Role of Ly49 Receptors on Natural Killer Cells During Influenza Virus Infection

By

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Abstract

Natural killer (NK) cells are lymphocytes of the innate immune system that play a major role in the destruction of both tumours and virally-infected cells. The cytotoxicity of NK cells is tightly controlled by signals received through activating and inhibitory receptors. NK cells express a variety of inhibitory receptors such as Ly49 receptors. Ly49 receptors bind to class I MHC molecules that expressed on normal cells. The Ly49 inhibitory receptors negatively regulate NK cells-mediated target cell lysis. Using Ly49-deficient (NKC^{KD}) mice we show that Ly49-KD NK cells successfully recognize and kill influenza virus-infected cells and that NKC^{KD} mice exhibit better survival than wild-type mice. Moreover, influenza virus infection has a propensity to upregulate cell surface expression of MHC class I on murine lung epithelial cells *in vivo* but not *in vitro*, which serves as a ligand for inhibitory NK cell receptors. Significantly, we demonstrate increased lung damage of WT-mice versus NKC^{KD} mice after influenza virus infection as determined by histological analyses. This data indicated that absence of Ly49 inhibitory NK receptors greatly enhances survival of infected mice, however the exact mechanism of the increased survival of NKC^{KD} mice is not clear at this time. This study demonstrated a critical role for NK cells and Ly49 receptors in the pathogenesis of influenza virus in mice.

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ADCC**: Antibody-dependent cellular cytotoxicity**
ALAK: Adherent lymphokine activated killer
β2m: beta-2-microglobulin
BAL: bronchoalveolar lavage
B6: C57Bl/6
CD: Cluster of differentiation
Clr: C-type lectin related
CMV: Cytomegalovirus promoter
DAP10 or 12: DNAX-activating protein of 10 or 12kDa
DC: Dendritic cell
FasL: Fas ligand
FBS: Fetal bovine serum
Fc: Fragment, cristallizable
FITC: Fluorescein isothiocyanate
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HA: Haemagglutinin
HEF: haemagglutinin-esterase-fusion protein
HLA: Human leukocyte antigen
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
ITAM: Immunoreceptor tyrosine-based activating motif
ITIM: Immunoreceptor tyrosine-based inhibitory motif
KD: Knockdown
KIR: Killer immunoglobulin-like receptors
LLT1: Lectin-like transcript 1
M1: matrix protein
M2: integral membrane protein
mAb: Monoclonal antibody
MCMV: Murine cytomegalovirus
MHC-I: Major Histocompatibility Complex Class I
MICA/B: MHC class I polypeptide-related sequence A/B
MIP: Macrophage-inflammatory protein
MULT1: Murine UL16-binding protein-like transcript 1
NA: neuraminidase
NK cell: Natural killer cell
NKC: Natural killer complex
NKC^{KD}: Natural killer complex knockdown
NP: nucleocapsid protein
NS: nonstructural proteins
PRR: pattern recognition receptors

PBS: Phosphate buffered saline
pDC: Plasmacytoid dendritic cell
PFU: Plaque-forming unit
Rae-1: Retinoic acid early inducible-1
SHIP: SH2-containing inositol polyphosphate 5-phosphatase
SHP-1 or 2: SH2-containing protein tyrosine phosphatase-1or 2
Tg: Transgene
Th1: T helper type 1
TLR: Toll-like receptor
TNF: Tumour necrosis factor
TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand
ULBP: UL-16 binding protein
WT: Wild-type

Introduction

1. Natural killer (NK) cells

NK cells were first discovered in 1975 as lymphocytes of the innate immune system that can kill leukemia cells *in vitro* without previous sensitization (1). Since then, NK cells have been revealed to play an important role in the early defense against certain viruses and cancers (2). Recently, NK cells have been implicated in the regulation of adaptive immune responses following an inflammatory response through elimination of specific antigen-activated T cells (3, 4). In contrast to T and B cells, NK cells do not express rearranged antigen-specific receptors. Alternatively, NK effector function is tightly controlled by the combination of signals received through germ-line-encoded receptors with inhibitory or activating functions that can recognize ligands on their cellular targets (2).

Inhibitory receptors, such as the inhibitory Ly49 family, killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs) and CD94-NKG2A receptors, bind to class I MHC (MHC-I), or MHC-I-like molecules and signal through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail (Fig.1). Some transformed and virus-infected cells tend to down-regulate normal expression of MHC-I in order to avoid recognition by $CD8^+$cytotoxic T lymphocytes and so become susceptible to NK cell-mediated killing. Activating NK receptors, such as NKG2D, KIR2DS2 and CD94-NKG2C are structurally related to inhibitory receptors but lack ITIMs (5).

Figure 1

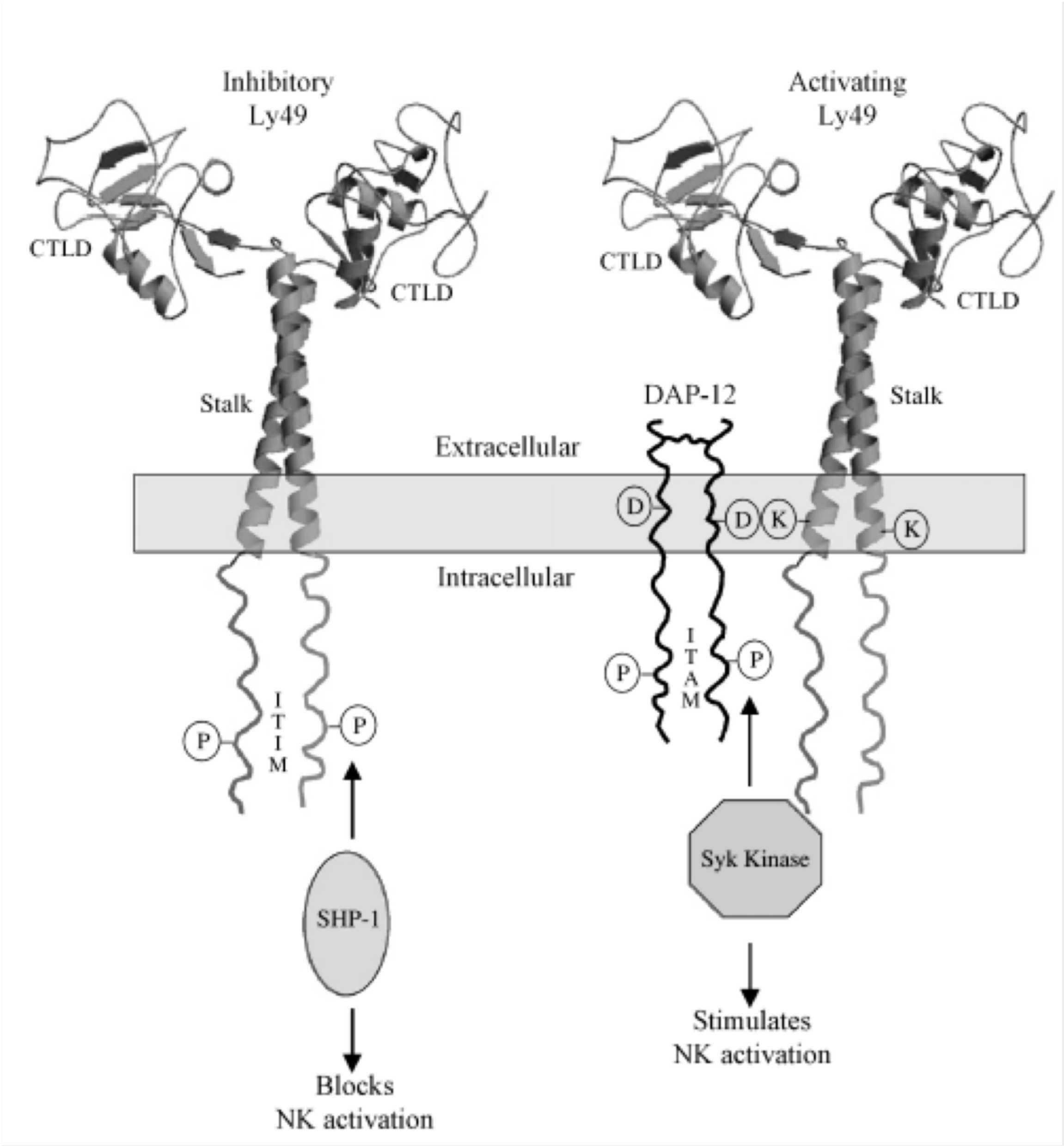

Inhibitory Ly49
Activating Ly49
CTLD
CTLD
CTLD
CTLD
Stalk
Stalk
DAP-12
Extracellular
Intracellular
D
D
K
K
P
P
P
P
ITIM
ITAM
SHP-1
Syk Kinase
Blocks NK activation
Stimulates NK activation

Figure 1. Inhibitory and activating Ly49 receptor signalling pathways. Inhibitory Ly49 receptors contain an ITIM motif in their cytoplasmic tail. Upon ligand binding, the ITIM becomes phosphorylated and leads to the recruitment of the tyrosine phosphatase SHP-1, thereby preventing NK cell activation. Activating Ly49 receptors associate with the adaptor molecule, DAP-12, which is characterized by the presence of ITAM motif in its cytoplasmic tail. Engagement of Ly49 activating receptors with its ligand promotes the phosphorylation of ITAM which in turn stimulates recruitment and activation of Syk kinase. This initiates a signaling cascade leading to activation of NK cell function. Adapted from (6) with permission from Nature Publishing Group.

They associate with signaling molecules such as DAP12, CD3ζ, or FcγR, which signal through immunoreceptor tyrosine-based activating motifs (ITAMs) (Fig.1). Additionally, NK cell express the low-affinity Fc receptor (CD16), thus enabling them to detect antibody-coated target cells and to exert antibody-dependent cellular cytotoxicity (ADCC) (5).

NK cell kill their target cells by either of two mechanisms that require direct cell–cell contact. Primarily, NK cell use cytoplasmic granule toxins known as perforin and granzymes, which are stored in cytoplasmic granules and are secreted by exocytosis, and together they induce apoptosis of the target cell. NK cells can also kill target cells using the Fas Ligand (FasL)-dependent pathway to kill Fas^+ target cells, Fas/FasL interactions, resulting in apoptosis of the target cell (5, 7).

Furthermore, upon stimulation, NK cell can secrete potent levels of cytokines, especially interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-10, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and chemokines such as CC chemokine ligand 3 (CCL3), CCL4 and CCL5 (8). Studies have also demonstrated a critical role of several cytokines in the induction of NK-cell cytotoxicity. These include interleukin-12 (IL-12), IL-18, IL-15, and type I interferons (9). Specifically, IL-12 release by mature dendritic cells (DCs) leads to production of IFN-γ by NK cells, which increases the surface expression of MHC-I and MHC-II on surrounding cells and results in their subsequent recognition by cytotoxic T cells. Moreover, IFN-γ further activates macrophages and dendritic cells, and enhances T helper type 1 (Th1) activation and polarization by inducing T-bet expression (10, 11).

1.1. Missing-self hypothesis

The observation made by Klas Kärre and collaborators that the absence or altered expression of MHC-I molecules on the surface of transformed or virally infected cells renders these cells especially susceptible to NK cell cytotoxicity led to the “missing-self” hypothesis, which proposed that NK cells recognize and destroy cells lacking normal expression of MHC-I molecules on the cell surface. This is due to the absence of inhibitory signals from inhibitory receptors that recognize ‘self’ MHC-I molecules. Thus, cells that express normal levels of host MHC-I molecules are protected from NK cell cytotoxicity (12). The missing-self hypothesis is supported by the finding that transformed and virally-infected cells sometimes down-regulate MHC-I surface expression (2), thus making them susceptible to NK cell attack (13). An important validation of the “missing self” hypothesis was demonstrated by transfecting MHC-I- deficient target cells with MHC-I gene encoding D^d, K^b, and K^k haplotypes. As a result, these cells became protected from lysis by NK cells (14). Further support for the “missing self” hypothesis was demonstrated by the finding that typical rejection of MHC-I deficient bone marrow cells by wild type mice is confirmed to be NK cell-mediated (15). In mice, the capability of an NK cell to recognize the lack of self-MHC-I expression on target cells is linked to Ly49 inhibitory receptors (16). In 1989, Yokoyama and his colleagues identified an inhibitory receptor called Ly49 that is expressed on a subset of mouse

NK cells and is responsible for blocking NK cell activation. They purified and compared Ly49^{+} NK cells with Ly49^{-} NK cells and incubated them with target cells from H-2^{d} and H-2^{k} backgrounds. Yokoyama *et al* found that target cells were very susceptible to killing by the Ly49^{-} NK cell subsets but were not killed by the Ly49^{+}. Interestingly, monoclonal antibody (mAb) against Ly49 or the α1 and α2 domains of the H-2D^{d} molecule were able to restore killing of resistant target cells by Ly49^{+}NK cells, suggesting that interactions between MHC-I and Ly49 inhibitory receptor transmit an inhibitory signal that will turn off NK cell activation (16).

1.2. Human NK cells

Human NK cells constitute approximately 10% of peripheral blood lymphocytes. They can be divided into two subsets based on their cell surface density of CD56. The majority of NK cells are CD56^{dim} cells, which express high levels of CD16 (FcgRIII), have high cytolytic activity, play key roles in natural and Ab-mediated cell cytotoxicity and make up around 90% of circulating NK cells. On the other hand, 10% of human NK cells are CD56^{bright} cells, which express low or no levels of CD16, and produce abundant cytokines (such as IFN-γ). Therefore, the traditional phenotype of human circulating NK cells are CD3^{-}CD16^{+} CD56^{dim} or CD3^{-} CD16^{-} CD56^{bright} (17, 18).

A balance between opposing signals delivered by the activating and inhibitory receptors are responsible for the regulation of effector functions of NK cells. The inhibitory receptors specific for MHC-I (in human called HLA) consist of three structurally distinct families: the killer cell Ig-like receptors (KIR), immunoglobulin-like transcripts (ILTs), and the killer cell lectin-like receptors (KLR) (2). Interestingly, KIR receptors were reported to be expressed on a considerable fraction of CD56^{dim} CD16^{+} NK cells, whereas the CD56^{bright} CD16^{-} NK subset expresses CD94/NKG2A and lacks KIR receptors. Both NK cell subsets express the activating receptor NKG2D, which recognizes the MHC-I-related molecules MICA and MICB, and they also express the natural cytotoxicity receptors (NCRs) NKp46 (19).

Tissue distribution of these two major NK cell subsets depends on distinct expression of chemokine receptors. CD56^{dim} CD16^{+} cytotoxic NK cells can be attracted from blood to peripheral tissues by several chemokines released during inflammatory responses because of the expression of CXCR1 and CX3CR1. In contrast, CD56^{bright} CD16^{-} cytokine-secreting NK cells express CD62L and CCR7, the receptor for CCL19 and CCL21 chemokines, which allow migration from the bloodstream into the lymph nodes (20, 21).

1.3. Murine NK cells

Murine NK cells were initially characterized as a population of lymphocytes expressing the NK1.1 antigen. Later on, However, NK1.1 was found to be only expressed in a few mouse strains, such as C57Bl/6 (B6), NZB, CE, FVB, and Swiss outbred mice, but not in BALB/c, CBA, C3H, or 129 mice (22). Mouse NK cells are identified by the lack of the CD3/TCR complex and by the expression of CD49b (DX5), CD11b, CD27, and NKp46 (23). Therefore, in NK1.1^{-} mouse strains, NK cells are commonly identified using DX5, a mAb that recognizes CD49b, despite the expression of CD49b on some T cells, mast cells, and granulocytes. (23). Interestingly, NKp46 was shown to be conserved between humans, all strains of mice tested, and in three species of non-human primates. This makes NKp46 the best universal marker for NK cells across mammalian species. However, Nkp46 was also shown to be expressed by a very small subset of human and mouse T lymphocytes, including NKT cells (24, 25).

