells promoting tissue repair and a return to steady state

following influenza infection (135, 136). However, despite their apparent importance to anti-influenza immunity the mechanism through which NK cells recognize and become activated by influenza infected cells has been largely neglected. Studies by Mandelboim *et al*. demonstrated that the NK cell activating receptors NKp46 (in humans and mice) and NKp44 (in humans) are able to directly interact with the influenza hemagglutinin protein which is expressed on the viral envelope and on the surface of infected cells (11, 92, 137). They have further identified that this interaction is mediated by the influenza HA protein recognizing and binding to α2,3 (recognized primarily by avian derived strains) and α2,6 (recognized primarily by human derived strains) sialic acid residues present on the extracellular domain of the NKp46 and NKp44 receptors (87, 88, 101, 137). Further studies have also established NKG2D as a likely co-receptor which works synergistically with NKp46 toward NK cell activation during influenza infection, but does not mediate activation on its own (96). Interestingly, although NKp46 has been shown to associate with influenza HA proteins in a variety of ways little has been done to examine how HA intrinsic differences may affect NKp46 binding and NK cell activation.

Two HA intrinsic differences which have previously been shown to have immunological importance are receptor binding specificity and the accumulation of additional glycosylation sites over time as an immune evasion mechanism (91, 138). Hemagglutinin receptor specificity is conferred by the presence of specific, conserved amino acids organized in a particular tertiary structure at and around the receptor binding site (86, 139, 140). Similarly, glycans are added enzymatically in the ER and golgi to specified amino acids which are part of an enzyme-specific recognition sequence with the overall glycosylation pattern dependent the protein sequence and the biochemical machinery of the host cell (141). Studies by Du *et al*. utilized non-infectious virus like particles (VLPs) derived from disparate influenza viruses to show that HAs from different viral strains differentially activate NK cells (97). The authors suggested that more "virulent" strains of influenza induced greater NK cell activation, however they did not speculate on what virulence factors may be involved (97). Similarly, Achdout *et al*. showed that both avian and human derived influenza A viruses induced NK cell activation and cytolysis of infected cells. Furthermore, they found that the disparate HAs interacted with different areas of the NKp46 receptor, with

A/Puerto Rico/8/34 (H1N1) derived HA interacting primarily through O-linked glycans attached to the T225 residue while A/Vietnam/1203/04 (H5N1) derived HA interacted through both the T225 and N216 residues (93). Together these studies suggest that influenza strain virulence as well as sialic acid binding preference correlate with NK cell activation, however neither study interrogated the effects of NKp46-HA binding on NK activation.

Based on the studies described above we hypothesized that avian derived HAs, which possess an α2,3 sialic acid binding preference, would have a higher binding affinity for NKp46 and greater NK activating potential. We additionally hypothesized that the natural accumulation of glycosylation sites on influenza HAs would result in decreased binding to NKp46 due to steric hindrance at the HA receptor binding site, which we would expect to decrease NK activating potential. In order to investigate ability of various disparate influenza HAs of avian and human origin to bind to human NKp46, we expressed and purified recombinant human NKp46-Ig fusion protein (rNKp46) in the human HEK293T cell line. Utilizing this rNKp46 we interrogated the relative binding affinity of various viral HAs via ELISA. We also used this rNKp46 protein to analyze its ability to bind cells infected with disparate influenza viruses or HEK293T cells stably expressing influenza derived HA via flow cytometry. Finally, we investigated the ability of HEK293T cells infected with various influenza viruses to stimulate NK cells *in vitro*. NK cell activation is often measured by % cytotoxicity when co-cultured with a known number of target cells, intracellular expression of IFNγ, surface expression of the marker CD69, and/or the internalization of labeled anti-CD107a (LAMP-1) antibody (11, 96, 97, 142, 143). CD69 is an activation marker that is not expressed on naïve NK cells, but is quickly upregulated following activation (142). Similarly, CD107a is not normally expressed on the cell surface, but becomes exposed when activated NK cells degranulate allowing it to serve as a surrogate for cytotoxicity (143). We find that in support of our hypothesis, avian derived HAs have a higher relative binding affinity compared to human derived HAs. Additionally, we found that more highly glycosylated HAs had decreased binding to rNKp46. Interestingly, and contrary to previous studies, we found that rNKp46 was able to bind to influenza HA in a sialic acid binding independent manner. Together these data show that the number of glycosylation sites on the viral HA as well as HA sialic acid

binding preferences can alter NKp46-HA interactions and therefore NK cell activation. They also suggest an alternative, and as of yet uncharacterized, interaction between human NKp46 and influenza HA which can mediate binding.

Materials and Methods

Transfection of HEK293T cells

HEK293T cells were grown in growth media to 80-90% confluency in 6 well plates. Once confluency was achieved, cells were transfected using Lipofectamine 2000 reagent per manufacturer's instructions. Briefly, 2.5µg of plasmid DNA (pcDNA3.1) containing the gene of interest and a G418 resistance gene was diluted into 300uL Opti-MEM media containing 9uL Lipofectamine 2000 Reagent, mixed and allowed to incubate at RT for 5 minutes. Growth media was removed from wells and replaced with Opti-MEM containing Lipofectamine and plasmid DNA and cells were incubated for 48 hrs at 37°C at which point fresh selection media (growth media containing 500ug/mL G418 (genetecin) (Takara, Mountain View, CA)) was added to the wells. Cells were grown under selection for 1.5 weeks and surviving cells were assayed for HA expression via flow cytometry.

Determination of NKp46 binding affinity via ELISA

0.1µg of recombinant HA derived from A/Vietnam/1203/2004 (H5), A/Indonesia/5/2005 (H5), A/Anhui/1/2005 (H5), A/Solomon Islands/3/2006 (H1), A/California/04/2009 (H1), A/Perth/16/2009 (H3), or A/Brisbane/10/2007 (Influenza Reagent Resource, Manassas, VA) were bound to Corning costar 96-well flat bottom EIA plates (Corning, Corning, NY) overnight in bicarbonate buffer at 4°C. The following morning, wells were washed 3x with PBS, incubated with various concentrations of rNKp46-Fc overnight at 4°C. The next morning, wells were washed 3x with PBS, incubated with HRP-conjugated anti-human Fc antibody (Company, Location) for 1hr at 4°C, washed 3x with PBS, and analyzed 30 minutes after addition of 3,3′,5,5′-Tetramethylbenzidine (TMB) using a BioTek Synergy 2 plate reader (Winooski, VT). Wells not coated with HA or not incubated with rNKp46-Fc served as background controls.

Enrichment of human NK cells from PBMCs

Human blood was collected from healthy donors and PBMCs were isolated using a histopaque 1077 (Sigma-Aldrich, St. Louis, MO) density gradient according to manufacturer's instructions and provided by the laboratory of Dr. Balazs Rada (University of Georgia). Following receipt of PMBCs, we enriched for NK cells using Miltenyi NK Cell Isolation Kit and Miltenyi LS columns (Miltenyi Biotec, San Diego, CA) following manufacturer's instructions. Enrichments were tested for purity via flow cytometry and showed >90% purity for $CD3^{-}CD56^{+}CD16^{+}NKp46^{+}$ lymphocytes.

Infection of HEK293T cells and NK cell stimulation assays

HEK293T cells were infected or mock infected for 8 hours in growth media (DMEM + 10% FBS) with 1 MOI of A/ Puerto Rico/8/34 (H1N1), A/Hong Kong/1/68 x A/Puerto Rico/8/34 2:6 reassortant (x31, H3N2), A/Vietnam/1203/05 (H5N1) or A/Anhui/1/05 (H5N1). Expression of HA on the surface of infected cells was confirmed by flow cytometry. For NK cell stimulation assays, infected cells were co-cultured at a 1:4 (E:T) ratio for 8 hrs in growth media in the presence of APC-conjugated anti-human CD107a and GolgiStop (BD Biosciences, San Jose, CA) with human NK cells column purified using Miltenyi NK Cell Isolation Kit and Miltenyi LS columns (Miltenyi Biotec, San Diego, CA) following manufacturer's instructions. Analysis of NK cell stimulation was determined via flow cytometry as described below.

Flow Cytometry

To visualize HA expression on infected HEK293T cells, single cell suspensions of infected HEK293T cells were incubated with murine anti-HA immune serum (diluted 1:100 in FACS buffer) generated against the various influenza strains for 20 min at 4°C, followed by incubation with FITC-conjugated anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) for 20 min at 4°C. Alternatively, cells were incubated with purified recombinant human-NKp46 (200µg/mL) for 1hr at 4°C. To assess NK cell activation, single cell suspensions of NK and HEK293T cells were obtained from previously described co-cultures, centrifuged at 16,000 RCF for 5 min and stained for 20 minutes at 4°C with a combination of

FITC-conjugated anti-CD56, PerCP-Cy5.5-conjugated anti-NKp46, PE-conjugated anti-CD69, APC/Cy7-conjugated anti-CD3. All antibodies were purchased from Tonbo Biosciences (San Diego, CA) or BD Biosciences (San Jose, CA). Data was acquired using an LSR II with FacsDiva software (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star INC, Ashland, OR).

Results

Influenza A infection increases NK cell activation and effector function in an NKp46 dependent manner

Previous studies have shown that NK cells are able to kill target cells following incubation with influenza infected cells and that NK cells can become functionally activated by influenza infected DCs via NKp46 stimulation (11, 96). We wanted to confirm that NK cells are directly activated by infected target cells which are not professional APCs, and that this activation is mediated by NKp46 ligation. To this end, we infected HEK293T cells with a human origin H3N2 influenza A virus and co-incubated the infected cells with freshly purified donor human NK cells. Human NK cells can be categorized by CD56 expression level, with $CD56^{dim}$ cells representing cytotoxic cells while $CD56^{bright}$ cells primarily produce IFNγ (144). In our study we assessed expression of the activation marker CD69 and uptake of CD107a, a marker of cytotoxicity and degranulation, as well as expression of IFNγ on both CD56 bright and dim populations. Human NK cells co-incubated with uninfected HEK293T cells showed significantly upregulated CD69, demonstrated internalization of CD107a, and produced significantly more IFNγ compared to NK cells incubated in media alone (Fig 3.1). This is likely due to the fact that HEK293T cells are a human tumor cell line and likely display aberrant expression of MHC-I molecules as well as stress ligands recognized by NK cell activating receptors. Co-incubation of human NK cells with influenza infected HEK293T cells significantly upregulated CD69 and significantly increased intracellular IFNγ production compared to uninfected controls and also demonstrated internalization of CD107a (Fig 3.1). This indicates that while co-culture of uninfected HEK293T cells with NK cells leads to NK cell activation, the expression of influenza proteins by HEK293T target cells significantly increases their activation potential. Next, we pre-incubated influenza infected cells with rNKp46 protein followed by co-culture with donor NK cells and

found that while pre-incubation with rNKp46 did not significantly decrease the proportion NK cells expressing CD69, there was a clear decrease in CD107a uptake among cytotoxic NK cells and a significantly decreased ability of $CD56^{bright}$ NK cells to produce IFNγ compared to NK cells co-cultured with infected HEK293T cells that had not been pre-incubated with rNKp46 (Fig 3.2). Additionally, we found that infected HEK293T cells pre-incubated with rNKp46 still showed increased ability to activate NK cells (increased expression of CD69 and IFNγ as well as increased internalization of CD107a) compared to NK cells incubated in media alone (Fig 2.3). This is likely due in part to the inherent ability of HEK293T cells to activate human NK cells (as demonstrated in Fig 3.1), and the levels of CD69 and IFNγ expression as well as CD107a internalization on NK cells pre-incubated with rNKp46 may represent the basal level of the donor's NK cells when co-incubated with HEK293T cells. However, further experiments will need to be done in order to determine if infected HEK293T cells pre-incubated with rNKp46 demonstrate increased ability to activate NK cells when compared to uninfected HEK293T cells as this experiment did not directly compare the two groups. Together these data indicate that influenza infected HEK293T cells have a significantly greater potential to activate NK cells and this potential is mediated, at least in part, by interaction with NKp46. It must be noted, however, that these data are representative of two independent experiments for figure 3.1 and of a single experiment for figure 3.2 (all experiments done with biological duplicates). Given the potential for significant variation of NK cell activation and effector function within the human population, these experiments will need to be repeated in the future to increase the sample size.

