Influenza Virus Evades NK Cell Responses by Enhancing Ly49:MHC-I Interactions

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the Doctorate in Philosophy degree
in Microbiology and Immunology

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Abstract

Natural killer (NK) cells are a type of innate immune cell that can identify and eliminate viral infected cells and cancer cells. NK cells express an array of inhibitory and activating receptors such as natural cytotoxicity receptors, the mouse Ly49 or human KIR family, and NKR-P1 family. The integration of signals that NK cells receive through these receptors controls their activation and ability to kill target cells. Both Ly49 and KIR recognize MHC-I molecules on healthy cells Ly49:MHC-I engagement is essential for functional NK cell development. In the absence of these interactions NK cell is consider as 'uneducated' or 'unlicensed'. Ly49 receptor interactions with MHC-I are critical in an effective NK cell response against cancer. However, the role of unlicensed NK cells in NK-mediated control of viruses is poorly understood.

Using NKC^{KD} mice, we sought to determine how the loss of Ly49:MHC-I education, and the concomitant loss of inhibition via MHC-I, affected survival against influenza infection. In this study, we show that Ly49-deficient mice exhibit lower viral load and greater protection than WT mice when infected with influenza. However, this protection was lost when Ly49I was transgenically restored to these mice. Similarly, MHC-I-deficient mice, that also lack educated NK cells, were resistant to influenza infection, and lost this protection when NK cells were depleted before challenge. Based on the markedly reduced inflammation in the Ly49-deficient mice compared to the WT, we conclude that the Ly49-deficient NK cells are swifter and more effective in clearing influenza, resulting in less viral burden and consequentially less need for a dangerously aggressive inflammatory response. Furthermore, influenza infection enhanced MHC-I expression on lung epithelial cells, which could be responsible for inhibition of NK cells. Consequently, blockade of inhibitory

Ly49C/I receptors protected WT mice from lethal influenza infection. Additionally, Perforin-deficient NKC^{KD} succumbed to the infection demonstrating that NK cell directly eliminate influenza-infected cells. Collectively, these results confirm that influenza is capable of inhibiting NK cells through MHC-I engagement of KIR/Ly49, and suggests that blocking this interaction may provide a viable therapeutic avenue for severe influenza cases.

these results challenge our understanding of basic NK cell function and suggest that, rather than subdividing NK cells into 'licensed' and 'unlicensed' based on their expression of self-specific Ly49 receptors, a more accurate depiction of these NK subsets would be 'cancer-specialized' and 'pathogen-specialized'. While further work is required to fully test this paradigm of cancer- and pathogen-specialized NK cells, I hope that my findings will stimulate a new appreciation for the role of NK cells in virus control, and lead to a better understanding of this critical immune cell.

Acknowledgements

Foremost, I would like to express my sincere gratitude to my parents, my wife, daughters, and my siblings, my friends for their love and support throughout my life. They have been my inspiration and motivation for continuing to improve my knowledge and move my career forward.

I offer my sincerest gratitude to my advisor and mentor, Dr. Andrew Makrigiannis, his wide knowledge, analytical and his intuitive thinking has been a great inspiration for me. He has guided me throughout this journey with patience, support, and encouragement while also allowing me the chance to independently shape my research skills and become a better researcher.

Besides my advisor, I would like to thank the rest of my thesis committee Dr. Seung-Hwan Lee and Dr. Robin Parks for their encouragement, support, and insightful comments.

My sincere thanks also go my fellow lab-mates Mir Munir A. Rahim, Megan Tu, Elias Abou-Samra, Andrew Wight, and Angela Troke, It has been a pleasure working with you and for all the fun we have had. I have learned a lot from each one of you thank you very much. I wish you nothing but future success both professionally and personally.

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
BM	Bone marrow
CD	Cluster of differentiation
CLP	Common lymphoid progenitors
Clr	C-type lectin-related
Clr-b	C-type lectin-related protein b
CTL	Cytotoxic T lymphocytes
DAP12	DNAX-activating proteins of 12 kDa
DCs	Dendritic cells
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
FasL	Fas ligand
GFP	Green fluorescent protein
HA	Hemagglutinin
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
HS	Heparin sulfate
HSC	Hematopoietic stem cell
HSV	Herpes simplex virus

ICU	Intensive care unit
IELs	Intestinal epithelial lymphocytes
IFN-γ	Interferon-γ
IL-1	Interleukin-1
ITAMs	Immunoreceptor tyrosine-based activation motifs
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
KIRs	Killer Immunoglobulin-like Receptors
KLRA	Killer cell lectin-like receptor subfamily A
LCMV	Lymphocytic choriomeningitis virus
LLT1	lectin-like transcript-1
M1	Matrix 1
mAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
MCA	Methylcholanthrene
MCMV	Murine cytomegalovirus
MHC-I	Major histocompatibility complex class-I
MICA	MHC-I chain-related proteins A
mRNA	messenger RNA
MULT1	murine ULBP-like transcript 1
NA	Neuraminidase
NCR	Natural cytotoxicity receptor
NKC	NK gene complex

NKG2D	Natural Killer Group 2D
NP	Nucleoprotein
NS1	Non-structural protein 1
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
RAE-1	Retinoic acid early-inducible 1
RAET1	Retinoic acid early transcripts-1
RCMV	Rat cytomegalovirus
RNP	Ribonucleoprotein
SHP-1	Src homology 2 domain-containing protein tyrosine phosphatase 1
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
ULBP	UL16-binding proteins
WHO	World Health Organization

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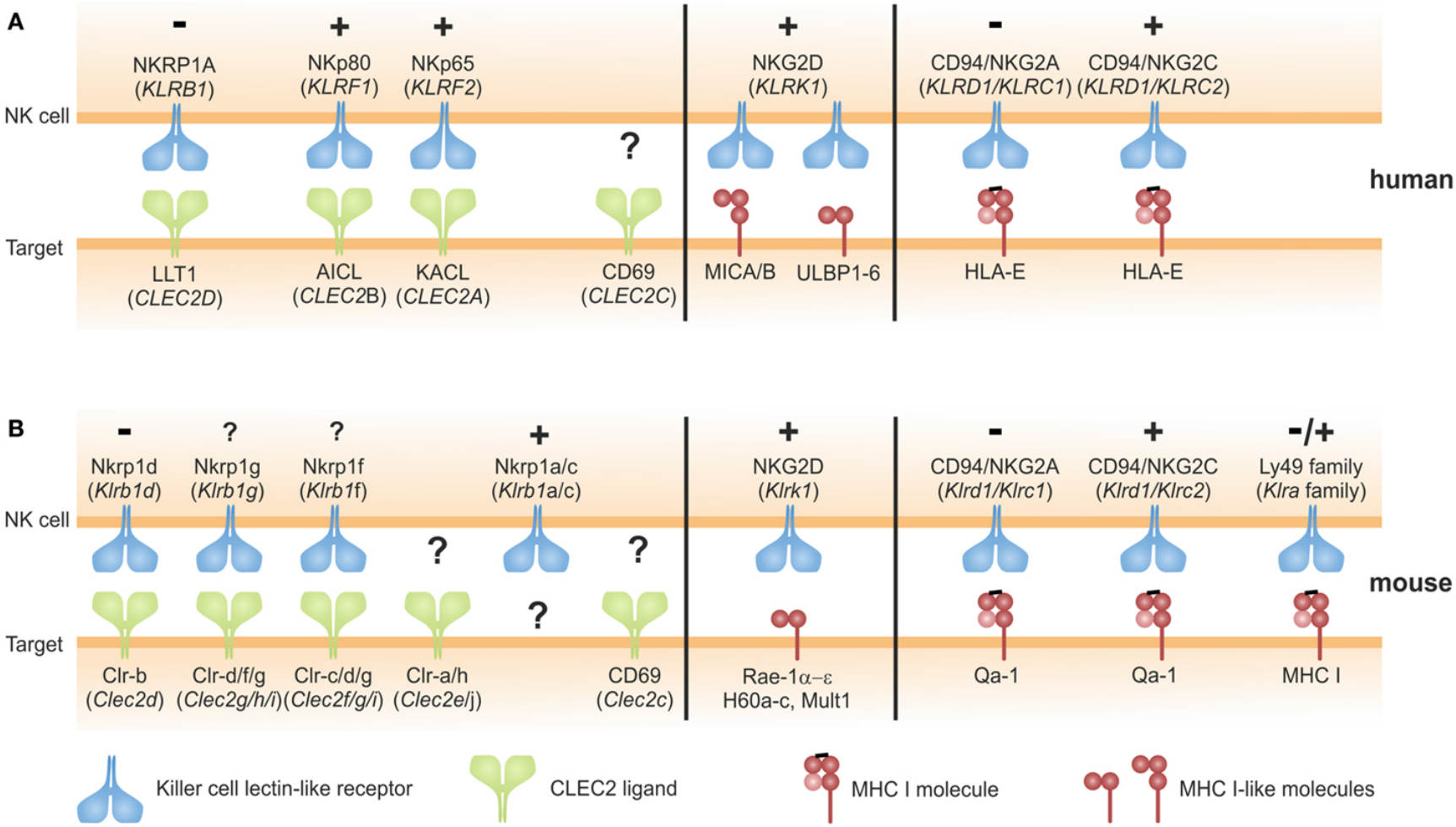
Introduction

1. Natural killer (NK) cells

In 1975, NK cells were identified in mice as large granular leukocytes (1). These cells have the capability to recognize and destroy transformed, virus-infected, and antibody-coated cells without prior sensitization in both rodents and humans (1, 2). NK cells are capable of distinguishing healthy from unhealthy cells using an array of activating and inhibitory receptors (Figure 1) (3). These receptors assist NK cells to sense their environment and respond to alterations caused by pathogens or tumors. Importantly, the effector functions of NK cells are controlled via a net balance of signals from inhibitory and activating receptors (4). Like cytotoxic T lymphocytes (CTL), NK cells destroy target cells using two central pathways: the perforin/granzyme pathway and various death receptor–mediated pathways, which include Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (5). Furthermore, upon activation, NK cells secrete several effector cytokines such as interferon-γ (IFN-γ), besides several other immunoregulatory cytokines that shape the adaptive and innate immune responses (6). The importance of NK cells is demonstrated in reports of individuals lacking functional NK cells, who suffer from life-threatening infections such as herpes simplex virus (HSV) and human cytomegalovirus (HCMV) infections (7). Additionally, in an 11-year follow-up study, it was revealed that lower peripheral blood NK cell cytotoxicity associates with an enhanced risk of several cancers (8). These observations determine that NK cells are a fundamental part of the immune cells that plays a major role against tumors and viral infection.


A
-
+
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NKRP1A
(KLRB1)
NKp80
(KLRF1)
NKp65
(KLRF2)
NKG2D
(KLRK1)
CD94/NKG2A
(KLRD1/KLRC1)
CD94/NKG2C
(KLRD1/KLRC2)
NK cell
?
human
Target
LLT1
(CLEC2D)
AICL
(CLEC2B)
KACL
(CLEC2A)
CD69
(CLEC2C)
MICA/B
ULBP1-6
HLA-E
HLA-E
B
-
?
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-/+
Nkrp1d
(Klrb1d)
Nkrp1g
(Klrb1g)
Nkrp1f
(Klrb1f)
Nkrp1a/c
(Klrb1a/c)
NKG2D
(Klrk1)
CD94/NKG2A
(Klrd1/Klrc1)
CD94/NKG2C
(Klrd1/Klrc2)
Ly49 family
(Klra family)
NK cell
?
?
?
mouse
Target
Clr-b
(Clec2d)
Clr-d/f/g
(Clec2g/h/i)
Clr-c/d/g
(Clec2f/g/i)
Clr-a/h
(Clec2e/j)
CD69
(Clec2c)
Rae-1α–ε
H60a-c, Mult1
Qa-1
Qa-1
MHC I
Killer cell lectin-like receptor
CLEC2 ligand
MHC I molecule
MHC I-like molecules

Figure 1. Schematic representation of NK cell receptors and their ligands in humans and mice.

Activating and inhibitory human (A) and mouse (B) NK cell receptors and their ligands are shown. Symbols "+" indicate activating receptors and "–" indicate inhibitory receptors. Figure is taken from (9).

1.1. NK cell development

NK cell is generated from common lymphoid progenitors (CLP), that give rise to all lymphocyte subsets. NK cell development primarily takes place in the bone marrow (BM) (10), as ablation of the bone marrow microenvironment inhibited the function and most importantly development of NK cells. NK cells subsequently migrate and undergo terminal differentiation in the periphery, where they become mature with defined effector signatures as a means of making tissue-specific and functionally distinct subsets of NK cells (11-13). Depend on sequential acquisition of NK cell surface markers, development and differentiation of human NK cell can be divided into five main stages (14, 15). In stage I, cells ($CD34^+CD117^-CD94^-CD16^-CD10^+$) do not express CD122 and do not respond to IL-15 *ex vivo*, even though IL-15 is identified to induce NK cell differentiation, functional maturation, and survival (16). Interestingly, the stage I population was also detected in peripheral blood and extramedullary tissue such as the decidua, liver, and mucosa-associated lymphoid tissue (MALT), which likely originated within human bone marrow and then migrated to these tissues for further development (17). Freud *et al* found and identified stage I cell subsets in human decidua that, in the presence of either appropriate cytokines or upon co-culture with decidua-derived stromal cells, can be differentiated into mature cytotoxic NK cells *ex vivo* (18, 19). Stage II cells ($CD34^+CD117^+CD94^-CD16^-$) are known as IL-15-responsive $CD34^+CD45RA^+CD117^+$ integrin $\beta7^+$ NK cell precursors (14, 15). Under suitable conditions stage II subsets can develop into T cells, dendritic cells (DCs), and the stage III cells *in vitro* (15). Human NK cell differentiation then proceeds through stage III ($CD34^-CD117^+CD161^+CD94^-LFA\text{-}1^+$). The stage III cell population cannot develop into T cells or DCs *ex vivo.* They are considered immature NK cells because they can neither produce IFNγ

nor cytotoxic granules, which is a hallmark for mature NK cells (15). Stage III cells will then finally give rise to stage IV ($CD3^-$ $CD34^-CD117^{+/-}CD94^+CD16^-$), also defined as $CD56^{bright}$ NK cells, and finally the cells proceed to stage V ($CD34^-CD117^-$ $CD94^{+/-}CD16^+$ $KIR^{+/-}$), also known as $CD56^{dim}$ NK cells (10, 14, 15). Killer Immunoglobulin-like Receptors (KIRs) start to be expressed at minimal level at stage IV, and highest level of expression is seen at stage V when NK cell become fully mature. The $CD56^{bright}$ NK cells make up approximately 5 to 15% of circulating NK cells in humans, and have an immunoregulatory role by producing the majority of NK immunoregulatory cytokines while playing a limited role in cytolytic responses. In contrast, $CD56^{dim}$ NK cells are fully mature, comprise up to 90% of total NK cells in peripheral blood, have potent cytolytic capacity, and can also contribute significantly to early cytokine production (20-22).

Likewise, murine NK cell development undergoes four stages depend on sequential expression of specific cell surface markers (23). Surface expression of CD11b and CD27 can be used to define the four-stage model of NK cell maturation as follows: $CD11b^-$ $CD27^-$ $\rightarrow$ $CD11b^-$ $CD27^{hi}$ $\rightarrow$ $CD11b^{hi}$ $CD27^{hi}$ $\rightarrow$ $CD11b^{hi}$ $CD27^{lo}$. Mature mouse $CD11b^{hi}$ NK cells could be subdivided into $CD27^{hi}$ and $CD27^{lo}$. $CD11b^{hi}$ $CD27^{hi}$ NK cells express slightly lesser percentage of inhibitory Ly49 receptors, display a greater effector function and are potent cytokine producers, whereas $CD11b^{hi}$ $CD27^{lo}$ NK cells display greater restriction by self-MHC-I, possesses a higher threshold to stimulation, and are long-lived (11, 23, 24). Since most NK lineages can be found outside the BM in both humans and mice (14), more studies have to be conducted to clearly understand NK cell development and maturation outside of the BM and determine whether immature NK cells could have an immune function in these tissues.

1.2. NK cell receptors

NK cells express a diverse array of inhibitory and activating receptors that regulate their effector functions when ligated with their target molecules (4). There are three major groups of inhibitory receptors expressed by NK cells: I) Inhibitory human KIR and rodent Ly49 receptor families, which are divergent in structure but perform similar functions and recognize classical MHC class-I (MHC-I) molecules as their ligand. MHC-I is present on entire nucleated cells (25). II) The CD94/NKG2A inhibitory receptor, a heterodimer that bind to non-classical HLA-E in humans and MHC-I—Qa-1^b in mice (26). III) Inhibitory mouse NKR-P1B and human NKR-P1A, that recognize C-type lectin-related protein B (Clr-b) and lectin-like transcript-1 (LLT1) respectively (27). NK cell inhibitory receptors comprise long cytoplasmic tails containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which are phosphorylated upon binding between the receptor with its ligand, leading to the recruitment of SHP-1 and SHP-2, tyrosine phosphatases (Figure 2). This results in the dephosphorylation of substrates that are critical for NK cell activation that then inhibits NK cell activity (25, 28, 29). In contrast, activating receptors, like NKG2D, besides activating members of NKR-P1, KIR and Ly49 receptor families, lack the ITIMs and acquire a charged residue in their transmembrane domain that mediates interaction with signal transduction adaptor molecules such as DNAX-activating proteins of 12 kDa (DAP12) (30, 31). These signal transducer molecules have immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails (Figure 1). Phosphorylated ITAMs recruit the Syk tyrosine kinase that initiates an intracellular signalling cascade events resulting in NK cell activation (32).

Activating ligands that have the ability to trigger NK cell responses can be proteins encoded by pathogens or normal self-proteins that have become upregulated upon cellular stress such as infection or transformation. The presence of these ligands has the potential to activate NK cells (33, 34).

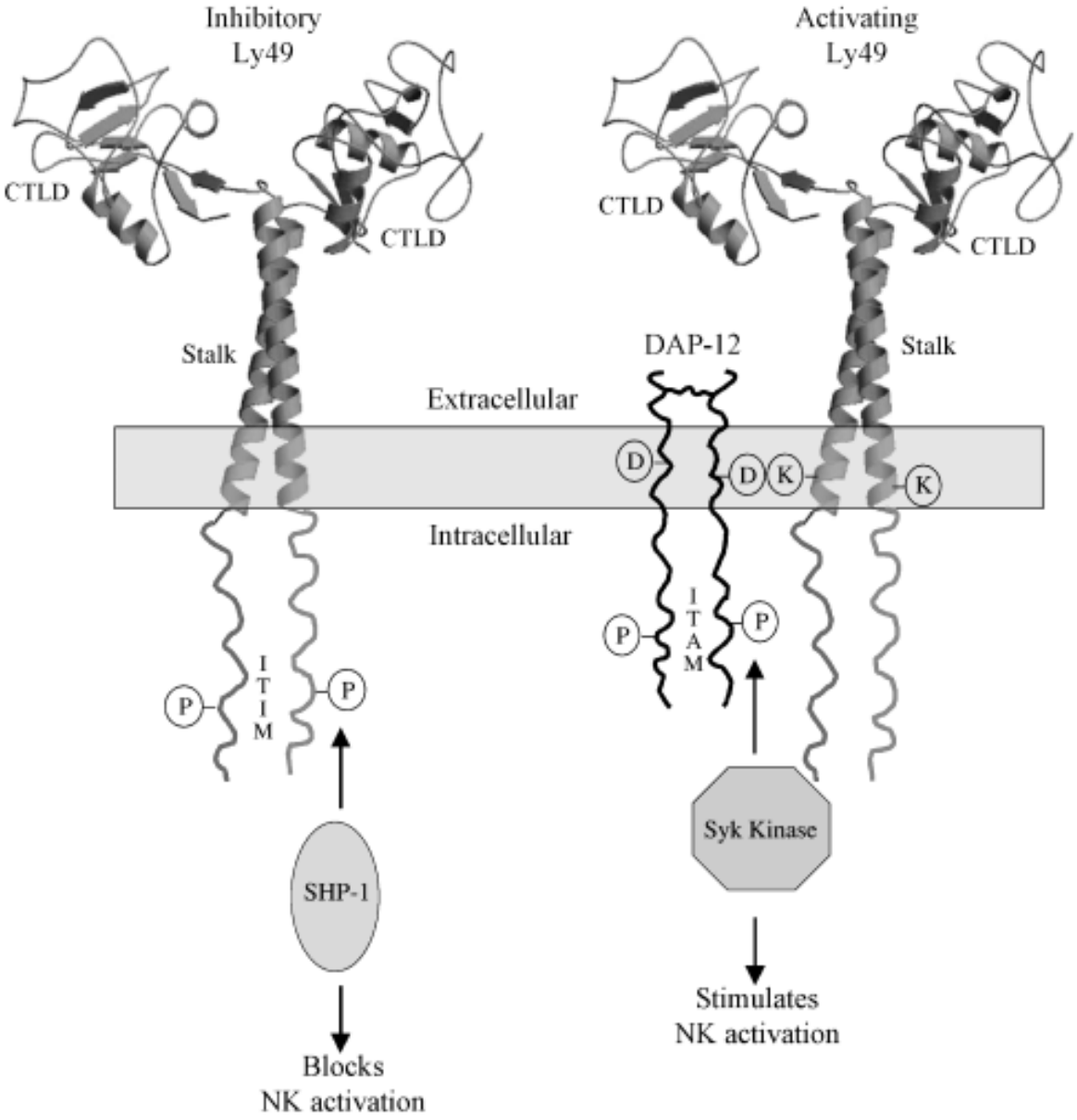

Inhibitory
Ly49
CTLD
CTLD
Stalk
Activating
Ly49
CTLD
CTLD
Stalk
DAP-12
Extracellular
Intracellular
D
D
K
K
P
P
ITIM
P
P
ITAM
SHP-1
Syk Kinase
Blocks
NK activation
Stimulates
NK activation

Figure 2. NK cell inhibitory and activating receptor signalling mechanisms.

The Ly49 receptor is a lectin-like type II transmembrane disulfide-bonded homodimer. Engagement of a Ly49 inhibitory receptor with its ligand results in the phosphorylation of its ITIM motif and recruitment of SHP-1, tyrosine phosphatase, that then inhibits NK cell activation. On the other hand, phosphorylation of DAP-12 ITAMs is trigger upon ligand binding to its activating receptors, resulting in the recruitment and activation of Syk kinase, which in turn enhances NK cell response. Figure is taken from (35).

1.2.1. NKp46 (Ncr1)

NKp46 is a member of the natural cytotoxicity receptor (NCR) family, that in humans also contains NKp30, and NKp44. NKp46 is the only member of NCRs known to be expressed in mice (36). NKp46 is a type I transmembrane protein belonging to the Ig superfamily, and is characterized by two extracellular C2-type Ig-like domains and a short tail (37). The transmembrane domain contains an arginine residue that signals NK cell activation via ITAM-containing adapter proteins (FcϵRI and CD3ζ) upon receptor engagement. NKp46 is evolutionarily conserved among mice and human, and is distinctively present on all NK cell subsets and has been used as a pan NK cell marker (38). Several ligands have been demonstrated that can be recognized by NKp46 including: hemagglutinin (HA) of sendai and influenza viruses (39), HA-neuraminidase of newcastle disease virus (40), the intermediate filament protein vimentin that is expressed on mycobacterium tuberculosis-infected monocytes (41), and target cell-expressed heparin sulfate (HS) (42). Collectively, these determine the importance of NKp46 in the recognition of pathogen-associated ligands which in turn would limit the progress of an infection. Using NKp46-deficient mice, numerous reports have shown that it plays a major role in limiting various primary tumor cell metastases, including melanoma, lymphoma, and carcinoma; nevertheless, the ligands that are recognised by NKp46 on these tumor cells are yet unknown (43). There are two mouse models that are used to study NKp46 function. The first model, generated by Mandelboim's group ($Ncr1^{gfp/gfp}$), replaced exons 5–7, which contain the transmembrane and cytoplasmic domains of Ncr1, with GFP (44). The second model ($Ncr1^{Noé/Noé}$) was generated by a random mutation, W32R, in position 32, which impaired cell surface expression of NKp46 (45). Although NK cells in $Ncr1^{Noé/Noé}$ show hyperactivity

toward MCMV-infected cells in comparison to NK cells from WT mice *in vitro* and *in vivo*, there were no variances in NK cell responses between WT and Ncr1$^{gfp/gfp}$ mice. These opposing results were recently studied by Mandelboim's group (46), where they expressed the WT and mutant Ncr1-Noé construct in BW reporter system, in which ligation of Ncr1 with its ligand leads to IL-2 secretion. Ncr1-Noé receptor was expressed normally on the surface of BW cells, but the expression level was lower than the WT Ncr1 and was downregulated completely after a week in culture. In the presence of influenza-infected cells, WT and mutant Ncr1-Noé BW reporter cells produced IL-2 at high levels. Interestingly, after blocking influenza-hemagglutinin, a ligand for NKp46, with anti-HA1 antibody, IL-2 production was reduced, suggesting an interaction between Ncr1-Noé and HA molecules (46). This proves that, unlike Ncr1$^{gfp/gfp}$ that lack exons 5–7, W32R mutation is not enough to interfere with the interaction between NKp46 and influenza-HA.

1.2.2. The natural killer group 2d (NKG2D)

NKG2D is a homodimeric type II transmembrane glycoprotein that serves as an activating receptor. Two adaptor molecules, DAP12 and DAP10, were recognized to associate with NKG2D. (47). NKG2D is expressed on activated CD8$^+$ T cells, NK cells, NKT cells, and subsets of T cells in humans and mice (48). NKG2D bind to "stress-inducible" molecules, which structurally similar to MHC-I molecules. These stress molecules are absent or hardly present on normal tissues, do not bind β2-microglobulin or present antigen to T cells, and their expression is activated through viral infection, cellular transformation, and/or DNA damage (47). Thus, NKG2D has been shown to be involved in antiviral immunity and tumor clearance. In humans, two main NKG2D ligands families have been identified that include MHC-I chain-related proteins A and B (MICA and MICB) as

well as UL16-binding proteins (ULBP), also known as retinoic acid early transcripts-1 (RAET1) (49). To date, identified mouse NKG2D ligands include the retinoic acid early-inducible 1 family of proteins (RAE-1α to ε), H60 family (H60a, b, and c), and murine ULBP-like transcript 1 (MULT1) (49).

1.2.3. NKG2/CD94 family

The NKG2/CD94 family includes two activating receptors, NKG2C and NKG2E , and one inhibitory receptor, NKG2A (50). NKG2A is an inhibitory receptor that form heterodimers with CD94 on activated $CD8^+$ T cells and NK cells. CD94-NKG2A interacts with non-classical MHC-Ib molecules, Qa-1 in mice and HLA-E in humans (50, 51). The cytoplasmic domain of NKG2A contains one ITIM in mice and two in humans (52). Accordingly, interaction between CD94-NKG2A and its ligand inhibits NK cell function. Both human and murine NKG2A share a high degree of similarity in their extracellular domains (50, 52). NKG2A is one of the first inhibitory receptors that is expressed on developing fetal NK cells (14, 23). Macrophages are known to enhance NK cell cytotoxic function against tumor cells, and a recent report showed that macrophages protect themselves from being attacked by NK cells via upregulation of a NKG2A ligand on their surface (53). Interestingly, in rheumatoid arthritis, blockade of NKG2A-HLA-E interactions *in vitro* enhanced NK cell cytotoxicity toward fibroblast-like synoviocytes, located inside joints in the synovium (54). Similar results were obtained *in vitro* using several tumor cell lines including: erythroleukemia, cervical cancer, ovarian cancer, and multiple myeloma cell lines (55).

NKG2C and NKG2E lack ITIM in their cytoplasmic tails and are associated noncovalently with DAP12(56). Both activating receptors bind and recognize non-classical

MHC-I (HLA-E or Qa-1) molecules; however, their affinity for binding is much lower than the inhibitory NKG2A(57). NKG2C^{+} NK cells can be detected at high levels in HCMV-seropositive (HCMV^{+}) individuals compared with HCMV-seronegative (HCMV^{-}) individuals(58). NKG2C^{+} NK cells from HCMV^{+} seropositive people express a lower level of NKG2A inhibitory receptors and show high responsiveness to HCMV-infected cells(59). Likewise, studies have shown that NKG2E plays a significant role against viral infections in mice, especially against Ectromelia virus (60).

1.2.4. NKR-P-1 family

NKR-P1 play a crucial role in tumor and pathogen recognition. NKR-P1 receptors are conserved across many mouse species, and are expressed on NK cells and subsets of T cells (61). Ligands for NKR-P1 receptors are members of the C-type lectin-related (Clr) family (62). *Clr* genes are genetically interspersed amongst the *Nkrp1* genes (63). This family includes activating receptors, such as NKR-P1A, NKR-P1F and NKR-P1C, in addition to inhibitory receptors, such as NKR-P1B and NKR-P1G (64). NKR-P1B is expressed on nearly 60% of NK cells in the spleen of C57BL/6 mouse. NKR-P1B potently inhibits NK cell effector functions upon Clr-b engagement (65). Clr-b is present on hematopoietic cells, with detectable mRNA levels in almost all tissues except the brain (66). Very few studies have explored the significance of the NKR-P1B:Clr-b receptor–ligand interaction in the discrimination of healthy versus pathogen-infected target cells (65). NKR-P1B has been shown to play a significant role in NK cell-mediated immune surveillance against cells that have lost Clr-b expression (65). NKR-P1B-deficient (*Nkrp1b*^{KO}) mice exhibit diminished rejection of Clr-b-deficient cells compared to wild type (WT) mice (65). A recent study has determined that murine cytomegalovirus (MCMV) infection results in

downregulation of Clr-b expression *in vitro* (67), leading to enhanced NK cell cytotoxicity toward target cells. Of note, this downregulation required live virus since UV-inactivated virus did not modulate Clr-b expression (67). Rat CMV (RCMV) expresses a homologue of Clr-b, which can interact with rat NKR-P1B to inhibit NK cells (68). This common modulation of Clr-b expression on infected cells by multiple species of CMV suggests that viruses are able to subvert the NKR-P1B:Clr-b recognition system through various evasion mechanisms to escape detection.

1.2.5. Human killer immunoglobulin-like receptors (KIRs)

KIR receptors are expressed on the surface of human NK cells and subsets of T cells (69). KIR family includes highly polymorphic inhibitory and activating receptors that are essential in controlling NK cell responses (70). KIRs bind to the classical MHC-Ia (HLA-A, HLA-B, or HLA-C) molecules, which are present on the surface of all healthy nucleated cells (69). KIRs are categorized based on their number of immunoglobulin-like extracellular domains (2D and 3D) (71). Activating KIRs have a short cytoplasmic tail, referred to as "S" such as KIR2DS3, and is associated with DAP12. Inhibitory KIRs are categorized by their long cytoplasmic tail, referred to as "L" such as KIR2DL1, which contains ITIM motifs (71).

The KIR gene family currently consists of 15 gene loci (*KIR: 3DL1/S1, 2DL1, 3DL2, 2DL2/L3, 3DL3, 2DL5A, 2DL5B, 2DL4, 2DS1* to *2DS5,* and two pseudogenes, *2DP1* and *3DP1*) (72). Four framework genes, *KIR3DL2, KIR3DP1*, *KIR3DL3,* and *KIR2DL4* are present in every individual, with very few exceptions (71, 72). Most KIRs can bind to a set of HLA-I ligands, for example inhibitory KIR3DL2 receptor can recognize and bind to HLA-A3 and HLA-A11 alleles (73). HLA-Cw2 is recognized by both activating KIR2DS1

receptors and inhibitory KIR2DL1 receptors; however, the affinity of KIR2DS1 to HLA-C molecules is much lower than KIR2DL1 (74).

1.2.6. The Ly49 family

Ly49 receptors, also known as killer cell lectin-like receptor subfamily A (KLRA), are lectin-like type II transmembrane disulfide-bonded homodimers. It is a highly polymorphic and polygenic gene family that encodes activating and inhibitory receptors (63). Comparable to human KIR, mice Ly49 receptors bind to MHC-I in a peptide-dependent manner (75, 76). Although crystallography studies have shown that Ly49 bind to MHC-I molecules at a position underneath the peptide binding groove, the requirement for peptide selectivity has been previously well described (77). Franksson *et al* (75) used TAP-deficient RMA-S cells and a variety of H-2K^{b}-restricted peptides, such as ovalbumin-derived OVA 257–264 (pOVA), VSV nucleoprotein 52–64 (pVSV), EF1α 412–420 (pEF), and SEV nucleoprotein 324–332 (pSEV) to show that only pOVA, at the optimal MHC-I inducing/stabilizing concentration, protected RMA-S cells from killing by Ly49C^{+} NK cells (75). This MHC-I peptide selectivity has also been shown for mouse Ly49I and rat Ly49i2 (75, 78, 79); however, the mechanism is unknown. This selectivity may play a critical function in NK cell memory formation, since Ly49C/I^{-} NK cells show no sign of memory(80). In addition to classical MHC-I, recently, inhibitory Ly49A has been shown to recognize and bind to a non-classical MHC-I molecule, H2-M3, with comparable affinity as the classical H-2D^{d} molecule (81). More studies have to be conducted to determine whether other Ly49 receptors might also recognize non-classical MHC-I.

Ly49 receptors are present on CTL, NK cells, NK-T cells, intestinal epithelial lymphocytes (IELs), and cells of the myeloid lineage (82). Whereas NK cells express the

majority of the Ly49 receptors, Ly49B is exclusively expressed by macrophages (83), whereas Ly49Q is expressed by plasmacytoid dendritic cells and neutrophils (84). Likewise, Ly49E is detected only on neonatal NK cells and is downregulated on adult NK cells (85). *Ly49* gene haplotypes have been comprehensively studied among four inbred mouse strains commonly used in the lab by the Makrigiannis lab (Figure 3) (63). The C57BL/6 *Ly49* gene haplotype encodes for two activating (*Ly49h, d*) and eight inhibitory receptors (*Ly49f, a, q, c, i, g, j, e*) (86). The 129 haplotype encodes for three activating (*Ly49p, r, u*) and nine inhibitory receptors (*Ly49e, o, i1, q1, s, g, t, ec2, v*)(87). Adult mouse NK cell can expresses up to six different Ly49 receptors in random combinations, with an average of two to three receptors expressed by each NK cell (88). Ly49 expression on NK cells reaches its optimal level by 6–8 weeks after birth, and each of these receptors can bind to several but not all alleles of MHC-I (82). For instance, inhibitory Ly49C can distinguish and bind to H-2K^{b} and H-2D^{b} but not H-2L^{d}; Ly49G2, Ly49A, and Ly49F with H-2L^{d}; and Ly49I with the H-2K^{b} haplotype (63, 79, 82). Different specificities for MHC-I enable NK cell to recognize several target that lacking various self-MHC-I molecules without causing autoimmunity.

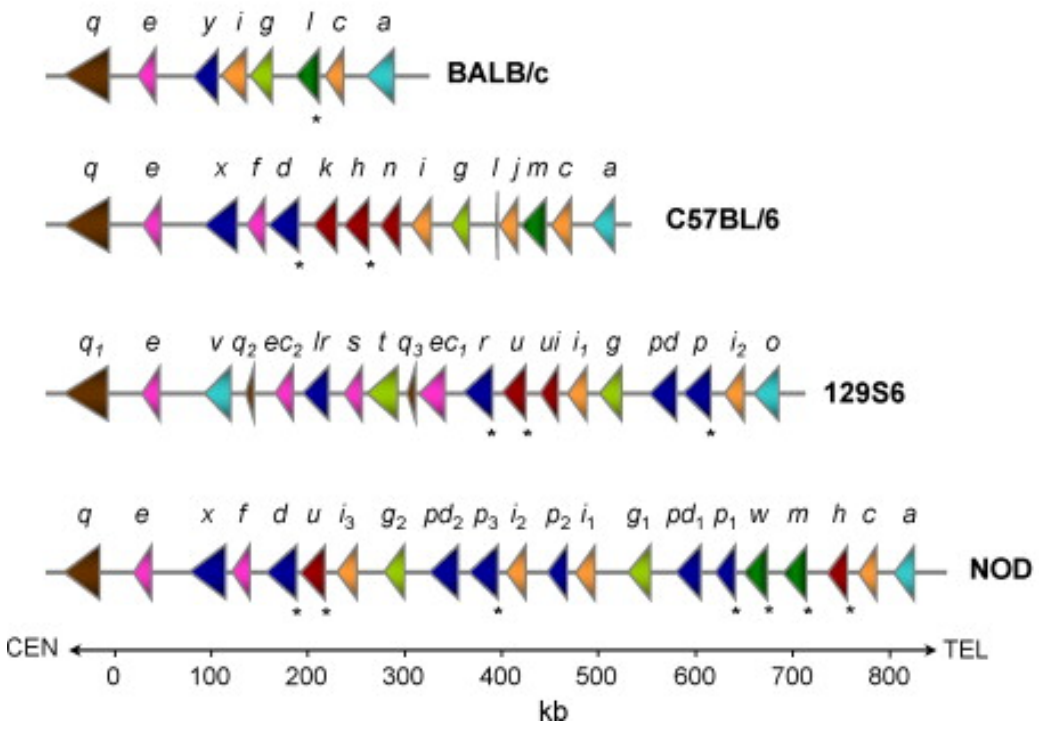

q e y i g l c a
BALB/c
q e x f d k h n i g l j m c a
C57BL/6
q1 e v q2 ec2 lr s t q3 ec1 r u ui i1 g pd p i2 o
129S6
q e x f d u i3 g2 pd2 p3 i2 p2 i1 g1 pd1 p1 w m h c a
NOD
CEN
TEL
0 100 200 300 400 500 600 700 800
kb

Figure 3. Ly49 receptor haplotypes among various inbred mouse strains.
All *Ly49* genes are located on chromosome six within the NK gene complex (NKC). Different mouse strains (C57BL/6, BALB/c, NOD, 129S6) have been described to carry different *Ly49* haplotypes, with varying numbers of activating and inhibitory genes. Each haplotype also contains one or more pseudogenes (ψ). Activating receptors, shown with an asterisk '*', include Ly49L, H, D, P, U, R, M, and W, and the inhibitory receptors include Ly49A, C, E, I, O, J, G, F, T, S, V, E and Q. Each *Ly49* allele can differ in expression level and functional capacity. Figure is taken from (89).

1.3. Education of NK cells

Among the initial models to explain NK cell education is the missing-self hypothesis, in which NK cells can identify then lyse a target cell that is deficient in a specific MHC-I-allele recognized via the Ly49 (89, 90). Although this hypothesis is true at some level, autoimmune disease was not detected in MHC-I-deficient mouse models furthermore NK cells do not target self-tissues for destruction, although they are normal in number (91, 92). Likewise, because inhibitory receptors cannot be found equally expressed on NK cells, small percentage of NK cells don't express any inhibitory Ly49 receptors and yet they do not initiate autoimmune disease (63, 88). This raised the question of how NK cells acquire tolerance to healthy peripheral tissues. Yokoyama and his group reported that only NK cells that express Ly49C and/or Ly49I in C57BL/6 mice (which express the H-2K^b and H-2D^b ligands for Ly49C/I) are actually functional or "licensed". Only licensed NK cells respond and lyse target cells that lack self-MHC-I, whereas other NK cells were hyporesponsive or "unlicensed" (93). For NK cells to be fully responsive or licensed, an interaction between inhibitory KIR/Ly49 and its specific MHC-I is required throughout NK cell maturation (3). Moreover, beside its ability to sense MHC-I down-regulation on a target cell via its inhibitory receptors, NK cells are also educated to use their activating receptors to lyse any target cells that up-regulate stress-induced ligands on target cells (31, 33, 54, 70, 93). Direct engagements between Ly49 inhibitory receptors and its ligand contribute to NK cell education (93). Consequently, NK cells from Ly49- or MHC-I–deficient mice are generally known as non-functional or hyporesponsive, and thus these mice do not develop autoimmune disorders (94-96).