Many NK cell receptors have been shown to activate or inhibit NK cell function, such as those belonging to the NK-RP1, NKG2, and Ly49 families, which are encoded in the NK gene complex (NKC) found on chromosome 6 in mice and on chromosome 12p13.1 in humans (Fig.2). Resistance or susceptibility of certain mouse strains to mouse cytomegalovirus (MCMV) were linked to a gene encoding Ly49H that is located in the NKC region. B6 mice express the Ly49H allele and are resistant to MCMV, whereas BALB/c mice, which lack Ly49H, are highly susceptible to MCMV infection (26). Many studies have shown that the NKC appears to be a highly polymorphic region. Allelic variability of various NKC loci has been demonstrated in inbred mice with consequences for NK cell function (27).

Figure 2

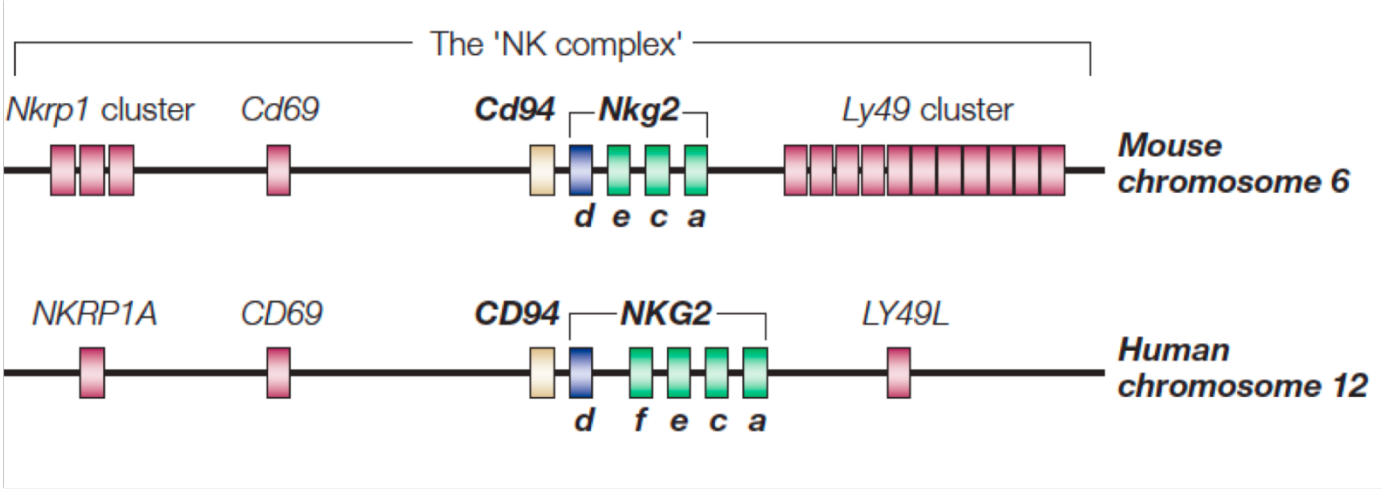

The 'NK complex'
Nkrp1 cluster
Cd69
Cd94
Nkg2
Ly49 cluster
Mouse chromosome 6
d e c a
NKRP1A
CD69
CD94
NKG2
LY49L
Human chromosome 12
d f e c a

Figure 2. Human and mouse natural killer complex (NKC). The natural killer gene complex is located on chromosome twelve in humans and chromosome six in mice. This genetic region encodes many C-type lectin NK receptors, which occur in both inhibitory and activating forms. Adapted from (28) with permission from Nature Publishing Group.

1.4. NK cell receptors

1.4.1. General properties of NK cell receptors

NK cells express a variety of activating and inhibitory receptors including Ly49 in mice or KIR in humans, NKG2D, CD94–NKG2 heterodimers, as well as natural cytotoxicity receptors (e.g. NKp46). These receptors use opposing signaling motifs to inhibit or stimulate activation of NK cells, with the negative signal mediated by MHC-I-specific inhibitory receptors being dominant over the activating signals. Inhibitory receptors allow NK cells to survey tissues for normal MHC-I expression and protect healthy cells from inappropriate NK cell mediated killing (2).

NK cell inhibitory receptors such as inhibitory Ly49, KIR, and CD49/NKG2A signal through immunoreceptor–tyrosine-based inhibitory motifs (ITIM) which are present in the cytoplasmic tail. ITIM motifs have the consensus sequence of Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val, where ‘X’ represents any amino acid. The tyrosine residue in the ITIM is a critical element for mediating inhibitory function. Ligation of these inhibitory receptors with the MHC-I molecule leads to tyrosine phosphorylation of the ITIM. Phosphorylated tyrosine in the ITIM serves as a docking site for recruitment of the protein tyrosine phosphatase Src homology region 2-containing protein tyrosine phosphatase (SHP)-1. Recruited SHP-1 leads to the disruption of activating signaling cascades (29).

Unlike the inhibitory receptors that contain an ITIM in their cytoplasmic tail, activating NK cell receptors such as Ly49H and NKG2D do not contain cytoplasmic domains capable of transducing signals. Instead, activating receptor signals are mediated by the association of transmembrane adaptor proteins, such as DAP10 and DAP12, that contain an immunoreceptor tyrosine-based activation motif (ITAM), defined by the sequence (D/E)XXYXX(L/I)X6–8YXX(L/I). Engagement of these receptors leads to phosphorylation of the ITAM tyrosine residue by Src family kinases and subsequent recruitment and activation of spleen tyrosine kinase (Syk), ultimately leading to NK cell activation (30).

1.4.2. CD94/NKG2:

Structurally, CD94 and NKG2 belong to type II integral membrane glycoproteins that contain an extracellular C-type carbohydrate recognition domain. The CD94 protein was shown to covalently associate with distinct members of the NKG2 family, forming functionally distinct heterodimers. These receptors are expressed predominantly on NK cells and a subset of $CD8^+$ T cells, and vary in function as an inhibitor or activator depending on which type of NKG2 is expressed, either the activating (NKG2C, NKG2E) or inhibitory (NKG2A/B) isoforms (2). The ligand for CD94/NKG2 is the non-classical MHC molecule Qa-1^b in mice and its homologue, HLA-E, in humans. HLA-E and Qa-1b molecules present peptides derived from the leader sequences of other MHC-I molecules (23, 31).

NKG2A molecules have longer cytoplasmic tails containing two ITIMs. Engagement of CD94/NKG2A with its ligand results in the inhibition of NK cell cytotoxicity (32). On the other hand, NKG2C and NKG2E have short intracellular regions and lack ITIM motifs, but were found to associate with DAP12 for proper expression and initiation of activating signals upon receptor cross-linking (33). Although the activating CD94-NKG2C and CD94-NKG2E heterodimers bind to the same ligand, their affinity for non-classical MHC-I molecule is much lower than the inhibitory CD94-NKG2A (34). Interestingly, CD94-NKG2E plays an important role in NK cell-mediated resistance to the Orthopoxvirus ectromelia (35). Moreover, NKG2C was found to be able to recognize and kill HIV-infected cells (36). During HIV infection, the expression of the NKG2C receptor on NK cells as well as its ligand was shown to be upregulated, which could indicate its protective role during HIV infection (37, 38)

1.4.3. NKG2D

NKG2D is a type II trans-membrane protein that is also a member of the C-type lectin family. The NKG2D gene exists within the NKC on human chromosome 12 and on mouse chromosome 6. Although the NKG2D gene is located next to the other NKG2 genes in the NKC, NKG2D displays only limited sequence homology to other NKG2 family members and it does not form heterodimers with CD94. NKG2D is expressed as a homodimer and signals through association with the adaptor protein DAP10 in humans and with both DAP10 and DAP12 in mice and which also helps in stabilizing its surface expression. NKG2D is constitutively expressed on all NK cells, subsets of T cells, activated CD8$^+$T and macrophages (39).

Several ligands have been identified for human NKG2D, including major histocompatibility complex class I chain related molecules A and B (MICA and MICB) and UL-16 binding proteins (ULBP-1, -2, -3, -4, and -5). On the other hand, mouse NKG2D binds to Rae-1α, Rae-1β, Rae-1γ, Rae-1δ, Rae-1ε, and histocompatibility antigen 60 (H60). These self-molecules have been shown to be expressed in response to several stress conditions such as viral infections and DNA damage. Engagement of NKG2D with one of these self-molecules activates NK cell cytotoxicity and induces cytokine production (40).

In vivo and *in vitro* studies have demonstrated that mice are able to eliminate tumor cells expressing NKG2D ligands. In human cancer patients, NKG2D ligands are constitutively expressed in multiple types of tumors, including AML (acute myeloid leukemia), ALL (acute lymphatic leukemia), CML (chronic myeloid leukemia), and CLL (chronic lymphatic leukemia) (40).

1.4.4. NKp46

One important family of activating receptors that is expressed on NK cells is the natural cytotoxicity receptor (NCR) family, which includes NKp30, NKp44 and NKp46. NKp46 is a type I transmembrane glycoprotein with two extracellular C2-type Ig-like domains. NKp46 contains a charged amino acid in its transmembrane domain which associates with ITAM-bearing adaptor molecules CD3ζ and/or FcεRIγ (41). NKp46 is expressed in both human and mouse NK cells. Indeed, NKp46 is considered a major NK lysis receptor and plays a dominant role in the activation of NK cells against various targets. Furthermore, it is involved in the clearance of both tumor and virus-infected cells (42). Interestingly, hemagglutinin molecules of influenza virus and the haemagglutinin–neuraminidase of parainfluenza virus were identified as the first specific NKp46 ligands (43). Although the NKp46 receptor can recognize and kill tumor cells, the nature of the ligand is still unknown (44).

Mice lacking NKp46 fail to clear influenza virus and do not survive the infection (45). A recent study has demonstrated that NKp46^{high} NK cells were more efficient at controlling HCV-infected hepatocytes than NKp46^{low} NK cells (46).

1.4.5. Killer immunoglobulin-like receptors (KIRs)

KIRs are type I trans-membrane glycoproteins that are expressed on human NK cells and on small subsets of T cells. KIRs are named based on their extracellular domain (2D and 3D), which reflect the number of immunoglobulin-like domains. Moreover, KIRs consist of both inhibitory and activating receptors, Activating KIRs are characterized by their short cytoplasmic tail, whereas inhibitory KIRs have a long cytoplasmic tail (2). The KIR gene family contains 15 genes and 2 pseudogenes with substantial allelic diversity. KIR genes are closely linked on human chromosome 19q13.4 within the leukocyte receptor complex (LRC) (47).

Studies of KIR genotypes have demonstrated variations between individuals in their KIR gene content. Based on the genetic diversity and allelic polymorphism of KIRs at the level of the locus, two main KIR haplotypes can be distinguished: A and B. Generally, A haplotypes encode mostly for inhibitory KIRs (KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2, and KIR3DL3) and include KIR2DS4 and KIR2DL4 as the only activating KIRs, whereas B haplotypes are defined by the presence of one or more of the following genes: KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, and KIR3DS1, most of which are activating. Only three common genes (called framework) are shared by all haplotypes KIR3DL3, KIR2DL4, and KIR3DL2 (48-50)

Ligands for several KIRs have been defined, and every receptor appears to recognize a set of classical HLA class I molecules. The inhibitory KIRs contain ITIM motifs in their cytoplasmic domains and they bind to MHC-I molecules with varying allelic specificity. For example, KIR2DL receptors were found to predominantly recognize the HLA-C alleles, whereas KIR3DL2 binds HLA-A3 and HLA-A11. One the other hand, activating receptors possess a lysine residue in their transmembrane domain allowing for association with the DAP12 molecule. In contrast, KIR2DL4 which has a long cytoplasmic tail, associates with FcεR1γ, an adaptor molecule containing an ITAM, to transduce an activating signal (51-53). Interestingly, some pairs of activating and inhibitory receptors, such as KIR2DS1 and KIR2DL1, recognize the same ligand (HLA-C); however, activating KIRs interact with HLA class-I molecules with lower affinity than their inhibitory counterparts (54).

1.4.6. Ly49 family

Ly49 family members are type II transmembrane glycoproteins, are part of the C-type lectin superfamily, and form disulphide-linked homodimers. Ly49 genes are encoded in the NKC on mouse chromosome 6 (55). The Ly49 receptors are extremely polymorphic, with variations in gene number and in multiple allelic forms (Fig.3). Mapping experiments and complete sequencing of the Ly49 gene cluster from different inbred mouse strain genomes (C56BL/6, 129/J, BALB/c, and NOD mice) showed distinct numbers of Ly49 genes in each mouse strain.

Figure 3

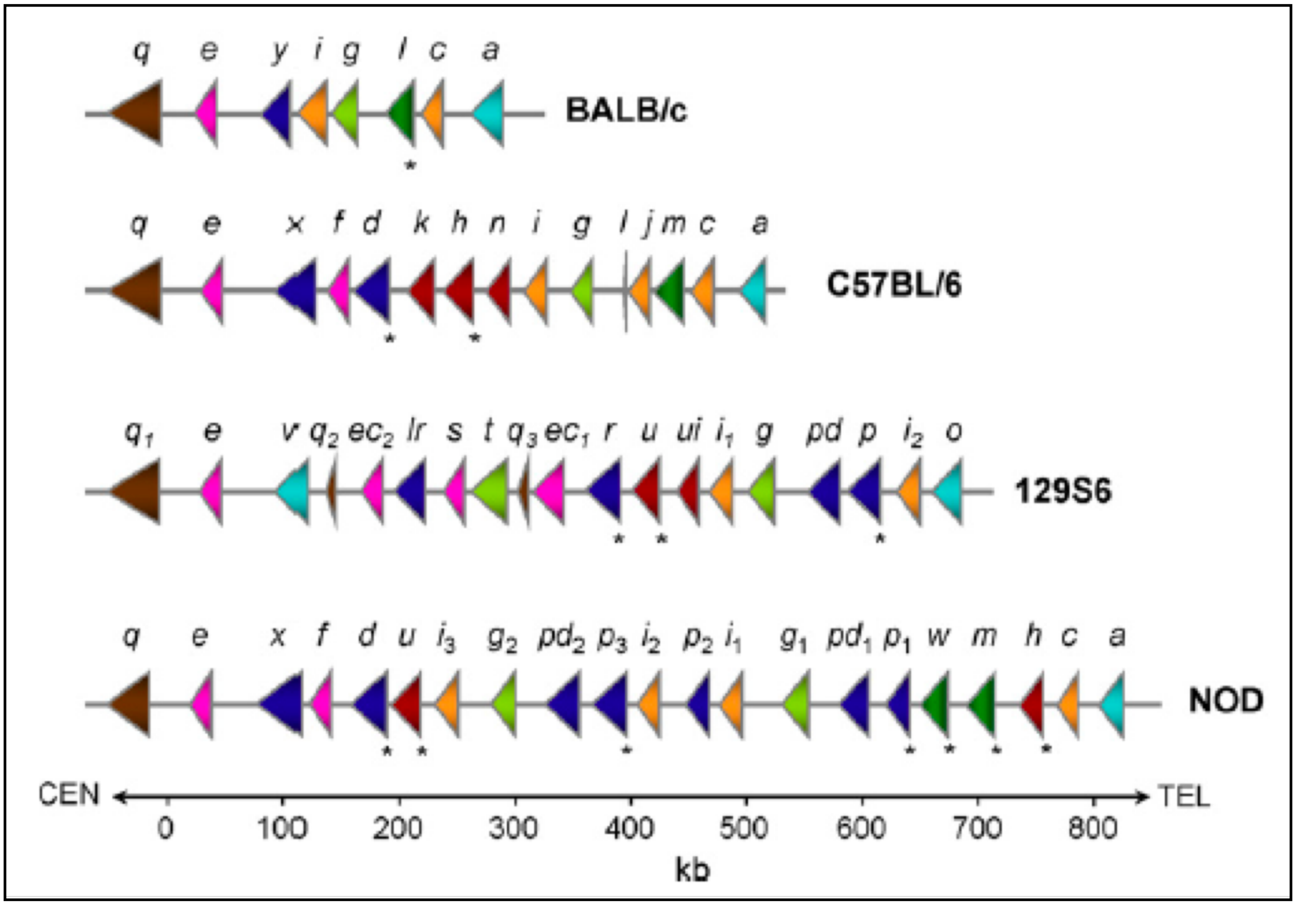

q e y i g l c a
BALB/c
q e x f d k h n i g l j m c a
C57BL/6
q1 e v q2 ec2 lr s t q3 ec1 r u ui i1 g pd p i2 o
129S6
q e x f d u i3 g2 pd2 p3 i2 p2 i1 g1 pd1 p1 w m h c a
NOD
CEN
TEL
0
100
200
300
400
500
600
700
800
kb

Figure 3. Content of the Ly49 haplotypes in different inbred mouse strains. The number of Ly49 genes varies dramatically between inbred mice strains. Different inbred mice strains contain different Ly49 haplotypes. Inhibitory receptors (Ly49A, B, C, E, F, G, I, J, O, Q, S, T and V), activating receptors (Ly49D, H, L, M, P, R, U and W), and the rest are pseudogenes that do not code for any functional proteins. Adapted from (56) with permission from Elsevier.