Recombinant NKp46 preferentially binds to avian derived HAs

Human NKp46 has previously been shown to bind to both human and avian origin influenza HA proteins through specific sialic acid moieties on the NKp46 ectodomain and mediate NK cell activation and cytolysis (11, 93). Additionally, it was also determined that human origin HAs recognized glycans attached to a single amino acid (T225) whereas avian origin HAs recognized sialic acid residues attached to two distinct amino acids (T225 and N216) and that avian origin influenza viruses may have a greater potential to activate human NK cells (93, 97). To test if NKp46 preferentially binds to avian derived influenza HAs

over human origin HAs, we utilized an ELISA by coating a 96-well plate with recombinant influenza HAs (rHA), incubating these wells with rNKp46 protein, and then detecting the amount of rNKp46 bound to the plate. We found that avian origin HAs do display a larger amount of rNKp46 compared to human origin HAs (Fig 3.3). Interestingly, there appeared to be a hierarchy of binding ability with A/Vietnam/1203/04 (H5) (VN/04) exhibiting the highest level of binding followed by other avian origin (H5) HAs, human origin H1 HAs exhibiting intermediate levels of binding, and human origin H3 HAs exhibiting the lowest level of binding. Additionally, we infected the human derived HEK293T cells with avian and human origin influenza viruses to test the ability of NKp46 to bind infected cells. We found that while the majority of HEK293T cells were infected, as demonstrated by surface expression of viral HA, cells infected with avian origin viruses (H5) had increased NKp46 binding compared to human origin viruses (H1 and H3) (Fig 3.4). Similar to our previous findings, cells infected with VN/04 (H5N1) had the highest levels of NKp46 binding while A/Aichi/2/68 (H3N2) displayed little binding. Interestingly, the ability of VN/04 (H5) to bind to NKp46 was markedly greater than that of A/Anhui/1/2005 (H5) (AN/05) (~3x) according to our binding ELISA (Fig 3.3), however the difference was greatly diminished when testing NKp46 binding to infected cells (Fig 3.4). This suggests that differences in binding ability observed using recombinant HAs may not be representative of the HAs binding ability when expressed on the surface of human cells. Although the HEK293T cells were infected with the same MOI of the different viruses at the same time, it is also possible that the HA protein is synthesized and/or expressed on the surface of infected cells at a different rate for these viruses, allowing for AN/05 to have more HA protein expressed on the cell surface. Further studies will need to be done utilizing a monoclonal antibody that is capable of recognizing both viral HAs to control for possible discrepancies in HA expression levels. While this data demonstrates that avian origin HAs do indeed possess a greater ability to bind NKp46 protein compared to human origin HAs, there may be differences even among related HAs within the same clade. This suggests that while α2,3 or α2,6 sialic acid binding preference likely influences NKp46 binding potential, it is not the sole determining factor.

Increased glycosylation of influenza HA, but not receptor binding specificity impacts NKp46 binding

Another factor which could impact the ability of NKp46 to interact with influenza HA is the level of glycosylation on the HA head. Influenza A viruses naturally accumulate additional glycosylation sites over time on the head of the HA protein via antigenic drift as a mechanism to evade humoral immunity (91, 145). While the accumulation of glycosylation sites on the influenza HA head is thought primarily to shield the virus against sterilizing immunity via neutralizing antibodies, these modifications can also decrease HA binding affinity and avidity (91). Since NKp46 binding is thought to be dependent on HA binding to sialic acids, we hypothesized that addition of glycosylation sites would result in decreased NKp46 binding.

To interrogate the impact of HA glycosylation on NKp46 receptor binding, we utilized an influenza HA from a human origin virus (A/Aichi/2/68 (HK68)) as a parental strain to which we added point mutations that have previously been shown to confer additional glycosylation sites to human H3 viruses naturally circulating through the human population (145). Using a plasmid contacting a parent HA sequence, we generated a mutant plasmid to express a HA with four additional, naturally occurring glycosylation sites (HK68+4) through site directed mutagenesis. Additionally, we generated a mutant HA plasmid which had the same number of glycosylation sites as the parent, but contained a Y98F mutation (HK68-Y98F) which has previously been described to eliminate the HAs ability to bind to sialic acid (146, 147). We anticipated that the Y98F mutant would serve as a negative control as previous studies indicated that the NKp46-HA interaction was mediated solely by HA recognition of sialic acid residues. Once our plasmids were generated, we transfected them into a human cell line HEK293T, selected for cells expressing our various HAs, and confirmed surface expression of HA via flow cytometry (Fig 3.5A, C). Once we confirmed that the majority of our selected cells were stably expressing influenza HA (HK68, HK68+4, or HK68-Y98F respectively) (Fig 3.5A, C), we assessed their ability to bind to rNKp46 protein via flow cytometry. We found that, in support of our hypothesis the cells expressing the HK68+4 mutant HA had decreased binding of rNKp46 (~1/2 reduction) compared to cells expressing the parental HK68 HA (Fig 3.5A, B). Interestingly, although the HK68-Y98F mutant exhibited decreased NKp46 binding (~60% over background) compared to the parent HA, it was able to bind rNKp46 protein above background levels (Fig 3.5C). Since this mutation has previously been demonstrated to ablate the ability of the HA to

bind sialic acids, this points to a novel mechanism mediating NKp46-HA interactions. These data demonstrate that natural accumulation of glycosylation sites on influenza HA proteins decreases their ability to be recognized by human NK cells via the NKp46 receptor and suggest an uncharacterized interaction between NKp46 and viral HA which could mediate NK cell activation.

Discussion

Although historically neglected, the contribution of NK cells to the anti-influenza immune response has recently begun to be elucidated. Early studies showed that depletion of NK cells in animal models resulted in increased morbidity and decreased survival following influenza A infection (134, 148). Later studies identified the NK cell activating receptor NKp46 as a possible mediator of NK cell protection as mice genetically modified to delete this gene also showed decreased survival following infection (103). A plethora of animal and human studies also indirectly support the importance of NKp46 expressing NK cells following influenza A infection (98-100). Additionally, studies by Mandelboim *et al*. and Draghi *et al*. showed that influenza HA directly interacts with the NKp46 receptor and that this interaction mediates NK cell immune functions (11, 96). However, the molecular determinants of the NKp46-HA interaction or their contributions to NK cell activation are as yet incompletely understood.

Here we examined the contribution of HA sialic acid binding preferences as well as HA glycosylation to NKp46 binding. Previous studies have suggested that avian origin influenza virus HA proteins may have a higher propensity for NKp46 binding when compared to human origin viruses and that this could result in increased NK cell activation follow infection with avian origin viruses (93, 97). Utilizing recombinant NKp46 and influenza HA proteins we found that avian origin viruses do indeed have a greater potential for binding human NKp46 (Fig 3.3). Although we did not directly compare the ability of human and avian origin HAs to activate NK cells, we do demonstrate the importance of NKp46 ligation for NK cell activation following influenza infection (Fig 3.2). Given the importance NKp46 ligation plays in NK cell activation following influenza infection and that avian origin HAs had a higher binding affinity for NKp46, it is likely that avian origin HAs possess a greater potential for activation of NK cells compared to human origin HAs (96, 97). However, further studies utilizing equivalent amounts of human and avian

origin influenza HAs to activate NK cells will need to be conducted in order to test this hypothesis. Differences in sialic acid binding preference of disparate influenza HAs represent one possible explanation for differential recognition of NKp46. However, our studies utilizing a mutant HA which is incapable of sialic acid binding found that the NKp46-HA interaction can be mediated independent of sialic acid binding albeit at a lower affinity (Fig 3.5). Previous studies have utilized point mutations in the NKp46 receptor to demonstrate the importance of specific amino acids as potential sites mediating NKp46-HA interactions via expression of host sialic acid residues, however, to our knowledge this is the first example of sialic acid independent binding of NKp46 and influenza HA (Fig 3.5) (149, 150). It is unclear why mutations at these sites appear to ablate both sialic acid mediated and independent interactions with influenza HA, however it is possible that the inserted mutations altered the structure of the NKp46 protein in an unpredicted way which alters the receptors ability for binding or signaling.

In addition to sialic acid binding preferences, the number of glycosylation sites present on the influenza HA could alter NKp46 binding potential. Previous studies have shown that the natural accumulation of glycosylation sites on the HA of circulating influenza strains decrease viral fitness and inhibit the ability of the HA to bind sialic acids (91, 145). To this end, we compared the ability of a mutant HA containing four additional, naturally occurring glycosylation sites to bind rNKp46 with the parent HA binding rNKp46. We found that addition of glycosylation sites did indeed decreased the ability of the mutant HA to bind rNKp46, supporting our hypothesis that natural accumulation of glycosylation on influenza HA proteins decreases NKp46 recognition and likely leads to decreased NK cell activation (Fig 3.5). While it is unclear how influenza HA glycosylation affects NK cell activation *in vivo*, this data in conjunction with our previous data suggest that viruses which have higher levels more glycosylation will activate NK cells less efficiently. This in turn could lead to milder symptoms during the infection as well as increased generation of CD8 T_{RM} (Chapter 2) (134). Additionally, the accumulation of glycosylation on influenza HAs is thought to promote generation of a broadly protective anti-influenza antibody responses specific to the HA stalk (91). Therefore, use of more heavily glycosylated HAs in influenza vaccines

represents a promising method to induce broadly protective, long lived T and B cell mediated immunological memory.

Altogether these studies demonstrate that inherent differences in influenza HA – either sialic acid binding or glycosylation level – can alter interactions with the NK cell activating receptor NKp46 and thus NK cell activation. Given that NK cells have been implicated in suppressing the development of anti-viral immunological memory, these studies present potential mechanisms which could be exploited to generate better, more broadly protective influenza vaccines (112, 151). However, further studies are needed to determine the impact of these HA differences *in vivo* as well as further characterize the NKp46-HA interaction and the molecular mechanisms that contribute to NKp46 ligation.

