

Elucidating the roles of GATA3 and IL-10 in the development and function of Natural Killer cells during murine cytomegalovirus infection

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Abstract

Natural killer (NK) cells are lymphocytes of the innate immune system that exhibit cytotoxic functions against virus-infected cells and cancer cells. To achieve their complete functional capacity, NK cells need to differentiate through several stages of development. The maturation of NK cells is initiated in the bone marrow and is a prerequisite to equip the NK cell with migratory capabilities for the seeding of peripheral organs where they can protect the host. Several transcription factors have been shown to guide NK cell development. In this study, using NK cell-specific *Gata3*-deficient mice, I demonstrated that the transcription factor GATA3 plays an essential role in NK cell terminal differentiation required for bone marrow egress, resulting in decreased NK cell frequencies in the periphery at the steady-state. Despite the immature phenotype of NK cells in the absence of GATA3, these NK cells displayed enhanced proliferation during murine cytomegalovirus (MCMV) infection, indicating that immature NK cells are a reservoir of highly proliferative cytotoxic cells.

Although numerous studies have demonstrated the critical role of NK cells in enhancing inflammatory responses through the production of pro-inflammatory cytokines such as IFN γ and TNF α , few studies have described the regulatory functions of NK cells. Using IL-10-GFP reporter mice, I showed that NK cells are major producers of the anti-inflammatory cytokine IL-10 during the early stages of MCMV infection, prompting further investigation into the contribution of NK cell-derived IL-10 on the inflammatory response against MCMV. Using NK cell-specific *Il10*-deficient mice, I demonstrated that NK cell-derived IL-10 regulates the function of CD4 and CD8 T cells in a model of sustained infection, and prevents liver damage, suggesting that NK cells produce IL-10 to limit immune-associated pathologies.

These studies will advance our understanding of how NK cells develop and mediate their antiviral responses, while preventing host tissue damage.

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
BM	Bone marrow
CDC	Cholesterol dependent cytolysins
CLP	Common lymphoid progenitors
CM	Conditioned media
CMP	Common myeloid progenitor
cNK	Conventional natural killer
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR4	C-X-C motif chemokine receptor 4
DC	Dendritic cell
DD	Death domain
DISC	Death-inducing signaling complex
E4BP4	E4-binding protein 4
Eomes	Eomesodermin
FADD	Fas-associated protein with death domain
FasL	Fas ligand
Flt3L	Fms-like tyrosine kinase 3 ligand
GAS	Gamma-activated site
γ_c	Common gamma-chain
G-CSF	Granulocyte colony-stimulating factor
GPCR	G-protein coupled receptor
H&E	Hematoxylin and eosin

HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HSC	Hematopoietic stem cells
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
IFN γ	Interferon-gamma
IL-2R α	Interleukin-2 receptor α subunit
IL-2R β	Interleukin-2 receptor β subunit
IL-7R α	Interleukin-7 receptor α subunit
IL-10	Interleukin-10
ILC	Innate lymphoid cell
ILC2	Innate lymphoid cells type 2
iNK	Immature Natural Killer cell
iNOS	Inducible nitric oxide synthase
ISG	Interferon-stimulated genes
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JAK	Janus kinase
LAMP-1	Lysosomal-associated membrane protein 1
LCMV	Lymphocytic choriomeningitis virus
MACPF	Membrane attack complex/perforin domain
MCK2	MCMV-encoded chemokine 2
MCMV	Murine cytomegalovirus
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex

mNK	Mature Natural Killer cell
MPP	Multipotent progenitor
mTOR	Mammalian target of rapamycin
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nfil3	Nuclear factor interleukin-3
NK	Natural killer
NKP	NK cell precursor
NLR	Nucleotide-binding oligomerization domain-like receptor
ORF	Open reading frame
PD-1	Programmed death-1
PDPK1	Phosphoinositide-dependent protein kinase 1
PFU	Plaque forming unit
PRR	Pattern recognition receptor
Rae-1	Retinoic acid early inducible 1
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
S1P	Sphingosine-1-phosphate
S1P5	Sphingosine-1-phosphate receptor 5
SCF	Stem cell factor
Serpin B9	Serine protease inhibitor B9
SHP-1	Src homology region 2 domain-containing phosphatase-1
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF- β	Transforming growth factor beta
Th1	T helper cell type 1

TLR	Toll-like receptor
TMH	Transmembrane helical region
TNF	Tumor necrosis factor
TRADD	TNFR1-associated death domain protein
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell

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Chapter 1: General Introduction

1.1. Innate and adaptive immune responses

1.1.1. Innate response

Interactions between a host organism and its surrounding pathogens occur on a regular basis. Fortunately, a robust and effective immune response is in place to protect the host against a multitude of invading microbes (1). The first line of defense against microbial pathogens is achieved by anatomical and physiological barriers such as the skin, the mucus layers in the lung and gut, the acidic environment in the stomach, and the presence of lysozyme in the saliva and tears (1, 2). In case these barriers fail to limit the entry of a foreign microbe inside the body, the innate immune system is equipped with a plethora of additional defense mechanisms that can successfully eliminate the microbe. The innate immune system is able to eliminate the pathogen through humoral responses such as the complement system or production of antimicrobial peptides (3, 4). Granulocytes, for example neutrophils that are among the first innate immune cells to respond to infections, contribute in reducing the microbial burden via phagocytosis, thereby engulfing the pathogen and subjecting it to destruction via acidification of the phagosome which triggers the action of proteases and release of reactive oxygen species (ROS) (5).

1.1.2. Recognition of pathogens by innate immune cells

Cellular components of the innate immune responses rely on germline-encoded receptors that recognize conserved molecular structures common among pathogens, such as flagellin, lipopolysaccharide, and double-stranded RNA (6). These motifs are ligands for pattern recognition receptors (PRRs) expressed on innate immune cells such as macrophages and dendritic cells (DCs). Examples of PRRs are Toll-like receptors (TLRs) and NOD-like

receptors (NLRs). The ligand-binding domain of the TLRs and NLRs at the C-terminal end consists of leucine-rich repeats, which take the shape of a horseshoe (7). While TLRs are found both on the plasma membrane and intracellular membranes, NLRs are only found in the cytosol. Signaling downstream of TLRs and NLRs will ultimately lead to the translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) into the nucleus where the transcription of pro-inflammatory cytokines will be initiated.

1.1.3. Cytokines and chemokines

The magnitude of inflammatory responses is regulated by cytokines and chemokines, which orchestrate the activation and trafficking of immune cells (8). Cytokines control many aspects of the immune system, from supporting the development and differentiation of immune cells, to promoting the expansion and effector functions of immune cells (9). Cytokines are produced by different cell types, can act on the same cell type or a different cell type (even of non-hematopoietic origin), and can undertake either autocrine or paracrine signaling. Chemokines regulate the chemotaxis of cells to and from different organs and allow their circulation through the bloodstream and lymphatic system (10). They are important in regulating the homeostatic distribution of cells in various organs at steady-state and for recruiting immune cells to the site of inflammation. Chemokines attract immune cells by signaling through G-protein coupled receptors (GPCRs) composed of seven transmembrane helices (11).

1.1.4. Induction of adaptive immune response

Dendritic cells are antigen-presenting cells that are vital in collecting microbial particles from the site of inflammation and migrating to secondary lymphoid tissues to present

the antigen to T cells allowing their priming and triggering the adaptive immune response (12). The adaptive immune response relies on somatic recombination of the genome through the rearrangement of hundreds of gene segments to generate millions of different receptors (13). These receptors can recognize diverse structures or peptides, which allows the detection of an extensive repertoire of molecular structures. Unlike the innate immune cells, specific T cell clones and B cell clones are few in numbers in naïve conditions and require up to several days to be primed and expand to reach high numbers (2). For this reason, whereas the innate immune response is immediate and takes a few hours, the adaptive immune response may take up to several days to be fully activated. T cells express T cell receptors (TCRs) to recognize peptides presented on MHC molecules, while B cells express immunoglobulins as B cell receptors (BCRs) that can recognize specific moieties or epitopes. The specificity of immunoglobulin can be enhanced through somatic hypermutations in activated B cells. The adaptive immune response is particularly important because it generates immunological memory (13). Therefore, upon reencounter of the same pathogen, the adaptive immune response will respond much faster and at a higher magnitude.

1.2. NK cell development

1.2.1. NK cell differentiation from precursor cells

In mice, NK cells mainly develop in the bone marrow, although some studies suggest that tissue-specific NK cells can develop within peripheral organs such as the liver, thymus, and uterus (14-16). Hematopoietic stem cells (HSCs) are maintained in the bone marrow niche via the action of stem cell factor (SCF) and Fms-like tyrosine kinase 3 ligand (Flt3L) which are growth factors that signal through c-kit and Flt3 receptors, respectively (17). HSCs

differentiate into multipotent progenitors (MPPs), which give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). The CMPs further differentiate into granulocytes, platelets, and red blood cells. On the other hand, the CLPs differentiate into B cells, T cells, NKT cells, NK cells, and innate lymphoid cells (ILCs). As a marker of commitment to the lymphoid lineages, the CLPs will acquire expression of the IL-7 receptor α subunit (IL-7R α , CD127) (Figure 1.1A). The transition of the CLPs into the NK cell lineage first occurs through the upregulation of the IL-2R β subunit (CD122), which gives rise to the NK cell precursors (NKPs). NKPs then differentiate into immature NK (iNK) cells by upregulating the activating receptor NK1.1 (Figure 1.1A). The iNK cells undergo terminal maturation into mature NK (mNK) cells upon acquisition of the integrin CD11b (Mac-1).

NK cell maturation can be dissected based on the expression of CD27, a member of the tumor necrosis factor (TNF) superfamily of receptors, and CD11b, giving rise to 4 different NK cell subsets (18). The least mature subset of NK cells lacks both receptors (CD27-CD11b-) (Figure 1.1B). This subset differentiates further by upregulating CD27 (CD27+CD11b-), followed by CD11b (CD27+CD11b+). The latter subset will then lose the expression of CD27 but will maintain CD11b (CD27-CD11b+) and will be defined as the most mature subset. Interestingly, the CD27+CD11b+ subset of NK cells was shown to exhibit stronger effector functions than the CD27-CD11b+ population, which is believed to be due to a higher state of intrinsic regulation at the latest stage of maturation as exemplified by the increased expression of inhibitory receptors, such as Ly49C/I and KLRG1, in the CD27-CD11b+ subset of NK cells (19).

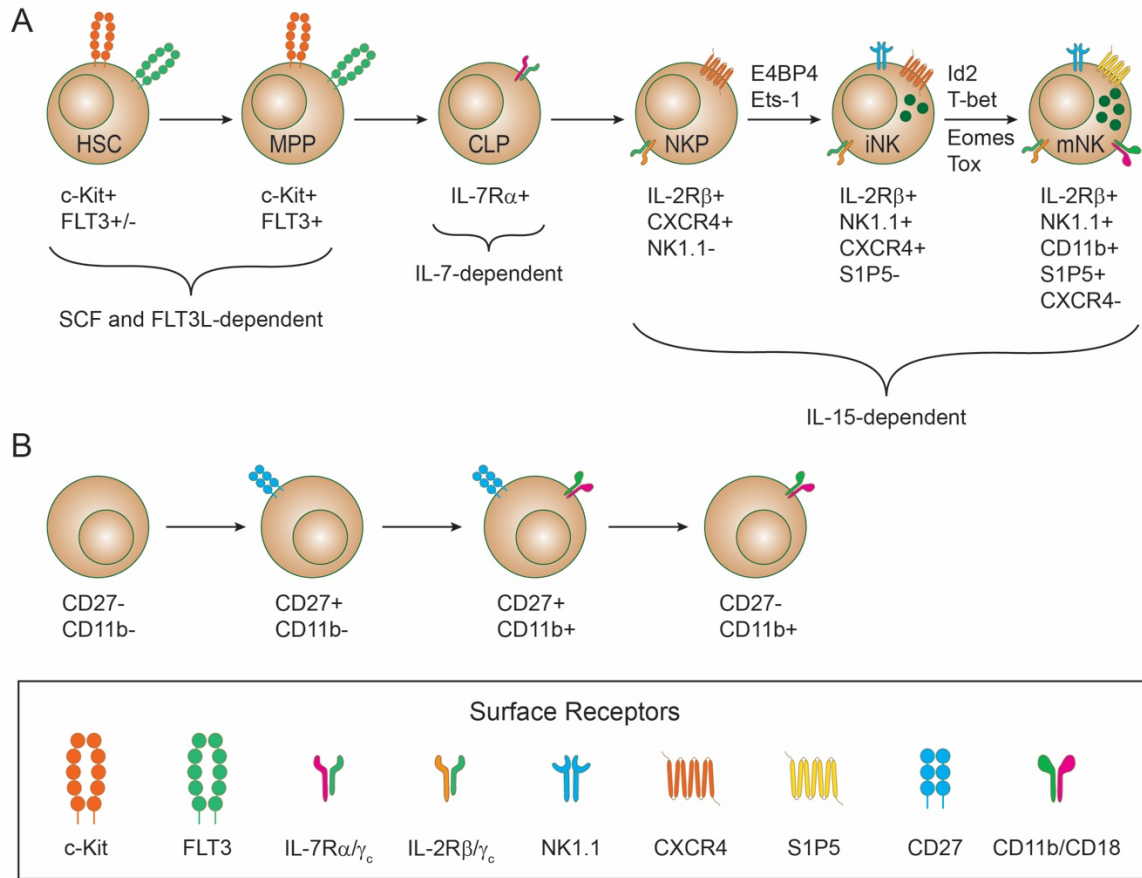


Figure 1.1. NK cell developmental program in the bone marrow. (A) Hematopoietic stem cells (HSCs) differentiate into multipotent progenitors (MPPs), while being sustained through the action of SCF and FLT3L cytokines. Common lymphoid progenitors (CLPs) are derived from MPPs and are characterized by the acquisition of IL-7Rα subunit. The commitment of the CLPs to the NK cell lineage is associated with the expression of IL-2Rβ subunit to give rise to NK cell precursors (NKPs). The differentiation of NKPs into NK1.1-expressing immature NK (iNK) cells is dependent on the transcription factors E4BP4 and Ets-1. Terminal maturation of NK cell into mature NK (mNK) cells is associated with the acquisition of the integrin CD11b and is dependent on several transcription factors, including Id2, T-bet, Eomes, and Tox. Maturation of iNK cells into mNK cells is accompanied by the sequential loss of CXCR4 expression and gain of S1P5 expression, which are essential chemokine receptors that dictate NK cell retention in the bone marrow and egress to peripheral organs. **(B)** The maturation of NK cells can be divided into 4 stages in the following order: DN (CD27⁻CD11b⁻), CD27 SP (CD27⁺CD11b⁻), DP (CD27⁺CD11b⁺), CD11b SP (CD27⁻CD11b⁺).

1.2.2. Cytokine requirement for NK cell development

IL-2 and IL-15 are potent inducers of NK cell proliferation (20). Both IL-2 and IL-15 signal through the IL-2R β chain (CD122) and the common γ chain (γ_c) (CD132) and activate the JAK3-STAT5 signaling pathway (21). However, IL-2 and IL-15 have distinct α receptor chain, IL-2R α , and IL-15R α , respectively. While IL-2R α (also known as CD25) is upregulated on activated NK cells, IL-15R α expression is not induced on NK cells. Instead, IL-15 cytokine is trans-presented to NK cells by surrounding cells that express the IL-15R α , such as dendritic cells (22). Indeed, adoptive transfer experiments have demonstrated that wild type NK cells fail to persist in an IL-15R α -deficient host, but that IL-15R α -deficient NK cells are able to survive in a wild type host (23). Interestingly, mice lacking any of the components involved in IL-15 signaling, either IL-15 cytokine itself or any of the three IL-15 receptor subunits (IL-15R α , IL-2R β , or γ_c), have NK cell deficiencies (24-27), indicating that IL-15 plays an essential and nonredundant role in NK cell development.

1.2.3. Homeostatic migration of NK cells

Chemokines and adhesion molecules are an integral part of NK cell trafficking throughout their developmental stages (28-30). Apart from their critical role during inflammation, these molecules are involved in the retention of NK cells in the bone marrow at steady-state as well as their migration from the bone marrow to peripheral organ. Accordingly, the expression of chemokine receptors on NK cells is differentially regulated during their maturation. In fact, the localization of NK cells in the bone marrow and their egress to peripheral organs is controlled by the coordinated expression of receptors for the chemoattractants CXCL12 and S1P (31). These molecules have different spatial distribution and attract different NK cell subsets based on their maturation status. CXCL12 is a chemokine

that is constitutively expressed in the bone marrow and is produced by CXCL12-abundant reticular (CAR) cells (32). CXCR4, the receptor for CXCL12, is expressed by NKP and iNK cells but its expression is gradually decreased as the NK cells mature (33, 34) (Figure 1.1A). AMD3100, an antagonist of CXCR4, was shown to promote the mobilization of hematopoietic stem cells from the bone marrow to the blood and was suggested to be used as a tool for HSC transplantation (35). Sphingosine-1-phosphate (S1P) is a lipid produced in the bone marrow sinusoids and allows the migration of cells that express S1P receptors. mNK cells acquire the S1P5 receptor which allows them to be trafficked from the bone marrow to the bloodstream via S1P (36). In fact, S1P levels are high in the blood and lymph but low in the bone marrow (37). Thus, immature NK cells are retained in the bone marrow through the CXCR4-CXCL12 axis. However, once the NK cells mature, they lose expression of CXCR4 and are no longer attracted to the bone marrow parenchyma. Instead, they are attracted by the S1P levels in the sinusoidal blood vessels, through their increased expression of S1P5 receptor.

1.2.4. Critical transcription factors in the differentiation of NK cells

Through single gene deletions in mice, studies have unraveled the role of several transcription factors in promoting the differentiation of NK cells (38). The transcription factor E4-binding protein 4 (E4BP4), also known as nuclear factor interleukin-3 (Nfil3), is essential for the development of NK cells specifically, but not for that of other immune cell types (39, 40). E4BP4-deficient mice show reduced frequencies of iNK cells and lack mNK cells, suggesting that E4BP4 is required for the differentiation of NKP to iNK, as well as the maturation of iNK to mNK (Figure 1.1A). The indispensable role of E4BP4 in NK cell differentiation is corroborated by evidence showing that E4BP4 signals downstream of IL-15 receptor (39), suggesting that IL-15 is essential for NK cell development through the E4BP4-

dependent mechanism. Moreover, the transcription factor Id2 is a repressor that was shown to be required for the development of NK cells (41). While Id2-deficient mice have normal numbers of NKPs and iNK cells in the bone marrow, the iNK fail to differentiate into mNK cells (42).

The T-box transcription factors T-bet and Eomesodermin (Eomes) are important in NK cell development. T-bet, important in the development of T helper 1 cells, was shown to be critical for the generation of liver-resident NK cells, which are characterized by the expression CD49a and TRAIL, and lack of CD49b (DX5) (43). Eomes was essential for the generation of bone marrow-derived mNK cells (43). Other transcription factors that are critical for NK cell development include Ets-1 and Tox. Ets-1-deficient mice exhibited severe NK cell deficiency as evidenced by their reduced numbers of iNK and mNK cells (44, 45). Tox-deletion in mice leads to a deficiency in mNK cells (46).

1.3. GATA3

1.3.1. GATA3 structure

In mammals, there are six members of the GATA family of transcription factors. GATA1, GATA2, and GATA3 are essential in hematopoiesis, whereas GATA4, GATA5, and GATA6 are important in the development of other tissues such as the heart (47). GATA proteins recognize the consensus DNA sequence WGATAR (W = A/T, R = A/G) (48, 49). GATA3, which has a molecular weight of 48 kDa, was first discovered in 1991 when it was shown to regulate the expression of the TCR α gene (50). GATA3 contains two transactivation domains (TA1 and TA2) and two zinc finger motifs that are critical for GATA3-mediated functions (51, 52). The complete crystal structure of GATA3 protein has not been determined

yet. However, the crystal structure of the C-terminal zinc-finger motif bound to DNA has been successfully generated (53). According to this report, GATA3 proteins would be able to dimerize to interact with DNA.

1.3.2. Role of GATA3 in the development of multiple tissues

GATA3 plays a critical role in the development of numerous organs and cell types (54). The whole-body deletion of GATA3 leads to embryonic lethality by day 12, with malformation of the central nervous system, kidneys, and adrenal glands (55-57). In addition, GATA3 deficiency leads to impaired differentiation of cells in the skin, associated with a defect in lipid synthesis (58, 59). Another report demonstrated that GATA3 regulates the differentiation of adipocytes (60). Moreover, GATA3 is important in the differentiation of luminal cells in the mammary gland (61, 62). The Barakat syndrome in humans, also known as hypoparathyroidism, sensorineural deafness, and renal disease (HDR) syndrome, is caused by an autosomal dominant mutation in the GATA3 gene and is associated with hearing loss and reduced functions of the parathyroid glands and kidneys (63, 64). Interestingly, hearing loss was also reported in GATA3 haploinsufficient mice (65).

1.3.3. Role of GATA3 in hematopoiesis

In regard to the role of GATA3 in the development of leukocytes, this transcription factor is critical for the development of T cell at an early precursor stage (66), but its requirement at later stages is specific to different T cell subsets. In particular, GATA3 is required for the differentiation of T helper 2 (Th2) cells and their production of IL-5 and IL-13 cytokines (67, 68). Interestingly, the innate lymphoid cells type 2 (ILC2), which are functionally similar to Th2 cells, also require GATA3 for their differentiation. Both ILC2 and

Th2 are major players in promoting the humoral response to infections (69, 70). One report demonstrated that GATA3 can guide T cell differentiation by repressing the developmental program of B cells (71).

It was previously shown that NK cells develop in the absence of GATA3. However, these NK cells displayed an immature phenotype, with impaired IFN γ production and a defect in homing to the liver (72). In this report, the authors generated hematopoietic chimeras by the adoptive transfer of hematopoietic stem cells from *gata3*-deficient embryos into alymphoid recipient mice (*Rag*^{-/-} γ *c*^{-/-}). The ability of these cells to seed the organs and to differentiate was assessed, while bypassing the need for the cells to exit the bone marrow to reach the bloodstream and seed the peripheral organs. Although this model provided valuable insights into the role of GATA3 in NK cell development and function, this model does not accurately recapitulate the *in vivo* settings in wild type mice, where NK cells initially develop in the bone marrow. Another report demonstrated that GATA3 is required for the development of thymic NK cells, which are characterized by their expression of IL-7R α (16).

1.4. NK cell function

1.4.1. Recognition of “missing-self”

In the early 1970s, studies found that lymphocytes from T cell depleted rodents could induce the killing of cancer cells, and were believed to be B cells (73, 74). Further studies determined that a new class of lymphocytes, other than T cells and B cells, can kill tumor cells without prior immunization (75, 76). In 1975, independent studies led by Kiessling and Herberman identified these cells as Natural Killer cells (77, 78). Later in the 1980s, NK cells were also found to play an important role in controlling viruses (79-81).

Although the essential role of NK cells in eliminating tumor cells and virus-infected cells has been well established, the mechanisms by which NK cells recognize their target cells was initially a mystery. Earlier reports demonstrated that target cells that were sensitive to T cell-mediated killing were resistant to NK cell cytotoxic functions, while target cells that were sensitive to NK cell-mediated killing were resistant to T cells (82). In addition, treatment with type I IFNs, which increases MHC class I expression, reduced the susceptibility of target cells to NK cell-mediated lysis (83). Based on these observations, while completing his PhD thesis in the early 1980s, Dr. Klas Kärre postulated the “missing-self” hypothesis (84). The notion of “self” is attributed to molecules produced by the host organism, while “non-self” are molecules produced by foreign entities and recognized by the immune system. The “missing-self” hypothesis speculates that NK cells can sense the absence of “self” MHC class I molecules on the target cells by means of inhibitory receptors expressed on NK cells. Therefore, a healthy cell would express normal levels of MHC class I that inhibit NK cell function. However, tumor cells or virus-infected cells that downregulate MHC class I molecules to evade recognition by T cells would be detected by NK cells. Indeed, by generating lymphoma mutants that lacked expression of MHC class I, Kärre and colleagues demonstrated the ability of NK cells to preferentially kill MHC class I-deficient cancer cells (85).

The “missing-self hypothesis” has been revisited with the notion that NK cells need to be stimulated through their activating receptors in order to kill the target cells (86). The absence of signaling through inhibitory receptors is not sufficient to induce killing, as the inhibitory signals act merely to regulate the signals from activating receptors. Thus, if the target cell expresses MHC class I molecules but lacks activating ligands, as is the case for a healthy cell, the NK cell will refrain from killing the target cell (Figure 1.2A). However, if

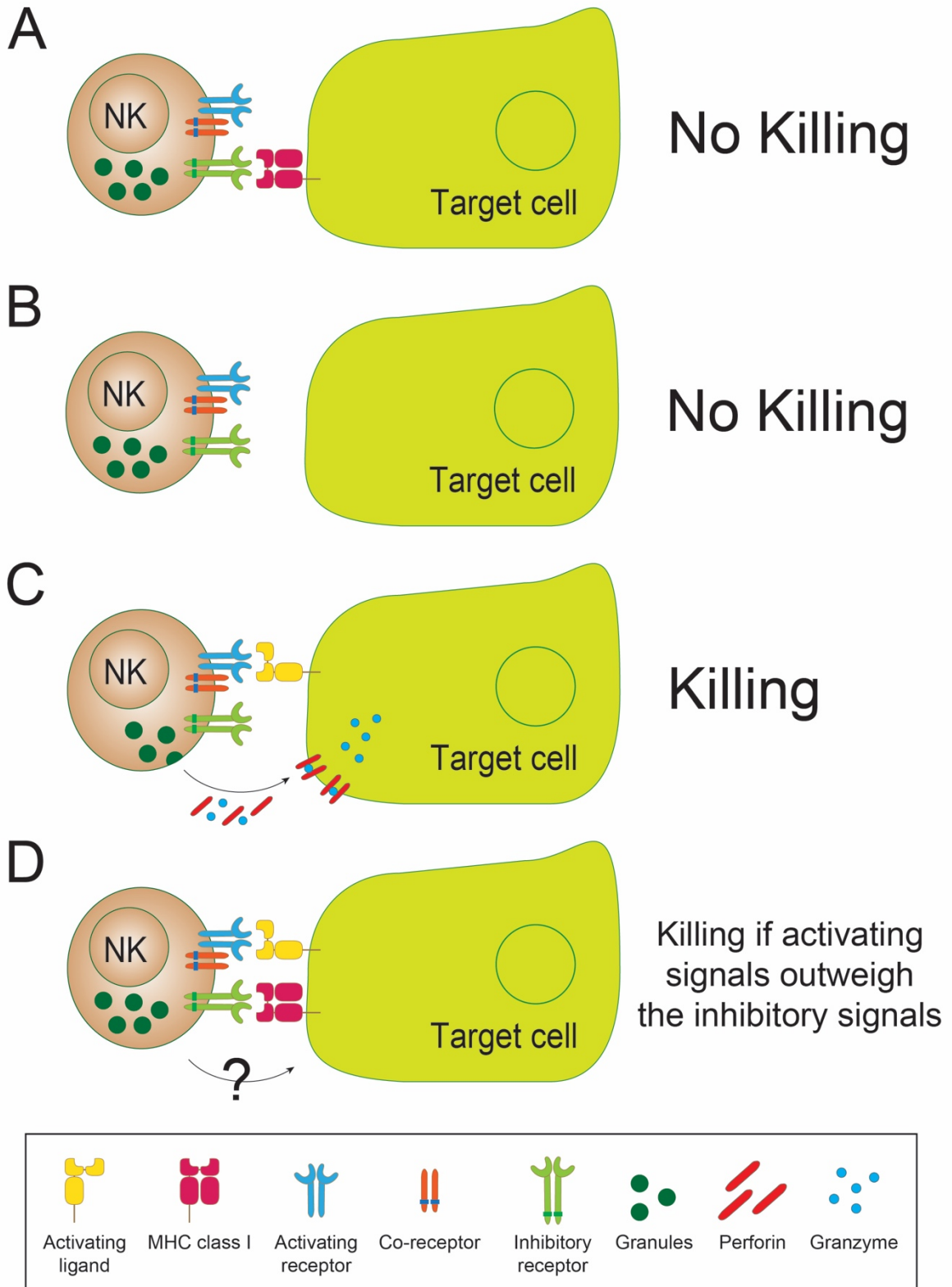


Figure 1.2. NK cell recognition of the target cell. NK cells express an array of inhibitory and activating receptors. Inhibitory receptors recognize the presence of MHC class I molecules and inhibit the cytotoxic functions of NK cells, whereas activating receptors recognize stress ligands produced by the target cell or microbial proteins. NK cells sense the absence of “self” through their inhibitory receptors but require signaling through their activating receptors to mediated lysis of the target cell. **(A)** In the presence of MHC class I molecules but in the absence of activating ligands, NK cell-mediated lysis does not occur. **(B)** In the absence of both MHC class I molecules and activating ligands, NK cell-mediated lysis does not occur. **(C)** In the absence of MHC class I molecules and in the presence of activating ligands on the target cell, this balance favors NK cell-mediated lysis of the target cell. **(D)** In the presence of both MHC class I molecules and activating ligands on the target cell, the balance between the signals emanated from the inhibitory and activating receptors on NK cells will dictate the outcome.

the target cell lacks MHC class I molecules but does not express activating ligands, the NK cell will not be able to recognize this cell (Figure 1.2B). In ideal conditions, the target cell would lack MHC class I but express activating ligands, in such case, the NK cells are activated through their activating receptors, while their inhibitory receptors are not engaged and would not inhibit NK cell function, leading to NK cell-mediated killing (Figure 1.2C). In many cases, the ability of the NK cell to kill its target will be determined by the balance of signals emanated from both the inhibitory or activating receptors. Therefore, in order for NK cells to kill their target, the signals from activating receptors need to overcome the signals from the inhibitory receptors (Figure 1.2D).