For instance, the B6 Ly49 cluster is composed of genes encoding two activating receptors (Ly49D, H), eight inhibitory receptors (Ly49Q, E, F, I, G, J, C, A), and five pseudogenes that do not code for any functional proteins. In contrast, the 129-Ly49 cluster is composed of three genes encoding activating receptors (Ly49R, U, P), nine inhibitory receptors (Ly49Q1, E, V, EC2, S, T, I1, G, O), and seven pseudogenes (56). The majority of the Ly49 receptors are expressed by mature NK cells with the exception of Ly49E, Ly49B, and Ly49Q. Ly49E is expressed on fetal NK cells and not on mature NK cells. On the other hand, Ly49B and Ly49Q are expressed by macrophages and plasmacytoid dendritic cells, respectively (57, 58).

Although mouse Ly49 receptor families are not structurally related to human KIRs, the mouse Ly49 were shown to be functionally equivalent to human KIRs. The ligands for Ly49 receptors have been demonstrated to be either MHC- I molecules or MHC- I related molecules that are expressed by pathogens upon infection. Individual Ly49 receptors can recognize several, but not all, polymorphic MHC-I alleles. For example, Ly49A can recognize and bind to H-2D^d, H-2D^k, and H-2D^p, but not to H-2D^b, H-2L^d, or H-2K^b. As a consequence, Ly49A is not functional in B6 mice, which express only two types of MHC- I molecules (H-2K^b and H-2D^b) (59). Nevertheless, both Ly49C and Ly49I are functional in B6 mice since they are able to recognize and bind to H-2K^b molecules. If a target cell does not express the specific MHC-I molecule that would be recognized by the available inhibitory Ly49 receptors on a given subset of NK cells, that cell will be lysed (60)

Inhibitory Ly49 receptors

The inhibitory Ly49, such as Ly49A, Ly49C, Ly49I, and Ly49G, contain ITIMs in their cytoplasmic domains that become phosphorylated in response to receptor ligation with its MHC-I ligand on the target cell. This leads to recruitment of SHP-1 phosphatase, and results in dephosphorylation and deactivation of signaling proteins such as the nucleotide exchange factor VAV1, which is involved in the NK activation cascade, thus blocking NK activation signals (61).

Many tumors have been shown to express sufficient levels of self-MHC-I that can be recognized by Ly49 inhibitory receptors, thus allowing them to escape lysis by NK cells. For example, in H-2^b strains of mice (such as B6 mice) the C1498 leukemia cells bearing self-MHC-I will grow smoothly without being killed by NK cells because of the strong interaction between the Ly49 inhibitory receptors (Ly49 C and I) and the MHC-I on the tumor. However, blockade of Ly49C and I inhibitory receptors in B6 mice using F(ab')$_2$ fragments of the 5E6 monoclonal antibody (mAb) resulted in increased cytotoxicity against these types of tumors and decreased tumor cell growth *in vitro* and *in vivo* (62).

Attempting to evade T-cell recognition, transformed and virally infected cells tend to down-regulate MHC- I from the surface of infected cells. This induces NK-mediated target cell lysis because of loss of inhibitory signals via self-MHC-I recognizing receptors which normally inhibit NK cell cytotoxicity (63).

Ly49 inhibitory receptors and NK cell education:

NK cell education is mediated by the interaction between Ly49 inhibitory receptors and MHC-I during NK cell development, conferring to them the ability to discriminate between normal cells and stressed “missing-self” cells. Studies have shown that NK cells from mice that lack MHC-I or Ly49 receptors during NK cell development are widely considered as hyporesponsive or non-functional (64, 65). As a consequence, these NK cells become unable to kill or reject MHC-I-deficient tumor cells *in vitro* or *in vivo* (64, 66), although these mice have normal numbers of NK cells. The interaction between MHC-I and Ly49 receptor is required for NK cell education, and results in the ability of NK cells to kill and reject MHC-I-deficient cells *in vitro* and *in vivo*. Interestingly, work from our lab has shown that Ly49 deficient NK cells can recognise and kill MHC-deficient cells that express stress ligands for the activating NKG2D receptor, demonstrating that Ly49-deficient NK cells are not completely hyporesponsive (66).

Activating Ly49 receptors

Activating Ly49 lack ITIM sequences. Rather, their cytoplasmic domain associates with the ITAM-containing adaptor molecules DAP12. Upon engagement of an activating Ly49 receptor, the ITAMs of the associated DAP12 become phosphorylated, most likely by Src family kinases (including Lck, Fyn, Src, Yes, Lyn and Fgr), leading to the recruitment of protein tyrosine kinases ZAP-70 or Syk, which in turn initiates a cascade of signaling events leading to NK activation. When NK cells engage with healthy cells that express normal levels of MHC-I, inhibitory receptor signals are dominant over activating receptor signals by recruiting tyrosine phosphatases such as SHP-1/SHP-2/SHIP-1 to dephosphorylate relevant kinases, preventing auto-aggression and thus maintaining NK self-tolerance (30, 67)

NK cells from B6 mice express activating Ly49D receptors that are capable of recognizing and killing target cells that express the H-2D^d allele of MHC-I molecules. Ly49D$^+$ NK cells in B6 mice play an important role in rejection of bone marrow cells that were obtained from BALB/c mice, which express H-2D^d molecules (68). Additionally, depletion of the Ly49D$^+$ NK subset in B6 mice results in increased engraftment of BALB/c bone marrow cells in recipient mice (69). Interestingly, Ly49D was also shown to recognize the hamster MHC-I molecule Hm1-C4 that is normally expressed by Chinese hamster ovary (CHO) cells, thus making these cells extremely susceptible to lysis by Ly49D$^+$ NK cell. Correspondingly, lysis of this target cell can be specifically inhibited using antibody against Ly49D receptors to block the interaction between the receptor and its ligand (70, 71).

Several types of Ly49 receptors (such as Ly49H and Ly49I) were found to bind to viral MHC-I like molecules. Interestingly, both the Ly49H activating receptor (expressed in B6 mice) and the Ly49I inhibitory receptor (expressed in 129/J mice) bind specifically to m157 (72), an MCMV-encoded glycoprotein with MHC-I-like homology. Binding of Ly49H receptor to m157 makes B6 mice resistant to MCMV infection, whereas 129 mice are not. Moreover, Ly49H-deficient C57BL/6 mice were shown to be susceptible to MCMV infection (27, 73). On the other hand, although BALB/c mice are susceptible to MCMV infection, BALB/c-Ly49H transgenic mice become resistant to the infection (74), thus demonstrating the importanceof Ly49 receptors during viral infection.

2. Influenza A virus

2.1. General features and classification

The influenza viruses are classified as members of the Orthomyxoviridae family, which are defined as enveloped viruses with a segmented, negative single-stranded RNA (ssRNA) genome that contain 7–8 gene segments. Based on the antigenic character of influenza virus nucleoproteins, influenza viruses are divided into three types, influenza A, influenza B and influenza C viruses. Influenza A and B viruses contain 8-gene segments whereas influenza C virus contains 7-gene segments. Structurally, unlike influenza A and B which express two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), influenza C virus expresses only a single glycoprotein, the haemagglutinin-esterase-fusion (HEF) protein, which possesses both HA and NA functions (75, 76)

Influenza C viruses cause only mild upper respiratory tract infection. However, influenza A and B viruses can cause severe human illness including upper and lower respiratory tract infection and pneumonia. Influenza A can spread among both humans and animals including pigs, horses, minks, marine mammals and birds, and are associated with the major human pandemics. Influenza B and C affect humans predominantly, nevertheless these viruses have also been isolated from seals and pigs (75, 76).

Influenza A viruses are further classified into many different subtypes based on genetic and antigenic differences in their HA and NA surface glycoproteins. To date, sixteen subtypes of HA (H1–H16) and 9 antigenic subtypes of NA (N1–N9) have been identified, all of which have been isolated from avian hosts. Theoretically, 144 possible different combinations of HA with NA protein could be found. Over one hundred subtype combinations have been identified in birds so far (77, 78). Human influenza viruses of the subtypes H1N1 and H3N2 are the major causes of annual epidemics in the human population. Additionally, avian viruses H5N1, H7N7, H9N2, and H7N3 were also reported to infect humans (79).

2.2. Biology and life-cycle of influenza A virus

The influenza A viruses are highly polymorphic and are visualized as spherical or filamentous. It is an enveloped virus with the outer layer composed of a plasma membrane obtained after its budding from an infected host cell (80). The influenza A virus genome contains eight negative single-stranded RNA segments, that encode for HA, NA, viral matrix protein (M1), integral membrane protein (M2), nucleocapsid protein (NP), the RNA polymerase complex (PA, PB1, and PB2), and nonstructural proteins (NS1 and NS2). Each genome segment is packaged in the virus in complex with the nucleoprotein (NP) and associates with the viral polymerase complex to form viral ribonucleoprotein complexes (vRNPs) (81). HA mediates influenza viral attachment and fusion to the target cell membrane by binding to sialic acid residues that are expressed on the target cell. Therefore, HA has an important role in determining host tropism. Human influenza virus has a HA receptor-binding specificity for sialic acid in α (2-6)-linkage [Neu5Ac (α2-6) Gal], whereas avian influenza virus has higher specificity for α (2-3)-linkage [Neu5Ac (α2-3) Gal]. In parallel with these preferential binding properties, human airway epithelial cells were found to express mainly α (2-6)-linkage, and duck trachea and intestine contain mainly α (2-3)-linkage. Moreover, in the pig trachea, epithelial cells contain both linkages which explains the ability of human and avian viruses to infect pigs (82, 83). Upon binding to sialic acid on the cell surface of the target cell, the virus is internalized into the endosome by receptor-mediated endocytosis. The low pH of the endosome activates the influenza M2 protein to pump in more protons (H^+) into the vesicle, which acidifies the viral interior and facilitates M1 dissociation from RNPs and release of the viral RNP segments into the cytoplasm. The RNPs are then imported into the nucleus, which is the major site for influenza virus transcription and replication. Viral RNA serves as a template for synthesis of mRNA and cRNA. Newly synthesized HA and NA proteins are transported to the cell surface where they integrate into the cell membrane and initiate the budding event. Later on, the newly synthesized RNPs bind to M1 which induces export of the complex from the nucleus to the cytoplasm (76, 84-86). In the cytoplasm, the interaction between M1 coupled with RNPs and the cytoplasmic domains of HA and NA, which serve as docking sites for M1, trigger the assembly of viral components at the lipid rafts and thus signals for exclusion of host proteins from the budding site. The last stage in the influenza A virus replication cycle is mediated by NA, which cleaves sialic acid residues from viral proteins, and prevents HA-receptor interaction and aggregation of the new viruses. This allows the release of newly formed virion particles from the host cell surface to begin a new round of infection (76, 84-86).

2.3. Innate response to influenza virus infections

Both the innate and adaptive immune responses are responsible for host defense against influenza infection. Members of the adaptive immune response, including T and B cells, are important in clearance and prevention of influenza infection; however, it takes approximately 5 to 7 days before antigen-specific antibodies and T cells appear in the lung. Thus, during that time period, the innate immune cells, which include NK cells, alveolar macrophages, and DCs play a critical role in host defense against virus infection by limiting influenza virus replication and by enhancing the rapid development of adaptive responses (87). Pathogens, such as viruses or bacteria, express several distinct ligands, known as pathogen associated molecular patterns (PAMPs), which are essential for survival and pathogenicity. These molecular patterns are typically present on the pathogens' surface or their nucleic acids, such as genomic viral DNA or RNA, and bacterial gram-negative outer membrane lipopolysaccharides (LPS).

Innate immunity plays an important role in the rapid recognition and elimination of invading pathogens through germ-line encoded pattern recognition receptors (PRRs) that recognize the molecular signature PAMPs. PRRs are expressed intracellularly or extracellularly on the cell surface, such as some members of the Toll-like receptor (TLR) family. The stimulation of PRRs leads to induction of several extracellular activation cascades such as complement pathways and various intracellular signaling pathways, leading to inflammatory responses that are essential for effective clearance of evading pathogens. Most of the innate immune cells such as macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, and NK cells, express one or more of the PRRs (88-90).

Influenza A virus primarily infects lung epithelial cells and then spreads to nearby epithelial cells and alveolar macrophages. Being a lytic virus, numerous influenza virus particles are released in the extracellular space and are exposed to innate PRRs and to the PRR in infected epithelial cells themselves. Infected lung epithelial cells can detect influenza virus replicative intermediate double-stranded RNA (dsRNA) using its Toll-like receptor 3 (TLR3), resulting in the production of type I interferon (IFN-I) (91, 92). IFN-I, which includes, IFN-α and IFN-β, are major components of the innate immune response that limit influenza viral infections and drives the adaptive immune response to the site of infection by enhancing the presentation and recognition of influenza virus antigens. Moreover, IFNs stimulate induction of several antiviral genes that interfere with influenza virus replication and thus contribute to cellular resistance to influenza virus infection. For example, IFN-I induces expression of the human MxA protein, which is capable of binding to the RNA polymerase subunit of the influenza virus and thus prevents virus replication (93-95). Moreover, IFNs significantly enhance NK cell activity leading to NK cell proliferation and production of cytotoxic granules that kill target cells. IFNs also enhance DC differentiation and activation (96, 97). Interestingly, plasmacytoid DC are able to detect and recognize influenza virus single-stranded RNA (ssRNA) using TLR7 leading to robust induction of type I interferons (98).

In the resting state, alveolar macrophages negatively regulate NK cell activity by secreting inhibitory cytokines such as prostaglandins and transforming growth factor (TGF-β) (99, 100). Studies have demonstrated that pulmonary NK cells isolated via bronchoalveolar lavage (BAL) or from lung tissue were not able to lyse

NK-sensitive target cells (101). However, incubation of pulmonary NK cell with IFN-I or IL-2 were enough to restore NK cell activity (101, 102). Interestingly, NK cells from mice lacking IFN-I receptors are unable to kill the MHC-I deficient cell line (RMA-S) in comparison to wildtype NK cells after stimulation with IFN-I. However both types of NK cells were able to recognize and kill a cell line that expresses a ligand for the NK cell activating receptor, NKG2D (102). This demonstrates the ability of NK cells to kill target cells that express stress ligands without previous activation. Moreover, these reports demonstrate the importance of IFN-Is as an early and critical regulator of NK cell activation and proliferation (102, 103).

Upon influenza virus infection, infected macrophages produce large amounts of monocyte chemoattractants, particularly the CC chemokines (such as CCL2), that recruit large numbers of NK cells to the lung within the first few days of infection (104-106). Abundant secretion of IFN-I by activated and infected macrophages and DCs augments NK cell cytotoxicity. Furthermore, direct interactions between influenza virus infected-macrophages or DCs with NK cells strongly stimulates NK cytotoxicity and induction of IFN-γ production, which has an important role in macrophage and DC activation and Th1 cell proliferation (107, 108).