Acknowledgements

We would like to thank Dr. Balazs Rada of the University of Georgia for supplying us with human donor PBMCs. We would also like to thank Ivelisse Resto-Garray for her contributions to this work in purifying the rNKp46 fusion protein and performing NKp46 binding assays. Additionally, we would like to thank Dr. David Steinhauer of Emory University for providing A/Aichi/2/1968 HA containing plasmid we used to generate our HA mutants. Recombinant H5 HA with Histidine Tag, from Influenza A/Vietnam/1203/2004 (H5N1), FR-39, H5 HA with Histidine Tag, from Influenza A/Indonesia/5/2005 (H5N1), FR-59, H5 HA with Histidine Tag, from Influenza A/Anhui/1/2005 (H5N1), FR-86, H1 HA with Histidine Tag, from Influenza A/Solomon Islands/3/2006 (H1N1), FR-67, H1 HA with Histidine Tag, from Influenza A/California/04/2009 (H1N1), FR-180, H3 HA with Histidine Tag, from Influenza A/Perth/16/2009 (H3N2), FR-472, and H3 HA with Histidine Tag, from Influenza A/Brisbane/10/2007 (H3N2), FR-61, were obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA

Figures

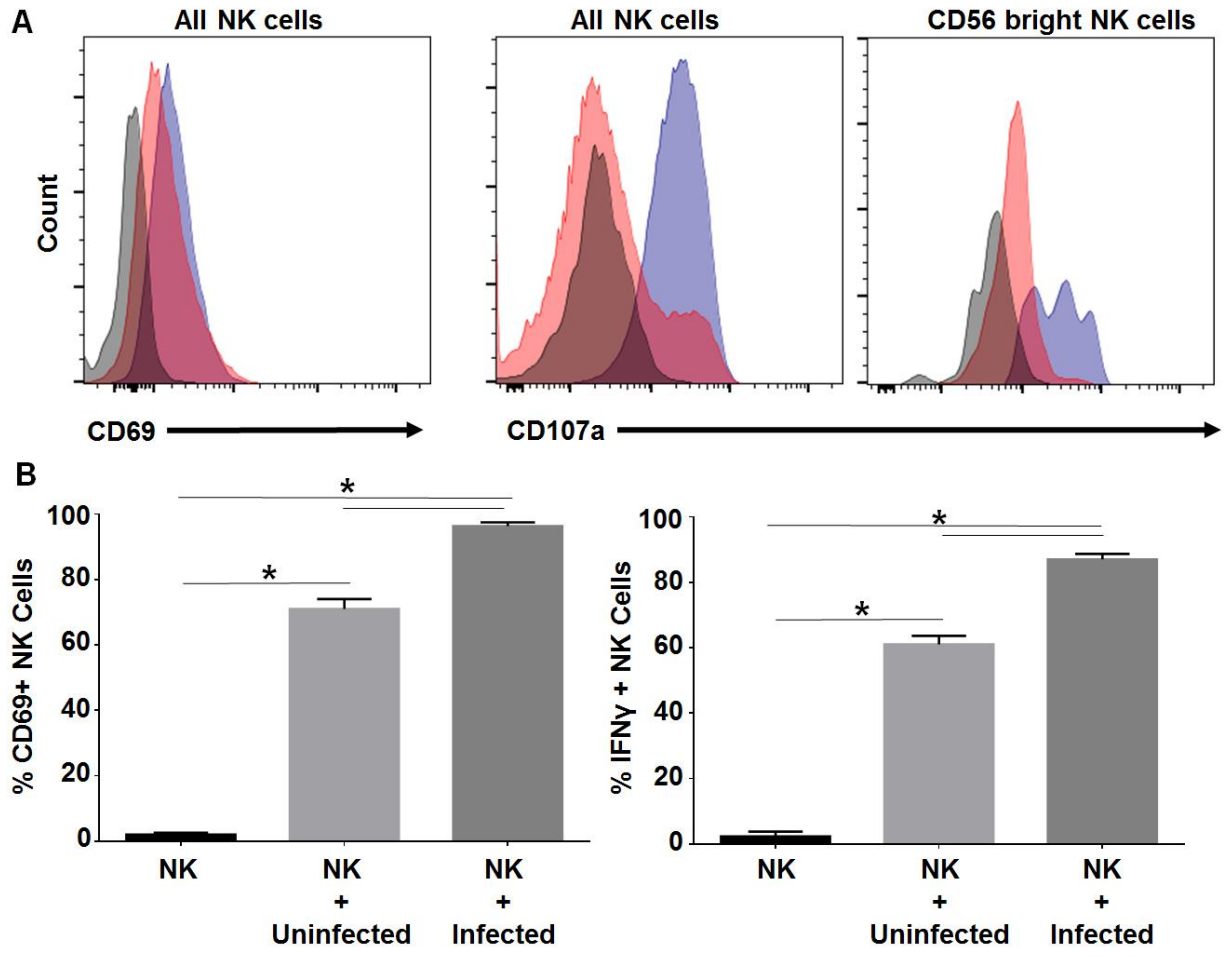

A
All NK cells
All NK cells
CD56 bright NK cells
Count
CD69
CD107a
B
% CD69+ NK Cells
100
80
60
40
20
0
*
*
NK
NK + Uninfected
NK + Infected
% IFNγ + NK Cells
100
80
60
40
20
0
*
*
NK
NK + Uninfected
NK + Infected

Figure 3.1. Influenza infection increases activation and effector function of NK cells. Human NK cells were incubated with infected and uninfected HEK293T cells in the presence of monensin and anti-CD107a antibody. (A) NK cells (grey) and NK cells incubated with HEK293T cells infected (blue) or uninfected (red) with HKx31 influenza virus were analyzed for expression of activation marker CD69 and internalization of degranulation marker CD107a by all NK cells (left; middle) and CD107a internalization by the $CD56^{bright}$ subset (right). (B) CD56 bright NK cells were analyzed for expression of activation marker CD69 (left) and IFNγ (Right). Graphs are representative of 2 independent experiments done with biological duplicates. * = $p < 0.05$.

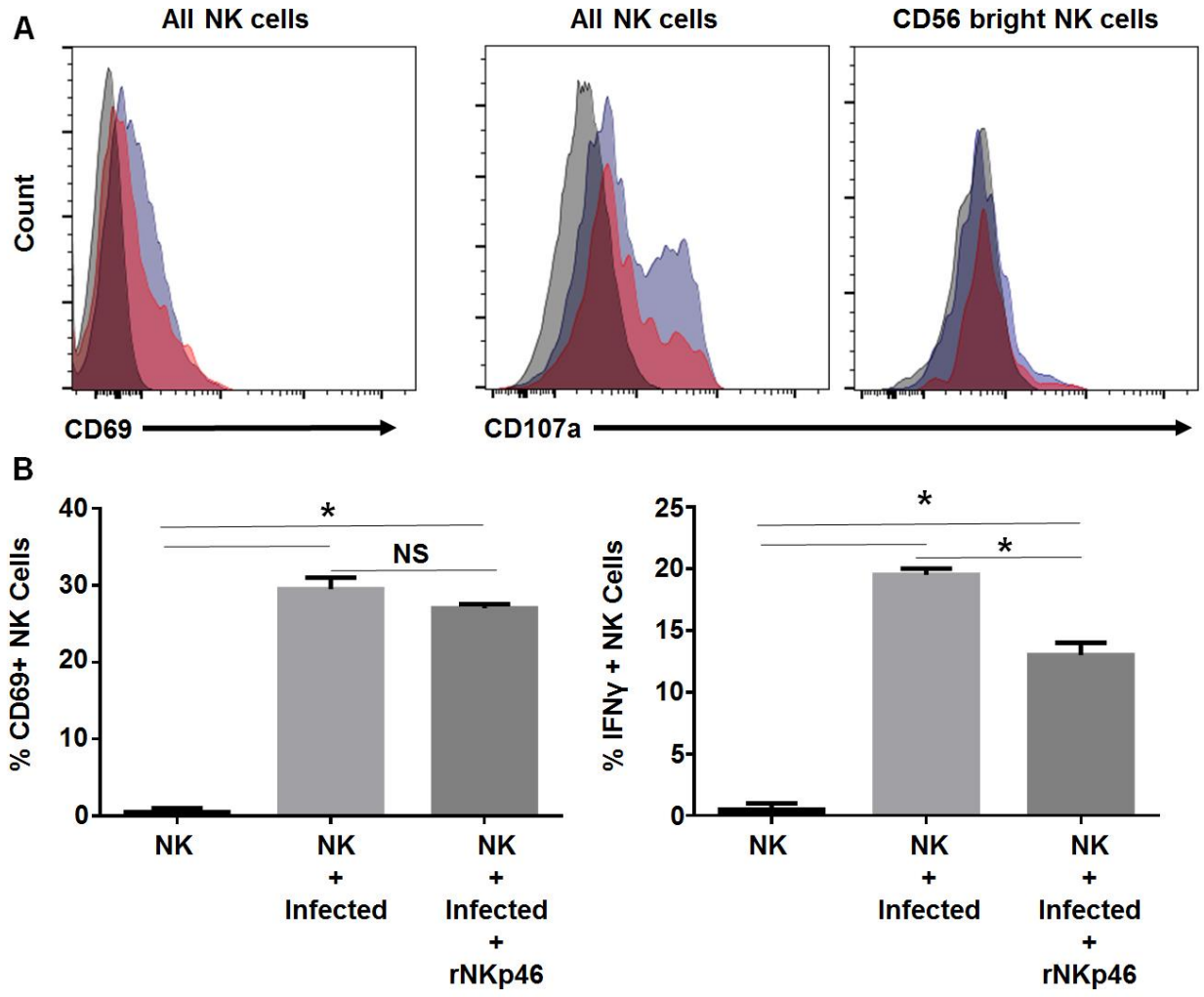

A
All NK cells
All NK cells
CD56 bright NK cells
Count
CD69
CD107a
B
% CD69+ NK Cells
40
30
20
10
0
*
NS
NK
NK + Infected
NK + Infected + rNKp46
% IFNγ + NK Cells
25
20
15
10
5
0
*
*
NK
NK + Infected
NK + Infected + rNKp46

Figure 3.2. Pre-incubation of infected HEK293T cells with rNKp46 decreases NK cell activation. (A) Infected HEK293T cells were incubated in the presence (red) or absence (blue) of rNKp46 prior to co-incubation with human NK cells and analyzed for expression of CD69 and internalization of CD107a for all NK cells (left; middle) and CD107a internalization by the $CD56^{bright}$ subset (right). (B) CD56 bright NK cells were analyzed for expression of CD69 and IFNγ. Graphs are representative of 1 experiment done with biological duplicates. * = $p < 0.05$; NS = not significant.

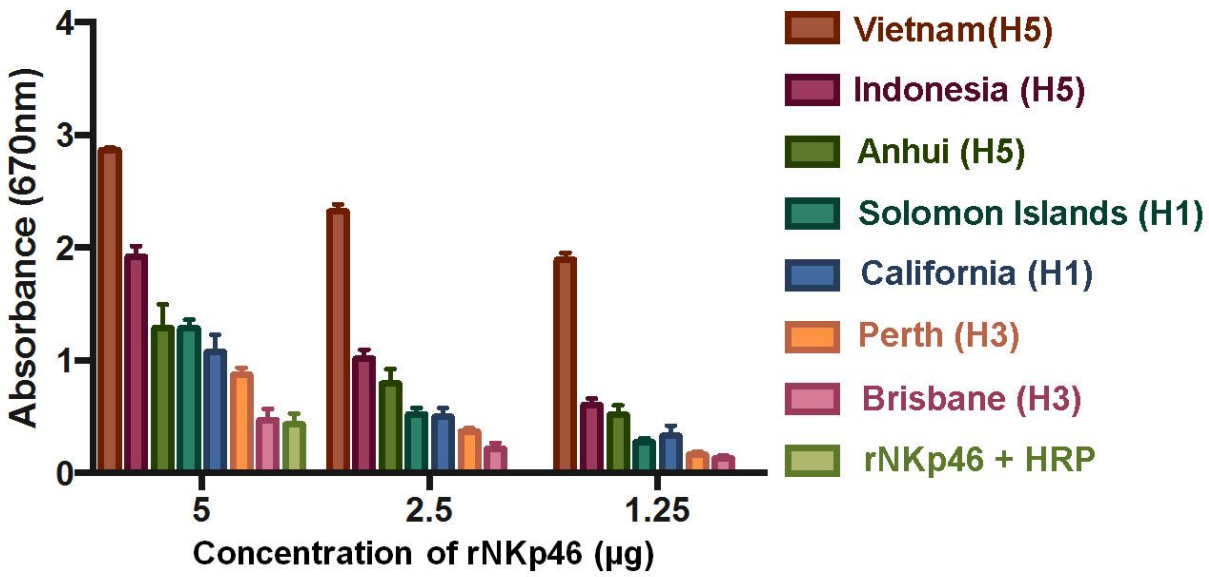

Absorbance (670nm)
4
3
2
1
0
5
2.5
1.25
Concentration of rNKp46 (µg)
Vietnam(H5)
Indonesia (H5)
Anhui (H5)
Solomon Islands (H1)
California (H1)
Perth (H3)
Brisbane (H3)
rNKp46 + HRP

Figure 3.3. Binding of rNKp46 to recombinant HAs. A 96 well plate was coated with the indicated recombinant HAs and then incubated with indicated concentrations of recombinant NKp46 overnight at 4ºC. A HRP-conjugated α-human Fc mAb and 3,3′,5,5′-Tetramethylbenzidine (TMB) were used as secondary antibody and as substrate, respectively. Graphs are representative of 3 independent experiments done in triplicate.