1.4.2. Activating and inhibitory receptors

Seminal work conducted by Yokoyama and colleagues had identified the first NK cell inhibitory receptors in mice, Ly49A (87). Other inhibitory and activating receptors were later discovered in both humans and mice (88, 89). The cytoplasmic domains of inhibitory receptors contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) that recruit phosphatases such as SHP-1, SHP-2, and SHIP1, which dephosphorylate signaling proteins downstream of activating receptors (86). Even though inhibitory and activating NK cell receptors share a similar extracellular binding structure, activating receptors do not contain ITIM motifs. Instead, they have short cytoplasmic tail and are associated with co-adaptor proteins (such as DAP-12) that contain immunoreceptor tyrosine-based activation motifs (ITAMs). The ITAMs recruit and activate kinases including Syk and ZAP-70, which will activate the cytotoxic functions of NK cells. Activating receptors on NK cells recognize stress-induced ligands or microbial proteins on the surface of target cells. In mice, known stress ligands are the family of retinoic acid early inducible 1 (Rae-1) proteins (isoforms α to ϵ), H-60, and MULT1 which

interact with the activating receptor NKG2D on NK cells (90, 91). Examples of activating receptors which recognize viral proteins are NKp46 which recognizes hemagglutinin of influenza virus (92), and Ly49H which recognizes m157 of murine cytomegalovirus (described in section 1.5.4). Recently, the activating receptor NK1.1 was shown to recognize the m12 of murine cytomegalovirus, a viral protein that also inhibits NK cell function by binding the inhibitory receptor NKR-P1B (93).

Another mode of recognition by NK cells is the mechanism of antibody-dependent cell-mediated cytotoxicity (ADCC). In this case, NK cells can recognize a target cell that is opsonized by an antibody bound to a particular antigen on the cell surface. NK cells accomplish their ADCC function by recognizing the Fc region of the antibody through their low-affinity Fc receptor CD16 (94).

1.4.3. Mechanism of NK cell-mediated lysis through perforin and granzymes

Upon recognition of the target cells, NK cells mediate their cytotoxic functions through several different mechanisms, including perforin/granzymes, TRAIL, and FasL (95). In 1994, Kagi and colleagues demonstrated that T cells and NK cells from perforin-deficient mice exhibited reduced cytotoxicity (96). Since then, the perforin/granzyme pathway has been considered as the major mechanism of NK cell-mediated killing. In fact, deficiencies in perforin lead to an impaired ability to control tumors and infections (97, 98). For instance, perforin-deficient mice are much more susceptible to MCMV infection in which case the virus can persist longer in the host (99, 100).

Perforin molecules are contained within granules that are trafficked inside the cytosol in a coordinated manner by the cytoskeleton towards the synapse at the interface of the

effector/target junction, and are released via exocytosis (98). Granule exocytosis depends on Rab27a, a GTPase enzyme involved in vesicular trafficking and secretion (101). A cytosolic Ca^{2+} influx is also important to mobilize specific proteins involved in granule secretion (102).

Perforin is essential for granules mediated lysis of the target cells. Perforin forms pores to allow the uptake of granzymes (103). The crystal structure of perforin has revealed its similarity with cholesterol dependent cytolysins (CDCs) of bacteria (98, 104), a virulence factor that forms a pore on its target cell (105). A model of pore formation by CDCs has been suggested (106), and perforin seems to assemble as a pore in a manner similar to that of CDCs. Perforin is composed of a membrane attack complex/perforin (MACPF) domain that contains two transmembrane helical (TMH) regions involved in piercing the plasma membrane of the target cell.

Granules also contain granzymes, which are released together with perforin when NK cells are activated to kill. Although there are 10 granzymes in mice, granzymes A and B were shown to be the main inducers of NK cell-mediated lysis, while granzyme B was shown to be the most potent one (107). Granzyme B mediates its function by activating caspases to induce apoptosis (108).

1.4.4. NK cell resistance to perforin and granzyme-mediated suicide

Intriguingly, NK cells are resistance to their own weapon (109). They are able to avoid self-destruction by their own secreted granules, allowing them to kill more than one target cell. However, the mechanism that prevents NK cells from being targeted by the perforin molecules that they release is still not completely elucidated. An interesting observation was reported by Balaji and colleagues when they found that cytotoxic T lymphocytes (CTLs) are more susceptible to death upon granule exocytosis in the presence of cathepsin inhibitors (110).

They also showed that CTLs express cathepsin B on their surface upon activation and that cathepsin B can cleave perforin *in vitro*, suggesting that cathepsin B prevents the suicide of CTLs upon degranulation. However, a later study showed that CTLs from cathepsin B-deficient mice are not more susceptible to self-destruction via granules exocytosis (111), indicating that another member of the cathepsin family of protease may be responsible. More recently, the lysosomal-associated membrane protein 1 (LAMP-1, CD107a), a protein expressed on the surface membrane of granules, was shown to protect NK cells from degranulation-mediated death (112). Moreover, NK cells and CTLs, which share similar effector functions, were shown to express the serine protease inhibitor B9 (serpin B9) which inhibits granzyme B (113, 114).

1.4.5. Mechanism of NK cell-mediated lysis through death receptors

Alternatively, NK cells can kill their target cell using mechanisms through death receptors, including Fas and TNF-related apoptosis-inducing ligand (TRAIL) mediated mechanisms (95). In fact, recent work has demonstrated that NK cells first rely on perforin/granzymes to kill their target cells, but once their granules are depleted, they start to use TNF death receptor family members-mediated signaling such as Fas-FasL interaction (115). The signaling pathways emanated from these death receptors have been well documented (116, 117). FasL expressed by NK cells binds to Fas receptor on the target cells and activates apoptosis. TRAIL on NK cells can bind to either TRAIL-R1 (DR4) or TRAIL-R2 (DR5) on the target cell. The intracellular regions of these death receptors contain a death domain (DD). Engagement of the Fas receptor or TRAIL receptors on the target cell with their respective ligand on NK cells will recruit an adaptor protein that also contains a DD called Fas-associated protein with DD (FADD). The DD of the death receptor directly binds to the

DD of FADD. FADD then recruits caspase-8 and caspase-10, and together they assemble into the death-inducing signaling complex (DISC). DISC undergoes autoproteolytic cleavage that activates the caspases. The active forms of caspase-8 and caspase-10 will activate the executioner caspase-3, caspase-6, and caspase-7 which will trigger the apoptosis pathway.

1.4.6. Cytokines produced by NK cells

NK cells are able to mediate their effector functions through the secretion of soluble factors. The main pro-inflammatory cytokines produced by NK cells are interferon-gamma ($\text{IFN}\gamma$) and tumor necrosis factor-alpha ($\text{TNF}\alpha$) (118). The name “interferon” was given to a group of cytokines that share the ability to interfere with viral infection. Indeed, $\text{IFN}\gamma$ interferes with many steps of viral life cycle, even though its anti-viral activity is less potent than those of type I IFNs (119). In addition, $\text{IFN}\gamma$ can mediate antitumor functions (120). Upon engagement of $\text{IFN}\gamma$ to its receptor, JAK1 and JAK2 are activated and phosphorylate the cytoplasmic tail of the receptor, generating a docking site for the recruitment of STAT1 molecules (121). The STAT1 molecules are phosphorylated and dimerize before translocating to the nucleus where they bind to gamma-activated site (GAS) elements and induce the transcription of several interferon-stimulated genes (ISGs). Signaling induces the upregulation of many antiviral and pro-apoptotic genes (122). $\text{IFN}\gamma$ promotes the exposure of the adaptive immune cells to antigen by upregulation of MHC Class I molecules on target cells (123). $\text{IFN}\gamma$ will also upregulate MHC Class II molecules on APCs (124) and increase the antimicrobial and tumoricidal functions of macrophages (125, 126). $\text{IFN}\gamma$ activates Th1 cells through upregulation of T-bet, which further promotes $\text{IFN}\gamma$ production by creating a positive feedback loop (127, 128). Activation of Th1 cells enhances the response of CTLs (129).

TNF α , as the name suggests, was first discovered as a molecule that induces necrosis in tumors (130, 131). There are two known receptors of TNF α , TNFR1, and TNFR2, which have distinct signaling pathways (132). Unlike TNFR2, TNFR1 is a death receptor because it contains a death domain. Signaling through TNFR1 can induce both pro-apoptotic and anti-apoptotic pathways, whereas TNFR2 only induces pro-survival signals (133). TNFR1 induces cell death through recruitment of TNFR1-associated death domain protein (TRADD) and caspase-8 activation leading to apoptosis. On the other hand, signaling through TNFR2 leads to the recruitment of TNF receptor-associated factor 2 (TRAF2) and activation of NF- κ B. TNFR1 is expressed on most cell types, whereas TNFR2 is predominantly expressed in immune cells (134). TNF α stimulates the production of pro-inflammatory cytokines and enhances the function of macrophages (135-137). TNF α -deficient mice are susceptible to *Leishmania major* and *Streptococcus pneumoniae* infections (138, 139). In addition, TNFR1-deficient mice are susceptible to *Listeria monocytogenes* infection (140).

1.4.7. Cytokines that stimulate NK cells effector functions

Cytokines that enhance the effector functions of NK cells include IL-2, IL-15, IL-12, IL-18, and IL-21 (141). Not only is IL-15 essential in NK cell development and homeostasis, but we have also shown that IL-15 is able to prime NK cells by lowering the threshold of NK cell activation during inflammation (142). Therefore, NK cells that are initially primed with IL-15 will have enhanced effector functions upon further stimulations. Although not essential for NK cell development, IL-2 is structurally and functionally very similar to IL-15 (143). The *in vitro* culture of NK cells requires either IL-2 or IL-15 (144), highlighting the importance of these mitogenic cytokines in promoting NK cell survival and growth. IL-12 and IL-18 activate NK cells to produce IFN γ when these cytokines are used alone, however, together they

synergize to produce greater amounts of IFN γ (145, 146). IL-12 and IL-18 both have been shown to increase the expression of the IL-2R α chain on NK cells, thereby increasing the responsiveness of NK cell to IL-2 dependent proliferation (147). In fact, NK cells that are preactivated *in vitro* with a combination of IL-15, IL-12, and IL-18 are sustained longer *in vivo* and show enhanced ability to control tumor (148). Tumor control by IL-18 is mediated through Fas-FasL, whereas tumor control by IL-12 is mediated through perforin/granzymes (149). The mammalian target of rapamycin (mTOR) is an important metabolic activator of NK cell effector functions (142, 150, 151). We have recently shown that IL-18 is a potent inducer of the metabolism of NK cells through the upregulation of nutrient transporters, enabling increased uptake of leucine for enhanced mTOR activation (152). IL-21 was shown to limit NK cell proliferation but increase NK cell cytotoxicity and IFN γ production (153, 154).

1.5. Murine cytomegalovirus (MCMV)

1.5.1. MCMV structure and replication

MCMV is a member of the *Herpesviridae* family of viruses. Structurally, this herpesvirus is composed of an icosahedral capsid, a tegument, and a lipid bilayer envelope (155). Within the capsid lies a linear double-stranded DNA genome of approximately 230 kbp in length that encodes up to 170 open reading frames (ORFs) (156). MCMV enters the cell through a complex of envelope proteins composed of glycoprotein H (gH), gL, and gO (157, 158). MCMV entry is mediated by binding to heparan sulfate proteoglycans on the host cell (159). The genomic replication occurs in the nucleus.

The replication cycle of MCMV can be divided into three phases: the immediate-early (IE), early (E), and late (L) phases (160). During the early phase of replication, MCMV induces

the expression of IE genes (m36, m38.5, and m41.1) that inhibit the apoptosis of the infected cell and provide the virus with sufficient time to replicate. The viral protein m36 inhibits the activation of caspase-8 (161). The viral proteins m38.5 and m41.1 block the action of cellular pro-apoptotic proteins Bax and Bak, respectively, and prevent the permeabilization of the mitochondrial membrane and release of cytochrome c (162, 163). The late phase of replication occurs after 24 hours, and the production of infectious virions requires at least 48 hours (160). As it replicates in the nucleus, MCMV causes the infected cell to have an enlarged nucleus, leading to cellular swelling (164). Following an extended period of production of infectious virions, CMV will induce cell death to terminate the replication cycle (165).

1.5.2. MCMV pathogenesis

Human cytomegalovirus (HCMV) infection is asymptomatic in the immunocompetent host but can cause disease in immunocompromised individuals as an opportunistic infection, as evidenced during pregnancy, in recipients of organ transplants, and in HIV-infected patients who progress to AIDS (166). In mice, MCMV can cause symptomatic disease, however the severity of the disease depends on different factors such as the viral dose, the route of delivery, the mouse strain, and the age of the mice (167).

Cytomegalovirus is strictly specific to the host species that it infects, hence MCMV only infects mice. The transmission of CMV occurs through body fluids, such as saliva, urine, and breast milk (168, 169). It can enter the body through mucosal surfaces such as the upper respiratory tract, the epithelium of the gastrointestinal tract, and the epithelium of the genitourinary tract. MCMV spreads to many organs such as the lung, spleen, liver, and pancreas (170, 171). It can infect numerous cell types, including endothelial cells (172), epithelial cells (173), reticular fibroblasts in the spleen, and hepatocytes (174, 175). In

immunocompromised mice, MCMV can also infect retinal glial cells (176, 177) and bone marrow stromal cells. Infection of bone marrow stromal cells leads to reduced cellular functions characterized by decreased expression of essential factors of hematopoiesis such as SCF and granulocyte colony-stimulating factor (G-CSF) (178). Monocytes/macrophages are a major reservoir of MCMV replication and act as the source of viral dissemination (170, 174, 179). Nonetheless, infected macrophages play an important role in initiating the antiviral responses required for viral clearance (180). A recent study showed that dendritic cells are also involved in MCMV dissemination (181). Moreover, MCMV gene m131/129 codes for MCMV-encoded chemokine 2 (MCK2), a chemokine homolog that promotes viral dissemination to the salivary gland (182).

In susceptible mice, MCMV infection can cause severe damage to the spleen and liver, leading to death (171). This damage can be caused by the direct cytopathic effect of MCMV (164) or as a result of excessive immune cell activation and increased pro-inflammatory cytokines such as TNF α , leading to increased cell death in the infected tissue (183-185). Following the acute phase of MCMV infection, the virus is able to maintain an active replication in the salivary glands for several weeks (186). Eventually, if efficiently controlled by the immune system, MCMV remains latent in the host and production of infectious virions is absent within the spleen, salivary gland, lung, and pancreas several months after initial infection (187, 188). Endothelial cells and macrophages were shown to be a reservoir for MCMV latent infection (189, 190). Reactivation of the virus may occur when the immune system is compromised (187).

Due to the strict species-specificity of CMV, the study of MCMV in mice has been an important model to better understand the biology and pathogenesis of HCMV in humans. MCMV and HCMV share many analogous genes that are similarly regulated and encode viral

proteins that have similar functions (156, 191). In addition, they cause similar pathogenesis based on the cells and organs that are infected (167). Both MCMV and HCMV lead to the resolution of productive infection, establish latency in the host, and can be reactivated when the immune system is compromised (191, 192). MCMV infection of irradiated mice is also a good model to study HCMV infection in patients suffering from hematopoietic malignancies who undergo hemato-ablation followed by hematopoietic cell transplantation (HCT), since both viruses cause pneumonia (193, 194). Moreover, the use of MCMV infection has been a valuable tool in preclinical studies to test the efficacy of new antiviral drugs including ganciclovir, foscarnet and cidofovir (195). These antiviral drugs are also potent against other herpesviruses. Therefore, MCMV infection is an excellent model that recapitulates the features of human disease caused by HCMV.

HCMV is a leading cause of congenital infection (196). However, MCMV is not transmitted to the placenta in immunocompetent hosts (197-199), making it an inadequate model to study congenital infection. To circumvent this difficulty and mimic congenital disease caused by HCMV, previous studies have relied on injecting the virus directly into the placenta (200), or infecting newborn mice (201, 202), nonetheless a better model that would allow natural transmission of the virus to the fetus is warranted.

1.5.3. Immune response to MCMV infection

The host sensors of foreign pathogens, such as TLR2, TLR3, and TLR9, have been shown to be essential for the control of MCMV *in vivo* (203-205). MCMV infection induces the expression of multiple pro-inflammatory cytokines, including IL-6, IL-12, IFNs and TNF α that are produced at a peak level on day 1.5 post-infection (206-208). Many immune cells are

important in the control of MCMV, including neutrophils, macrophages, DCs, NK cells, NKT cells, CD4 T cells, and CD8 T cells (209).

1.5.4. NK cell response to MCMV infection

MCMV infection has been a useful tool to study the response of NK cells to pathogens (210). This virus induces strong antiviral responses by NK cells. In fact, NK cells are essential in the control of MCMV infection in the early phase of infection (81). Likewise, humans who lack NK cells are highly susceptible to infections by herpesviruses (79). NK cell response during MCMV infection has been well documented and is characterized by a strong induction of the pro-inflammatory cytokine IFN γ , granule-mediated cytotoxicity, and robust proliferation. Notably, IFN γ and perforin have been shown to be critical in NK cell-mediated suppression of MCMV infection (99, 211, 212).

MCMV causes an acute infection in MCMV-resistant strains such as C57BL/6 mice but can persist in MCMV-susceptible strains like BALB/c mice. This difference in disease manifestation is in part due to a distinct expression of activating receptors on NK cells. In C57BL/6 mice, approximately 50% of NK cells express the activating receptor Ly49H that recognizes the MCMV protein m157 on the surface of infected cells and provides resistance of the host against MCMV (213-215). In BALB/c mice, Ly49H receptor is absent on NK cells, making this strain particularly susceptible to MCMV. The m157-Ly49H interaction induces specific activation and proliferation of the Ly49H-expressing subset of NK cells. Transgenic expression of Ly49H conferred resistance to cytomegalovirus in genetically susceptible mice (216). Other strains have other activating receptors, such as Ly49P that provides resistance against MCMV infection in hosts of the H-2D^k haplotype (217).

1.5.5. Mechanisms of immune evasion by MCMV

MCMV employs various strategies to evade immune recognition (218). For instance, MCMV encodes the M27 protein, which inhibits the action of type I and type II IFNs by downregulating STAT2 (219). Although the viral protein m157 activates NK cells through the Ly49H activating receptor, m157 is structurally homologous to MHC class I molecules and is able to inhibit NK cells through their Ly49I inhibitory receptor in specific strains of mice (220). In addition, the MCMV proteins m138, m145, m152, and m155 induce the downregulation of stress ligands, which are recognized by the activating receptor NKG2D on NK cells (221). MCMV has also evolved to evade from recognition by CD8 T cells, through expression of m06 and m152 proteins, which interfere with antigen presentation by downregulating MHC class I molecules (218, 222).

1.6. Immunoregulation during viral infection

1.6.1. Immune cells involved in immunopathology during viral infection

While tissue damage during viral infection can be a result of direct cytopathic effect, lesions are often caused by immune-mediated tissue injury, a scenario that is more prominent during chronic infections. In this case, proper regulation of the extent of inflammation is critical. A balance between pro-inflammatory and anti-inflammatory immune responses that prevents tissue damage while allowing viral clearance would be an ideal situation (223). Cellular players that have been shown to cause excessive immune response leading to tissue damage when dysregulated are CTLs (99). These cells can directly kill the target cells and produce pro-inflammatory cytokines such as IFN γ and TNF α (224). To counter the excessive inflammatory responses, the immune system is armed with regulatory cells that dampen these

responses. Such cellular players include regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and regulatory NK cells.

Tregs are a subset of CD4 T cells that are characterized by the expression of IL-2R α and the transcription factor Foxp3 (225). Tregs have been shown to contribute to dampening inflammatory responses during several infection models while reducing tissue injury (226, 227). For example, Tregs reduce tissue damage in the cornea caused by herpes simplex virus (HSV) infection (228), prevent lung damage during respiratory syncytial virus infection (229), and protect against virus-induced lesions in the central nervous system (230). Tregs accomplish their suppressive functions through production of anti-inflammatory cytokines and expression of inhibitory receptors (225), by consuming and depleting IL-2 (231, 232), by suppressing the antigen presentation function of DCs (233), and by regulating the effector functions of CD8 T cells during infection (234, 235).

MDSCs are a heterogeneous population of immature myeloid cells that exhibit immunosuppressive functions and thereby are potent regulators of tissue injury (236). MDSCs have been shown to promote viral persistence through suppression of T cells (237). MDSCs achieve their suppressive functions through production of inducible nitric oxide (iNOS) and ROS, which inactivate the T cell receptor on CD8 T cells and disrupt the binding to antigenic peptides presented on MHC molecules (238). Through the expression of arginase-1, MDSCs also limit the availability of L-arginine, which is important for the proliferation of T cells (239). MDSCs were shown to suppress antigen-specific CD8 T cells during Friend retrovirus (FV) infection (240). Recently, it was shown that Hepatitis B virus (HBV) induces the expansion of MDSCs to regulate T cell functions (241), and this expansion of MDSCs correlated with reduced liver damage (242).

Several reports have suggested the regulatory role of NK cells (243). For instance, NK cells can kill DCs or activated T cells. NK cells were shown to kill immature DCs *in vivo* via a TRAIL-dependent mechanism and, as a result, hindered T cell priming (244). During MCMV infection, NK cells kill virus-infected DCs, thereby reducing T cell exposure to antigen-presenting cells (245). Another study demonstrated that IL-2 stimulated NK cells can kill activated T cells *in vitro* (246). Moreover, in a model of sustained MCMV infection using perforin-deficient mice, NK cells produce IL-10 to regulate CD8 T cell activation and prevent lethal immunopathology (99).

1.6.2. Pro-inflammatory cytokines involved in immunopathology during viral infection

Pro-inflammatory cytokines such as IL-12 and IFN γ may contribute to tissue damage during infection by increasing the functions of Th1 cells, leading to enhanced activation of CTLs (223). However, some cytokines such as TNF α can also directly induce tissue damage via induction of programmed cell death of infected cells (247). TNF α mediates liver inflammation and induces the death of hepatocytes during MCMV (183-185). Type I IFNs production by macrophages was also shown to contribute to lung injury during influenza virus infection, through induction of TRAIL in an autocrine fashion (248). Activation of innate immune cells through PRRs can also contribute to tissue damage. For example, the use of a TLR4 antagonist was shown to prevent death from influenza virus infection, in part through protection from lung injury (249).

1.6.3. Anti-inflammatory cytokines that regulate immunopathology during viral infection

Regulatory cells like Tregs will exert their functions in part through the production of anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor beta (TGF- β). IL-10 is induced at later phases of the immune response to help resolve inflammation and prevent tissue damage caused by excessive immune activation (250). IL-10 assembles as a dimer with each monomer consisting of 6 alpha helices (251). Signaling occurs through a heterotetrameric complex composed of the IL-10 receptor alpha (IL-10R α) and beta (IL-10R β) subunits (252). Signaling through IL-10R activates Jak1 and Tyk2 kinases, resulting in the recruitment and phosphorylation of the transcription factor STAT3 (250). Phosphorylated STAT3 molecules dimerize and are translocated into the nucleus to activate the transcription of specific genes involved in immunosuppression (253). Deficiencies in IL-10 or IL-10 receptor cause inflammatory bowel disease (IBD) characterized by spontaneous inflammation in the gut of aged mice (254, 255), highlighting the importance of IL-10 in maintaining gut homeostasis. IL-10 has been shown to be produced by numerous cell types including myeloid cells, T cells, B cells and NK cells (256). IL-10 exerts pleiotropic functions on immune cells as the effect of IL-10 depends on the cell type and the inflammatory context (257). In particular, IL-10 is known to inhibit antigen presentation by reducing the expression of MHC molecules on antigen-presenting cells (APCs) such as macrophages and dendritic cells, which can ultimately lead to reduced T cells priming (258, 259).

IL-10 and TGF- β are known to be key modulators of tissue injury. Similar to Treg-depleted wild type mice, IL-10-deficient mice are more susceptible to immune-mediated ocular lesions during HSV infection (260). During influenza virus infection, effector T cells

produce IL-10 to prevent lung damage (261). TGF- β inhibits the effector functions of CD8 T cells (262) and protects against the death of infected neurons in the brains of reovirus-infected mice (263). Moreover, TGF- β 1 induces IL-6 to prevent lung injury during influenza virus infection (264). Notably, IL-6 prevents immune-mediated lung damage during respiratory syncytial virus (RSV) by promoting the maturation of Tregs (265). Although they can limit tissue injury, IL-10 and TGF- β can be detrimental to the host by preventing an effective antiviral response by T cells, thereby allowing viral persistence (266, 267).

1.6.4. Immune exhaustion during viral infection

The first evidence of T cell exhaustion during viral infection was a study using lymphocytic choriomeningitis virus (LCMV) and demonstrating the maintenance of antigen-specific T cells with reduced or absent effector functions (268). Exhaustion of T cells is associated with reduced cytokine production, cytotoxicity, and proliferation, and increased inhibitory receptors and apoptosis (269). Inhibitory receptors that have been shown to promote T cell exhaustion include programmed death-1 (PD-1), CTLA-4, LAG3, and Tim-3. Notably, blocking signaling from these inhibitory receptors restores T cell function (270). While these molecules may contribute to persistent infections, they may also prevent excessive immune responses that can lead to tissue injury (271). These inhibitory receptors have been implicated in suppressing the immune system, including CD8 T cells and NK cells, in the tumor microenvironment (272). Notably, recent immunological approaches to treat cancer by blocking these inhibitory receptors, mainly PD-1 and CTLA-4 (273, 274), have shown unprecedented successful results. This treatment termed checkpoint blockade is actively being explored in developing effective cancer therapies.

Chapter 2: Natural killer cell-specific Gata3 ablation identifies the maturation program required for bone marrow exit and control of proliferation

Preface

The following chapter contains original research demonstrating that GATA3 is required for NK cell terminal maturation and egress from the bone marrow. This research was originally published in *The Journal of Immunology*:

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The specific contributions of each author to this paper are listed below:

Alaa Kassim Ali: Performed experiments, analyzed the data, designed the study, and wrote the manuscript.

Jun Seok Oh: Performed experiments, including the isolation of splenic leukocytes.

Eric Vivier: Provided the *NKp46^{iCre}* knock-in mice and reviewed the manuscript.

Meinrad Busslinger: Provided the *Gata3^{fllox}* mice.

Seung-Hwan Lee: Designed the study, wrote the manuscript and obtained the funding.

2.1. Abstract

Natural killer (NK) cells are innate lymphocytes capable of eliciting an innate immune response to pathogens. NK cells develop and become mature in the bone marrow (BM) before they migrate out to peripheral organs. While the developmental program leading to mature NK cells has been studied in the context of several transcription factors, the stage-specific role of GATA3 in NK cell development has been incompletely understood. Using *NKp46-Cre-Gata3^{fl/fl}* mice in which *Gata3* deficiency was induced as early as the immature stage of NK cell differentiation, we demonstrated that GATA3 is required for the NK cell maturation beyond the CD27 single-positive stage and is indispensable for the maintenance of liver-resident NK cells. The frequencies of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice were found higher in the bone marrow but lower in peripheral organs compared to control littermates, indicating that GATA3 controls the maturation program required for bone marrow egress. Despite the defect in maturation, upon MCMV infection, NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice expanded vigorously, achieving NK cell frequencies surpassing those in controls, and therefore provided comparable protection. The heightened proliferation of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice was cell-intrinsic and was associated with enhanced upregulation of CD25 expression. Taken together, our results demonstrate that GATA3 is a critical regulator for NK cell terminal maturation and egress out of the bone marrow, and that immature NK cells present in the periphery of *NKp46-Cre-Gata3^{fl/fl}* mice can rapidly expand and provide a reservoir of NK cells capable of mounting an efficient cytotoxic response upon virus infection.