3. Role of NK cells during influenza virus infection

Mice and hamsters depleted of NK cells show increased morbidity and mortality during influenza virus infection, which demonstrates the important role of NK cells during influenza virus infection (109). Activated NK cells are recruited to the lung two days after influenza virus infection and peak at day 5 and 7 (110), whereas influenza virus replication peaks within 2 to 3 days after infection and declines significantly by day 5 (111). The protective function of NK cells during influenza virus infection was partially explained by the report that the NK cell activating receptor (NKp46) can recognize influenza hemagglutinin on virally infected target cells and that this recognition is crucial for protecting mice against lethal doses of influenza virus (43, 45). Binding of NKp46 activating receptor to influenza virus hemagglutinin on infected cells triggers the NK cell to lyse the infected cell and consequently, limits viral infection and replication (43, 45, 112). As stated earlier, influenza virus-infected monocytes and dendritic cells robustly enhance NK cell cytotoxicity by producing high levels of IFN-I and by direct contact. Recent studies have demonstrated that influenza virus infected monocytes and dendritic cells express high level of the stress ligand UL16-binding protein (ULBP)1–3, which is recognized by NK cell activating receptor NKG2D, and results in enhanced cytolytic activity of NK cells toward influenza virus-infected cells and increased IFN-γ production (108, 113). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induces apoptosis of several tumor cells but not normal cells (114). Interestingly, influenza A virus infection was shown to induce TRAIL expression on lung NK cells, but not on T cells 4 days post-influenza virus infection (115). Blocking of this TRAIL resulted in significantly increased virus titer (115). Therefore NK cells expressing TRAIL may play an important role in the immune response to influenza A virus infection. Additionally, NK cells are able to kill influenza virus-infected cells that are bound by specific antibody (Ab) (116). The protective function of M2-specific antibodies that recognize and bind to M2 protein expressed on the surface of influenza virus-infected cells depends mainly on the presence of NK cells *in vivo* (117). NK cells mediate this protection via antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells express CD16 which binds to the Fc portion of antibodies and induces NK cell lytic function (118).

3.1. How do influenza viruses escape NK cell recognition?

In response to NK cell cytolytic function, influenza virus has developed several evasion strategies to escape NK cell recognition. Interestingly, influenza viruses have developed a strategy to down-regulate NK cell activating receptors (ex: NKp46) *in vitro* and *in vivo*. Studies have demonstrated that incubating influenza virus particles with NK cells resulted in significant NKp46 down-regulation from the cell surface (119). In agreement with this finding, incubation of fresh or IL-2-activated NK cells with influenza virions or hemagglutinin resulted in significant inhibition of NK cell cytotoxicity towards influenza virus-infected macrophages (120).

Additionally, influenza virus was found to be able to bind directly to sialic acid residues that are normally expressed on the surface of NK cells. As a result, NK cells become extremely susceptible to influenza virus infection, which results in NK cell apoptosis (91). Influenza virus uses clathrin-dependent endocytosis to enter NK cells. Although, some influenza viral components are synthesized in NK cells, no infectious virus is detected. This phenomenon is called abortive infection (91). *In vivo* infection of lung NK cells by influenza A virus has also been reported (121). NK cells express sialic acids containing α-2, 3 and α-2, 6 linkages that facilitate influenza virus binding and entry. Additionally, this infection resulted in significantly lower cytotoxicity by influenza-infected NK cells than uninfected NK cells against the NK-sensitive target cells YAC-1 and RMA-S (121).

NK cell activity is regulated by a variety of both activating, such as NKp46, and inhibitory receptors, such as the inhibitory Ly49 family in mice and the KIR family in humans. Upon engagement of both activating and inhibitory receptors by a target cell, the outcome is determined by the net balance of signals, which determines whether the NK cell becomes activated to kill the target cell or not (122). Surprisingly, influenza virus infection was shown to induce reorganization and accumulation of MHC-I molecules in the lipid raft microdomains of infected cells leading to increased binding of the NK cell inhibitory receptors KIR2DL1 and resulting in the inhibition of NK cell cytotoxicity (123, 124). Other reports demonstrated that influenza virus infection causes up-regulation of MHC-I molecules on infected A549 human alveolar epithelial cell-line, which results in the inhibition of NK cell cytotoxicity (125).

4. NK gene complex Knockdown (NKC^{KD}) mice

Our lab has generated the NKC^{KD} mouse model in which approximately 80% of NK cells do not express Ly49 receptors compared to NK cells in WT mice (66). NKC^{KD} were generated by targeting the promoter region of Ly49o with a floxed neomycin selection cassette that was then electroporated into 129-background embryonic stem (ES) cells carrying a Ly49Q ^{lox} allele. Thisstrategy was devised to delete the entire Ly49 gene clluster using Cre-mediated recombination of flanking LoxP sites. ES cells were electroporated with a CMV-Cre plasmid to induce *in vitro* Cre-mediated removal of the neomycin cassette. After generating the mice, it was found that the deletion of the entire Ly49 gene cluster was unsuccessful. Unexpectedly, multiple copies of the targeting construct were integrated as a concatemer upstream of the Ly49 gene cluster. Flow cytometry analysis demonstrated that cell surface expression of Ly49, NKG2/CD94, and KLRI were significantly downregulated in NKC^{KD} mice in comparison to WT mice.

NKC^{KD} NK cells exhibit defective in killing MHC-I-deficient targets cells *in vitro* and *in vivo*. However, NK cells from NKC^{KD} mouse are able to recognize and kill MHC-I-deficient targets cells that express a ligand for NKG2D receptors as efficiently as WT NK cells. Therefore, NKC^{KD} mice serve as a viable model to study the role of Ly49 inhibitory receptors during infectious disease.

5. Hypothesis

Upregulation of cell surface expression of MHC-I on influenza infected cells may inhibit NK cell function through interaction with mouse Ly49 inhibitory receptors. We hypothesis that Ly49-deficient (NKC^{KD}) mice would exhibit better survival against influenza infection than wild-type mice due to a lack of inhibitory receptors.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.Ly49^{129} congenic and B6.NKC^{KD} mice have been described previously (21,26). All mice were maintained in a specific-pathogen-free environment. All breeding and manipulations performed on animals were in accordance with university guidelines and approved by the University of Ottawa animal ethics committee.

Cell lines

Lung epithelial cell line (TC-I) is a tumor cell line derived from C57BL/6 mice, and was produced by cotransforming E6 and E7 antigens of human papillomavirus 16 (HPV-16) and activated ras oncogenec-Has-Ras into mouse lung epithelial cells. TC-1 was provided by James Herman (Johns Hopkins Oncology Center, Johns Hopkins Medical School, Orleans, USA).

Influenza virus

The human HIN1 prototype strain, A/FM/1/47 (FM), was epidemic in Fort Monmouth, New Jersey, USA in 1947 (126). FM virus was adapted to the mouse by twelve serial lung passages to produce a virulent variant, A/FM/1/47-MA (FM-MA), that grows to a high titre in vivo and in vitro (127). FM-MA virus was kindly provided by Dr. Earl Brown (University of Ottawa, Ottawa, Canada).

Influenza virus infection

Groups of male and female mice (6-8 weeks old) were anesthetized with isoflurane and inoculated intranasally with 500 and 1050 plaque forming units (PFU), diluted in sterile PBS for a total inoculation volume of 50 ul, of mouse-adapted A/FM/1/47 H1N1 strain of influenza A virus (FM-MA virus). Influenza-infected mice were housed in a level 2 confinement area for the duration of the experiment.

To infect a lung epithelial cell line (TC-I), 4 x10^6 cells were washed twice with 1x PBS in a 15ml tube and spun down for 5min at 500xg. Cells were infected with 24x10^6 PFU of FM-MA virus in 100µL volume of PBS, theoretically in order to ensure efficient infection of >99% of all TC-1 cells, we infected cell at a multiplicity of infection (MOI)=6, for 1h at 37°C. Following incubation, infected cells were resuspended in 8 ml of RPMI medium supplemented with 100U/mL penicillin and 100µg/mL streptomycin without FBS and transferred to a Petri dish. Infected cells were further incubated for 4h, 8h or 15h at 37°C and 5% CO_2.

Adherent lymphokine activated killer (ALAK) cell culture

Spleens of indicated mice were crushed on a 70**µ**M cell strainer using a 10ml syringe plunger and then washed with 1x PBS. Cells were transferred into 15ml tubes and spun down for 5min at 500xg. Cell pellets were resuspended in 5mL/spleen of ACK lysis solution (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA;

pH 7.2-7.4) for 5min at room temperature to lyse red blood cells. After 5min incubation 10ml of 1x PBS was added followed by centrifugation for 5min at 500xg. Splenocytes were cultured in 15mL RPMI medium supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 100μg/mL streptomycin, 1mM sodium pyruvate, 100μM non-essential amino acids, 10mM HEPES, 50μM 2-β-mercaptoethanol and 1000U/mL human IL-2 (NK medium), and incubated for three days at 37ºC with 5% CO_2. After 3 days, the medium was removed without disturbing the adherent cells and was replaced with 15mL of fresh NK medium with 1000U/mL human IL-2. Adherent cells were grown for 3 more days at 37ºC with 5%.

Lung epithelial cell isolation

Lungs of the indicated mice were removed and minced in 5ml RPMI with 2mg/mL collagenase D (Roche), followed by incubation for 1 hour at 37 ºC with agitation. The minced pieces were crushed on a 70**μ**m cell strainer using a 10ml syringe plunger and washed with 1x PBS. Cells were transferred to a tube and spun down for 5min at 500xg. Cell pellets were resuspended in 5mL/lung of ACK lysis solution for 5min at room temperature to lyse red blood cells. After 5min of incubation, 10ml of 1x PBS was add to the cells followed by centrifugation for 5min at 500xg. Cell pellets were resuspended in 10ml of RPMI medium supplemented with 10% FBS, 2mM L-glutamine,100U/ml penicillin and 100μg/mL streptomycin (complete RPMI).

Antibodies and flow cytometry

The following antibodies specific for various cell surface markers were obtained from commercial sources: anti-mouse CD18 (LFA-1), anti-mouse CD326 (EpCAM), anti-mouse MHC-I (H-2K^b) and anti-mouse MHC-I (H-2D^b) (eBioscience, San Diego, CA, USA), Allophycocyanin-Streptavidin (APC-SA) was purchased from eBioscience.

To stain lung epithelial cells for flow cytometry analysis, 1.5x10^6 lung cells were transferred to FACS tubes containing 1mL FACS buffer (1x PBS, 0.5% BSA and 0.02% NaN_3) and spun down for 5min at 500xg. Supernatants were removed and 1µL of rat serum was added to the cells, followed by vortexing. The tubes were incubated at 4ºC for 15 min. Antibodies appropriately diluted in FACS buffer (at 1:50 ratio) were added to the cells and incubated at 4ºC for 20 min. Cells were then washed with 1mL FACS buffer and if necessary, secondary staining with APC-SA was performed in FACS buffer as above. Cell fluorescence data was acquired with a CyAN-ADP flow cytometer (Beckman Coulter) and analyzed with Kaluza software (DB Biosciences, New Jersey, USA).

In vitro cytotoxicity assays

In vitro cytotoxicity was measured using the standard 4h ^{51}Cr-release assay. Target cells were harvested with 1mM EDTA in 1x PBS and counted. Appropriate number of target cells were spun down for 5min at 500xg and resuspended in 80µL of 1x PBS containing 100µCi of ^{51}Cr in the form of Na_2CrO_4 (Perkin Elmer, Boston, MA Cells were incubated for 1h at 37ºC with gently mixing every 15min. After the incubation, cells were washed twice with complete RPMI and once with NK media, resuspended in 500µl NK medium and counted. ^{51}Cr-loaded target cells were then resuspended in NK medium at a cell concentration of 5.0x10^4 cells/mL.

ALAK cells were harvested with 10ml of 1mM EDTA in 1x PBS, counted and resuspended in NK medium at a concentration of 2.5x10^6 cells/mL. ALAK cells were seeded in 96-well V-bottom plate in triplicate at specific effector/target (E/T) ratio. To the 1st 3 wells of a row, assigned for 50:1 E/T ratio, 100µL of ALAK cells (2.5x10^5 cells/well) was added and to the remaining 9 well of the same row 100µL of NK medium was added. To the 2nd 3 well, 100µL of ALAK cells was added to obtain a total volume of 200µL (100µL of ALAK cells+100µL of NK medium). The contents of the 2nd 3 wells were mixed, using a multichannel pipetteman, and 100µl was transferred to the 3rd 3 wells. Similarly, the contents of the 3rd 3 wells were mixed and 100µl was transferred to the 4th 3 wells. Lastly, the contents of the 4th 3 wells were mixed and 100µL was discarded leaving 100µL ALAK cells in the wells. The following E/T ratios were obtained through serial dilutions: 50:1, 25:1, 12.5:1 and 6.25:1. At this point, 100µL of ^{51}Cr-labelled target cells (5.0x10^3 cells/well) was added to every well containing ALAK cells. An additional 6 wells received 100 µL ^{51}Cr-labelled target cells to be used for maximal release and minimal release in triplicates. To the minimal release wells, 100µL of NK media was added, while the maximal release wells received 100µL of 10% SDS. The plate was incubated at 37ºC for 4 hours. At the end of incubation period, the plate was centrifuged at 1500rpm for 5min and 100µL of the supernatant from each well was transferred to titer tubes without disrupting the pellet. The ^{51}Cr released into the supernatant from lysed target cells was determined using a gamma counter (Perkin Elmer, Boston, MA).

The % ^{51}Cr released was calculated as follows:

$$\% \, ^{51}\text{Cr release} = \frac{(\text{experimental release} \;-\; \text{average of minimal release})}{(\text{average of maximal release} \;-\; \text{average of minimal release})} \times 100$$

Plaque assay for Influenza virus

Mice infected with FM-MA virus were sacrificed and lungs were removed in pre-weighed 2 ml tubes on ice. Tubes containing the lungs were weighed again to determine the weight of the lungs, The lungs were homogenized in 1ml of sterile 1x PBS using Bead beater. Lung homogenates were centrifuged at 5,000×g for 5min. Clear supernatants were transferred to clean 1.5ml microcentrifuge tubes and kept at -80°C.

Viral titre from lung homogenates was quantified on Madin-Darby Canine Kidney Cells (MDCK) cells, kindly provided by Dr. Earl Brown (University of Ottawa, Ottawa, Canada), that were seeded in 6-well plates two days in advance to obtain a confluent cell monolayer. Serial dilutions of lung homogenates ranging from 10^{-1} to 10^{-6} were prepared in 1x PBS on ice. Confluent MDCK monolayers in 6 well plates were washed twice with 1x PBS and infected in duplicates with 100 µl of the different virus dilutions. The plates were incubated at 37°C for 30 min to allow virus adsorption and were gently agitated every 15 min to spread the virus. Meanwhile the overlay consisting of 10ml of 1.3% agarose, 10ml of 2x MEM, 0.52ml of 7.5% $NaHCO_3$, and 4ul trypsin TPCK (5mg/ml), sufficient for one 6-well plate, was prepared and kept warm. At the end of incubation period, 3ml of the overlay was added to each well. Plates were incubated at 37°C for 3 days. On day 3, plaques were fixed by adding 3ml of Carnoy's fixative (three parts Methanol with one part Acetic Acid (v/v)) to each well for 1 day at room temperature. Plates were then washed with tap water to remove the overlay gel and 2ml of 0.1% crystal violet was added to each well for 1h. Plates were washed gently and the plaques were counted. Virus titer was expressed as the number of plaque forming units per gram of lung (pfu/gm).