1.4. NK cell responses

NK cell activities are controlled by a complex array of germline-encoded inhibitory and activating receptors. Therefore, the balance of signals from these receptors governs NK cell effector functions (70, 97). If the NK cell receives stronger inhibitory signals it will be inhibited; conversely, when the activating signal is predominant then that NK cell will be activated. Though NK cells are a type of the innate immune cells, they also display several characteristics of the acquired immune system's lymphocytes. Activated NK cells can undergo clonal expansion of pathogen-specific cells (98, 99) and the initiation of long-lasting memory cells (100, 101). NK cells lyse target cells primarily by perforin/granzyme molecules released via the granule exocytosis pathway (70). Perforin perforates the cell membrane to form pores through which granzyme can enter inside the cell (102). Granzyme B induces target-cell apoptosis by activating caspases (especially caspase-3) (102). NK cells furthermore destroy target cells via death receptor-mediated apoptosis using TNF family members, including TRAIL and FasL (103, 104). Moreover, activated NK cells can secret high levels of chemokines and cytokines in order to enhance recruitment and activation of adaptive and innate immune cells (105). Among the most prominent chemokines and cytokines produced by NK cells are interleukin-1 (IL-1), IL-10, IL-8, IL-5, IL-12, IL-13, IL-15, TNF-α, IFN-γ, and the growth factors RANTES, MIP-1α, GM-CSF, and MIP-1β (6). Likewise, innate and adaptive cells produce several chemokines and cytokines, such as IL-12, IL-15, IL-23, IL-21, and IFN-α, which have the ability to activate NK cell cytotoxicity and recruit them to the site of infection or inflammation.

1.5. NK cell responses to viral infections

Unlike adaptive immune cells, NK cells do not undergo genetic recombination to attain receptor specificity for a particular ligand; instead NK cells use a sophisticated system of germline-encoded inhibitory and activating receptors to recognise infected cells (25). NK cells contribute significantly in host defense to viral infections. Upon activation, NK cells control infection by killing the infected cells, and by recruiting and activating further leukocytes to the site of infection (106). Several reports have demonstrated that human NK cells fight against members of the poxvirus, herpesvirus, and papillomavirus families (107-109). Patients who are lacking NK cells and/or their functions suffer from severe recurrent viral infections, particularly ones caused by HSV, EBV, and HCMV (110). A long-term longitudinal study of a 13 year old female patient who was diagnosed with NK cell deficiency demonstrated the importance of NK cells during viral infection (7). The patient was first diagnosed with varicella pneumonia and chicken pox infection. Following recovery from the infection, she was diagnosed with NK cell deficiency but normal antibody and T cell responses. A few years later, she developed primary life-threatening human cytomegalovirus (HCMV) infection and abnormal sensitivities to herpes viruses (7). In another report, two patients from the same family exhibited chronic active Epstein-Barr virus (EBV) infection and viral-based respiratory illnesses, which was found to be due to defective NK cell cytotoxicity in these patients (111). These reports demonstrated the significance of NK cell function in limiting viral infections.

Likewise, antibody-dependent NK cell depletion reports have demonstrated the significance of mouse NK cells in controlling several viral infections, particularly murine cytomegalovirus (MCMV) and influenza virus (112, 113). Additionally, NK cells activity

have been detected in a number of several viral infections, such as lymphocytic choriomeningitis virus (LCMV), HSV, Coxsackie virus, and others (106).

1.5.1. NK cells in CMV infection

Cytomegalovirus (CMV) is a herpes virus that can infect almost anyone, usually without producing symptoms. However, when the immune system is weakened, such as in HIV/AIDS patients and transplant recipients, CMV can cause serious disease such as retinitis, colitis, pneumonia, and encephalitis. It can also cause serious disease in babies who were infected with CMV infection before birth (114).

Interestingly, viral genomes encode for several molecules that found to be activating ligands for NK cell (106, 115). Ly49H in C57BL/6 mice were the first NK cell receptors shown to specifically recognize and bind to MCMV m157 viral protein, a viral MHC-I homolog (116). Ly49H receptor expression in B6 mice confers resistance to MCMV infection. Likewise, during MCMV infection the Ly49P activating receptor protects the MA/My mouse strain (117). Ly49H engagement by m157 results in vigorous NK cell activation as demonstrated by the release of perforin, granzyme, cytokines, and chemokines, together with a robust $Ly49H^{+}$ cell proliferation (118). Notably, not all mouse strains are protected from MCMV infection since expression of Ly49H is limited to certain strains (119). To further confirm the role of the Ly49H receptor during MCMV infection, Ly49H-deficient mice with a B6 background or Ly49H-blocking antibodies were used (120). The absence or blockade of Ly49H results in susceptibility to MCMV, thus highlighting the importance of the Ly49H activating receptor in limiting viral pathology (120). Interestingly, inhibitory Ly49I receptors from the 129 mouse strain interact with MCMV m157 viral protein, that in turn inhibited NK cell activity (121). Some variants of m157 protein also

bind to inhibitory Ly49C receptors in B6 mice, but because MCMV clearance in B6 mice is mediated mostly by Ly49C/I^{-} unlicenced NK cells, the virus clearance is not affected (118). Adoptive transfer of Ly49C/I^{-} Ly49H^{+} NK cell into MCMV-infected neonate mice, which do not have mature NK cells, protected them from infection superior than Ly49C/I^{+} Ly49H^{+} NK cells (118). Moreover, selective depletion of the Ly49C/I^{-} Ly49H^{+} NK in B6 mice significantly enhanced viral titer in compression to Ly49H^{+} Ly49C/I^{+} NK depletion. Interaction between MHC-I and Ly49C/I limits the licensed NK activation (118). The significance of unlicensed NK cell in MCMV infection was further confirmed in MHC-I deficient mouse models (*B2m*$^{-/-}$ mice) that possess just unlicensed NK cells. Interestingly, unlicensed NK cell from *B2m*$^{-/-}$ mice can control MCMV infection better than WT mice (118, 122). A new report showed that adoptive transfer of Ly49C/I^{+} NK subsets into MCMV-infected NOD.SCID.IL-2γ receptor^{KO} mice, provides efficient MCMV protection (123), but is subject to regulation by regulatory T cells (Treg). Selective depletion of Treg or TGF-β blockade in B6 mice impressively augments licensed NK cell antiviral responses (123).

The MCMV-encoded m04 protein interacts with the inhibitory Ly49A receptor in BALB/c, CBA/J, and C57BL/6 mice (124). Control of m04-deficient (Δm04) MCMV strain by NK cells *in vivo* was significantly enhanced in comparison to WT MCMV, as a consequence of lacking the inhibitory signals delivered by Ly49A:m04 interaction. Moreover, the presence of m04 impairs the proliferation of NK cells, whereas infection with Δm04-MCMV strain enhanced NK cells proliferation (124). Additionally, down-regulation of cellular ligands for activating NKG2D receptors was also reported following MCMV infection. Viral proteins such as m152, m145, and m155 were found to reduce the expression of *RAE-1* gene products, MULT-1 and H60, respectively (125). With the exception of a few

mouse strains that possess Ly49H or Ly49P activating receptor, MCMV encoded proteins allowed MCMV to evade recognition by NK cells.

In humans, a report on HCMV-infected myeloid DCs demonstrated the importance of NKp46 in recognizing infected cells. NKp46 blocking significantly reduces the release of NK cell cytotoxic granules and cytokines (126). Moreover, HCMV infection enhanced expression of NKG2D ligands on infected cell, which increased NK cell killing (127). However, HCMV has developed numerous evasion strategies to avoid NK cell cytotoxicity. HCMV encoded MHC-I homologs UL142 and UL18 are recognized by inhibitory NK cell receptors (128, 129). Moreover, HCMV p65 viral protein was found to prevent NK cell killing *in vitro* by direct interaction with the activating NKp30 receptor, consequently this interaction reduce NK cell-mediated target cell death (130). Additionally, HCMV infection enhances up-regulation of HLA-E, which binds to NKG2A thus inhibiting NK cell cytotoxicity (131).

1.5.2. NK cells in hepatitis C virus infection

HCV is an enveloped, positive-sense RNA virus of the Hepacivirus genus within the Flaviviridae family. Most of HCV-infected individuals develop chronic disease with a risk for progressive liver fibrosis, cirrhosis and liver failure (132). Epidemiological studies have identified associations between inhibitory KIR2DL3 receptors and their ligand, HLA-C1, which could directly impact the resolution of HCV infection (133). This data strongly proposes that licensed NK cells play a major role in HCV infection. Activated human NK cells were successfully able to lyse hepatoma cells carrying HCV replicons in a perforin/granzyme-dependent manner (134). Moreover, co-culture of NK cells with HCV replicon-containing hepatocytes showed that NK cells repressed HCV RNA expression

(135). Neutralization of IFN-γ or blocking IFN-γ receptors abolished the anti-HCV activity of NK cell, demonstrating that besides direct killing, NK cells can suppress HCV RNA expression in an IFN-γ-dependent manner (135). Several reports have indicated that NK cells in patients chronically infected with HCV are hyporesponsive (136). However, patients who are using IFN-α as a therapy have active NK cells, as demonstrated by an increase in NK cell cytotoxicity, and elevated expression of NKG2D, NKp46, and TRAIL (137), indicating that failure to eliminate HCV may result from impaired NK cell function. Liver NK cells express greater levels of inhibitory NKG2A receptors than blood NK cells. Some studies have indicated that HCV virus induces upregulation and stabilization of HLA-E, a ligand for NKG2A, on HCV-infected hepatocytes, thereby inhibiting NK cell-mediated killing. Accordingly, blockade of the interaction between CD94/NKG2A and HLA-E enhanced NK cell cytotoxicity toward HCV-infected cells (138). Consequently, individuals with spontaneous clearance of HCV infection have fewer NK cells expressing NKG2A/C/E compared to patients with chronic infection (136). HCV envelope protein 2 (E2) was also found to bind to CD81, which belongs to a family of molecules called tetraspanins, on NK cells to inhibit their cytotoxicity (139). Moreover, HCV NS2 and NS5B proteins downregulate expression of MICA and MICB, ligands for NKG2D, on HCV-infected hepatoma cells, which in turn help HCV to avoid NK cells cytotoxicity (140). Circulating NK cells of HCV patients also have a reduced expression of NKG2D. A recent study has demonstrated that HCV-NS5A protein enhances NKG2D down-regulation in a TGFβ concentration-dependent manner (141). TGFβ is over-produced in HCV patients and was found to be secreted by monocytes in the presence of HCV-NS5A protein (141).

2. Influenza virus

Influenza virus is a member of the orthomyxoviridae family. It is an enveloped virus that contains a negative-sense single-stranded segmented RNA genome, which is divided into three groups: A, B, and C (142). Two of the segments code for the surface proteins neuraminidase (NA) and hemagglutinin (HA) that project from the virion. These proteins are used in classifying the influenza virus into sub-divisions, such as H1N1 and H5N1. At present 18 HA (H1-H18) and 11 NA (N1-N11) subtypes are known (143). According to the World Health Organization (WHO) standards on naming and the classification of influenza viruses, isolate is named by the kind, place of origin, isolation number, and when is isolated, separated by a slash punctuation, such as A/New Caledonia/20/1999 (H1N1), where A refers to the type, New Caledonia to the place of origin, 20 to isolation number, and 1999 to the year of isolation (144). Influenza A virus infects a broad variety of mammals, including domesticated as well as wild birds, swine, humans, and other mammals (145). However, waterbirds (including primarily anseriformes and charadriiformes, most prevalent in dabbling ducks) are recognized as the natural reservoirs of influenza A viruses (146). Influenza is associated with high global mortality and morbidity annually, especially in the infants, elderly, and those with chronic diseases (146). Symptoms range from headache, muscle or body aches, fatigue, cough, sore throat, sneezing, and nasal discharge. People who have the flu often feel some or all of these symptoms (147). Most influenza-infected individuals recover in a few days or can take up to two weeks for a full recovery. However, host factors, such as medical conditions, can increase the severity of illness leading to influenza-induced lung edema and inflammation that can be life-threatening and can result in death (147).

2.1. Influenza virus life cycle

The influenza virus has eight fragments of RNA which encode for several genes: the two glycoproteins neuraminidase (NA) and hemagglutinin (HA), non-structural protein 1 (NS1/NSP1), nucleoprotein (NP), non-structural protein 2 (NS2), matrix 1 (M1), matrix 2 (M2), ion channel, and the four polymerase proteins: polymerase basic protein 1 (PB1), polymerase acidic protein (PA), polymerase basic protein 2 (PB2). Each segment is encapsidated by the viral NP to form a ribonucleoprotein (RNP) particle, and each RNP carries its own RNA polymerase protein complex (PB1, PB2, and PA) (143, 145).

Entry into the host cell starts by binding of hemagglutinin to sialic acid, a glycoprotein presents on the surface of the host cell. Of note, two main types of sialic acid receptors are found in different species. Human respiratory epithelial cells express α(2,6) and α(2,3) linkage, mainly found in lower respiratory tract, whereas in birds it is an α(2,3) linkage (148). Accordingly, most human viruses preferentially bind to the α(2,6) linkage, while avian viruses bind to the α(2,3) linkages (148). α(2,3) linkage are abundant in the mouse respiratory tract, which is the reason why most human influenza A virus strains must be adapted for efficient replication in mice (149). On the other hand, epithelial cells of the pig trachea express both α(2,3) and α(2,6) linked sialic acids (150). Thus, pigs could be infected with avian influenza as well as human virus, which in several cases have resulted in the emergence of a new virus strain (150).

The hemagglutinin is composed of two subunits: HA1, receptor binding domain, and HA2, fusion peptide that facilitates merging of the host endosomal membrane with the viral membrane (151, 152). After attachment of HA1 to the sialic acid containing receptor, the

influenza is internalized into the endosome of the host cell via receptor-mediated endocytosis (153). The acidic pH in the endosome triggers HA2 to fuse the viral membrane with endosomal membrane. The acidic environment of the endosome will activate the M2 ion channel, which allows for proton transport into the virion core and its acidification (153). This acidic environment in the virion leads to dissociation between M1 and viral RNP complexes, which subsequently gets released into the cytoplasm and is then imported into the nucleus to start synthesis of messenger RNA (mRNA) and viral RNA replication (153). The negative-sense viral RNAs are transcribed into positive-sense RNA (mRNAs), which serve as templates to synthesize multiple new copies of negative-sense viral RNAs, (−) vRNA → (+) cRNA → (−) vRNA (153, 154). Transcribed mRNA is carried to the cytoplasm for translation into viral proteins in the endoplasmic reticulum (ER) (155). The newly synthesised M2, NA and HA are glycosylated in the Golgi apparatus then transported and expressed on the cell membrane (156). Folding of viral core proteins occurs in the cytosol, whereas the newly synthesized viral RNA is packaged the RNA polymerase components and with NP. The recently assembled virus releases from the cell surface enveloped in host plasma membrane containing M2, NA and HA viral proteins. when the virus is released, by surface budding, host proteases cleave HA into HA1 and HA2, thus activating influenza virus infectivity, and allowing for new infection and viral replication (153, 156).

2.2. The Immune Response to Influenza Infection

Adaptive and innate immunity against influenza virus plays a central part in controlling illness that might be generated as a consequence of influenza virus infection (157). Lung epithelial cells are the primary target for influenza virus; however, the virus

subsequently infects alveolar macrophages, resident DCs, and NK cells (158-161). Within hours following initial virus infection, the infected cells begin a series of events to activate and recruit immune cells in order to limit and control the infection (162). The immune system has various mechanisms by which the presence of influenza virus is detected which initiate a strong immune response (162).

Pattern-recognition receptors (PRR), including some toll-like receptors (TLR) family members of the, contribute directly in the detection of influenza infection in cells. TLR-3 and TLR-7 recognize influenza's double stranded RNA (dsRNA) in the cytoplasm of an infected cell and single stranded RNA (ssRNA) in the endosome of an infected cell, respectively (162). Activation of TLRs induces the production of several types of cytokines and chemokines such as IFN-α and IFN-β, IP-10, and MIP-1β (157). These chemokines and cytokines recruit and activate macrophages, NK cells, and DCs to the site of the infection, which in turn promote viral clearance (157, 162).

CCL2 secretion by influenza-infected epithelial cells enhances recruitment of alveolar macrophages and monocytes to the site of infection (159). Activated macrophages produce high levels of TNF-α, which has potent anti-influenza virus activity, and become highly-phagocytic (163). Depletion of alveolar macrophages resulted in reduction of antibody titers and reduced numbers of virus-specific $CD8^+$ T cells post-infection, indicating the importance of alveolar macrophages during the infection (164). Moreover, alveolar macrophages are a target for influenza infection: upon infection, alveolar macrophages produce high levels of pro-inflammatory cytokines such as TNF-α (165). Unfortunately, TNF-α has been linked to severe lung pathology following influenza infection (166), suggesting that alveolar macrophages can pose a negative effect during the infection. Thus,

infiltration of macrophages into the lung during the infection correlates with pathology and morbidity (167).

DCs are also important in activating innate and adaptive immune cells; most importantly, DCs are required for suitable induction of effector $CD8^+$ T cell responses (157, 162). Lung DCs can acquire virus-derived antigens either via direct infection or indirectly by sampling the surrounding environment (168, 169). Upon activation, DCs release several types of chemokines and cytokines—such as IFN-I, IP-10, IL-6, and IL-12, that have the ability to enhance the antiviral capability of infected cells and the recruitment of inflammatory cells to the site of the infection (170). DCs also undergo a process of maturation, during which the antigen-bearing DCs migrate to the lymph node, where they present antigen to naive CD8 T cells via MHC-I. DCs reach peak numbers at 18 h post-infection, and return to baseline by 48 h post-infection (171). Selective depletion or blockade of DC migration to the lymph nodes resulted in increased viral load and impaired pulmonary $CD8^+$ T cell responses, which in turn resulted in mouse mortality (171).

Influenza-specific $CD8^+$ T cells start to infiltrate into the lung by day 6-7, reaching peak numbers by 9-10 days post infection; these cells are characterized by IFN-γ secretion (172). T cells eliminate influenza-infected cells via direct killing in a perforin- and Fas ligand (FasL)-dependent manner which in turn inhibits virus replication and spreading (173, 174). Additionally, $CD8^+$ T cells enhance the broader anti-viral response by expressing multiple cytokines such as IFN-γ, TNF-α and IL2 that have the ability to activate other immune cells (173, 174). Importantly, some types of influenza-specific $CD8^+$ T cells were found to recognize conserved epitopes of internal influenza proteins such as NP, M1, and

PA; thus, those influenza-specific $CD8^+$ T cells can be protective against various influenza strains (173, 174).

Notably, however, efficient influenza virus clearance is not exclusively relay on CTL (175). Mice lacking functional $CD8^+$ T cells — *b2m*$^{-/-}$ mice — or mice depleted of $CD8^+$ T cells were able to control lung viral loads similarly to WT mice (175). This suggests that other immune cells can functionally protect against influenza in the absence of $CD8^+$ T, although $CD8^+$ T cells are known to have a potent role during the infection.

3. NK cells in influenza infection

NK cells in Human and mouse represent a higher percentage of resident lymphocytes in healthy lungs than in other tissues (176, 177). Within two days following influenza infection, NK cells can be detected in high numbers in the respiratory tract (178). However, the contribution of NK cells in influenza infection is still not clear. Initial reports have found that NK cells depletion in hamsters or mice renders these animals highly susceptible to lethal influenza infection (112, 179). Another study has shown that NK cells cause severe lung pathology during influenza infection (180). The discrepancy of the results might be due to the use of different viral doses for infection, which might impact NK cell activity and cytotoxicity. A recent study showed that at a lethal infection dose of influenza (PR8) virus, depletion of NK cells increased mouse survival, whereas at a lower dosage, survival is decreased following NK cell depletion (178). A similar finding was also demonstrated with LCMV infection (181). Thus, the exact contribution of NK cells in influenza infection remains elusive. The importance of NK cells in influenza infection was first reported in volunteers who were infected intranasally with human influenza virus (182). Shortly

following influenza virus infection, NK cell activity is increased in association with the induction of interferon, which is known to increase the activity of NK cells, in comparison to individuals who received placebo (182). The physiologic contribution of NK cells in influenza infection was further demonstrated following influenza virus vaccination in humans. The rate of IFN-γ^+ NK cells was enhanced in response to inactivated whole influenza virus stimulation (183). Influenza vaccination has also been shown to prevent postoperative NK cell dysfunction, which in turn prevents postoperative metastases in mouse models, and enhances NK cell activity in cancer patients (184). Interestingly, during the first six months, healthy volunteers vaccinated with the annual influenza vaccine developed memory NK cells with augmented IFN-γ antigen-specific recall responses (185). These data together demonstrate the importance of NK cells in influenza infection.

Human and mouse NK cells can identify influenza virus HA on influenza-infected target cells by their activating NKp46 receptors (39, 44). This recognition is required to kill influenza-infected cells, since blocking this interaction inhibits NK cell cytotoxicity. Moreover, mice lacking NKp46 expression succumb to influenza infection, although NK cells recruitment into the infected lung was not affected (44). Additionally, human NK cells were found to identify and destroy influenza-infected DC by using both NKp46 and NKG2D activating receptors, which recognized influenza HA and UL16-binding proteins (ULBP1), respectively (158). NK cell cytotoxicity toward infected cells was prevented in the existence of neutralizing mAbs against NKp46 and NKG2D (158). Stimulation of NK cells cytotoxicity by ADCC against infected cells expressing influenza M2 has also been reported *in vivo*, in which NK cell depletion enhances virus replication (186). Likewise, anti-M2 mAb enhanced human NK cell-mediated ADCC upon recognizing M2-expressing cells (187).

Altogether, these documents clearly determine that NK cells have potent activity for controlling influenza infections in both humans and mice.

3.1. Evasion of NK cell responses by influenza virus

While NK cells can detect and destroy influenza-infected cells, the influenza has adopted various evading strategies, allowing it to evade NK cell cytotoxicity. Thus, influenza virus is capable to replicate efficiently in the lung and cause pulmonary damage. Additionally, two day following infection, NK cells start to be activated and recruited to the infected lung, which sometimes can take up to five days, thus giving the virus time to replicate and increase the viral load (44). This vigorous replication of virus overcomes the ability of NK cells to limit virus infection (44, 178, 180).

The NKp46 receptor plays an fundamental role in the defense against influenza virus via the recognition of the viral HA, in which NKp46-HA interaction enhances NK-mediated killing in a sialic acid-dependent manner (188). Recent reports have shown that influenza virus uses its NA to remove sialic acid residues that are present on NKp46 activating receptors and in turn impairs the direct interaction between HA and NKp46, which impairs NK-mediated killing (189). NA inhibitors are used to block virus budding, leading to increase NKp46-dependent killing (189). Moreover, mutations in influenza HA help the virus to escape NKp46 recognition. Target cells that are infected with the recent H3N2 influenza virus were able to impair NK-mediated lysis, whereas those infected with older virus isolates were more susceptible to NK-mediated lysis (190).

Another mechanism that is used by influenza to escape NK cell cytotoxicity is by directly infecting and killing NK cells (161). Several influenza protein products have been detected in human and mouse NK cells infected with influenza virus. Influenza virus was

shown to enter inside human NK cells *in vitro,* via clathrin- mediated endocytosis (161, 191). Influenza virus infection enhanced NK cell apoptosis, causing significant reduction in NK cell cytotoxicity. This observation was further confirmed in influenza-infected patients, in which the quantity of peripheral NK cells was drastically decreased in severely infected patients (192). Likewise, mice that were infected with influenza virus showed a reduction in NK cell number and activity, including reduced cytotoxicity against different target tumor cells (191).

Influenza virus has also been shown to rearrange MHC-I expression on infected cells in a way that enhances their binding to inhibitory human NK cell receptors, including LIR-1 and KIR2DL1, leading to NK cell inhibition (193). Studies of KIR gene involvement in influenza infection have demonstrated that the frequency of inhibitory KIR2DL5 gene in hospitalized cases with severe and mild 2009 pandemic H1N1 influenza infection is significantly higher than healthy controls (194). Moreover, the frequency of KIR2DL3 and KIR2DL2 besides their ligand were remarkably enhanced in influenza H1N1-infected patient in intensive care unit (ICU) (195). Thus, disruption of interactions between MHC-I and KIR in humans or Ly49 in mice during influenza infection could relieve NK cells inhibition to benefit the host.

4. Hypothesis

Enhanced MHC-I upregulation on influenza-infected cells would prevent licensed NK cells from killing them; however, unlicensed NK cells would show strong activation toward influenza-infected cells. Thus, unlicensed NK cells in our Ly49-deficient (NKC^{KD}) mouse model would display superior protection in response to influenza infection and prevent lethal influenza virus infection.

Materials and Methods

Mice

C57BL/6 (B6; H-2^b) and B6.129P2-B2m^{tm1}Unc/J (*B2m*$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.Ly49^{129} congenic, B6.NKC^{KD}, B6.Ly49Q^{KO}, and B6.NKC^{KD}-Ly49I^{tg} mice have been described in detail previously(84, 96, 196) Clr-b-deficient (*Ocil*$^{-/-}$) and NKR-P1B-deficient (*Nkrp1b*$^{-/-}$) mice were previously described(65, 197). IFN-I receptor 1 deficient mice (*Ifnar1*$^{-/-}$, C57BL/6 background) were kindly provided by Dr. Subash Sad (University of Ottawa, Canada). B6.NKC^{KD}-Ly49I^{tg} mice have been backcrossed for 10 generations to B6.NKC^{KD} mice. Perforin-deficient (*Prf*$^{-/-}$, C57BL/6 background) mice were kindly provided by Dr. Seung-Hwan Lee (University of Ottawa, Canada). Perforin-deficient B6.NKC^{KD} mice were produced by mating B6.Prf$^{-/-}$ with B6.NKC^{KD} mice. Genotyping was performed by polymerase chain reaction (PCR) using specific primers.

Ethics statement

All mice were housed in a specific-pathogen-free (SPF) environment. All animal breeding and manipulations were carried out in accordance with the principles published in the Canadian Council on Animal Care's "Guide to the Care and Use of Experimental Animals" and with the Animals for Research Act, R.S.O. 1990, c.22, s. 17(1-3), and were approved by the University of Ottawa animal care committee (protocol BMI-2049).

Influenza virus

The mouse-adapted Influenza A/FM/1/47-MA (FM-MA) strain was generated by adapting the human HlN1, A/FM/1/47 (FM) strain to the mouse by serial passage in mouse lungs to produce a virulent variant (198). The virus was kindly provided by Dr. Earl Brown (University of Ottawa, Canada). The stock of the virus was divided into aliquots (20 µl) and stored at -80°C; each aliquot was used once.

Influenza virus infection

Groups of age- and sex-matched mice at 6–8 weeks of age were used for all experiments. Mice were transferred into a containment level 2 (CL-2)-biohazard isolated room, anesthetized with isoflurane, and the FM-MA virus was administered intranasally in a total volume of 50 µl in a sterile phosphate-buffered saline (PBS) at 600 or 1050 PFU. Influenza-infected mice were maintained in a CL-2 biohazard area for the duration of the experiment. Survival and weight loss of infected mice were tracked over a period of 14 days. Mice were weighed daily and those with a weight loss of >25% of the initial bodyweight were euthanized and documented as dead, along with mice that were found dead. For regular experiments mice were euthanized 3-, 5-, or 7-days post-infection.

Cell lines

YAC-I was purchased from the American Type Culture Collection (ATCC). A549—an adenocarcinomic human alveolar basal epithelial cell line—was kindly provided by Dr. Earl Brown (University of Ottawa, Canada).

A549 infection

A549 cells were seeded into 6-well plates (0.5×10^6 per well) and kept overnight for attachment in 4ml of complete DMEM medium. The next day, medium was removed and cells washed twice with PBS, and influenza was added at a multiplicity of infection (MOI) of 1.0 in serum-free DMEM for 45 minutes. Then, virus was aspirated, new complete DMEM (4ml) was added to each well, and the plates were incubated for 18 hours at 37°C and 5% CO_2.

Plaque assay of influenza virus

Mice infected with FM-MA virus were sacrificed and lungs were weighed before homogenization in 1 ml of sterile 1x PBS using a bead beater (MM 200, Retsch, Germany). Lung homogenates were centrifuged at 5,000x g for 5 min. Clear supernatants were transferred to clean 1.5 ml microcentrifuge tubes (3 aliquots/sample) and kept at -80°C. Viral loads of infected mice were determined by plaque assay, as described previously (26). Briefly, a week before the assay, MDCK—Madin-Darby Canine Kidney Cells, an influenza

virus tissue culture,—were cultured for two days in a T25 flask using MEM+10% FBS (MEM: Minimum Essential Medium). After two days, the flask was washed twice with PBS and then 2 ml Trypsin+EDTA were added and the plate was incubated for 20 minutes at 37°C. Cells were pelleted, then seeded in a 6 well plate and incubated at 37°C for 2 days.

On the day of infection, 10-fold serial dilutions of the lung homogenates in 1x PBS were prepared on ice ranging from 10^{-1} to 10^{-6} of the initial stock. Confluent MDCK cell monolayers were rinsed twice with 1x PBS, inoculated with 100 µl/well of the viral dilutions in duplicates, and incubated at 37°C for 30 minutes to let the virus adsorb to the MDCK cell monolayer, agitating it occasionally. Then, the inoculum was removed and the influenza-infected MDCK cell monolayer was covered with 3 ml of overlaying medium containing: agarose 1.3%, 2x MEM (without serum), 7.5% $NaHCO_3$, and Trypsin TPCK (5mg/ml). The plates were incubated at 37°C for 3 days. After incubation, plates were fixed using 3 ml/well of methanol-acetic acid fixation (three parts methanol with one part acetic acid (v/v)) for 1 hour to 1 day at room temperature. The overlay gels were then removed and the plates were washed twice with PBS, and stained with 0.1% crystal violet solution for 1 hour. The plates were finally washed gently with water and plaques were counted.

In vitro NK cell assays

For intracellular interferon (IFN)-γ and CD107a measurement and staining, lung lymphocytes were incubated with or without YAC-1 cells at 1:1 ratio for 4 hours in the presence of brefeldin A, monensin, and anti-CD107a mAb (eBioscience). For intracellular IFN-γ staining, the plate was then centrifuged at 1,500 rpm for 5 minutes, cells were washed,

FC blocked, and stained for cell surface receptors, as described below. Cells were then re-suspended in 150 µl of BD Cytofix/CytoPerm solution in the plate and incubated for 30 minutes in the dark at room temperature. The plate was centrifuged at 1,500 rpm for 5 minutes and cells were washed twice with 200 µl BD Perm/wash buffer and stained with anti-IFN-γ staining antibody in 1x Perm/Wash buffer. This stain was incubated for 30 minutes in the dark at room temperature. Following two more washes in 1x Perm/Wash buffer, the cells were re-suspended in 200µl FACS buffer and kept at 4°C before acquisition on a flow cytometer.

Lung epithelial cell and lymphocyte isolation

Lungs were removed and minced in 5 ml RPMI (serum free media) with 0.5 mg/ml collagenase D (Roche), and then incubated for 1 hour at 37ºC, with agitation every 15 minutes. Lung pieces were then crushed and pressed through a 70 µm cell strainer, and washed with 1X PBS containing 5 mM EDTA. Red blood cells were lysed by resuspending cells in 5 ml of ACK lysis solution (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 5 minutes on ice. After washing, cells were counted and ready for surface staining, as described below.

For lymphocyte isolation, cell pellets were re-suspended in 4 mL of 67.5% Percoll and transferred into a 10 ml tube and then carefully overlaid with 2 mL of 45% Percoll, cells were then centrifuged at 800 g at 4°C for 20 minutes. The 45% Percoll layer (≈ 1.5 ml) was removed, leaving the lymphocyte layer intact in the middle. Lymphocytes were washed, and

then cell pellets were resuspended in 1 ml of 1X PBS and transferred into 15 ml tube. Cells were counted and ready for staining or stimulation.

Tetramer staining

Phycoerythrin (PE)-conjugated influenza A nucleocapsid protein ($NP_{311-325}$, QVYSLIRPNENPAHK) and non-structural protein ($NS2_{114-121,}$ RTFSFQLI) tetramers were kindly provided by the National Institutes of Health (NIH) Tetramer Core Facility (TCF) at Emory University (Emory University Vaccine Center, Atlanta, GA). PE-conjugated-H-$2K^b$ $NS2_{114-121}$ tetramer was used to stain influenza virus-specific $CD8^+$ T cells, while PE-conjugated-I-A^b $NP_{311-325}$ tetramer was used to stain influenza virus-specific $CD4^+$ T cells. To detect influenza virus-specific $CD8^+$ T and $CD4^+$ T cells, tetramer staining was performed using 1 μg of $NS2_{114-121}$ or $NP_{311-325}$ tetramers that were incubated with $0.5x10^6$ lung lymphocytes for one hour at 37°C, respectively. Various lymphocyte subsets were characterized by flow cytometry using fluorochrome-conjugated mAbs to CD3, CD8, CD4, and NKp46, as described below.

In vivo mAb treatments

Anti-IFN-γ mAb (clone XMG1.2), anti-NK1.1 mAb (clone PK136), and $F(ab')_2$ 5E6 (anti-Ly49C/I) were used for *in vivo* mouse treatments. Each mouse was injected intraperitoneally with 200 μg of mAb two days prior to influenza virus infection, on the day of infection, and every two days post-infection until day 10 p.i.

Antibodies and flow cytometry

The following antibodies were obtained from commercial sources as follows: anti-mouse CD326 (EpCAM), CD18 (LFA-1), 5E6 (anti-Ly49C/I), MHC-I (H-2K^{b}), 4D11 (anti-Ly49G), CD4, CD8, CD3, TCRβ, NKp46 (CD335), NKG2A (16a11), NKG2D (CD314), NKG2A/C/E (20d5), CD11b, CD27, CD107a (1D4B), and IFN-γ (XMG1.2). Live/Dead cell staining reagent was purchased from eBioscience (eBioscience, San Diego, CA, USA).

PK136 (anti-NK1.1) hybridoma was a kind gift from Dr. James Carlyle (Sunnybrook Research Institute, Toronto, ON). 5E6 (anti-Ly49C/I^{B6}) hybridoma was a kind gift from Dr. Charles Sentman (Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire). XMG1.2 (anti-IFN-γ) hybridoma was a kind gift from Dr. Subash Sad (University of Ottawa, Ottawa, ON).

For flow cytometry analysis, cells were washed in FACS buffer (1x PBS, 0.5% BSA and 0.02% NaN_3) in a 96 well plate. Supernatants were removed and cells were blocked with Fc block (anti-CD16/CD32), followed by vortexing and incubation at 4ºC for 15 minutes. Antibodies appropriately diluted in FACS buffer (total volume of 50 µl) were added to the cells and incubated at 4ºC for 20 minutes. Cells were then washed with FACS buffer. Supernatants were removed and if required, secondary staining with labeled streptavidin-biotin (LSaB) method was performed in FACS buffer as above. Cells were then resuspended in 200 µl of FACS buffer and kept at 4ºC before acquisition on a flow cytometer. Cell fluorescence data was acquired with a CyAN-ADP flow cytometer (Beckman Coulter) and analyzed with Kaluza software (Beckman Coulter, New Jersey, USA).

Purification and modification of mAbs

Hybridoma cells were grown in Dulbecco's Modification of Eagle's medium (DMEM) supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 100 μg/ml streptomycin, and 100 U/ml penicillin for three days in a humidified incubator at 37°C and 5% CO_2. After three days, hybridoma cells were removed from the plate by softly pipetting. Hybridoma cells were resuspended in 10 ml of DMEM and split into three or four fresh culture dishes and incubated for further two to three days at 37°C and 5% CO_2. The splitting procedure was repeated several times until a 500 ml volume was reached. Hybridoma cells were then incubated in large tissue culture flasks for a maximum of 10 days or until the media turned yellow. Then, cultured cells were collected in 100 ml centrifuge containers, and supernatants were transferred into fresh tubes. Supernatants were filtered through a 0.45μm filter. Protein G sepharose chromatography (ExalphaBiologicals, Inc, USA) was used to isolate monoclonal antibody (mAb). After elution, antibody was washed twice with cold 1X PBS (pH 7.4) using an ultracel-100 kDa membrane with an Amicon ultra-15 centrifugal filter unit (EMD Millipore Corporation, MA, USA). The solution was centrifuged at 2,500 g at 4°C, and concentrated to 500 μL. Concentration of monoclonal Ab was determined by SDS-PAGE gel and by spectrophotometric measurement at 280 nm, and diluted in 1X PBS to achieve 1mg/ml concentration.

$F(ab')_2$ fragment purification

5E6 mAb was washed twice with 100 mM sodium acetate solution (pH 4.0), using an ultracel-100 kDa membrane and Amicon ultra-15 centrifugal filter unit (EMD Millipore Corporation, MA, USA). The solution was centrifuged at 2,500 g at 4°C, and concentrated to 1mg/ml. Pepsin solution was made in the same buffer (100 mM sodium acetate) and was used at a 1:40 pepsin:antibody mass ratio for 10 h at 37°C in a shaking water bath set at medium speed. 1 M Tris (pH 8.0) was added to the fraction to stop the reaction and the digested mAb was transferred into an ultracel-50 kDa centrifugal filter unit (EMD Millipore Corporation, MA, USA). The Fc fragments were washed twice with 1x PBS buffer (pH 7.4) and then concentrated. Protein A affinity chromatography was used to purify 5E6 $F(ab')_2$ fragments; concentration was determined by spectrophotometric analysis at 280 nm and the fragments was analyzed by SDS-PAGE gel.

Lung histopathology

Mice were inoculated intranasally as described earlier. Lungs of the infected mice were collected 7 days post-infection. Lung tissues were fixed in 10% of neutral buffered formalin (NBF) at 4°C for 48 hours, with a buffer change after 24 hours. Lung tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) (Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Canada). Images were examined and scored by a pathologist (Dr. Harman Sekhon, University of Ottawa, Ottawa, Canada) blinded to the assay.

Statistical analysis

Statistical analysis was performed using a two-tailed Student's t-test or a one-way ANOVA with Bonferroni post-hoc test for comparisons between groups. Survival statistical analysis was performed using the Kaplan Meier log rank test. Statistical analyses were performed with GraphPad Prism software (GraphPad, San Diego, USA). A p-value <0.05 was considered statistically significant.