2.2. Introduction

Natural killer cells are an important component of the innate immune system. They have gained much attention because of their ability to kill transformed cells without prior sensitization to antigens (77, 78). NK cells can induce the activation of other immune cells by producing pro-inflammatory cytokines such as IFN- γ and TNF- α (275). In addition, NK cells mediate the direct killing of target cells by secreting cytotoxic granules that contain perforin and granzymes (211, 276). Because NK cells play a major role in the early control of viral infection and cancer, understanding the molecular mechanism by which NK cells fully develop and become functionally mature is of great importance. NK cells primarily undergo the development and maturation program in the BM. When they become mature, NK cells migrate through the bloodstream and seed into peripheral organs such as the spleen, liver, and lungs where they can provide early protection against infection (277, 278). In the BM, NK precursor cells (NKP) express the IL-2 receptor β -chain (CD122), and this receptor is maintained in all the stages of NK cell development. CD122 in combination with the common γ -chain (CD132) forms the IL-2/15 receptor, allowing NK cells to be responsive to IL-15. NK cell development depends on IL-15 and the downstream signaling molecules JAK1/3 and STAT5 (142, 279, 280). Immature NK (iNK) cells can be identified by the expression of NK1.1 and these cells further develop into mature NK (mNK) cells as they acquire CD11b. Notably, NK cells in the BM differentiate through 4 stages of maturation, which are associated with the acquisition of the surface markers CD27 and CD11b (18, 19). In the first stage, NK cells lack both CD27 and CD11b receptors. In the second and third stages, NK cells acquire CD27 and CD11b respectively. In the last stage of maturation, NK cells become negative for the expression of

CD27. Therefore, NK cells develop as follows: CD27⁻CD11b⁻, CD27⁺CD11b⁻, CD27⁺CD11b⁺ and CD27⁻CD11b⁺.

Numerous elegant studies using immune cell-specific deficient mice or *in vitro* NK cell differentiation have defined factors responsible for the developmental process of NK cells (278, 281, 282). Examples of those transcription factors include Id2, E4bp4 (also known as Nfil3), T-bet, and Eomes. Id2 is an antagonist of the transcription factor TCF3, which promotes the development of B cells and myeloid cells (283). Eomes was known to bind the CD122 promoter region, and significantly lower CD122 expression and reduced responsiveness to IL-15 was observed in NK cells from Eomes-deficient mice (284). E4bp4 seems to function upstream of Eomes, so that E4bp4 deficiency caused severe defects in NK cell development (40, 285). A recent paper demonstrated that NK cells deficient in 3-phosphoinositide-dependent protein kinase 1 (PDPK1), a kinase downstream of PI3K and upstream of mTOR, are unable to differentiate into NK cells *in vivo* (286). Rescue of the defect of PDPK1 by ectopic expression of E4bp4 or Eomes suggests that PDPK1 signaling is critical for NK cell development via induction of E4bp4 and Eomes. Lastly, NK cell development is altered in the absence of T-bet, resulting in increased numbers of NK cells in the BM and reduced peripheral NK cell numbers. T-bet and Eomes seem to play roles in regulating CD122 in NK cells (284, 287).

Several tissue-specific NK cell subsets have been identified, and their origins and differentiation programs are current topics of interest. Not only critical for T cell development, the thymus also generates a unique NK cell population called thymic NK cells. In contrast to the dispensable role of IL-7 for conventional natural killer (cNK) cells, thymic NK cells express IL-7R α and are highly dependent on IL-7 (16). More recently, liver-resident NK cells defined as CD49b⁻ and CD49a⁺ were identified. They appeared phenotypically immature by

expressing TNF-related apoptosis-inducing ligand (TRAIL) (15), but showed relatively low proliferative capacity at the steady-state (43). Adoptive transfer of hepatic mononuclear cells rescued the liver-resident NK cell population in lethally irradiated recipient mice, demonstrating that liver-specific hematopoietic progenitor cells are the origin of hepatic CD49b⁻ CD49a⁺ NK cells (288). The liver-resident NK cells produce large amounts of TNF- α and IL-2 and less IFN- γ , suggesting their unique function (289). Notably, these cells have been demonstrated to exhibit memory potential in a model of hapten-specific contact hypersensitivity (288). Furthermore, a recent study of transcriptomic analysis indicated that cNK cells, thymic NK cells, and liver-resident NK cells are all generated as distinct lineages (289).

From a family of six transcription factors termed GATA binding protein, GATA3 contains two zinc-finger motifs that bind the consensus sequence [A/T]GATA[A/G] promoter elements (290). GATA3 is known to play crucial roles in a variety of immune cells in a developmental-stage and cell-lineage specific fashion (67). For instance, GATA3 is important in the development and maintenance of T cells (66, 67, 291). Notably, GATA3 plays a critical role in the differentiation of type 2 helper T cells and their ability to secrete cytokines such as IL-4, IL-5, and IL-13. GATA3 is also expressed in the NK cell lineage, and its role is restricted to the NK cell maturation, as GATA3 deficiency did not result in a major defect in NK cell frequencies but led to reduced T-bet expression accompanied with defective IFN- γ production and liver-specific homing of NK cells (72). Similar to T cell's developmental requirement for GATA3, thymic NK cells expressing IL-7R α require GATA3 (16) and are absent in *Vav-Cre-Gata3^{fl/fl}* mice (289). However, cNK cells and liver-resident NK cells are still present in the *Vav-Cre-Gata3^{fl/fl}* mice (289, 292) in which *Gata3* deficiency is induced in all hematopoietic cells at the early stage of development, indicating the dispensable role of GATA3 in their early

development. Therefore, the lineage- and stage-specific roles of GATA3 during NK cell development without the influence from other cell types are warranted. Here, we generated a conditional *Gata3* deficient mouse using NKp46-Cre mice in order to dissect the role of *Gata3* at the late stage of NK cell development. We demonstrate that GATA3 is required for NK cell maturation beyond the acquisition of CD27. The incomplete maturation of NK cells due to GATA3 deficiency resulted in a defect in BM egress, and this was associated with decreased NK cell frequencies in peripheral organs. Surprisingly, in spite of the intrinsic defect in NK cell maturation and accompanying decrease of NK cell frequencies in the periphery, comparable protection between NK cell-specific *Gata3* knockout mice and control littermates was observed during MCMV infection. This protection was due to enhanced expansion of NK cells in the periphery of *NKp46-Cre-Gata3^{fl/fl}* mice and was associated with enhanced upregulation of CD25 expression. Therefore, our result suggests that immature NK cells found in peripheral organs can proliferate vigorously and spread, and could provide a reservoir of efficient effector NK cells during a virus infection.

2.3. Materials and Methods

2.3.1. Mice and genotyping

NKp46^{iCre} knock-in (293), *Gata3^{flox}* mice (55, 56), and CD45.1⁺ Ly49H^{-/-} mice (294) were housed in specific pathogen-free environment. The *Ly49h*-intron primers were D6Ott151-F: 5'-GTGCTACCACTGAAAACCATTG-3', D6Ott151-R: 5'-CTGTCTCTTGAGTCACCTGCAC-3' (295). The *Gata3* floxed and *Gata3* deleted alleles were genotyped using the following primers, forward: 5'-GTCAGGGCACTAAGGGTTGTT-3', floxed-reverse: 5'-TGGTAGAGTCCGCAGGCATTG-3', deleted-reverse: 5'-TATCAGCGGTTTCATCTACAGC-3' (55). To identify the specificity of *Gata3* deletion by Cre recombinase, T cells, B cells, and NK cells from spleen and NKT cells from liver of *NKp46-Cre-Gata3^{fl/fl}* mice were isolated and sorted using MoFlo Astrios (Beckman Coulter). Purities of sorted cell populations were >96%. For PCR, genomic DNA was extracted from each purified cell population. Unless otherwise stated, all experiments were carried out using littermate controls from the *NKp46-Cre^{+/-}* X *Gata3^{fl/fl}* X *NKp46-Cre^{-/-}* X *Gata3^{fl/fl}* mating. All mice used for experiments were between 6 and 12 weeks old. All procedures were approved by and conducted in accordance with the institution's animal guidelines of the University of Ottawa.

2.3.2. Cell isolation

Spleen and blood were harvested and single-cell suspension was obtained following red blood cell lysis and filtration through 70µm filter. Leucocytes were isolated from liver using Percoll gradient centrifugation. BM cells were extracted from tibia and femur.

2.3.3. Antibodies and flow cytometry

The following monoclonal antibodies were used: α -GATA3 (TWAJ), α -NK1.1 (PK136), α -CD3 (17A2 and 145-2C11), α -CD27 (LG.7F9), α -CD11b (M1/70), α -Ly49D (4E5), α -Ly49G2 (4D11), α -Ly49H (3D10), α -NKG2D (CX5), α -NKG2A-B6 (16a11), α -CD62L (MEL-14), α -KLRG1 (2F1), α -TCR- β (H57-597), α -CXCR4 (2B11), α -IFN- γ (XMG1.2), α -CD45.2 (104), α -CD25 (PC61.5), α -CD49b (DX5), α -CD107a (1D4B) from eBioscience, α -GATA3 (L50-823), α -Ly49C/I (5E6), α -CXCR3 (CXCR3-173), α -STAT4 (8/Stat4), α -IgG1 (A85-1), α -pSTAT4 (38/p-Stat4), α -CD49a (Ha31/8), α -CD19 (1D3), α -Ki-67 (B56), α -BrdU (3D4) from BD Biosciences, α -IL-12R β 2 (305719), α -IL-18R α (112614) from R&D Systems, mouse IgG1, kappa (MOPC-21) and rat IgG2b, kappa (RTK4530) from BioLegend, Fixable Far Red Live/Dead from Invitrogen. For intracellular IFN- γ measurements *in vivo*, leukocytes from BM, spleen, and liver were harvested post-infection at D1.5 and incubated in RP-10 media (RPMI-1640, 10% FBS, 1X penicillin/streptomycin, 1% L-Glutamine, 10 mM HEPES, 50 μ M β -mercaptoethanol) containing 5 μ g/ml brefeldin A for 4 hrs, followed by staining for intracellular IFN- γ using BD Cytofix/Cytoperm protocols. Intracellular staining of GATA3 and Ki-67 was carried out using Foxp3 staining kit (eBioscience). Surface staining of CXCR4 was performed at 37°C for 10 minutes. Cells were acquired using FACS Cyan ADP and analyzed using Kaluza software v2 (Beckman Coulter) and FlowJo (Tree Star Inc.).

2.3.4. Induced egress of NK cells from BM to the blood

NK cell exit from BM and labeling of sinusoidal lymphocytes were achieved as described previously (31). Briefly, AMD3100 (Sigma-Aldrich) was given intraperitoneally (150 μ g/mouse) one hour before sacrifice. To label sinusoidal lymphocytes, mice were injected

intravenously with 1 μ g of anti-CD45.2 mAb conjugated with biotin (eBiosciences). Mice were sacrificed 2 minutes after antibody injection. Then, cells were isolated from the blood and BM and stained with Streptavidin-PE before being analyzed using FACS Cyan ADP & Kaluza software v2 (Beckman Coulter).

2.3.5. Transwell migration assay

Transwell assay was performed as previously described (31, 36). Briefly, 5- μ m pore transwell inserts were used to analyze the migratory response of BM cells to CXCL12. BM cells were pretreated with or without 50 μ M of AMD3100 for 90 minutes in RPMI 1640 medium supplemented with 1% FBS. Cells were then washed and counted. Using the same media, 50 ng/mL CXCL12 was prepared and 500 μ L per well was added into a Transwell-24 well plate (Corning). The inserts were placed inside the wells and 5×10^5 cells were loaded into each insert. After incubating the plate at 37°C for 3 hours, the migrated cells were collected. The cells were counted, and the frequency of NK cells was determined by flow cytometry. Cell migration was calculated as percentage of input NK cells.

2.3.6. IFN- γ production upon *in vitro* stimulation

Spleens were harvested, and single-cell suspension of splenic leukocytes were enriched by negative selection using the EasySep Mouse NK cell enrichment kit (Stemcell Technologies). Cells were then seeded in triplicates in RP-10 media on antibody-coated plates or cultured with different combinations of recombinant human (rh) IL-2 (1000 U/ml, NIH/NCI BRB Preclinical Repository), recombinant murine (rm)IL-12 (50 ng/ml, eBioscience) and rmIL-18 (100 ng/ml, Peprotech). After 1 hour of stimulation, brefeldin A was added to a final

concentration of 5 µg/ml and cells were incubated for 4 hours followed by staining for intracellular IFN-γ using BD Cytofix/Cytoperm protocols.

2.3.7. MCMV infection, cytokine quantification and viral titration

Smith strain MCMV stocks were generated in our laboratory from infected salivary glands of BALB/c mice and viral titers determined by standard plaque assays (295). Mice were infected with 5,000 plaque-forming units (PFUs) of MCMV intraperitoneally. To measure viral burdens in organs, spleens and livers from infected mice were homogenized by MagNA Lyser (Roche Applied Science) and the lysates were appropriately diluted and overlaid on mouse embryonic fibroblasts cells in duplicates in 2% DMEM (Dulbecco Modified Eagle's Medium, 2% FBS, 1X penicillin/streptomycin, 1% L-Glutamine, 10 mM HEPES). Plaques were counted 3 days later and represented as log PFU/gram of organ. Blood serum from naïve or infected mice were appropriately diluted and analyzed for the production of mouse IFN-γ and IL-12 using Cytometric Bead Array kit (BD Biosciences). Samples were prepared according to the manufacturer's instructions, acquired on FACS Cyan ADP (Beckman Coulter) and analyzed using the BD FCAP Array Software (BD Biosciences).

2.3.8. NK cell cytotoxicity assay

NK cell cytotoxicity was measured by analyzing the anti-tumor activity of NK cells against YAC-1 target cells. Splenic leukocytes were isolated from *Gata3^{fl/fl}* or *NKp46-Cre-Gata3^{fl/fl}* mice that were either naïve or infected with MCMV. To ensure equal numbers of NK cells were used, spleen populations were surface stained for NK and T cells and each sample adjusted to appropriate concentrations in sterile NK media (RPMI-1640, 10% FBS, 1X penicillin/streptomycin, 1% L-Glutamine, 10 mM HEPES, 50 µM β-mercaptoethanol, 1%

Non-Essential Amino acids, 1 mM Sodium Pyruvate). YAC-1 cells were cultured in sterile RP-10 media and labeled with 100 μ Ci of ^{51}Cr for 1 hr at 37°C, washed three times with PBS, and made to a concentration of 5×10^4 cells/ml in NK medium. In V-bottom 96-well plates, 100 μ L of YAC-1 cells were added in triplicates and incubated for 4 hrs with 100 μ L of effector cells with effector: target ratios ranging from 0.3:1 to 10:1. Supernatants were then used to quantify the amount of ^{51}Cr released due to specific lysis of YAC-1 targets by NK cells, and counted using 2470 WIZARD² Automatic Gamma Counter (Perkin Elmer). NK cytotoxicity was calculated according to the formula: % specific ^{51}Cr release = [(experimental release - minimum release) / (maximum release - minimum release)] $\times$ 100.

2.3.9. *In vitro* assay for NK cell proliferation

Spleens were harvested under sterile conditions, and single-cell suspension of splenic leukocytes was generated after red blood cell lysis and filtration through 70 μ m nylon mesh. Cells were enriched by negative selection using the EasySep Mouse NK cell enrichment kit (Stemcell Technologies) and labeled with Cell Proliferation Dye eFluor 450 (eBioscience). Cells were then cultured in 96-well plates in triplicates in RP-10 media with different concentrations of rhIL-2 or rmIL-15 (eBioscience). To measure BrdU incorporation, BrdU was added to the culture at a final concentration of 10 μ M, 24 hours before harvesting the cells.

2.3.10. CD25 induction and STAT4 phosphorylation

To evaluate CD25 induction on NK cells, 5E5 total splenocytes were stimulated in a 96-well plate with rmIL-12 (10 ng/ml, eBioscience) and/or rmIL-18 (10 ng/ml, Peprotech) for 24 hours. To measure total STAT4 levels, cells were first stained using purified α -STAT4 antibody followed by secondary staining using anti-IgG1 conjugated to PE. To detect

phosphorylated STAT4, cells were rested at 37°C for 4 hours. Cells were then stimulated with rmIL-12 (10 ng/ml) and rmIL-18 (50 ng/ml) for 30 minutes before staining. To measure STAT4 and pSTAT4, cells were fixed with Biolegend Fix/Perm buffer and permeabilized with pre-chilled 100% methanol before intracellular staining.

2.3.11. Adoptive transfer

Spleen cells from *Gata3^{fl/fl}* or *NKp46-Cre-Gata3^{fl/fl}* mice (CD45.2⁺) were isolated and equal numbers of Ly49H⁺ NK cells were transferred into Ly49H^{-/-} recipients (CD45.1⁺). Ly49H^{-/-} recipients were either uninfected or given 3,000 PFU MCMV i.p. one day following adoptive transfer. Leukocytes from spleen and liver of recipient mice were harvested on day 5 post-infection.

2.3.12. Quantitative real-time PCR

NK cells from *Gata3^{fl/fl}* or *NKp46-Cre-Gata3^{fl/fl}* mice were enriched by negative selection using the EasySep Mouse NK cell enrichment kit (Stemcell Technologies) and sorted using MoFlo Astrios (Beckman Coulter). Purities of sorted NK cells were >98%. Total RNA was extracted from the sorted NK cells using TRIZOL reagent (GIBCO BRL) according to the manufacturer's instructions. Using 35ng of total RNA, cDNA was reverse transcribed in a 20µl reaction using First strand cDNA synthesis kit (Thermo Scientific). For quantification of Gapdh and target genes by real-time PCR, cDNA was added to a 20µl reaction of FastStart Universal SYBR Green Master (Rox) (Roche) and amplified using ViiA 7 Dx Real-Time PCR Instrument (Applied Biosystems). The expression of target genes was normalized to Gapdh levels. The primer sequences for Gapdh are F: 5'-ACCACAGTCCATGCCATCAC-3' and R: 5'-TCCACCACCCTGTTGCTGTA-3', for Id2 are F: 5'-ATGAAAGCCTTCAGTCCGGTG-

3' and R: 5'-AGCAGACTCATCGGGTCGT-3', for T-bet are F: 5'-CTAAGCAAGGACGGCGAATGT-3' and R: 5'-GGCTGGGAACAGGATACTGG-3', for E4bp4 are F: 5'-GAACTCTGCCTTAGCTGAGGT-3' and R: 5'-ATTCCCGTTTTCTCCGACACG-3', for Ets1 are F: 5'-TGGAATGTGCAGATGTCCCG-3' and R: 5'-TGAGCATGCTCGATACCGTA-3', for Irf2 are F: 5'-CGATTATTCAACTGACGGGCTTTC-3' and R: 5'-GCATTCGCATCCGTTCCAC-3', for Eomes are F: 5'-GATGTACGTTACCCAGAAT-3' and R: 5'-ATCGTAGTTGTCCCGGAAGC-3', for Tox are F: 5'-CTACTGCAGTAGTCGGTACTCC-3' and R: 5'-CTGAAGCAGCTAGCAGCATAC-3'.

2.3.13. Statistical analysis

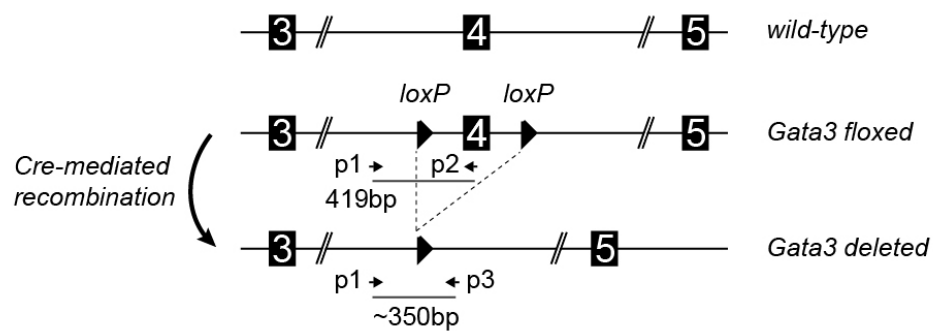
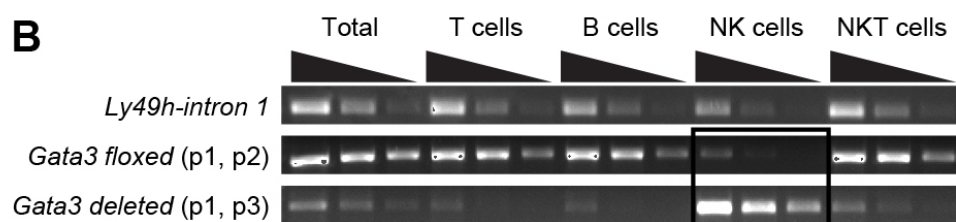
Significance of results was determined by two-tailed unpaired student *t* tests ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) and graphed using Prism Version 5 (Graph Pad software).

2.4. Results

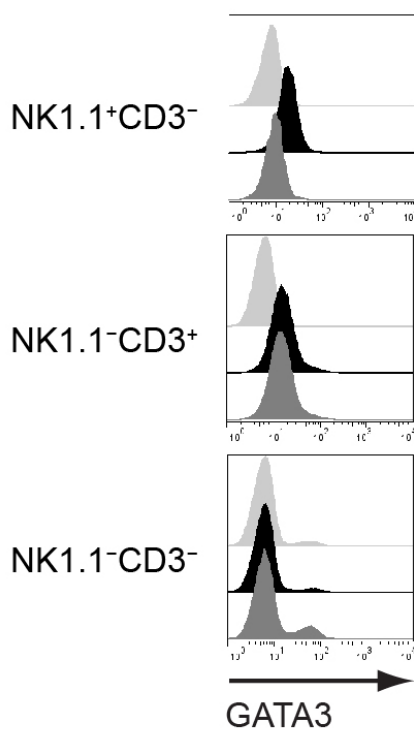
2.4.1. Generation of *NKp46-Cre-Gata3^{fl/fl}* mice

The complete ablation of *Gata3* leads to embryonic lethality due to noradrenaline deficiency and improper kidney development (55, 296). Developmental stage-specific roles of GATA3 in diverse immune cells have been demonstrated (67). To investigate the role of GATA3 at the late stage of development of NK cells, we generated *NKp46-Cre-Gata3^{fl/fl}* mice using a mouse expressing the Cre recombinase under the *NKp46* promoter. Compared to *Vav-Cre-Gata3^{fl/fl}* mice, *NKp46-Cre* expression is induced at a stage of NK cell development that follows the acquisition of NK1.1, therefore allowing to study the role of GATA3 at a late stage of development. In a previous report, *Gata3^{fl/fl}* mice that carry *loxP* sites flanking exon 4 of the *Gata3* locus have been successfully used to generate conditional *Gata3*-deficient mice (Figure 2.1A) (55, 297). To determine whether Cre-mediated recombination occurred only in cNK cells in the spleens of *NKp46-Cre-Gata3^{fl/fl}* mice, we extracted genomic DNA from sorted T, B, NK and NKT cells isolated from *NKp46-Cre-Gata3^{fl/fl}* mice and determined the Cre-mediated recombination by genomic DNA PCR. The *Gata3* floxed allele was present among sorted T cells, B cells, and NKT cells but only weakly amplified from sorted NK cells (Figure 2.1B). Contrarily, the *Gata3* deleted allele was predominately detected from sorted NK cells but not in other populations. Intron 1 of *Ly49h* gene was used as control and showed similar genomic abundance between the different sorted populations. These results indicate that the genomic deletion of *Gata3-exon 4* occurred predominantly in splenic NK cells of *NKp46-Cre-Gata3^{fl/fl}* mice.

We also evaluated GATA3 protein expression in NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice and their control littermates that carry *loxP*-flanked *Gata3* alleles but lack *NKp46-Cre*

A*Gata3* locus**B****C**

Legend:
 ■ Isotype control
 ■ *Gata3^{fl/fl}*
 ■ *NKp46-Cre-Gata3^{fl/fl}*

**D**

Legend:
 ■ Isotype control
 ■ *Gata3^{fl/fl}*
 ■ *NKp46-Cre-Gata3^{fl/fl}*

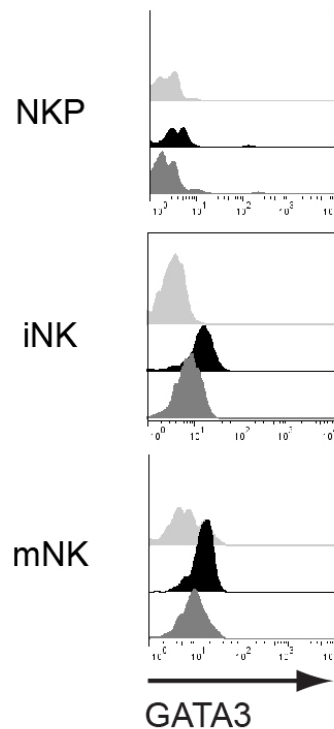


Figure 2.1. The deletion of *Gata3* gene (exon 4) occurs predominantly in NK cells. Genomic deletion of *Gata3* and protein expression of GATA3 in *NKp46-Cre-Gata3^{fl/fl}* mice and control littermates. (A) In *Gata3^{fl/fl}* mice, exon 4 of *Gata3* gene is flanked with *loxP* sites. Crossing *Gata3^{lox}* mice with *NKp46-Cre* mice leads to Cre-mediated recombination resulting in exon 4 deletion. The diagram is not on scale. (B) Sorted T, B, NK, and NKT cells were genotyped to determine the presence of *Gata3* floxed allele, and *Gata3* deleted allele. Semi-quantitative genomic PCR was performed on serial 5-fold dilutions of DNA from the different cell populations. (C) GATA3 protein level was measured by flow cytometry in splenic NK cells (NK1.1⁺CD3⁻), T cells (NK1.1⁻CD3⁺), and non-NK/non-T cells (NK1.1⁻CD3⁻) from *NKp46-Cre-Gata3^{fl/fl}* and *Gata3^{fl/fl}* mice. (D) GATA3 protein level was measured by flow cytometry in BM NK precursor cells (NKP; CD3⁻CD122⁺NK1.1⁻CD11b⁻), immature NK cells (iNK; CD3⁻CD122⁺NK1.1⁺CD11b⁻) and mature NK cells (mNK; CD3⁻CD122⁺NK1.1⁺CD11b⁺) from *NKp46-Cre-Gata3^{fl/fl}* and *Gata3^{fl/fl}* mice. Data are from one experiment representative of at least two independent experiments.

(*Gata3^{fl/fl}* mice). GATA3 is known to be expressed in mature T cells but not in B cells or myeloid cells (297). Accordingly, T cells expressed high levels of GATA3, and this expression was at a similar intensity as NK cells from control mice (Figure 2.1C). Non-T/non-NK cells which are composed of B cells and myeloid cells did not express GATA3 as expected. Notably, NK cells from *NKp46-Cre-Gata3^{fl/fl}* showed reduced expression of GATA3 compared to NK cells from control mice. To determine at which stage of NK cell differentiation GATA3 expression is abrogated in *NKp46-Cre-Gata3^{fl/fl}* mice, we measured the expression of GATA3 in NKP, iNK and mNK cells in the BM. GATA3 was not expressed in NKP cells, but was induced at the iNK stage and retained its expression in mNK cells (Figure 2.1D). However, in *NKp46-Cre-Gata3^{fl/fl}* mice, GATA3 expression was reduced in both iNK and mNK cells, suggesting that *NKp46-Cre*-induced *Gata3* downmodulation occurs as early as the iNK cell stage. Taken together, the *NKp46-Cre-Gata3^{fl/fl}* mouse is an adequate model to study the role of GATA3 in the development and maturation of NK cells.

2.4.2. GATA3 is required for NK cell maturation and exit from the BM

Previously, GATA3 deficiency at the hematopoietic stem cell stage was shown to promote the homing of NK cells into the liver (72). To determine whether GATA3 influences the distribution of NK cells within different compartments, we examined the NK cell frequencies in the BM, spleen, and liver of *NKp46-Cre-Gata3^{fl/fl}* mice. Compared to their control littermates, NK cell frequencies and numbers in *NKp46-Cre-Gata3^{fl/fl}* mice were found higher in the BM but lower in the spleen and liver (Figure 2.2A, B), suggesting a defect in the egress of NK cells from the BM to peripheral organs in the absence of GATA3.