Lung histopathology

Groups of mice were inoculated intranasally with 500 pfu of FM-MA virus as described above. Lungs were collected 5 days post-influenza virus infection and fixed in 10% neutral buffered formalin (25 mL) for 48h. Subsequently, lungs were embedded in paraffin, sectioned at a thickness of 4μm and stained with hematoxylin and eosin (H&E) (Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Canada). Slides were examined by a qualified pathologist to score histopathologic changes in the lungs (Dr. Harman Sekhon, University of Ottawa, Ottawa, Canada)

Statistical analysis

Two-tailed student’s *t*-test and ANOVA statistical analysis was performed using GraphPad Prism software. Data are presented as mean value ± standard deviation (SD) and a p value <0.05 was considered statistically significant.

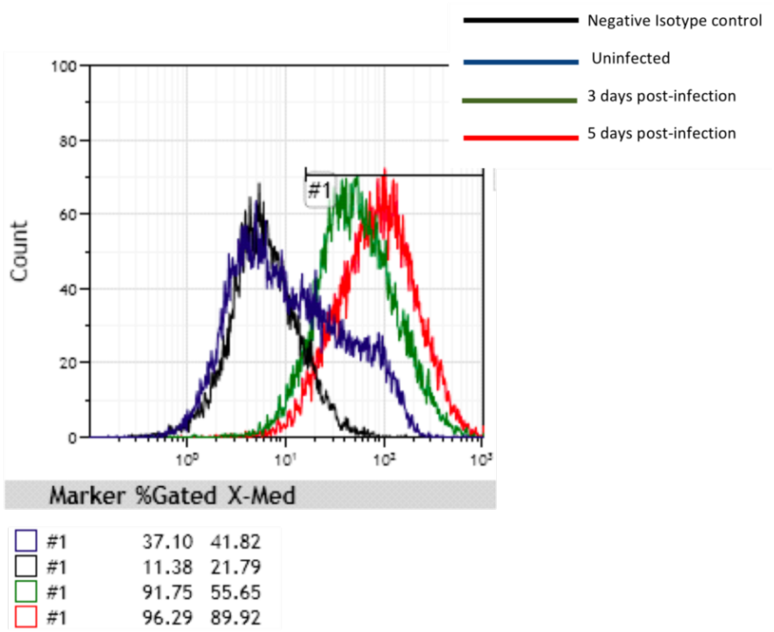
Results

Influenza A virus induces MHC class I upregulation on lung epithelial cells upon infection *in vivo*

It is well established that *in vitro* influenza virus inhibits NK cell cytotoxicity by enhancing the binding of NK cell inhibitory receptors to MHC-I on influenza virus-infected human lung epithelial cells (123-125, 128). Therefore, we decided to determine whether influenza virus infection could modulate MHC-I expression on mouse lung epithelial cells. To the best of our knowledge, there is no solid evidence for *in vivo* or *in vitro* modulation of MHC-I expression on mouse lung epithelial cells following influenza virus infection. Most of the studies have been performed using human cell lines, yet there is no clear answer whether influenza virus infection results in modulation of MHC-I expression on lung epithelial cells. To this end, B6 mice, which express H2D^b and H2K^b MHC-I alleles (59), were infected intranasally with 5000 pfu of FM-MA virus, and MHC-I expression was determined on EpCAM$^+$ (CD326) lung epithelial cells (129) on day 3 and 5 post-infection (p.i.) by flow cytometry. In the uninfected mice, H2K^b and H2D^b expression was detected on a small proportion of lung epithelial cells (**Fig. 4A and B**). However, upon infection with influenza virus, the proportion of lung epithelial cells expressing H2K^b and H2D^b was dramatically increased (**Fig. 4A and B**). Interestingly, MHC-I expression was higher on day 5 as compared to day 3 p.i., as determined by a positive shift in fluorescence intensity (**Fig. 4A, B**).These data demonstrate that, influenza virus infection induces upregulation of MHC-I expression on lung epithelial cells which increases with the duration of infection.

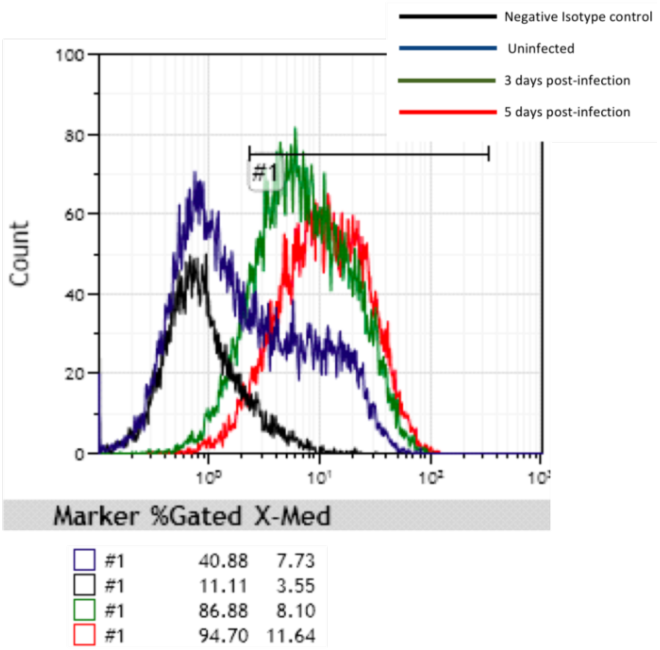
Figure 4

A


H2Db expression on mouse lung epithelial cells

Marker	%Gated	X-Med
☐ #1	37.10	41.82
☐ #1	11.38	21.79
☐ #1	91.75	55.65
☐ #1	96.29	89.92

B


H2kb expression on mouse lung epithelial cells

Marker	%Gated	X-Med
☐ #1	40.88	7.73
☐ #1	11.11	3.55
☐ #1	86.88	8.10
☐ #1	94.70	11.64

Figure 4. Influenza A virus induces MHC-I upregulation on lung epithelial cells. B6 mice were infected intranasally with 5000 pfu of FM-MA virus. Single-cell suspension was prepared from uninfected lungs and those infected with the virus for 3 and 5 days. Cells were stained with antibodies against H2K^b H2D^b and CD326 (EpCAM; epithelial cell marker), and analysed by flow cytometry. Surface expression of H2D^b (A) and H2K^b (B) were determined on EpCAM$^+$ lung epithelial cells and percent of positive cells and mean fluorescent intensity (MFI) are indicated. The following fluorochrome-conjugated mAb were used in this experiment: FITC-anti-LFA-1, PE-anti-EpCAM, efluro450-anti-H-2K^b, biotin-anti-H-2D^b and APC-streptavidin. The percentage of positive cells and mean fluorescence intensity are calculated by subtracting the isotype control from the specific signal.

Increased survival of influenza A virus-infected NKC^{KD} mice

It has been shown that binding of NK cell inhibitory receptors to MHC-I molecules is enhanced after influenza virus infection, which significantly decreases NK cell cytotoxicity toward influenza virus-infected cells (125). 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. To determine whether Ly49 interaction with MHC-I molecules are relevant to influenza infection *in vivo*, we inoculated WT and NKC^{KD} mice, which have a significantly lower expression of Ly49 receptors on NK cells, with 1050 pfu FM-MA virus intranasally. The animals were observed daily over 2 weeks and sacrificed when moribund. Infected mice exhibited signs of illness and body weight loss by day 4 p.i. Death due to infection began occurring on day 8 p.i., at which time mice had lost over 25% of their body weight (**Fig. 5A**). By day 10 p.i., surviving mice began to gain weight (data not shown). At the end of 2 weeks p.i., almost 90% of WT mice had succumbed to the infection while 60% of NKC^{KD} mice had survived (**Fig. 5B**, *$p>0.05$). These data show that, NKC^{KD} mice survive influenza virus infection better than the WT mice, indicating a possible role for Ly49-MHC-I interaction in the pathogenesis of influenza virus in mice.

Figure 5

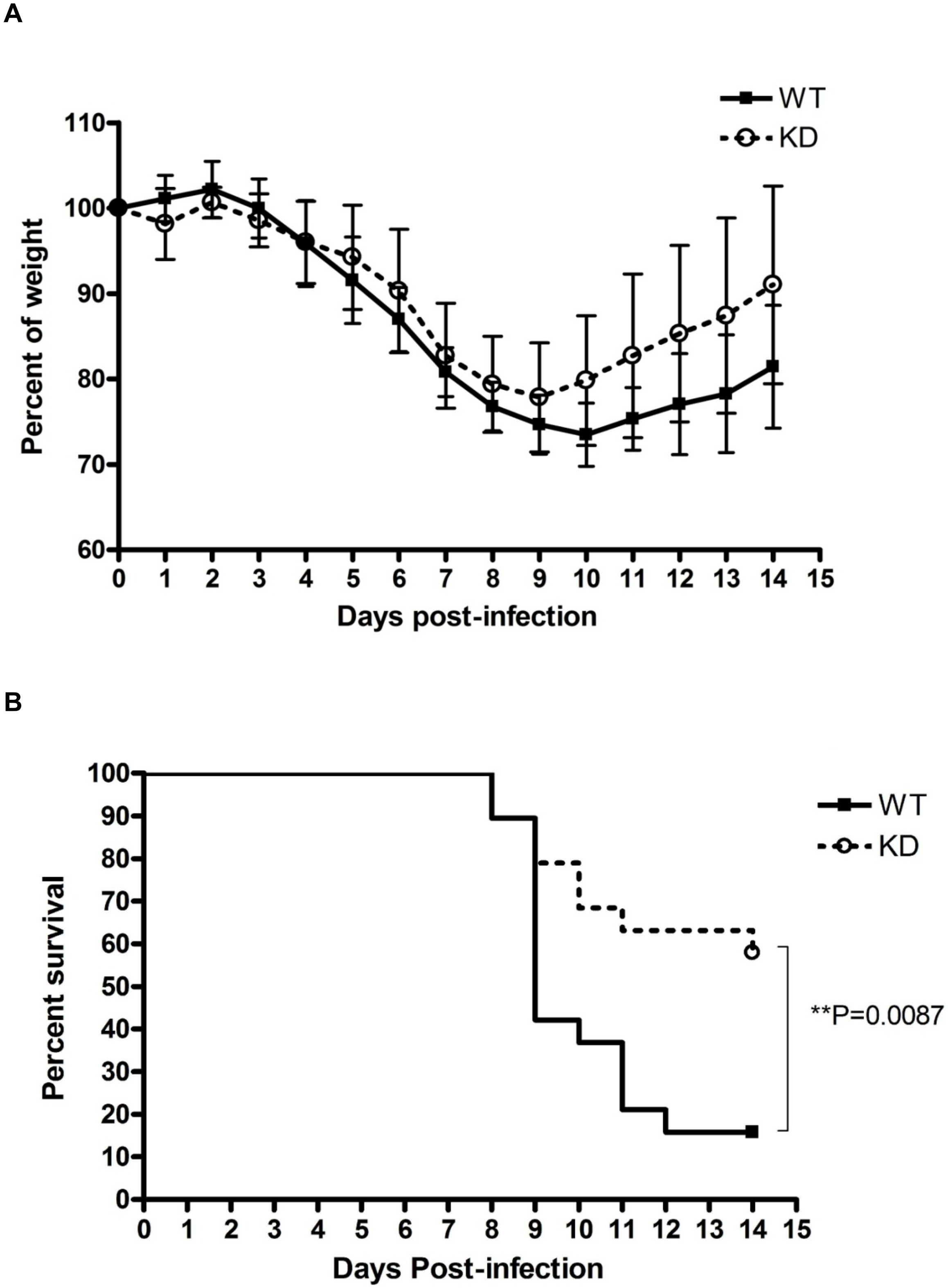

A
WT
KD
Percent of weight
110
100
90
80
70
60
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days post-infection
B
WT
KD
Percent survival
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post-infection
**P=0.0087

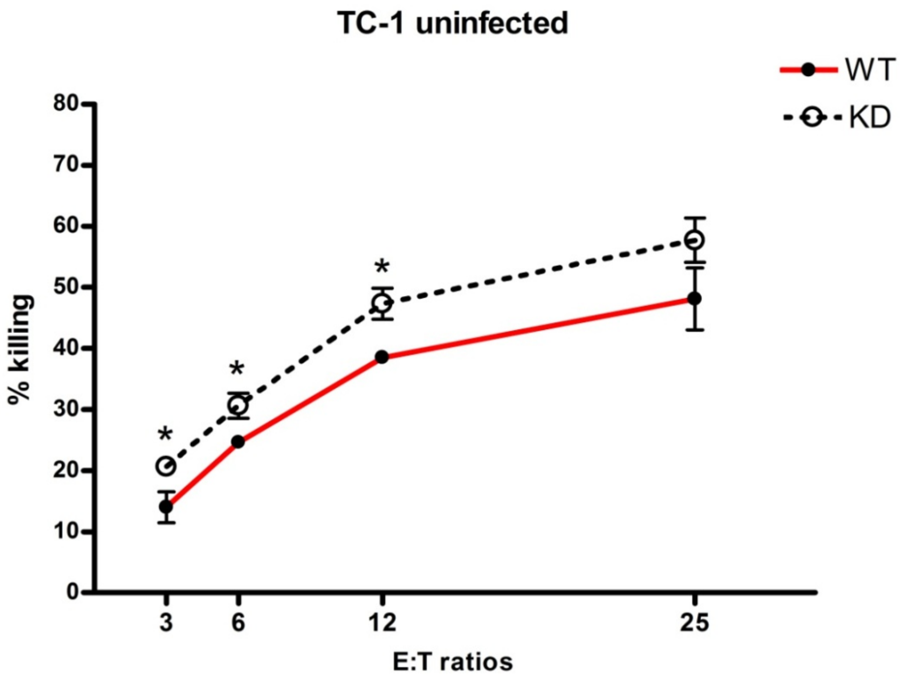
Figure 5. Better survival of influenza-infected NKC^{KD} mice. WT mice and NKC^{KD} mice were inoculated intranasally with 1050 pfu of FM-MA virus and monitored for 2 weeks. Data are pooled from two independent experiments. In the first experiment, two groups of mice (ten each) were infected. In the second experiment, two groups of mice (nine each) were infected. All mice were 6-8 weeks old. Average percentage of body weight +/- SD (A), and percent of surviving mice (B) are shown. WT: wild-type, KD: NKC^{KD}. $p < 0.05$ was calculated by log-rank test.