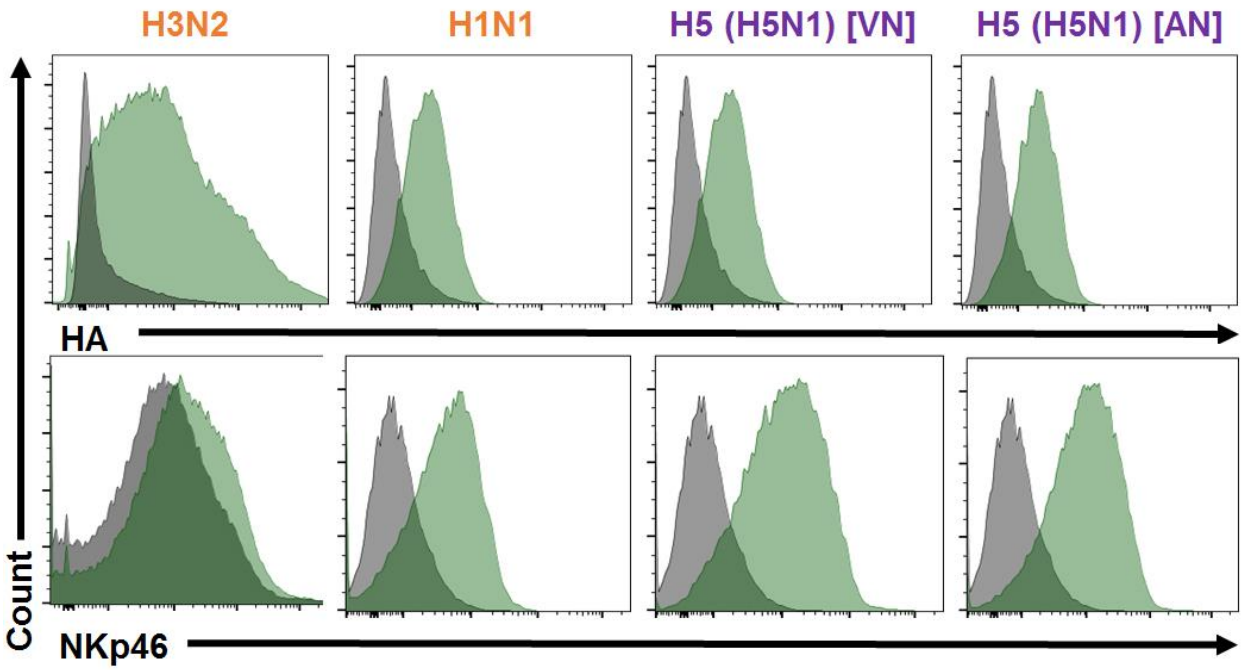

H3N2
H1N1
H5 (H5N1) [VN]
H5 (H5N1) [AN]
HA
Count
NKp46

Figure 3.4. Recombinant NKp46 binds to virally infected HEK293T cells. HEK239T cells were infected or mock infected overnight with 1 MOI of indicated virus. Cells were then incubated with anti-HA antiserum (Top) or with 20ug recombinant NKp46-huFC (Bottom) and binding was detected using anti-Fc antibodies via flow cytometry. Uninfected cells (Top) and infected cells incubated with 0ug NKp46 (Bottom) were used as controls (grey). All viruses (A/HK/1/68, A/VN/1203/04, A/Anhui/1/05) are on a BSL2 A/PR/8/34 backbone. Graphs are representative of 2 independent experiments done with biological duplicates.

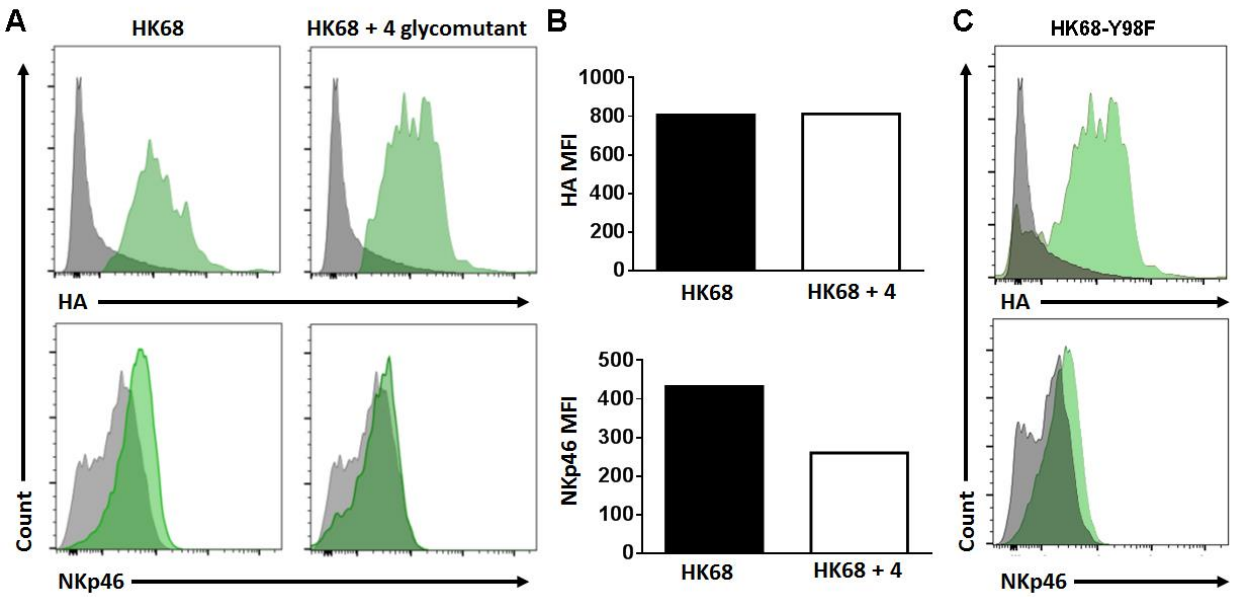

A
HK68
HK68 + 4 glycomutant
HA
Count
NKp46
B
HA MFI
1000
800
600
400
200
0
HK68
HK68 + 4
NKp46 MFI
500
400
300
200
100
0
HK68
HK68 + 4
C
HK68-Y98F
HA
Count
NKp46

Figure 3.5. Hemagglutinin glycosylation level but not ability to bind sialic acid impacts NKp46 binding. HEK293T cells were transfected with viral hemagglutinin from A/Hong Kong/1/68 (HK68), a HK68 glycosylation mutant that adds 4 additional glycosylation sites, or a Y98F mutant (HK68-Y98F) that lacks the ability to bind to sialic acid residues[6] and selected for stable transfection. (A) Expression of viral HA on the surface of transfected (green) HEK293T cells compared to non-transfected (grey) controls (top) and binding of rNKp46 protein to transfected HEK239T cells (green) or non-transfected (grey) controls (bottom). (B) Median fluorescent intensity of viral HA expressed on the surface of transfected HEK293T cells (top) and rNKp46 protein (bottom) bound to transfected HEK239T cells. (C) Expression of viral HA on the surface of transfected (green) cells or non-transfected (grey) controls (top) and binding of rNKp46 protein (bottom) to transfected cells (green) or non-transfected (grey) controls. Graphs are representative of 2 independent experiments done with biological duplicates.

CHAPTER 4

Discussion and future directions

The immune response to influenza virus infection is a complex and multifaceted process which involves a multitude of interactions between various distinct immune cells. To date most anti-influenza research has focused on the development of humoral and cellular immune responses and factors which directly impact immunological memory development while other accessory cells have been largely ignored. Natural Killer cells represent one such neglected cell type which can directly impact primary influenza infections as well as influence the adaptive immune response (148, 151). NK cells compose a large proportion of lymphocytes present in the lung and respiratory airways during steady-state, putting them in a position to be first responders against respiratory infections such as influenza (10). In fact, NK cells have been implicated in control of early influenza viral replication and production of important pro-inflammatory cytokines following infection (120, 134). After viral clearance NK cells have also been shown to be important for tissue repair and returning the lung to a state of tolerance through the production of IL-22 and IL-10 (50, 135). Additionally, NK cells have been implicated in suppressing both B and T cell responses to systemic viral infections such as LCMV and MCMV directly via lysis of activated cells and indirectly through NK-DC crosstalk (9, 151). Thus, NK cells are uniquely positioned to modulate the adaptive immune response to influenza infection through direct contact with adaptive immune cells, interactions between NK cells and DCs, as well as production of pro-inflammatory and anti-inflammatory cytokines.

Here we interrogated the contribution of NK cells to the generation of the anti-influenza CD8 T cell response and subsequent memory formation. We found that depletion of NK cells prior to influenza infection resulted in increased numbers of influenza-specific CD8 T cell in the lung draining lymph node as well as resident memory CD8 T cells in the lung (Fig 2.4). Previous studies indicate that these T_{RM} cells are essential mediators of heterosubtypic protection against subsequent influenza infections, while infiltrating memory CD8 T cells can mediate deleterious lung immunopathology (64). Importantly, we found that the increased number of CD8 T_{RM} in NK depleted mice was able to mediate heterosubtypic

protection against a secondary influenza challenge with fewer infiltrating memory CD8 T cells accumulating within the lung compared to NK sufficient controls (Figure 2.2). While memory CD8 T cells are crucial for protection against disparate influenza viruses, they have also been implicated as a major cause of lung immunopathology and thereby contributors to secondary bacterial infection (5, 64, 65, 105, 152). Thus, NK cells present a potential mechanism to modulate the anti-viral CD8 T cell response during influenza vaccination and produce a larger pool of cross-reactive T_{RM} cells which protect against a broad range of subsequent viral infections with a decreased risk of secondary bacterial infection.

While our studies in chapter 2 utilized an NK cell depletion model to investigate the contribution of NK cells to the generation of CD8 T cell memory, NK cell depletion is neither practical nor safe in the context of human vaccination. Therefore, in order to modulate NK cell activation to improve the adaptive immune response to influenza infection we must first gain a better understanding of how NK cells interact with and become activated by the influenza virus. It is possible that with a greater understanding of the factors that contribute to NK cell activation by influenza viruses, novel treatments or inhibitors could be developed to interfere with NK cell activation during vaccination or shortly after infection and thus increase the generation of broadly protective CD8 T_{RM} cells in the lung and lung airways. Similarly, elucidating the factors of NK cell activation may help to explain discrepancies in disease severity and outcome between different influenza strains and subtypes as more severe disease has been correlated with a dampening of the NK cell response (99, 153). The NK cell activating receptor, NKp46, has been shown to directly interact with the influenza hemagglutinin protein and this interaction can mediate NK cell activation resulting in inflammatory cytokine production and cytolysis (Fig 3.1; Fig 3.2) (11). This NKp46-HA interaction is thought to be the result of the influenza HA binding to sialic acid residues attached to specific amino acids on the NKp46 receptor (137, 150). However, contrary to previous studies we found that influenza HA is able to interact with the NKp46 receptor independently of sialic acid binding (Fig 3.5). Since other studies have utilized point mutations in a recombinant NKp46 receptor to establish the importance of specific amino acids in the recognition of influenza HA, it is possible that in addition to removing potential glycosylation sites on NKp46 the structure of the receptor was altered. Alterations to the structure of NKp46

through mutation could block other sites of NKp46-HA interaction and interfere with NKp46 ligation. Alternatively, the mutated amino acids could be essential to NKp46-HA binding in a sialic acid independent manner. Further research will be required fully elucidate the molecular nature of the NKp46-HA interaction, possibly requiring x-ray crystallography of HA bound to NKp46.