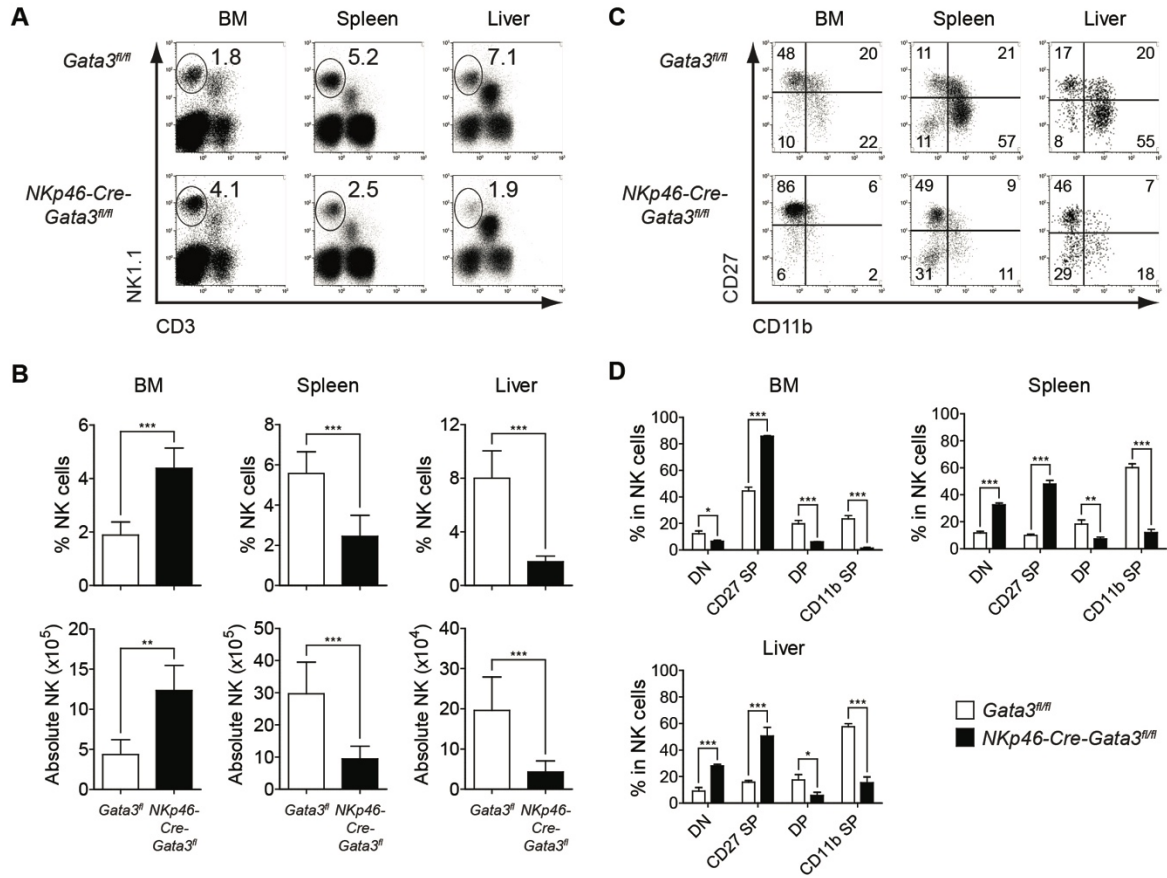


Figure 2.2. NK cell frequencies and maturation are perturbed in the absence of GATA3. Flow cytometric analysis of NK cell populations in the bone marrow, spleens, and livers of *Nkp46-Cre-Gata3^{fl/fl}* and *Gata3^{fl/fl}* mice. (A) Representative plots of NK cell (NK1.1⁺CD3⁻) frequencies. (B) Percentage and number of NK cells (NK1.1⁺CD3⁻ or NK1.1⁺TCR-β⁻) in *Gata3^{fl/fl}* (white bars) or *Nkp46-Cre-Gata3^{fl/fl}* (black bars) mice. (C) Representative plots of CD27⁻CD11b⁻ (DN), CD27⁺CD11b⁻ (CD27 SP), CD27⁺CD11b⁺ (DP) and CD27⁻CD11b⁺ (CD11b SP) populations among NK cells (NK1.1⁺CD3⁻). (D) Percentages of DN, CD27 SP, DP and CD11b SP populations in *Gata3^{fl/fl}* (white bars) or *Nkp46-Cre-Gata3^{fl/fl}* (black bars) mice. For (A) and (B), data were pooled from at least 2 independent experiments, with five to nine mice per group. For (C) and (D), data are from one experiment representative of at least two independent experiments, with three mice per group. Data represent mean + standard deviation. *p < 0.05; **p < 0.01; ***p < 0.001.

To investigate the role of *Gata3* in the NK cell maturation process, we assessed the maturation of NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice. NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice displayed an immature phenotype, and this was characterized by an increase in double-negative (DN) and CD27 single-positive (CD27 SP) populations and a decrease in double-positive (DP) and CD11b single-positive (CD11b SP) subsets. This was shown in all three organs tested (Figure 2.2C, D). Therefore, GATA3 is important for the differentiation of NK cells from CD27 SP to DP.

2.4.3. GATA3 is required for the development of CD49a+ liver-resident NK cells

The newly identified CD49b- CD49a+ liver-resident NK cells are known to be generated from hepatic hematopoietic progenitors (288). Since a defect in the homing of NK cells into the liver has been identified in chimeric mice using *Gata3*-deficient fetal liver cells (72), the dramatic reduction of NK cells in the livers shown in the previous report might not be due to the lack of hepatic progenitor cells. Alternatively, GATA3 is required for the development of liver-resident NK cells from hepatic progenitor cells. To test this possibility, both cNK cells and liver-resident NK cells, which express CD49b and CD49a respectively, were analyzed in *NKp46-Cre-Gata3^{fl/fl}* mice. A reduction in the numbers of cNK cells was found in *NKp46-Cre-Gata3^{fl/fl}* mice, which can be explained by the inability of these cells to home into the liver (Figure 2.3A, B). Interestingly, the numbers of liver-resident NK cells were also reduced in *NKp46-Cre-Gata3^{fl/fl}* mice, indicating that GATA3 is required for the development of liver-resident NK cells. The impaired ability of cNK cells to migrate from the BM to the liver in combination with the defective development of liver-resident NK cells

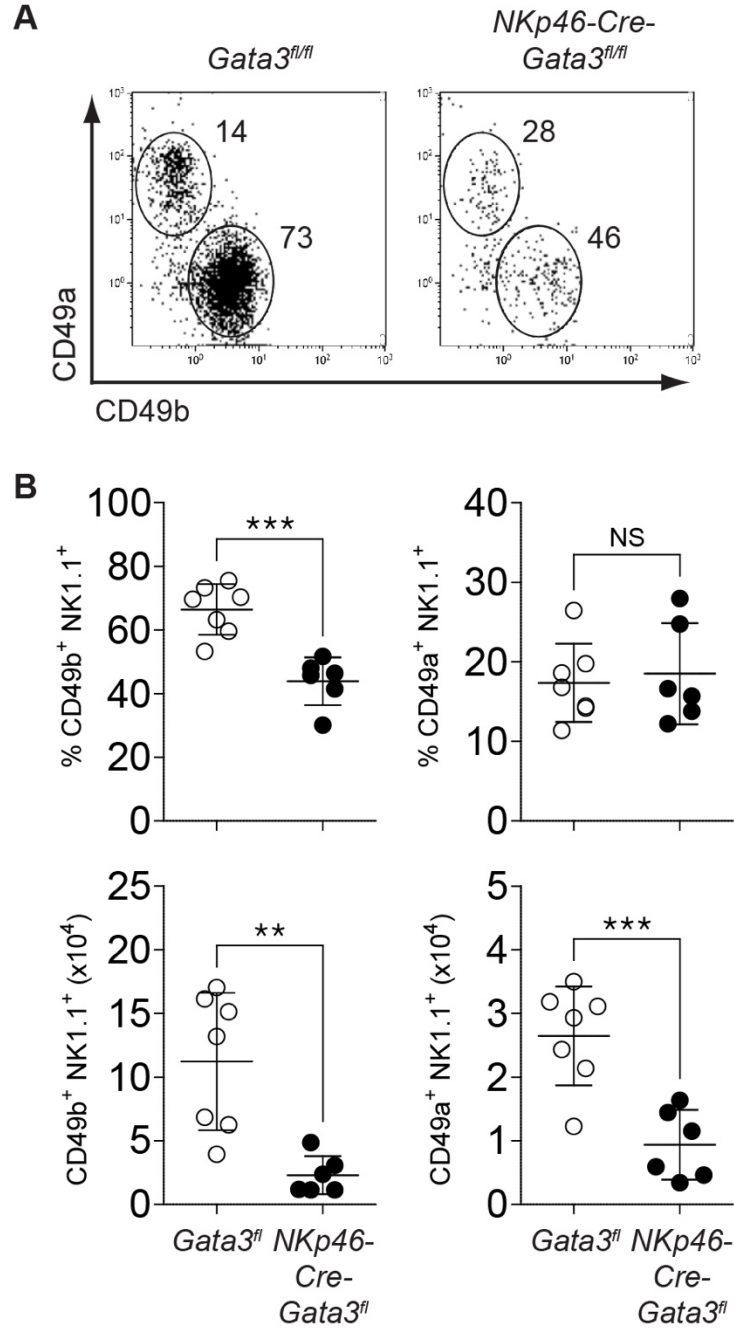


Figure 2.3. Numbers of liver-resident NK cells are reduced in *NKp46-Cre-Gata3^{fl/fl}* mice. (A) Representative plots of liver-resident NK cells (NK1.1⁺CD3⁻CD49b⁻CD49a⁺) at the steady state. (B) Percentages and numbers of cNK or liver-resident NK cells in *Gata3^{fl/fl}* (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice. For (A), data are from one experiment representative of two independent experiments. For (B), data were pooled from two independent experiments, with six to seven mice per group. Data represent mean + standard deviation. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

in the absence of GATA3 is consistent with the dramatic reduction of NK1.1+CD3- cell frequencies in the livers of *NKp46-Cre-Gata3^{fl/fl}* mice (Figure 2.2A, B).

2.4.4. GATA3 modulates the expression of transcription factors involved in NK cell development

The critical roles of GATA3 in the development of diverse immune cells suggest that GATA3 might function indirectly by positively or negatively modulating other transcription factors. To gain insight into the mechanism underlying the GATA3-governed regulation of transcription factors, we measured the mRNA level of transcription factors known to drive NK cell development. *Gata3*-deficient NK cells showed reduced expression of *Id2*, *T-bet*, *E4bp4* (Figure 2.4A). Down-modulation of these transcription factors could be in line with developmental defects observed in *NKp46-Cre-Gata3^{fl/fl}* mice. Notably, defects in NK cell maturation in *Id2*-deficient or *E4bp4*-deficient NK cell progenitor cells are the most severe and result in a maturation blockade occurring before NK1.1 acquisition in the BM (39, 40, 42), different from the accumulation of NK1.1+ immature NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice, suggesting GATA3 involvement in multiple stages of NK cell development. The NK cell developmental defect in *T-bet*-deficient mice showed increased numbers of NK cells in the BM and reduced peripheral NK cell numbers, similar to *NKp46-Cre-Gata3^{fl/fl}* mice (298). Paradoxically, mRNA expression of *Tox*, which is required for NK cell maturation, was found abundant in NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice (46). Taken together, this result suggests a complex interplay between GATA3 and these transcription factors during the development of NK cells.

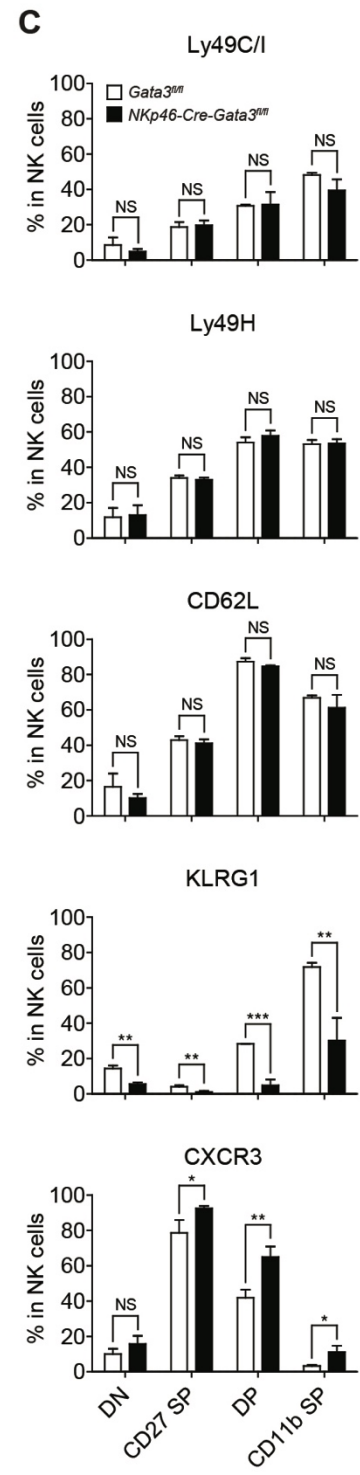
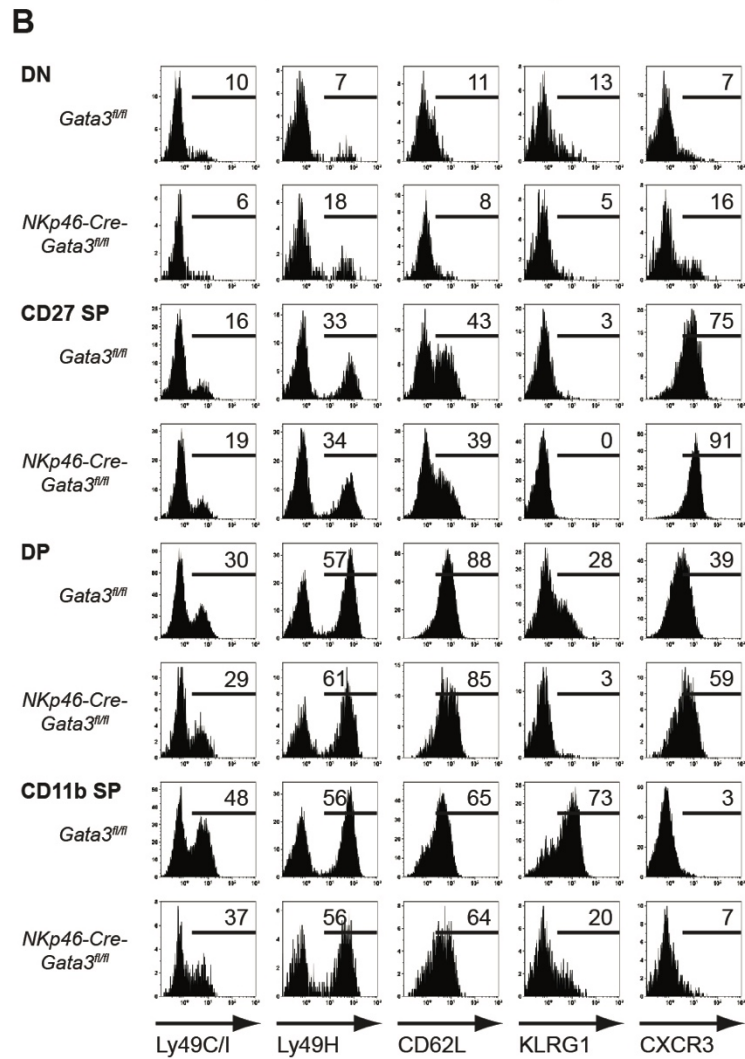
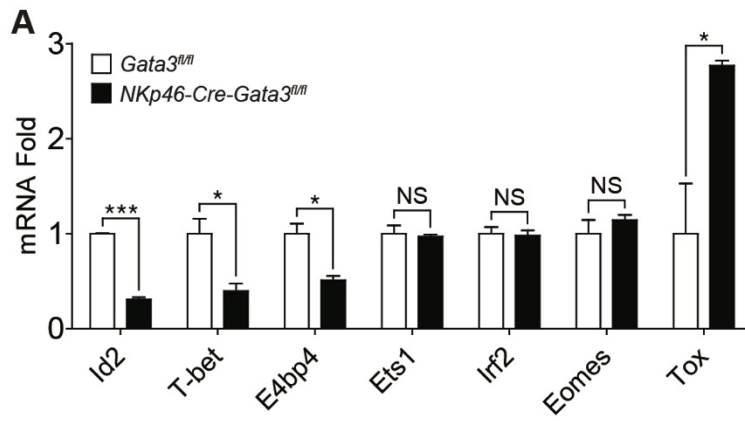


Figure 2.4. Transcript levels of several transcription factors and surface expression of Ly49 receptors and molecules involved in cell adhesion in NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice and control littermates. (A) Total RNA was extracted from splenic NK cells and the expression of several transcription factors was quantified by quantitative real-time PCR. Graph depicts relative mRNA levels in NK cells from *Gata3^{fl/fl}* (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice. (B and C) Flow cytometric analysis of several receptors on NK cells from *NKp46-Cre-Gata3^{fl/fl}* and *Gata3^{fl/fl}* mice comparing DN, CD27 SP, DP, and CD11b SP subsets. For (A), data are from one experiment representative of two independent experiments, with two replicates per group. For (B and C), data are from one experiment representative of two independent experiments, with three mice per group. Data represent mean + standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.4.5. The expression of molecules involved in cell adhesion is dictated by GATA3 on NK cells

To understand GATA3-mediated control in NK cell development, we decided to further characterize the expression of several surface receptors on NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice and their control littermates. In *NKp46-Cre-Gata3^{fl/fl}* mice, the expression of inhibitory and activating receptors was slightly reduced in the spleen and liver (Figure 2.5A, B). Surprisingly, *NKp46-Cre-Gata3^{fl/fl}* mice exerted dramatically perturbed expression of molecules involved in cell adhesion such as CD11b, and CD62L on NK cells (Figure 2.5A, C). Known to associate with CD18 to form an integrin (299), CD11b was poorly expressed on NK cells in all three compartments analyzed from *NKp46-Cre-Gata3^{fl/fl}* mice. The proportion of NK cells expressing L-selectin (CD62L) was also decreased in these mice. Interestingly, NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice showed increased expression of the chemokine receptor CXCR3. Although the role of CXCR3 on NK cell migration at the steady-state is unclear, it has been shown to be important for the recruitment of NK cells into tumors (300). Thus, the altered expression of molecules involved in cell adhesion may explain the defective ability of NK cells to exit the BM in *NKp46-Cre-Gata3^{fl/fl}* mice.

Because CD27+CD11b- immature NK cells are overrepresented in total NK cells, we decided to compare the phenotypes in subsets of distinct maturation stages based on the expression of CD27 and CD11b. The expression pattern of most surface receptors was similar in NK cell subsets of equivalent maturation stages when comparing *NKp46-Cre-Gata3^{fl/fl}* mice with their control littermates (Figure 2.4B, C), indicating that the differential expression can be explained by higher proportions of CD27+CD11b- immature NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice. In addition, the expression of the inhibitory receptor KLRG1 was almost

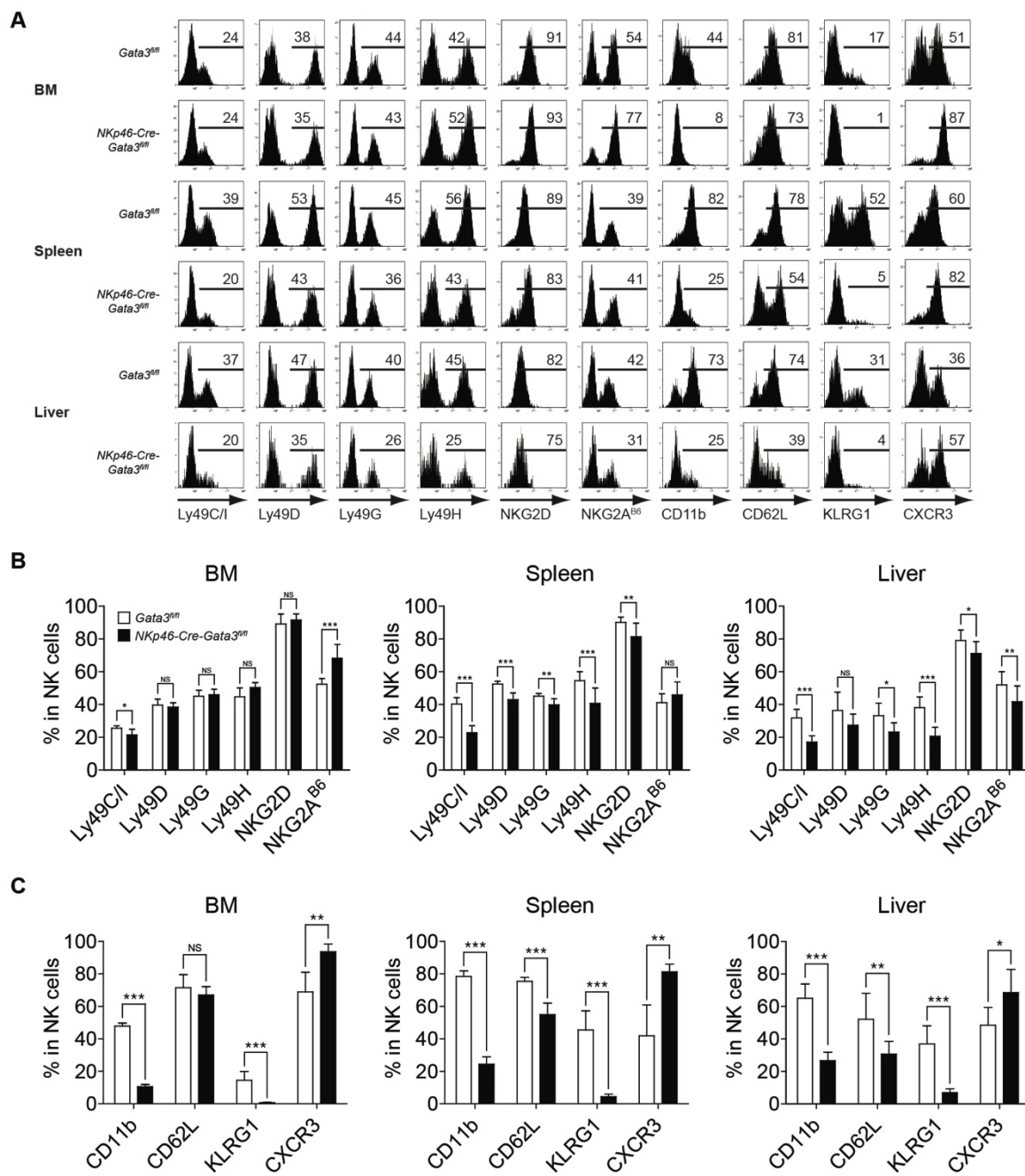


Figure 2.5. Surface expression of several activating and inhibitory receptors and molecules involved in cell adhesion are reduced on NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice. Flow cytometric analysis of several activating and inhibitory receptors and adhesion molecules on NK cells from *NKp46-Cre-Gata3^{fl/fl}* and *Gata3^{fl/fl}* mice. (A) Representative plots of surface receptors in bone marrow, spleens, and livers of *NKp46-Cre-Gata3^{fl/fl}* or *Gata3^{fl/fl}* mice. (B and C) Percentage of NK cells (NK1.1⁺CD3⁻ or NK1.1⁺TCR- β ⁻) expressing the indicated receptors in *Gata3^{fl/fl}* (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice. Data were pooled from at least two independent experiments, with five to twelve mice per group. Data represent mean + standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

completely abrogated on NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice (Figure 2.5A, C). However, the expression of KLRG1 was drastically different in DP and CD11b SP subsets, suggesting that KLRG1 expression is directly regulated by GATA3.

2.4.6. GATA3-deficient NK cells localized in the BM can be mobilized to the blood following treatment with CXCR4 antagonist

Among various chemokine receptors, the critical role of CXCR4 on the retention of NK cells in the BM was suggested (31). The expression of CXCR4 is decreased in mature NK cells making these cells less responsive to CXCL12, a CXCR4 ligand highly expressed in the BM environment (29, 31, 33, 301). Injection of mice with the CXCR4 antagonist AMD3100 resulted in the recruitment of immature NK cells from the BM to the blood (31). Given that NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice are immature and that these NK cells show a defect in egress from the BM to the periphery, we sought to determine whether the use of AMD3100 would allow NK cells to exit the BM in *NKp46-Cre-Gata3^{fl/fl}* mice. Mice were injected intraperitoneally with AMD3100 for one hour and were then injected intravenously with anti-CD45.2 monoclonal antibody two minutes before sacrifice in order to label sinusoidal lymphocytes. PBMCs and BM were analyzed. In control littermates, the proportion of NK cells in BM sinusoids was not altered following the injection of AMD3100 (Figure 2.6A, B). However, NK cell frequencies within BM sinusoids were increased in AMD3100-treated *NKp46-Cre-Gata3^{fl/fl}* mice. Similarly, the fold change of the absolute NK cell number in BM sinusoids upon AMD3100 treatment was heightened in *NKp46-Cre-Gata3^{fl/fl}* mice (Figure 2.6C). This was associated with increased number of NK cells in the blood (Figure 2.6D), even though the effect was not as dramatic as in BM sinusoids. As shown previously (31), the immature CD27 SP NK cells were selectively recruited to the BM sinusoids and the blood

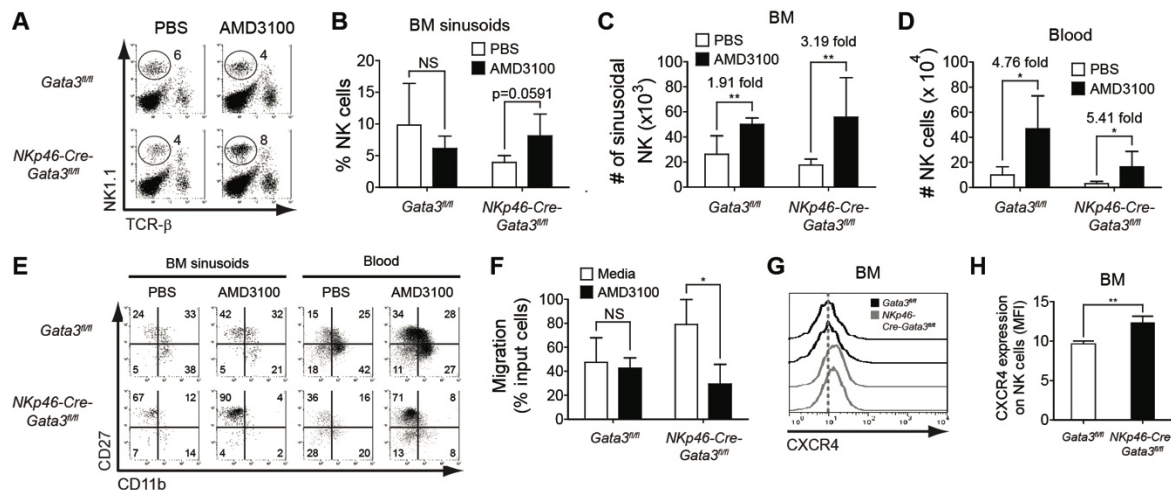


Figure 2.6. NK cells from *NKp46-Cre-Gata3*^{fl/fl} mice can be recruited to the blood upon blockade of CXCR4. Flow cytometric analysis of BM and blood NK cells in *NKp46-Cre-Gata3*^{fl/fl} or *Gata3*^{fl/fl} mice treated for 1 hour with PBS (control) or AMD3100 and injected intravenously with biotin-conjugated anti-CD45 mAb for the final 2 minutes. (A) Representative plot of NK cell frequencies among CD45⁺ cells in the bone marrow. (B) Percentage of NK cells among CD45⁺ cells in the bone marrow, (C) Number of sinusoidal NK cells (CD45⁺ NK cells) in the bone marrow, (D) Number of NK cells in the peripheral blood of PBS-treated (white bars) or AMD3100-treated (black bars) mice were measured. (E) Representative plots of CD27 and CD11b expression in gated CD45⁺ (sinusoidal) NK cells from BM. (F) NK cell migration in response to CXCL12 was assessed following BM pretreatment with or without AMD3100. (G and H) MFI of CXCR4 expression of BM NK cells. For (A-D), data were pooled from three independent experiments, with two to three mice per group. For (F), data are from one experiment representative of two independent experiments, with four replicates per group. For (G and H), data are from one experiment representative of two independent experiments, with three mice per group. Data represent mean + standard deviation. **p* < 0.05; ***p* < 0.01.

upon CXCR4 antagonist AMD3100 treatment (Figure 2.6E), suggesting that antagonizing CXCR4 rescues the defective egress of immature *Gata3*-deficient NK cells from the BM.