Results

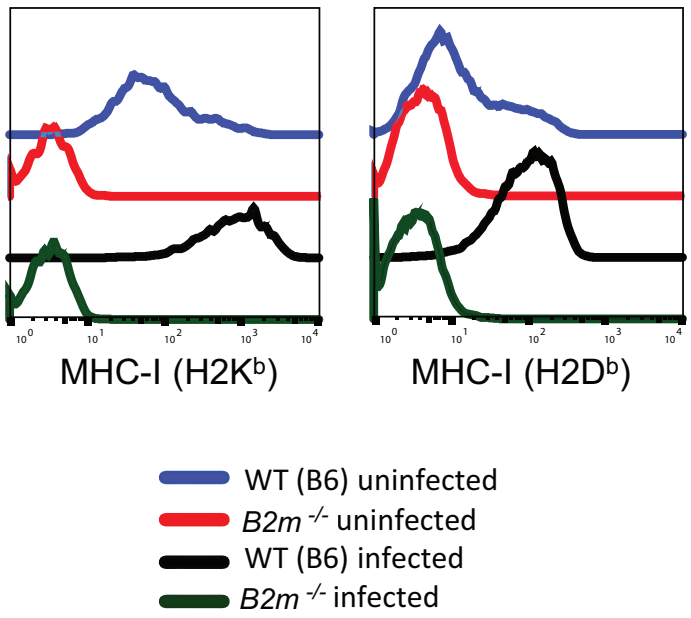
1- Influenza virus enhances MHC-I modulation on mouse pulmonary epithelial cells in an IFN-I independent manner

In vitro studies have indicated that influenza infection enhanced engagement of NK cell inhibitory KIR2DL1 receptor, to influenza-infected human cell line in MHC-I dependent manner and that KIR:MHC-I interactions on NK cells was coupled with remarkable decrease in NK cell responses (191, 193). Enhanced MHC-I expression has also been demonstrated on the human alveolar basal epithelial cells (A549) following human influenza infection (199). Specifically, it has been shown that influenza infection activates tumor suppressor p53 protein, which in turn activates the endoplasmic reticulum aminopeptidase 1 (ERAP1) which enhances MHC-I expression on infected cells *in vitro* (199). My goal was to investigate whether the MHC-I:Ly49 engagement contribute in the virulence of influenza infection *in vivo*; this interaction is the mouse analogue of the human KIR:MHC-I interaction. Firstly, I wanted to demonstrate if expression of MHC-I is modulated on mice pulmonary epithelial due to influenza infection. Groups of *B2m*$^{-/-}$ (MHC-I-deficient) and WT mice were either left untreated or inoculated with mouse-adapted (MA) influenza virus variant A/Fort Monmouth (FM)/1/47 H1N1, also known as the FM-MA (H1N1) virus. *B2m*$^{-/-}$ mice were used as a control. Five days post-infection lungs were harvested, and single cell suspensions were prepared by collagenase D digestion. The presence of lung leukocytes in the samples would give a false-positive result; to avoid this issue, a lung single cell suspension was stained with antibody specific to the mouse CD18 (LFA-1), which is expressed on all leukocytes. The cells were also stained with antibodies specific to the epithelial cell adhesion molecule (EpCAM) and either H-2K^{b} or 2D^{b}, the two alleles of MHC-Ia expressed in these mice. As expected, infected and uninfected *B2m*$^{-/-}$ mice show no sign of MHC-I modulation (Figure

4A). Lungs from uninfected WT mice were used to determine the percentage of baseline MHC-I expression on mouse pulmonary epithelial cells. Interestingly, the MHC-I expression was dramatically increased five days post-influenza virus infection. Next, I wanted to test whether the induction of MHC-I was IFN-I-dependent or not. It is identified that influenza virus infection enhances IFN-I production, which contribute in shaping the immune response, including upregulating MHC-I expression to increase antigen presentation capacity leading to T cell activation. To test whether the induction of MHC-I is due to IFN-I secretion, WT and IFNAR$^{-/-}$ (mice lacking IFN-I receptors) mice were either uninfected or infected with influenza and maintained for five days. Interestingly, interrupting IFN-I signaling during influenza infection has no effect on MHC-I modulation on pulmonary epithelial cells (Figure 4B). These results demonstrate that independently of IFN-I, influenza virus seems to enhance expression of MHC-I, most likely to escape NK cell recognition by enhancing Ly49:MHC-I interactions, leading to NK cell inhibition.

Figure 4

A


MHC-I (H2K^b)
MHC-I (H2D^b)
WT (B6) uninfected
B2m $^{-/-}$ uninfected
WT (B6) infected
B2m $^{-/-}$ infected

B

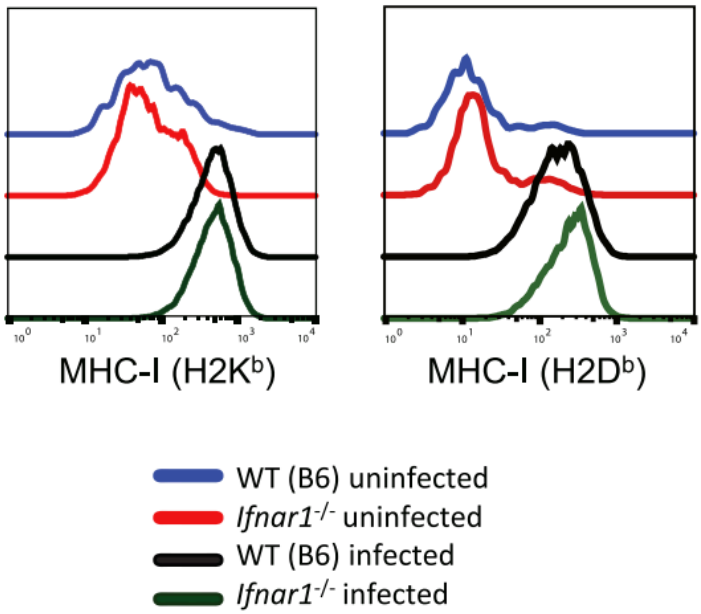

MHC-I (H2K^b)
MHC-I (H2D^b)
WT (B6) uninfected
Ifnar1$^{-/-}$ uninfected
WT (B6) infected
Ifnar1$^{-/-}$ infected

Figure 4. Influenza virus enhances upregulation of MHC-I on lung epithelial cells *in vivo*.

(A, B) 600 PFU of influenza was used to infect sex and age-matched $B2m^{-/-}$, $IFNAR1^{-/-}$, and WT (B6) mice or left untreated for 5 days. Mice were culled at designated time points, lungs were homogenized, and single-cell suspensions were prepared. Expression of $H\text{-}2D^b$ or $H\text{-}2K^b$ on pulmonary epithelial cells (EpCAM—epithelial cell marker) was tested by flow cytometry using specific antibodies against each of these markers. Antibody specific to LFA-1 was used to gate out lung leukocytes.

2- Unlicensed NK cells respond better to influenza infection

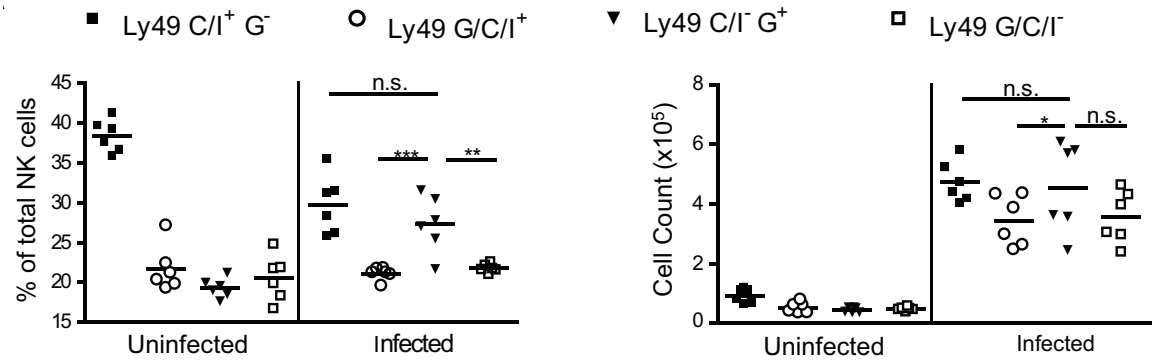
Enhanced MHC-I modulation on pulmonary epithelial cells inhibit the cytotoxic function of licensed NK cells which in turn support influenza illness. Because WT (B6) mice express the $H2K^b$ and $H2D^b$ haplotype of MHC-I, only NK cells that express Ly49C or/and Ly49I are considered as fully functional or "licensed" according to the licensing hypothesis (93, 95, 200). The rest are considered as unlicensed NK cells. To address the relative impact of enhanced MHC-I upregulation on different NK cell subsets, the proliferation capacity of lung NK cells was determined. WT (B6) mice were either uninfected or infected with FM-MA. Five days following the infection, lungs were harvested and lung single cell suspensions were prepared and stained with anti-Ki-67, a marker of cell proliferation. NK cells were characterized depending on the presence or absence of the licensing markers Ly49C/I, and the inhibitory receptor Ly49G. At the steady state, licensed $Ly49C/I^+G^-$ NK cells are dominant in the lungs of uninfected mice, representing around 40% of total lung NK cells (Figure 5A). Each of the other three NK cell subsets represent around 20% of total NK cells. However, following influenza infection the percentage of licensed $Ly49C/I^+G^-$ NK cells decreased and the percentage of unlicensed $Ly49C/I^-G^+$ increased.

Next, we examined whether these variances are associated with differences in proliferation capacity of the indicated NK cell subsets. Lungs of uninfected mice showed no significant differences in the proliferation rate between unlicensed NK cell subsets ($Ly49C/I^-$ G^- and $Ly49C/I^-$ G^+) and their licensed counterparts ($Ly49C/I^+$ G^- and $Ly49C/I^+$ G^+). However, fewer cells of the licensed NK cells, $Ly49C/I^+$ G^- (30%) and $Ly49C/I^+$ G^+ (40%), proliferated compared to the unlicensed NK cells, $Ly49C/I^-$ G^- (50%) and $Ly49C/I^-$ G^+ (55%), in the lungs of infected mice (Figure 5B). It seems that enhanced

MHC-I upregulation inhibits the licensed NK cell subsets' proliferation. On the other hand, the proliferation capacity of unlicensed NK cells was not affected. Of note, unlicensed NK cells make up almost half of the total NK cells, and although they may not play a major role in fighting tumors, these cells—under suitable circumstances–might contribute in immune defense during viral infection.

Figure 5

A


Ly49 C/I+ G-
Ly49 G/C/I+
Ly49 C/I- G+
Ly49 G/C/I-
% of total NK cells
Cell Count (x10^5)
n.s.
***
**
*
Uninfected
Infected

B

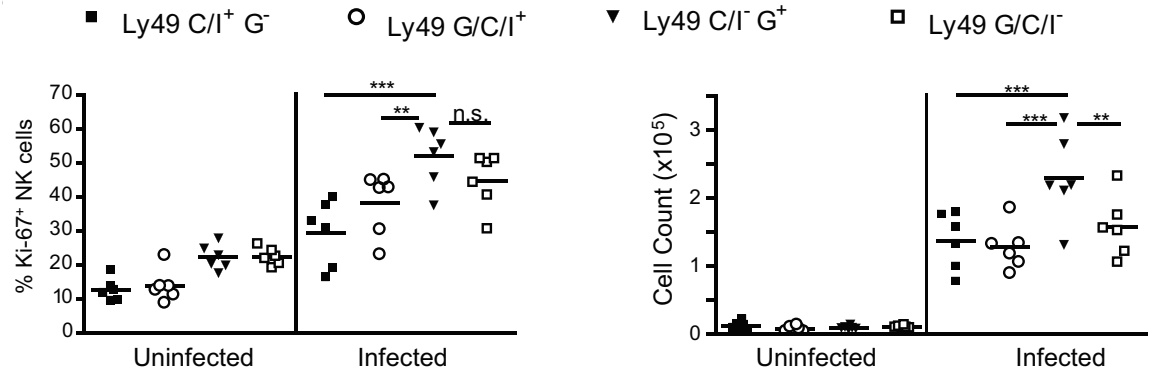

Ly49 C/I+ G-
Ly49 G/C/I+
Ly49 C/I- G+
Ly49 G/C/I-
% Ki-67+ NK cells
Cell Count (x10^5)
***
**
n.s.
Uninfected
Infected

Figure 5. Unlicensed NK cells respond better to influenza virus infection.

(A, B) WT mice were either uninfected or infected with 600 PFU influenza virus. Five days post-infection, lungs were collected and single cell suspensions prepared and stained with antibodies against TCRβ, NKp46, Ly49G, Ly49C/I, and Ki-67 mAbs. The horizontal line indicates the mean values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was conducted using a one-way ANOVA, Bonferroni correction was used as post-hoc test.

3- NK cells from NKC^{KD} mice have a mature phenotype

Our lab has generated Ly49-deficient (KC^{KD}) mice, in which around 80% of its NK cells are uneducated, so they cannot recognize MHC-I upregulation or downregulation on a target cell (96, 201). Thus, unlike WT mice, a tumor that does not express MHC-I grows faster in NKC^{KD} mice and cannot be rejected (201). Nevertheless, both WT and NKC^{KD} mice can identify and eliminate a tumor cell that expresses a ligand for NKG2D (96, 201). Ly49 deficiency in these mice did not make them better than WT mice at rejecting this kind of tumor. On the other hand, these Ly49-deficient mice are a good model to determine the contribution of MHC-I:Ly49 engagement and unlicensed NK cells in influenza illness. First, I wanted to make sure that lung NK cells from these mice are mature and express a normal level of activating NKp46 and NKG2D receptors, which are involved in recognizing and killing of influenza-infected cells (39, 158). To this end, lung-derived lymphocytes from uninfected WT and NKC^{KD} mice were stained and analyzed by flow cytometry. As shown in (Figure 6A), NK cells from NKC^{KD} mice significantly lost Ly49 expression: only 10.51% of gated lung NK expressed Ly49G, I_1, O, R, and V in comparison to 70.80% of lung NK cells from WT mice (Figure 6A). Because the WT (B6.Ly49^{129}) mice used in these experiments have the NKC locus from 129S1 mice (96), only NK cells that express Ly49O, Ly49I_1, or Ly49V are considered as licensed NK cells (analogous to Ly49C/I in C57BL/6 mice), emphasizing that most NK cells in the NKC^{KD} mouse model are unlicensed. NKp46 and NKG2D are expressed normally on pulmonary NK cells of both mice groups (Figure 6B and 6C), indicating that they acquire the properties necessary to recognize and eliminate influenza-infected cells. Mature NK cells are characterized as CD11b^{+} CD27^{-}. Flow data show that most pulmonary NK cells of NKC^{KD} and WT mice are fully mature (Figure 6D),

suggesting that Ly49 is not required for NK cell to become fully mature. Taken together, these data show that lung NK cells from NKC^{KD} mice are mature with normal NKp46 and NKG2D expression.

Figure 6

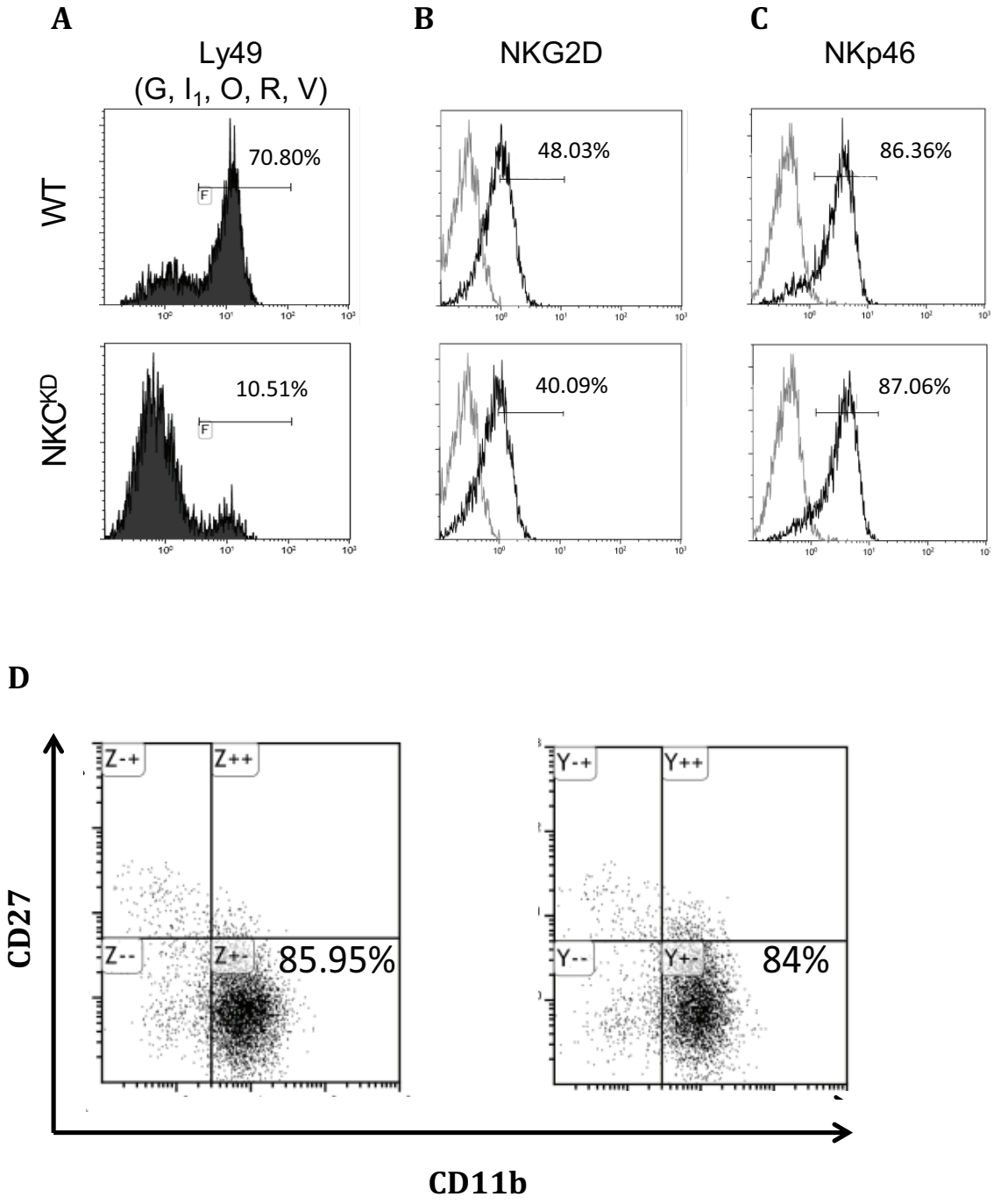

A
Ly49
(G, I1, O, R, V)
B
NKG2D
C
NKp46
WT
NKCKD
70.80%
48.03%
86.36%
10.51%
40.09%
87.06%
D
CD27
CD11b
Z-+
Z++
Z--
Z+-
85.95%
Y-+
Y++
Y--
Y+-
84%

Figure 6. NKC^{KD} mice show normal maturation and expression of activating receptors on the lung NK cells.

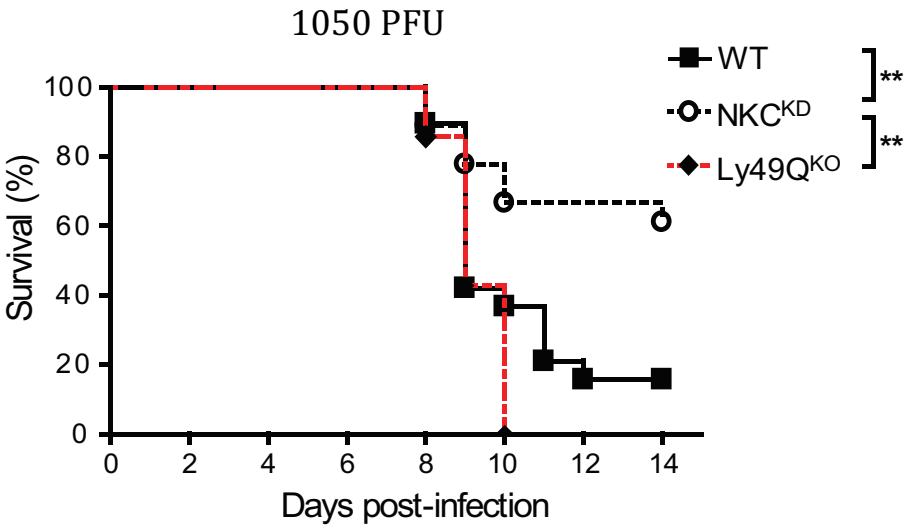
(A-D) lungs of uninfected WT and NKC^{KD} were harvested and single-cell suspensions were prepared and stained with a combination of antibodies against Ly49O/V/R (4E5), Ly49G (4D11) and Ly49I (14B11) (A), NKG2D (B), NKp46 (C), CD11b, and CD27 (D), to detect their expression on lung NK cells. Isotype antibody is shown as gray line.

4- NKC^{KD} mice are resistant to influenza infection

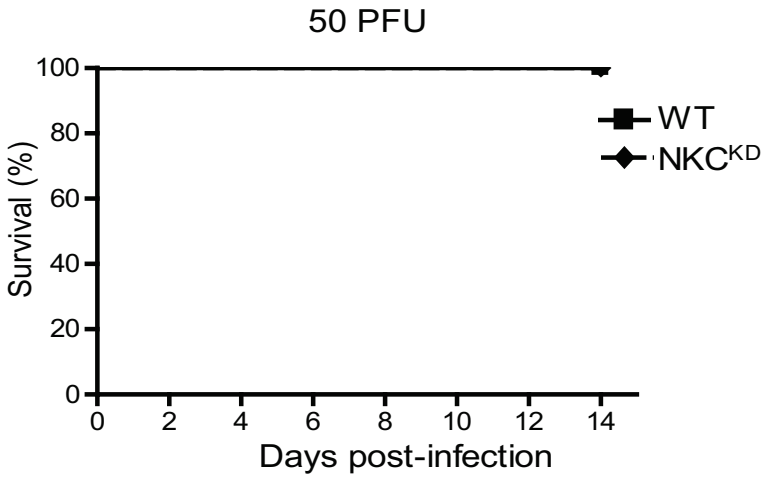
To test if MHC-I:Ly49 interaction can contribute in the severity of the infection, 1050 PFU of FM-MA virus was used to infect NKC^{KD} and WT mice, andthen the mice were checked daily for two weeks. Mice were euthanized when they lost 25% of their pre-inoculation body weight. Mice start to die eight days after influenza infection. Remarkably, nearly all WT mice (90%) succumbed to the infection whereas just 40% of NKC^{KD} died from the infection (Figure 7A). Induction of MHC-I expression on mouse pulmonary epithelial cells might negatively regulate licensed NK cell cytotoxicity; thus, unlicensed NK cells might be more beneficial for host survival. Previously, our lab has shown that the Ly49Q inhibitory receptor is expressed predominantly on pDCs, and that this receptor is important in IFN-I production by pDCs (84). NKC^{KD} mice also lack the expression of Ly49Q (96). To make sure the phenotype that I observed is not due to the inability of pDCs to produce IFN-I, I infected groups of WT, NKC^{KD}, and Ly49Q^{KO} mice and checked daily. All Ly49Q^{KO} mice died from the infection 10 days post-infection (Figure 7A); accordingly, the Ly49Q receptor does not seem to contribute in protection against influenza virus infection. Mice lacking pDCs in influenza infection showed that this cell subset plays a minor role in limiting influenza virus infection (202). Next, I wanted to determine how WT and NKC^{KD} would respond to higher and lower doses of influenza virus infection. At lower dose, 50 PFU of influenza, both groups survived the infection (Figure 7B). However, at very high dose, 5000 PFU, both groups succumbed to the infection (Figure 7C). These results demonstrate that, although NKC^{KD} mice cannot control tumors and spontaneous lymphoma growth (201), NKC^{KD} mice behave much better than WT mice during influenza infection.

Figure 7

A


1050 PFU
WT
NKC^KD
Ly49Q^KO
**
**
Survival (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Days post-infection

B


50 PFU
WT
NKC^KD
Survival (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Days post-infection

C

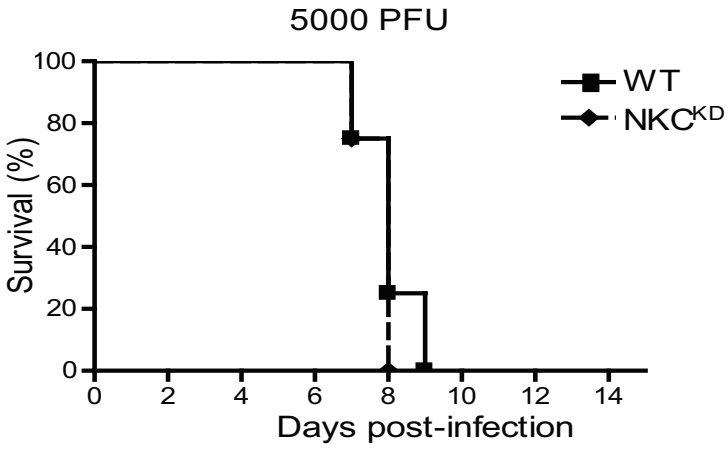

5000 PFU
WT
NKC^KD
Survival (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Days post-infection

Figure 7. NKC^{KD} mice survive better than WT mice following infection.

(A) 1050 PFU of influenza was used to infect sex and age-matched NKC^{KD}, WT, and $Ly49Q^{KO}$ mice, and then mice were checked for two weeks. Pooled from 2 experiments (n = 19 in each group). (B,C) 50 or 5000 PFU of influenza were used to infect sex and age-matched mice (n=5/group) then mice were checked for two weeks. The percentage of surviving mice is shown. $*p < 0.05$, and $**p < 0.01$. Statistical analysis was performed with the log rank test.

5- Depletion of unlicensed NK cells renders NKC^{KD} mice susceptible to influenza infection

The enhanced survival of NKC^{KD} mice following influenza virus infection raised an important question, whether this survival is actually due to the hypofunctional characteristics of unlicensed NK cells in these mice. According to the NK cell education hypothesis, interaction between Ly49 and its self-MHC-I during maturation is important for the NK cell to become fully functional (93, 200). As we reported previously, NK cells from these mice cannot eliminate tumors that have lost MHC-I expression (201), suggesting that NK cells might have no role in limiting influenza virus infection as well. Previous study has indicated that NK cells contribute to the severity of influenza virus infection by enhancing lung pathology and consequently increase influenza virus–induced morbidity and mortality (180); therefore, the hypofunctional NKC^{KD} NK cells would be beneficial for the host. To confirm whether the survival of NKC^{KD} mice is due to the functional status of their NK cells, I intended to deplete NK cells in these mice during the infection. There are two ways to deplete NK cell in a given mouse strain, either through using anti-NK1.1 mAb, which targets the NKR-P1C activating receptor, or anti-asilo-GM1 Ab (203, 204). The disadvantage of using anti-asilo-GM1 Ab is that it is expressed on activated $CD8^+$ and $CD4^+$ T cells. However, because not all mouse strains express the NKR-P1C receptor, anti-asilo-GM1 is still being used to deplete NK cells. Because NKC^{KD} mouse do not express NKR-P1C receptor, I used anti-asilo-GM1 Ab to deplete NK cells in these mice. In this experiment we used minimal doses of anti-asilo-GM1 Ab to avoid T cell depletion (Figure 8A). To determine specific depletion of NK cells, I injected a group of NKC^{KD} mice with anti-asilo-GM1 Ab two days before influenza infection, day 0, and then each 3 days till day 6. A

second group of infected mice were left untreated. Seven days post-infection, mice that were treated with anti-asilo-GM1 Ab completely lost NK cells in the lung ($NKp46^+$ $TCR\beta^-$ cells) (Figure 8A), while the percentage of T cells were not reduced ($TCR\beta^+$ $NKp46^-$ cells). With this result, I injected a group of NKC^{KD} mice with anti-asilo-GM1 Ab a day before influenza infection, on day 0, and then each 3 days until day 9 following the infection. Uninfected mice was injected with anti-asilo-GM1 Ab and used as a control, to make sure the mice do not die because of the injection. Remarkably, all NK cell-depleted NKC^{KD} mice died by day 10 (Figure 8B), while influenza-infected NKC^{KD} mice survived the infection. This result determined that unlicensed NK cells in NKC^{KD} mice are functional and their existence is important for the mice to control influenza virus infection.

Figure 8

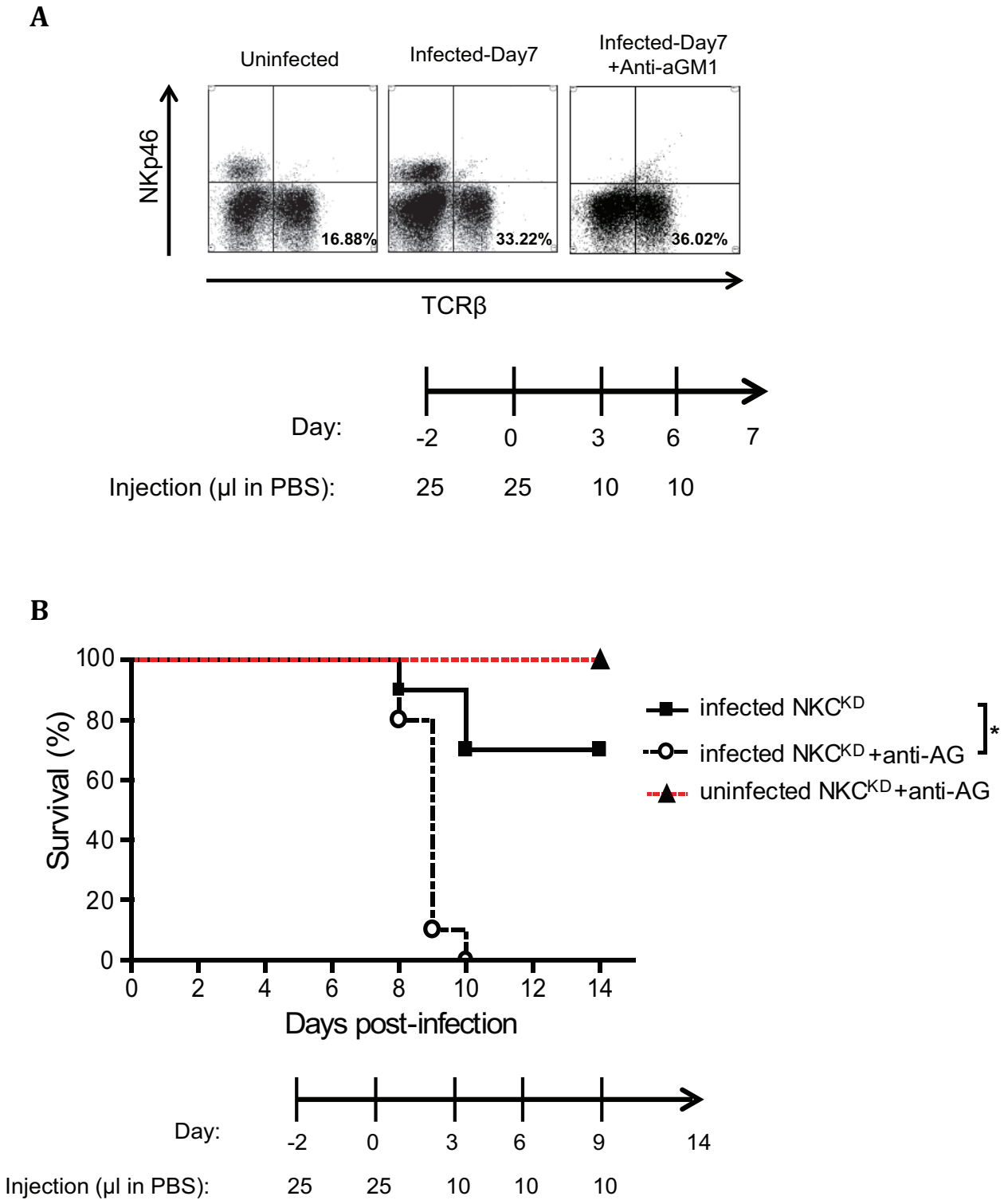

A
Uninfected
Infected-Day7
Infected-Day7
+Anti-aGM1
NKp46
16.88%
33.22%
36.02%
TCRβ
Day:
-2
0
3
6
7
Injection (μl in PBS):
25
25
10
10
B
Survival (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Days post-infection
infected NKC^KD
infected NKC^KD +anti-AG
uninfected NKC^KD +anti-AG
*
Day:
-2
0
3
6
9
14
Injection (μl in PBS):
25
25
10
10
10

Figure 8. NK cells are required to protect NKC^{KD} mice from influenza infection.

(A) Influenza-infected mice were treated with anti-AsialoGM1 Ab at the indicated times to deplete NK during influenza infection. The proportion of $NKp46^{+}$ and $TCR\beta^{+}$ cells indicated on each plot. (B) 2 groups of NKC^{KD} were inoculated with 600PFU of influenza and checked for 2 weeks. One group of infected mice were treated with anti-Asialo-GM1 Ab at the indicated times, and the other was left untreated. A group of uninfected mice was also treated with anti-asialo-GM1 Ab, to determine the effects of multiple anti-asialo-GM1 Ab injections on animal survival. The percentage of surviving mice is shown. $*p < 0.05$. Statistical analysis was performed with the log rank test.

6- Greater numbers of unlicensed NK cells associated with enhanced protection of NKC^{KD} mice

Next, I wanted to further assess the activity of NK cells that do not express Ly49 ($Ly49^-$) in the NKC^{KD} and WT mice after infection with FM-MA. Both groups were infected and maintained for five days. At the indicated time point, lungs from infected mice were harvested and single cell suspensions were prepared. Cells were co-cultured with or without the NK-sensitive YAC-I cell line, which expresses a ligand for NKG2D, for 4 hours. Following incubation, cells were stained with antibodies specific to IFN-γ and CD107 markers that indicate NK cell functional activity. In the presence of YAC-1 stimulation, similar proportions of $Ly49^-$ NK cells produce IFN-γ in WT and NKC^{KD} mice (Figure 9A). However, in comparison to the WT mice, there was a notably greater quantity of $Ly49^-$ IFN-γ^+ NK cells in NKC^{KD} mice because of the lacking of Ly49 receptors in these mice (Figure 9A). The lung of NKC^{KD} mice showed significantly higher numbers of $Ly49^-$ $CD107a^+$ NK cells than WT mice (Figure 9B). The increase in the absolute number of activated unlicensed NK cells in the lung of NKC^{KD} mice might contribute positively in controlling influenza virus infection. These cells do not receive an inhibitory signal from expressed MHC-I, which gives them an advantage over licensed NK cells. Importantly, unlike WT mice, the percentage of $Ly49G^+$ NK cells did not change before and after influenza infection in the lungs of NKC^{KD} mice (Figure 9C), indicating that Ly49G does not play an important role in protecting NKC^{KD} mice from influenza infection.

Figure 9

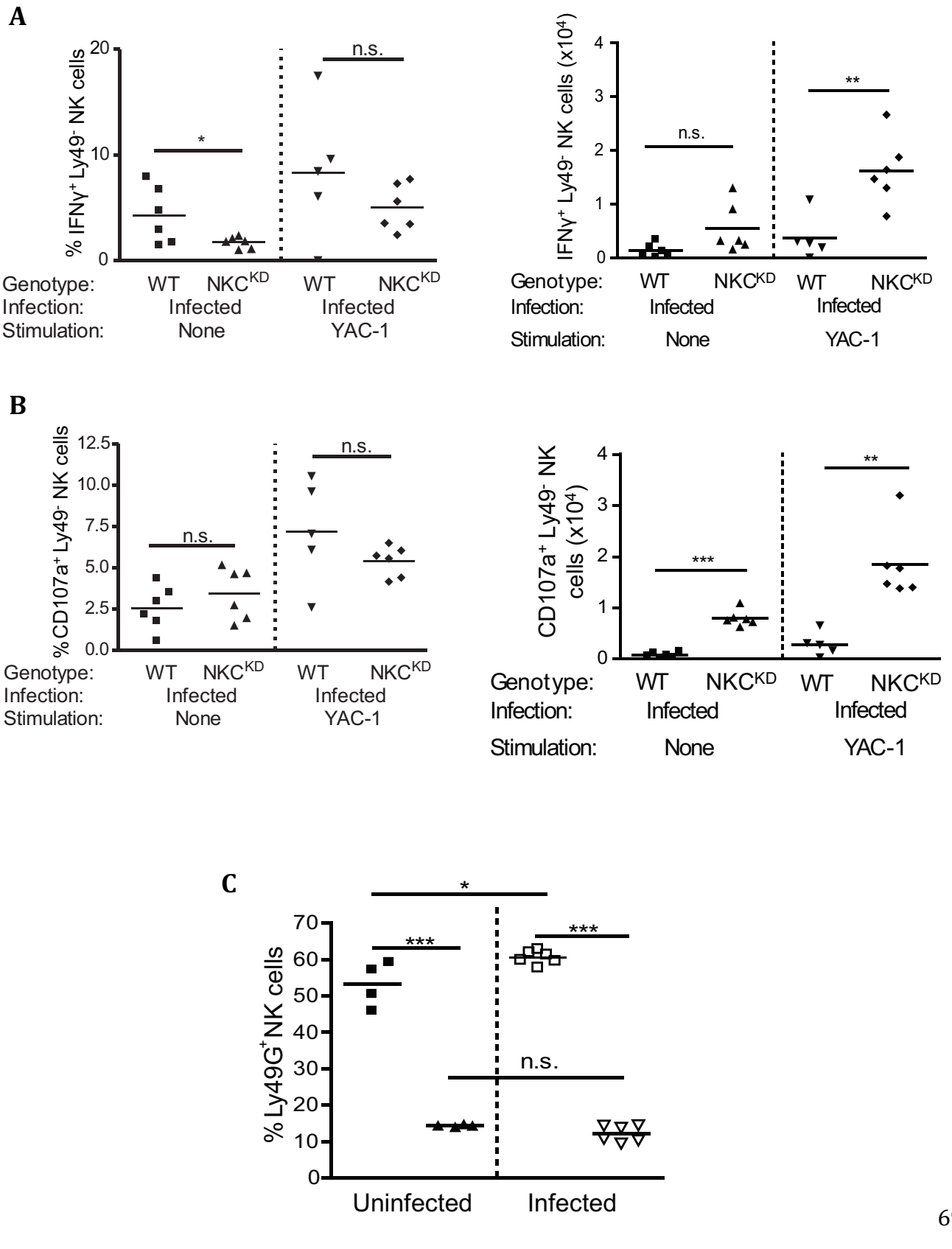

A
% IFNγ+ Ly49- NK cells
20
10
0
*
n.s.
Genotype: WT NKCKD WT NKCKD
Infection: Infected Infected
Stimulation: None YAC-1
IFNγ+ Ly49- NK cells (x10⁴)
4
3
2
1
0
n.s.
**
Genotype: WT NKCKD WT NKCKD
Infection: Infected Infected
Stimulation: None YAC-1
B
%CD107a+ Ly49- NK cells
12.5
10.0
7.5
5.0
2.5
0.0
n.s.
n.s.
Genotype: WT NKCKD WT NKCKD
Infection: Infected Infected
Stimulation: None YAC-1
CD107a+ Ly49- NK cells (x10⁴)
4
3
2
1
0
***
**
Genotype: WT NKCKD WT NKCKD
Infection: Infected Infected
Stimulation: None YAC-1
C
% Ly49G+ NK cells
70
60
50
40
30
20
10
0
*
***
***
n.s.
Uninfected
Infected

Figure 9. Higher numbers of activated Ly49-deficient NK cells in NKC^{KD} mice.