Although the NKp46-HA binding can occur independent of HA sialic acid recognition, it does appear that sialic acid recognition increases the potential for these two proteins to interact as evidenced by decreased, but not completely ablated, binding of rNKp46 to Y98F mutant HA compared to wild type HA. It is therefore possible that different sialic acid binding preferences of disparate influenza HAs mediate differential binding to the NKp46 receptor. Avian influenza viruses show a preference for binding α2,3 sialic acids while human origin viruses show a α2,6 binding preference (87, 88). The NKp46 receptor has been shown to express both α2,3 and α2,6 sialic acids with avian origin viral HA recognizing two distinct binding sites of the NKp46 receptor and human origin HA recognizing one (93). It is therefore possible that avian origin viruses possess an increased ability to activate human NK cells compared to human origin viruses (97). In chapter 3 we showed that avian origin influenza HAs bind recombinant human NKp46 more readily than human origin HAs (Fig 3.4; Fig 3.5). This may be due to the fact that avian origin HAs are able to recognize more binding sites on the NKp46 protein, that avian HAs have a higher affinity for the NKp46 protein, or some combination of the two. Since we see binding discrepancies both between different HA subtypes (H5 vs. H1 vs. H3) and within the subtypes themselves (H5 VN vs. H5 AN), it is likely that disparate HAs have different binding affinities for NKp46 and that the difference in binding affinity contributes to difference observed in our binding assays. For instance, the avian HA derived from A/Vietnam/1203/2004 differs from the A/Anhui/1/2005 derived HA a number of amino acid residues that could affect sialic acid binding affinity. Differences at the amino acid residues 190, 197, 216, 228 and 243 could contribute to differential binding between these two HAs as they occur near amino acid residues known to impact sialic acid recognition and binding owing to their proximity to the sialic acid binding pocket (147). Importantly, these discrepancies in relative binding affinity cannot be ascribed solely to differences in HA subtype and binding affinity suggesting that other factors are also involved in mediating

the NKp46-HA interaction. However, our assays only probed relative binding affinity of various avian and human origin HAs for NKp46. Therefore, further studies are necessary to determine exact binding affinities and avidities for the disparate influenza HAs interacting with NKp46.

In addition to sialic acid binding preferences, the overall level of glycosylation on the HA head of disparate influenza viruses can vary significantly (91). Increased glycosylation is capable of interfering with the ability of the HA to recognize and bind sialic acids (145). While it is thought that natural accumulation of glycosylation sites on influenza HA primarily functions to evade antibody mediated neutralization, it is possible that these additional glycosylation sties also interfere with the ability of NKp46 to recognize the viral HA (91). In chapter 3 we demonstrated that additional glycosylation of the influenza HA can reduce NKp46 binding (Fig 3.5). Similarly, we found that the A/Vietnam/1203/2004 derived HA bound to recombinant NKp46 protein to a greater extent than A/Anhui/1/2005 derived HA (Fig 3.3; Fig 3.4). One possible explanation for this binding discrepancy is that A/Vietnam/1203/2004 derived HA has fewer predicted glycosylation sites when compared to A/Anhui/1/2005 derived HA. This increased glycosylation could negatively impact the ability to A/Anhui/1/2005 derived HA to bind NKp46 as we have shown additional glycosylation decrease NKp46 recognition and binding (Fig 3.5). Due to the importance of NKp46 binding to NK cell activation by influenza infected cells, it is likely that HA glycosylation level also impacts the ability to the HA to stimulate NK cell activation and effector functions (11, 96). This in turn could impact the generation of CD8 T cell memory as evidenced in chapter 2.

In humoral immunity, HA glycosylation level is thought to contribute to the concept of original antigenic sin – where the first infection has an outsized impact on subsequent antibody responses and can results in overproduction of non-neutralizing antibodies by memory B cells – as influenza HAs with higher levels of glycosylation tend to result in more neutralizing antibodies targeting more highly conserved HA stalk epitopes rather than epitopes on the HA head (91, 154). Based on this hypothesis, children who are first exposed to a heavily glycosylated HA should develop a larger repertoire of broadly protective stalk antibodies than those exposed to a less glycosylated variant of the same influenza strain. Similarly, as we have shown in chapter 3 that HA binding to NKp46 is decreased as HA glycosylation increases and that

NKp46 ligation is critical for NK cell activation it is likely that the HA glycosylation level can also impact the CD8 T cell response and memory formation. In chapter 2 we demonstrated that NK cells actively suppress CD8 T cell memory generation and that the presence of NK cells diminishes the T_{RM} subset of CD8 T cell memory which is important for viral protection. It is therefore possible that primary exposure to a heavily glycosylated influenza strain will lead to decreased NK cell activation (via hindrance of the NKp46-HA interaction) and thus increased generation of broadly CD8 T cell memory in addition to the aforementioned predisposition toward protective stalk antibodies.

Although NK cells are normally thought of as innate lymphoid cells, recent studies suggest that NK cells are capable of taking on a memory like phenotype following infection with a number of different viruses including influenza A (155). Murine models have indicated that influenza is able to stimulate the generation of CCR2 expressing NK cells which home to the bone marrow following the resolution of influenza infection, but traffic to the lung and exert increased cytotoxic and cytokine producing capabilities upon secondary viral challenge (156). Interestingly, immunization with influenza virus like particles containing influenza HA was sufficient to generate partial protection from viral challenge in $RAG^{-/-}$ mice three months post-vaccination, suggesting a protective role for these cells in the absence of an adaptive immune response (157). Similarly, a recent human study found that influenza-specific memory like NK cells were generated and survived for up to 6 months following influenza vaccination, and that these NK cells were likely targeting the viral HA via NKp46 (158). While the NK cell contribution to the primary influenza immune response has been minimally investigated, these studies suggest that memory NK cells may have a role in control of and protection from subsequent viral infections. To this end our data would suggest that avian origin influenza viruses likely develop more memory NK cells as they have higher relative binding affinity for NKp46 (Fig 3.2) and appear to more readily stimulate NK cells (97). Furthermore, our studies suggest that more heavily glycosylated influenza HAs would produce fewer memory NK cells due to decreased ligation of NKp46. Although the importance of memory NK cells in protection of immunocompetent persons against future viral infections is likely small given the speed and efficiency of memory B and T cell responses (especially T_{RM} CD8 T cells) they may help protect people

with primary B and T cell deficiencies due to genetic disease or infection with HIV (159, 160). Additionally, it is unclear if the immunomodulatory functions of NK cells continue to shape the adaptive immune response beyond the primary infection. CD8 T_{RM} cells become functionally reactivated and produce pro-inflammatory cytokines within 2 days post challenge making them de facto first responders (Fig 2.3; Fig 2.4). Memory T cell derived IFN-γ has also been shown to be important for activation of human NK cells with influenza A virus *in vitro* (161). It is therefore unlikely that NK cells play a large role in shaping the adaptive immune response to influenza A infection after primary exposure, however further studies are needed to confirm this hypothesis.

It is clear that NK cells play important roles in the anti-influenza immune response. On the one hand they can limit early viral replication and contribute to tissue repair (134, 135, 162). On the other hand, NK cells also limit the generation of CD8 T cell memory and protective CD8 T_{RM} cells (Chapter 2). Therefore, in the context of vaccination NK cells are not only dispensable but also deleterious, and the inhibition of the anti-influenza NK cell response should boost long term, protective CD8 T cell immunity with very little risk. The NKp46 receptor represents an obvious target for limiting the anti-influenza CD8 T cell response given that it directly interacts with influenza HA proteins and appears to be the major activating receptor through which influenza viruses activate NK cells (Chapter 3) (11, 96). While there are currently no known small molecule inhibitors which block the NKp46-HA interaction that could be added as components of influenza vaccines, alterations of HA receptor binding specificity and/or HA glycosylation levels could serve as mechanisms to decrease NK cell activation and boost immunological memory. In chapter 3 of this dissertation we demonstrated that avian origin influenza viruses with an α2,3 binding preference have greater potential to bind rNKp46 compared to human origin viruses and that increased glycosylation of the influenza HA decreases rNKp46 binding ability. Utilizing this knowledge and in conjunction with studies characterizing the molecular determinants of influenza HA receptor binding preference as well as studies mapping natural accumulation of glycosylation on influenza HA proteins it may be possible to design live-attenuated or subunit vaccines which do not induce strong NK cell response, but do produce long lived, broadly protective cellular and humoral immune responses. We believe that the

work presented in this dissertation will serve an important role highlighting the importance of NK cells to rational vaccine design and contribute toward the development of a broadly neutralizing influenza vaccine.

References

1. WHO. 2014. Influenza (Seasonal) Fact Sheet N211.
2. Fields, B. N., D. M. Knipe, and P. M. Howley. 2007. *Fields' Virology*. Wolters Kluwer Health/Lippincott Williams & Wilkins.
3. Doherty, P. C., and A. Kelso. 2008. Toward a broadly protective influenza vaccine. *The Journal of clinical investigation* 118: 3273-3275.
4. Doherty, P. C., W. Allan, M. Eichelberger, and S. R. Carding. 1992. Roles of alphabeta and gammadelta T Cell Subsets in Viral Immunity. *Annual review of immunology* 10: 123-151.
5. Hogan, R. J., E. J. Usherwood, W. Zhong, A. D. Roberts, R. W. Dutton, A. G. Harmsen, and D. L. Woodland. 2001. Activated Antigen-Specific CD8+ T Cells Persist in the Lungs Following Recovery from Respiratory Virus Infections. *The Journal of Immunology* 166: 1813-1822.
6. Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan. 2009. T cell activation. *Annual review of immunology* 27: 591-619.
7. Cox, M. A., S. M. Kahan, and A. J. Zajac. 2013. Anti-viral CD8 T cells and the cytokines that they love. *Virology* 435: 157-169.
8. Keppler, S. J., K. Rosenits, T. Koegl, S. Vucikuja, and P. Aichele. 2012. Signal 3 Cytokines as Modulators of Primary Immune Responses during Infections: The Interplay of Type I IFN and IL-12 in CD8 T Cell Responses. *PLoS ONE* 7: e40865.
9. Cook, K. D., S. N. Waggoner, and J. K. Whitmire. 2014. NK Cells and Their Ability to Modulate T Cells during Virus Infections. 34: 359-388.
10. Wang, J., F. Li, M. Zheng, R. Sun, H. Wei, and Z. Tian. 2012. Lung natural killer cells in mice: phenotype and response to respiratory infection. *Immunology* 137: 37-47.

11. Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 409: 1055-1060.

12. Kiessling, R., E. Klein, and H. Wigzell. 1975. Natural killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *European journal of immunology* 5.

13. Kiessling, R., E. Klein, H. Pross, and H. Wigzell. 1975. Natural killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *European journal of immunology* 5: 117-121.

14. Sun, J. C., and L. L. Lanier. 2011. NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nature reviews. Immunology* 11: 645-657.

15. Montaldo, E., P. Vacca, L. Moretta, and M. C. Mingari. 2014. Development of human natural killer cells and other innate lymphoid cells. *Seminars in immunology* 26: 107-113.

16. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68: 869-877.

17. Shinkai, Y., G. Rathbun, K.-P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall, and F. W. Alt. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68: 855-867.

18. Lanier, L. L. 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nature immunology* 9: 495-502.

19. Spits, H., D. Artis, M. Colonna, A. Diefenbach, J. P. Di Santo, G. Eberl, S. Koyasu, R. M. Locksley, A. N. J. McKenzie, R. E. Mebius, F. Powrie, and E. Vivier. 2013. Innate lymphoid cells - a proposal for uniform nomenclature. *Nature reviews. Immunology* 13: 145-149.

20. Haller, O., and H. Wigzell. 1977. Suppression of Natural Killer Cell Activity with Radioactive Strontium: Effector Cells Are Marrow Dependent. *The Journal of Immunology* 118: 1503-1506.

21. Freud, A. G., J. Yu, and M. A. Caligiuri. 2014. Human natural killer cell development in secondary lymphoid tissues. *Seminars in immunology* 26: 132-137.