NKC^{KD} NK cells show enhanced cytotoxity against influenza virus-infected lung epithelial cells *in vitro*

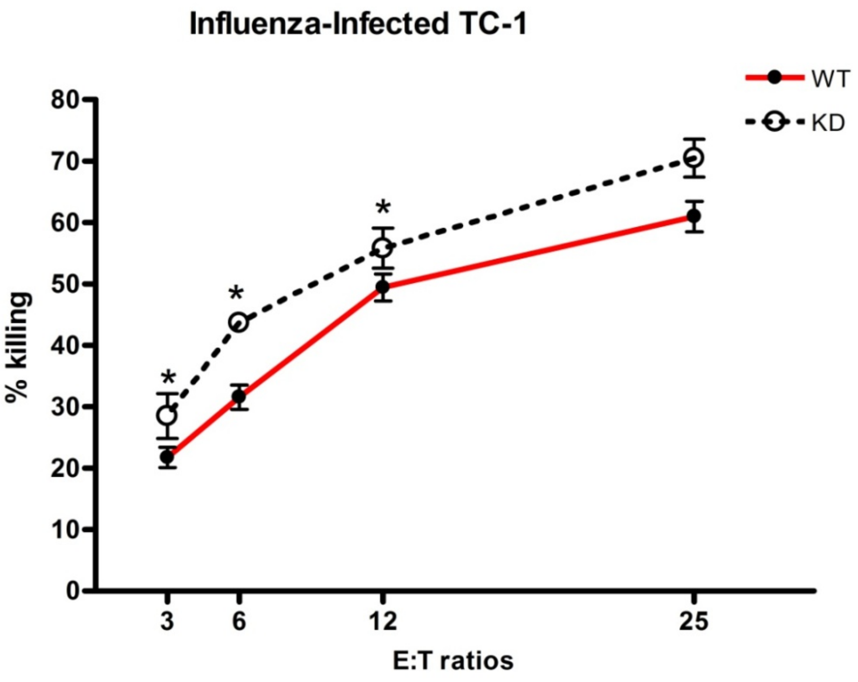
Better survival of influenza-infected NKC^{KD} mice led us to ask whether NK cells from NKC^{KD} mice can eliminate influenza virus-infected lung epithelial cells better than the NK cells of WT mice. We determined NK cytotoxicity against influenza virus-infected lung epithelial cell line, TC-1, which has been shown to be a good target for NK cells (129). IL-2 activated splenic NK cells from NKC^{KD} and WT mice were examined for their killing of influenza virus-infected TC-1 using the standard 4h chromium-release cytotoxicity assay. NK cells from NKC^{KD} mice were able to recognize and lyse uninfected TC-1 cells significantly better than NK cells from WT mice (**Fig.6A**). However, this difference was not statistically significant at high E:T ratio (25:1) (**Fig. 6A**). NK cells from both WT and NKC^{KD} mice showed increased killing of influenza virus-infected TC-1 as compared to uninfected cells (**Fig. 6A and B**). NKC^{KD} NK cells were again significantly more efficient at killing influenza virus-infected TC-1 as compared to WT NK cells (**Fig. 6B**). Flow cytometry analysis of MHC-I expression on TC-1 cells showed uniformly high expression of H2K^{b} and H2D^{b}, which did not increase further upon influenza virus infection (**Fig. 6C and D**). These data suggest that influenza virus infected-lung epithelial cells (TC-1) are more susceptible to killing *in vitro* by NK cells lacking the inhibitory Ly49 receptors. This may be due to high expression of MHC-I on TC-1 cells which could inhibit WT NK cells through interaction with the inhibitory Ly49 receptors.

Figure 6

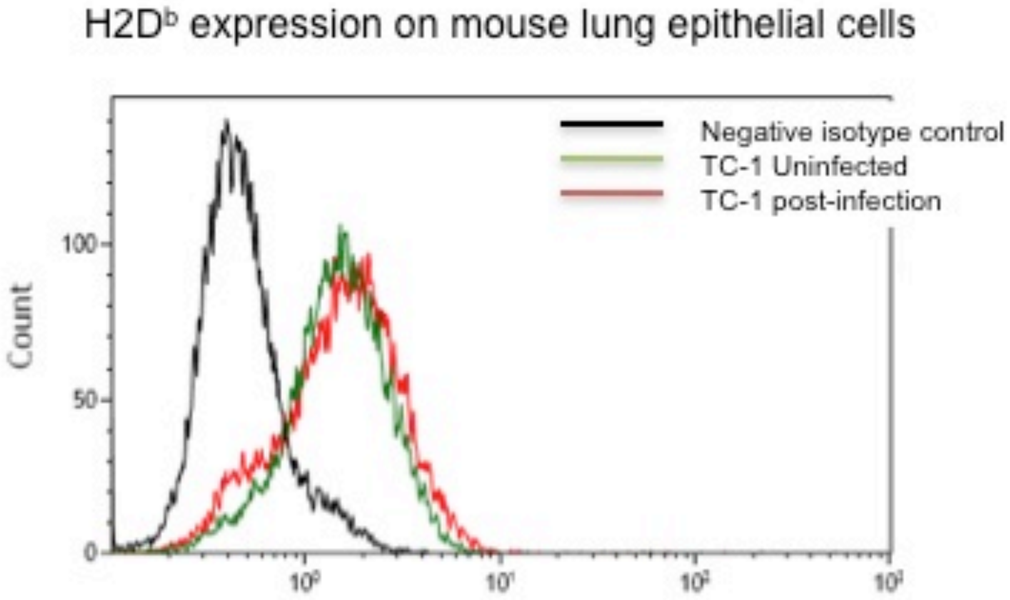
A


TC-1 uninfected

B


Influenza-Infected TC-1

C


H2D^b expression on mouse lung epithelial cells

D

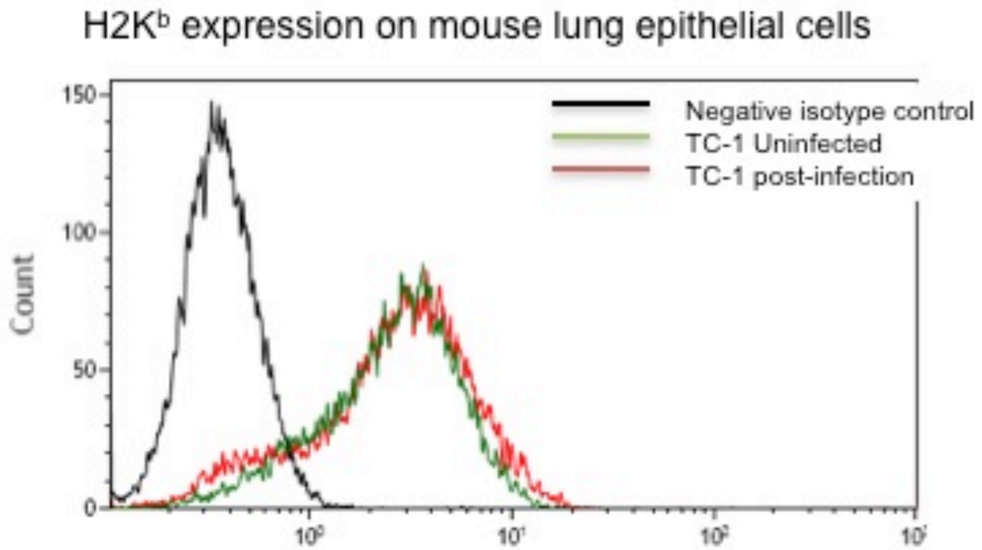

H2K^b expression on mouse lung epithelial cells

Figure 6. NK cells from NKC^{KD} mice kill influenza virus- infected TC-1 cells better than WT mice *in vitro*. TC-1 cells were infected with FM-MA virus at MOI=6 for 9h, in order to ensure efficient infection of >99% of all TC-1 cells theoretically (130). IL-2-activated NK cell cytotoxicity towards uninfected (A) and infected (B) TC-1 cells was performed using the standard 4h ^{51}Cr-release assay at various effector to target cell ratios (E:T). Data are shown as the average of triplicate wells +/- SD. **p*<0.05 was calculated by Student's *t*-test. MHC-I expression, H2D^b (C) and H2K^b (D), on uninfected and infected TC-1 cells was determined by flow cytometry. This experiment was performed at least 3 times with similar results. One mouse used for each group per cytotoxicity experiment. ALAK cells were plated in triplicate samples.

Similar lung influenza virus titers in NKC^{KD} and WT mice

Increased cytotoxicity of NKC^{KD} NK cells towards infected lung epithelial cells may lead to more efficient elimination of virus-infected cells from lungs, and lower viral load in the lungs of infected mice. To determine lung viral titres, WT and NKC^{KD} mice were infected intranasally with 500 pfu of FM-MA virus and lungs were harvested on day 3 and 5 p.i. Virus titer of the lung homogenate was determined using a virus plaque assay. High viral load was detected in the lungs of WT and NKC^{KD} mice 3 days p.i., which decreased equally in both groups of mice on day 5 p.i. (**Fig. 7**). Although, viral titer in NKC^{KD} mice was slightly lower than WT mice, particularly on day 5 p.i., this difference was not statistically significant, possibly due to the small number of mice used in this assay. The results so far indicate that NKC^{KD} NK cells are not better than WT NK cells at controlling viral load in the lungs of influenza infected mice.

Figure 7

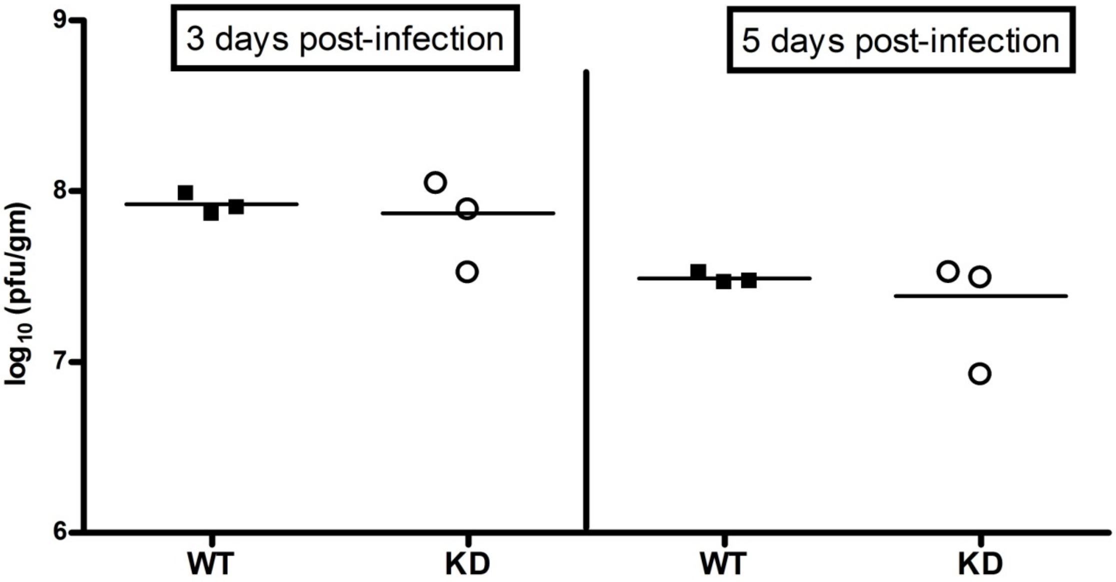

3 days post-infection
5 days post-infection
9
8
7
6
$\log_{10}$ (pfu/gm)
WT
KD
WT
KD

Figure 7. **Similar viral loads in the lungs of WT and NKC^{KD} mice infected with influenza virus**. WT and NKC^{KD} mice were infected intranasally with 500 PFU of FM-MA virus. Lungs were collected at day 3 and day 5 p.i. and virus titre was assessed in lung homogenates by plague assay on MDCK cells. Virus titre is presented as pfu/gm of lung tissue. Horizontal bars represent mean values. WT, wild-type; KD, NKC^{KD}.

More severe lung pathology in WT mice vs. NKC^{KD} mice during influenza infection

Influenza virus infection causes severe lung pathology leading to respiratory distress and mortality (131). To examine lung pathology in WT and NKC^{KD} mice, lungs were collected 7 days after the mice were infected intranasally with 500 pfu of FM-MA virus. Lung sections were prepared and stained with hematoxylin and eosin (H&E). Microscopic examination of H&E stained lung sections showed more severe alveolar damage, leukocyte infiltration and pulmonary edema in WT mice compared to the NKC^{KD} mice (**Fig. 8**). These data suggest that influenza virus infection induces more severe lung pathology in WT mice compared to NKC^{KD} mice, and may explain the increased mortality in WT mice compared to NKC^{KD} mice during influenza virus infection.

Figure 8

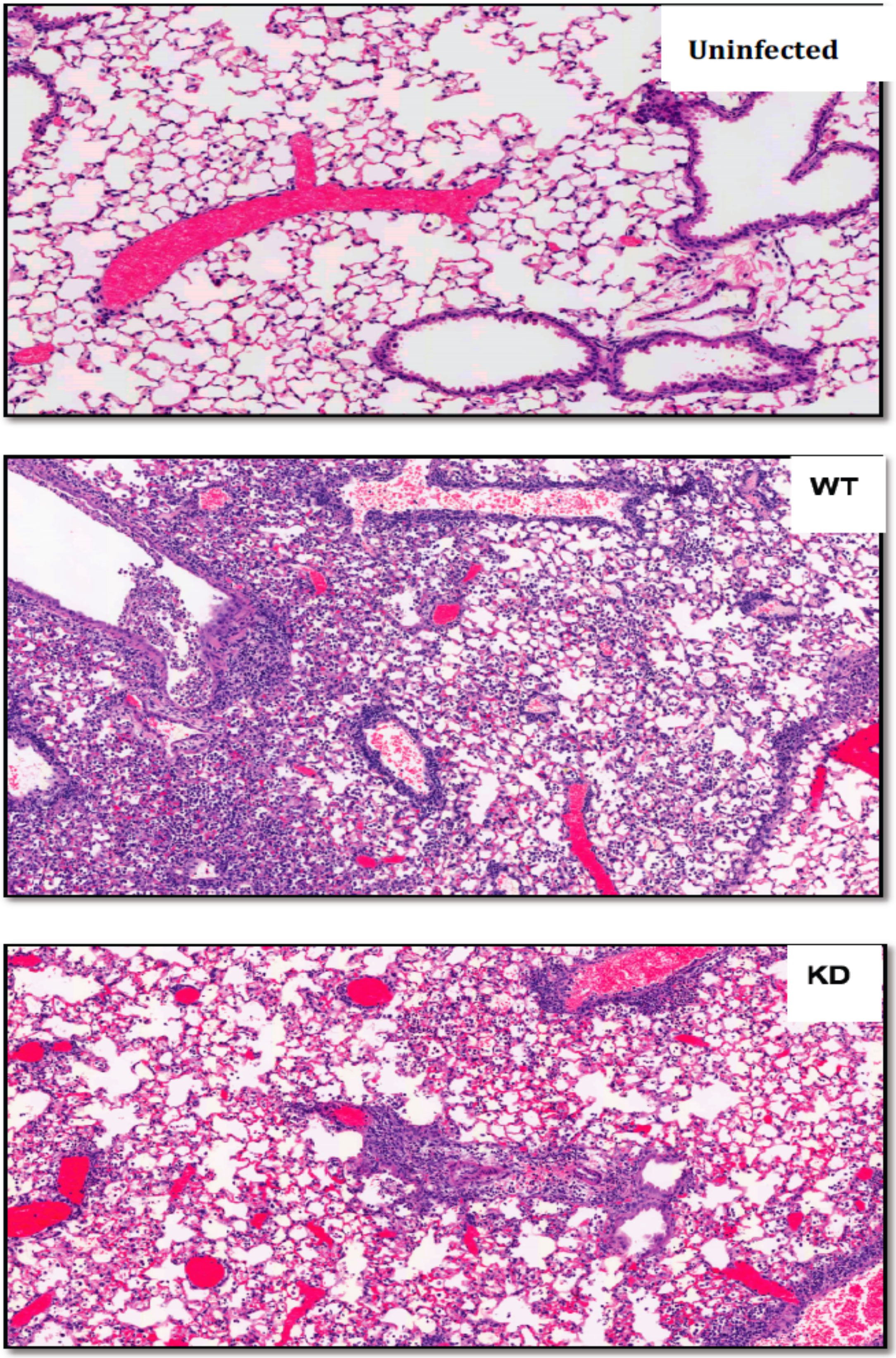


Figure 8. More severe lung pathology in influenza infected WT mice than NKC^{KD} mice. WT and NKC^{KD} mice were inoculated intranasally with 500 PFU of FM-MA virus. On day 7 p.i., lungs were fixed in neutral-buffered 10% formalin and sections were stained with H&E. H&E stained lung tissue from uninfected and influenza infected WT and NKC^{KD} mice are shown at 50X magnification. WT, wild-type; KD, NKC^{KD}. This experiment was performed at least 3 times with similar results. Three mice used for each group per experiment. All mice were 6-8 weeks old.

Discussion

The effector function of NK cells is dependent on a balance between activating and inhibitory signals delivered through various receptors such as the activating NKp46 and inhibitory Ly49 (2). Our lab has generated the NKC^{KD} mouse model in which approximately 80% of NK cells do not express Ly49 receptors compared to NK cells in WT mice (66). The majority of NK cells from NKC^{KD} mice cannot recognize MHC-I modulation on target cells because they are educated independently of MHC-I expression (66). Therefore, NKC^{KD} mice serve as a viable model to study the role of MHC-I inhibitory receptors during viral infection.