(A,B) 600 PFU of influenza was used to infect sex and age-matched NKC^{KD} and WT mice. Mice were culled at day 5 post-infection, lungs were harvested, and single-cell suspensions were prepared. Lymphocytes were either unstimulated or co-cultured with YAC-I cells at 1:1 cell ratio in presence of brefeldin A and incubated for 4 h. Lymphocytes were then stained with antibodies against NKp46, TCRβ, Ly49O/V/R (4E5), Ly49G (4D11), Ly49I (14B11), IFN-γ, and CD107a. (C) Sex and age-matched NKC^{KD} and WT mice were infected with 600 PFU influenza virus. Mice were culled at day 5 post-infection, lungs were harvested, and single-cell suspensions were prepared stained with antibodies against NKp46, TCRβ, Ly49G (4D11), and KI-67. Each dot represents one mouse. The horizontal line indicates the mean values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was conducted using a one-way ANOVA, the Bonferroni correction was used as post-hoc test.

7- Ly49 deficiency results in less severe lung pathology than WT mice following influenza infection

Influenza virus infection, if it does not get controlled, causes severe pulmonary damage and eventually death (205-208). To determine whether Ly49:MHC-I interaction resulted in severe lung pathology due to an inability of NK cells to limit viral infection, I decided to compare the lung histopathology of NKC^{KD} and WT mice following influenza infection. I inoculated a group of NKC^{KD} and WT mice with influenza virus, 600 PFU. Seven days following influenza infection, lungs were collected and sections were prepared for hematoxylin and eosin (HE) staining. Compared with NKC^{KD} mice, WT mice showed severe lung damaged associated with bronchi filled with cellular debris, lymphocytic infiltration, diffuse alveolar damage, and pulmonary edema (Figure 10). Examination of the pathological changes in the lung tissues indicated that NKC^{KD} mice were better at controlling influenza virus infection, which in turn resulted in less lung damage. It is likely that the inflammatory response in the WT mice is prolonged as these mice also have more pulmonary edema that may be related to decreased re-absorption during the organization phase, or continuous leakage of fluids from the damaged alveolar walls. Taken together, histological examination of the pulmonary tissue indicated that NKC^{KD} mice developed less damage in the lung which could be due to better control of influenza infection and less cellular infiltration to the lungs.

Figure 10

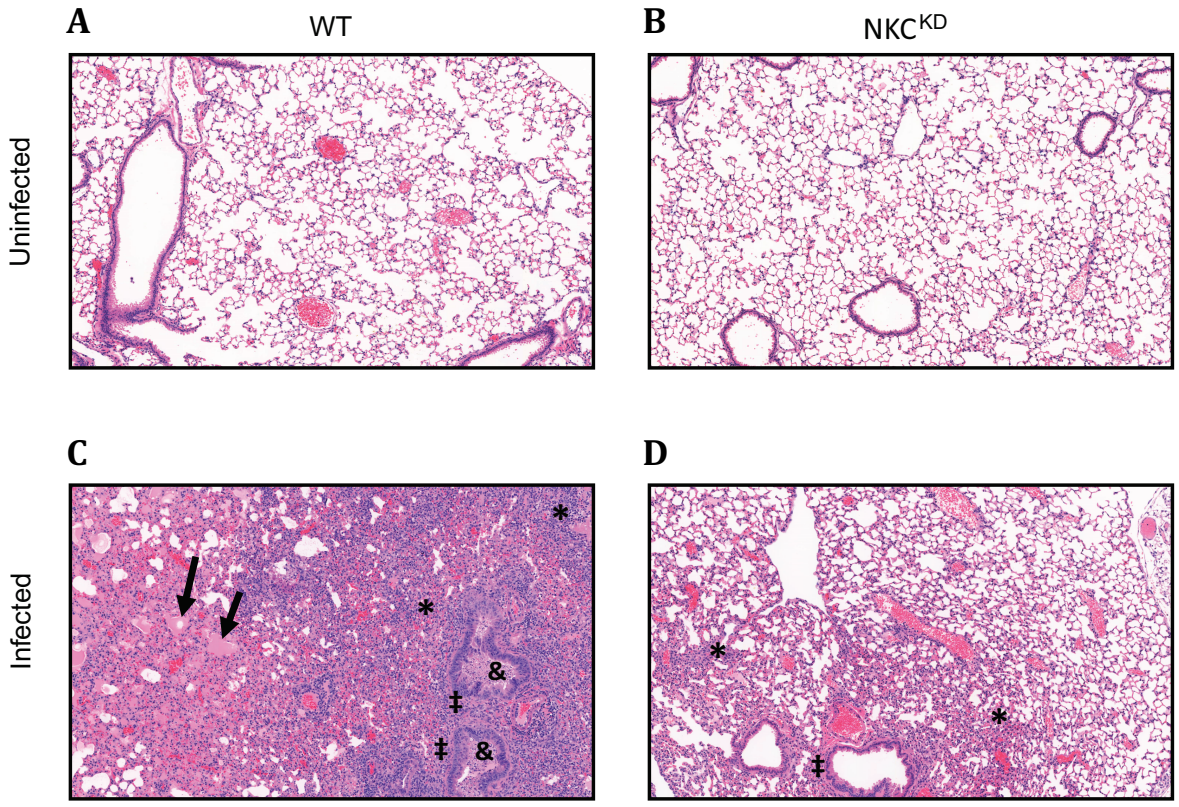


Figure 10. Ly49-deficient mice have less pulmonary damage than WT mice.

Uninfected or 600 PFU of influenza was used to infect WT (A and C) and NKC^{KD} (B and D) mice. Seven days following the infection, lungs were collected, fixed in 10% formalin (NBF), and stained using standard hematoxylin and eosin protocol. 100x magnification images were captured. Lungs of WT mice showed bronchi filled with cellular debris '&', lymphocytic, damage and neutrophilic infiltrate '‡', diffuse alveolar '*', and pulmonary edema '!'.

,

8- Ly49-deficient mice control influenza infection better than the WT mice

Next, I wanted to determine the mechanism behind the survival of NKC^{KD} mice and the mortality of the WT mice from the influenza virus infection. Thus, I investigated whether the pathology in the lung of WT mice correlates with an increase in the influenza virus replication in the lungs. Enhanced MHC-I modulation on mouse pulmonary epithelial cells after infection could inhibit licensed NK cells' cytotoxic function. Thus, Ly49-deficient mice are better at controlling influenza virus replication, which in turn protects NKC^{KD} mice from the infection. If the Ly49:MHC-I engagement inhibits NK cell killing, NKC^{KD} mice should have less viral load than WT mice. To test this hypothesis, groups of mice were infected with influenza and checked daily. Lungs of infected mice were harvested at different time points: day 3, 5, and 8 post-infection. Lungs were homogenized and viral titers were determined by plaque assay. Similar levels of virus load were detected in the lungs from both group of mice on day 3 post-infection (Figure 11A), endorsing that the onset of the infection was that same. Five days following infection, the level of influenza was significantly lesser in the lungs of NKC^{KD} mice in comparison to the WT mice (Figure 11C). At day 8 (Figure 11B), both groups showed a similar viral titer, most probably because, by this time after infection, activated T cell had been recruited to the infected lungs. These results indicate that controlling influenza infection at early time points is important to avoid lung pathology which is most likely due to lymphocyte recruitment and cytokine production. Also, these results demonstrate that unlicensed NK cells in NKC^{KD} mice contribute in limiting influenza virus replication.

Figure 11

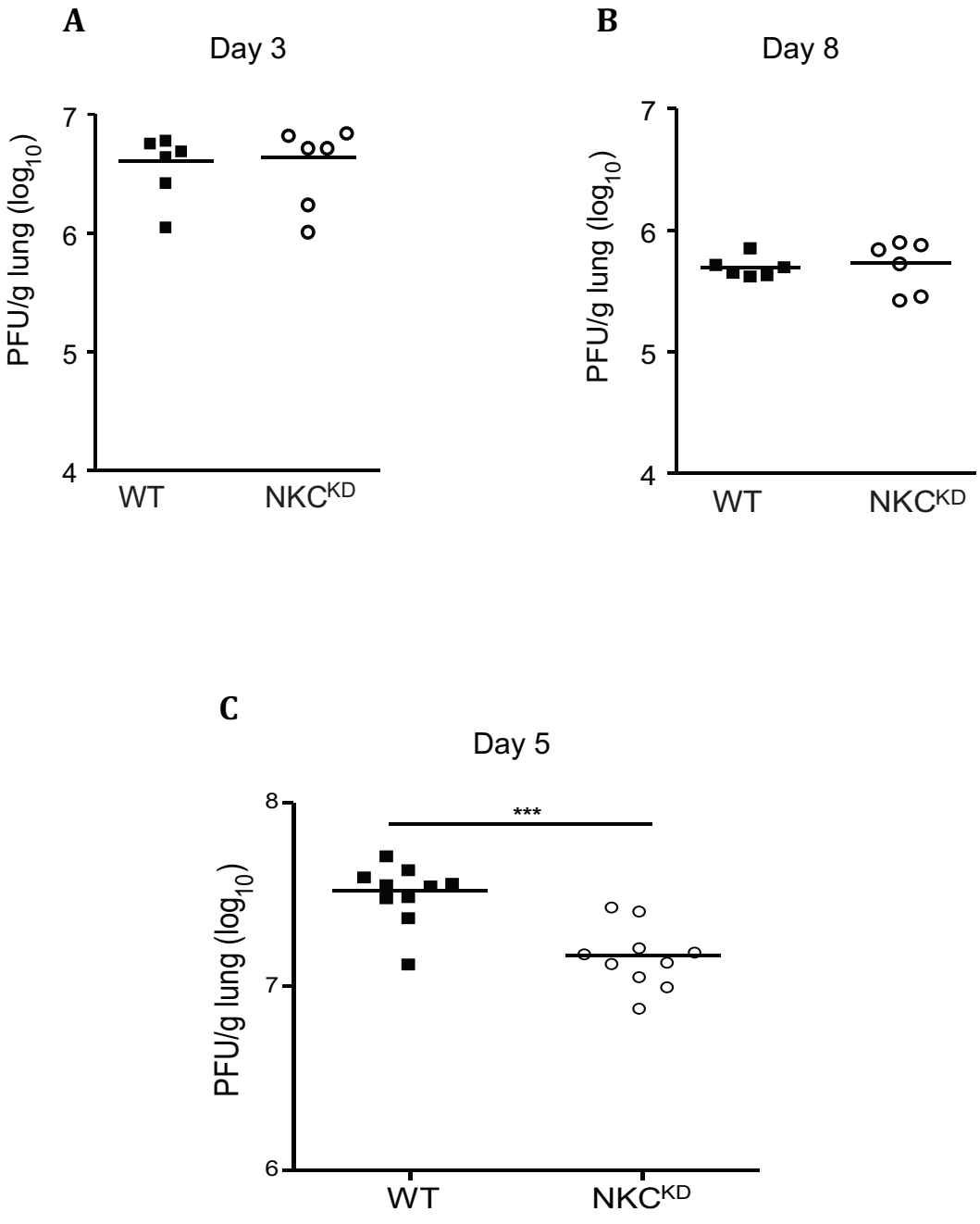

A
Day 3
PFU/g lung (log10)
7
6
5
4
WT
NKCKD
B
Day 8
PFU/g lung (log10)
7
6
5
4
WT
NKCKD
C
Day 5
***
PFU/g lung (log10)
8
7
6
WT
NKCKD

Figure 11. Ly49-deficient mice have less viral load in the lung.

Mice were infected intranasally with 600 PFU of influenza. On days 3 (A), 5 (C) and 8 (B) following the infection, lungs were collected, weighed, and homogenized to determine lung viral titers by plaque assay on MDCK cells (PFU/g of lung tissue). Pooled data from three independent experiments are shown (A, C) n=6/group, (B) n=10/group. Each dot represents one mouse. The horizontal line indicates the mean values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was performed using Student's t-test.

9- Early recruitment of NK cells but not CD8 or CD4 T cells to the lung is associated with better survival

Unlike the innate immune response, adaptive immune responses require at least a week to be recruited to the site of an infection. Moreover, lung histopathology of WT and NKC^{KD} mice showed increased numbers of lymphocytes accumulating in the lungs following the infection (Figure 10). Therefore, I wanted to investigate whether $CD4^+$ and $CD8^+$ T cells were involved in limiting influenza virus infection in the NKC^{KD} mice. Five days following influenza infection, I found that viral load is significantly decreased in the lungs of NKC^{KD} mice (Figure 11C). To determine whether $CD4^+$ and $CD8^+$ T cells involve in early responses to influenza virus infection, groups of mice were either uninfected or infected with 600 PFU of influenza for 5 and 7 days. Mouse infections were performed 2 days apart so that the lungs from day 5- and day 7-infected mice were harvested at the same time. Recruitment of lymphocytes to the lung were determined using flow cytometry. Influenza-specific $CD8^+$ and $CD4^+$ T cells were detected using $NP_{(311-325)}$ and $NS2_{(114-121)}$ tetramers, respectively. At resting state, the number and percentage of NK cells are dominant over the influenza-specific $CD4^+$ and $CD8^+$ T cells (Figure 12). Five days post-infection, the number and percentage of NK cells notably increased in the lungs of both groups (Figure 12E and F), while the numbers for influenza-specific $CD4^+$ and $CD8^+$ T cells are still low (Figure 12 A-D). This suggests that NK cells played a major role in the early defense against influenza virus infection. Moreover, the number and percentage of total NK cells between mice groups were almost the same, suggesting that the protection in NKC^{KD} mice was due to Ly49-deficiency (Figure 12E and F). At day 7 post-infection, a more significant increase in the quantity of lung NK cells and influenza-specific $CD8^+$ T cells were found in WT mice

more than the NKC^{KD} mice (Figure 12B). Failure to control the influenza virus infection at early time points by NK cells of WT mice may have resulted in the expansion or recruitment of more NK cells and influenza-specific $CD8^+$ T cells to the lungs of WT mice in order to control the infection.

Figure 12

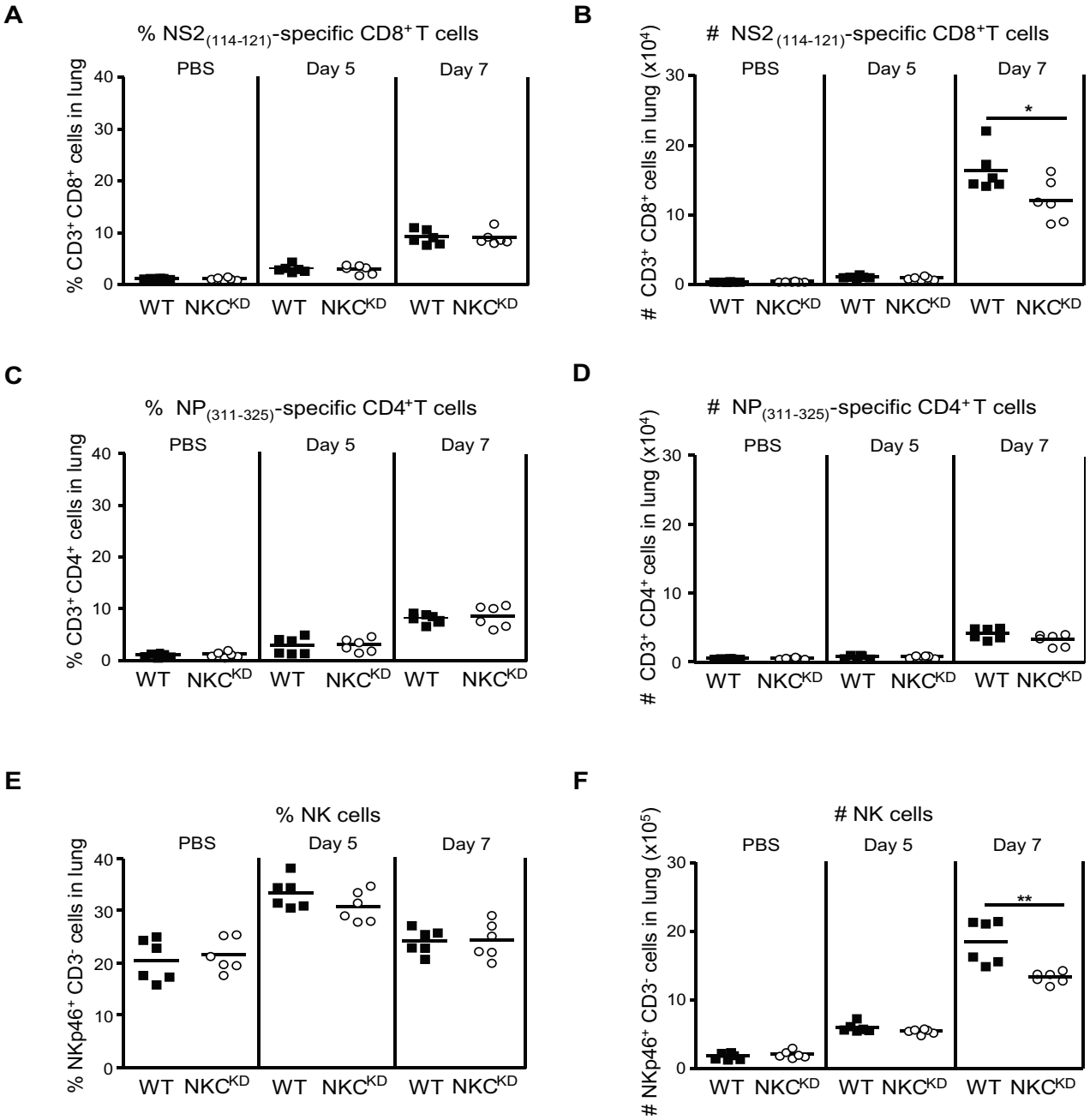

A
% NS2(114-121)-specific CD8+ T cells
PBS
Day 5
Day 7
% CD3+ CD8+ cells in lung
WT NKCKD WT NKCKD WT NKCKD
B
NS2(114-121)-specific CD8+T cells
CD3+ CD8+ cells in lung (x10^4)
*
C
% NP(311-325)-specific CD4+T cells
% CD3+ CD4+ cells in lung
D
NP(311-325)-specific CD4+ T cells
CD3+ CD4+ cells in lung (x10^4)
E
% NK cells
% NKp46+ CD3- cells in lung
F
NK cells
NKp46+ CD3- cells in lung (x10^5)
**

Figure 12. NK cells dominate in the lung at early time points post-infection.

(A-F) Lungs from 3 groups of WT and NKC^{KD} mice—one uninfected group and two groups of mice infected with 600 PFU of influenza virus for 5 and 7 days—were harvested at the same time. Single-cell suspensions were prepared and stained using anti-NKp46, anti-CD3, anti-CD4, anti-CD8 mAbs, and I-Ab–NP311-325 and K^b-NS2114−121 tetramers. Absolute counts and frequencies of cell populations are indicated. Statistical analysis was performed using Student's t-test. The horizontal line indicates the mean values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

10- Significant increase in the chemokine and cytokine levels in the lung of WT mice

Severe influenza illness is characterized by enhanced cytokine levels, which contributes to influenza virus-mediated immunopathology in the lung (209). Severe lung damage and increased influenza virus titer were observed in the lungs of WT mice in comparison to NKC^{KD} mice (Figure 10 and 11C). Next, I wanted to determine the level of several chemokines and cytokine in the lungs of both mice groups following infection with influenza. Lungs tissues were harvested five days following the infection—when there are differences in viral load and there is still no T cell involvement—and lung homogenates were prepared for cytokine and chemokine measurement. Most of the cytokine and chemokine levels were the same in both groups of mice (Figure 13A-J), again suggesting that NKC^{KD} mice respond normally to the infection. Interestingly, the level of cytokines including IFN-γ, TNF-α, IL-17, and chemokines including MIP-1β, MCP-3, and MCP-1 were notably increased in the lungs of WT mice (Figure 14A-F). These results suggest that, unlike WT mice, the ability of NKC^{KD} mice to limit virus infection early resulted in less lung pathology due to reduced inflammation and cytokine/chemokine production in the lungs.

Next, I wanted to determine whether NK cells participate directly in producing these cytokines. Using flow cytometry I measured the intracellular level of IFNγ and TNFα (Figure 14G and 14H). These two were chosen as they were the only relevant cytokines that have antibodies validated for flow cytometry. In our assays, only a small proportion of lung NK cells produced TNF-α and IFN-γ following influenza infection. Other cells might be involved in the secretion of these chemokines and cytokines upon infection in the lungs. However, regardless of the cytokine source, I believe that the immunopathology that I

detected in the lung is because NK cells of WT mice cannot efficiently control the influenza virus infection at early time points leading to increased inflammation and lung pathology compared to the NKC^{KD} mice.

Figure 13

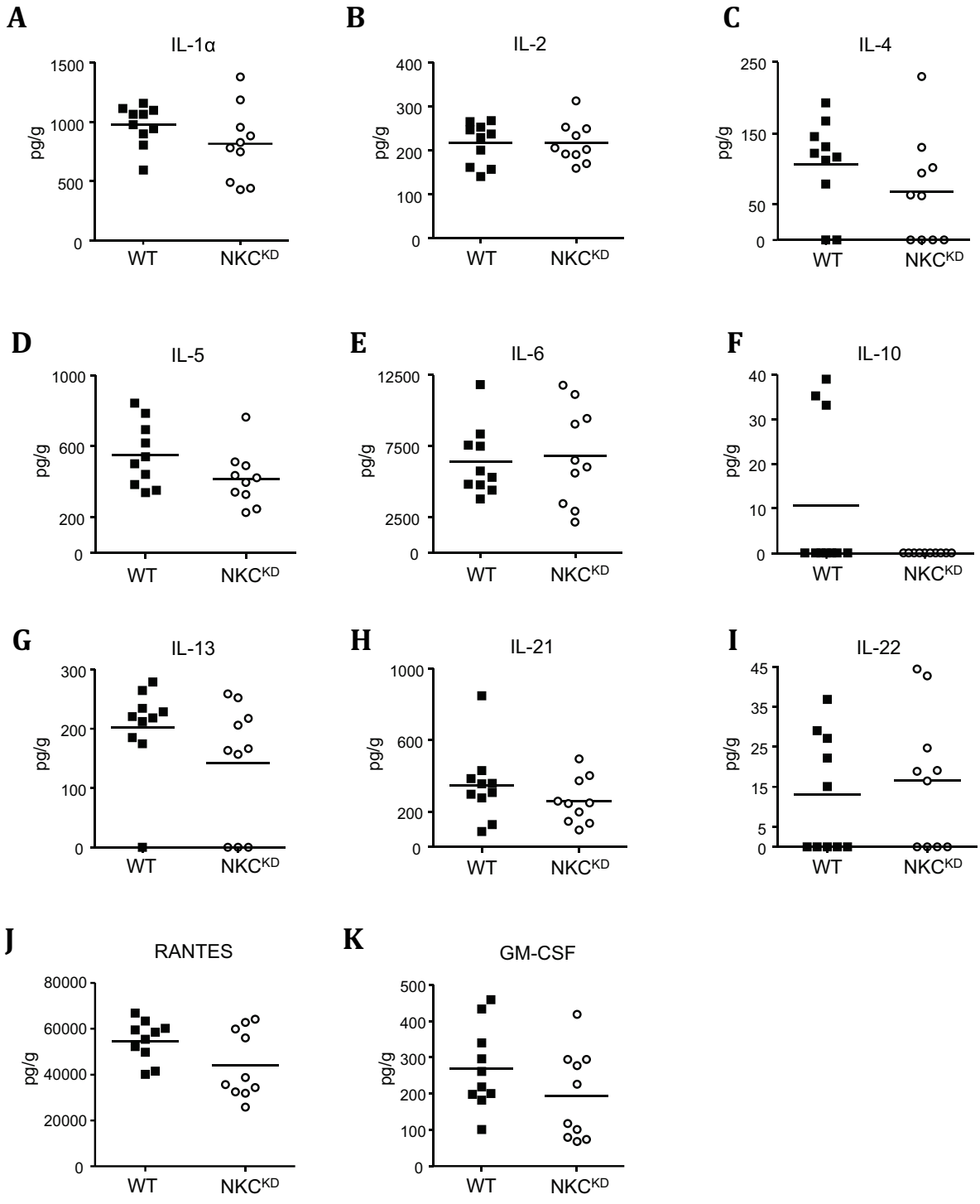

A
IL-1α
pg/g
1500
1000
500
0
WT
NKC^KD
B
IL-2
pg/g
400
300
200
100
0
WT
NKC^KD
C
IL-4
pg/g
250
150
50
0
WT
NKC^KD
D
IL-5
pg/g
1000
600
200
0
WT
NKC^KD
E
IL-6
pg/g
12500
7500
2500
0
WT
NKC^KD
F
IL-10
pg/g
40
30
20
10
0
WT
NKC^KD
G
IL-13
pg/g
300
200
100
0
WT
NKC^KD
H
IL-21
pg/g
1000
600
200
0
WT
NKC^KD
I
IL-22
pg/g
45
35
25
15
5
0
WT
NKC^KD
J
RANTES
pg/g
80000
60000
40000
20000
0
WT
NKC^KD
K
GM-CSF
pg/g
500
400
300
200
100
0
WT
NKC^KD

Figure 13. Similar levels of some chemokines/cytokine in the lungs of influenza-infected NKC^{KD} and WT mice.

(A-K) 600 PFU of influenza was used for infection. Five days following the infection lungs were collected, weighed, and homogenized to determine cytokine and chemokine levels. Pooled data from 3 experiments are shown (n=10/group). Each dot represents one mouse. The horizontal line indicates the mean values. Statistical analysis was performed by Student's t-test.

Figure 14

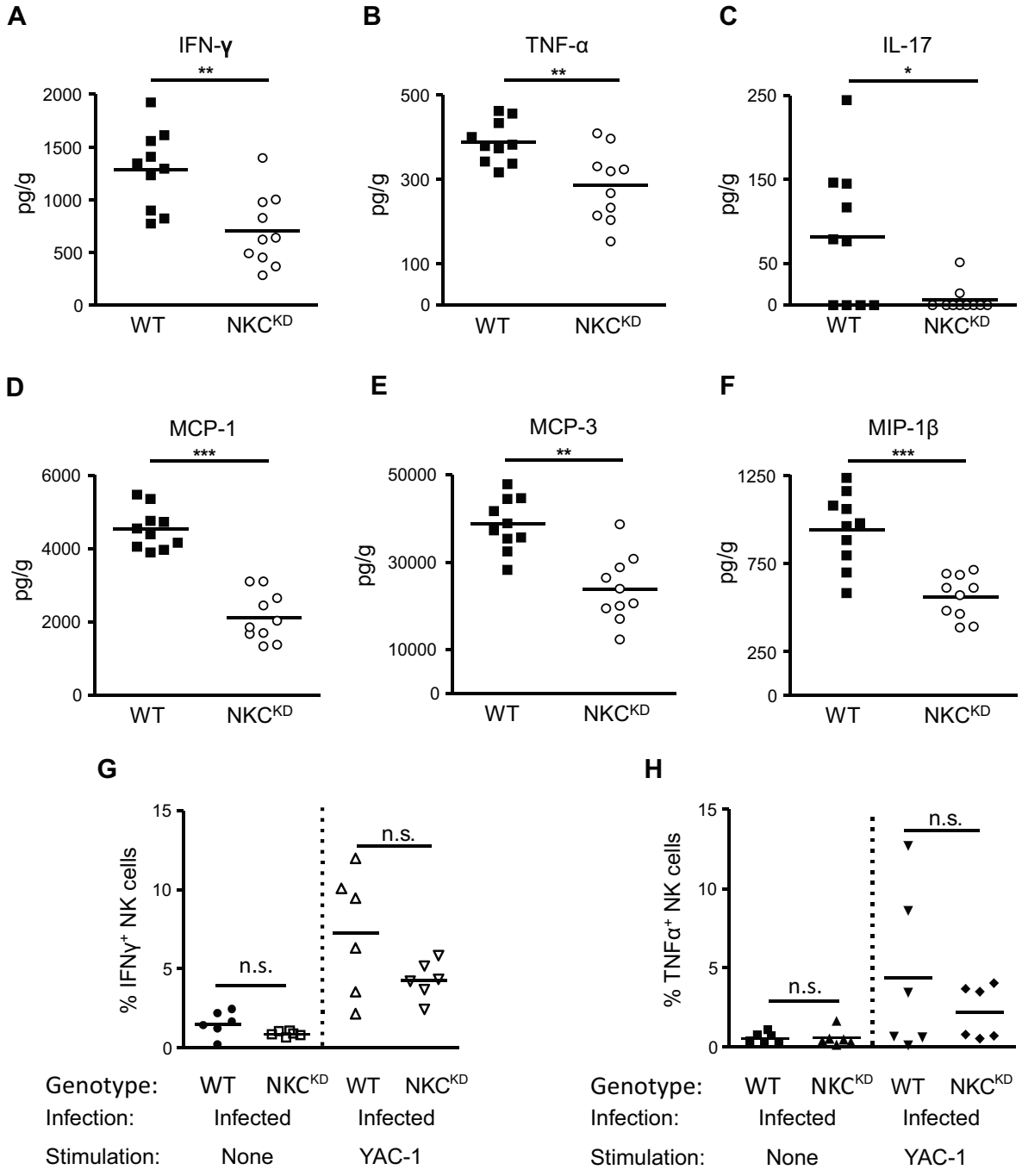

A
IFN-γ
**
pg/g
2000
1500
1000
500
0
WT
NKC^KD
B
TNF-α
**
pg/g
500
300
100
0
WT
NKC^KD
C
IL-17
*
pg/g
250
150
50
0
WT
NKC^KD
D
MCP-1
***
pg/g
6000
4000
2000
0
WT
NKC^KD
E
MCP-3
**
pg/g
50000
30000
10000
0
WT
NKC^KD
F
MIP-1β
***
pg/g
1250
750
250
0
WT
NKC^KD
G
% IFNγ+ NK cells
15
10
5
0
n.s.
n.s.
Genotype: WT NKC^KD WT NKC^KD
Infection: Infected Infected
Stimulation: None YAC-1
H
% TNFα+ NK cells
15
10
5
0
n.s.
n.s.
Genotype: WT NKC^KD WT NKC^KD
Infection: Infected Infected
Stimulation: None YAC-1

Figure 14. Differences in the levels of some cytokines and chemokines in the lungs of WT and NKC^{KD} mice following influenza infection.

600 PFU of influenza was used for infection. (A-F) Five days following the infection lungs were collected, weighed, and homogenized to determine cytokine and chemokine levels. Pooled data from 3 independent are shown (n=10/group). Each dot represents one mouse. The horizontal line indicates the mean values. (G,H) 600 PFU of influenza was used to infect sex and age-matched mice. Lungs were harvested 5 days after the infection, single-cell suspensions were stained with antibodies against NKp46, TCRβ, IFN-γ, and TNF-α. Each dot represents one mouse. The horizontal line indicates the mean values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was conducted using a one-way ANOVA, Bonferroni correction was used as post-hoc test.

11- NKC^{KD}-Ly49I^{tg} mice succumbed to influenza infection

Enhanced MHC-I expression, lets influenza virus to avoid detection and elimination via licensed NK cells by enhancing the inhibitory MHC-I:Ly49 interaction. Thus, mice that do not express Ly49 are better at controlling influenza virus infection. Beside Ly49 deficiency, NKC^{KD} mouse model additionally express minor levels of the NKG2A/C/E family. Moreover, because the NKC^{KD} mouse model was made using ES cells that were originally isolated from 129S1 mice, despite back-crossing these mice onto the C57BL/6 background, they likely carry some genomic segments from 129S1 mice (96). To this end, I wanted to make sure the phonotype that I see in the NKC^{KD} mice is related to Ly49-deficiency and not because of other factors. Doing so would further prove the contribution of licensed NK cells in the illness of influenza infection. To analysis this, my colleague and I managed to re-express the inhibitory Ly49I transgene into NKC^{KD} mice by backcrossing Ly49I^{tg} mice to NKC^{KD} mice. All NK cells in NKC^{KD}-Ly49I^{tg} mice express inhibitory Ly49I receptors, therefore, they are all licensed NK cells. Groups of NKC^{KD}-Ly49I^{tg}, NKC^{KD}, and WT mice were infected and the mice were checked daily for two weeks. As I showed earlier, almost all WT mice died from the infection and only 40% of NKC^{KD} mice died (Figure 15). Interestingly, all NKC^{KD}-Ly49I^{tg} mice succumbed to the infection 10 days post-infection (Figure 15). This result demonstrates that the protection I observed in NKC^{KD} during influenza virus infection is due to Ly49-deficiency and not because of NKG2A/C/E or other 129S1 factors. Also, licensed NK cells contribute significantly to the severity of influenza illness.

Figure 15

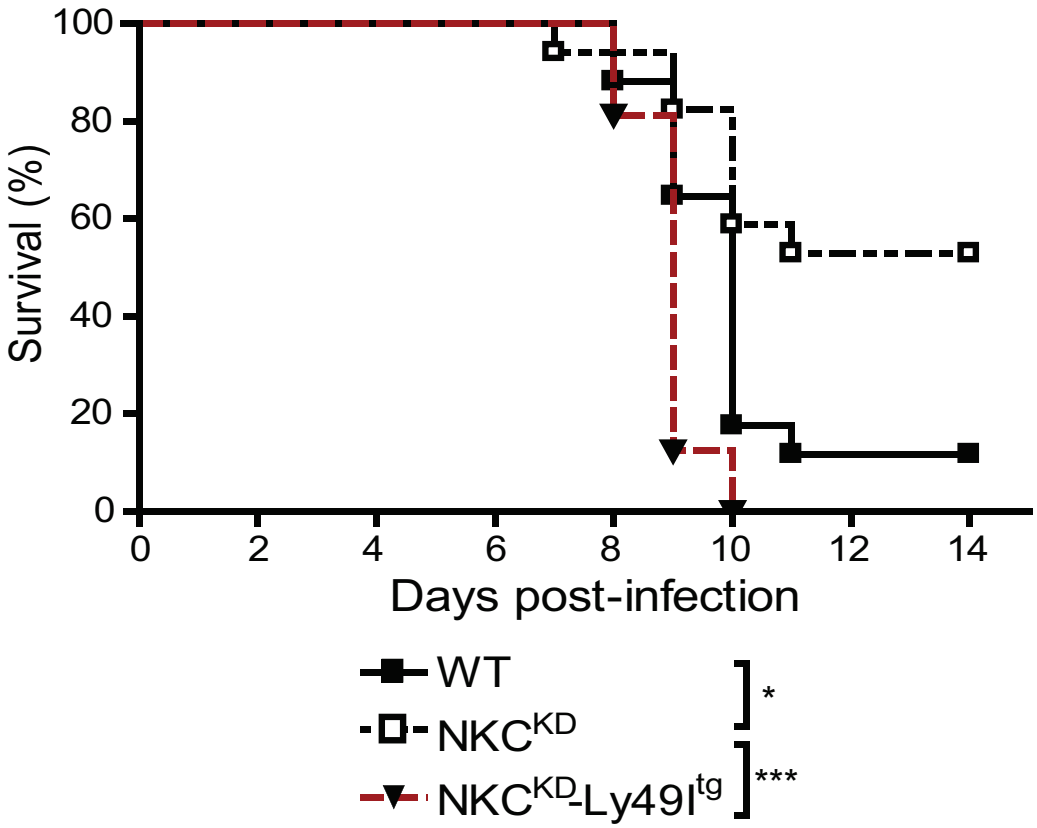

100
80
60
40
20
0
Survival (%)
0
2
4
6
8
10
12
14
Days post-infection
WT
NKC^KD
NKC^KD-Ly49I^tg
*
***

Figure 15. Ly49-deficiency protects mice from influenza infection.

600 PFU of influenza was used to infect sex and age-matched NKC^{KD}, NKC^{KD}-$Ly49I^{tg}$, and WT mice, and then the mice were checked for two weeks. Pooled data from 2 experiments (n = 16/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was performed with the log rank test.

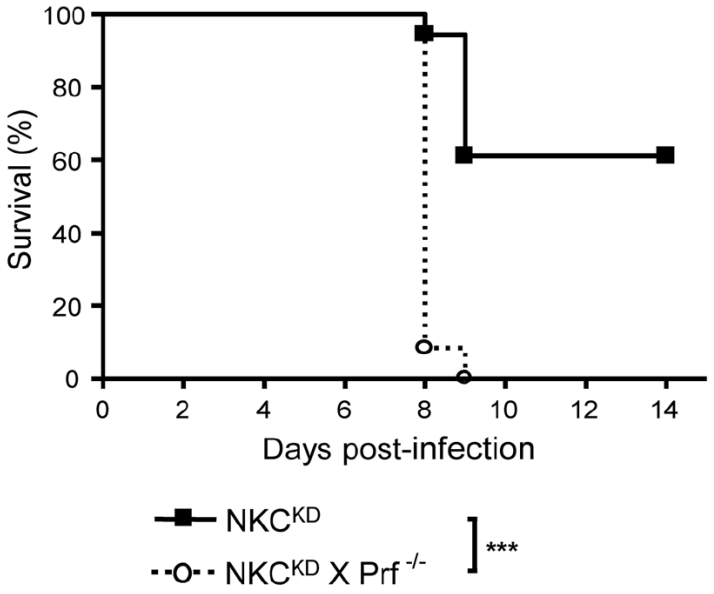
12- Perforin and IFN-γ are required for NKC^{KD} mouse survival following influenza virus infection

Thus far, I have determined that uneducated NK cells involve significantly in limiting virus replication, consequently inhibiting lung pathology and death. Next, I wanted to determine the mechanism by which unlicensed NK cells protect NKC^{KD} mice against influenza infection. NK cells kill target cells directly using perforin/granzyme, or indirectly by secreting cytokines—importantly, IFN-γ, that contributes directly in activating subsets of adaptive and innate immune cells (210). To elucidate the mechanism for the protection, I backcrossed perforin-deficient (Prf$^{-/-}$) mice to NKC^{KD} mice to generate unlicensed NK cells that lack perforin. A group of NKC^{KD} and NKC^{KD}xPrf$^{-/-}$ mice were infected and observed for two weeks with daily monitoring. Interestingly, all NKC^{KD}xPrf$^{-/-}$ mice died from the infection eight days following the infection (Figure 16A), suggesting that unlicensed NK cells require perforin to eliminate influenza virus-infected cells directly.

Next, I wanted to determine whether IFNγ is required for NKC^{KD} mouse protection following influenza virus infection. Because IFNγ$^{-/-}$ mice display reduced macrophage and resting NK activity, I decided not to backcross it to NKC^{KD} mice. I rather decided to use anti-IFN-γ mAb to neutralize IFNγ in NKC^{KD} mice 2 days before the infection, day 0, and every other day till day eight post-infection. Uninfected mice were used as a control and were also injected with anti-IFNγ mAb. Around 90% of IFNγ-depleted NKC^{KD} mice died from the infection (Figure 16B), suggesting that IFNγ is also important in protecting the mice from the infection.