22. Lysakova-Devine, T., and C. O'Farrelly. 2014. Tissue-specific NK cell populations and their origin. *Journal of leukocyte biology*.

23. Freud, A. G., and M. A. Caligiuri. 2006. Human natural killer cell development. *Immunological reviews* 214: 56-72.

24. Kamizono, S., G. S. Duncan, and M. G. Seidel. 2009. Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. *The Journal of …*.

25. Suzuki, H., G. S. Duncan, H. Takimoto, and T. W. Mak. 1997. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor chain. *The Journal of experimental medicine* 185: 499-506.

26. Cichocki, F., E. Sitnicka, and Y. T. Bryceson. 2014. NK cell development and function – Plasticity and redundancy unleashed. *Seminars in immunology* 26.

27. Barton, K., N. Muthusamy, C. Fischer, and C. N. Ting. 1998. The Ets-1 transcription factor is required for the development of natural killer cells in mice. *Immunity*.

28. Samson, S. I., O. Richard, M. Tavian, and T. Ranson. 2003. GATA-3 promotes maturation, IFN-γ production, and liver-specific homing of NK cells. *Immunity*.

29. Kallies, A., S. Carotta, and N. D. Huntington. 2011. A role for Blimp1 in the transcriptional network controlling natural killer cell maturation. *…*.

30. Gordon, S. M., J. Chaix, L. J. Rupp, J. Wu, S. Madera, J. C. Sun, T. Lindsten, and S. L. Reiner. 2012. The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. *Immunity* 36.

31. Held, W., M. Kijima, G. Angelov, and S. Bessoles. 2011. The function of natural killer cells: education, reminders and some good memories. *Curr Opin Immunol* 23: 228-233.

32. Pegram, H. J., D. M. Andrews, M. J. Smyth, P. K. Darcy, and M. H. Kershaw. 2011. Activating and inhibitory receptors of natural killer cells. *Immunology and cell biology* 89: 216-224.

33. Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet. 2005. *A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules*.

34. Joncker, N. T., N. C. Fernandez, E. Treiner, E. Vivier, and D. H. Raulet. 2009. NK Cell Responsiveness Is Tuned Commensurate with the Number of Inhibitory Receptors for Self-MHC Class I: The Rheostat Model. *The Journal of Immunology* 182: 4572-4580.

35. Shifrin, N., D. H. Raulet, and M. Ardolino. 2014. NK cell self tolerance, responsiveness and missing self recognition. *Seminars in immunology* 26: 138-144.

36. Vivier, E., E. Tomasello, M. Baratin, and T. Walzer. 2008. Functions of natural killer cells. *Nature immunology* 9: 503-510.

37. Cichocki, F., J. Miller, S. Anderson, and Y. Bryceson. 2013. Epigenetic regulation of NK cell differentiation and effector functions. *Frontiers in Immunology* 4.

38. Fauriat, C., E. O. Long, H.-G. Ljunggren, and Y. T. Bryceson. 2010. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 115: 2167-2176.

39. Moser, B., M. Wolf, A. Walz, and P. Loetscher. 2004. Chemokines: multiple levels of leukocyte migration control☆. *Trends in immunology* 25: 75-84.

40. Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume. 2004. Interferon-γ: an overview of signals, mechanisms and functions. *Journal of leukocyte biology* 75: 163-189.

41. Farrar, M. A., and R. D. Schreiber. 1993. The molecular cell biology of interferon-gamma and its receptor. *Annual review of immunology* 11: 571-611.

42. Wajant, H., K. Pfizenmaier, and P. Scheurich. 0000. Tumor necrosis factor signaling. *Cell Death Differ* 10: 45-65.

43. Seo, S. H., and R. G. Webster. 2002. Tumor Necrosis Factor Alpha Exerts Powerful Anti-Influenza Virus Effects in Lung Epithelial Cells. *Journal of virology* 76: 1071-1076.

44. Sun, J. C. 2010. Re-educating natural killer cells. *The Journal of experimental medicine* 207: 2049-2052.

45. Walzer, T., M. Bléry, J. Chaix, N. Fuseri, L. Chasson, S. H. Robbins, S. Jaeger, P. André, L. Gauthier, L. Daniel, K. Chemin, Y. Morel, M. Dalod, J. Imbert, M. Pierres, A. Moretta, F. Romagné, and E. Vivier. 2007. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proceedings of the National Academy of Sciences* 104: 3384-3389.

46. Horowitz, A., D. M. Strauss-Albee, M. Leipold, J. Kubo, N. Nemat-Gorgani, O. C. Dogan, C. L. Dekker, S. Mackey, H. Maecker, G. E. Swan, M. M. Davis, P. J. Norman, L. A. Guethlein, M. Desai, P. Parham, and C. A. Blish. 2013. Genetic and Environmental Determinants of Human NK Cell Diversity Revealed by Mass Cytometry. *Science Translational Medicine* 5: 208ra145.

47. B. Lodoen, M., and L. L. Lanier. 2006. Natural killer cells as an initial defense against pathogens. *Current Opinion in Immunology* 18.

48. Ivanova, D., R. Krempels, J. Ryfe, K. Weitzman, D. Stephenson, and J. Gigley. 2014. NK Cells in Mucosal Defense against Infection. *BioMed Research International* 2014.

49. Cooper, M. A., T. A. Fehniger, A. Fuchs, M. Colonna, and M. A. Caligiuri. NK cell and DC interactions. *Trends in immunology* 25: 47-52.

50. Mehrotra, P. T., R. P. Donnelly, S. Wong, H. Kanegane, A. Geremew, H. S. Mostowski, K. Furuke, J. P. Siegel, and E. T. Bloom. 1998. Production of IL-10 by Human Natural Killer Cells Stimulated with IL-2 and/or IL-12. *The Journal of Immunology* 160: 2637-2644.

51. Gray, J. D., M. Hirokawa, K. Ohtsuka, and D. A. Horwitz. 1998. Generation of an Inhibitory Circuit Involving CD8+ T Cells, IL-2, and NK Cell-Derived TGF-β: Contrasting Effects of Anti-CD2 and Anti-CD3. *The Journal of Immunology* 160: 2248-2254.

52. Cerboni, C., A. Zingoni, M. Cippitelli, M. Piccoli, L. Frati, and A. Santoni. 2007. Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK- cell lysis. *Blood* 110: 606-615.

53. Takao, S., T. Ishikawa, K. Yamashita, and T. Uchiyama. 2010. The Rapid Induction of HLA-E Is Essential for the Survival of Antigen-Activated Naive CD4 T Cells from Attack by NK Cells. *The Journal of Immunology* 185: 6031-6040.

54. Bot, A., S. Bot, and C. A. Bona. 1998. Protective Role of Gamma Interferon during the Recall Response to Influenza Virus. *Journal of virology* 72: 6637-6645.

55. Topham, D. J., R. A. Tripp, and P. C. Doherty. 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *The Journal of Immunology* 159: 5197-5200.

56. Klein, L., B. Kyewski, P. M. Allen, and K. A. Hogquist. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature reviews. Immunology* 14: 377-391.

57. Obar, J. J., and L. Lefrançois. 2010. Early events governing memory CD8+ T-cell differentiation. *International immunology* 22: 619-625.

58. Daniels, M. A., and E. Teixeiro. 2015. TCR Signaling in T Cell Memory. *Frontiers in Immunology* 6: 617.

59. Obar, J. J., E. R. Jellison, B. S. Sheridan, D. A. Blair, Q. M. Pham, J. M. Zickovich, and L. Lefrancois. 2011. Pathogen-induced inflammatory environment controls effector and memory CD8+ T cell differentiation. *J Immunol* 187: 4967-4978.

60. Foulds, K. E., M. J. Rotte, and R. A. Seder. 2006. IL-10 Is Required for Optimal CD8 T Cell Memory following Listeria monocytogenes Infection. *The Journal of Immunology* 177: 2565-2574.

61. Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4: 1191-1198.

62. Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature immunology* 1: 426-432.

63. Shane, H., and K. Klonowski. 2014. Every breath you take: The impact of environment on resident memory CD8 T cells in the lung. *Frontiers in Immunology* 5.

64. Wu, T., Y. Hu, Y.-T. Lee, K. R. Bouchard, A. Benechet, K. Khanna, and L. S. Cauley. 2014. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *Journal of Leukocyte Biology* 95: 215-224.

65. Woodland, D., R. J. Hogan, and W. Zhong. 2001. Cellular immunity and memory to respiratory virus infections. *Immunologic Research* 24: 53-67.

66. Sung, S.-S. J., S. M. Fu, C. E. Rose, F. Gaskin, S.-T. Ju, and S. R. Beaty. 2006. A Major Lung CD103 (αE)-β7 Integrin-Positive Epithelial Dendritic Cell Population Expressing Langerin and Tight Junction Proteins. *The Journal of Immunology* 176: 2161-2172.

67. Edelson, B. T., W. Kc, R. Juang, M. Kohyama, L. A. Benoit, P. A. Klekotka, C. Moon, J. C. Albring, W. Ise, D. G. Michael, D. Bhattacharya, T. S. Stappenbeck, M. J. Holtzman, S.-S. J. Sung, T. L. Murphy, K. Hildner, and K. M. Murphy. 2010. Peripheral CD103(+) dendritic cells form a unified subset developmentally related to CD8α(+) conventional dendritic cells. *The Journal of experimental medicine* 207: 823-836.

68. Ho, A. W. S., N. Prabhu, R. J. Betts, M. Q. Ge, X. Dai, P. E. Hutchinson, F. C. Lew, K. L. Wong, B. J. Hanson, P. A. Macary, and D. M. Kemeny. 2011. Lung CD103+ Dendritic Cells Efficiently Transport Influenza Virus to the Lymph Node and Load Viral Antigen onto MHC Class I for Presentation to CD8 T Cells. *The Journal of Immunology* 187: 6011-6021.

69. Hao, X., T. S. Kim, and T. J. Braciale. 2008. Differential Response of Respiratory Dendritic Cell Subsets to Influenza Virus Infection. *Journal of virology* 82: 4908-4919.

70. Legge, K. L., and T. J. Braciale. 2003. Accelerated Migration of Respiratory Dendritic Cells to the Regional Lymph Nodes Is Limited to the Early Phase of Pulmonary Infection. *Immunity* 18: 265-277.

71. Yoon, H., K. L. Legge, S.-s. J. Sung, and T. J. Braciale. 2007. Sequential Activation of CD8+ T Cells in the Draining Lymph Nodes in Response to Pulmonary Virus Infection. *The Journal of Immunology* 179: 391-399.

72. GeurtsvanKessel, C. H., M. A. M. Willart, L. S. van Rijt, F. Muskens, M. Kool, C. Baas, K. Thielemans, C. Bennett, B. E. Clausen, H. C. Hoogsteden, A. D. M. E. Osterhaus, G. F. Rimmelzwaan, and B. N. Lambrecht. 2008. Clearance of influenza virus from the lung depends on migratory langerin^{+} CD11b^{-} but not plasmacytoid dendritic cells. *The Journal of experimental medicine* 205: 1621-1634.

73. Belz, G. T., C. M. Smith, L. Kleinert, P. Reading, A. Brooks, K. Shortman, F. R. Carbone, and W. R. Heath. 2004. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proceedings of the National Academy of Sciences of the United States of America* 101: 8670-8675.

74. Belz, G. T., C. M. Smith, D. Eichner, K. Shortman, G. Karupiah, F. R. Carbone, and W. R. Heath. 2004. Cutting Edge: Conventional CD8α+ Dendritic Cells Are Generally Involved in Priming CTL Immunity to Viruses. *The Journal of Immunology* 172: 1996-2000.

75. Aldridge, J. R., C. E. Moseley, D. A. Boltz, N. J. Negovetich, C. Reynolds, J. Franks, S. A. Brown, P. C. Doherty, R. G. Webster, and P. G. Thomas. 2009. TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. *Proceedings of the National Academy of Sciences* 106: 5306-5311.