There are several lines of evidence indicating that viruses have developed mechanisms to evade NK cell recognition and killing, such as selective upregulation of MHC-I expression on the surface of virally-infected cells which in turn could inhibit NK cell cytotoxicity (132-136). MHC-I upregulation has been demonstrated following infection with hepatitis C virus, parainfluenza virus, rhinovirus, flavivirus, coronavirus, and paramyxovirus. Furthermore, in several studies this upregulation was associated with significant inhibition in NK cell cytotoxicity and is most probably due to the interaction between the MHC-I molecule and inhibitory receptors on NK cells (132-136).

1. MHC-I expression

In the current study, we show for the first time that influenza virus infection induces upregulation of MHC-I on the surface of mouse lung epithelial cells *in vivo* (**Figure 4**). The proportion of cells that express H-2D^{b} class-I molecule on the surface of mouse lung epithelial cells increased from 26% in uninfected mice to 85% 5 days post-influenza virus infection (**Figure 4A**). As well, expression level of the H-2D^{b} molecule on lung epithelial cells increased from 20% in uninfected mice to 68% 5 days post influenza virus infection (**Figure 4A**). Likewise, the proportion of lung epithelial cells that express H-2K^{b} increased from 30% to 84% within the same period of time, and the expression level of H-2K^{b} from 4% to 8% (**Figure 4B**). In agreement with our *in vivo* work, Coombs and colleagues found that expression of MHC-I protein on influenza virus-infected human lung A549 cells was significantly increased 24 hours post-infection (125). Moreover, this upregulation was also associated with downregulation of several proteins that are responsible for endogenous protein presentation via MHC-I, which would result in reduction of influenza antigen presentation on the surface of infected cells in order to avoid a T cell response.

However, *in vitro* influenza virus infection of mouse lung epithelial cells, TC-1, did not result in upregulation of MHC-I expression (**Figure 6C and D**), suggesting that upregulation of MHC-I is specific to influenza virus infection *in vivo* and may be induced by various cytokines produced in response to the infection. Our results are in disagreement with the *in vitro* results of Coombs and colleagues in which infection of human lung epithelial cells induces upregulation of MHC-I *in vitro* (7), however this opposing observation could be explained by taking into account differences in influenza virus strains, Coombs used influenza virus A/PR/8/34 at MOI of 7 while we used a different strain and at MOI of 6. Also, we used a mouse lung epithelial cell line which is different from the human lung epithelial cell line utilized by Coombs et al. Moreover, they cultured the cells for 24 h while we cultured the cells for 12 h in order to do the killing assay. Collectively, this could explain the difference in MHC-I expression between Coombs and our result. Moreover, the production of some cytokines that are responsible for MHC-I upregulation could be defective *in vitro*, and therefore would result in no detectable change in the level of MHC-I molecules on influenza virus-infected TC-1 cells. This is in agreement with a previous study which showed that enhanced expression of MHC-I molecules on influenza virus-infected human cells is IFN-dependent (137). Influenza virus-infected human macrophages upregulate MHC-I expression *in vitro*; however, neutralization of IFN-I using mAb prevented MHC-I upregulation, implicating the importance of IFN-I in upregulation of MHC-I expression.

Enhanced expression of MHC-I molecules on the surface of influenza virus-infected cells was shown to augment MHC-I binding to the inhibitory KIR receptors expressed on human NK cells. This binding inhibited NK cell-mediated cytotoxicity against influenza virus-infected cells *in vitro* (123, 128). These findings suggest that upregulation of MHC-I on infected cells might allow influenza virus to escape recognition by NK cells and facilitates viral transmission and replication, in turn contributing to viral pathogenesis. NK cells from NKC^{KD} mice are expected to be resistant to influenza virus infection since they express significantly lower levels of inhibitory Ly49 receptors, and as a result, there would be no interaction between the NK cells from NKC^{KD} mice and the upregulated MHC-I on mouse lung epithelial cells.

2. NKC^{KD} mice exhibit better survival than WT mice

NKC^{KD} mice exhibit better survival than WT mice when infected with a lethal dose of influenza virus (**Figure 5B**). NKC^{KD} mice survive the influenza virus infection possibly due to the lack of NK cell inhibition which is normally mediated by Ly49-MHC-I interaction. This study is in agreement with previous *in vitro* work by Achdout *et al* which showed that influenza virus infection causes reorganization of MHC-I into lipid raft microdomains on the surface of influenza virus-infected cells. Accumulation of MHC-I molecules in lipid rafts increased binding of KIR2DL1-Ig to the influenza virus-infected cells, which is associated with inhibition of NK cell cytotoxicity toward the infected cells. These results suggest that influenza virus use MHC-I-KIR interaction as an evading mechanism which facilitates influenza virus pathogenicity (123). Our data indicates that inhibitory Ly49 receptors play an important role in the pathogenesis of influenza virus infection in mice, whereby lack of these receptors leads to more rapid recovery from infection, however the exact mechanism is not clear at this time. MHC-I-Ly49 interaction could be blocked using antibodies specific for Ly49 inhibitory receptors *in vivo* to further demonstrate the role of this interaction during influenza virus infection. It has been shown that blocking MHC-I-Ly49 interaction using F(ab') 2 fragments of mAb resulted in increased cytotoxicity against tumor cells *in vitro* and *in vivo* (62). In agreement with previous studies, we have demonstrated that NK cells are important in host defences against influenza virus infection (45). Previous work has shown that depletion of NK cells prior to influenza virus infection significantly increases morbidity and mortality in hamsters and mice (109). Severely ill patients who were diagnosed with 2009 influenza A (H1N1) infection exhibited a reduction in the number of NK cells but not cytotoxic $CD8^+$ T cells in the lungs, demonstrating the importance of NK cells during influenza viral infections (138). Noyola and his group found that patients with mild and severe pandemic influenza A (H1N1) 2009 virus infections had significantly higher frequencies of inhibitory KIR2DL5 gene expression in comparison to control individuals (139). Though the ligand for KIR2DL5 is unknown, the presence or absence of interaction between KIR2DL5 and its ligand could be responsible for this effect. Moreover, Luo and her group found that KIR2DL2 and KIR2DL3 allotypes and their cognate ligands, HLA-C1 and HLA-C2, respectively, were significantly enriched in H1N1/2009 intensive-care unit (ICU) patients in comparison to healthy individuals (140). The authors concluded that potential associations of specific inhibitory KIR receptors and their MHC-I ligands during severe influenza A virus infections would explain the observed decrease in NK cell activity and increase in virus pathogenicity. If Ly49 inhibitory receptors are responsible for the progression of lethal viral infections in WT mice, transgenic expression of an inhibitory Ly49, such as Ly49I, in NKC^{KD} mice should make them susceptible to influenza virus infection. Ly49I transgenic NKC^{KD} mice can be generated by backcrossing NKC^{KD} mice to Ly49I transgenic mice. The presence of Ly49I is expected to restore MHC-I-Ly49 interaction in NKC^{KD} NK cells, resulting in a phenotype similar to WT mouse when infected with influenza virus. For a more in-depth analysis of the role of Ly49 inhibitory receptors and their MHC-I ligands in influenza virus infection, β-2-microglobulin-deficient mice (β-2-M$^{-/-}$), which lack MHC-I expression, can be used to support our finding. NK cells from β-2-M$^{-/-}$ mice are hyporesponsive against tumor cells (64) and lack CD8^{+} T cells. Recent work has shown that in response to mouse cytomegalovirus (MCMV) infection, NK cells in MHC-I–deficient mice proliferate and produce IFN-γ as efficiently as WT mice (141). Moreover, depletion of NK cells from MHC-I–deficient mice prior to MCMV infection resulted in increased virus titer in the salivary glands (142). These studies suggest that NK cells in MHC-I–deficient mice are responsive against viral infection despite being hyporesponsive towards tumor cells. MHC-I–deficient mice were also shown to survive influenza virus infection even though these mice do not have cytotoxic CD8^{+} T cells (143). This could be due to reduced immunopathology which is normally caused by activated CD8^{+} T cells. Alternatively, NK cells in these mice are not inhibited due to the lack of MHC-I-Ly49 interaction and thus are more efficient in eliminating influenza virus infected lung epithelial cells, as we have noticed in the NKC^{KD} mice. This could be answered by depleting NK cells from MHC-I–deficient mice and studying their survival upon influenza virus infection. If NK cells in these mice are more efficient in eliminating virus infected cells than the WT NK cells, then their depletion should make these mice as susceptible to influenza infection as the WT mice.

3. NKC^{KD} NK cells are slightly better at killing influenza infected cells *in vitro.*

Furthermore, we have shown that NKC^{KD} NK cells have slightly better cytotoxicity function against influenza virus-infected TC-I epithelial cells than WT NK cells in an *in vitro* cytotoxicity assay (**Figure 6B**). In this assay, theoretically, in order to ensure efficient infection of >99% of all TC-1 cells, we infected the cells with influenza virus at an multiplicity of infection of 6 (130). We observed high cytotoxic activity of WT NK cells against uninfected TC-1 as well as influenza virus-infected TC-I cells, although this was consistently lower than the cytotoxic activity of NKC^{KD} NK cells (**Figure 6A and B**). No upregulation of MHC-I on TC-I cells following influenza virus infection was observed. As a result, WT NK cells will not receive the inhibitory signals that would have been expected if MHC-I expression was upregulated upon virus infection. Achdout and colleagues showed that NK cell cytotoxicity towards influenza virus infected cells was inhibited shortly after influenza infection, and this inhibition was due to increased binding of the inhibitory KIR2DL1 receptor to MHC-I, which is expressed on the surface of influenza virus infected cells (123). As a result, the influenza virus would further replicate and continue to release new virions leading to severe illness.

4. Influenza virus titres are similar in NKC^{KD} and WT mice

Although, NKC^{KD} NK cells are slightly better at killing influenza infected cells *in vitro*, however, the difference in the viral titres measured in the lungs of infected WT and NKC^{KD} mice **(Figure 7)** was not statistically significant. There was reduced viral load in the lungs of NKC^{KD} as compared to WT mice on day 5 post-infection; however, a small sample size may have been the reason for the result not being significant. This issue can be resolved by using a larger cohort of mice and infecting them with a lower viral dose. Alternatively, viral titre measurements can be performed at earlier time points after infection. Another explanation could be that although there is a difference between WT and NKC^{KD} NK cells in killing influenza infected cells *in vitro* (**Figure 6B**), this may not be the case *in vivo*. Hence, the viral titres are similar in both mice.

5. Possible role of cytokines in lung pathology

Histological examination of lung sections from influenza virus infected WT and NKC^{KD} mice showed pathological changes such as leukocyte infiltration and lung edema (**Figure 8**), which is characteristic of influenza virus infection (131). Both of these pathological changes were more severe in the lungs of WT mice as compared to those of NKC^{KD} mice. The higher mortality rate in influenza virus infected WT mice compared to NKC^{KD} mice is believed to be due to severe lung damage. Higher infiltration of lymphocytes into the lung of WT mice than NKC^{KD} mice could be due to the abundant levels of cytokines and chemokines secreted by

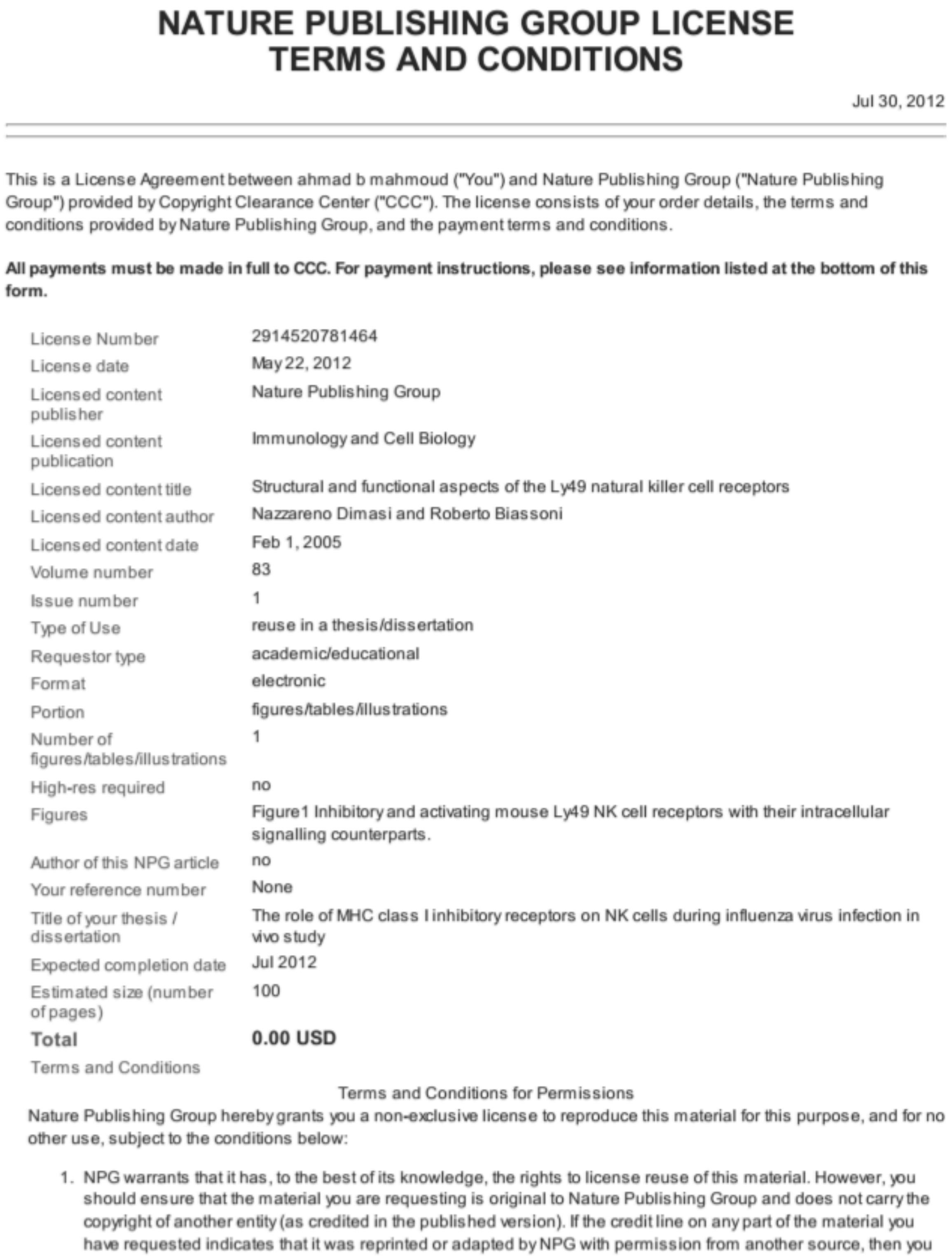
NK cells from WT mice upon stimulation, such as MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ (144), which in turn enhances recruitment of various inflammatory cells to the lung. There has been shown to be a correlation between increased levels of these cytokines and lung pathology (145). Moreover, NK cells could also have a direct role in the pathogenesis of influenza infection by producing abundant TNF-α, which have been directly linked to lung damage upon influenza virus infection (146). On the other hand, NK cells from NKC^{KD} mice are considered to be uneducated similarly to β-2-M$^{-/-}$ KO mice (66). Uneducated NK cells are well known to produce less cytokines than educated NK cells upon stimulation with tumour cells (147). Lanier and colleagues showed that Ly49H^{+} Ly49C/I^{-} NK cells produced significantly more IFN- γ than Ly49H^{+} Ly49C/I^{+} NK cells after stimulation with RMA cells expressing m157 molecule, which is a ligand for Ly49H. Moreover, Ly49H^{+} Ly49C/I^{-} NK were demonstrated to control MCMV infection better than Ly49H^{+} Ly49C/I^{+} NK cells (148). Although NK cells in NKC^{KD} mice can recognize and kill influenza virus infected cells (**Figure 6B**), it is possible that since NKC^{KD} mice do not express the inhibitory Ly49 receptors important for NK cell education (66) their ability to secrete cytokines and chemokines may be affected compared to WT mice.