Figure 16

A


100
80
60
40
20
0
Survival (%)
0
2
4
6
8
10
12
14
Days post-infection
NKC^KD
NKC^KD X Prf -/-
***

B

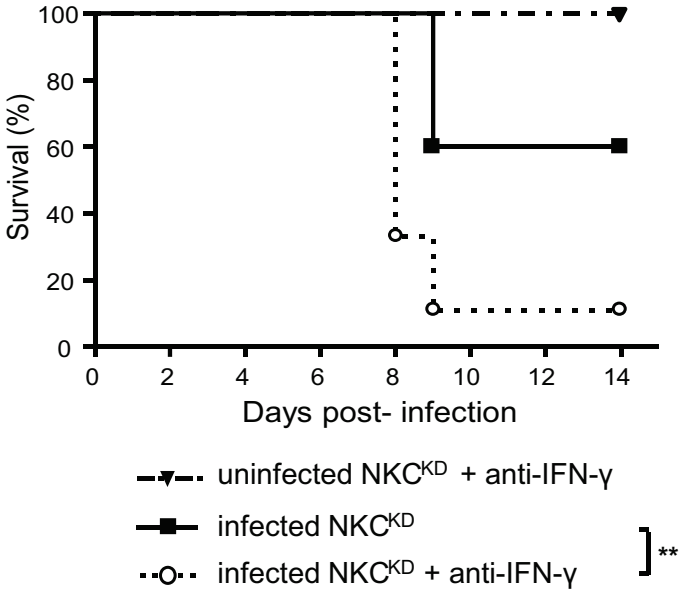

100
80
60
40
20
0
Survival (%)
0
2
4
6
8
10
12
14
Days post- infection
uninfected NKC^KD + anti-IFN-γ
infected NKC^KD
infected NKC^KD + anti-IFN-γ
**

Figure 16. NKC^{KD} mice require perforin and IFN-γ to be protected from infection.

600 PFU of influenza was used to infect (A) Sex and age-matched NKC^{KD} (n=18) and NKC^{KD}xPrf$^{-/-}$ (n=12) or (B) uninfected NKC^{KD}+anti-IFN-γ mAb (n=6), infected NKC^{KD} (n = 10), and NKC^{KD}+anti-IFN-γ mAb (n=18) mice were checked for two weeks. Pooled data from 2 experiments are presented. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was performed with the log rank test.

Next, I wanted to determine the effect of perforin- and IFN-γ-deficiency in NKC^{KD} mice on influenza virus replication. Groups of NKC^{KD}, IFN-γ-neutralized NKC^{KD}, and $NKC^{KD}xPrf^{-/-}$ mice were infected and checked daily. Five and seven days post-infection, lungs were collected and lung tissue homogenates were prepared for plaque assays to determine viral titers. Virus load at day 5 and 7 in the lungs of influenza-infected NKC^{KD} mice were almost the same as before (Figure 17). Additionally, virus loads in the lungs of NKC^{KD} mice at day five and seven were significantly lower than IFNγ-neutralized NKC^{KD} and $NKC^{KD}xPrf^{-/-}$ mice. At day 5 post-infection, there were no significant difference between viral titers in the lungs of IFN-γ-depleted NKC^{KD} and $NKC^{KD}xPrf^{-/-}$ mice. However, at day 7, influenza virus titer in $NKC^{KD}xPrf^{-/-}$ mice was significantly higher than the other groups. These results suggest that perforin and IFNγ are important in limiting influenza virus infection. However, perforin appears to be important both at early and late times following infection since it is known to be an effector molecules produced by both NK and T cells to kill target cells.

Figure 17

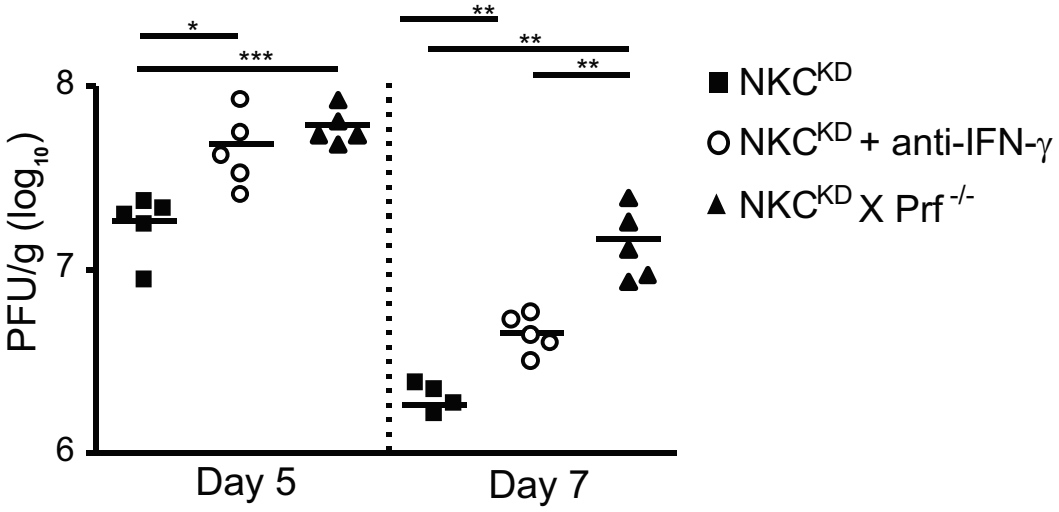


Figure 17. Perforin and IFN-γ deficiencies enhance viral titers.

600 PFU of influenza was used to infect NKC^{KD}, $NKC^{KD}xPrf^{-/-}$, and NKC^{KD}+anti-IFN-γ mice (n=5/group). Five days following the infection lungs were collected, weighed, and homogenized to determine lung viral titers by plaque assay on MDCK cells (PFU/g of lung tissue). Each dot represents one mouse. The horizontal line indicates the mean values. *p < 0.05, **p < 0.01, ***p < 0.001. Statistical analysis was performed using Student's t-test.

14- MHC-I-deficient mice confer robust resistance to influenza infection in a NK cell-dependent manner

My data has strongly suggest that the interaction between MHC-I:Ly49 enhanced the harshness of influenza infection, suggesting that unlicensed NK cells contribute in limiting the infection. Next, I hypothesized that MHC-I-deficient mice, in which all NK cells are unlicensed, would be also protected from the infection. Influenza virus would not be able to inhibit NK cell cytotoxic function via enhancing MHC-I:Ly49 interactions in these mice; thus, MHC-I-deficiency would be beneficial for the host. One disadvantage of *B2m*$^{-/-}$ (MHC-I deficient) mice is that they do not possess functional CLT. Consequently, I thought to deplete NK cells in this mouse model to further determine the function of NK cells in the infection. Groups of WT, *B2m*$^{-/-}$, and NK-depleted- WT and *B2m*$^{-/-}$ mice were infected and survival was checked for two weeks. NK cell-depleted and undepleted WT mice succumbed to the infection by day 9 post-infection (Figure 18). Interestingly, like Ly49-deficient mice, 60% of MHC-I-deficient mice survived the infection (Figure 18), again demonstrating that MHC-I:Ly49 engagement enhances the illness of influenza infection. Also, NK cell depletion in *B2m*$^{-/-}$ confirmed again that NK cells, and specifically unlicensed NK cells, contribute directly in protection against influenza infection (Figure 18).

Figure 18

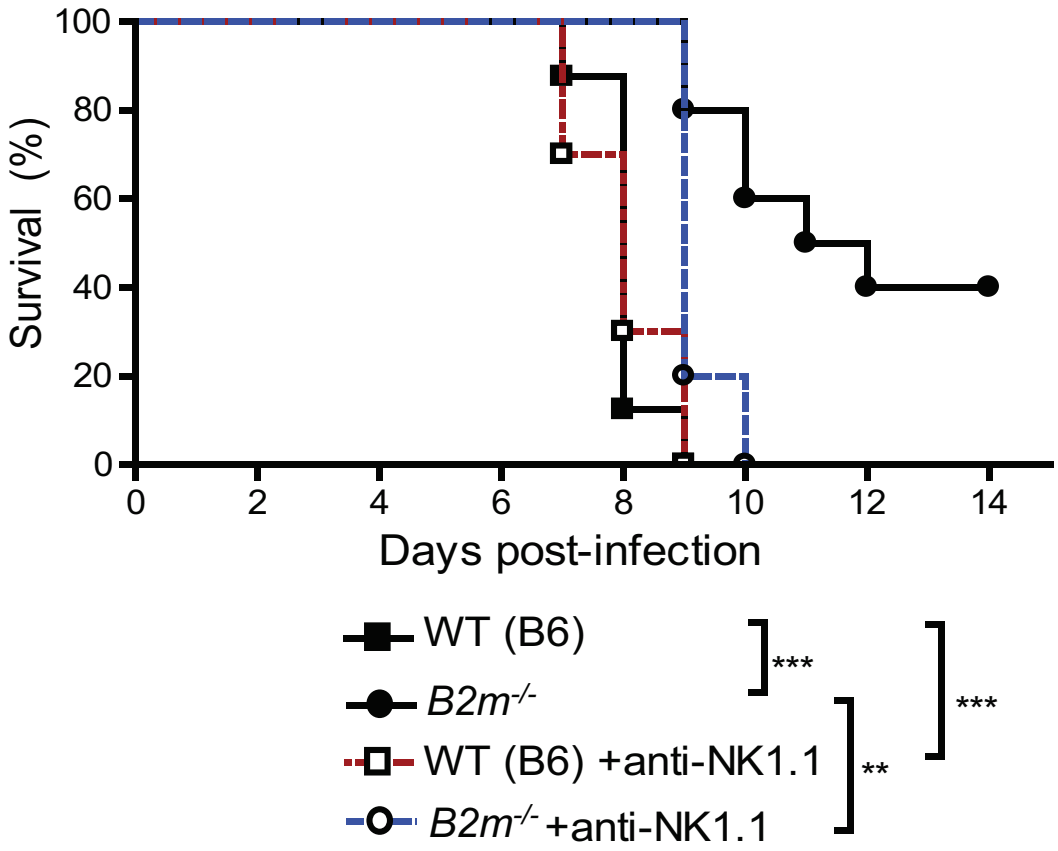

100
80
60
40
20
0
Survival (%)
0
2
4
6
8
10
12
14
Days post-infection
WT (B6)
B2m-/-
WT (B6) +anti-NK1.1
B2m-/- +anti-NK1.1
***
**
***

Figure 18. Unlicensed NK cells control influenza infection.

600 PFU of influenza was used to infect sex and age-matched WT (n=18), WT+anti-NK1.1 mAb, *B2m*$^{-/-}$, and *B2m*$^{-/-}$+anti-NK1.1 mAb and checked for two weeks. Pooled data from 2 experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was performed with the log rank test.

15- Blockade of the Ly49:MHC-I interaction protects mice from influenza infection

Overall, the results presented above suggests that blocking the engagement between Ly49:MHC-I could save WT mice from influenza infection. As mentioned earlier, NK cells from WT (B6) express two self-Ly49 inhibitory receptors, Ly49C and Ly49I (93, 118). The monoclonal antibody, 5E6, is believed to interact with Ly49C and Ly49I (211), although a new report suggests that it only interacts with Ly49I (212). I hypothesized that blocking the engagement between self-Ly49 inhibitory receptors and upregulated MHC-I would save WT mice from influenza illness. To validate this proof-of-concept, I generated F(ab')2 5E6 mAbs to interrupt the engagement between Ly49:MHC-I. $F(ab')_2$ mAbs only block, while using whole mAbs will deplete NK cells. $F(ab')_2$ mAbs were intraperitoneally injected two days, day 0, and every other day following the infection. Mice were infected and monitored daily for clinical signs of sickness and loss of body weight for two weeks. Only 10% of untreated WT mice survived the infection while 30% of $F(ab')_2$ treated WT mice were significantly protected from the infection (Figure 19). This result confirms our finding that MHC-I:Ly49 engagement contribute in the severity of influenza infection. Using an antibody that can block the interaction of both Ly49C and I with MHC-I may result in even better protection against influenza virus infection.

Figure 19

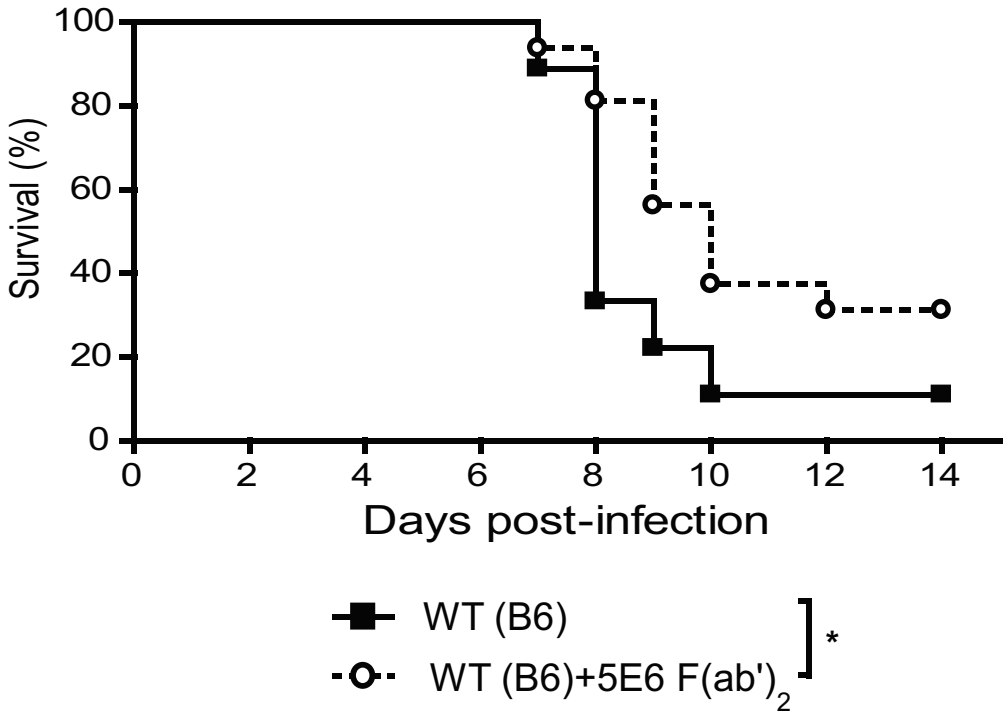

100
80
60
40
20
0
Survival (%)
0
2
4
6
8
10
12
14
Days post-infection
WT (B6)
WT (B6)+5E6 F(ab')2
*

Figure 19. F(ab')$_2$ 5E6 mAb treatment is protective in WT mice post-infection.

600 PFU of influenza was used to infect sex and age-matched WT and WT+anti-Ly49C/I F(ab')$_2$ 5E6 mAb**,** and mice was checked for two weeks. Pooled data from two experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was performed with the log rank test.

16- There is no association between NKG2A expression and protection

$F(ab')_2$ blocking Ly49:MHC-I engagements significantly protected WT mice from influenza illness; however, the protection was not as high as that observed with the loss of Ly49 expression. I assumed that other inhibitory receptors might also be involved in inhibiting NK cell function during influenza infection, which might explain why we saw only modest protection. NK cells express an array of inhibitory and activating receptors including the inhibitory NKG2A receptor. Next, I wanted to determine whether NKG2A is involved during influenza infection by determining the expression of NKG2A on WT (B6) and *B2m*$^{-/-}$ mice before and after influenza infection. Groups of WT and *B2m*$^{-/-}$ mice were infected and then five days post infection, lungs were harvested and single cell suspensions were prepared and stained with anti-NKG2A or anti-NKG2A/C/E mAbs to be analyzed by flow cytometry. The data showed no significant difference in the expression of NKG2A between WT and *B2m*$^{-/-}$ mice before and after the infection (Figure 20A). Interestingly, the expression of NKG2A/C/E is higher in *B2m*$^{-/-}$ than WT mice (Figure 20B). Similar expression of NKG2A before and after the infection suggests that NKG2A might not contribute in the illness of influenza virus.

Figure 20

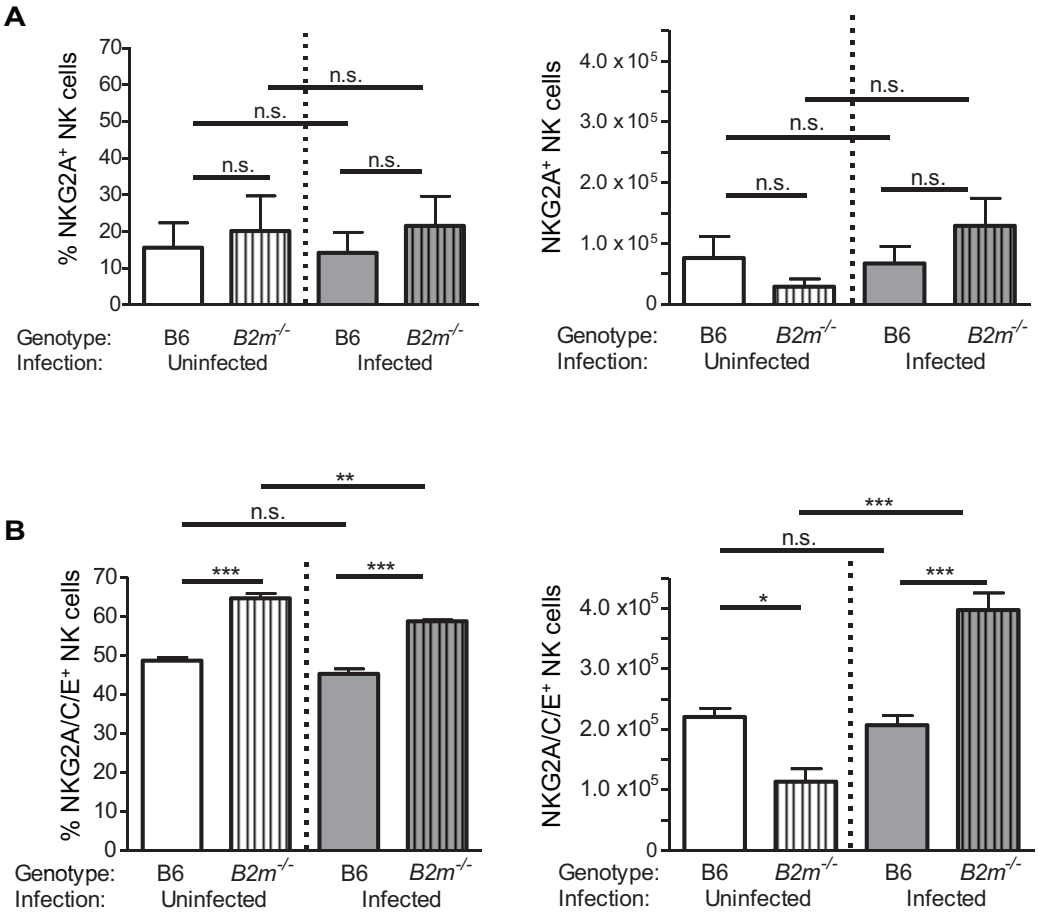

A
% NKG2A+ NK cells
70
60
50
40
30
20
10
0
n.s.
n.s.
n.s.
n.s.
Genotype: B6 B2m-/- B6 B2m-/-
Infection: Uninfected Infected
NKG2A+ NK cells
4.0 x 10^5
3.0 x 10^5
2.0 x 10^5
1.0 x 10^5
0
n.s.
n.s.
n.s.
n.s.
Genotype: B6 B2m-/- B6 B2m-/-
Infection: Uninfected Infected
B
% NKG2A/C/E+ NK cells
70
60
50
40
30
20
10
0
**
n.s.
***
***
Genotype: B6 B2m-/- B6 B2m-/-
Infection: Uninfected Infected
NKG2A/C/E+ NK cells
4.0 x10^5
3.0 x10^5
2.0 x10^5
1.0 x10^5
0
***
n.s.
*
***
Genotype: B6 B2m-/- B6 B2m-/-
Infection: Uninfected Infected

Figure 20. NKG2A plays no role during influenza infection.

(A,B) 600 PFU influenza virus was used to infect sex- and age-matched *B2m*$^{-/-}$, WT(B6), NKC^{KD}, and WT mice. Mice were euthanized at day 5 post-infection, lungs were harvested, and single-cell suspensions were stained with antibodies against NKp46, TCRβ, NKG2A, and NKG2A/C/E. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was performed by one-way ANOVA, Bonferroni correction was used as post-hoc test.

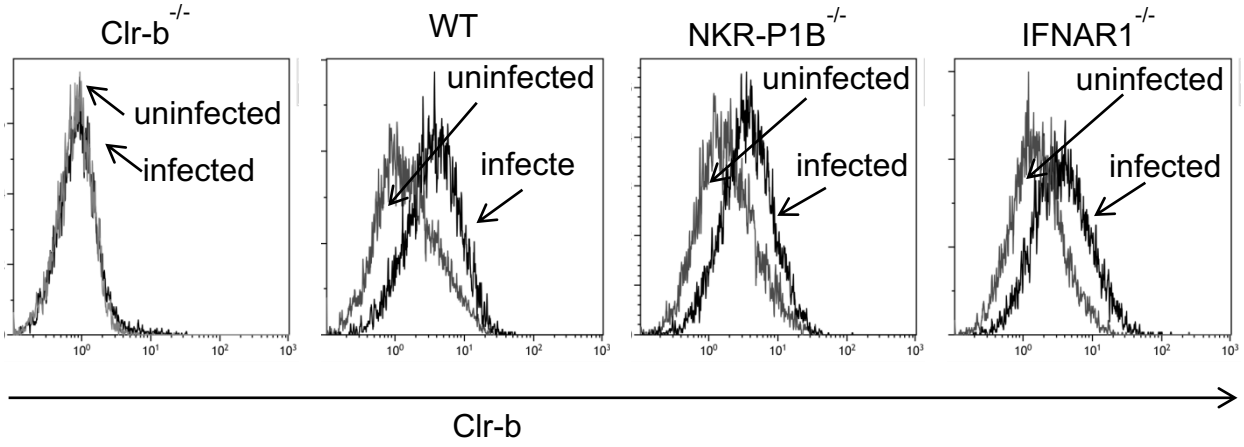
17- Influenza virus enhances mouse Clr-b and human LLT1 expression on lung epithelial cells

Although NKC^{KD} and $B2m^{-/-}$ can survive the infection, not all mice were protected. Moreover, the $F(ab')_2$ blockade suggested that other inhibitory receptor might also be involved in the severity of influenza infection. The Natural Killer gene Complex (NKC) contains 4 main families of receptors: Ly49, NKG2, NKRP-1, and Clr (52, 63). NKG2A seems to have no role during influenza infection; however, this does not rule out other major NK cell inhibitory receptors such as NKR-P1B, which binds to Clr-b (65). Clr-b is expressed on almost all tissues except the brain, and its interaction with NKR-P1B results in NK cell inhibition (65, 66). To determine the role of NKR-P1B:Clr-b interactions in influenza illness, first I wanted to test whether influenza infection would modulate Clr-b expression on lung epithelial cells. Groups of $Clr\text{-}b^{KO}$ and WT mice were uninfected or infected with 600 PFU of influenza virus for 5 days. $Clr\text{-}b^{KO}$ mice were used as a negative control. Lungs were harvested, single cell suspensions were prepared and stained to be analyzed by flow cytometry. Gating on lung epithelial cells, I determined that Clr-b expression is upregulated in response to influenza infection (Figure 21A). Next, I wanted to determine if this upregulation was a in response to IFN-I produced during the infection. 600 PFU of FM-MA was used to infect WT, $NKR\text{-}P1B^{KO}$, $Clr\text{-}b^{KO}$, and $IFNAR1^{-/-}$ mice with. Five days post-infection, the expression of Clr-b was still upregulated on lung epithelial cells of WT, $NKR\text{-}P1B^{KO}$, and $IFNAR1^{-/-}$ but not on the control $Clr\text{-}b^{KO}$ mice (Figure 21A). This suggests that NKR-P1B:Clr-b interaction may play a role during the infection. Influenza virus was indicated to upregulate MHC-I expression *in vitro* on a human lung epithelial cell line (199); I thought influenza virus might also upregulate LLT1, the human analog for mouse Clr-b. To

test this hypothesis I infected human lung epithelial cell line, A549, with influenza virus at MOI=1 for 18 hours. Next, cells were fixed and stained with anti-LLT1 mAb, and analyzed with flow cytometry. Similar to Clr-b, LLT1 was upregulated on the influenza-infected lung epithelial cell line (Figure 21B), suggesting that the interaction between Clr-b or LLT1 with their inhibitory receptor on NK cells could increase the severity of influenza infection, as was observed with Ly49 inhibitory receptors.

Figure 21

A


Clr-b-/-
WT
NKR-P1B-/-
IFNAR1-/-
uninfected
infected
infecte
Clr-b

B

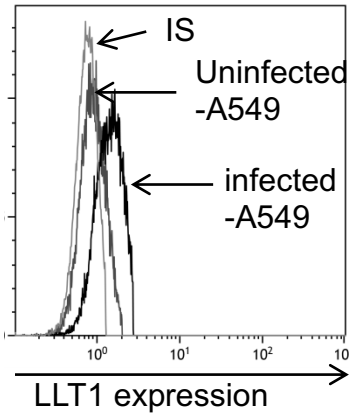

IS
Uninfected
-A549
infected
-A549
LLT1 expression

Figure 21. Influenza virus induces CLR-b and LTT-1 upregulation.

(A) 600 PFU influenza virus was used to infect sex- and age-matched WT, NKR-P1B^{KO}, Clr-b^{KO}, and Ifnar1$^{-/-}$ mice or left untreated for 5 days. Mice were culled at designated time points, lungs were homogenized, and single-cell suspensions were prepared. Surface expression of Clr-b on lung epithelial cells was tested by flow cytometry using specific antibodies against each of these markers. Anti-LFA-1 mAb was used to gate out lung leukocytes. (B) Human lung epithelial cell line, A549, was infected with influenza virus at MOI=1 for 18 hours. Cells were then stained with anti-LLT1 mAb and analyzed by flow cytometry to determine surface expression of human LLT1 on A549 cells.

18- NKR-P1B^{+} and NKR-P1B^{-} NK cells respond to influenza infection

Next, I wanted to determine the expression level of NKR-P1B on lung NK cells. Lungs were harvested from groups of WT, NKR-P1B^{KO}, and Clr-b^{KO} mice. Lung single cell suspensions were prepared and stained to be analyzed by flow cytometry. Almost 73 % of lung NK cells of WT mice express NKR-P1B, whereas almost 70 % of lung NK cells of Clr-b^{KO} mice express NKR-P1B (Figure 22A and 22B). Then, I wanted to determine whether NKR-P1B^{-} NK cells are as functional as NKR-P1B^{+} NK cells in WT mice. 600 PFU of influenza virus was used to infect WT mice for 3 and 5 days. Lung lymphocytes from infected and uninfected mice were co-cultured with YAC-I cells or left untreated for four hours. In the absence of YAC-I minimal background IFN-γ production was observed (Figure 22C), and there were no significant differences in the level of IFN-γ in both groups. In the present of YAC-I, our data showed that both NKR-P1B^{+} and NKR-P1B^{-} NK cells were activated and produced IFN-γ in respond to YAC-1 following influenza infection (Figure 22D). Again there were no significant differences in the levels of IFN-γ production between NKR-P1B^{+} and NKR-P1B^{-} NK cells following the infection. These data show that NKR-P1B^{-} NK cells are responsive during influenza infection and might be play a role during the infection.

Figure 22

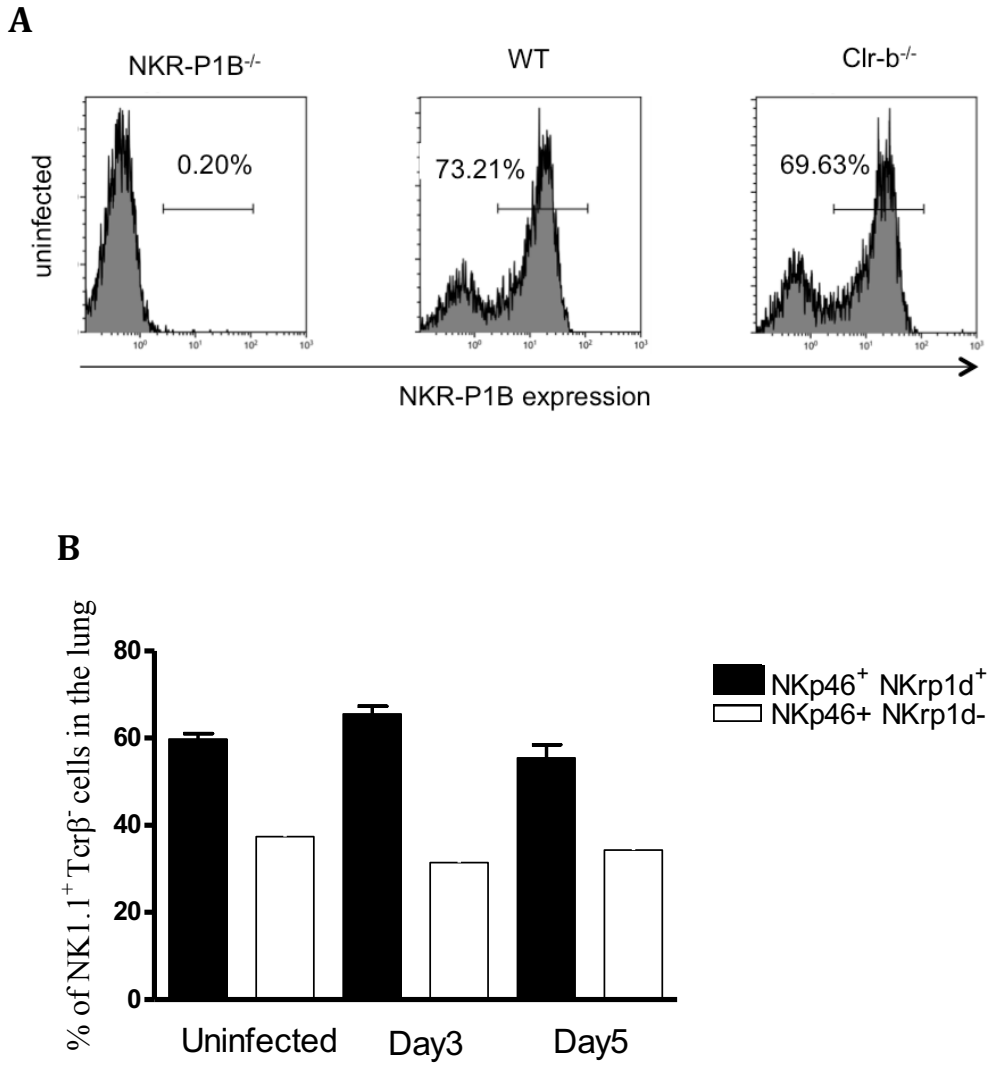

A
NKR-P1B-/-
WT
Clr-b-/-
uninfected
0.20%
73.21%
69.63%
NKR-P1B expression
B
% of NK1.1+ Tcrβ- cells in the lung
80
60
40
20
0
Uninfected
Day3
Day5
NKp46+ NKrp1d+
NKp46+ NKrp1d-

Figure 22

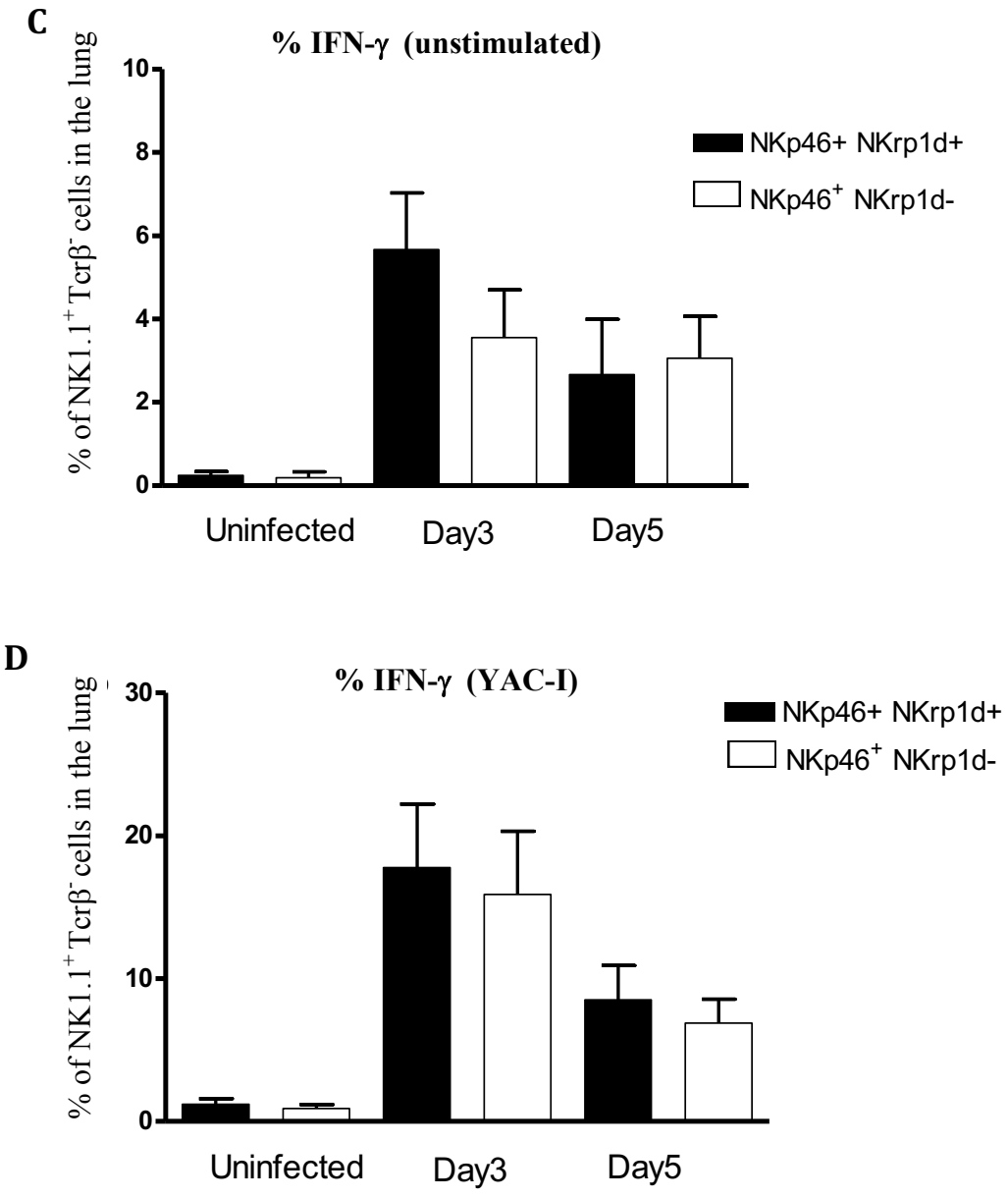

C
% IFN-γ (unstimulated)
% of NK1.1+ Tcrβ- cells in the lung
10
8
6
4
2
0
NKp46+ NKrp1d+
NKp46+ NKrp1d-
Uninfected
Day3
Day5
D
% IFN-γ (YAC-I)
% of NK1.1+ Tcrβ- cells in the lung
30
20
10
0
NKp46+ NKrp1d+
NKp46+ NKrp1d-
Uninfected
Day3
Day5

Figure 22. Lung NKR-P1B^{+} and NKR-P1B^{-} NK cells respond to influenza infection.

(A) Lungs of uninfected WT, NKR-P1B^{KO}, and Clr-b^{KO} mice were harvested and single-cell suspensions were prepared and stained with antibodies against NKp46, TCRβ, and NKR-P1B to detect their expression on lung NK cells. (B) Percentage of NKR-P1B^{+} and NKR-P1B^{-} NK cells in the lungs of uninfected, Day3- and Day5-infected WT mice. (C,D) 1x10^{6} lung lymphocytes were incubated in a 1:1 ratio with YAC-I, or left without treatment for 4 hours. Cells were then stained with anti-NKp46, anti-TCRβ, anti- NKR-P1B, and anti-IFN-γ mAbs and analyzed by flow cytometry. Pooled data from three independent experiments are shown (n=6/group).

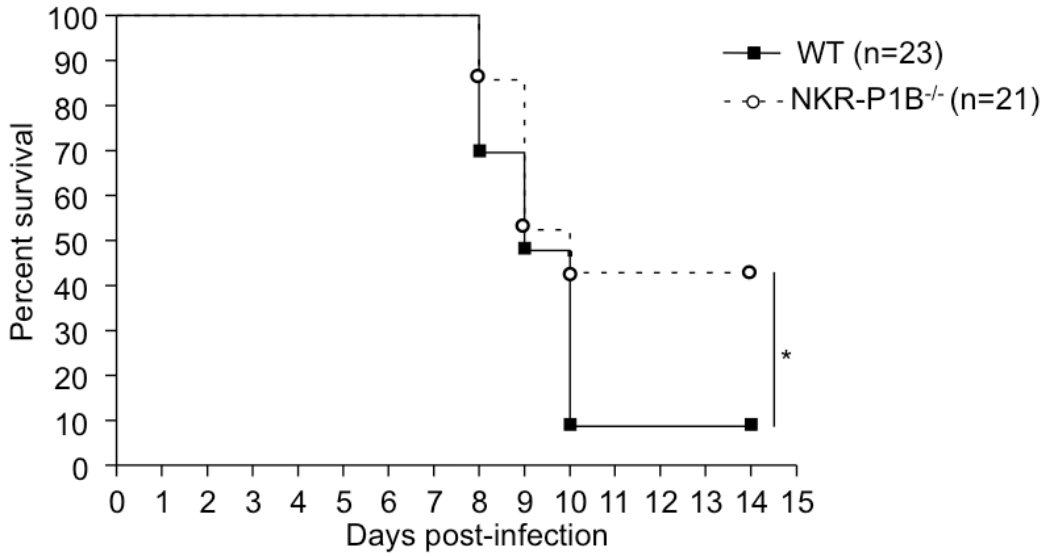
19- NKR-P1B-deficient mice are resistant against influenza infection

Elevated expression of Clr-b might lead to inhibition of NK cells due to the engagements with inhibitory NKR-P1B receptors. To test if the NKR-P1B engagements with Clr-b ligand is associated with severity of influenza infection, WT and NKR-P1B$^{-/-}$ were infected and the mice were monitored daily. All of the WT mice died from the infection on day 8 post-infection, and 45% of NKR-P1B$^{-/-}$ mice survived the infection (Figure 23A). This result suggests that interrupting the interaction between NKR-P1B and Clr-b molecules is beneficial for the host during influenza infection. Also, this interaction might also have a negative impact on our previous F(ab')$_2$-mediated blockade of the Ly49:MHC-I interaction, leading to only a partial protection against influenza infection.

Next, I asked, since NKR-P1B-deficient mice can survive the infection, whether Clr-b^{KO} mice could survive influenza infection too. I infected a group of WT and Clr-b^{KO} mice and the mice were monitored for up to 2 weeks post-infection. All WT mice died from the infection while 30% of Clr-b mice survived (Figure 23B). Clr-b^{KO} mice have less active NK cells in the resting state (213), which might reflect on the survival as well. Taken together, NKR-P1B and Clr-b play a role in enhancing the severity of influenza infection.