The ability of BM NK cells to migrate in response to the CXCR4 ligand, CXCL12, was assessed *in vitro* using the transwell migration assay. Pretreatment with AMD3100 reduced the chemotaxis of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice but did not affect the migration of NK cells from control littermates (Figure 2.6F), suggesting that chemotaxis to CXCL12 is responsible for the retention of immature NK cells of *NKp46-Cre-Gata3^{fl/fl}* mice in the BM. Notably, NK cells in the BM of *NKp46-Cre-Gata3^{fl/fl}* mice showed higher expression of CXCR4 (Figure 2.6G, H). Taken together, GATA3 is required for the CXCR4 downmodulation associated with mature NK cell phenotype and CXCR4-mediated retention is a major mechanism for the abundant NK cell proportion in the BM of *NKp46-Cre-Gata3^{fl/fl}* mice.

2.4.7. The requirement of GATA3 for IFN- γ -production by NK cells

It was previously shown that in the absence of GATA3, NK cells are defective in producing IFN- γ when stimulated through the surface receptor CD11b or using IL-12 and IL-18 (72). The defective IFN- γ production might be due to the reduced expression of surface CD11b or cytokine receptors. In addition, the ability of *Gata3*-deficient NK cells to produce IFN- γ has not been thoroughly shown via stimulation of NK activating receptors. Thus, we tested whether cross-linking of the activating receptors NK1.1 and Ly49H would result in reduced production of IFN- γ in the absence of GATA3. The expression of surface NK1.1 was comparable between *NKp46-Cre-Gata3^{fl/fl}* and control mice. *In vitro* stimulation through NK1.1 resulted in similar frequencies of NK cells producing IFN- γ for both *NKp46-Cre-Gata3^{fl/fl}* and control mice (Figure 2.7A, B), indicating that stimulation through an activating

receptor can lead to normal cytokine production by NK cells in the absence of GATA3. When stimulated through Ly49H, slightly less NK cells were able to produce IFN- γ in the absence of GATA3 but this might be explained by the moderate reduction of Ly49H⁺ NK cell proportions in *NKp46-Cre-Gata3^{fl/fl}* mice (Figure 2.5A, B). The role of GATA3 on IFN- γ production was also investigated upon cytokine stimulation. When NK cells were stimulated with different combinations of cytokines such as IL-2, IL-12, and IL-18, *Gata3*-deficient splenic NK cells exhibited a reduced ability to produce IFN- γ . NK cell degranulation evaluated by the LAMP-1 expression was largely comparable between NK cells from *NKp46-Cre-Gata3^{fl/fl}* and control mice. Taken together, GATA3 is largely dispensable for induction of effector functions in response to stimulation of activating receptors, but is required for efficient IFN- γ production in response to cytokines.

2.4.8. NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice expand robustly during MCMV infection

During murine cytomegalovirus (MCMV) infection, cytokine levels in the serum peak at day 1.5 whereas the proportion of proliferating NK cells reaches its peak at day 3 (99, 208). To determine whether the defect in cytokine production observed *in vitro* can also be seen *in vivo*, we measured the production of IFN- γ by NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice upon challenge with MCMV. Following infection with MCMV, increased NK cell frequencies were found in the livers, presumably due to cell migration to the liver (302). Fewer NK cells in the spleens and livers of *NKp46-Cre-Gata3^{fl/fl}* mice produced IFN- γ *in vivo* at day 1.5 post MCMV infection (Figure 2.8A, B). IFN- γ -production at day 1.5 of MCMV infection is largely mediated by IL-12 (303, 304). However, IFN- γ and IL-12 levels in the serum of

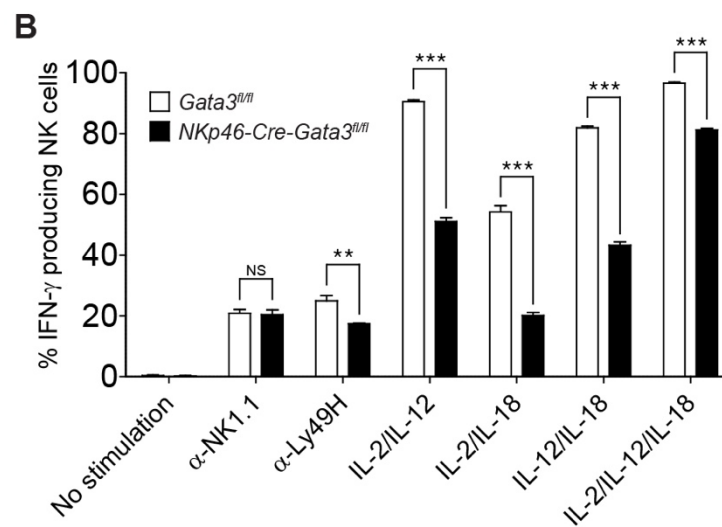
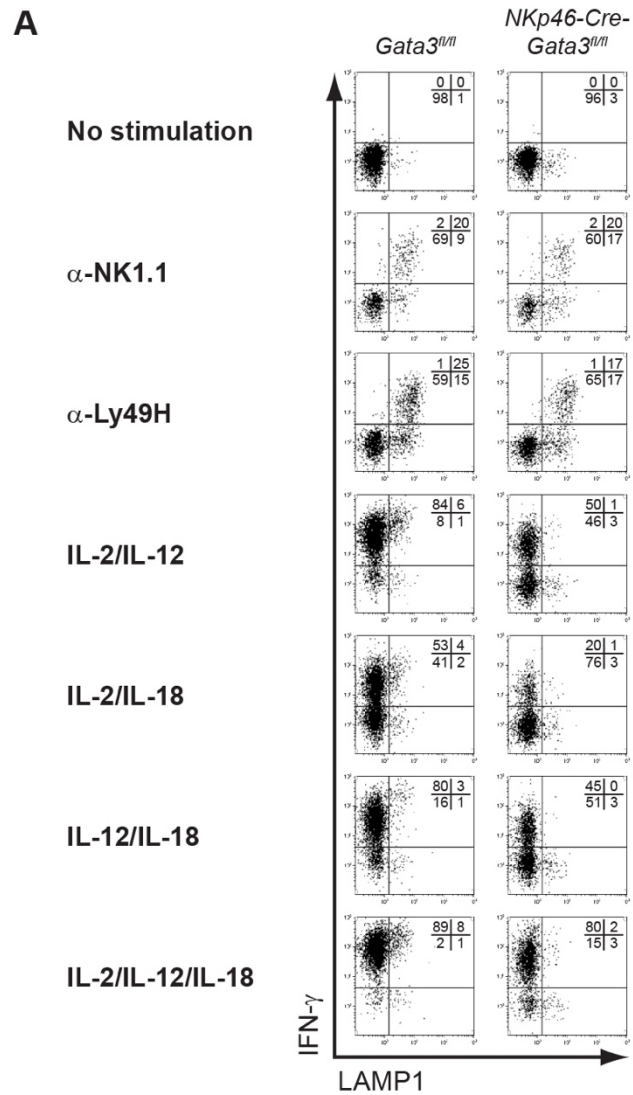


Figure 2.7. IFN- γ production of splenic NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice. IFN- γ production is measured in NK cells from *NKp46-Cre-Gata3^{fl/fl}* and *Gata3^{fl/fl}* mice following cytokine stimulation or stimulation of activating receptors. (A) Representative plots of intracellular IFN- γ in NK cells from *NKp46-Cre-Gata3^{fl/fl}* or *Gata3^{fl/fl}* mice upon stimulation with the indicated antibodies or cytokines. (B) Percentage of IFN- γ -producing NK cells (NK1.1⁺TCR- β ⁻) from control (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice upon stimulation with the indicated antibodies or cytokines. Data are from one experiment representative of two independent experiments, with three replicates per group. Data represent mean + standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

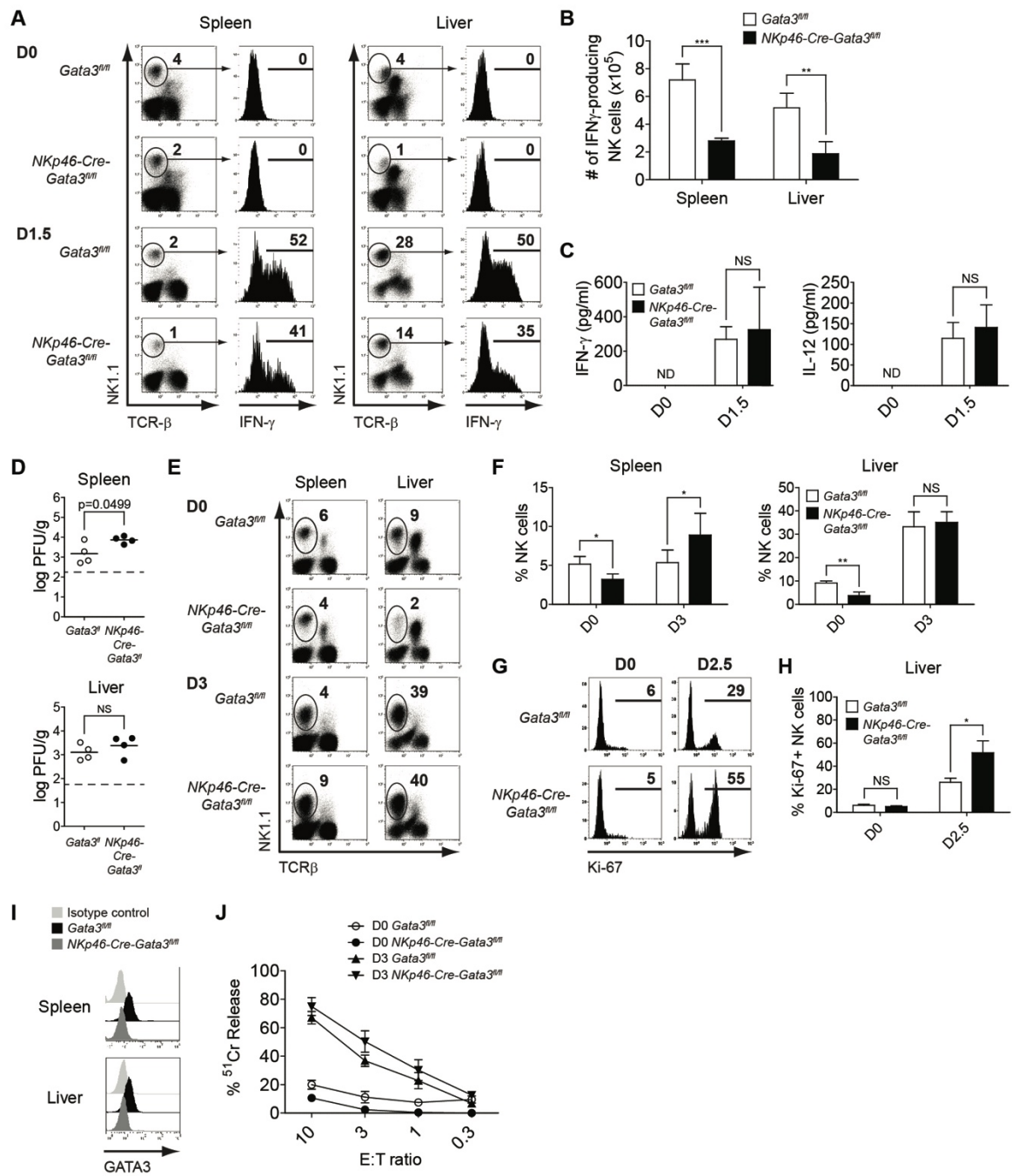


Figure 2.8. Upon MCMV infection, NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice expand robustly. *NKp46-Cre-Gata3^{fl/fl}* or *Gata3^{fl/fl}* mice were either uninfected or given 5,000 PFU MCMV i.p. and sacrificed on day 1.5, day 2.5 or day 3 post-infection for the following analyses: (A) NK cells from spleens and livers of naïve (D0) or MCMV-infected mice (D1.5) were stained for IFN- γ production. (B) Number of NK cells producing IFN- γ in spleen and liver of *Gata3^{fl/fl}* (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice. (C) Serum levels of IFN- γ and IL-12 in indicated groups at D0 and D1.5 post-infection. (D) Viral burden in spleen and liver of *Gata3^{fl/fl}* (white circles) or *NKp46-Cre-Gata3^{fl/fl}* (black circles) mice at D3 post-infection. (E) Representative plots of NK cell frequencies (NK1.1⁺TCR- β ⁻) in spleens and livers of naïve (D0) or MCMV-infected (D3) mice. (F) Percentage of NK cells (NK1.1⁺TCR- β ⁻) in *Gata3^{fl/fl}* (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice at D0 and D3 post-infection. (G and H) Percentage of Ki-67⁺ NK cells in the liver of naïve and infected mice at D2.5. (I) GATA3 expression in NK cells from *NKp46-Cre-Gata3^{fl/fl}* or *Gata3^{fl/fl}* mice at D3 post-infection. (J) The cytotoxicity of NK cells of *NKp46-Cre-Gata3^{fl/fl}* or *Gata3^{fl/fl}* mice against YAC-1 target cells is depicted. For (A-E) and (G-J), data are from one experiment representative of two independent experiments, with two to five mice per group. For (F), data were pooled from two independent experiments, with three to nine mice per group. Data represent mean + standard deviation. For (D), horizontal lines indicate the mean. ND; not detected. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

NKp46-Cre-Gata3^{fl/fl} mice were similar to their control littermates (Figure 2.8C). When we compared IFN- γ expression in subsets of distinct maturation stages, CD27 SP and DP NK cells were the most efficient at producing IFN- γ (Figure 2.9A, B).

We next investigated whether this defect in IFN- γ production in combination with the reduced NK cell frequencies in the periphery would render the *NKp46-Cre-Gata3^{fl/fl}* mice highly susceptible to viral infection. Interestingly, we observed only a slightly higher viral burden in the spleens and similar viral burden in the livers of *NKp46-Cre-Gata3^{fl/fl}* mice at day 3 following infection (Figure 2.8D). In order to explain this unexpected resistance in *NKp46-Cre-Gata3^{fl/fl}* mice, we analyzed the NK cell populations in these mice. Surprisingly, despite their reduced numbers in the spleen and liver at the steady-state, following MCMV infection, NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice were able to expand to similar frequencies as NK cells from control littermates (Figure 2.8E, F). In particular, the proportions of NK cells in the liver were greatly increased and were associated with increased proliferation of liver NK cells at day 2.5 post-infection based on Ki-67 staining (Figure 2.8G, H). CD27 SP and DP NK cells showed the highest levels of proliferation (Figure 2.9E, F). When we compared Ki-67 expression in subsets of distinct maturation stages, the expression was similar in NK cell subsets of equivalent maturation stages, indicating that the higher proportion of immature NK cells contained in total NK cells of *NKp46-Cre-Gata3^{fl/fl}* mice explain the increased Ki-67 expression. To exclude the possibility that these expanded cells originate from residual GATA3⁺ NK cells in which the Cre recombination failed to occur, we measured GATA3 expression in the expanded NK cells from day 3 post MCMV infection. The expanded NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice did not express GATA3 (Figure 2.8I). Not only were NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice able to expand during MCMV infection, they were also able to execute their cytotoxic functions as shown by killing assay (Figure 2.8J).

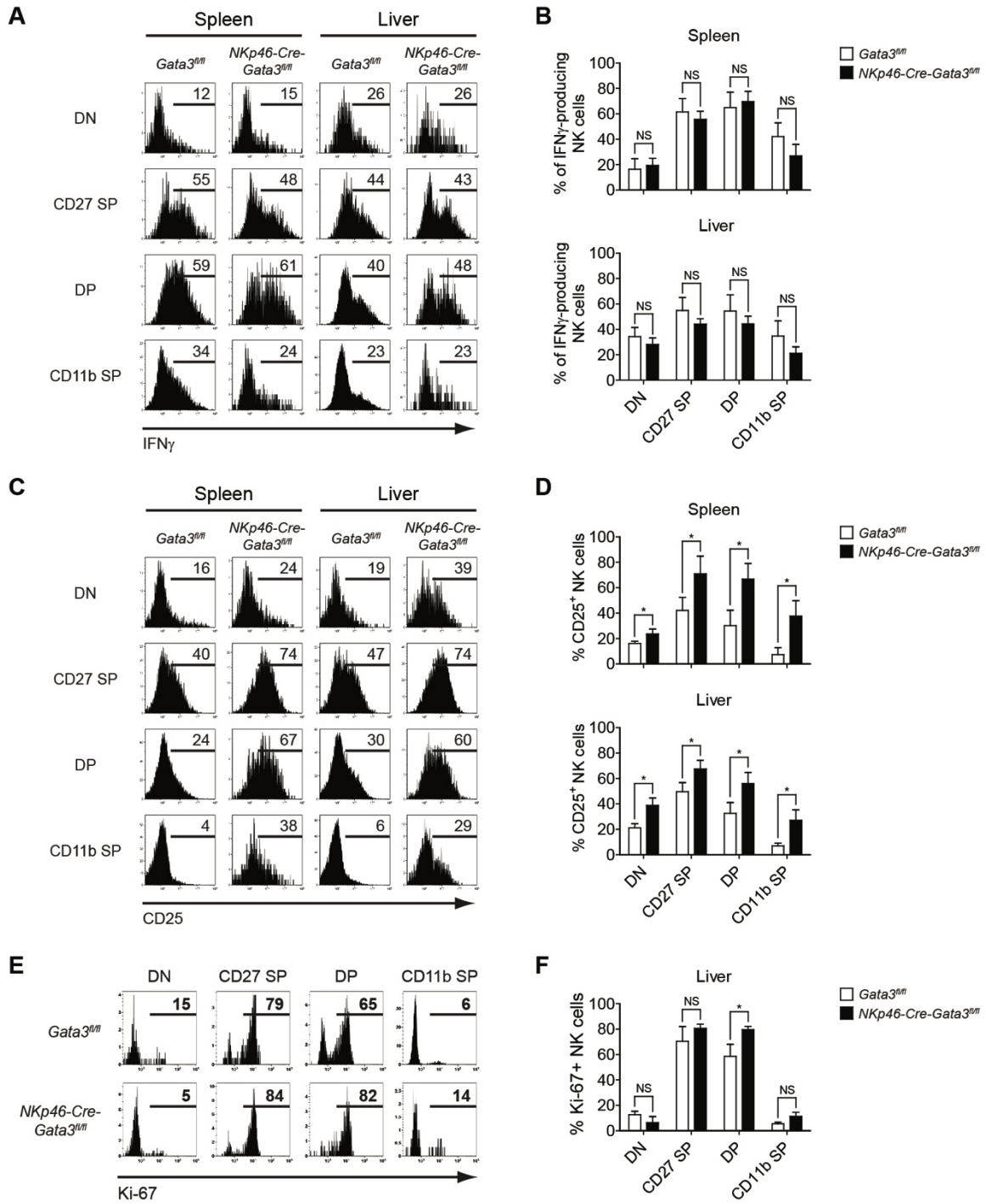


Figure 2.9. IFN- γ production and CD25 expression in NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice upon MCMV infection. *NKp46-Cre-Gata3^{fl/fl}* or *Gata3^{fl/fl}* mice were either uninfected or given 5,000 PFU MCMV i.p. and sacrificed for the following analyses: NK cell subsets from naïve or MCMV-infected mice were stained for (A and B) IFN- γ production and (C and D) CD25 expression at day 1.5 post MCMV infection. (E and F) Representative plots of Ki-67 expression in different subsets of liver NK cells at day 2.5 post MCMV infection. Data are from one experiment representative of two independent experiments, with three mice per group. Data represent mean + standard deviation. *p < 0.05.

2.4.9. The enhanced proliferation of NK cells from *NKp46-Cre-Gata3^{fl/fl}*

mice is cell-intrinsic

In order to be able to bind to IL-2 with high affinity and therefore to respond to low levels of physiological IL-2 concentrations *in vivo*, NK cells need to express IL-2R α (CD25) to complete the heterotrimeric high-affinity IL-2 receptor composed of IL-2R α , β and the common γ chain (305). During MCMV infection, IL-12 and IL-18 can induce the upregulation of CD25 (147). Following MCMV infection, NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice expressed higher levels of CD25 than NK cells from control mice (Figure 2.10A, B, and Figure 2.9C, D) which could explain the enhanced proliferation of these cells. To exclude the effect of differential inflammation during MCMV infection, we cultured naïve splenic NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice and control littermates in the presence of IL-12 and IL-18 to measure the upregulation of CD25 *in vitro*. NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice were more efficient at upregulating CD25 as compared with NK cells from control littermates (Figure 2.10C, D), and this was not due to increased levels of IL-12 in the serum (Figure 2.8C). Interestingly, the enhanced CD25 expression was observed in spite of reduced IL-18R α expression on GATA3-deficient NK cells (Figure 2.10E, F). IL-12R β 2 expression was similar between the *NKp46-Cre-Gata3^{fl/fl}* and control mice. CD27 SP and DP NK cells populations were the most efficient at upregulating CD25 upon stimulation with IL-12 and IL-18 respectively (Figure 2.11A, B). Similar to the Ki-67 expression, CD25 expression in all subsets in both *NKp46-Cre-Gata3^{fl/fl}* and control littermates was equivalent, indicating that the higher proportion of immature NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice explain the increased CD25 expression. The reduced expression of IL-18R α might explain the decreased CD25 expression

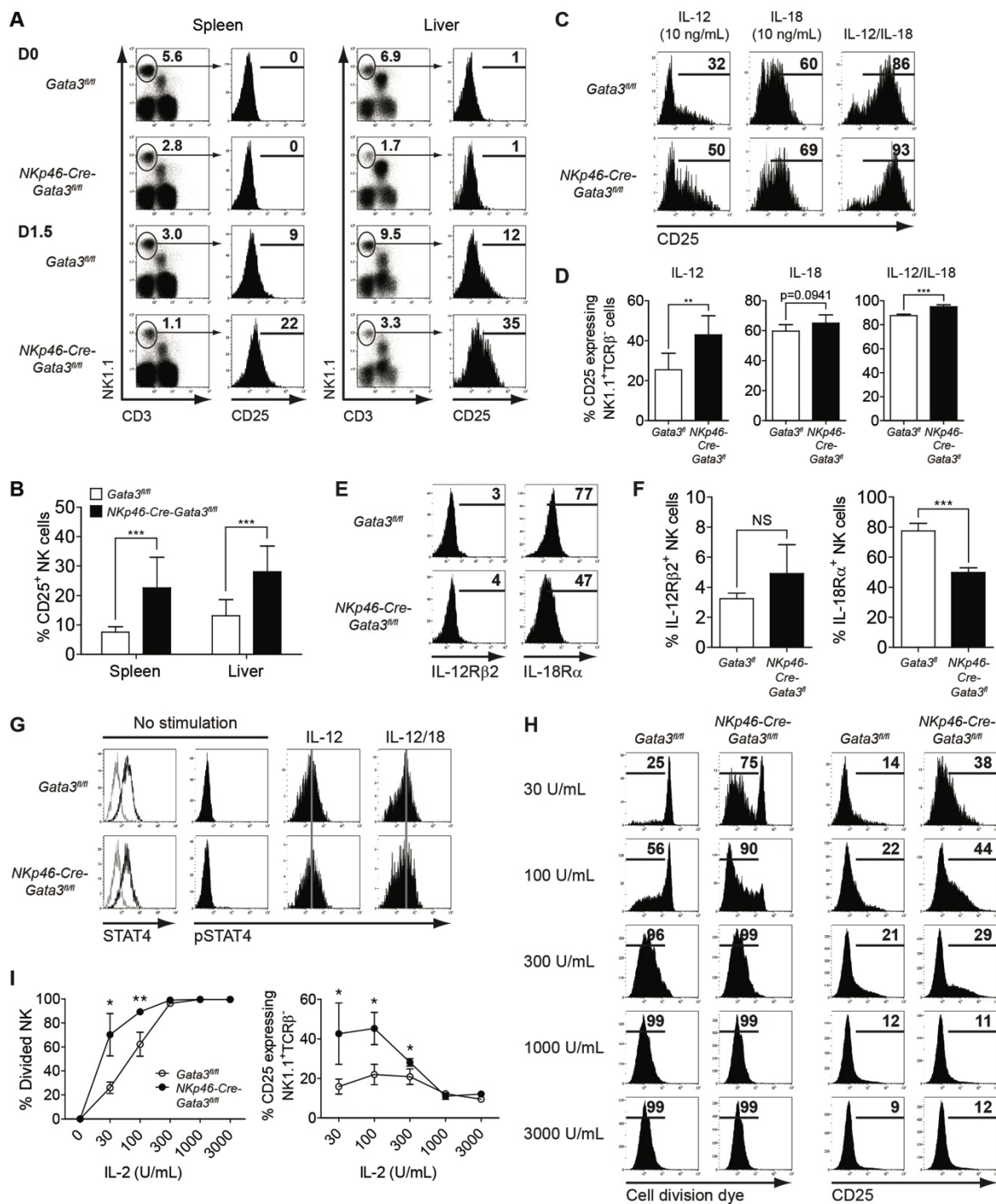


Figure 2.10. NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice express higher levels of CD25 upon activation and show enhanced proliferation upon stimulation with low doses of IL-2 *in vitro*. (A and B) *NKp46-Cre-Gata3^{fl/fl}* or *Gata3^{fl/fl}* mice were either uninfected or given 5,000 PFU MCMV i.p. and sacrificed on day 1.5 post-infection. NK cells from spleens and livers of naïve (D0) or MCMV-infected mice (D1.5) were surface stained for CD25. (C) Representative plots of CD25 expression in NK cells following stimulation with IL-12 and/or IL-18 for 24 hours. (D) Percentage of CD25-expressing NK cells from *Gata3^{fl/fl}* (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice when stimulated with IL-12 and/or IL-18. (E) Representative plots of IL12R β 2 and IL-18R α expression on unstimulated NK cells. (F) Percentage of IL12R β 2- and IL-18R α -expressing unstimulated NK cells from *Gata3^{fl/fl}* (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice. (G) Total STAT4 and pSTAT4 expression in unstimulated or IL-12- and IL-18-stimulated NK cells from *Gata3^{fl/fl}* or *NKp46-Cre-Gata3^{fl/fl}* mice. (H and I) Enriched NK cells from spleens of *NKp46-Cre-Gata3^{fl/fl}* or *Gata3^{fl/fl}* littermates were labeled with a cell division dye and stimulated with IL-2. (H) Histograms depict the proliferation and CD25 expression of NK cells following 5 days of culture with the indicated concentrations of IL-2. (I) Percentage of divided NK cells and CD25-expressing NK cells when cultured for 5 days with the indicated concentrations of IL-2. For (A, C, E, G, H, and I), data are from one experiment representative of two independent experiments, with three replicates per group for (I). For (B, D, F), data were pooled from two independent experiments, with six to nine mice per group for (B) and (F), and with six replicates per group for (D). Data represent mean + standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

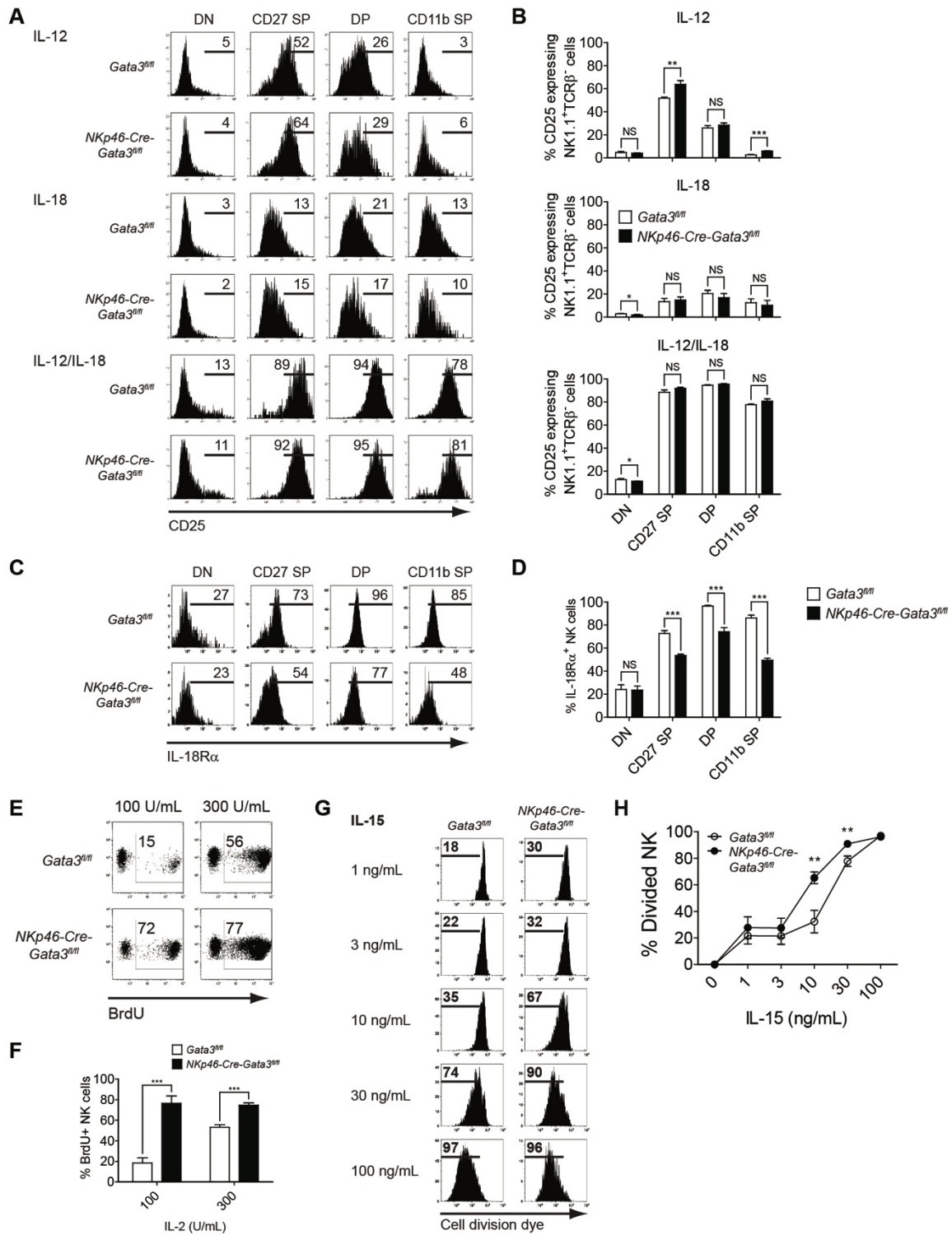


Figure 2.11. CD27 SP and DP NK cells subsets show efficient upregulation of CD25 upon stimulation with IL-12 and IL-18, while NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice show enhanced proliferation upon stimulation with either IL-2 or IL-15. (A and B) CD25 expression on different subsets of NK cells following stimulation with IL-12 and/or IL-18 for 24 hours. (C and D) IL-18R α expression on different subsets of NK cells. (E and F) BrdU incorporation *in vitro* upon culture with low doses of IL-2. (G and H) Histograms depict the proliferation of NK cells following 5 days of culture with the indicated concentrations of IL-15. For (B, F, and H), data are from one experiment representative of two independent experiments, with triplicates per group. For (D), data are from one experiment representative of two independent experiments, with three mice per group. Data represent mean + standard deviation. *p < 0.05; **p < 0.01; ***p < 0.001.

upon IL-18 stimulation on NK cells of *NKp46-Cre-Gata3^{fl/fl}* mice (Figure 2.11C, D).