A second potential explanation may be related to the better recognition and killing of influenza virus-infected alveolar macrophages by NK cells from NKC^{KD} mice than WT mice. Upon influenza virus infection, alveolar macrophages become activated and produce robust amounts of TNF-α and NOS$_2$ which contribute to lung damage and destruction (149). Moreover, reduced cytokine production by uneducated NK cells from NKC^{KD} mice could also affect alveolar macrophage recruitment since alveolar macrophages are recruited to the site of influenza virus infection via CCR2 (149), in response to MCP-1, which is also secreted by NK cells.

NK cells are required for the induction of cytotoxic T cells during influenza virus infection (150), most probably through cytokine production. We have previously shown that there is no significant difference in IFN-γ levels between NKC^{KD} and WT mice upon stimulation (66); however, production of other cytokines and chemokines such as TNF-α or IL-10 from NKC^{KD} mice most likely increased or decreased in compare to WT mice, as a result, recruitment of cytotoxic T cells to the site of infection would be affected. Cytotoxic T cells are also known to be associated with lung pathology through abundant production of TNF-α (151). Several lines of evidence suggest that TNF-α production contributes to lung immunopathology following influenza virus infection (151, 152). Neutralization of TNF-α using anti-TNF-α antibody resulted in reduced recruitment of inflammatory cells to the lungs as well as reduced lung epithelial cell death following both influenza virus and respiratory syncytial virus (RSV) infections (153). Since NKC^{KD} mice show less severe lung immunopathology than WT mice, it would be interesting to determine the contribution of TNF-α towards lung pathology in WT and NKC^{KD} mice following influenza virus infection.

In conclusion, we show in this study that influenza virus infection induced MHC-I upregulation on murine lung epithelial cells *in vivo*. Furthermore, NK cells from NKC^{KD} mice are possibly hyperresponsive toward influenza virus infected cells compared to NK cells from WT mice. In addition, NKC^{KD} mice are significantly more resistant to influenza virus infection than WT mice. However, influenza virus was cleared from the lungs of both mouse groups at similar rates. Interestingly, influenza virus infection induced more severe lung pathology in WT mice than NKC^{KD} mice, and may be why there is better survival of NKC^{KD} mice than WT mice. These results suggest that absence of Ly49 inhibitory NK receptors can somehow induce less lung damage following influenza virus infection. This study suggests an important role for NK cells and Ly49 receptors in the pathogenesis of influenza virus in mice.

Appendices



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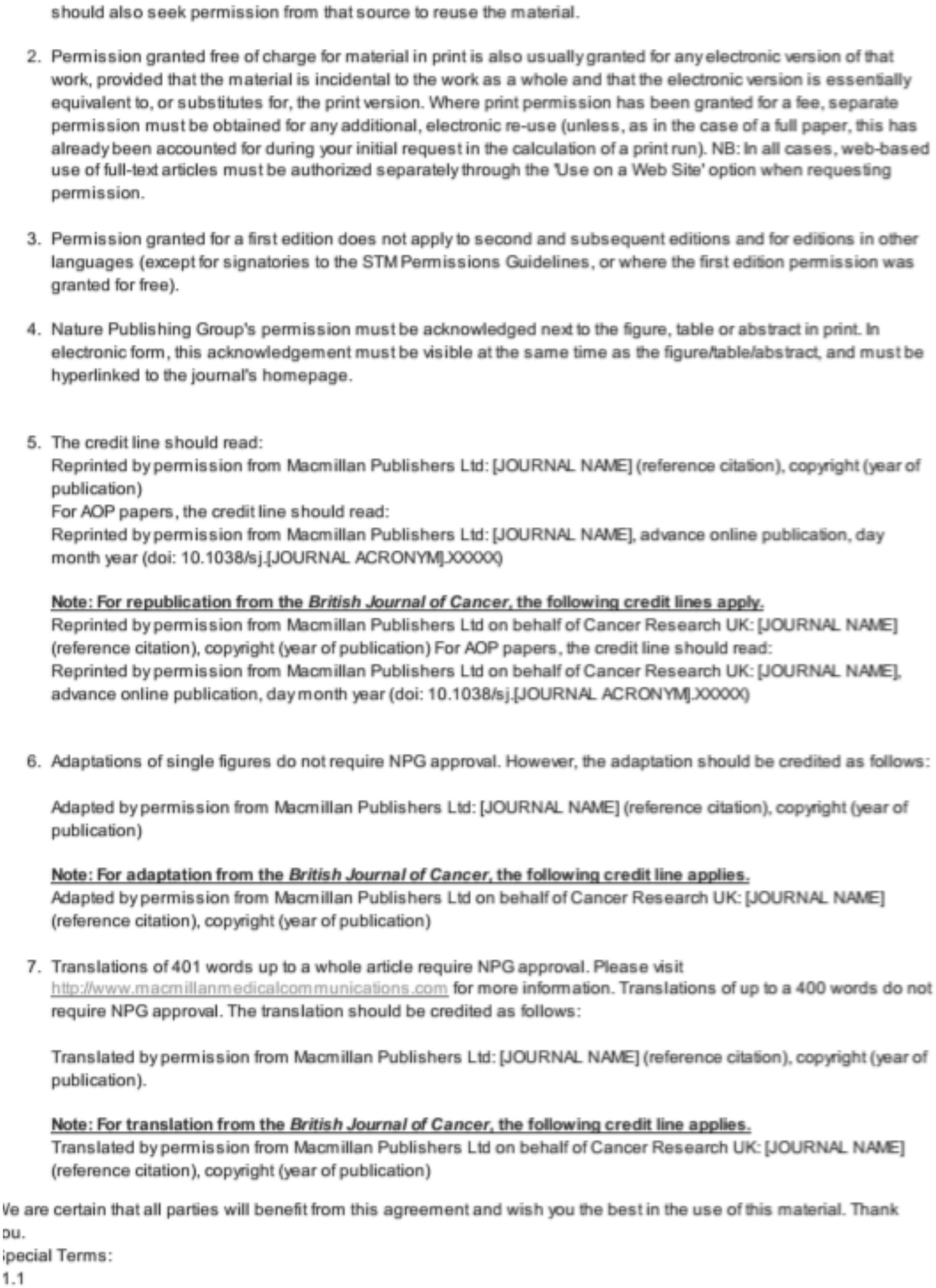

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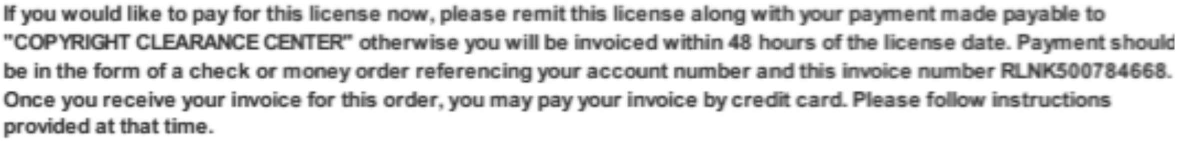

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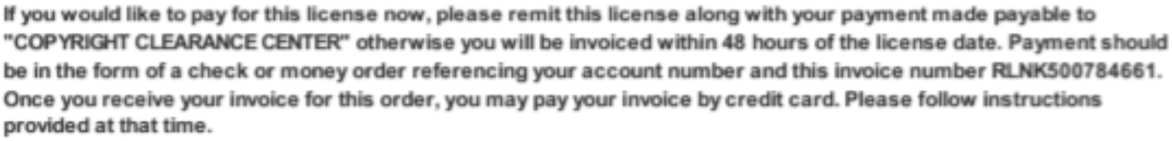

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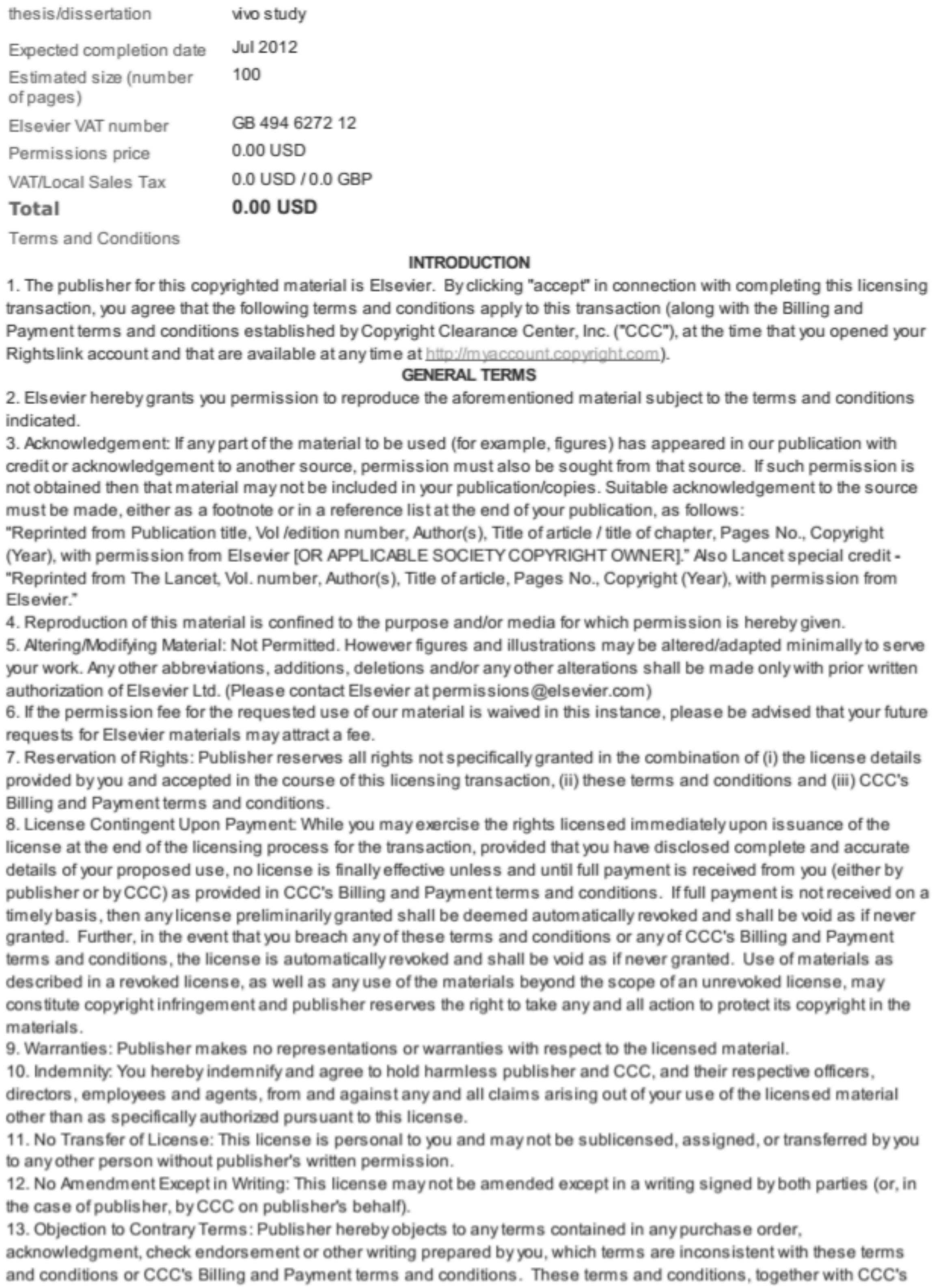

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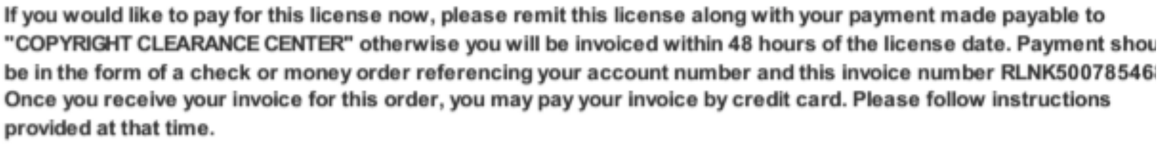

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Curriculum Vitae

AHMAD BAKUR MAHMOUD

OBJECTIVE

To pursue postgraduate studies in Virus Infection to gain research and analytical skills that will enable me to continue my career as a lecturer in Taiba University, the Kingdom's most recently commissioned university.

EDUCATION

- A second-year Master's degree candidate in Microbiology and Immunology program, 2010-2012, at University of Ottawa, ON , Canada

  Research Project: Role of Class I MHC Receptors on Natural Killer Cells During Influenza Infection

- Science stream unclassified student (senior year undergraduate course), Laurentian University, Sudbury, ON, 2010
- English Language program, PLI, Toronto, ON, 2009

- Bachelor of Medical Sciences, 2006
  College of Medicine and Medical Sciences
  Umm Al – Qura University, Makkah (Mecca), Saudi Arabia

  Research Project: Use of Biomarkers in the Diagnosis of Prostate Cancer

PROFESSIONAL EXPERIENCE

- Candidate Lecturer, 2007- Present
  Department of Virology
  College of Applied Medical Sciences
  Taiba University, Saudi Arabia

- Senior Laboratory Technician, September 2006 – April, 2007
  Royal Commission Hospital
  Jubail Industrial City, Saudi Arabia

Area of Responsibility:

- Coordinating departmental development
- Preparing and updating section procedure manuals
- Monitoring of quality control within the Department
- Assisting the establishment and maintenance of a Q.M / Q.I. program
- Organize the day-to-day activities of the section

- Rotating Medical Internship, June 2005 – June 2006
  Department of Pathology and Laboratory Medicine
  King Abdulaziz Medical City, Jeddah, Saudi Arabia

  Area of Rotation & Experience:

  - Medical Microbiology
  - Biochemistry & Hormones
  - Hematology & Blood bank
  - Parasitology
  - Serology
  - Molecular Biology

CONFERENCES, SYMPOSIA & PRESENTATIONS

- M.Sc. Oral Award, Dept. Biochemistry, Microbiology and Immunology, University of Ottawa, ON, Canada, 2012.

- Microbiology and Immunology Seminar Symposium. Dept. Biochemistry, Microbiology and Immunology, University of Ottawa, ON, Canada, 2012.

- Microbiology and Immunology Seminar Symposium. Dept. Biochemistry, Microbiology and Immunology, University of Ottawa, ON, Canada, 2011.

- Emerging Pathogens Research Centre (EPRC) - Symposium on World AIDS Day. University of Ottawa, ON, Canada, 2011.

- 4th Scientific Conference for Medical Students (GCC Region), January 21 – 24, 2006
  Muscat, Sultanate of Oman

  - Presentation: “Use of Biomarkers in the Diagnosis of Prostate Cancer”
  - Workshop attended: “Rabid Serologic and Molecular Methods in the Diagnosis of Infection Diseases”

- International Saudi Symposium of Pediatric Hematology / Oncology, Nov. 21 – 23, 2005
  Jeddah, Saudi Arabia

- Lecture Presented: “Platelet Antigen in Health and Disease,” July, 2005
  Blood Bank Department
  King Abdulaziz Medical city, Jeddah

- Training Committee: Course Discussing Uses of Antimicrobial Drug, June 13 – 14, 2005
  Faculty of Medicine and Medical Sciences
  Umm Al – Qura University, Makkah (Mecca)

- Seminar: The Future of Medical Laboratory Technology, April 27 – 28, 2005
  Al – Khobar, Saudi Arabia. (27 & 28 April, 2005)

- Lecture Presented: “Rabies,” 2002
  Department of Virology
  Umm Al – Qura University, Makkah (Mecca)

Publications:

1- Bélanger S, Tu MM, Rahim MM, **Mahmoud AB**, Patel R, Tai L-H, Troke AD, Wilhelm BT,Landry J-R, Zhu Q, Tung KS, Raulet DH, and Makrigiannis AP. Impaired natural killercell self-education and 'missing-self' responses in Ly49-deficient mice. Blood.2012; 120:592-602. (Recommended by the Faculty of 1000 as a 'must read')