Figure 23

A


100
90
80
70
60
50
40
30
20
10
0
Percent survival
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days post-infection
WT (n=23)
NKR-P1B-/- (n=21)
*

B

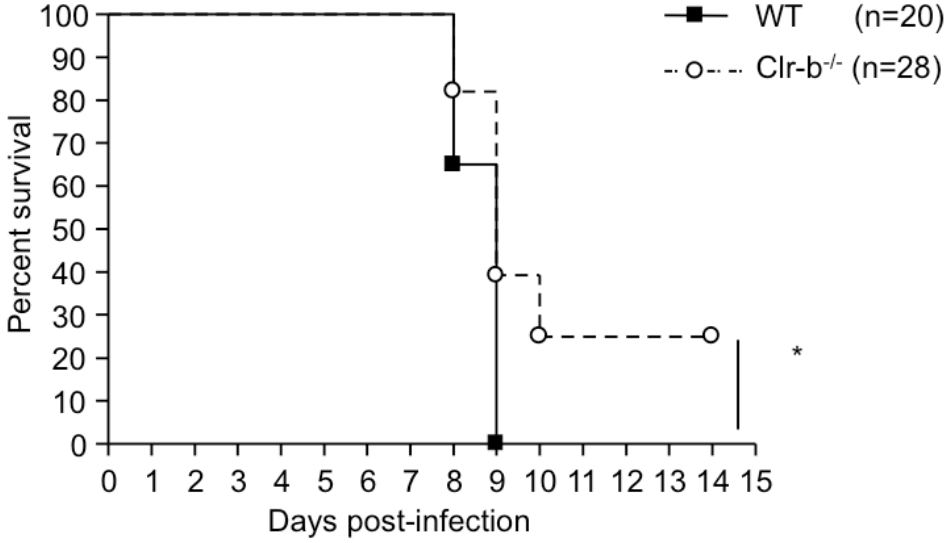

100
90
80
70
60
50
40
30
20
10
0
Percent survival
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days post-infection
WT (n=20)
Clr-b-/- (n=28)
*

Figure 23. NKR-P1B- and Clr-b-deficient mice are resistant against influenza virus infection.

(A, B) 600 PFU influenza was used to infect sex- and age-matched WT, NKR-P1B^{KO}, and Clr-b^{KO} mice were infected with influenza virus 600 PFU influenza virus and monitored for two weeks. Pooled data from 2 experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was performed with the log rank test.

20- NKR-P1B-deficient mice have less severe lung pathology than WT mice following influenza infection

Lung pathology is a characteristic of severe influenza illness. The survival of NKR-P1B mice led us to ask if the mice would develop less pulmonary damage than WT mice, or whether the mice survived for a different reason. I infected a group of WT and NKR-P1B^{KO} mice with 600 PFU of influenza. Seven days post infection, lungs were collected and fixed. The fixed lungs were paraffin embedded, sectioned, and stained with H&E. Lung histopathology showed that uninfected WT and NKR-P1B mice display normal lung histology (Figure 24). However, infected WT mice showed more severe lung pathology. Infected WT mice showed more alveolar septal infiltrate and damage, perivascular connective tissue, and peribronchial connective tissue. These data demonstrated that the interaction between NKR-P1B and Clr-b enhances lung pathology during influenza infection.

Next, I wanted to identify the level of several cytokines in the lungs of both groups following infection with influenza. Lungs tissues were harvested 5 days following the infection—again to avoid T cell involvement—and lung homogenates were prepared for cytokine measurement. The level of TNF-α was similar in both groups of mice (Figure 25B). Interestingly, IFN-γ levels were significantly higher in the lungs of WT mice compared to the NKR-P1B^{KO} mice (Figure 25A). These results suggest that better survival of NKR-P1B^{KO} mice is linked with reduced pulmonary pathology caused by influenza virus infection as compared to the WT mice.

Figure 24

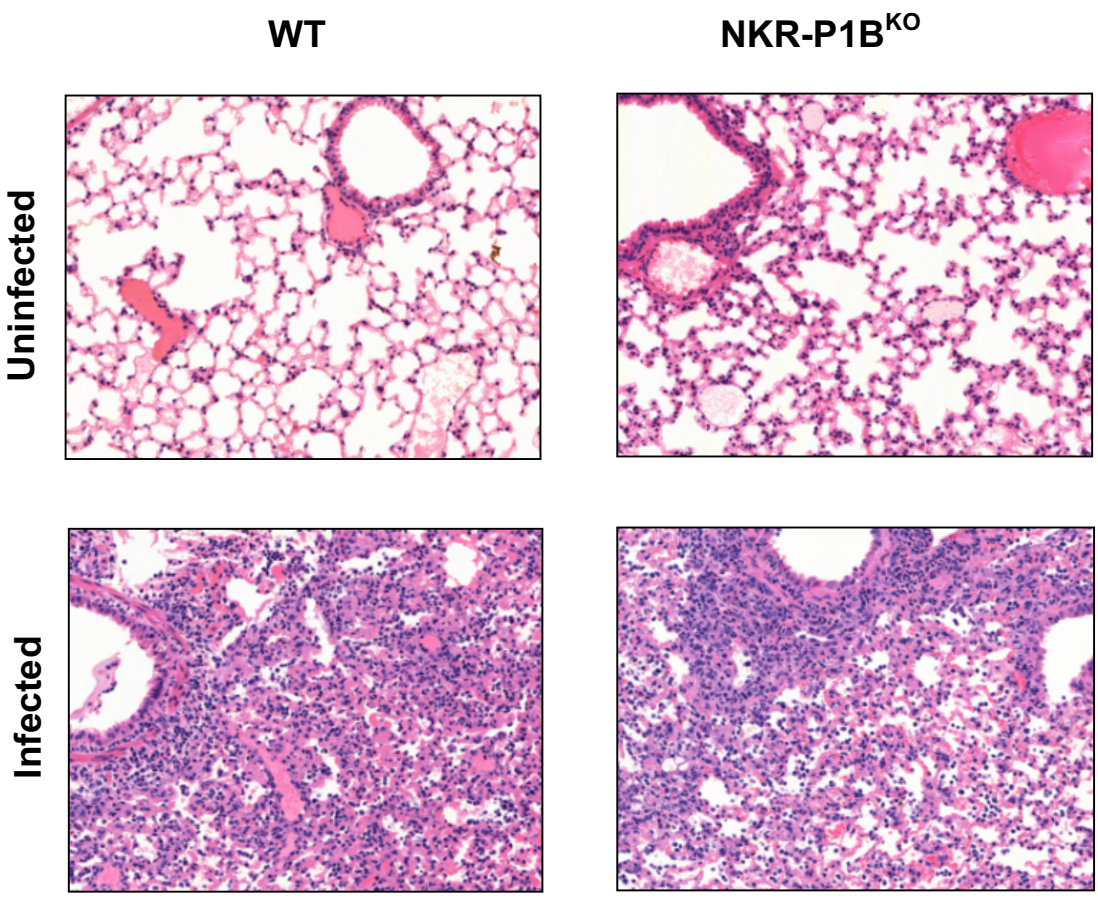


Figure 24. NKR-P1B deficiency is beneficial for the host during influenza infection.

600 PFU influenza was used to infect NKR-P1B^{KO} and WT mice. Seven days following the infection lungs were collected, fixed in 10% formalin (NBF), and stained using standard hematoxylin and eosin protocol, captured at 100X magnification.

Figure 25

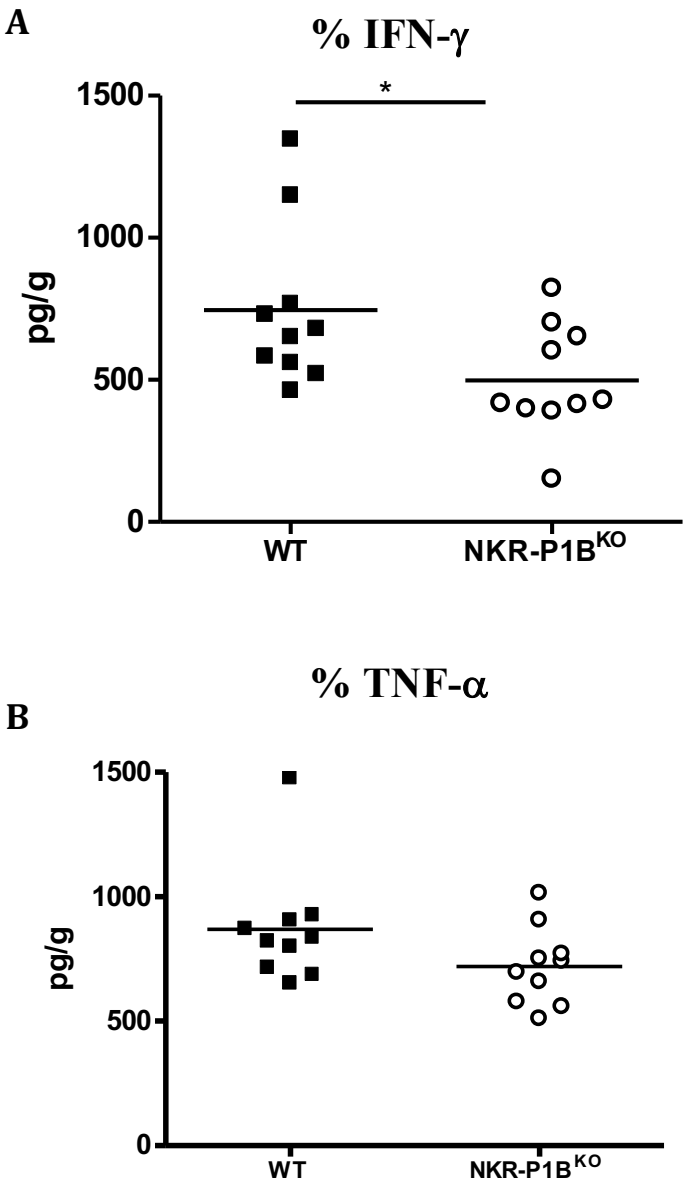

A
% IFN-γ
*
1500
1000
500
0
pg/g
WT
NKR-P1B^KO
B
% TNF-α
1500
1000
500
0
pg/g
WT
NKR-P1B^KO

Figure 25. NKR-P1B-deficient mice have less IFN-γ production in the lung.

Five days following influenza infection, lungs were collected, weighed, and homogenized to determine cytokine and chemokine levels. IFN-γ (A) and TNF-α (B) level per gram of lung tissue is shown. Pooled data from 3 experiments are shown (n=10/group). Each dot represents one mouse. The horizontal line indicates the mean values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis was conducted using a one-way ANOVA, Bonferroni correction was used as post-hoc test.

Discussion

NK cells can distinguish and eliminate virus-infected cells and cancer cells, while leaving normal cells untouched. Upon activation, NK cells produce cytotoxic granules, perforin and granzyme, that kill target cells, accompanied by cytokines and chemokines to recruit and activate other immune cells (70, 105, 214). The engagement between self-Ly49 inhibitory receptors with its cognate ligand is required for NK cells to be fully functional, as well as a way to categorize NK cells into two main subsets, licensed and unlicensed (93, 96, 215). Viruses have developed several strategies to evade innate and adaptive immune responses, which can lead to the enhanced severity of a viral infection. Herein, my work has shown that in order to evade NK cells, influenza virus is able to enhance MHC-I induction on mouse pulmonary epithelial cells in an IFN-I-independent manner. The engagement between upregulated MHC-I and its self-Ly49 inhibitory receptor inhibits the cytotoxicity of licensed NK cells against influenza-infected cells. Thus, unlike WT mice, MHC-I- deficient and Ly49-defecient mice are protected from influenza infection.

Unlicensed NK cells are responsive during viral infection

While the licensing hypothesis is broadly accepted, very few studies have explored the role of each NK cell subset in different viral and bacterial infections. Although, the hypofunctional feature of unlicensed NK cells is widely recognized (93), I provide new evidence showing that unlicensed NK cells contribute significantly in limiting influenza illness in both MHC-I-deficient and Ly49-deficient mice, wherein the majority of NK cells, if not all, are considered unlicensed (96, 201, 216). Activation of NK cells is controlled by several groups of inhibitory and activating surface receptors (6). Several studies have shown that unlicensed NK cells, which lack inhibitory receptors for MHC-I, have reduced natural

cytotoxicity against tumors and so they respond poorly to stimulation through their activating receptors (93, 216). In this study, I have demonstrated that as a consequence of influenza virus, unlicensed NK cells (Ly49 C/I$^-$ NK cell subsets) in WT mice proliferate and respond significantly better than licensed NK cells (Ly49 C/I$^+$ NK cell subsets) (Figure 5B). The increased proliferation of unlicensed NK cells during influenza infection is most likely because they do not engage with their inhibitory ligand, MHC-I, which gives them an advantage over licensed NK cells. My findings are compatible with Orr et al., wherein their study showed that the presence of unlicensed NK cells (Ly49C/I$^-$) is associated with superior protection of WT mice against MCMV infection (118). MCMV-infected cells express the m157 protein of MCMV that can be recognized by NK cell's activating Ly49H receptor (120, 121). Indeed, unlicensed NK cells can kill tumor cells expressing m157 better in MHC-I-dependent manner, while licensed NK cells are inhibited by MHC-I expression (118). Likewise, following MCMV infection, unlicensed NK cells proliferate more than licensed NK cells in MHC-I-dependent manner (118). Additionally, *in vivo Listeria monocytogenes* infection enhanced unlicensed NK cells to produce IFN-γ to a level similar to licensed NK cells (216). Thus, together these data suggest that unlicensed NK cells are critical to the host during viral and bacterial infections, whereas licensed NK cells are most likely inhibited due to MHC-I expression. Although the education of NK cells through MHC-I is widely appreciated, several labs including ours have obtained results which show that unlicensed NK cells are highly active in certain situations that cannot be answered through the licensing hypothesis (118, 216, 217), suggesting that NK cell education is more complicated than what has been previously thought. NK cell education is suggested to be completed in the bone marrow, thus any NK cells outside the bone marrow can either be

licensed or unlicensed. Elliott et al. indicated that although NK cells from mice lacking MHC-I expression are hypofunctional, even to stimulation through its activating receptor, however adaptive transfer these cells into WT host was able to reprogram the NK cells that express self-inhibitory Ly49 to become functional again and acquire a licensed phenotype. These NK cells were now able to secrete IFN-γ and perforin in response to stimuli (218). Another interesting result showed that pre-incubating human licensed and unlicensed NK cells with IL-2 or IL-15 made both of them responsive to external stimuli at comparable levels (219). Moreover, when primed with polyinosinic:polycytidylic acid (poly I:C), a mimic of viral double-stranded RNA, in the WT and Ly49-deficient mice, unlicensed NK cells respond efficiently against MHC-I-deficient tumor cells to a level comparable to the licensed NK cells (96, 201). Suggesting that, under suitable conditions unlicensed NK cells can be reprogramed to become functional and responsive to the surrounding environment. Viruses, particularly influenza virus, is known to enhance cytokine production (166, 220), and that would result in reactivating and boosting unlicensed NK cells cytotoxic response in Ly49- and MHC-I- deficient mice following infection. This could explain why in my study unlicensed NK cells proliferate and respond significantly more than licensed NK cells (Figure 5B). Moreover, I showed that unlicensed NK cells in both Ly49-deficient mice produced IFN-γ and TNF-α at a level comparable to licensed NK cells (Figures 14G and 14H), confirming that these cells actually are active and responsive to the influenza infection. Although both licensed and unlicensed NK cells secrete IFN-γ and degranulate at similar level (Figures 9A and 9B), the enhanced number of unlicensed NK cells in NKC^{KD} and $B2m^{-/-}$ made these mice significantly save from influenza illness. To further demonstrate the ability of unlicensed NK cells to response to influenza infection, I depleted unlicensed NK

cells from NKC^{KD} and *B2m*$^{-/-}$ in influenza infection which resulted in the mice succumbing to the infection rapidly (Figures 8B and 18). These data strongly prove the responsiveness of unlicensed NK cells in the infection, which I believe is due to enhanced cytokines production. Studies have shown that activating receptors, such as NKG2D, can activate unlicensed NK cell cytotoxic functions (96, 201). Therefore, Rae1β-expressing cells can be recognised and killed by $NKG2D^{+}$ unlicensed NK cells at a level similar to licensed NK cells *in vitro* and *in vivo* (96, 201). Additionally, antibody-dependent cell-mediated cytotoxicity (ADCC) pathway enhances the cytotoxic function of unlicensed human NK cells against the surface disialoganglioside GD2-expressing neuroblastoma cells (217). NK cell activating receptors such as NKp46 and NKG2D contribute in detecting and eliminating influenza-infected cells (44, 158). Engagement of these receptors along with cytokines production may enhance unlicensed NK cell cytotoxic functions. Taken together these results imply that the hypofunctional state of unlicensed NK cells is only true for MHC-I modulation on a target cell; however, the unlicensed NK cells are highly functional against a "dangerous" self-stressed cell. The responsiveness of "unlicensed" NK cells to viral and bacterial infection explain why they represent almost half the number of all NK cells in a WT mouse (221). Interestingly, in the intestine of WT mice, most of the NK cells lack the expression of self-Ly49 receptors and are therefore suggested to be "unlicensed" (222). This could be due to the presence of complex bacterial communities in the intestine, and so these organisms need to be kept under control by strong, locally present immune cells which lack inhibitory receptors. Whereas, "licensed" NK cells seem to be mostly specific for eliminating cells that have lost MHC-I expression.

It is important to mention that the reaction of unlicensed NK cells to influenza infection did not result in autoimmunity by attacking self-tissues. In this study, I showed that five days following influenza infection, the lung of Ly49-deficient mice look better than the lung of WT mice with no sign of autoimmunity. Again this result suggests that unlicensed NK cells cannot recognize MHC-I modulation on normal nor infected cells, thus their reactivation did not induce self-attack. Another explanation could be that unlicensed NK cells get inhibited by a ligand that binds to a receptor other than MHC-I, which prevents self-attack. NK cell education is complicated and require more studies to clearly understand how NK cells are educated. To make the education hypothesis even more complex, there is a lot of evidence showing that licensed NK cells can develop memory to specific peptides including influenza and HIV (223). More studies have to be conducted to understand where and how NK cells are educated. The process of how licensed NK cells can develop memory is unknown, but this highlights the importance of licensed NK cells during infection, and it would be a great idea to test the contribution of memory licensed NK cells in influenza infection.

Influenza virus enhances Ly49:MHC-I engagement to inhibit NK cells cytotoxicity

NK cells play a major role in fighting different types of viruses such as influenza virus, HIV, and CMV, to name a few. In order to evade T cell responses, several viruses such as CMV downregulate MHC-I expression on infected cells. Herein, I found that following influenza infection MHC-I expression is enhanced the lung. A recent study has shown that human influenza infection causes MHC-I upregulation on lung epithelial cell

lines (199). MHC-I:Ly49 interaction is known to inhibit NK cell cytotoxicity, suggesting that influenza virus might induce this upregulation to evade NK cell responses. During an infection, both the infected-cells and the innate immune cells produce IFN-α, which has the ability to enhance MHC-I upregulation. However, in the absence of a functional IFN-I pathway, such as in Ifnr1$^{-\backslash-}$ mice, I have confirmed that influenza infection still enhances MHC-I expression *in vivo* (Figure 4B). Unfortunately, I was not able to determine whether this upregulation is specific for influenza-infected cells or if it is a general upregulation. Besides IFN-I, MHC-I upregulation can be induced by IFN-γ production, which is heavily produced in the lung after the infection (Figure 14A). Further work is required to identify the role of IFN-γ in the upregulation of MHC-I using IFN-γ^{ko} mice or neutralizing IFN-γ during the influenza infection. An alternative explanation would be that influenza virus might itself induce this upregulation following the infection as an evasion mechanism. In humans, influenza virus upregulates MHC-I expression on influenza-infected A549 cells, in a P53-dependent manner (199). The nucleoprotein (NP) and non-structural protein 1 (NS1) of influenza virus have been shown to interact with P53 protein (224, 225), which may play a role in the activation of P53 signalling and MHC-I upregulation in the infected cells. NS1 has various accessory functions during infection, and has been shown to interact with several host proteins that are important in regulating viral and host gene expression (226). Infecting mice with influenza virus lacking NS1 would let us know whether or not NS1 has a role in MHC-I upregulation during the infection.

Besides influenza virus, enhanced MHC-I expression was also detected following several virus infections such as: dengue virus (227), respiratory syncytial virus (RSV) (228), west nile virus (229), rhinovirus (230) and hepatitis c virus (HCV) (231). HCV-infected liver

cells, HepG2, and infected-primary human hepatocytes show enhanced MHC-I expression in the presence of HCV-core protein. Interestingly, similar to influenza virus, MHC-I was upregulated in a P53-dependent manner, in which P53 induced TAP1 (Transporter associated with antigen processing 1) activation, resulting in enhanced MHC-I expression (231). It is known that P53 is important to enhance MHC-I modulation by activating TAP1(232). Since most tumor cells do not have a functional P53, MHC-I expression is often downregulated on tumor cells (232). Importantly, HepG2 cells expressing the HCV-core protein were resistant to NK cell cytotoxicity, unlike parental HepG2 cells, in an MHC-I-dependent manner. Treatment of the parental HepG2 cells with IFN-γ also enhanced MHC-I modulation and protected it from NK cell responses (231). Likewise, MHC-I upregulation following dengue virus infection enhanced NK cell inhibitory receptor binding, which in turn inhibited NK cell cytotoxic functions (233). It has been previously reported that influenza stimulates reorganization of MHC-I molecules on the infected cells (193). This reorganization of MHC-I expression enhanced NK cell inhibitory receptor binding, which in turn inhibited NK cells cytotoxicity (193, 234). These findings agree with my result in which NK cells that express self-Ly49 inhibitory receptors proliferate and respond significantly lesser than the Ly49-deficient NK cells, most probably because upregulated MHC-I expression and binding with the inhibitory Ly49 receptors inhibits licensed NK cell proliferation (Figure 5B). Ly49-deficient mice resist influenza virus infection most possibly because NK cells in NKC^{KD} mice do not receive inhibitory signals, thus enhancing their cytotoxic responses (Figure 7A). This suggests that the engagement between upregulated inhibitory Ly49 receptors and its ligand inhibit licensed NK cells from responding to the infection. The percentage and number of unlicensed NK cells in WT mice is significantly

lower than in Ly49-deficient mice (Figures 6A and 9), therefore, they are unable to offer same level of protection as in the Ly49-deficient mice.

A previous study showed that NK cells contribute significantly in lung pathology following influenza infection, thus depleting NK cells resulted in lower mortality in the influenza-infected mice (180). The hyperactivity of NK cells might be responsible for the immune pathology, thus NK cell depletion protected mice from influenza infection. Accordingly, one would assume that because of the hyporesponsive nature of the unlicensed NK cells in NKC^{KD} mice (96), these mice will suffer lesser lung immune pathology and better protection from the infection. However, depleting NK cells from NKC^{KD} mice during influenza infection demonstrated that unlicensed NK cells are actually functional and are required to protect the NKC^{KD} mice from the infection (Figure 8B). Although the frequency of activated NK cells in WT and NKC^{KD} mice are almost the same, the quantity of IFN-γ^+ and degranulating unlicensed NK cells are significantly higher in the lung of NKC^{KD} mice in compression to the lung of WT mice (Figure 9A). Higher number of activated unlicensed NK cells in the lung of NKC^{KD} mice is associated with better survival.

In my assays, I did not detect any differences in IFN-γ production and degranulation between the licensed and the unlicensed NK cells in WT mice after the infection. These assays were done by incubating NK cells with YAC-I cells to induce NK cell activation. YAC-I cells express a ligand for the NKG2D activating receptor, and has low surface MHC-I expression level (235), making them an excellent target for the activation of licensed NK cells. This may have resulted in our inability to see activation differences between the licensed and unlicensed NK cells following influenza infection. Use of better target cells such as, influenza-infected primary lung epithelial cells of adult mice or alveolar

macrophage, might better reflect what is happening during influenza infection *in vivo*. Moreover, it is important to determine how exactly NK cells recognize influenza-infected cells, since the importance of NKp46 activating receptors is still questionable(44, 45).

MHC-I:Ly49 interaction enhances severity of influenza virus infection

Our pulmonary histopathology studies showed that WT mice develop severe lung pathology in comparison to Ly49-deficient mice. The lungs of WT mice showed significant lymphocytic infiltration along with pulmonary edema, and the presence of cell debris (Figure 10). On the other hand, Ly49-deficient mice showed less lung inflammation in comparison to WT lungs. Severe influenza infection is known to enhance lung damage either due to enhanced virus replication in the lung or due to severe immune responses, or a combination of both (145, 166). NK cells have been suggested to a play a negative role during influenza infection, which contributes to the severity of the lung pathology (180). Our studies show that NK cells are required in controlling influenza infection, and unlicensed NK cells are confer better protection against the infection than the licensed NK cells. During infection, epithelial cells express high levels of MHC-I which can directly inhibit killing by the licenced NK cells, but not unlicensed NK cells (Figure 4). Influenza viral load in the lung of Ly49-deficient mice were significantly lower than WT mice (Figure 11C). Lung pathology and viral load results in WT mice suggest that because licenced NK cells cannot eliminate influenza-infected cells as efficient as unlicensed NK cells, there is a higher leukocyte recruitment into the lung in order to assist in fighting against the infection. Lymphocyte recruitment along with higher viral load in the lung of WT mice may have induced severe lung damage seen in these mice. In humans, inhibitory KIR2DL3 and KIR2DL2 and their

cognate ligands, were enriched in H1N1/09 patients, suggesting that the interaction between NK inhibitory KIR receptors and their ligand in influenza infection contribute to greater disease severity (195). Moreover, another study showed that the frequency of KIR2DL5 allotype in patients with severe H1N1/09 pandemic influenza infection were higher than in the control population (194). These findings suggest that similar to the MHC-I:Ly49 interaction, severe influenza illness in human may be due to the MHC-I:KIR interaction during the infection. Nevertheless, these reports were done by using small patient cohorts and thus further analyses are required using larger groups of subjects to truly define the clinical relevance of MHC-I:KIR engagement in the harshness of the infection. Additionally, functional mechanisms dependent on KIR2DL2, KIR2DL3 and KIR2DL5 and epidemiological associations have not yet been defined. An *in vitro* study showed that following influenza virus infection, the engagement of KIR2DL1-Ig to influenza-infected cells is significantly enhanced in an MHC-I-dependent manner (193). Enhanced KIR2DL1-Ig binding to MHC-I might result in preventing NK cell responses against influenza-infected cells, confirming that KIR:MHC-I engagement is not beneficial for the host during influenza virus infection. Interestingly, several hours post-infection, *in vitro* infected cells were shown to express high levels of influenza-hemagglutinin, that bind to NKp46-Ig fusion proteins, and became vulnerable to targeting by human NK cells (193). It is known that NK cell effector function is determined by a net balance of signals that they receive from the inhibitory and activating receptors, and in this case, it is possible that the NKp46:hemagglutinin activating signals are dominate over the MHC-I:KIR inhibitory signals (193). These results suggest that at early time points, NK cells cannot eliminate influenza-infected cells because the inhibitory signal from MHC-I:Ly49/KIR interaction

would be dominant. However, during later phase of infection, the activating signal from NKp46: hemagglutinin becomes dominant, which activates the NK cell response against the influenza-infected cells. Nonetheless, the presences of a large target pool at later stages of the disease may result in a strong NK immune response that could contribute to the lung immunopathology. This situation could also apply to the WT mice infected with influenza virus. In the Ly49-deficient mice, the activating signals are expected to dominate from the beginning of the infection, resulting in a better control of infection and preventing excessive lung immunopathology due to hyperactive NK and/or other immune cells (Figure 10B and 10D).

Transgenic expression of self-Ly49I into NKC^{KD} mice ($NKC^{KD}xLy49I^{tg}$) forced the expression of inhibitory Ly49I receptor on all NK cells in this mouse model. NK cells of the $NKC^{KD}xLy49I^{tg}$ mice have been shown to be able identify and eliminate tumors superior than NK cells of NKC^{KD} mice *in vitro* and *in vivo* (96, 201). Unlike NKC^{KD} mice, $NKC^{KD}xLy49I^{tg}$ mice could not survive the infection (Figure 15), confirming that the presence of self-inhibitory Ly49 contribute significantly to the morbidity during influenza infection. Re-expression of self-inhibitory Ly49 into influenza-resistant NKC^{KD} mice restores MHC-I:Ly49 interactions. Thus enhanced MHC-I expression would inhibit their cytotoxic function, resulting in lethal influenza infection. This data again shows the significance of NK cells in the infection. Additionally, this observation suggests that during influenza infection NK cells can either protect from infection, or enhance illness, depending on the presence or absence of interaction between inhibitory self-Ly49 receptors and MHC-I.

To further confirm that MHC-I:Ly49 interaction is not beneficial for the host during influenza infection, I showed that mice also lacking MHC-I can survive influenza infection better than WT mice (Figure 18). It was originally thought that since these mice also lack $CD8^+$ T cells, they do not develop severe lung immunopathology during influenza virus infection and thus show better survival than the WT mice. However, NK depletion renders these mice susceptible to influenza infection, confirming the importance of NK cells during the infection and thus ruling out the possibility that the absence of T cells protected the mice (Figure 18). Similarly, unlisniced NK cells have been shown to respond strongly to MCMV infection (236). These results show that the presence of either MHC-I or Ly49 is not beneficial for the host during influenza infection. The level of protection in MHC-I- or Ly49-deficient mice appears to be due to the presence of large numbers of NK cells that do not get inhibited by the MHC-I:Ly49 interactions. In the WT mice on the other hand, there are significantly lower numbers of NK cells which do not express self-inhibitory receptor and do not receive the inhibitory signals form Ly49:MHC-I interactions during influenza virus infection, hence showing higher susceptibility to the infection.

Blockade of the engagement between Ly49 and its ligand using $F(ab)_2$ fragments of the 5E6 mAb resulted in better survival of WT mice post-influenza infection (Figure 19). 5E6 $F(ab)_2$ blockade might enhance cytotoxic response of lisniced NK cells against influenza-infected cells, thus blockade of NK self-inhibitory receptors would stimulate antiviral reactions *in vivo* which enhances mouse survival. Survival of WT mice treated with 5E6 $F(ab)_2$ mAb confirm the inability of the $Ly49^+$ NK cells to lyse and eliminate influenza-infected cells. Blockade of Ly49C and I using 5E6 $F(ab)_2$ mAb also enhanced NK cell cytotoxicity toward acute myeloid leukemia cells, C1498, and EL4 lymphoma cells *in vitro*

(237). Moreover, mice treated with 5E6 $F(ab)_2$ mAb have significantly enhanced survival when challenge with a lethal dose of C1498 leukemia cells (237). These result confirm that tumor- or virus-infected cells that bear MHC-I induce a strong negative signal via the inhibitory Ly49 receptors, which in turn enhances tumor or virus growth. Correspondingly, these results demonstrate that MHC-I:Ly49 interaction on licensed NK cells can be responsible for their decreased antiviral and antitumor activity, and that blockade of self-Ly49 receptors increases their cytotoxic effects. Since MHC-I:Ly49 interaction play a significant role in inhibiting NK cell function, several viruses such as MCMV and HCMV encode a homologue of MHC-I that enables them to evade NK cell cytotoxicity (238, 239). Recombinant MCMV which does not encode for the MHC-I mimic, m144, replicate significantly slower than WT MCMV in mice. As well, NK cell depletion restores recombinant MCMV replication (238), indicating that NK cells are responsible for the control of the recombinant MCMV strain lacking the expression of m144 MHC-I mimic.

One of the concerns with the blockade of self-Ly49/KIR receptor is that it might enhance NK cell's autoreactivity against self-cells, as host MHC-I would not engage with NK cell inhibitory Ly49 receptors. However, Koh et al., has shown that using 5E6 $F(ab)_2$ mAb to block self-Ly49:MHC-I interactions did not result in an increased autoreactivity of NK cells *in vivo* (237). Based on this concept, anti-KIR-blocking mAb enhanced NK cell cytotoxicity toward MHC-I-expressing tumor cells deprived of killing self-cell in a KIR transgenic mice model (240). Humanized anti-KIR mAb is presently being examined in clinical trials and NK cells show no autoreactivity and remain tolerant in treated patients (241). Anti-KIR mAb can be used to determine whether blockade of MHC-I:KIR would be favourable in influenza infection in humans.

Although blockade of MHC-I:Ly49 interaction protected WT mice during influenza infection, however the mortality rate was still high. Other inhibitory receptors on NK cells may also be involved in the harshness of influenza illness in the WT mice in a way similar to the Ly49 receptors. We have shown similar NKG2A expression on NK cells in WT and MHC-I-deficient mice (Figure 20A), implying that it might not participate in the severity of the infection. Other studies have indicated that NKG2A on NK cells does not fundamental in influenza infection (242, 243).

Increased lymphocyte recruitment into the lung of WT mice enhanced mortality

At baseline, prior to influenza infection, NK cells represent almost 15-20% of total lymphocytes in the lung of both WT and NKC^{KD} mice (Figure 12E and 12F). NK cells play a major role in fighting against pathogen and because lungs are frequently exposed to a large number of airborne pathogens due to daily inhalation, NK cells are present in the lung at a high number and ready to respond to any infection. Five days post-infection, NK cells increased rapidly in the lungs (Figure 12E and 12F). Until day 5 post-infection, influenza-specific T cells were not detected in the lungs (Figure 12A-12D), confirming that the differences we see in the viral load between the two mouse groups is due to the NK cell response. Importantly, early recruitment of NK cells to the lungs is associated with better survival and lower influenza virus load in the lung. NK cell numbers and percentages were almost the same between WT and NKC^{KD} mice, however, NKC^{KD} mice have more Ly49-deficient NK cells, which are more beneficial for the host than WT NK cells. On day 7 post-

infection, both the number and percentage of NK cells and influenza-specific $CD8^+$ T cells increased in the lung of WT mice but not NKC^{KD} mice. This suggests that the inability of NK cells in WT mice to eliminate the infection at an early time post-infection enhanced infiltration of more inflammatory cells into the lung, possibly leading to a more severe lung pathology and mortality of the infected WT mice.

Severe influenza infection is characterized by elevated the virus load, and high levels of proinflammatory cytokines in the lung, a condition known as "cytokine storm", which play a role in human as well as mice mortality during influenza infection (209). Increased adaptive and innate immune cell recruitment into the lung of WT, but not NKC^{KD} mice, would be expected to result in enhanced cytokine and chemokine production in the lungs, contributing to severe lung pathogenesis following infection. The levels of IFN-γ, TNF-α, IL-17, MCP-1 (CCL2), MCP-3 (CCL7), and MIB-1β (CCL4) were significantly higher in the lung of WT, but not NKC^{KD} mice (Figures 14A-14F). IFN-γ and TNF-α are mainly produced by NK cells, Th1 cells, $CD8^+$ T cells, macrophages, and dendritic cells (DCs) (210, 244-248). MCP-1 and MCP-3 are known to induce migration of activated NK cells toward the inflammation (249). These two chemokines were produced at higher levels 5 days post-infection in WT mice which may explain why there are more NK cells in the WT mice in comparison to NKC^{KD} mice on day 7 post-infection (Figure 12E and 12F). Influenza-infected macrophages have been shown to produce MCP-1, MCP-3, and MIP-1β, along with other chemokines (166). Moreover, in response to influenza infection, lung epithelial cells produce MCP-1 in order to recruit more immune cells (166). Additionally, MIP-1β and MCP-1 have been found in nasopharyngeal secretions of "experimentally" influenza-infected human volunteers (250). In mice, neutralizing MCP-1during invasive aspergillosis,

a fungal lung infection, resulted in a significant reduction in NK cell recruitment into the lung, which enhanced pathogen burden in the lungs, and increased mouse mortality(251). Thus, the presence of these chemokines seems to be important in fighting against influenza virus infection, however, over production of these chemokines would recruit more inflammatory cells into the lung resulting in increased lung damage.

NK cells isolated from infants with IFN-γ deficiency have a reduction in NK cell activity against the leukemia cell line (Molt 4F), and suffer from recurrent infections(252). Interestingly, *in vivo* neutralization of IFN-γ during influenza infection reduced cell recruitment into the lung (253). This could be due to the role of IFN-γ to activate the innate immune cells, especially macrophages, which produce chemokines that further enhance cell recruitment to the site of infection. However, a complete neutralization of IFN-γ may not be beneficial to the host, since NKC^{KD} mice were no longer protected from influenza infection when IFN-γ was neutralization (Figure 16B). Thus, IFN-γ appears to be important during an immune response against influenza virus infection but if produced in excessive quantities, it may lead to enhanced lung pathology and lethality due to the infection.

TNF-α, on the other hand, has been linked to lung immunopathology during influenza infection(254). TNF depletion during influenza virus infection reduced lung immuno-pathology and enhanced mice survival without affecting virus load(255). Similarly, influenza-infected TNF receptor 1 (TNFR1)-deficient mice exhibit significantly less weight lost in comparison to WT mice, and again the levels of the virus did not change(220). TNFR1- deficient mice show minimal lung inflammation and less necrosis in comparison to the lungs of WT mice(256). Intracellular IFN-γ and TNF-α staining of NK cells 5 days post-infection showed that they are not the main producer of these cytokines during the infection

(Figure 14G and 14H). Other leukocytes such as alveolar macrophage or DCs could be the main producers since they are also the first line of defence and respond early during infection. Macrophages, DCs, and myeloid cells are known to be a good target for influenza virus during infection (160, 257). Influenza-infected myeloid cells can be targeted by activated NK cells (158). It is possible that NK cells from NKC^{KD} mice can target these cells more powerfully than the WT NK cells, thereby, reducing their numbers, and consequently, the amount of chemokines and cytokines released by them. Although NK cells themselves may not contribute much to the cytokine and chemokine pool of the infected lungs, their major role appears to be the elimination of infected cells, which seems to be more efficient in the Ly49-deficient mice compared to the WT mice. This in turn would result in lesser lung inflammation and lung immunopathology.

Unlicensed NK cells require the perforin cytotoxic pathway for protection

NK cells eliminate target cells by two central mechanisms: i) perforin and granzymes that are produced from cytotoxic granules, or ii) death receptor activation pathway, such as FasL and TRAIL. Herein, I found that in the absence of perforin, NKC^{KD} mice mortality rates increased following influenza virus infection (Figure 16A). Cytolysis by perforin-dependent pathway is required to protect NKC^{KD} mice from the infection, suggesting that direct targeting of infected cells by NK cells are required. The perforin-dependent pathway is used by cytotoxic T cells along with NK cells. As I have determined earlier, influenza-specific T cells start to increase in numbers in the lung by day 7 post-infection, whereas the mice start to die by day 8, suggesting that perforin-deficient T cells have a minimal impact on mouse survival. Perforin-deficient NKC^{KD} mice have significantly higher viral load in the

lungs at day 5 and 7 post-infection (Figure 17). Again, this result demonstrates the importance of perforin during the infection. Although I show that perforin is very important in fighting against influenza infection, TRAIL and FasL have been shown to be important during influenza infection as well (258-260). It was observed that 4 days post-infection, almost 40% of lung NK cells express TRAIL and that number increases to 80% of lung NK cells by day 7 post-infection (261). TRAIL-deficient mice and anti-TRAIL monoclonal antibody enhanced viral load in the lung and augmented mouse death due to influenza infection (259, 261). NK cells additionally produce IFN-γ to exert direct and indirect antiviral effects, with observed elevated levels in the lung. Thus, neutralizing IFN-γ in NKC^{KD} mice during influenza infection made mice susceptible to infection (Figure 16B). Moreover, neutralizing IFN-γ in NKC^{KD} mice increased influenza viral load in the lung (Figure 17), but to a level lower than perforin-deficient NKC^{KD} mice.