STAT4 is required for CD25 induction upon IL-12 stimulation during MCMV infection (147). Expression of total STAT4 was similar between the two groups (Figure 2.10G), and stimulation with IL-12 and IL-18 led to similar phosphorylation levels of STAT4 in NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice. Because NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice expand robustly during MCMV infection *in vivo*, we next sought to determine whether naïve splenic NK cells are more responsive than control NK cells when stimulated with IL-2 *in vitro*. NK cells from *NKp46-Cre-Gata3^{fl/fl}* showed enhanced proliferation upon stimulation with low doses of IL-2 (Figure 2.10H, I and Figure 2.11E, F). It could be explained by the increased upregulation of CD25 on NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice compared with those from control littermates. Indeed, enriched NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice displayed higher expression of CD25 when cultured for 5 days at low doses of IL-2 (Figure 2.10H, I). Therefore, splenic NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice exhibited intrinsically enhanced CD25 expression and proliferative ability. Similar hyper-responsiveness of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice was observed in response to IL-15 (Figure 2.11G, H).

During MCMV infection, the increased proliferation of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice may result from higher viral load and/or higher inflammation compared with control littermates. Therefore, to investigate whether the enhanced proliferative potential of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice is cell-intrinsic, we adoptively transferred equal numbers of Ly49H⁺ NK cells from either control or *NKp46-Cre-Gata3^{fl/fl}* (CD45.2⁺) spleen into Ly49H-deficient (CD45.1⁺) recipient mice. The recipient mice were either infected with MCMV one day later or left uninfected before being sacrificed 5 days post-infection (Figure 2.12A). In both spleen and liver, we observed greater expansion of donor NK cells when these

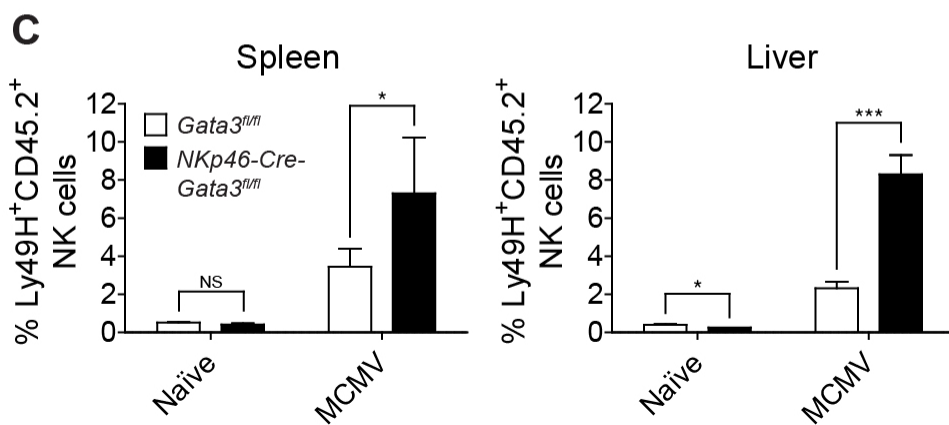
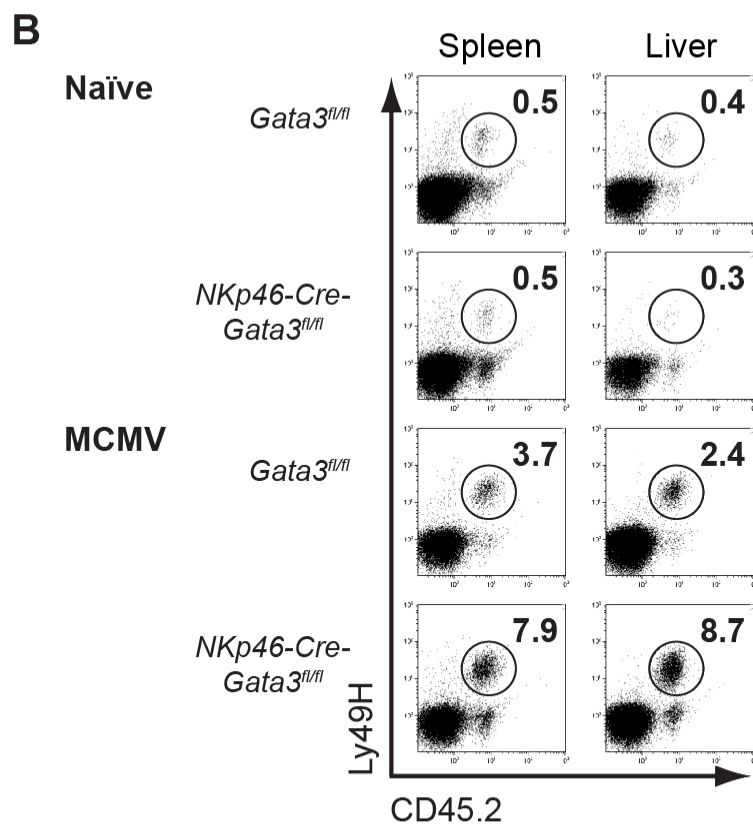
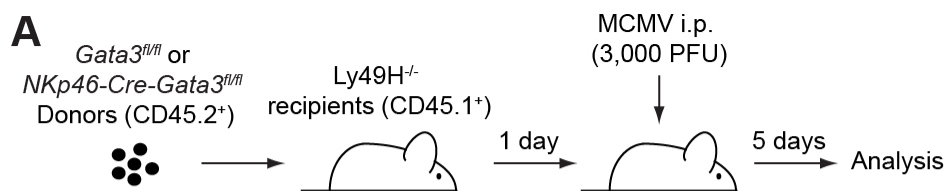


Figure 2.12. NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice show enhanced expansion in a cell-intrinsic manner. (A) Diagram of chimera generation and experimental design. Spleen cells from *Gata3^{fl/fl}* or *NKp46-Cre-Gata3^{fl/fl}* mice (CD45.2⁺) were isolated and transferred into Ly49H^{-/-} recipients (CD45.1⁺). Ly49H^{-/-} were either uninfected or given 3,000 PFU MCMV i.p. one day following adoptive transfer. Leukocytes from spleen and liver of recipient mice were harvested on day 5 post-infection. (B) Representative plots of transferred cells (Ly49H⁺CD45.2⁺) in spleen and liver of recipient mice at D5, gated on DX5⁺TCRβ⁻ cells. (C) Percentage of transferred cells from *Gata3^{fl/fl}* (white bars) or *NKp46-Cre-Gata3^{fl/fl}* (black bars) mice in spleens and livers of recipient mice at D5. Data are from one experiment representative of two independent experiments, with two to four mice per group. Data represent mean + standard deviation. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

cells originated from *NKp46-Cre-Gata3^{fl/fl}* mice (Figure 2.12B, C), indicating that NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice show an intrinsically enhanced proliferative capability.

2.5. Discussion

In the study, we investigated the NKp46-Cre driven *Gata3*-deficient mice. Compared to *Vav-Cre-Gata3^{fl/fl}* mice for which *Gata3*-deficiency is induced in all hematopoietic cells at the early stage of development (292), *NKp46-Cre-Gata3^{fl/fl}* mice provide a mouse model that allows us to study the roles of GATA3 at a late stage of NK development in a cell-intrinsic manner. We identified a critical role of GATA3 in the NK cell maturation stage associated with seeding of peripheral organs, highlighted by increased numbers of NK cells in the BM and decreased numbers in the peripheral organs of *NKp46-Cre-Gata3^{fl/fl}* mice. Interestingly, intact frequencies of NK1.1+ cNK cells were found in the spleens of *Vav-Cre-Gata3^{fl/fl}* mice, suggesting that *Gata3*-deficiency at the early stage of NK cell development might allow an alternative pathway of BM egress (292). Otherwise, the developmental defect in other immune cells in the absence of GATA3 might alleviate the defect in NK cell maturation.

Liver-resident NK cells are different from cNK but share many characteristics with ILC1s as they express NK1.1, NKp46, and CD49a, and require T-bet for their development (43, 288, 289, 306, 307). In contrast to its dispensable role in the development of cNK precursor cells, GATA3 is important in the development of multiple ILCs. GATA3 was initially identified to be essential in ILC2 maintenance in mice and humans (71, 308, 309). More recently, GATA3 was shown to be essential for the generation of the common progenitor cells for IL-7R α -expressing ILCs. In the studies using *Vav-Cre-Gata3^{fl/fl}* mice or chimeric mice generated with *Gata3*-deficient fetal liver hematopoietic precursor cells, several ILC subsets including ILC2s and ILC3s were severely affected in the absence of GATA3 during their development (292, 310). A higher proportion of liver-resident NK cells were present in *Vav-Cre-Gata3^{fl/fl}* mice compared to controls, however the absolute numbers of those cells were

not provided (289). Therefore, our data showing reduced numbers of liver-resident NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice is rather surprising. It is possible that deletion of other immune cells due to the early deletion of *Gata3* might provide an advantageous environment for the generation of liver-resident NK cells. Similarly, the frequencies of ILC1s were found decreased in the absence of GATA3 (307).

NK cells developing in the absence of GATA3 showed a defect in exiting the BM at the steady-state. NK cells displayed an immature phenotype characterized by an accumulation of the CD27⁺CD11b⁻ subset in the BM, spleens, and livers, demonstrating that GATA3 is required for the NK cell maturation beyond the CD27 single-positive stage. More recently, *Ikzf3* was reported to be required for the maturation in the spleen of CD27-CD11b⁺ NK cells, suggesting that IKZF3 regulates the maturation of NK cells subsequent to GATA3 (311). Interestingly, the immature NK cells exhibited lower expression of adhesion molecules such as CD11b and CD62L. It has been shown that NK cells that enter the BM sinusoids are mainly mature NK cells, which express CD11b (31). During development, the BM egress of NK cells is mainly regulated by two independent events, CXCR4 desensitization and the sphingosine-1-phosphate receptor 5 (S1P5) engagement (31). The chemokine receptor CXCR4 was shown to retain immature NK cells in the BM, and CXCR4 expression is negatively correlated with NK cell maturation. Here, we tested the ability of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice to exit the BM by using AMD3100, a CXCR4 antagonist. Treatment of *NKp46-Cre-Gata3^{fl/fl}* with AMD3100 resulted in the egress of the immature CD27⁺ SP NK cells from the BM to the blood. Moreover, our transwell migration assay indicated that NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice exhibit reduced chemotaxis in response to CXCL12 upon AMD3100 treatment. Therefore, sustained CXCR4 expression on NK cells in the BM of *NKp46-Cre-Gata3^{fl/fl}* mice blocks the migration of NK from the BM to

the periphery. Antagonizing CXCR4 by AMD3100 could be used in clinical settings for patients who carry genetic defects that lead to low numbers of peripheral NK cells due to their incomplete maturation.

The migratory defects observed in NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice are similar to those in *S1P5*-deficient, *Prmd1*-deficient, and *T-bet*-deficient mice (36, 312, 313), showing higher proportions of NK cells in the BM and reduced proportions of NK cells in the periphery. T-bet acts upstream of Blimp1 in mouse NK cells (313), and T-bet expression is regulated by GATA3 (72) (Figure 2.4A). More importantly, S1P5 is a direct target of T-bet and its expression is significantly reduced on NK cells from *T-bet*-deficient mice (312). Thus, the reduced expression of *T-bet* observed in *NKp46-Cre-Gata3^{fl/fl}* mice might be responsible for the migratory defect of NK cells. Similarly, Foxo1, a negative regulator in the late-stage of NK cell maturation, represses T-bet (314). Unfortunately, we could not evaluate the expression of S1P5 on NK cells due to the lack of available good antibodies.

Our data indicated that NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice exhibit defective IFN- γ production when stimulated by cytokines, consistent with a previous report (72). At least, the defective IFN- γ production in response to IL-12 is not likely explained by the levels of cytokine receptor expression since the expression of the inducible IL-12R β 2 chain is comparable (315). The defect seems to be narrowed to the response to cytokines, since stimulation through activating receptors such as NK1.1 or Ly49H showed comparable IFN- γ production with control littermates. In addition, the cytotoxic function of NK cells from MCMV-infected *NKp46-Cre-Gata3^{fl/fl}* mice was intact, suggesting that stimulatory signals through activating receptors on NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice are largely intact and capable of inducing cytokine production and cytotoxicity.

In spite of having immature NK cells with reduced frequencies in the peripheral organs, *NKp46-Cre-Gata3^{fl/fl}* mice controlled the virus to a similar extent as their control littermates on day 3 post-infection. Remarkably, the frequencies of NK cells in spleens and livers of *NKp46-Cre-Gata3^{fl/fl}* mice reached similar numbers to NK cells from control littermates. The robust NK cell expansion can be explained by the enhanced proliferative capability of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice. The increased proliferative capability was cell-intrinsic as NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice divided more robustly in response to IL-2 or IL-15 than NK cells from control mice *in vitro*. Adoptive transfer experiments supported that the heightened proliferative ability of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice was cell-intrinsic. Finally, the enhanced proliferation of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice was associated with increased CD25 expression, which allowed NK cells to efficiently respond to low doses of IL-2. Similar to the aforementioned defective IFN- γ response to IL-12, the increased expression of CD25 is not explained by dysregulated IL-12 and/or IL-18 cytokine receptors. In spite of the reduced IL-18 receptor expression, IL-18 stimulation induced comparable levels of CD25 expression. Moreover, higher CD25 induction was observed on NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice while the level of STAT4, which is a critical transcription factor for inducing CD25 upon IL-12, was comparable, and STAT4 was phosphorylated at a similar degree upon IL-12/IL-18 stimulation. There have been indications that immature NK cells exhibit great proliferative capacity regardless of stimulation. In the BM, DX5⁺CD27⁺CD11b⁻ NK cells actively proliferate under steady-state conditions (19, 316). During MCMV infection, CD11b^{low} NK cells proliferate more than CD11b^{high} NK cells at day 2 post-infection (316). In addition, CD11b⁻ NK cells show enhanced proliferation as compared to CD11b⁺ NK cells when stimulated *in vitro* with IL-15 (317). Similarly, NK cells from *Ets1*-deficient mice showed reduced maturation and were hyperresponsive to IL-15 (45). Notably, a study also

demonstrated cell-specific gene regulation by *Gata3* by revealing a list of genes that are differentially regulated in ILC2 and Th2 cells (292). In contrast to CD25 upregulation on NK cells in our model during viral infection, GATA3 positively regulates CD25 expression on ILC2s, but not ILC3s (292). Similarly, *Gata3* deletion did not reduce the expression of *Klrg1* and *Id2* in ILC2s. Altogether, these results indicated the stage- and cell-specific roles of GATA3 during ILC development. Therefore, it might be interesting to know whether CD25 upregulation on NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice is regulated by developmental stage-specific epigenetic modifications in the *Cd25* locus. The differential binding of GATA3 on the NKG2A promoter region of human NK cells but not Jurkat T cells supports the influence of epigenetic regulations on the roles of GATA3 (318).

Taken together, we dissected the role of GATA3 in regulating the development of NK cells. Even though GATA3 is largely dispensable for the generation of conventional NK cells, it has a significant impact on the maturation of NK cells, resulting in a migratory defect in BM egress in the absence of GATA3. Notably, peripheral immature NK cells of *NKp46-Cre-Gata3^{fl/fl}* mice expand more than mature NK cells from control littermates during MCMV infection, allowing rapid accumulation of NK cells with intact cytotoxicity. Our data demonstrated that immature NK cells hold a higher intrinsic proliferative capability and can serve as a reservoir of cytotoxic NK cells upon infection, and therefore will advance our understanding of the antiviral NK cell response in individuals who have defects in NK cell maturation.

Chapter 3: Natural killer cell-derived IL-10 prevents liver damage during sustained murine cytomegalovirus infection

Preface

The following chapter contains original research showing that IL-10 produced by NK cells regulates T cell response and prevents liver damage during sustained MCMV infection. This research was originally published in *Frontiers in Immunology*:

Alaa Kassim Ali, Amandeep Kaur Komal, Saeedah Musaed Almutairi, and Seung-Hwan Lee. (2019) Natural killer cell-derived IL-10 prevents liver damage during sustained murine cytomegalovirus infection. *Front Immunol.* 10: 2688.

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The specific contributions of each author to this paper are listed below:

Alaa Kassim Ali: Performed experiments using *PKO-NKp46-Cre-Gata3^{fl/fl}* mice, analyzed the data, designed the study, and wrote the manuscript.

Amandeep Kaur Komal: Performed experiments using *NKp46-Cre-Gata3^{fl/fl}* mice to generate the data shown in Supplementary figures 1 to 5 in Appendix, analyzed the data, designed the study, and wrote the manuscript.

Saeedah Musaed Almutairi: Performed experiments.

Seung-Hwan Lee: Designed the study, wrote the manuscript, and obtained the funding.

3.1. Abstract

Natural Killer (NK) cells are lymphocytes of the innate immune response that play a vital role in controlling infections and cancer. Their pro-inflammatory role has been well established; however, less is known about the regulatory functions of NK cells, in particular, their production of the anti-inflammatory cytokine IL-10. In this study, we investigated the immunoregulatory function of NK cells during MCMV infection and demonstrated that NK cells are major producers of IL-10 during the early stage of infection. To investigate the effect of NK cell-derived IL-10, we have generated NK cell-specific IL-10-deficient mice (*NKp46-Cre-Il10^{fl/fl}*) displaying no signs of age-related spontaneous inflammation, with NK cells that show no detectable IL-10 production upon *in vitro* stimulation. In *NKp46-Cre-Il10^{fl/fl}* mice, the levels of IL-10 and IFN γ , viral burdens and T cell activation were similar between *NKp46-Cre-Il10^{fl/fl}* mice and their control littermates, suggesting that NK cell-derived IL-10 is dispensable during acute MCMV infection in immunocompetent hosts. In perforin-deficient mice that show a more sustained infection, NK cells produce more sustained levels of IL-10. By crossing *NKp46-Cre-Il10^{fl/fl}* mice with perforin-deficient mice, we demonstrated that NK cell-derived IL-10 regulates T cell activation, prevents liver damage, and allows for better disease outcome. Taken together, NK cell-derived IL-10 can be critical in regulating the immune response during early phases of infection and therefore protecting the host from excessive immunopathology.

3.2. Introduction

The immune system has evolved over time to defend the host against pathogens while protecting the host from collateral tissue damage. During infections, various immune cells are activated to exert their allotted roles in order to combat the evading microbes. The recognition of a pathogen by immune cells triggers a cascade of antimicrobial mechanisms that ultimately lead to the clearance of the pathogen (319). NK cells are lymphocytes of the innate immune response that play a critical role in controlling viruses during the early stage of infection (211, 320-322). Subsequently, T cells undergo clonal expansion in an antigen-specific manner upon recognition of a virus-infected cell, which results in the killing of infected cells via their cytotoxic activity. In addition, T cells are capable of providing long-term immunity against the pathogens through memory cell production (323, 324). However, the immune response exerted by either NK cells or T cells during microbial infections must be tightly regulated to maintain sufficient levels of inflammation for pathogen elimination while avoiding excessive tissue injury to the host (325).

IL-10 is a potent immunoregulatory cytokine that ameliorates excessive host-damaging immune responses. IL-10 interferes with the expression of major histocompatibility complex (MHC) and co-stimulatory molecules on antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs), and also limits the production of pro-inflammatory cytokines (326, 327). Various immune cell subsets, like DCs, macrophages, etc., produce IL-10, but regulatory T cells (Tregs) were known to be one of the major IL-10 producers (326). Notably, there have been studies showing IL-10 production by NK cells during various models of infections such as MCMV, *Toxoplasma gondii*, *Leishmania donovani*, and HIV (327-333).

IL-10 has paradoxical functions during viral infections. During acute influenza infection, blocking IL-10 response resulted in increased pulmonary inflammation and increased mortality (261). Moreover, IL-10 blockade during acute herpes simplex infection resulted in severe disease and enhanced stromal keratitis (260). Acute respiratory syncytial virus (RSV) and MCMV infections of IL-10-deficient mice are associated with increased weight loss and disease severity (334-336). Altogether, these studies demonstrate that IL-10 plays a critical role in limiting T cell-mediated inflammation and injury. On the contrary, blockade of IL-10 was often known to enhance T cell response, resulting in enhanced protection. IL-10 can interfere with protective immunity following a high-dose influenza challenge by inhibiting Th1 and Th17 responses, and blockade of IL-10 protects naïve mice against an otherwise lethal influenza challenge (337). Furthermore, during persistent LCMV infection, IL-10 deficiency resulted in a robust effector T cell response and the rapid elimination of the virus (266). Taken together, IL-10 can be beneficial to the host by protecting from excessive disease-mediated immunopathology, or it can be detrimental to the host by inducing immunosuppression and thereby allowing pathogen persistence.

Generally, the human cytomegalovirus (HCMV) infection is asymptomatic in healthy individuals but can result in fatal consequences in transplant recipients, immunocompromised individuals, and neonates (338). The murine cytomegalovirus (MCMV) is a herpesvirus that replicates in visceral organs such as the spleen and liver (339, 340). During the early phases of acute MCMV infection, NK cells and monocyte/macrophages are quickly recruited to the infected organs (341, 342), and IFN γ production by NK cell limits MCMV infection (343). During the later phases of MCMV infection, CD8 T cells (344) and CD4 T cells (345, 346) accumulate in organs such as the liver and participate in antiviral responses. Together, these immune responses are protective; however, they can lead to disease-mediated pathology if

inadequately regulated (167, 183, 185, 335, 347). Many studies have focused on characterizing the robust pro-inflammatory immune responses during MCMV infection; nonetheless, the signals involved in dampening these immune responses have not been completely elucidated.

Several reports have investigated the immunoregulatory role of IL-10 in the context of MCMV infection (99, 335, 348, 349). B cells produce IL-10 to prevent an exaggerated immune response by MCMV-specific CD8 T cells (350). Another study demonstrated that CD4 T cells production of IL-10 leads to increased viral burden, allowing MCMV to persist in mucosal areas such as the salivary gland (351). IL-10 was also shown to limit systemic levels of IFN γ , reduce T cell response, and increase the viral loads in the spleens of MCMV-infected mice (335). Administration of recombinant IL-10 in IL-10-deficient mice diminished the levels of pro-inflammatory cytokines and reduced liver pathology caused by MCMV infection (352). In order to evade recognition by T cells, MCMV induces the downregulation of MHC molecules on macrophages through an IL-10-dependent mechanism (353).

Previously, we proposed that the NK cell-derived IL-10 limits the magnitude of the CD8 T cell response in immunocompromised perforin-deficient (PKO) mice, which was demonstrated by blocking IL-10 during the peak response of IL-10 production by NK cells (99). Nevertheless, since several cell types are capable of producing IL-10 during infections, it remains unclear whether NK cell-derived IL-10 plays a definitive role in the regulation of inflammatory processes during sustained MCMV infection. In this study, we took advantage of a genetic approach and investigated the NK cell-mediated regulatory pathway during MCMV infection by generating an NK cell-specific IL-10-deficient mouse (*NKp46-Cre-Il10^{fl/fl}*). The MCMV infection resulted in a heightened immune response in the spleens and livers of *NKp46-Cre-Il10^{fl/fl}* mice, in mice deficient in perforin, but not under immunocompetent condition. While viral clearance was not improved, the augmented immune

response led to increased liver damage, suggesting the occurrence of excessive immune-mediated pathology.

3.3. Materials and Methods

3.3.1. Mice and genotyping

B6.129S6-*Il10*^{tm1Flv/J} mice (IL-10-GFP; *tiger*) and C57BL/6-*Prfl*^{tm1Sdz/J} (perforin-deficient, PKO) mice were purchased from The Jackson Laboratory, USA. IL-10-GFP mice were generated by insertion of an internal ribosomal entry site (IRES) and green fluorescence protein (GFP) element upstream of the polyadenylation site of the *Il10* gene as previously described (354). *Il10*^{fl/fl} mice were generated by inserting the loxP sequences between parts of the promoter region, transcription initiation site, and the first exon of *Il10* allele (355). The *NKp46*^{iCre} knock-in mice were generated by homologous recombination in which improved Cre (*iCre*) was inserted at the 3' end of the *Nkpa46* gene (356). The *Ly49h*-intron primers were *D6Ott151*-forward (F): 5'-GTGCTACCACTGAAAACCATG-3' and *D6Ott151*-reverse (R): 5'-CTGTCTCTTGAGTCACCTGCAC-3' (216). The *Il10* floxed and *Il10* deleted alleles were genotyped using the following primers: F, 5'-CCAGCATAGAGAGCTTGCATTACA-3'; floxed-R, 5'-TCCTCTTGGGATCCAGTTGT-3'; and deleted-R, 5'-GCTGCTTCTCCTGCTGAGTT-3'. Experiments were performed using littermate mice by mating *NKp46-Cre*^{+/-} × *Il10*^{fl/fl} mice with *NKp46-Cre*^{-/-} × *Il10*^{fl/fl} mice. All mice were bred and kept in the specific-pathogen-free animal facility at the University of Ottawa in agreement with guidelines and regulations of the Canadian Council on Animal Care. All procedures were approved by and conducted in accordance with the animal guidelines of the University of Ottawa. Unless indicated otherwise, all mice used for experiments were between the ages of 6-12 weeks old.

3.3.2. MCMV infection and virus titer determination

MCMV stocks (Smith strain) were generated in our laboratory from the salivary glands of infected BALB/c mice. To determine the kinetics of IL-10 production by NK cells and T cells, IL-10-GFP and PKO-IL-10-GFP were challenged with 3,000 or 5,000 PFU MCMV intraperitoneally. To study the role of IL-10 in immunocompetent mice, *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice were challenged with 12,000 or 50,000 PFU MCMV. To investigate the role of IL-10 in immunocompromised mice, *PKO-Il10^{fl/fl}* and *PKO-NKp46-Cre-Il10^{fl/fl}* mice were challenged with 3,000 or 5,000 PFU MCMV. For measuring the viral titers, organs from infected mice were homogenized by MagNA Lyser (Roche Applied Science) and the lysates were diluted and overlaid on mouse embryonic fibroblasts cells for 1 h at 37°C in 2% DMEM (DMEM medium supplemented with 2% FBS, 1× penicillin/streptomycin, 2 mM L-glutamine, 10 mmol HEPES, and 50 µmol 2-mercaptoethanol). After 1 h incubation, the virus was removed from the monolayers by aspiration. The monolayers were overlaid with 1 part of DMEM containing 2% low melting agar mixed with 3 parts of 13.5% DMEM (DMEM medium supplemented with 13.5% FBS, 1× penicillin/streptomycin, 2 mM L-glutamine, 10 mmol HEPES, and 50 µmol 2-mercaptoethanol). Three days later, the cells were fixed with 10% formalin for 10 min and stained with 1% Crystal Violet for 10 min. Plaques were counted and represented as log PFU/g of organs.