76. Tscharke, D. C., N. P. Croft, P. C. Doherty, and N. L. La Gruta. 2015. Sizing up the key determinants of the CD8+ T cell response. *Nature reviews. Immunology* 15: 705-716.

77. Lawrence, C. W., and T. J. Braciale. 2004. Activation, Differentiation, and Migration of Naive Virus-Specific CD8+ T Cells during Pulmonary Influenza Virus Infection. *The Journal of Immunology* 173: 1209-1218.

78. Stambas, J., C. Guillonneau, K. Kedzierska, J. D. Mintern, P. C. Doherty, and N. L. La Gruta. 2008. Killer T cells in influenza. *Pharmacology & Therapeutics* 120: 186-196.

79. Pleguezuelos, O., S. Robinson, A. Fernandez, G. A. Stoloff, and W. Caparrós-Wanderley. 2015. Meta-Analysis and Potential Role of Preexisting Heterosubtypic Cellular Immunity Based on Variations in Disease Severity Outcomes for Influenza Live Viral Challenges in Humans. *Clinical and Vaccine Immunology* 22: 949-956.

80. Araki, Y., M. Fann, R. Wersto, and N.-p. Weng. 2008. Histone Acetylation Facilitates Rapid and Robust Memory CD8 T Cell Response through Differential Expression of Effector Molecules (Eomesodermin and Its Targets: Perforin and Granzyme B). *The Journal of Immunology* 180: 8102-8108.

81. Kersh, E. N., D. R. Fitzpatrick, K. Murali-Krishna, J. Shires, S. H. Speck, J. M. Boss, and R. Ahmed. 2006. Rapid Demethylation of the IFN-γ Gene Occurs in Memory but Not Naive CD8 T Cells. *The Journal of Immunology* 176: 4083-4093.

82. Nogusa, S., B. W. Ritz, S. H. Kassim, S. R. Jennings, and E. M. Gardner. 2008. Characterization of age-related changes in natural killer cells during primary influenza infection in mice. *Mechanisms of ageing and development* 129: 223-230.

83. Gething, M.-J., K. McCammon, and J. Sambrook. Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. *Cell* 46: 939-950.

84. Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *The Journal of Cell Biology* 103: 1179-1191.

85. Wiley, D. C., and J. J. Skehel. 1987. The Structure and Function of the Hemagglutinin Membrane Glycoprotein of Influenza Virus. *Annual Review of Biochemistry* 56: 365-394.

86. Weis, W., J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel, and D. C. Wiley. 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* 333: 426-431.

87. Rogers, G. N., and J. C. Paulson. 1983. Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* 127: 361-373.

88. Rogers, G. N., T. J. Pritchett, J. L. Lane, and J. C. Paulson. 1983. Differential sensitivity of human, avian, and equine influenza a viruses to a glycoprotein inhibitor of infection: Selection of receptor specific variants. *Virology* 131: 394-408.

89. Connor, R. J., Y. Kawaoka, R. G. Webster, and J. C. Paulson. 1994. Receptor Specificity in Human, Avian, and Equine H2 and H3 Influenza Virus Isolates. *Virology* 205: 17-23.

90. Wilson, I. A., and N. J. Cox. 1990. Structural Basis of Immune Recognition of Influenza Virus Hemagglutinin. *Annual review of immunology* 8: 737-787.

91. Tate, M. D., E. R. Job, Y.-M. Deng, V. Gunalan, S. Maurer-Stroh, and P. C. Reading. 2014. Playing Hide and Seek: How Glycosylation of the Influenza Virus Hemagglutinin Can Modulate the Immune Response to Infection. *Viruses* 6: 1294-1316.

92. Arnon, T. I., M. Lev, G. Katz, Y. Chernobrov, A. Porgador, and O. Mandelboim. 2001. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *European journal of immunology* 31: 2680-2689.

93. Achdout, H., T. Meningher, S. Hirsh, A. Glasner, Y. Bar-On, C. Gur, A. Porgador, M. Mendelson, M. Mandelboim, and O. Mandelboim. 2010. Killing of avian and Swine influenza virus by natural killer cells. *Journal of virology* 84: 3993-4001.

94. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, and L. Moretta. 2001. ACTIVATING RECEPTORS AND CORECEPTORS INVOLVED IN HUMAN NATURAL KILLER CELL-MEDIATED CYTOLYSIS. *Annual review of immunology* 19: 197-223.

95. Glasner, A., A. Zurunic, T. Meningher, T. L. Rovis, P. Tsukerman, Y. Bar-On, R. Yamin, A. F. Meyers, M. Mandeboim, and S. Jonjic. 2012. Elucidating the mechanisms of influenza virus recognition by Ncr1. *PLoS One* 7: e36837.

96. Draghi, M., A. Pashine, B. Sanjanwala, K. Gendzekhadze, C. Cantoni, D. Cosman, A. Moretta, N. M. Valiante, and P. Parham. 2007. NKp46 and NKG2D Recognition of Infected Dendritic

Cells Is Necessary for NK Cell Activation in the Human Response to Influenza Infection. *The Journal of Immunology* 178: 2688-2698.

97. Du, N., J. Zhou, X. Lin, Y. Zhang, X. Yang, Y. Wang, and Y. Shu. 2010. Differential Activation of NK Cells by Influenza A Pseudotype H5N1 and 1918 and 2009 Pandemic H1N1 Viruses. *Journal of virology* 84: 7822-7831.

98. Forberg, H., A. G. Hauge, M. Valheim, F. Garcon, A. Nunez, W. Gerner, K. H. Mair, S. P. Graham, S. M. Brookes, and A. K. Storset. 2014. Early Responses of Natural Killer Cells in Pigs Experimentally Infected with 2009 Pandemic H1N1 Influenza A Virus. *PLoS ONE* 9: e100619.

99. Juárez-Reyes, A., D. E. Noyola, A. Monsiváis-Urenda, C. Alvarez-Quiroga, and R. González-Amaro. 2013. Influenza Virus Infection but Not H1N1 Influenza Virus Immunization Is Associated with Changes in Peripheral Blood NK Cell Subset Levels. *Clinical and Vaccine Immunology* 20: 1291-1297.

100. McNerney, M. E., K.-M. Lee, and V. Kumar. 2005. 2B4 (CD244) is a non-MHC binding receptor with multiple functions on natural killer cells and CD8+ T cells. *Molecular immunology* 42: 489-494.

101. Arnon, T. I., H. Achdout, N. Lieberman, R. Gazit, T. Gonen-Gross, G. Katz, A. Bar-Ilan, N. Bloushtain, M. Lev, and A. Joseph. 2004. The mechanisms controlling the recognition of tumor- and virus-infected cells by NKp46. *Blood* 103: 664-672.

102. Mandelboim, O., and A. Porgador. 2001. NKp46. *The International Journal of Biochemistry & Cell Biology* 33: 1147-1150.

103. Gazit, R., R. Gruda, M. Elboim, T. I. Arnon, G. Katz, H. Achdout, J. Hanna, U. Qimron, G. Landau, E. Greenbaum, Z. Zakay-Rones, A. Porgador, and O. Mandelboim. 2006. Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. *Nature immunology* 7: 517-523.

104. Fiore, A. E., T. M. Uyeki, K. Broder, L. Finelli, G. L. Euler, J. A. Singleton, J. K. Iskander, P. M. Wortley, D. K. Shay, J. S. Bresee, and N. J. Cox. 2010. Prevention and control of influenza with

vaccines: recommendations of the Advisory Committee on ImPreventimunization Practices (ACIP), 2010. *Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on ImPreventimunization Practices (ACIP), 2010*: 1-62.

105. Zhou, H., W. W. Thompson, C. G. Viboud, C. M. Ringholz, P.-Y. Cheng, C. Steiner, G. R. Abedi, L. J. Anderson, L. Brammer, and D. K. Shay. 2012. Hospitalizations Associated With Influenza and Respiratory Syncytial Virus in the United States, 1993–2008. *Clinical Infectious Diseases* 54: 1427-1436.

106. Joseph, C., Y. Togawa, and N. Shindo. 2013. Bacterial and viral infections associated with influenza. *Influenza and Other Respiratory Viruses* 7: 105-113.

107. Brown, L. E., and A. Kelso. 2009. Prospects for an influenza vaccine that induces cross-protective cytotoxic T lymphocytes. *Immunology and cell biology* 87: 300-308.

108. Plumlee, Courtney R., Brian S. Sheridan, Basak B. Cicek, and L. Lefrançois. 2013. Environmental Cues Dictate the Fate of Individual CD8+ T Cells Responding to Infection. *Immunity* 39: 347-356.

109. Cui, W., Y. Liu, Jason S. Weinstein, J. Craft, and Susan M. Kaech. An Interleukin-21-Interleukin-10-STAT3 Pathway Is Critical for Functional Maturation of Memory CD8+ T Cells. *Immunity* 35: 792-805.

110. Badovinac, V. P., K. A. N. Messingham, A. Jabbari, J. S. Haring, and J. T. Harty. 2005. Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. *Nat Med* 11: 748-756.

111. Lang, P. A., K. S. Lang, H. C. Xu, M. Grusdat, I. A. Parish, M. Recher, A. R. Elford, S. Dhanji, N. Shaabani, C. W. Tran, D. Dissanayake, R. Rahbar, M. Ghazarian, A. Brüstle, J. Fine, P. Chen, C. T. Weaver, C. Klose, A. Diefenbach, D. Häussinger, J. R. Carlyle, S. M. Kaech, T. W. Mak, and P. S. Ohashi. 2012. Natural killer cell activation enhances immune pathology and promotes chronic infection by limiting CD8+ T-cell immunity. *Proceedings of the National Academy of Sciences* 109: 1210-1215.

112. Rydyznski, C., K. A. Daniels, E. P. Karmele, T. R. Brooks, S. E. Mahl, M. T. Moran, C. Li, R. Sutiwisesak, R. M. Welsh, and S. N. Waggoner. 2015. Generation of cellular immune memory and B-cell immunity is impaired by natural killer cells. *Nat Commun* 6.

113. Soderquest, K., T. Walzer, B. Zafirova, L. S. Klavinskis, B. Polić, E. Vivier, G. M. Lord, and A. Martín-Fontecha. 2011. Cutting Edge: CD8+ T Cell Priming in the Absence of NK Cells Leads to Enhanced Memory Responses. *The Journal of Immunology* 186: 3304-3308.

114. Waggoner, S. N., M. Cornberg, L. K. Selin, and R. M. Welsh. 2011. Natural killer cells act as rheostats modulating antiviral T cells. *Nature* 481: 394-398.

115. Matrosovich, M., T. Matrosovich, W. Garten, and H. D. Klenk. 2006. New low-viscosity overlay medium for viral plaque assays. *Virology journal* 3: 63.

116. Verbist, K. C., C. J. Cole, M. B. Field, and K. D. Klonowski. 2011. A role for IL-15 in the migration of effector CD8 T cells to the lung airways following influenza infection. *Journal of immunology* 186: 174-182.

117. Andrews, D. M., M. J. Estcourt, C. E. Andoniou, M. E. Wikstrom, A. Khong, V. Voigt, P. Fleming, H. Tabarias, G. R. Hill, R. G. van der Most, A. A. Scalzo, M. J. Smyth, and M. A. Degli-Esposti. 2010. Innate immunity defines the capacity of antiviral T cells to limit persistent infection. *The Journal of experimental medicine* 207: 1333-1343.

118. Kos, F. J., and E. G. Engleman. 1996. Role of natural killer cells in the generation of influenza virus-specific cytotoxic T cells. *Cellular immunology* 173: 1-6.

119. Garrod, Kym R., Hélène D. Moreau, Z. Garcia, F. Lemaître, I. Bouvier, Matthew L. Albert, and P. Bousso. Dissecting T Cell Contraction In Vivo Using a Genetically Encoded Reporter of Apoptosis. *Cell Reports* 2: 1438-1447.