Influenza virus infection enhances inhibitory mouse Clr-b and human LLT-1 ligand expression on lung epithelial cells

Here I show that influenza virus infection enhances Clr-b expression on mouse lung epithelial cells, as well as human LLT1 on human cell lines (Figures 21A and 21B). Similar to MHC-I:Ly49 interaction, Clr-b:NKR-P1B interaction inhibits NK cell cytotoxic function (65). Clr-b is expressed normally on most tissues, and is known as a health marker that is downregulated during stress(27, 67). MCMV, ectromelia virus, and vaccinia virus infection have been shown to enhance MHC-I downregulation, that in turn augments NK cells activation (67, 262). Interestingly, unlike other viruses, influenza virus induces Clr-b upregulation in an IFN-I-independent manner (Figure 21A). This could partly explain why

blockade with 5E6 $F(ab)_2$ mAb did not completely rescue influenza-infected WT mice. To further confirm the role of Clr-b:NKR-P1B interaction during influenza infection, Clr-b^{KO} and NKR-$P1B^{KO}$ mice are protected from the infection (Figures 23A and 23B). These results imply that similar to Ly49:MHC-I engagement, Clr-b:NKR-P1B interaction contributes to the severity of influenza infection in WT mice. Influenza also enhanced human LLT1, which bind to the inhibitory NKR-P1A or CD161 receptor (263). LLT1:NKR-P1A interactions have been reported to inhibit human NK cell functions (264). Like influenza infection, human RSV has been shown to upregulate LLT1 expression on human bronchial epithelial cells, however the effect of this upregulation on human NK cells has not tested (265). These data suggest that Clr-b:NKR-P1B may protect influenza virus-infected cells from NK cell recognition and killing, which in turn enhances influenza-mediated illness.

Conclusion

In conclusion, this study demonstrated that NK cells play a major role in influenza virus infection. As an evasion mechanism, influenza virus enhances the expression inhibitory ligands for NK cell receptors, which can strongly inhibit the NK cell cytotoxic response toward influenza-infected cells. Unless we block the engagement between these inhibitory receptors and their ligand, influenza infection will be severe in the host. Blockade of the inhibitory receptor:ligand interactions has been shown to enhance NK cell-mediated antitumor responses, and I believe it could also enhance NK cell's antiviral activity. Moreover, this study showed that the NK cell education hypothesis is more complex than previously appreciated, and that the unlicensed NK cells can be highly responsive under certain conditions. Herein, I show that unlicensed NK cells are functional and contribute significantly in limiting influenza virus infection.

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Appendix

Appendix I: Permission to use Publication

Journal Information

Reflecting the full breadth of research on bacteria, fungi, parasites, prions and viruses, *PLOS Pathogens* publishes outstanding original research and commentary that significantly advance the understanding of pathogens and how they interact with their host organisms.

Scope

Bacteria, fungi, parasites, prions and viruses cause a plethora of diseases that have important medical, agricultural, and economic consequences. Moreover, the study of microbes continues to provide novel insights into such fundamental processes as the molecular basis of cellular and organismal function.

PLOS Pathogens reflects the full breadth of research in these areas by publishing outstanding original articles that significantly advance the understanding of pathogens and how they interact with their host organisms. Topics include (but are not limited to) adaptive and innate immune defenses as well as pathogen countermeasures, emerging pathogens, evolution, genomics and gene regulation, model host organisms, pathogen-cell biology, pathogenesis, prions, proteomics and signal transduction, rational vaccine design, structural biology, and virulence factors.

The journal will not consider purely descriptive studies, such as those that solely identify a new genomic sequence of a related pathogen or a series of related pathogens, the isolation of pathogen variants, or a new strain or type based only on sequence analysis or correlative studies of host and pathogen genotypes.

Genomics studies, which include functional predictions or inferences based on genome sequence analysis, will also require additional experimental validation that directly tests the prediction/inference and yields novel conclusions about mechanistic models or pathogenesis. Instances where inferences about potential functions can be supported by association studies of genotype-phenotype combined with existing functional data, may also be appropriate, pending approval by the editors. If a paper provides novel mechanistic insight but lacks functional validation, it may be considered on a case-by-case basis.

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Appendix II: Publication "Influenza Virus Targets Class I MHC-I Educated NK Cells for Immunoevasion"

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RESEARCH ARTICLE

Influenza Virus Targets Class I MHC-Educated NK Cells for Immunoevasion

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OPEN ACCESS

Citation: Mahmoud AB, Tu MM, Wight A, Zein HS, Rahim MMA, Lee S-H, et al. (2016) Influenza Virus Targets Class I MHC-Educated NK Cells for Immunoevasion. PLoS Pathog 12(2): e1005446. doi:10.1371/journal.ppat.1005446

Editor: Steven M. Varga, University of Iowa, UNITED STATES

Received: July 10, 2015

Accepted: January 19, 2016

Published: February 29, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by operating grant# MOP 62841 from the Canadian Institutes of Health Research (http://www.cihr-irsc.gc.ca/e/193.html) awarded to APM. APM is a Tier II Canada Research Chair Holder in Innate Pathogen Resistance. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

The immune response to influenza virus infection comprises both innate and adaptive defenses. NK cells play an early role in the destruction of tumors and virally-infected cells. NK cells express a variety of inhibitory receptors, including those of the Ly49 family, which are functional homologs of human killer-cell immunoglobulin-like receptors (KIR). Like human KIR, Ly49 receptors inhibit NK cell-mediated lysis by binding to major histocompatibility complex class I (MHC-I) molecules that are expressed on normal cells. During NK cell maturation, the interaction of NK cell inhibitory Ly49 receptors with their MHC-I ligands results in two types of NK cells: licensed ("functional"), or unlicensed ("hypofunctional"). Despite being completely dysfunctional with regard to rejecting MHC-I-deficient cells, unlicensed NK cells represent up to half of the mature NK cell pool in rodents and humans, suggesting an alternative role for these cells in host defense. Here, we demonstrate that after influenza infection, MHC-I expression on lung epithelial cells is upregulated, and mice bearing unlicensed NK cells (Ly49-deficient NKC^{KD} and MHC-I-deficient $B2m^{-/-}$ mice) survive the infection better than WT mice. Importantly, transgenic expression of an inhibitory self-MHC-I-specific Ly49 receptor in NKC^{KD} mice restores WT influenza susceptibility, confirming a direct role for Ly49. Conversely, $F(ab')_2$-mediated blockade of self-MHC-I-specific Ly49 inhibitory receptors protects WT mice from influenza virus infection. Mechanistically, perforin-deficient NKC^{KD} mice succumb to influenza infection rapidly, indicating that direct cytotoxicity is necessary for unlicensed NK cell-mediated protection. Our findings demonstrate that Ly49:MHC-I interactions play a critical role in influenza virus pathogenesis. We suggest a similar role may be conserved in human KIR, and their blockade may be protective in humans.

Author Summary

Influenza virus has developed a number of immune-evasion mechanisms to prolong its survival within the host. Development of functional NK cells is dependent on multiple factors such as the interaction between MHC-I and Ly49 receptors. NK cells that develop in the absence of these interactions are referred to as 'unlicensed' and represent up to half of the total number of NK cells. We show that significant MHC-I upregulation on lung epithelial cells following influenza virus infection most likely allows influenza virus to evade detection by licensed NK cells. Importantly, we demonstrate that unlicensed NK cells play a major role in protecting mice from influenza infection. Both Ly49- and MHC-I-deficient mice, which possess unlicensed NK cells, exhibit better survival than WT mice when infected with a lethal dose of influenza virus. Survival of Ly49-deficient mice is associated with reduced viral titers and lung pathology, compared to the infected WT mice. Moreover, disrupting the interaction between MHC-I and inhibitory Ly49 receptors protects WT mice from a lethal influenza virus infection. These results suggest that the so-called unlicensed NK cells, previously characterized as being hyporesponsive, actually possess potent antiviral activity, and are crucial for protection from influenza virus and possibly other viral infections.

Introduction

Influenza viruses are classified as members of the Orthomyxoviridae family, which are enveloped viruses with a segmented, negative, single-stranded RNA (ssRNA) genome that contains 7–8 gene segments. Structurally, influenza A virus expresses two surface glycoproteins, hemagglutinin and neuraminidase [1, 2]. Influenza A virus can cause severe human illness, including upper and lower respiratory tract infections and pneumonia, and is associated with major human pandemics. Seasonal influenza epidemics result in 250,000–500,000 deaths worldwide annually [3].

NK cells are innate lymphocytes that play a critical role in host defense against tumors and virus infection, both by directly eliminating them and by enhancing the rapid development of adaptive responses [4–6]. NK cells are important for protection against influenza virus infection in various animal models [5, 7, 8]. In response to NK cell cytolytic function, influenza virus has developed several evasion strategies to escape NK cell recognition [9, 10]. Importantly, influenza virus infection was shown to induce accumulation of MHC-I molecules in the lipid raft microdomains of infected cells, leading to increased binding of the NK cell inhibitory receptor KIR2DL1 and inhibition of human NK cell cytotoxicity *in vitro* [11, 12].

NK cell effector functions are tightly controlled by the combination of signals received through germline-encoded activating and inhibitory receptors [6, 13]. Mouse NK receptors include the Ly49, NKG2, and NKR-P1 families of receptors encoded in the Natural Killer gene Complex (NKC) on chromosome 6 [13, 14]. Inhibitory receptors engage molecular indicators of health, while activating receptors engage indicators of disease. By integrating these signals, the NK cell can appropriately spare or destroy a potential target [14]. Ly49 family members are type II transmembrane glycoproteins, part of the C-type lectin superfamily that forms disulphide-linked homodimers [15]. The mouse Ly49 are functionally equivalent to human killer-cell immunoglobulin-like receptors (KIR). The ligands for KIR and Ly49 receptors are self MHC-I molecules or MHC-I related molecules that are expressed by pathogens upon infection [5, 16, 17].

Beyond regulating NK cell killing, interactions between MHC-I and Ly49 receptors are required for NK cell education. The licensing hypothesis states that, to be fully functional, a developing NK cell must successfully engage a self-ligand with an inhibitory receptor [18, 19]. In a C57BL/6 mouse, this is canonically achieved by engagement of MHC-I by Ly49C and/or Ly49I. Accordingly, NK cells that do not express Ly49C/I, or cells from MHC-I-deficient or Ly49-deficient (NKC^{KD}) mice, are 'unlicensed', displaying attenuated responses to MHC-I-deficient tumors *in vitro* and *in vivo* [19–22]. NKC^{KD} mice also develop lymphomas earlier than WT mice, again suggesting a degree of dysfunction in unlicensed NK cells [22]. Despite being unlicensed, however, these Ly49C/I^- cells represent up to half of the population of mature NK cells in a healthy, WT mouse [9, 18, 23–25], suggesting a role for these cells in host defense.

Since these unlicensed cells are dysfunctional with regard to rejecting MHC-I-deficient tumors, their role in host defense may be in NK-mediated anti-pathogen activity. MHC-I-deficient ($B2m^{-/-}$) mice, which bear only unlicensed NK cells, exhibit robust NK cell responses and can control mouse cytomegalovirus (MCMV) infection as efficiently as WT mice [26, 27]. A publication by Orr et al. found that adoptively transferred unlicensed Ly49C/I^- Ly49$G2^+$ NK cells into MCMV-infected neonates enhanced their survival better than the licensed, Ly49C/I^+ Ly49$G2^+$ cells [28]. For a more in-depth analysis of the role of unlicensed NK cells in viral infection, we have used Ly49-deficient (NKC^{KD}) mice generated in our laboratory [20], in which approximately 80% of NK cells are unlicensed. Thus, NKC^{KD} mice serve as a model to study the role of unlicensed NK cells during viral infections. In this study, we explore the interactions of influenza virus with licensed and unlicensed NK cells. We present evidence that influenza effectively evades licensed NK cells, but is eliminated by unlicensed NK cells in a perforin-dependent manner. Importantly, genetic and physical disruption of Ly49 binding to its MHC-I ligands results in enhanced NK cell-mediated control of influenza virus infection *in vivo*, implicating virus-induced MHC-I expression as an immunoevasion strategy.

Materials and Methods

Mice

C57BL/6 (B6) and B6.129P2-$B2m^{tm1}$Unc/J ($B2m^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), $Ifnar1^{-/-}$ were obtained from Dr. Subash Sad (University of Ottawa, Ottawa, ON). B6.NKC^{KD}, B6.NKC^{KD}-$Ly49I^{tg}$, B6.$Ly49Q^{KO}$, and their congenic control, B6.$Ly49^{129}$ mice, have been previously described [20, 29]. Mice deficient in both Ly49 (NKC^{KD}) and perforin ($Prf^{-/-}$) were produced by mating B6.NKC^{KD} with B6.$Prf^{-/-}$ mice. B6.NKC^{KD}, B6.$Ly49Q^{KO}$ and WT controls were bred as homozygous pairs. B6.NKC^{KD}-$Ly49I^{tg}$ and B6.$Prf^{-/-}$ mice were bred as heterozygous mating pairs, and littermates were used for experimentation. Assessment of genotypes was performed by PCR.

Ethics statement

All mice were maintained in a specific-pathogen-free environment. All breeding and manipulations performed on animals were approved by the University of Ottawa animal care committee (protocol BMI-2049) in accordance with the principles published in the Canadian Council on Animal Care's "Guide to the Care and Use of Experimental Animals" and with the Animals for Research Act, R.S.O. 1990, c.22, s. 17(1–3).

Influenza virus infection

Groups of age and sex-matched mice (6–8 weeks old) were anesthetized with isoflurane and inoculated intranasally with 600 or 1050 PFU of mouse-adapted A/FM/1/47-MA (FM-MA)

strain influenza virus [30]. Influenza-infected mice were housed in a level 2 confinement area for the duration of the experiment. Body weight was measured daily. Animals were considered to be at endpoint if weight loss exceeded 25% of the body weight prior to infection, or if the animal was moribund. Viral loads of infected mice were determined by plaque assay, as described previously [30]. Virus titer is expressed as the number of plaque forming units per gram of lung (PFU/g).

In vivo mAb treatments

Anti-NK1.1 mAb (clone PK136), anti-IFN-γ mAb (clone XMG1.2), and anti-Ly49C/I $F(ab')_2$ mAb (clone 5E6) were injected i.p. into groups of age and sex-matched WT mice. 200 μg of mAb per mouse were injected i.p. two days prior to influenza virus infection, on the day of infection, and every two days post-infection until day 10 p.i. Anti-AsialoGM1 antibody (Wako Pure Chemical Industries, Osaka, Japan) was injected i.p. two days prior to influenza virus infection (25 μl), on the day of infection (25 μl), and every three days post-infection (10 μl) until day 10 p.i.

Lung epithelial cell isolation and staining

Lungs were removed and minced in 5 ml RPMI with 0.5 mg/ml collagenase D (Roche), followed by incubation for 1 h at 37°C with agitation. The minced pieces were crushed on a 70 μm cell strainer to prepare single cell suspensions for flow cytometry as previously described.

Antibodies and flow cytometry

Anti-mouse CD18 (LFA-1), CD326 (EpCAM), MHC-I ($H\text{-}2K^b$), 5E6 (anti-Ly49C/I), 4D11 (anti-Ly49G), CD8 (CD8β), CD4, CD3, TCRβ, NKp46 (CD335), NKG2D (CD314), NKG2A (16a11), NKG2A/C/E (20d5), CD27, CD11b, CD107a (1D4B), IFN-γ (XMG1.2), and Live/Dead stain were purchased from eBioscience (eBioscience, San Diego, CA, USA). Anti-NKG2D (CD314) was purchased from BioLegend (BioLegend, San Diego, CA, USA). Anti-mouse TCRβ chain was purchased from BD Biosciences (BD Biosciences, Mississauga, Ontario, Canada). PK136 (anti-NK1.1), 5E6 (anti-$Ly49C/I^{B6}$), and XMG1.2 (anti-IFN-γ) hybridomas were kind gifts from Drs. James Carlyle (Sunnybrook Research Institute, Toronto, ON), Charles Sentman (Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire), and Subash Sad (University of Ottawa, Ottawa, ON), respectively. Cell fluorescence data was acquired with a CyAN-ADP flow cytometer (Beckman Coulter) and analyzed with Kaluza software (Beckman Coulter, New Jersey, USA).

The levels of cytokines and chemokines in lung tissue homogenates were measured by bead array flow cytometry using the mouse Th1/Th2/Th17/Th22-13plex FlowCytomix multiplex kit and mouse chemokine 6plex kit (eBioscience, San Diego, CA, USA).

Tetramer staining

Streptavidin-PE conjugated influenza A non-structural protein $(NS2)_{114\text{-}121}$ (RTFSFQLI) and nucleocapsid protein $(NP)_{311\text{-}325}$ (QVYSLIRPNENPAHK) tetramers were kindly provided by the NIH Tetramer Core Facility at Emory University (Emory University Vaccine Center, Atlanta, GA). $5x10^5$ lung cells were stained with 1 μg of tetramer in 20 μL of cRPMI and incubated for 1 h at 37°C. Influenza virus-specific $CD8^+$ T cells were stained with $H\text{-}2K^bNS2_{114\text{-}121}$ (RTFSFQLI) tetramer, while influenza virus-specific $CD4^+$ T cells were stained with the $I\text{-}A^b$ $(NP)_{311\text{-}325}$ (QVYSLIRPNENPAHK) tetramer.

In vitro NK cell assay

Total lymphocytes isolated from the lungs were incubated with YAC-1 cells at 1:1 ratio or with phorbol 12-myristate 13-acetate (PMA, 10 μg/ml) and ionomycin (1 μg/ml) in the presence of anti-CD107a mAb and brefeldin A (eBiosience) for 4 h. Cells were stained for surface markers followed by intracellular staining for IFN-γ using IC fixation and permeabilization reagents (eBioscience) following manufacturer's instructions.

Purification and modification of mAbs

Individual hybridoma clones were cultured in DMEM supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture supernatants were then centrifuged (10,000x g for 20 min) and filtered through a 0.45 μm filter. Monoclonal antibodies (mAb) were purified using Protein G sepharose chromatography (ExalphaBiologicals, Inc, USA). Monoclonal Ab was dialyzed against 1x PBS buffer (pH 7.4) and then concentrated using an Amicon ultra-15 centrifugal filter unit with an ultracel-100 kDa membrane (EMD Millipore Corporation, MA, USA). Monoclonal Ab concentration was determined by SDS-PAGE gel and by spectrophotometric measurement at 280 nm.

To make 5E6 $F(ab')_2$ fragments [31], mAb was dialyzed twice against 100 mM sodium acetate solution (pH 4.0), digested using pepsin (Sigma-Aldrich, Ontario, Canada), and then dissolved in 100 mM sodium acetate solution (pH 4.0) at a 1:40 pepsin to mAb ratio for 10 h at 37°C. The digested mAb was dialyzed against 1x PBS buffer (pH 7.4) and then concentrated using an Amicon ultra-15 centrifugal filter unit with an ultracel-50 kDa membrane (EMD Millipore Corporation, MA, USA). $F(ab')_2$ fragments were then purified using protein A affinity chromatography. 5E6 $F(ab')_2$ fragment concentration was determined by SDS-PAGE gel and by spectrophotometric measurement at 280 nm. The purity of 5E6 $F(ab')_2$ fragments was determined by SDS-PAGE gel.

Lung histopathology

Lungs were collected from infected mice 7 days p.i. and fixed in 10% neutral buffered formalin (25 ml) for 24 h. Subsequently, lungs were embedded in paraffin, sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (H&E). Slides were examined under a microscope to score histopathologic changes in the lungs by a pathologist blind to the experimental conditions.

Statistical analysis

Statistical comparisons were made by a two-tailed Student's t-test, one-way ANOVA with Bonferroni post-hoc test, or Kaplan Meier survival statistical analysis (log rank test) using GraphPad Prism software (GraphPad, San Diego, USA). A p value <0.05 was considered statistically significant.

Results

Ly49-deficient mice are protected from lethal influenza virus infection

It is well established that influenza virus infection *in vitro* inhibits NK cell cytotoxicity by enhancing NK cell inhibitory receptor binding to MHC-I on infected human lung epithelial cells [11, 12]. It is possible that MHC-I has direct negative effects on NK cell activity during influenza virus infection in mice as well. To test this, we first determined whether influenza virus infection could modulate MHC-I expression on mouse lung epithelial cells. WT (B6)

mice were infected with 600 PFU of mouse-adapted influenza strain A/FM/1/47 (H1N1) (FM-MA) intranasally. MHC-I expression was determined on EpCAM^{+} (CD326) lung epithelial cells on day 5 post-infection (p.i.) by flow cytometry. In uninfected mice, low levels of MHC-I expression were detected on lung epithelial cells (Fig 1A). However, upon infection with influenza virus, lung epithelial cell expression of MHC-I was dramatically increased (Fig 1A). As a control, infected and uninfected MHC-I-deficient mice (*B2m*$^{-/-}$ mice) were included, and as expected, displayed no increase in MHC-I staining upon infection. Influenza virus infection is known to upregulate type I IFN (IFN-I) production, which in turn drives the upregulation of a variety of immunomodulatory proteins including MHC-I. To determine whether this upregulation is mediated by IFN-I production, we infected WT (B6) and mice deficient in the receptor for IFN-α and IFN-β (*Ifnar1*$^{-/-}$) with 600 PFU FM-MA virus intranasally. Lungs were harvested on day 5 p.i. Interestingly, expression levels of MHC-I were similar between WT (B6) and *Ifnar1*$^{-/-}$ mice (Fig 1B), confirming this upregulation was independent of IFN-I. These data demonstrate that influenza virus infection induces upregulation of MHC-I expression on lung epithelial cells. Increased expression of MHC-I on lung epithelial cells upon influenza virus infection may have implications for the inhibition of NK cells through interaction with inhibitory Ly49 receptors.

Recently, we reported that Ly49-deficient (NKC^{KD}) mice exhibit uncontrolled tumor growth and metastases [22]. Lacking licensed NK cells renders these mice highly susceptible to tumor formation, despite having otherwise normal mature NK cells. Flow cytometry analysis of lung lymphocytes showed that NK cells in the lungs of NKC^{KD} mice were mostly devoid of Ly49 expression (Fig 1C), but were otherwise predominantly mature cells (CD11b^{+} CD27^{low}) with normal expression of the activating receptors NKp46 and NKG2D (Fig 1C and 1D). To determine whether Ly49 interaction with MHC-I molecules is relevant to influenza infection *in vivo*, we inoculated WT and NKC^{KD} mice with 1050 PFU of FM-MA intranasally. The animals were observed daily for over two weeks, and sacrificed when moribund. Death due to infection began occurring on day 8 p.i. (Fig 1E). Two weeks p.i., almost 90% of the WT mice had succumbed to the infection, while unexpectedly, only 35% of the NKC^{KD} mice succumbed (Fig 1E, $^{**}p = 0.0072$). Next, we depleted NK cells in our mouse model to confirm the role of NK cells during influenza virus infection. This depletion was performed with anti-AsialoGM1 treatment instead of the standard NK1.1 treatment, as these mice express an allele of NKR-P1C that is not recognized by the NK1.1 antibody. Anti-AsialoGM1 is known to deplete some activated macrophages and CD8^{+} T cells at high doses [32, 33]. We used a dose that showed total NK depletion without T cell depletion (S1A Fig); however, depletion of activated T cells and activated macrophages remains a possibility. Depletion of NK cells from NKC^{KD} mice using anti-AsialoGM1 treatment (S1B Fig) resulted in a complete loss of protection (Fig 1F), indicating that NK cells from NKC^{KD} mice play a key role in protecting those mice from influenza. Very low (50 PFU) or high (5000 PFU) doses were too extreme to note any survival differences, but 600 PFU gave similar results to 1050 PFU (S2A Fig, Fig 1E and 1F), therefore, 600 PFU infection dose was used for the remainder of the study.

NKC^{KD} mice lack Ly49Q, which plays an important role in IFN-α production by plasmacytoid dendritic cells [29, 34]. To confirm that this survival advantage is not due to a loss of Ly49Q alone, we inoculated Ly49Q^{KO} mice with influenza virus. Like WT mice, Ly49Q^{KO} mice died 10 days post influenza virus infection (Fig 1E, $^{**}p = 0.0049$). NKC^{KD} mice also express lower levels of NKG2A/C/E. However, the inhibitory NKG2A is not believed to be involved in influenza protection by NK cells [35], and its expression is not altered in *B2m*$^{-/-}$ mice (S3A Fig), which are also protected from influenza virus (described below). In addition, while NKG2A/C/E expression is decreased in NKC^{KD} mice, it is increased in *B2m*$^{-/-}$ mice (S3B and S3C Fig). These data show that NKC^{KD} mice survive influenza virus infection better than WT

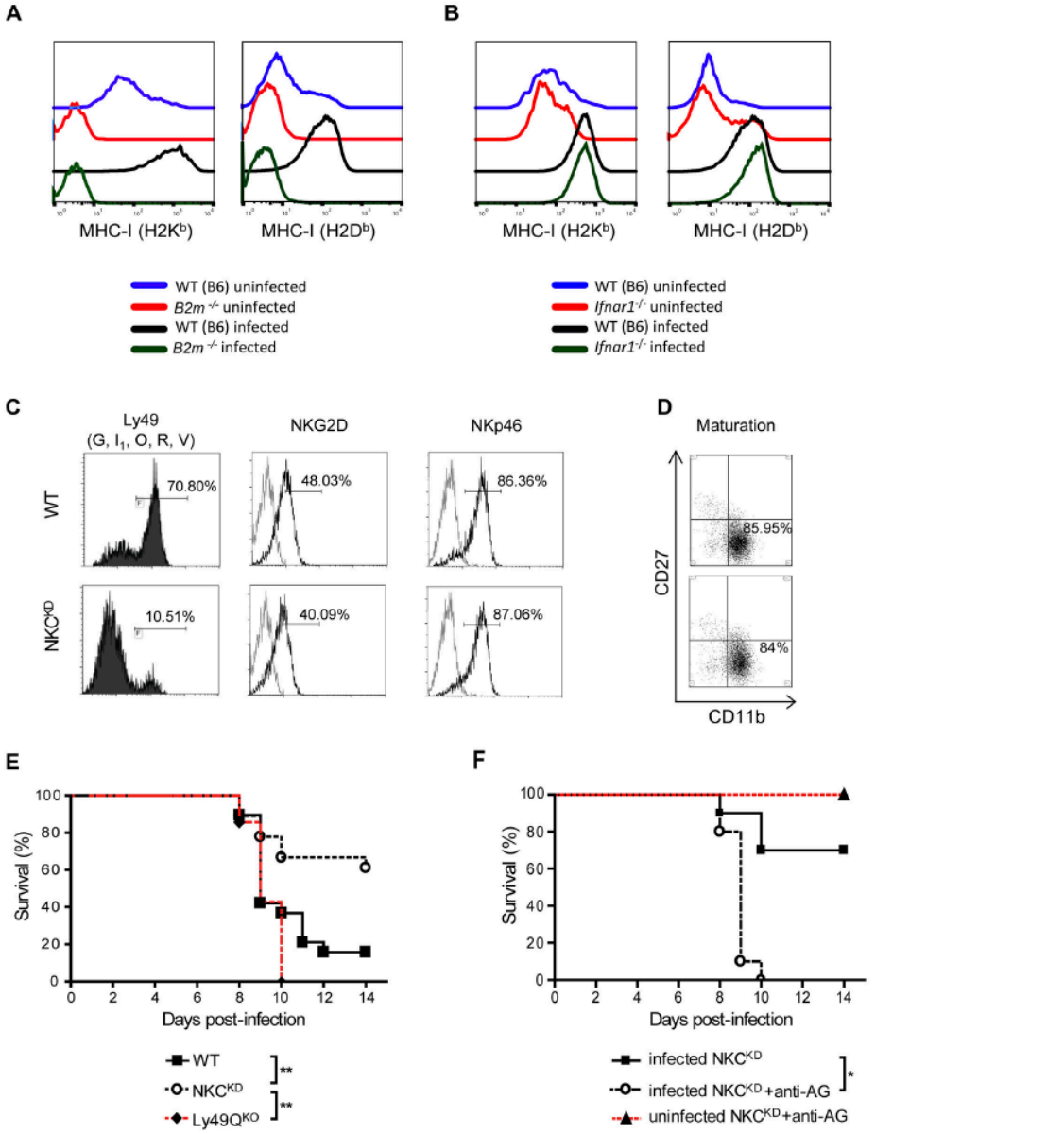

Fig 1. Ly49-deficient mice are protected from lethal influenza infection. (A, B) Groups of sex-matched WT (B6), *Ifnar1*$^{-/-}$ and *B2m*$^{-/-}$ mice were infected intranasally with 600 PFU of FM-MA virus. Single-cell suspensions were prepared from uninfected lungs and those infected with the virus for 5 days. Cells were stained with antibodies against H-2K^b or H-2D^b and CD326 (EpCAM—epithelial cell marker), and analyzed by flow cytometry. Surface expression of H-2K^b or H-2D^b was determined on EpCAM$^+$ lung epithelial cells. The following mAb were used in this experiment: anti-LFA-1, anti-EpCAM, anti-H-2K^b, and anti-H-2D^b. One representative image from each group is shown. This experiment was performed three times with similar results. **(C, D)** Ly49G, I$_1$, O, R, and

V expression was detected on lung NK cells of uninfected WT and NKC^{KD} mice using anti-NKp46, anti-TCRβ, and a combination of 4D11, 4E5 and 14B11 mAb. NKG2D, NKp46, CD11b, and CD27 expression was detected on lung NK cells, defined using anti-CD49b (DX5) and anti-TCRβ. The gray line represents staining with an isotype antibody. This experiment was performed three times with similar results. **(E)** Groups of age and sex-matched WT, Ly49Q^{KO}, and NKC^{KD} mice were infected with FM-MA virus (1050 PFU) and monitored for 2 weeks. Data are pooled from two independent experiments (n = 19 in each group). **(F)** Groups of age and sex-matched NKC^{KD} mice with or without NK depletion by anti-asialoGM1 were infected and monitored as above. A group of uninfected, NK-depleted mice was included as a control (n = 10 in each group). The percentage of surviving mice is shown. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Statistical analysis was performed with the log rank test.

doi:10.1371/journal.ppat.1005446.g001

mice in a Ly49Q- and NKG2A/C/E-independent manner. Better survival of NKC^{KD} mice compared to WT mice indicates a possible role for Ly49:MHC-I interactions in the pathogenesis of influenza virus in mice.

Higher numbers of unlicensed NK cells may confer survival advantage in Ly49-deficient mice

Our data implicate NK cells from NKC^{KD} mice, which are unlicensed due to a lack of Ly49 receptors, in better protection against influenza. To determine the responsiveness of unlicensed NK cells during influenza virus infection, we compared the function of different NK cell subsets in WT mice following infection. NK cells in WT mice can be divided into four populations based on their expression of Ly49C/I and Ly49G. While the licensed Ly49C/I^{+} G^{-} population dominates the lung microenvironment in the steady-state (Fig 2A, left), following infection, the unlicensed Ly49C/I^{-} G^{+} population shows more dramatic expansion (Fig 2A, right). This can be attributed in part to the greater number of proliferating cells in this subpopulation following infection (Fig 2B). NK cells were activated (IFN-γ^{+} and CD107a^{+}) upon infection with influenza virus, however, we observed equal levels of intracellular IFN-γ levels in all of these four NK cell subsets (S4 and S5 Figs). While we see an outgrowth of the Ly49G^{+} cells in the WT mice following infection, we observe no similar outgrowth from the residual Ly49G^{+} cells present in NKC^{KD} mice (Fig 2C), indicating that it is likely a lack of Ly49C/I, and not the presence of Ly49G, that is conferring the survival advantage in these animals.

Ly49-negative NK cells are not subject to inhibition via interaction with MHC-I molecules. A comparison of intracellular IFN-γ levels and CD107a (LAMP1) expression on this subset of NK cells in the WT and NKC^{KD} mice revealed a similar level of response in both mice following influenza infection (S6A and S6B Fig). However, a significantly higher number of IFN-γ^{+} and CD107a^{+} NK cells lacking expression of Ly49 are present in the NKC^{KD} mice, due to the disruption of Ly49 expression in these mice (Fig 2D and 2E). Therefore, the presence of a larger number of activated NK cells, which are not inhibited via interaction with the increased expression of MHC-I molecules on the lung epithelial cells in the influenza-infected mice (Fig 1A), may confer a survival advantage to NKC^{KD} compared to the WT mice, during an influenza virus infection.

WT mice exhibit higher lung viral loads in comparison to NKC^{KD}, resulting in severe lung immunopathology, following lethal influenza infection

Influenza virus infection causes severe lung pathology, leading to respiratory distress and mortality [36]. To examine lung pathology in WT and NKC^{KD} mice, lungs were collected 7 days p.i. with 600 PFU of FM-MA viruses. Microscopic examination of H&E stained lung sections showed more severe alveolar damage, leukocyte infiltration, and pulmonary edema in WT mice compared to NKC^{KD} mice (Fig 3A–3D). Similar results were obtained with 1050 PFU (S2B Fig). From these data, we attribute the increased mortality in WT mice over NKC^{KD} to

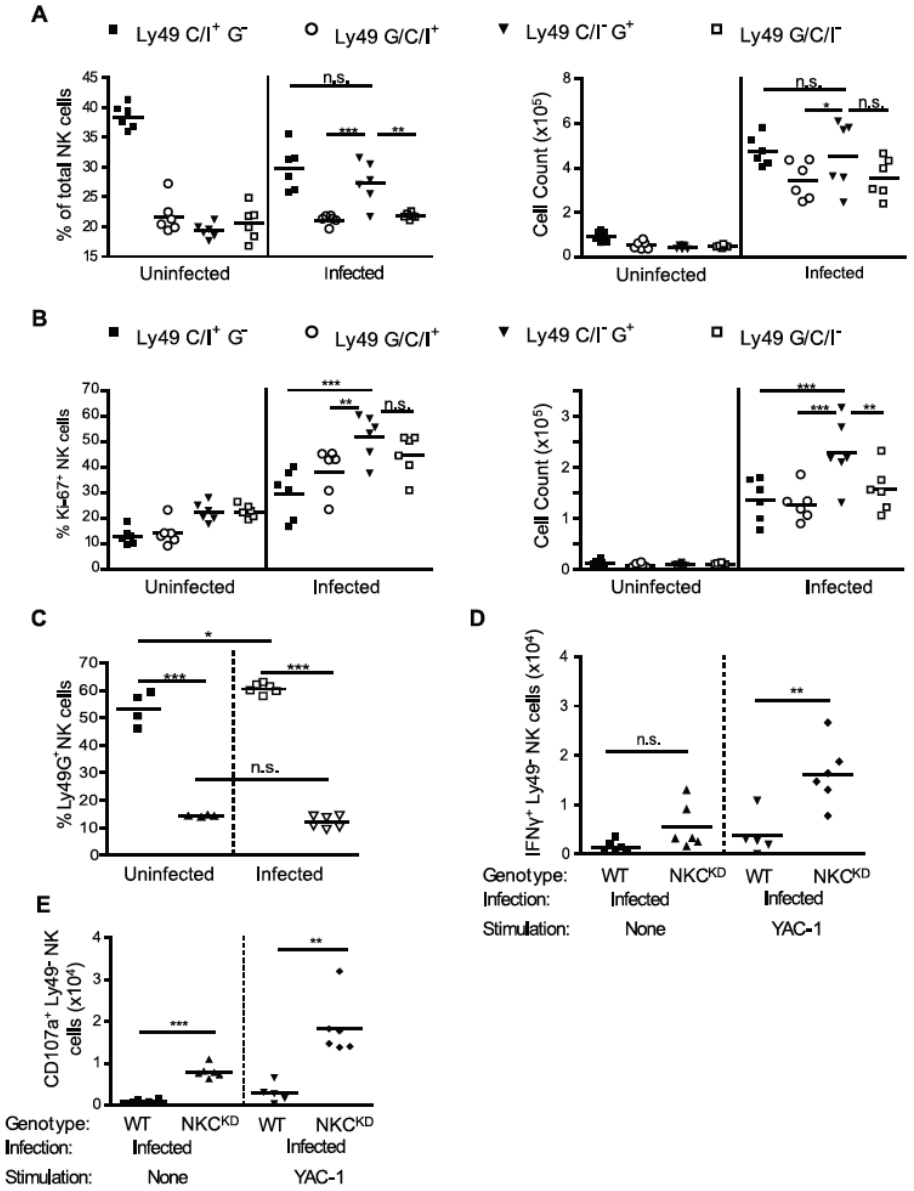

Fig 2. Increased numbers of activated, uneducated NK cells correlates with protection from influenza. Groups of age and sex-matched WT mice were infected as in Fig 1. On day 5 p.i., mice were sacrificed, and single cell suspensions prepared from the lungs. Flow cytometry was performed as above, using cells stained with a fixable viability dye, anti-NKp46, anti-TCRβ, anti-Ly49C/I (5E6), anti-Ly49G (4D11), anti-Ki-67, anti-IFN-γ, and anti-CD107a mAbs. Each symbol represents one mouse. Cell counts are given in absolute number calculated per lung. Horizontal bars represent mean values, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Statistical analysis was performed using a one-way ANOVA followed by a Bonferroni post-hoc test.

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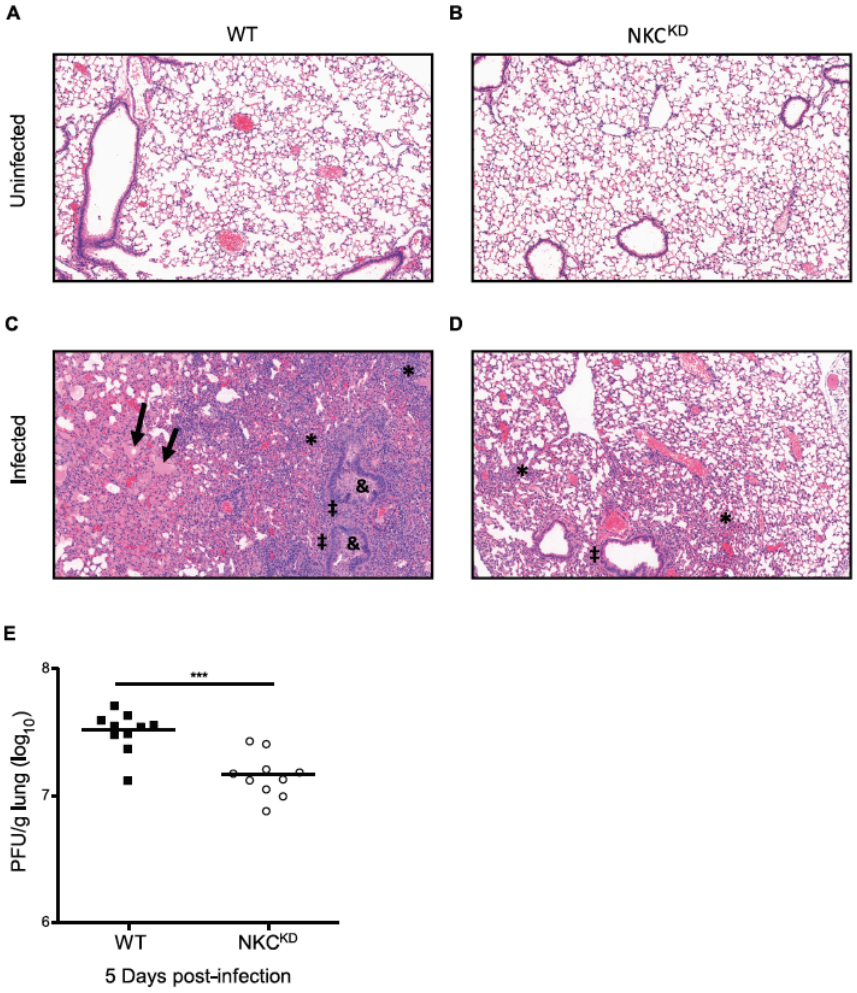

Fig 3. Lung immunopathology of influenza-infected WT and NKC^{KD} mice. (A-D) WT and NKC^{KD} mice were inoculated intranasally with 600 PFU of FM-MA virus. Lungs were collected from WT and NKC^{KD} mice 7 days p.i., fixed in neutral-buffered 10% formalin, sectioned, and stained with H&E. Images were acquired at 100x magnification. One representative image from each group is shown. Images were scored by a pathologist blind to the experimental conditions. Regions of tissue damage are indicated as follows: '→' pulmonary edema; '*' diffuse alveolar damage; '‡' lymphocytic and neutrophilic infiltrate; '&' bronchi filled with cellular debris. This experiment was performed three times with similar results. Two to three mice were used for each group per experiment. **(E)** Lungs from infected (600 PFU) age- and sex-matched WT and NKC^{KD} mice were collected on day 5 p.i., weighed, and virus titer (presented as PFU/g of lung tissue) was assessed in lung homogenates by plaque assay on MDCK cells. Data are pooled from three independent experiments (n = 10 in each group). Each symbol represents a single mouse. Horizontal bars represent mean values. ***$p < 0.001$. Statistical analysis was performed using Student's t-test.

doi:10.1371/journal.ppat.1005446.g003

more severe influenza-induced—and possibly immune-mediated—lung pathology. Next, we asked whether NKC^{KD} mice can eliminate influenza virus-infected cells more efficiently than WT mice, possibly leading to less viral burden and inflammation. To avoid survivor bias and ensure that the observed effect is due to innate immune responses, lungs were collected from infected WT and NKC^{KD} mice 5 days p.i., and viral titers were determined. Interestingly, viral titer in NKC^{KD} mice was significantly lower than in WT mice on day 5 p.i. (Fig 3E), while viral loads were equivalent between the two on days 3 and 8 p.i. (S7A Fig). This result indicates that NKC^{KD} mice are better than WT mice at controlling lung viral loads early during infection. Better control of the virus may lead to lower levels of inflammation and decreased lung injury following influenza virus infection in NKC^{KD} mice.