3.3.3. ALT and cytokine quantification

The presence of alanine aminotransferase (ALT) in the serum samples was determined using the Alanine Aminotransferase (ALT/GPT) Colorimetric Assay Kit (Elabscience, cat #: E-BC-K325). Samples were prepared according to the manufacturer's instructions, acquired on FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices) and analyzed using the

SoftMax Pro software (Molecular Devices). The production of cytokines IL-10, IFN γ , and TNF α in blood plasma or conditioned media was measured using Cytometric Bead Array (CBA) kit (BD Biosciences). Samples were prepared according to the manufacturer's instructions, acquired on LSRFortessa (BD Biosciences) and analyzed using the FCAP Array software (BD Biosciences).

3.3.4. Cell isolation, serum, and conditioned media (CM)

Approximately 40 μ L of peripheral blood was collected from the tail vein and washed with 1mL PBS. The erythrocytes were lysed using 1mL of Red Blood Cell Lysis Buffer (Roche) for about 30 seconds until the solution became clear. The cells were then washed with 9 mL of RPMI and filtered through a 70 μ m nylon mesh. Spleens were harvested, weighed, transferred onto a 70 μ m nylon cell strainer, and ground. A single-cell suspension of leukocytes was obtained following red blood cell lysis and filtration through a 70 μ m nylon mesh. For the isolation of hepatic lymphocytes, livers were harvested, weighed, and transferred onto the surface of 70 μ m nylon cell strainer and ground. The cells were washed three times with RPMI-1640 medium and suspended in 4 ml of 40% Percoll (GE Healthcare) and carefully overlaid onto 2 ml of 70% Percoll at room temperature (RT). The liver samples were then centrifuged at 2400 rpm for 25 min at RT with no breaks. The top fat layer was suctioned off using a Pasteur pipette, and the lymphocyte ring was collected and resuspended in RPMI-1640 medium. The isolation of lymphocytes from the salivary glands was performed as previously described (357). The number of viable cells was determined by trypan blue exclusion. Serum was obtained following centrifugation of whole blood collected in heparin tubes and stored at -20°C until further use for cytokine analysis. To prepare CM, purified NK cells were seeded in triplicates in a flat-bottom 96-well plate at 5×10^4 cells/well in RP-10 medium (RPMI-1640

medium supplemented with 10% FBS, 1× penicillin/streptomycin, 2 mM L-glutamine, 10 mmol HEPES, 50 µmol 2-mercaptoethanol) and stimulated with recombinant human (rh)IL-2 (1000 U/mL, NCI Preclinical Repository) and recombinant mouse IL-12 (50 ng/mL, eBioscience). After 18 h of incubation at 37°C, cell-free supernatants were collected and stored at -20°C until used for cytokine analyses.

3.3.5. Antibodies and flow cytometry

Single-cell suspensions (1×10^6 cells) was incubated at 4°C for 15 min with α -CD16/32 (clone 2.4G2, from Bioexpress) to reduce non-specific binding. Cells were labeled with various combinations of directly conjugated monoclonal antibodies and incubated at 4°C for 25 min. The following monoclonal antibodies were used: α -NK1.1 (PK136), α -CD3 (145-2C11), α -TCR β (H57-597), α -CD8a (53-6.7), α -CD49b (DX5), α -CD27 (LG.7F9), α -CD11b (M1/70), α -Ly49D (4E5), α -Ly49G2 (4D11), α -Ly49H (3D10), α -NKG2A-B6 (16a11), α -NKG2D (CX5), α -IL-10 (JES5-16E3), α -IFN γ (XMG1.2), and α -CD107a (1D4B) from eBioscience, α -CD19 (1D3), α -Ly49C/I (5E6), α -CD4 (RM4-5), and α -CD49a (Ha31/8) from BD Biosciences, Fixable Yellow Live/Dead from Invitrogen. For intracellular IL-10 and IFN γ measurements *in vivo*, leukocytes were harvested and incubated in RP-10 medium (RPMI-1640, 10% FBS, 1X penicillin/streptomycin, 1% L-Glutamine, 10 mM HEPES, 50 µM β -mercaptoethanol) containing 5 µg/ml brefeldin A for 4 hrs, followed by staining for intracellular IL-10 or IFN γ using BD Cytofix/Cytoperm protocol. The stained cells were acquired using LSRFortessa or FACSCelesta (BD Biosciences) and analyzed using Kaluza software v3 (Beckman Coulter) or FlowJo (Tree Star).

3.3.6. NK cell enrichment and cell sorting

NK cells were enriched from total splenocytes by negative magnetic separation using NK cell isolation kit II (MACS Miltenyi Biotec) according to the manufacturer's instructions. Total splenocytes were labeled with α -NK1.1, α -TCR β , and α -CD19 (to obtain T cells and B cells), while total hepatic leukocytes (to obtain NKT cells) and enriched NK cells were labeled with α -NK1.1 and α -TCR β . The labeled cells were then flow sorted by using MoFlo XDP-sorter from Beckman Coulter (Stem Core laboratories, OHRI, Ottawa) to obtain T cells, B cells, NK cells, and NKT cells. The purified NK cells were stimulated *ex vivo* with rhIL-2 (1000 U/ml) and IL-12 (50 ng/ml) in RP-10 medium for 18 h at 37°C and the conditioned media was harvested to measure IL-10 secretion. The purity of the fraction was >95%.

3.3.7. Histology

The colon samples were harvested from 6 months old mice. The samples were flushed with PBS by using syringe and needle and immersed in 4% paraformaldehyde (PFA) for fixing with slight shaking. Fixed samples were sent to the Department of Pathology and Laboratory Medicine at the University of Ottawa where the tissues were embedded and hematoxylin and eosin (H&E) stained. Slide pictures were taken at magnifications of 10 and 20.

3.3.8. Statistical analysis

Statistical significance between two groups was determined by two-tailed unpaired student *t* tests ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) using Prism Version 5 (Graph Pad Software). Statistical significance between more than two groups was determined by ANOVA. If the ANOVA rejected the null hypothesis, multiple comparisons were performed between two selected groups by a two-tailed unpaired *t*-test.

3.4. Results

3.4.1. NK cells are a major source of IL-10 during the early stages of MCMV infection in B6 immunocompetent mouse

To determine whether NK cells play an immunoregulatory role in immunocompetent mice by producing IL-10, we infected IL-10-GFP reporter mice (C57BL/6 background), which allows us to study the complete kinetics of IL-10 production during MCMV infection. Flow cytometric analysis showed that NK cells were predominantly expressing IL-10-GFP on D4 post MCMV infection in the peripheral blood (Figure 3.1A), whereas almost negligible IL-10-GFP expression was observed in T cells at that time. A similar trend was observed in the spleen and liver (Supplementary Figure 1 in Appendix). Recently, liver-resident NK cells defined as CD49a⁺CD49b⁻ have been shown to serve as sentinels in response to infection (289). The proportions of liver-resident NK cells were dramatically reduced upon MCMV infection, and the IL-10-GFP expression was lower than that of conventional CD49a⁺CD49b⁺ NK cells (Figure 3.2). Back gating analysis further confirms that NK cells in the peripheral blood are the major producer of IL-10 on D4 post infection. Among total IL-10-GFP expressing cells, 71% were NK cells, and only 17% were T cells (Figure 3.1B). The T cells expressing IL-10-GFP were comprised of both CD4 and CD8 T cells.

The complete kinetics of peripheral blood leukocytes by flow cytometry revealed that IL-10-GFP expression by NK cells starts as early as D2 with a peak observed on D4 post-infection (Figure 3.1C, D). After D4, the IL-10-GFP expression slowly starts to diminish in NK cells, and returns to basal levels by D10 of infection. Although GFP expression by T cells overlapped with NK cells, only a small proportion of T cells expressed IL-10 as compared to NK cells during the initial stages of infection. The IL-10-GFP expression by T cells reached a

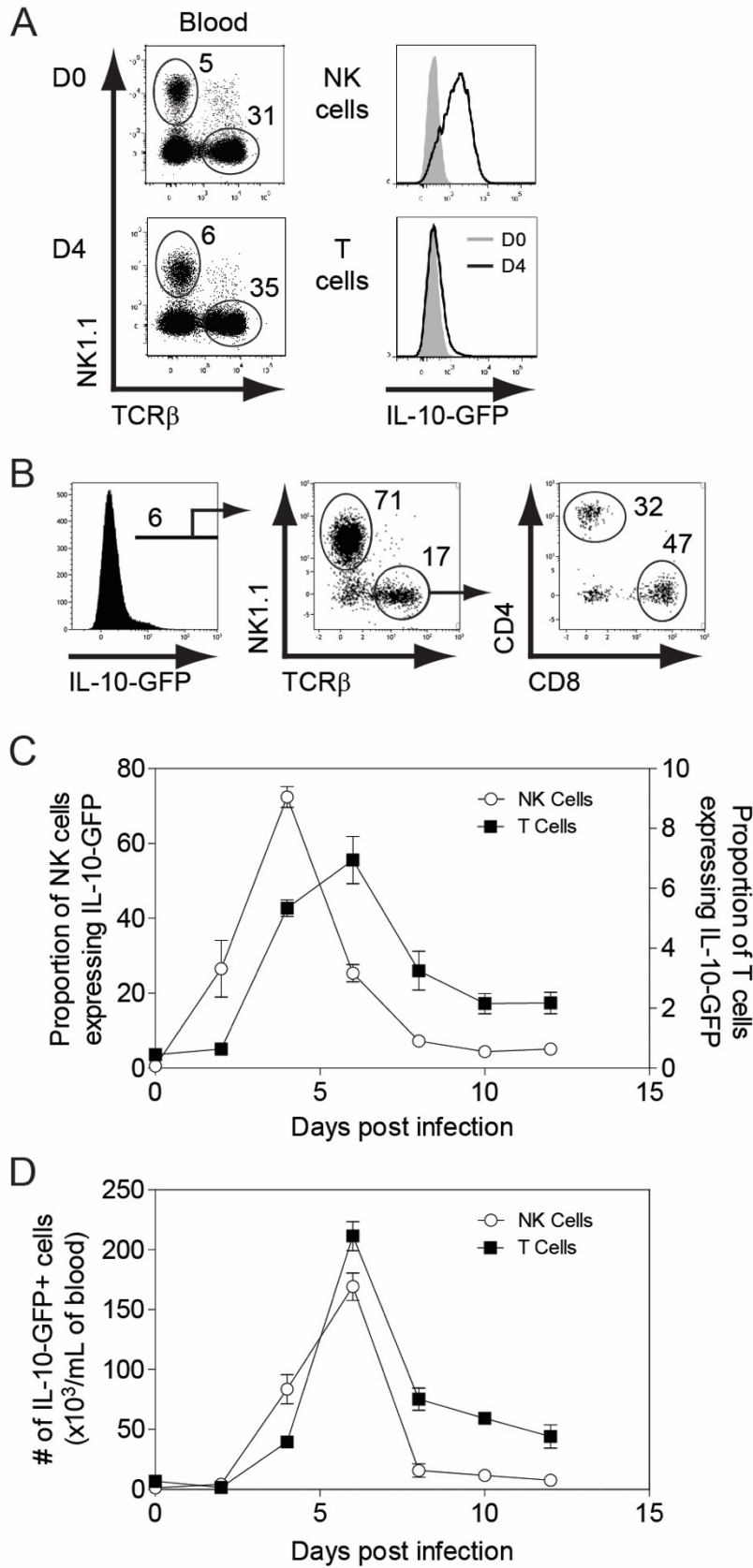


Figure 3.1. NK cells are major producers of IL-10 during the initial stages of MCMV infection. IL-10-GFP mice were either uninfected or challenged with 3,000 PFU MCMV i.p. and the peripheral blood leukocytes were analyzed on the indicated days. **(A)** The overlay plots depict IL-10-GFP expression in NK cells and T cells in the peripheral blood of uninfected (D0) and infected (D4) mice. **(B)** Back gating analysis representing IL-10 producing NK cells and T cells among total IL-10-GFP expressing cells. **(C, D)** The graphs represent the complete kinetics of IL-10-GFP expression throughout the course of MCMV infection. The proportion and number of IL-10-GFP expressing NK cells and T cells are obtained from total peripheral blood leucocytes (n = 3; two experiments). Data represent mean + SD.

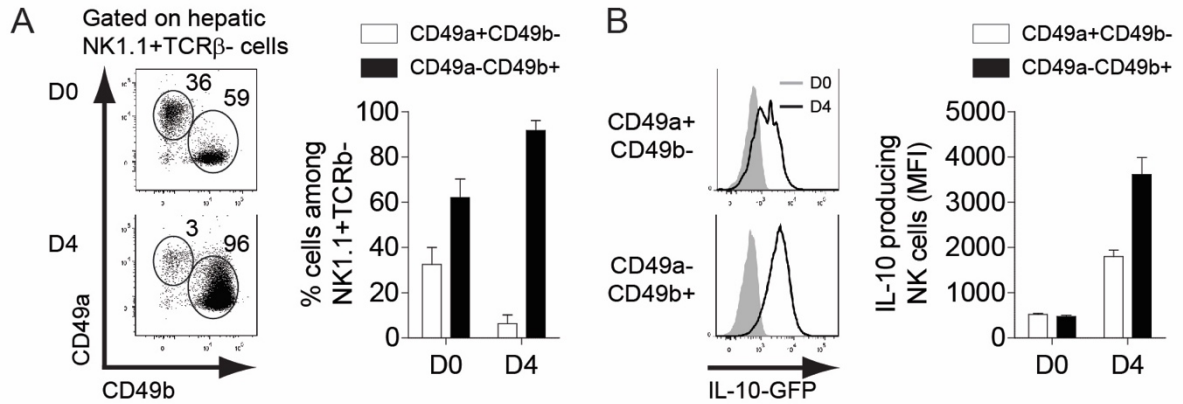


Figure 3.2. Frequencies of liver-resident NK cells are diminished during MCMV infection, while their production of IL-10 is lower than conventional NK cells. IL-10-GFP mice were either uninfected or challenged with MCMV i.p. and the hepatic lymphocytes were analyzed on the indicated days. **(A)** The proportion of liver-resident NK cells (CD49a+CD49b-) and conventional NK cells (CD49a-CD49b+) among total NK cells are shown in the livers of uninfected (D0) and infected (D4) mice (n = 4; two experiments). **(B)** The production of IL-10 by liver-resident NK cells and conventional NK cells in the livers of uninfected (D0) and infected (D4) mice (n = 4; two experiments). Data represent mean + SD.

peak on D6 before starting to decrease (Figure 3.1C, D). Approximately 7% of T cells were IL-10-GFP positive at D6 post-infection. Taken together, NK cells are the major producers of the regulatory cytokine IL-10 during the early stages of MCMV infection in immunocompetent mice. Furthermore, NK cells and T cells participate in a division of labor for maintaining constant levels of IL-10 during MCMV infection.

3.4.2. Generation and characterization of NK cell-specific *Il10*-deficient (*NKp46-Cre-Il10^{fl/fl}*) mice

The use of neutralizing antibodies against IL-10 or its receptor has been a commonly used approach to study its function. Since various cell populations can produce IL-10 during infection and different cell subsets possess IL-10 receptor, it is difficult to characterize the importance of cell-specific IL-10 production by using neutralizing antibodies. Complete IL-10-deficient (*Il10*^{-/-}) mice have also been used in previous studies to elucidate the role of IL-10 (358, 359), but the severe spontaneous inflammation observed in this mouse model limited its use for infection studies. To circumvent these disadvantages, we generated a mouse model that lacked *Il10* gene specifically in NK cells (*NKp46-Cre-Il10^{fl/fl}*) by crossing an *NKp46^{iCre}* knock-in mouse with an *Il10* floxed mouse (*Il10^{fl/fl}*).

To determine whether Cre-mediated recombination occurs specifically in NK cells, we flow-sorted T cells, B cells, and NK cells from spleens and NKT cells from livers of *NKp46-Cre-Il10^{fl/fl}* mice and extracted the genomic DNA to analyze the Cre-mediated recombination by PCR. The location of the primer sets used to identify the *Il10* floxed, and *Il10* deleted alleles are shown in Supplementary Figure 2A in Appendix. Notably, the *Il10* deleted allele was mainly observed in NK cells (Supplementary Figure 2B). Furthermore, *Il10* floxed allele was weakly amplified from NK cells but present in all other lymphocyte populations indicating

that Cre mediated recombination had occurred exclusively in NK cells but not in other cell populations such as T cells, B cells and NKT cells (Supplementary Figure 2B). Due to the presence of NK cells among total cells, a faint band of IL-10 deleted allele was observed in whole cells but none in T cells, B cells and only slightly in NKT cells. The *NKp46-Cre-Il10^{fl/fl}* mice were obtained at Mendelian frequency, were fertile and developed normally. Taken together, the PCR analysis confirmed that Cre-mediated recombination resulted in *Il10* gene deletion specifically in NK cells, whereas it was intact in all other lymphocyte populations.

The deletion of IL-10 in NK cells did not affect the proportion and number of immune cells at the steady-state, as the frequencies of lymphoid and myeloid subsets were comparable in the spleens of *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice (Figure 3.3A and data not shown). In addition, the maturation of NK cells was normal in *NKp46-Cre-Il10^{fl/fl}* mice (Figure 3.3B, C). The expression of activating and inhibitory receptors was also comparable between the two groups of mice (Figure 3.3D, E). It has been previously reported that the synergistic action of IL-2/IL-12 induces IL-10 production by NK cells (329, 360). Therefore, to confirm the loss of IL-10 protein in naïve NK cells from *NKp46-Cre-Il10^{fl/fl}* mice following *ex vivo* IL-2/IL-12 stimulation, we obtained NK cells with a purity of >95% by enrichment followed by cell sorting (Supplementary Figure 2C). The highly pure NK cells were subjected to *ex vivo* stimulation in the presence of IL-2/IL-12. Indeed, we could not detect any secreted IL-10 in the conditioned media of stimulated NK cells from *NKp46-Cre-Il10^{fl/fl}* mice (Supplementary Figure 2D). This further confirmed the loss of IL-10 production by NK cells in *NKp46-Cre-Il10^{fl/fl}* mice, whereas IL-10 production was intact in control *Il10^{fl/fl}* mice.

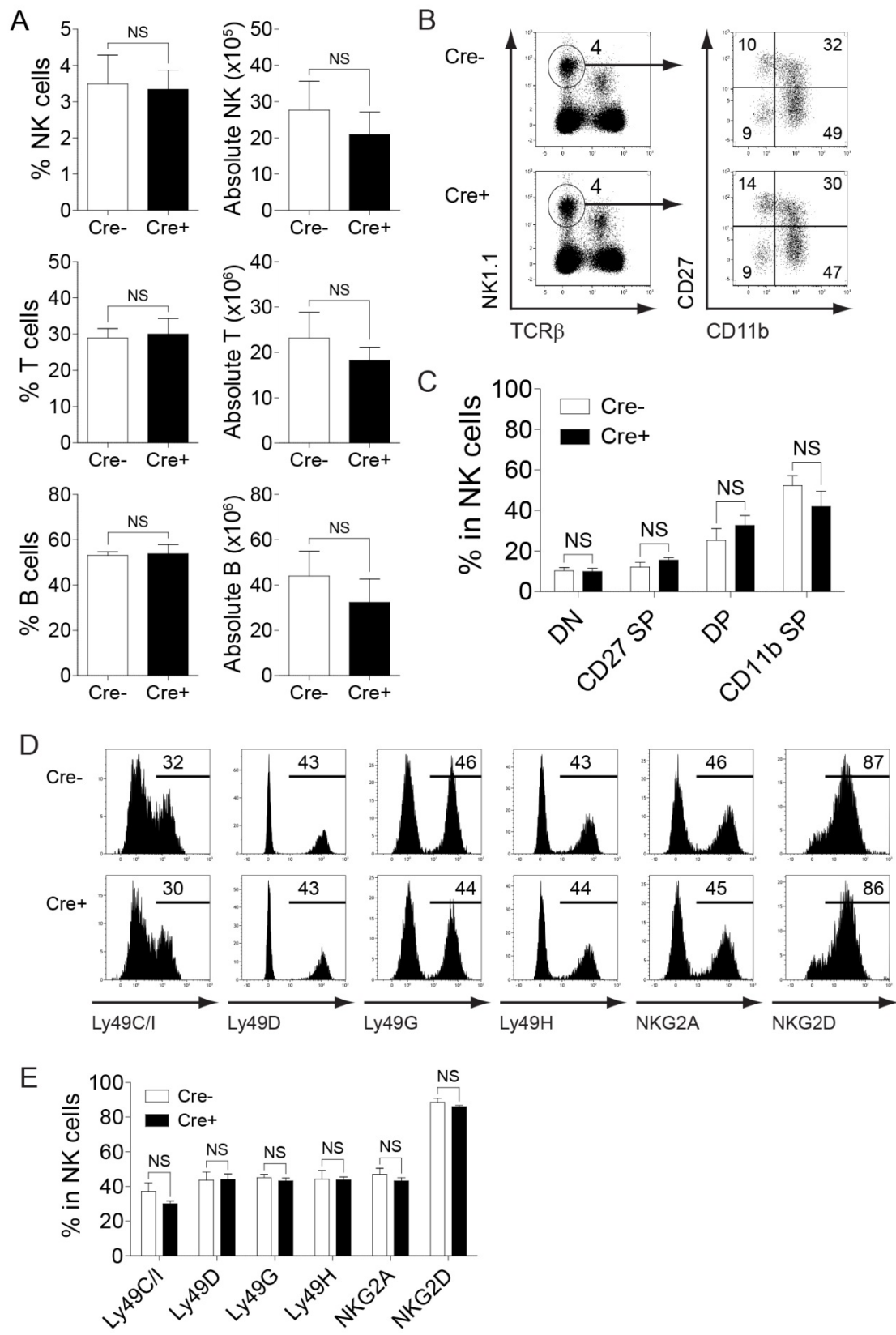


Figure 3.3. Phenotypic characterization of NK cells in the spleens of *Il10^{fl/fl}* (Cre-) and *NKp46-Cre-Il10^{fl/fl}* (Cre+) mice. (A) Proportions and absolute numbers of NK cells, T cells, and B cells in the spleens of *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice (three experiments; n = 7 for NK cells and T cells, n = 5 for B cells). (B) Representative plots of CD27⁻CD11b⁻ (DN), CD27⁺CD11b⁻ (CD27 SP), CD27⁺CD11b⁺ (DP) and CD27⁻CD11b⁺ (CD11b SP) populations among NK cells. (C) Proportions of DN, CD27 SP, DP and CD11b SP populations in *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice. (D) Representative plots of several activating and inhibitory receptors in NK cells of *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice. (E) Proportions of NK cells expressing the indicated surface receptors. Data are from one experiment representative of two independent experiments, with three mice per group. Data represent mean + SD. ns: non significant.

3.4.3. *NKp46-Cre-Il10^{fl/fl}* mouse does not show signs of spontaneous inflammation

Il10^{-/-} mice develop chronic enterocolitis characterized by excessive hyperplasia of the mucosal epithelium (359). Previous studies with *CD4-Cre-Il10^{fl/fl}* mice showed the spontaneous onset of inflammatory bowel disease (IBD) as mice aged, suggesting the crucial role of T cell-derived IL-10 in protecting mice from excessive inflammation. The most prominent histological changes have been observed in the colon of *CD4-Cre-Il10^{fl/fl}* mice (355). Although Tregs have been known to play a central role in suppressing immune inflammation, it is possible that additional IL-10 sources are required to restrain spontaneous inflammation. As NK cells from *NKp46-Cre-Il10^{fl/fl}* mouse do not produce IL-10, lack of this early regulatory function may render this mouse highly susceptible to spontaneous inflammation. Since the colon is the most affected part during gut inflammation as exemplified in earlier studies, we examined the colon histology in *NKp46-Cre-Il10^{fl/fl}* mice at 6 months of age and compared it with mice lacking IL-10 in T cells (*CD4-Cre-Il10^{fl/fl}*). H&E staining of colon from control and *NKp46-Cre-Il10^{fl/fl}* mice showed normal colon anatomy characterized by highly organized crypt structure and surface epithelium with no signs of spontaneous inflammation in *NKp46-Cre-Il10^{fl/fl}* mice as opposed to *CD4-Cre-Il10^{fl/fl}* mice that exhibited massive hyperplasia and disorganized crypt structure indicative of spontaneous inflammation as previously reported (355) (Supplementary Figure 3A in Appendix).

To evaluate the activation status of NK cells in young mice, enriched NK cells from 8 weeks old naïve control and *NKp46-Cre-Il10^{fl/fl}* mice were stimulated by various surface-bound stimulations such as α -NK1.1, α -Ly49H, and IL-2/IL-12 and their effector functions were analyzed. NK cells from control and *NKp46-Cre-Il10^{fl/fl}* mice showed comparable levels

of IFN γ production and LAMP-1 (a marker of degranulation) upon various stimulations (Supplementary Figure 3B, C), suggesting that NK cells from control and *NKp46-Cre-Il10^{fl/fl}* mice are phenotypically and functionally similar in the naïve state. Taken together, we have successfully generated an NK cell-specific *Il10*-deficient mouse that is free of spontaneous inflammation and hence is an ideal tool to elucidate the immunoregulatory role of NK cell-derived IL-10 during microbial infection.

3.4.4. *NKp46-Cre-Il10^{fl/fl}* mouse shows comparable viral clearance and T cell response during acute MCMV infection of immunocompetent mice

The ability of NK cells to produce IL-10 during MCMV infection was demonstrated above (Figure 3.1), however, whether NK cell-derived IL-10 can contribute to modulating the adaptive immune response and dictating the outcome of disease has never been clearly demonstrated. Having successfully generated an NK cell-specific *Il10*-deficient mouse model, we decided to investigate the role of NK cell-derived IL-10 during acute viral infection. We infected *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice with MCMV, and monitored body weight changes daily. By day 5 post-infection, the mice had lost as much as 25% of their initial body weight, before starting to gain back their weight. These mice were able to recover their initial weight by day 12 post-infection. NK cell-derived IL-10 did not seem to play a critical role in preventing the weight loss, as *NKp46-Cre-Il10^{fl/fl}* mice and their control littermates *Il10^{fl/fl}* displayed similar body weight change (Supplementary Figure 4A in Appendix). Notably, the presence of NK cell-derived IL-10 did not improve viral clearance, as *NKp46-Cre-Il10^{fl/fl}* mice and their control littermates *Il10^{fl/fl}* had a similar viral burden in their spleens at D5 post-infection (Supplementary Figure 4B). Moreover, *NKp46-Cre-Il10^{fl/fl}* mice and their control littermates *Il10^{fl/fl}* showed similar levels of the liver enzyme alanine aminotransferase (ALT)

in the serum (Supplementary Figure 4C), suggesting that NK cell-derived IL-10 does not regulate liver damage in immunocompetent mice.

To determine whether NK cell-specific IL-10 ablation can lead to a reduction in the systemic levels of IL-10 during viral infection, we measured the IL-10 levels in the peripheral blood of MCMV-infected mice. Interestingly, although we were able to successfully delete IL-10 in NK cells using *NKp46-Cre-Il10^{fl/fl}* mice as shown in figure 2, the IL-10 levels in the serum were only slightly decreased in *NKp46-Cre-Il10^{fl/fl}* mice at day 4 post-infection (Supplementary Figure 4D). In addition, the serum levels of IFN γ and TNF α were similar between the two groups. In order to determine whether NK cell-derived IL-10 can regulate T cell activation during infection, we measured the IFN γ production by T cells. However, in the absence of NK cell-derived IL-10, the production of IFN γ by T cells in the spleens of infected mice was not altered and only slightly increased in the liver (Supplementary Figure 4E). Taken together, these results suggest that the IL-10 produced by NK cells in immunocompetent mice during an acute MCMV infection is dispensable for viral clearance and is not critical in regulating the T cell response.