120. Ronni, T., T. Sareneva, J. Pirhonen, and I. Julkunen. 1995. Activation of Ifn-Alpha, Ifn-Gamma, Mxa, and Ifn Regulatory Factor-1 Genes in Influenza-a Virus-Infected Human Peripheral-Blood Mononuclear-Cells. *Journal of immunology* 154: 2764-2774.

121. Whitmire, J. K., J. T. Tan, and J. L. Whitton. 2005. Interferon-γ acts directly on CD8+ T cells to increase their abundance during virus infection. *The Journal of experimental medicine* 201: 1053-1059.

122. Badovinac, V. P., A. R. Tvinnereim, and J. T. Harty. 2000. Regulation of Antigen-Specific CD8+ T Cell Homeostasis by Perforin and Interferon-γ. *Science* 290: 1354-1357.

123. Tewari, K., Y. Nakayama, and M. Suresh. 2007. Role of Direct Effects of IFN-γ on T Cells in the Regulation of CD8 T Cell Homeostasis. *The Journal of Immunology* 179: 2115-2125.

124. Anderson, K. G., H. Sung, C. N. Skon, L. Lefrancois, A. Deisinger, V. Vezys, and D. Masopust. 2012. Cutting Edge: Intravascular Staining Redefines Lung CD8 T Cell Responses. *The Journal of Immunology* 189: 2702-2706.

125. Seder, R. A., P. A. Darrah, and M. Roederer. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nature Reviews Immunology* 8: 247.

126. Gerosa, F., A. Gobbi, P. Zorzi, S. Burg, F. Briere, G. Carra, and G. Trinchieri. 2005. The Reciprocal Interaction of NK Cells with Plasmacytoid or Myeloid Dendritic Cells Profoundly Affects Innate Resistance Functions. *The Journal of Immunology* 174: 727-734.

127. Sirén, J., T. Sareneva, J. Pirhonen, M. Strengell, V. Veckman, I. Julkunen, and S. Matikainen. 2004. Cytokine and contact-dependent activation of natural killer cells by influenza A or Sendai virus-infected macrophages. *Journal of General Virology* 85: 2357-2364.

128. Harty, J. T., and V. P. Badovinac. 2008. Shaping and reshaping CD8+ T-cell memory. *Nature reviews. Immunology* 8: 107-119.

129. Fang, P., V. Hwa, and R. G. Rosenfeld. 2006. Interferon-gamma-induced dephosphorylation of STAT3 and apoptosis are dependent on the mTOR pathway. *Exp Cell Res* 312: 1229-1239.

130. Stoycheva, D., K. Deiser, L. Stärck, G. Nishanth, D. Schlüter, W. Uckert, and T. Schüler. 2015. IFN-γ Regulates CD8+ Memory T Cell Differentiation and Survival in Response to Weak, but Not Strong, TCR Signals. *The Journal of Immunology* 194: 553-559.

131. Martin, M. D., T. C. Wirth, P. Lauer, J. T. Harty, and V. P. Badovinac. 2011. The impact of pre-existing memory on differentiation of newly recruited naïve CD8 T cells. *Journal of immunology* 187: 2923-2931.

132. Wirth, T. C., J. T. Harty, and V. P. Badovinac. 2010. Modulating numbers and phenotype of CD8(+) T cells in secondary immune responses. *European journal of immunology* 40: 1916-1926.

133. Djeu, J. Y., N. Stocks, K. Zoon, G. J. Stanton, T. Timonen, and R. B. Herberman. 1982. Positive self regulation of cytotoxicity in human natural killer cells by production of interferon upon exposure to influenza and herpes viruses. *The Journal of experimental medicine* 156: 1222-1234.

134. Abdul-Careem, M. F., M. F. Mian, G. Yue, A. Gillgrass, M. J. Chenoweth, N. G. Barra, M. V. Chew, T. Chan, A. A. Al-Garawi, M. Jordana, and A. A. Ashkar. 2012. Critical Role of Natural Killer Cells in Lung Immunopathology During Influenza Infection in Mice. *Journal of Infectious Diseases* 206: 167-177.

135. Kumar, P., M. S. Thakar, W. Ouyang, and S. Malarkannan. 2013. IL-22 from conventional NK cells is epithelial regenerative and inflammation protective during influenza infection. *Mucosal immunology* 6: 69-82.

136. Pociask, D. A., E. V. Scheller, S. Mandalapu, K. J. McHugh, R. I. Enelow, C. L. Fattman, J. K. Kolls, and J. F. Alcorn. 2013. IL-22 Is Essential for Lung Epithelial Repair following Influenza Infection. *The American Journal of Pathology* 182: 1286-1296.

137. Ho, J. W., O. Hershkovitz, M. Peiris, A. Zilka, A. Bar-Ilan, B. Nal, K. Chu, M. Kudelko, Y. W. Kam, H. Achdout, M. Mandelboim, R. Altmeyer, O. Mandelboim, R. Bruzzone, and A. Porgador. 2008. H5-Type Influenza Virus Hemagglutinin Is Functionally Recognized by the Natural Killer-Activating Receptor NKp44. *Journal of virology* 82: 2028-2032.

138. Bar-On, Y., A. Glasner, T. Meningher, H. Achdout, C. Gur, D. Lankry, A. Vitenshtein, Adrienne F. A. Meyers, M. Mandelboim, and O. Mandelboim. 2013. Neuraminidase-Mediated,

NKp46-Dependent Immune-Evasion Mechanism of Influenza Viruses. *Cell Reports* 3: 1044-1050.

139. Sauter, N. K., J. E. Hanson, G. D. Glick, J. H. Brown, R. L. Crowther, S. J. Park, J. J. Skehel, and D. C. Wiley. 1992. Binding of influenza virus hemagglutinin to analogs of its cell-surface receptor, sialic acid: analysis by proton nuclear magnetic resonance spectroscopy and x-ray crystallography. *Biochemistry* 31: 9609-9621.

140. Rogers, G. N., J. C. Paulson, R. S. Daniels, J. J. Skehel, I. A. Wilson, and D. C. Wiley. 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* 304: 76-78.

141. Rudd, P. M., and R. A. Dwek. 1997. Glycosylation: Heterogeneity and the 3D Structure of Proteins. *Critical Reviews in Biochemistry and Molecular Biology* 32: 1-100.

142. Fogel, L. A., M. M. Sun, T. L. Geurs, L. N. Carayannopoulos, and A. R. French. 2013. Markers of Nonselective and Specific NK Cell Activation. *The Journal of Immunology*.

143. Alter, G., J. M. Malenfant, and M. Altfeld. 2004. CD107a as a functional marker for the identification of natural killer cell activity. *Journal of Immunological Methods* 294: 15-22.

144. Poli, A., T. Michel, M. Thérésine, E. Andrès, F. Hentges, and J. Zimmer. 2009. CD56(bright) natural killer (NK) cells: an important NK cell subset. *Immunology* 126: 458-465.

145. Abe, Y., E. Takashita, K. Sugawara, Y. Matsuzaki, Y. Muraki, and S. Hongo. 2004. Effect of the Addition of Oligosaccharides on the Biological Activities and Antigenicity of Influenza A/H3N2 Virus Hemagglutinin. *Journal of virology* 78: 9605-9611.

146. Meisner, J., K. J. Szretter, K. C. Bradley, W. A. Langley, Z.-N. Li, B.-J. Lee, S. Thoennes, J. Martin, J. J. Skehel, R. J. Russell, J. M. Katz, and D. A. Steinhauer. 2008. Infectivity Studies of Influenza Virus Hemagglutinin Receptor Binding Site Mutants in Mice. *Journal of virology* 82: 5079-5083.

147. Bradley, K. C., S. E. Galloway, Y. Lasanajak, X. Song, J. Heimburg-Molinaro, H. Yu, X. Chen, G. R. Talekar, D. F. Smith, R. D. Cummings, and D. A. Steinhauer. 2011. Analysis of Influenza

Virus Hemagglutinin Receptor Binding Mutants with Limited Receptor Recognition Properties and Conditional Replication Characteristics. *Journal of virology* 85: 12387-12398.

148. Stein-Streilein, J., and J. Guffee. 1986. In vivo treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. *The Journal of Immunology* 136: 1435-1441.

149. Achdout, H., I. Manaster, and O. Mandelboim. 2008. Influenza virus infection augments NK cell inhibition through reorganization of major histocompatibility complex class I proteins. *Journal of virology* 82: 8030-8037.

150. Mendelson, M., Y. Tekoah, A. Zilka, O. Gershoni-Yahalom, R. Gazit, H. Achdout, N. V. Bovin, T. Meningher, M. Mandelboim, O. Mandelboim, A. David, and A. Porgador. 2010. NKp46 O-glycan sequences that are involved in the interaction with hemagglutinin type 1 of influenza virus. *Journal of virology* 84: 3789-3797.

151. Rydyznski, C. E., and S. N. Waggoner. Boosting vaccine efficacy the natural (killer) way. *Trends in immunology* 36: 536-546.

152. Moskophidis, D., and D. Kioussis. 1998. Contribution of Virus-specific CD8+ Cytotoxic T Cells to Virus Clearance or Pathologic Manifestations of Influenza Virus Infection in a T Cell Receptor Transgenic Mouse Model. *The Journal of experimental medicine* 188: 223-232.

153. Denney, L., C. Aitken, C. K.-F. Li, E. Wilson-Davies, W. L. Kok, C. Clelland, K. Rooney, D. Young, T. Dong, A. J. McMichael, W. F. Carman, and L.-P. Ho. 2010. Reduction of Natural Killer but Not Effector CD8 T Lymphoyctes in Three Consecutive Cases of Severe/Lethal H1N1/09 Influenza A Virus Infection. *PLoS ONE* 5: e10675.

154. Kim, J. H., I. Skountzou, R. Compans, and J. Jacob. 2009. Original Antigenic Sin Responses to Influenza Viruses. *Journal of immunology (Baltimore, Md. : 1950)* 183: 3294-3301.

155. O'Sullivan, Timothy E., Joseph C. Sun, and Lewis L. Lanier. 2015. Natural Killer Cell Memory. *Immunity* 43: 634-645.

156. van Helden, M. J. G., D. M. W. Zaiss, and A. J. A. M. Sijts. 2012. CCR2 Defines a Distinct Population of NK Cells and Mediates Their Migration during Influenza Virus Infection in Mice. *PLoS ONE* 7: e52027.

157. Paust, S., H. S. Gill, B.-Z. Wang, M. P. Flynn, E. A. Moseman, B. Senman, M. Szczepanik, A. Telenti, P. W. Askenase, R. W. Compans, and U. H. von Andrian. 2010. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nature immunology* 11: 1127-1135.

158. Dou, Y., B. Fu, R. Sun, W. Li, W. Hu, Z. Tian, and H. Wei. 2015. Influenza Vaccine Induces Intracellular Immune Memory of Human NK Cells. *PLoS ONE* 10: e0121258.

159. Conley, M. E., A. K. Dobbs, D. M. Farmer, S. Kilic, K. Paris, S. Grigoriadou, E. Coustan-Smith, V. Howard, and D. Campana. 2009. Primary B Cell Immunodeficiencies: Comparisons and Contrasts. *Annual review of immunology* 27: 199-227.

160. Edgar, J. D. M. 2008. T cell immunodeficiency. *Journal of Clinical Pathology* 61: 988-993.

161. He, X.-S., M. Draghi, K. Mahmood, T. H. Holmes, G. W. Kemble, C. L. Dekker, A. M. Arvin, P. Parham, and H. B. Greenberg. 2004. T cell–dependent production of IFN-γ by NK cells in response to influenza A virus. *The Journal of clinical investigation* 114: 1812-1819.

162. Guo, H., and D. J. Topham. 2010. Interleukin-22 (IL-22) Production by Pulmonary Natural Killer Cells and the Potential Role of IL-22 during Primary Influenza Virus Infection. *Journal of virology* 84: 7750-7759.