Ly49 deficiency results in significantly decreased levels of pro-inflammatory cytokine and chemokine levels in the lungs of NKC^{KD} mice after influenza virus infection

In addition to injury resulting from influenza virus replication, pro-inflammatory cytokines and chemokines have been suggested to play a pathogenic role in humans and animals infected with influenza virus [37, 38]. We have demonstrated previously that, similar to WT mice, NK cells from NKC^{KD} mice produce normal levels of cytokines upon stimulation with tumor cell lines, anti-NKp46 mAb, and after murine CMV (MCMV) infection [20]. To address the role of pro-inflammatory cytokines and chemokines in the pathogenesis of influenza viruses, we determined the cytokine and chemokine profile in the lungs of influenza-infected WT and NKC^{KD} mice by bead array flow cytometry, 5 days p.i. The majority of cytokines had similar baseline levels in WT and NKC^{KD} mice (S8 Fig). However, the most striking differences occurred in the levels of TNF-α, IFN-γ, IL-17, MCP-1, MCP-3, and MIP-1β, which were elevated significantly in lung homogenates of WT mice compared to the NKC^{KD} mice (Fig 4A–4F). These remarkable changes in pro-inflammatory cytokines and chemokines in the lungs of influenza virus-infected WT mice suggest their involvement in lung pathology. Furthermore, while we noticed a trend toward elevated IFN-γ and TNF-α in the WT lung NK cells themselves (Fig 4G and 4H), it is likely that other immune cells in the microenvironment are contributing to the cytokine profile we observe in the bulk lung extracts.

Early NK but not influenza virus-specific T cell increase in the lungs of influenza virus-infected mice

To demonstrate that the observed significant differences in cytokine levels and influenza virus load in the lungs of WT mice at day 5 p.i. is dependent on an NK cell response, we quantified the immune cell subsets responding to viral infection. We examined the NK cell and the virus-specific $CD8^+$ and $CD4^+$ T cell responses in the lungs of influenza virus–infected WT and NKC^{KD} mice 5 and 7 days p.i. Assessment of the percentage and absolute number of these lymphocyte subpopulations after influenza virus infection showed that a protective response to the infection within the first 5 days directly correlated with NK cell expansion (Fig 5A–5F), and not that of virus-specific $CD4^+$ or $CD8^+$ T cells. Substantial $CD4^+$ and $CD8^+$ T lymphocyte counts were only observed 7 days post-influenza virus infection (Fig 5A–5D); however, flow cytometry detected an expansion of the NK cell population in all mice 5 days p.i. (Fig 5E–5F). Notably, the percentage and the number of NK cells from both WT and NKC^{KD} mice show that there is no statistically significant difference between both groups 5 days p.i. However, the number of NK cells was significantly higher in WT mice ($^{**}p = 0.0031$) compared to NKC^{KD} mice (Fig 5F) 7 days p.i., most likely as a result of the reduction in virus load along with cytokine levels in the lungs of NKC^{KD} mice at day 5. These data strongly suggest that activation

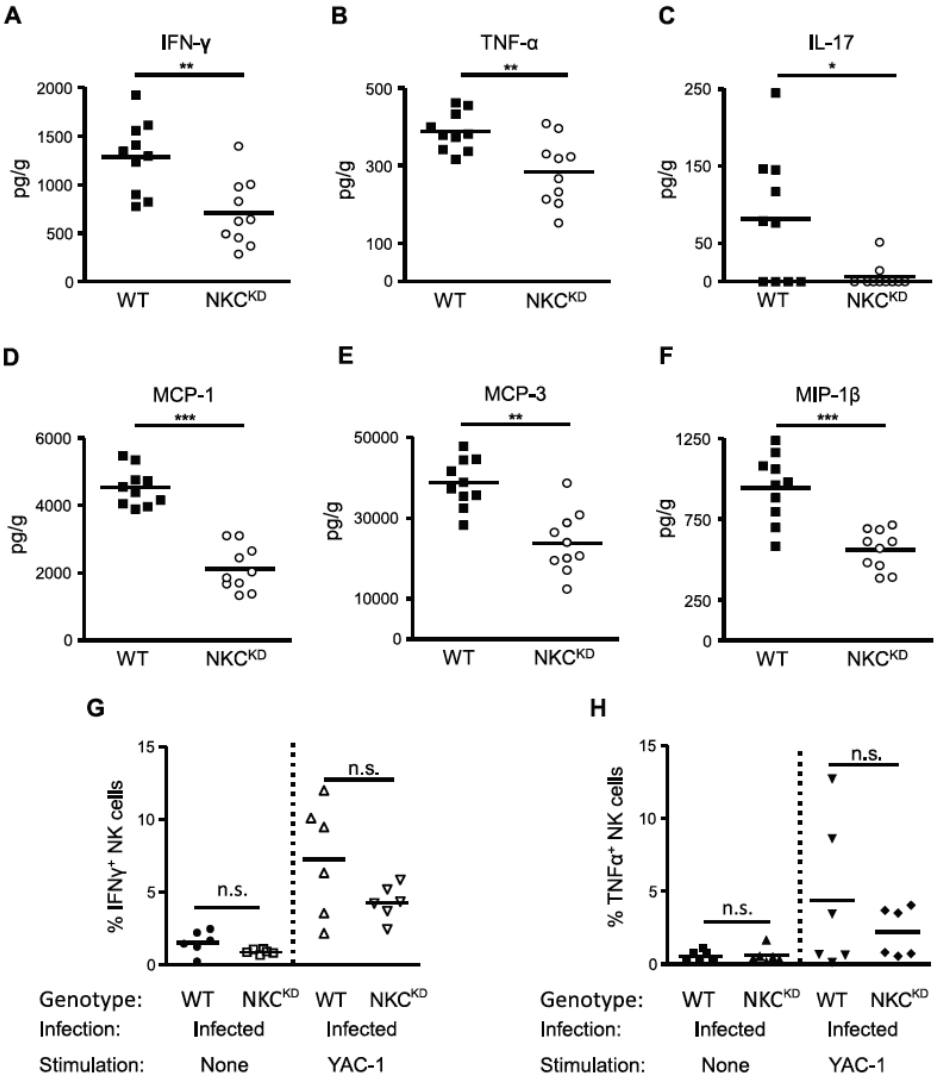

Fig 4. Cytokine and chemokine levels in the lungs of influenza virus-infected mice. Groups of age and sex-matched WT and NKC^{KD} mice were infected intranasally with 600 PFU of FM-MA virus. Lungs were collected on day 5 p.i., and lung tissue homogenates were prepared. The levels of **(A)** IFN-γ, **(B)** TNF-α, **(C)** IL-17, **(D)** MCP-1, **(E)** MCP-3, and **(F)** MIP-1β in the lung tissue homogenates were measured by flow cytometry using FlowCytomix Multiplex kits. Data are pooled from three independent experiments. **(G, H)** NK cells were isolated from infected lungs, stained, and analyzed by flow cytometry as above. Cells were stained with a fixable viability dye, anti-NKp46, anti-TCRβ, and anti-IFN-γ **(G)** or anti-TNF-α **(H)**. Horizontal bars represent mean values. Each symbol represents data from a single mouse. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Statistical analysis was performed using Student's t-test.

doi:10.1371/journal.ppat.1005446.g004

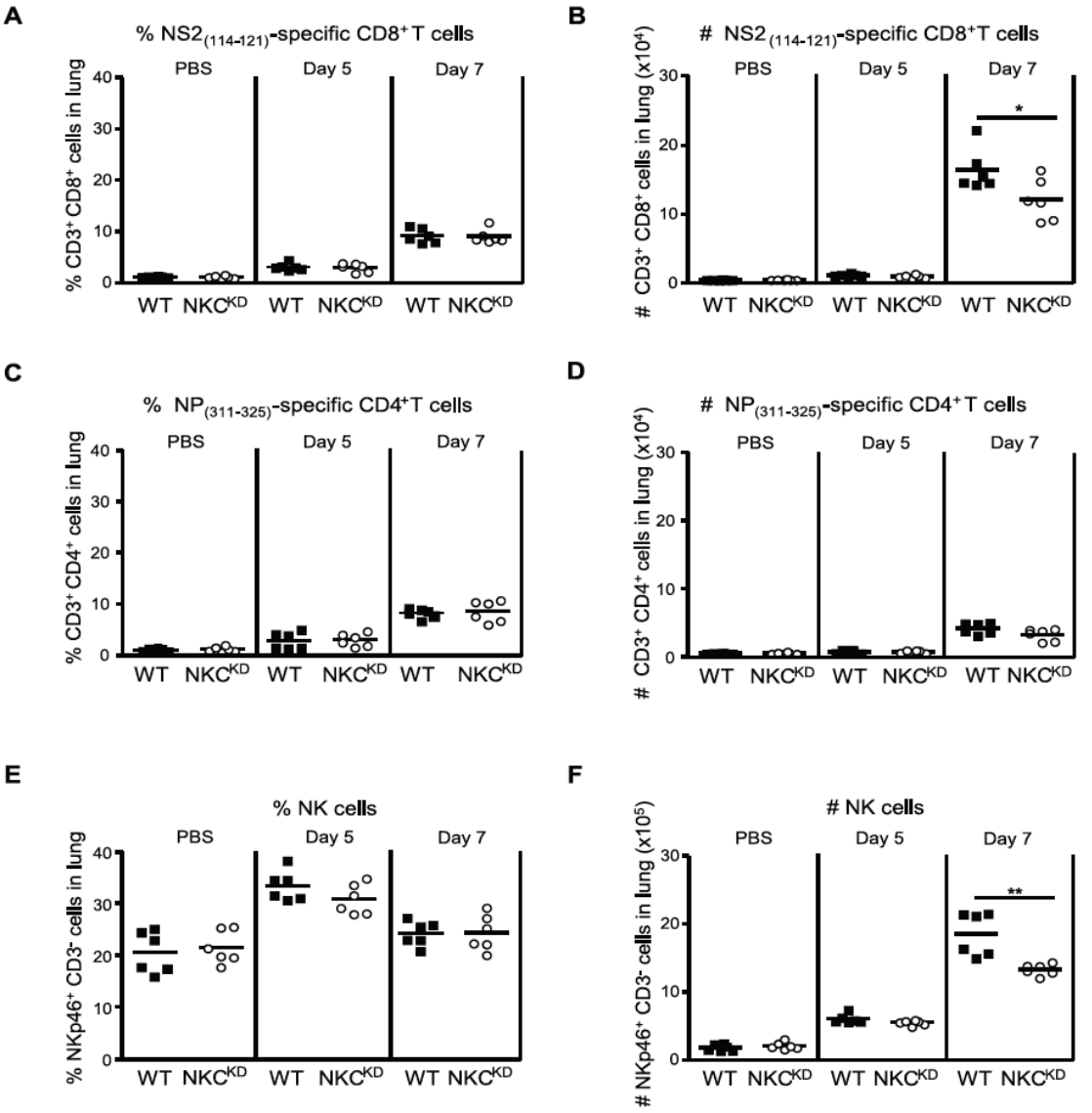

Fig 5. Lymphocyte recruitment to the lungs following influenza virus infection. WT and NKC^{KD} mice were infected intranasally with 600 PFU of FM-MA virus. Single-cell suspensions were prepared from uninfected lungs and those infected with the virus for 5 or 7 days. The following mAb were used in this experiment: anti-CD8, anti-CD3, anti-CD4, and anti-NKp46 mAb; as well, K^b-$NS2_{114-121}$ tetramers and I-A^b–$NP_{311-325}$ tetramers were used. Both the frequency (%) and absolute number (#) of cell populations is reported. **(A, B)** $NS2_{114-121}$ $CD8^+$ specific T cells were gated out of $CD3^+$ $CD8^+$ T cells. **(C, D)** $NP_{311-325}$ $CD4^+$specific T cells were gated out of $CD3^+$ $CD4^+$ T cells. **(E, F)** NK cells were detected as $CD3^-$ $NKp46^+$ cells. Data are pooled from two independent experiments (n = 6 in each group). Horizontal bars represent mean values. Each symbol represents data from a single mouse. *$p < 0.05$ and **$p < 0.01$. Statistical analysis was performed using Student's t-test.

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and increase in NK cell numbers within the first 5 days post-influenza virus infection enhances the antiviral response mediated by NK cells, and as a result, plays a substantial role in the initial control of influenza virus, especially by NKC^{KD} mice.

Direct contribution of Ly49 inhibitory receptors in the pathogenesis of influenza virus

To test the direct contribution of self-MHC-I-specific Ly49 inhibitory receptors in the pathogenesis of influenza virus, we introduced the inhibitory self-MHC-I-specific Ly49I transgene into NKC^{KD} mice through breeding as previously described [20]. We inoculated WT, NKC^{KD}, and NKC^{KD}-$Ly49I^{tg}$ mice with 600 PFU FM-MA virus intranasally. The animals were observed daily for over two weeks and sacrificed when moribund. Death due to infection began occurring on day 8 p.i. (Fig 6A). In agreement with our previous results, 90% of WT and 40% of NKC^{KD} mice succumbed by two weeks p.i. (Fig 6A, $^{*}p = 0.0147$). Remarkably, 100% of NKC^{KD}-$Ly49I^{tg}$ mice died ten days p.i. (Fig 6A, $^{***}p<0.0001$). Thus, Ly49 deficiency was definitively protective in NKC^{KD} mice, likely via the loss of MHC-I-specific NK cell inhibition. This finding raises an intriguing question as to whether MHC-I-deficient mice would also be protected from lethal influenza virus infection. To answer this question, we infected four groups of mice: WT (B6) and $B2m^{-/-}$ (MHC-I deficient), and WT and $B2m^{-/-}$ mice treated with anti-NK1.1 mAb to deplete NK cells. Remarkably, 50% of $B2m^{-/-}$ mice survived the infection, whereas all B6 mice and NK cell-depleted B6 mice died ten days p.i. (Fig 6B, $^{***}p<0.0001$). $B2m^{-/-}$ survival advantage was observed despite a similar viral load measured in the lungs of these mice on day 5 and 7 p.i. compared to the B6 mice (S7B Fig). NK cell activity (IFN-γ^{+} and $CD107a^{+}$) following *in vitro* stimulation was also similar in influenza virus-infected $B2m^{-/-}$ and WT (B6) mice (S6C and S6D Fig); however, NK cell inhibition via Ly49:MHC-I interaction is disrupted in $B2m^{-/-}$ mice. Interestingly, all NK cell-depleted $B2m^{-/-}$ mice succumbed to the infection as well (Fig 6B, $^{***}p = 0.0015$), indicating a direct role for MHC-I-unlicensed NK cells in controlling influenza virus infection in these mice. To validate this proof-of-concept, several groups have shown that blocking the interaction between Ly49C/I and their ligands enhances NK-mediated anti-cancer cytotoxic functions [39]. Likewise, it is possible that a functional blockade of Ly49C/I:MHC-I interactions might protect WT (B6) mice during influenza virus infection. To determine whether Ly49 interactions with MHC-I molecules are relevant to influenza infection *in vivo*, WT (B6) mice were treated two days prior to FM-MA infection, at the day of infection, and every two days after until day 10 p.i. with 200 μg of 5E6 $F(ab')_2$ mAbs, previously reported to block Ly49C/I:H-$2K^{b}$ interactions [39]. Blockade of Ly49C/I inhibitory receptors resulted in a significant increase in mouse survival when compared with untreated B6 mice (Fig 6C, $^{*}p = 0.0313$). These results demonstrate that preventing Ly49 inhibitory receptor interactions with their cognate MHC-I ligands is protective in mice against influenza virus infection.

NK cell-mediated early defense against influenza virus infection requires both IFN-γ and perforin

Our data strongly suggest that an early increase in NK cell numbers in the lungs during influenza virus infection enhances the antiviral response and the initial control of influenza virus, especially in NKC^{KD} mice. NK cells can directly limit virus replication by killing infected cells via the release of cytotoxic granules containing perforin and granzymes, or indirectly by producing IFN-γ, which plays a role in macrophage activation and in inhibiting viral replication.

We wanted to address whether perforin or IFN-γ protects NKC^{KD} mice against influenza virus infection. To determine whether the absence of perforin can be fatal for NKC^{KD} mice, we

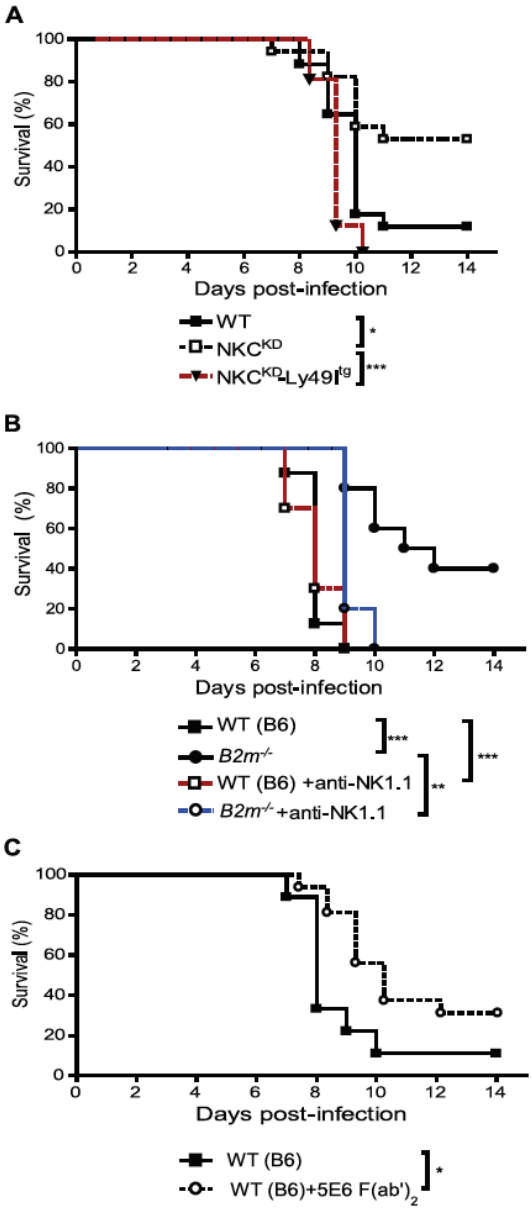

Fig 6. Involvement of Ly49 inhibitory receptors in the pathogenesis of influenza virus. (A) Groups of influenza-infected age and sex-matched WT, NKC^{KD}, and littermate NKC^{KD}-$Ly49I^{tg}$ mice were infected with 600 PFU of FM-MA virus, then monitored as before. Data are pooled from two independent experiments (n = 16 in each group). **(B)** Groups of influenza-infected age and sex-matched WT (B6) and $B2m^{-/-}$ mice were monitored as in Fig 1. One group each of WT or $B2m^{-/-}$ mice was treated with 200 μg anti-NK1.1 mAb per

mouse i.p. two days prior to influenza virus infection, on the day of infection (600 PFU), and every two days after until day 10 p.i. Data are pooled from two independent experiments (n = 10 in each group). **(C)** Groups of age and sex-matched WT (B6) mice were treated or not with 200 μg of 5E6 $F(ab')_2$ mAbs per mouse i.p. two days prior to influenza virus infection (600 PFU), on the day of infection, and every two days post-influenza virus infection until day 10 p.i. Data are pooled from two independent experiments (n = 16 in each group). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Statistical analysis was performed with the log rank test.

doi:10.1371/journal.ppat.1005446.g006

crossed NKC^{KD} mice to perforin-deficient mice ($Prf^{-/-}$) on the C57BL/6 background to obtain NKC^{KD}-$Prf^{-/-}$ mice. We inoculated NKC^{KD} and NKC^{KD}-$Prf^{-/-}$ mice with influenza virus intranasally. Interestingly, in contrast to NKC^{KD} mice, all NKC^{KD}-$Prf^{-/-}$ mice succumbed to the influenza infection (Fig 7A). Similarly, to address the role of IFN-γ, two groups of NKC^{KD} mice were inoculated with influenza virus intranasally. We neutralized IFN-γ in one group of influenza-infected mice using anti-IFN-γ mAb injections two days prior to and at the day of influenza infection, and every two days until day 10 p.i. As a control, a group of uninfected NKC^{KD} mice were treated with anti-IFN-γ mAb as well. Almost 90% of influenza virus-infected NKC^{KD} mice that were treated with anti-IFN-γ mAb died from the infection (Fig 7B). Both the NKC^{KD}-$Prf^{-/-}$ and anti-IFN-γ mAb-treated NKC^{KD} mice had higher lung viral titers compared to the NKC^{KD} control group on day 5 and 7, prior to the engagement of an adaptive immune response (Fig 7C). These data show that protective NK cell-mediated antiviral defenses in NKC^{KD} mice during influenza virus infection require both IFN-γ and perforin.

Discussion

In the current study, we demonstrate for the first time that disrupting the interaction between MHC-I and Ly49 inhibitory receptors on NK cells protects mice from lethal influenza virus infection. Here, we found that the proportion of lung epithelial cells that express MHC-I increased dramatically following influenza virus infection, in an IFN-I independent manner. We also provide evidence for the relevance of this upregulation in the severity of influenza virus infection, in which upregulation of MHC-I on lung epithelial cells in the presence of Ly49:MHC-I interactions allows influenza virus to escape recognition by NK cells, facilitating viral replication and mouse mortality. This demonstrates the direct contribution of Ly49:MHC-I interactions in the severity of influenza virus infection.

The human analogues of the mouse Ly49 receptors are the KIRs. Previous studies have reported that patients with either mild or severe pandemic influenza A H1N1/2009 virus infections had significantly higher levels of inhibitory KIR2DL5 gene expression in comparison to healthy individuals [40]. The presence or absence of interactions between NK cells expressing the KIR2DL5 inhibitory receptor and targets expressing its MHC-I ligand could be responsible for the severity of influenza virus infection in human patients. Moreover, another group observed that inhibitory KIR2DL2 and KIR2DL3 allotypes and their cognate ligands, HLA-C1 and HLA-C2, respectively, are significantly enriched in H1N1/2009 intensive-care unit patients in comparison to healthy individuals [41]. Taken together, these reports suggest that potential associations of specific inhibitory KIRs and their MHC-I ligands during severe influenza A virus infections may result in decreased NK function and increased virus pathogenicity. *In vitro* studies have shown that impairment of human NK cell activity is directly mediated by the accumulation of MHC-I molecules on the surface of influenza virus-infected cells, which in turn increases binding of the NK cell inhibitory receptor KIR2DL1 to the infected cells and inhibits human NK cell-mediated cytotoxicity against them *in vitro* [11, 12]. MHC-I upregulation by influenza virus may act as a mechanism to evade NK cell recognition by inhibiting NK cell functions via inhibitory Ly49 engagement. Disrupting this interaction between MHC-I and inhibitory NK cell receptors in humans may interfere with this and reduce the severity of an infection.

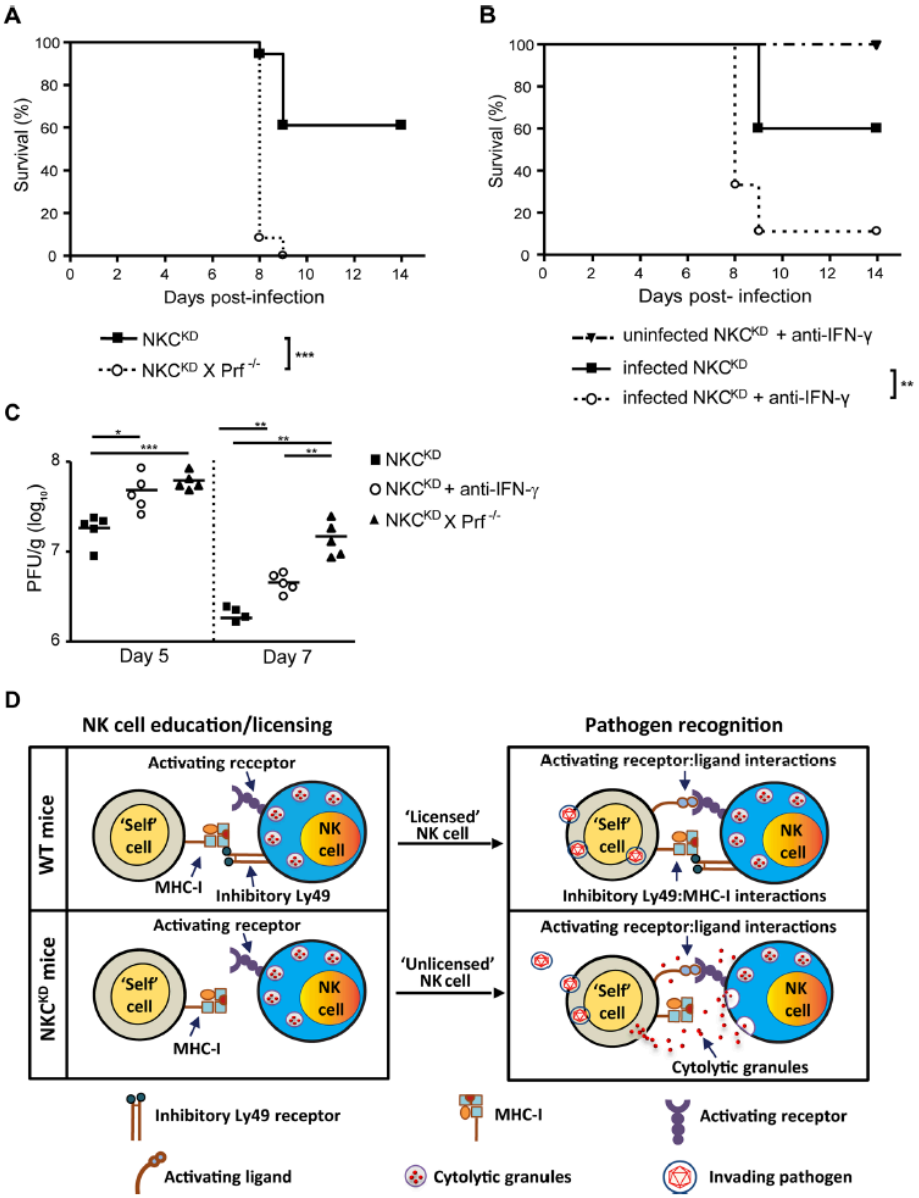

Fig 7. IFN-γ and perforin are required for protection against influenza virus infection. (A) Survival of infected NKC^{KD} (n = 18) and NKC^{KD}-Prf$^{-/-}$ (n = 12) mice (600 PFU) was monitored as before. Data are pooled from two independent experiments. **(B)** Groups of influenza virus-infected and uninfected NKC^{KD} mice (600 PFU) were treated or not with 200 μg anti-IFN-γ mAb per mouse i.p. two days prior to influenza virus infection, on the day of infection, and every two days post-influenza virus infection until day 10 p.i. Data are pooled from two independent experiments, with the following groups: infected NKC^{KD}+anti-IFN-γ (n = 18), infected NKC^{KD} (n = 10), and uninfected NKC^{KD}+anti-IFN-γ (n = 6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Statistical analysis was

performed with the log rank test. **(C)** Lungs from infected (600 PFU) age- and sex-matched WT and NKC^{KD} mice were collected on day 5 p.i., weighed, and virus titer (presented as PFU/g of lung tissue) was assessed in lung homogenates by plaque assay on MDCK cells. **(D)** Schematic representation of NK cell education/licensing and pathogen recognition by NK cells that express self-MHC-I-specific inhibitory Ly49 (licensed) and those that do not (unlicensed).

doi:10.1371/journal.ppat.1005446.g007

Ly49-defecient mice are able to survive an influenza virus infection specifically due to the lack of Ly49 inhibitory receptors; transgenic expression of a self-MHC-I-specific inhibitory Ly49 receptor in NKC^{KD} mice restores their influenza susceptibility to WT levels. This demonstrates the direct contribution of Ly49:MHC-I interactions in the severity of influenza virus infection. The presence of Ly49I restored the Ly49:MHC-I interaction in NKC^{KD} NK cells, and lead to significant mortality. While licensed NK cells are protective against MHC-I-deficient tumors [22], we show that unlicensed NK cells are chiefly responsible for protection from influenza, which may also be true with other viral infections. This could be explained by the lack of inhibitory Ly49 receptors which, if engaged by their MHC-I ligands on the virus-infected cells, could otherwise result in NK cell inhibition (Fig 7D). This hypothesis is supported by our F(ab')$_2$ fragment-mediated Ly49C/I blockade, which was found to be protective in WT mice against influenza virus infection, possibly by disrupting the Ly49C/I:MHC-I interaction. Protection conferred by the blocking of Ly49C/I receptors was smaller compared to the lack of Ly49 receptors (NKC^{KD}) or MHC-I ligands (*B2m*$^{-/-}$), possibly because of a suboptimal blocking in these experiment or the involvement of other inhibitory MHC-I receptors in influenza immunoevasion.

Our results from influenza virus infection in NKC^{KD} mice indicate that survival is associated with decreased cytokine and chemokine levels. It would be reasonable, therefore, to assume that unlicensed NK cells are 'protective' by being hyporesponsive, leading to a reduced immune response and consequentially less immune-mediated tissue damage. If this were true, a reduction in NK cell activity would be protective in mice challenged with influenza. However, our data show that reducing the activity of NK cells in NKC^{KD} mice, either by deleting perforin or by neutralizing IFN-γ, is detrimental to the animal's survival. One caveat to interpreting these data is that neither model is NK specific, and T cells will also be affected by a loss of perforin or IFN-γ. As we have shown, however, NK depletion in NKC^{KD} mice is sufficient to disrupt their viral resistance, making it difficult to discount NK-derived perforin and IFN-γ. Furthermore, although infected with equal amounts of virus, perforin-deficient and IFN-γ-neutralized NKC^{KD} mice show a significantly higher viral titer compared to the NKC^{KD} control mice on days 5 and 7 p.i., before a T cell response is established. In addition, the NKC^{KD} mice show a significantly reduced viral titer compared to the WT mice on day 5 p.i., implying that the NK cells in these mice are more efficient in clearing the virus. Taken together, these results indicate that the protection enjoyed by NKC^{KD} mice is not due to a decrease in NK cell activity but is in fact due to an increased efficiency at clearing the virus. This is also supported by increased viral lethality following the depletion of NK cells in *B2m*$^{-/-}$ mice. This increased efficiency of unlicensed NK cells against influenza agrees with previous results obtained with MCMV infections [28]. Our survival data indicate that the NK cells in the NKC^{KD} mice are more protective than in WT, suggesting that what we have called 'unlicensed' NK cells do in fact possess an efficient anti-viral activity. This could explain why, in a normal mouse immune system, up to half of the NK cells are 'unlicensed' with regard to self-Ly49 expression [18, 25]. If the two compartments were understood to provide immunity under different circumstances (tumor versus infection), there is a rationale for maintaining such a large pool of NK cells that, until now, were believed to be dysfunctional.

We propose that the increased efficiency of NK cells in clearing influenza virus in NKC^{KD} mice results in less need for an extended and toxic immune response, reducing the amount of

inflammatory cytokines known to cause severe lung damage and a high rate of mortality during influenza virus infection [38, 42]. Survival of MHC-I-deficient mice infected with influenza virus was previously attributed to the lack of cytotoxic $CD8^+$ T cells and hence reduced pulmonary damage in these mice [43]. Our data, on the other hand, show a dominant role for NK cells in the protection of these mice against influenza virus infection, since depletion of NK cells makes them susceptible. Consistent with our observations is the reported reduced frequency of NK cells in patients with severe H1N1/2009 infections; $CD8^+$ effector T cells were detected at normal levels in these patients [44].

Our study identifies the mechanisms by which influenza virus escapes recognition by NK cells. Disrupting this interaction between MHC-I and inhibitory NK cell receptors *in vivo* interferes with this evasion mechanism and, thus, alters the severity of the infection. Additionally, we have demonstrated a previously unidentified role for unlicensed NK cells of the innate immune system. These observations open up a new field of investigation related to NK cell education and encourage more precise elucidation of the nature of each of these functional NK subsets.

Supporting Information

S1 Fig. Validation of anti-asialoGM1 depletion in NKC^{KD} mice. (**A**) Flow cytometry was performed as in Fig 1 on cells from uninfected or infected NKC^{KD} mice to determine degree of NK-specific depletion in anti-AsialoGM1 treated animals. The proportion of $TCR\beta^+$ cells is indicated on each plot to show no effect of anti-AsialoGM1 treatment on the T cell compartment. A timeline of injections for this validation is also shown. (**B**) Timeline showing injection series and volume of anti-AsialoGM1 treatment of mice in Fig 1F.
(EPS)

S2 Fig. Survival and histopathology in WT and NKC^{KD} mice infected with influenza virus. (A) Groups of age- and sex-matched WT and NKC^{KD} mice (n = 5) were infected with influenza virus (50 and 5000 PFU) and monitored for 2 weeks. (B) WT and NKC^{KD} mice were inoculated intranasally with 1050 PFU of influenza virus. Lungs were collected from WT and NKC^{KD} mice 7 days p.i., fixed in neutral-buffered 10% formalin, sectioned, and stained with H&E. Images were acquired at 100x magnification. One representative image from each group is shown.
(EPS)

S3 Fig. Expression of NKG2A and NKG2A/C/E on WT, NKC^{KD}, and $B2m^{-/-}$ mice. NK cells were collected, processed, and analyzed as in Fig 1. The NKG2A-specific antibody was raised in 129-background mice, and so cannot be used in our NKC^{KD} mice. Data are pooled from two independent experiments, n = 6 mice per group. Mean ± SEM is shown, with $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Statistical analysis was performed by one-way ANOVA followed by a Bonferroni post-hoc test.
(EPS)

S4 Fig. Intracelluar IFN-γ levels and CD107a expression on NK cells from poly (I:C)-treated and influenza-infected mice. Mice were either injected with 15 μg poly (I:C) i.p. for 18 h or infected with influenza virus for 3 days. Cells isolated from spleen and lungs were unstimulated, co-cultured with YAC-I cells at 1:1 cell ratio, or cultured with 10 μg/ml PMA + 1 μg/ml ionomycin in presence of brefeldin A for 4 h. Intracellular IFN-γ (**A**) and CD107a (**B**) staining was performed as in the Materials and Methods. Data are pooled from two independent experiments. Statistical analysis was performed by two-tailed Student's *t*-test. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.
(EPS)

S5 Fig. No difference in IFN-γ production by uneducated or educated NK cells from influenza infected lungs. Cells were stained, processed, and analyzed as in Fig 1. IFN-γ expression was determined after four hours of (**A**) culture in RPMI supplemented with 10% FBS, penicillin/streptomycin, HEPES, non-essential amino acids, 2-mercaptoethanol, and brefeldin A, (**B**) co-culture as in (**A**) with YAC-1 target cells at 1:1 cell ratio, or (**C**) culture as in (**A**) with 10 μg/ml PMA + 1 μg/ml ionomycin. Data are pooled from two independent experiments (n = 6 mice in each), and mean ± SEM is shown. Statistical analysis was performed by one-way ANOVA.
(EPS)

S6 Fig. Similar expression of IFN-γ and CD107a on WT, NKC^{KD}, and *B2m*$^{-/-}$ mice. Cells from infected WT, NKC^{KD} (**A, B**), and *B2m*$^{-/-}$ (**C, D**) mice were collected, processed, co-cultured, and analyzed as in S3 Fig. Results are pooled from two independent experiments, with each symbol representing one mouse. Horizontal bars represent means, with $^{*}p < 0.05$. Statistical analysis was performed by a two-tailed Student's t-test.
(EPS)

S7 Fig. No difference in early or late viral titer between WT, NKC^{KD}, and *B2m*$^{-/-}$ mice. Virus titer was determined as in Fig 3. (**A**) No difference in early or late viral titers between WT and NKC^{KD} mice. (**B**) No difference in viral titer on day 5 or day 7 p.i. between WT and *B2m*$^{-/-}$ mice. Each symbol represents one mouse. Horizontal bars represent mean. Statistical analysis was performed by a two-tailed Student's t-test, with no significant differences found.
(EPS)

S8 Fig. Production of cytokines and chemokines in the lungs of influenza virus-infected mice. Groups of age and sex-matched WT and NKC^{KD} mice were infected intranasally with 600 PFU of FM-MA virus. Lungs were collected at day 5 p.i., and lung tissue homogenates were prepared. The levels of IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-21, IL-22, IL-27 RANTES and GM-CSF in the lung tissue homogenates were measured by flow cytometry using FlowCytomix Multiplex kits. Data are pooled from three independent experiments. Each symbol represents a single mouse. Statistical analysis was performed using Student's t-test.
(EPS)

Acknowledgments

We gratefully acknowledge the NIH Tetramer Core Facility (contract HHSN272201300006C) for supplying us with the $NS_{2114\text{-}121}$ (RTFSFQLI) and with $NP_{311\text{-}325}$ (QVYSLIRPNENPAHK) tetramers.

Author Contributions

Conceived and designed the experiments: ABM APM. Performed the experiments: ABM MMT AW MMAR. Analyzed the data: ABM HSS APM. Contributed reagents/materials/analysis tools: HSZ SHL EGB. Wrote the paper: ABM MMAR AW. Supervised all aspects of the study: APM.

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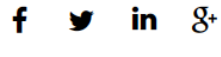

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