The salivary gland environment is highly immunosuppressive, making it an immune-privileged site for viral persistence (361). Both MCMV and HCMV are known for establishing a lifelong latent infection inside the salivary glands and therefore act as a natural source of horizontal viral transmission (338). Several studies have reported a role for IL-10 in maintaining the immunosuppressive environment inside the salivary glands (334, 348). To study the effect of NK cell-derived IL-10 in maintaining an immunosuppressive environment and viral persistence inside the salivary glands, we infected *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice and analyzed the T cell response in salivary glands on day 16 post-infection. Notably, we did not observe any difference in the viral burden or T cell response between the groups

(Supplementary Figure 5A, B in Appendix). In fact, using MCMV-infected IL-10-GFP mice, we observed that NK cells are poor producers of IL-10 in the salivary gland whereas CD4 T cells are major producers of IL-10 (Supplementary Figure 5C).

3.4.5. Perforin-deficient mice have more sustained production of IL-10

Since the need for regulatory pathways is well recognized during persistent infections in which immune cells are often highly activated, we decided to examine the importance of NK cell-specific IL-10 during a persistent MCMV model. To analyze the kinetics of IL-10 production by NK cells in perforin-deficient mice, we crossed perforin-deficient (PKO) mice with IL-10-GFP mice (PKO X IL-10-GFP). NK cells from perforin-deficient mice undergo extensive proliferation during MCMV infection, as compared to immunocompetent mice (Figure 3.4A). Notably, NK cells from perforin-deficient mice produced more sustained levels of IL-10 (Figure 3.4B). IL-10 production by NK cells in IL-10-GFP mice peaked at day 4 post-infection before declining rapidly by day 6 post-infection. However, NK cells in PKO X IL-10-GFP mice produced high amounts of IL-10 from day 4 to day 8 post-infection before starting to decrease. Similarly, T cells from PKO mice also produced more sustained levels of IL-10. In IL-10-GFP mice, IL-10 production by T cells was acute and peaked at day 6 post-infection. On the other hand, in PKO mice, IL-10 production by T cells was higher and peaked at day 8 post-infection. In addition, CD4 T cells showed a more sustained production of IL-10, while CD8 T cells showed an acute production of IL-10. Taken together, NK and T cells from PKO mice produce more sustained levels of IL-10 during MCMV infection, suggesting a critical role of IL-10 during persistent infection.

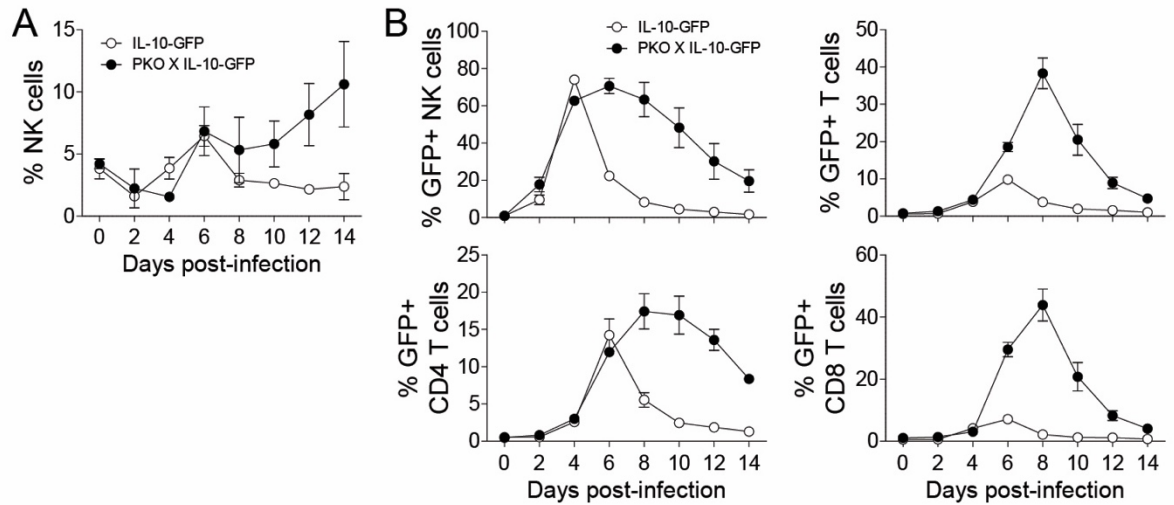


Figure 3.4. NK cells from *perforin*-deficient (PKO) mice express more sustained levels of IL-10 during MCMV infection. IL-10-GFP and PKO-IL-10-GFP mice were either uninfected or given 5,000 PFU MCMV i.p. and the peripheral blood leukocytes were analyzed on the indicated days. **(A)** The proportion of NK cells in the blood of IL-10-GFP and PKO-IL-10-GFP infected mice throughout the course of MCMV infection. **(B)** The graphs represent the complete kinetics of IL-10-GFP expression in NK cells and T cells from the peripheral blood of IL-10-GFP (n = 4) and PKO-IL-10-GFP (n = 4) infected mice at the indicated days post-infection (two experiments). Data represent mean + SD.

3.4.6. NK cell-derived IL-10 in perforin-deficient mice prevents liver damage during sustained MCMV infection

To investigate the role of NK cell-derived IL-10 in perforin-deficient mice during MCMV infection, we generated *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice under a perforin-deficient background to give rise to *PKO-NKp46-Cre-Il10^{fl/fl}* mice and their control littermates *PKO-Il10^{fl/fl}* which lack the Cre recombinase. These mice were infected with MCMV, and their body weight was monitored daily. NK cells from *PKO-NKp46-Cre-Il10^{fl/fl}* mice failed to produce IL-10 during MCMV infection, as measured by intracellular staining of IL-10 (Figure 3.5A, B). PKO mice challenged with MCMV suffered slightly increased body weight loss when NK cells were unable to produce IL-10 (Figure 3.5C). Notably, the inability of NK cell to produce IL-10 did not influence the viral burden in MCMV-infected PKO mice, as *PKO-NKp46-Cre-Il10^{fl/fl}* had similar virus titer in their spleens and livers as compared with their control littermates *PKO-Il10^{fl/fl}* at D5 post-infection (Figure 3.5D). Despite that, the absence of NK cell-derived IL-10 led to increased liver damage, based on the levels of the liver enzyme ALT in the serum of infected mice (Figure 3.5E), suggesting that NK cell-produced IL-10 prevents immunopathology.

Next, we investigated whether NK cell-specific IL-10 ablation in perforin-deficient mice leads to a reduction in the systemic levels of IL-10 during infection. Thus, we measured the IL-10 levels in the peripheral blood of MCMV-infected mice. Indeed, the IL-10 levels in the serum were reduced in *PKO-NKp46-Cre-Il10^{fl/fl}* mice at D4 post-infection, as compared to their control littermates (Figure 3.5F), however this difference was not observed at D7 post-infection. Notably, the levels of IFN γ and TNF α were increased in the serum of *PKO-NKp46-Cre-Il10^{fl/fl}* mice (Figure 3.5G, H), indicating that NK cell-derived IL-10 can regulate

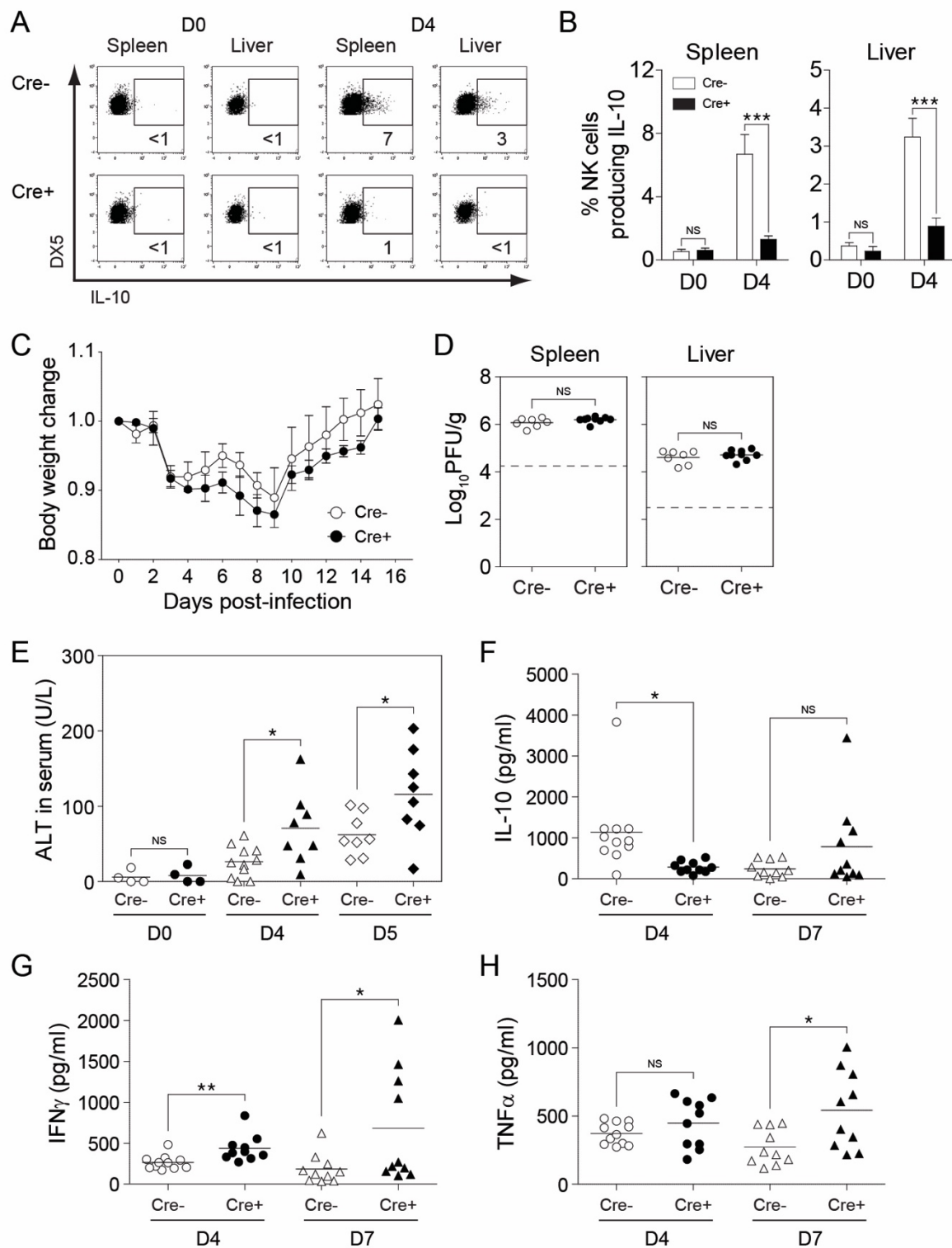


Figure 3.5. NK cell-derived IL-10 provides protection against liver damage during sustained MCMV infection. *PKO-Il10^{fl/fl}* (Cre-) and *PKO-NKp46-Cre-Il10^{fl/fl}* (Cre+) mice were either uninfected or given 3,000 PFU or 5,000 PFU MCMV i.p. for the following analyses: **(A, B)** Proportion of NK cells (DX5+TCR β -) expressing IL-10 in uninfected and infected mice at day 4 post-infection (two experiments; n = 4 for D0 Cre-, n = 4 for D0 Cre+, n = 7 for D4 Cre-, n = 5 for D4 Cre+). **(C)** Body weight change of *PKO-Il10^{fl/fl}* (n = 4) and *PKO-NKp46-Cre-Il10^{fl/fl}* (n = 3) infected mice relative to their initial body weight at day 0 post-infection (three experiments). **(D)** Viral titer in the spleens and livers of *PKO-Il10^{fl/fl}* (n = 7) and *PKO-NKp46-Cre-Il10^{fl/fl}* (n = 9) mice at day 5 post-infection (two experiments). **(E)** Serum levels of ALT enzyme at the indicated days post-infection (four experiments; n = 4 for D0 Cre-, n = 4 for D0 Cre+, n = 11 for D4 Cre-, n = 8 for D4 Cre+, n = 8 for D5 Cre-, n = 8 for D5 Cre+). **(F-H)** Serum levels of IL-10, IFN γ , and TNF α were measured at day 4 and day 7 post-infection (three experiments; n = 11 for D4 Cre-, n = 10 for D4 Cre+, n = 10 for D7 Cre-, n = 10 for D7 Cre+). Data represent mean + SD. ns: non-significant; *p < 0.05; **p < 0.01; ***p < 0.001.

the systemic levels of pro-inflammatory cytokines during a sustained MCMV infection. To determine whether the IL-10 produced by NK cells can directly regulate T cell activation, we measured the proportions of IFN γ -producing CD4 and CD8 T cells in the spleens and livers of infected mice. Notably, T cells showed increased production of IFN γ in *PKO-NKp46-Cre-Il10^{fl/fl}* mice, as compared to their control littermates (Figures 3.6A, B and Figure 3.7), indicating that NK cells produce IL-10 to control the activation status of T cells during MCMV infection.

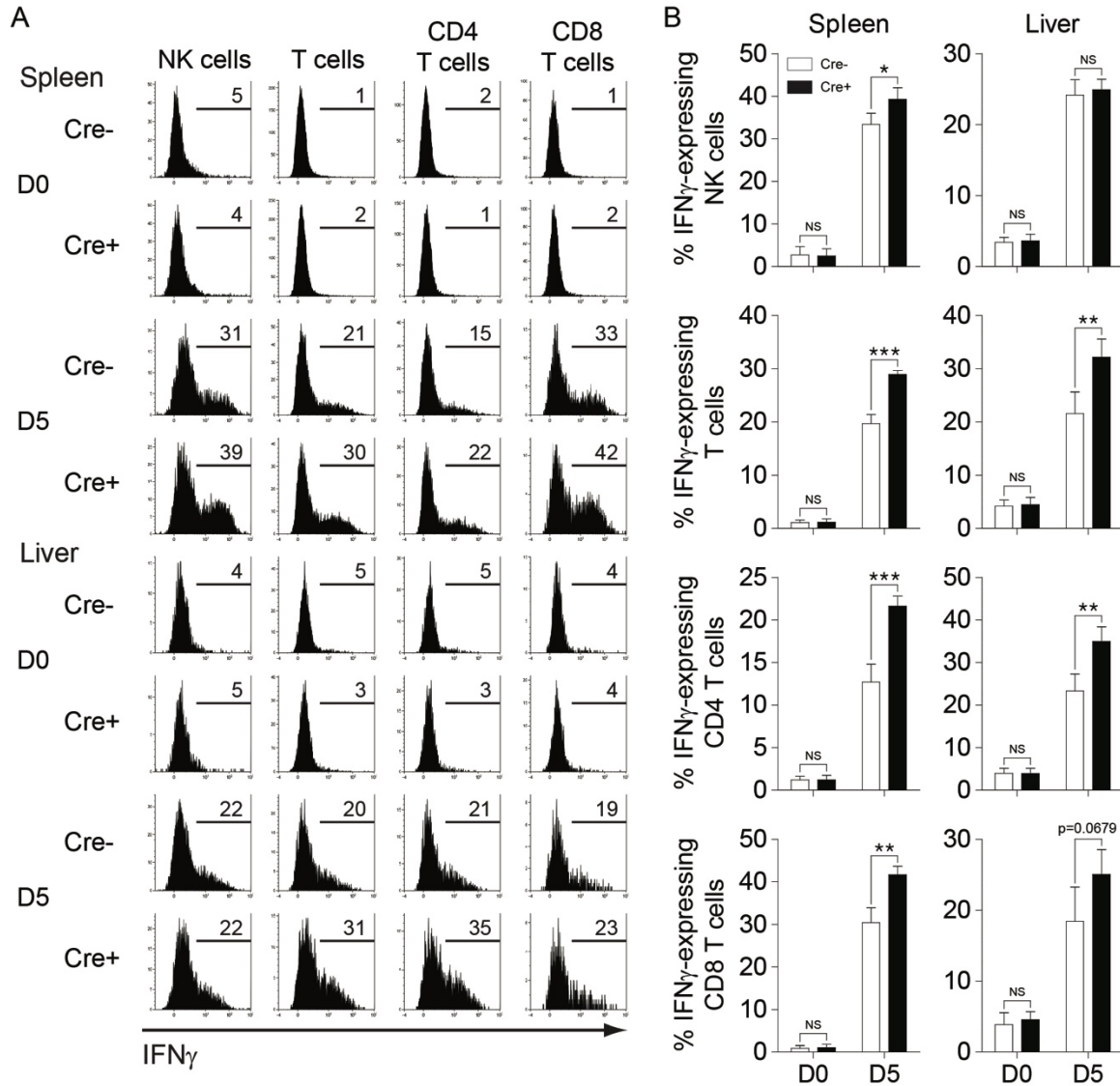


Figure 3.6. NK cell-derived IL-10 regulates T cell activation during sustained MCMV infection. *PKO-Il10^{fl/fl}* (Cre-) and *PKO-NKp46-Cre-Il10^{fl/fl}* (Cre+) mice were either uninfected or given 5,000 PFU MCMV i.p. (**A**, **B**) NK cells, CD4 T cells, and CD8 T cells from the spleens and livers of *PKO-Il10^{fl/fl}* (n = 4) and *PKO-NKp46-Cre-Il10^{fl/fl}* (n = 4) mice at day 5 post-infection were stained for IFN γ production (three experiments). Data represent mean + SD. ns: non-significant; *p < 0.05; **p < 0.01; ***p < 0.001.

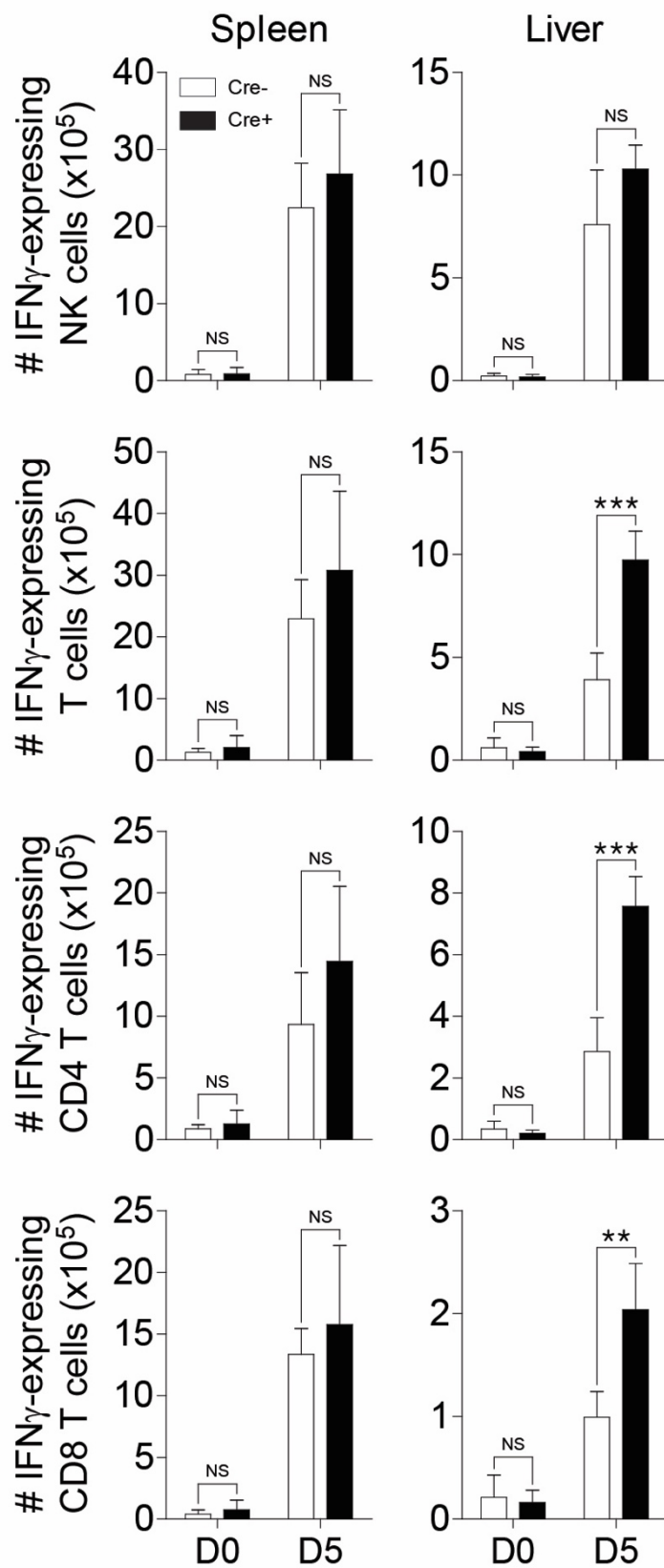


Figure 3.7. NK cell-derived IL-10 regulates the activation of T cells during sustained MCMV infection. *PKO-Il10^{fl/fl}* (Cre-) and *PKO-NKp46-Cre-Il10^{fl/fl}* (Cre+) mice were either uninfected or given 5,000 PFU MCMV i.p. Graphs indicate the number of NK cells, CD4 T cells, and CD8 T cells producing IFN γ in the spleens and livers of *PKO-Il10^{fl/fl}* (Cre-) and *PKO-NKp46-Cre-Il10^{fl/fl}* mice at day 5 post-infection. Data are from one experiment representative of three independent experiments, with four mice per group. Data represent mean + SD. ns: non-significant; **p < 0.01; ***p < 0.001.

3.5. Discussion

NK cells have been well recognized as potent pro-inflammatory cells due to their capability of secreting cytokines such as IFN γ and TNF α that limit microbial growth during the initial stages of infections (211). Furthermore, NK cell-derived IFN γ helps to shape the T cell response in the lymph nodes, possibly through a direct interaction between naïve T cells and NK cells migrating from the site of inflammation to secondary lymphoid organs or by an indirect effect on DCs (362). Notably, several reports have demonstrated that NK cells are potent producers of IL-10, suggesting their role in regulating the immune response (99, 363). In this study, we have demonstrated that NK cells are a key source of IL-10 during the early phases of MCMV infection and that NK cell-derived IL-10 is important to protect the host against liver damage during a persistent virus infection. To evaluate IL-10 production, we employed IL-10-GFP reporter mice (C57BL/6 background). The use of this IL-10-GFP reporter mouse to measure IL-10 expression is advantageous over the intracellular staining of IL-10 because the signals obtained by intracellular staining are generally weak and challenging to interpret. Since the *Gfp* gene is inserted in the 3' untranslated end of the *Il10* gene locus in the reporter mouse, *Gfp* expression is directly correlated to *Il10* gene expression (41). Such mice have been previously used to study the role of intestinal T cell-derived IL-10 in regulating gut inflammation (41), B cell-derived IL-10 during autoimmunity (46) and IL-10 mediated regulation of liver inflammation during acute MCMV infection (28).

Previously, we have demonstrated that IL-10 blockade in persistent MCMV infection resulted in an elevated CD8 T cell response, higher levels of circulating pro-inflammatory cytokines IFN γ and TNF α , and increased viral burden, which led to poor survival of the host (99). Since different immune cell types can participate in the production of circulating IL-10,

blocking IL-10 functions using neutralization antibodies against IL-10 limits our understanding of the role of IL-10 from a particular cell subset. To overcome the limitation, using the Cre induced recombination of *Il10* genomic locus, we generated *NKp46-Cre-Il10^{fl/fl}* mice to elucidate the regulatory role of NK cells during viral infection. In the *NKp46^{iCre}* knock-in mouse, Cre recombinase is expressed under the control of the NKp46 promoter, which is mainly found in NK cells (364-366). The faithful expression of the *Cre* gene in NK cells has been confirmed previously (356). *Il10* floxed mice carry *loxP* sites flanking exon 1 of the *Il10* gene and have been previously used to investigate the effect of T cell (355) and Treg (367) specific IL-10 ablation on the immune response. We confirmed the specific IL-10 deficiency in NK cells from *NKp46-Cre-Il10^{fl/fl}* mice at the genomic and protein level. Whether spontaneous inflammation develops in the *NKp46-Cre-Il10^{fl/fl}* mice was intriguing because both conventional *Il10*^{-/-} mice and T cell-specific *Il10*-deficient mice on the C57BL/6 background develop rectal prolapse and diarrhea that are indicators of severe spontaneous intestinal inflammation at 6 months of age (359). Although Tregs have been known to play a central role in suppressing immune-mediated inflammation, it is possible that additional sources of IL-10 are required to restrain spontaneous gut inflammation. Our analysis of NK cell adhesion molecules and activation status *in vitro* showed the absence of any aberrant inflammation in *NKp46-Cre-Il10^{fl/fl}* mice. Taken together, the *NKp46-Cre-Il10^{fl/fl}* mouse is a useful tool to study the regulatory role of NK cells and can be employed in various infection studies.

To dissect the immunoregulatory role of NK cells during infection, *NKp46-Cre-Il10^{fl/fl}* and their control littermates were infected with MCMV. No significant impact of NK cell-derived IL-10 on the T cell response and the disease outcome was observed. Despite the NK cell-specific IL-10 ablation, the serum levels of IL-10 were only slightly reduced, suggesting

that the production of IL-10 by other cell types such as macrophages, dendritic cells, and Tregs might compensate for the absence of NK cell-specific IL-10 and account for the similar phenotype observed. Previously, CD4 T cell-derived IL-10 was shown to be critical in establishing immunosuppression in the salivary gland, allowing for viral persistence (334, 348). Since NK cells are known to be present in the salivary gland (357), we investigated whether NK cells in the salivary gland could also play an immunosuppressive role to allow viral persistence. However, we observed comparable viral loads and IFN γ response of T cells on day 16 post-infection between *NKp46-Cre-Il10^{fl/fl}* mice and their control littermates, demonstrating that NK cell-derived IL-10 is dispensable in establishing immunosuppression in the salivary gland during MCMV infection.

Regulatory pathways on immune cells have been suggested to be more critical during persistent infections (269, 368). We have previously demonstrated that perforin-deficient mice show elevated virus burdens accompanied by the extensive proliferation of NK cells during MCMV infection (99). Interestingly, IL-10 production is positively regulated in proliferating NK cells via the epigenetic modification of the IL-10 locus (208). Hence, in NK cells that undergo proliferation, the IL-10 gene would be more accessible for transcription, allowing for increased IL-10 production. In order to investigate the importance of NK cell-specific IL-10 during a persistent MCMV model, we crossed perforin-deficient mice with IL-10-GFP reporter mice, and demonstrated that NK cells produce more sustained levels of IL-10 in mice deficient in perforin. Then, we showed that in *PKO-NKp46-Cre-Il10^{fl/fl}* mice, the absence of IL-10 by NK cells resulted in detrimental disease outcome, as exemplified by increased body weight loss, and worsened liver damage accompanied by highly activated T cells. Taken together, NK cell-derived IL-10 is important in regulating the T cell response and in protecting the host from excessive immune-mediated pathology during persistent infection.

A previous study conducted to characterize the inflammatory response in MCMV infected liver has supported the role of cytokines and chemokines in modulating immune-mediated pathology in infected organs (302). This study demonstrated elevated IFN γ production suggesting the role of pro-inflammatory cytokines in mediating pathology at the site of infection. It is conceivable that IL-10 being a potent regulatory cytokine can protect the organ architecture from the immune-mediated pathology by limiting the production of pro-inflammatory cytokines.

The immunoregulatory function of IL-10 production by numerous other cell types has been demonstrated. Previous work demonstrated that myeloid cells produce IL-10 to promote the survival of the host upon *Citrobacter rodentium* infection by limiting IL-23 production (369). In another study, B cell production of IL-10 was shown to regulate the activation of antigen-specific CD8 T cells and the expansion of plasma cells (350). Moreover, the loss of IL-10 function either by genetic deletion of *Il10* gene or by blocking IL-10 receptor results in the accumulation of IFN γ producing CD4 T cells and reduced viral burden, suggesting a role of IL-10 in maintaining an immunosuppressive environment (334, 348). In this report, our data demonstrated that NK cell-derived IL-10 is indispensable in protecting the host from liver damage during persistent infection. Therefore, inducing NK cell regulatory pathway during autoimmune diseases, transplantation-related disorders, and infections with overwhelming inflammation can help restrain excessive immune-mediated inflammatory diseases.

The NK cells are one of the predominant populations of lymphocytes found during early gestation. Interestingly, the human uterine NK cells (uNK) have also been shown to produce IL-10 via IL-2/IL-12 mediated stimulation. The current speculation about uNK derived IL-10 during pregnancy is the protection of fetus from the harmful maternal immune response by inhibiting production of deleterious pro-inflammatory cytokines (370). Therefore,

investigating the immunoregulatory functions of NK cells will generate valuable insights into NK cell-mediated modulation of innate and adaptive immune responses, improve our ability to monitor their role in immune suppression and viral persistence, and perhaps, provide new methods for NK cell-based therapies.

Chapter 4: General Conclusion

Conclusion

In contrast to T cells, the early development of conventional NK cells, which derive from the bone marrow, does not require the transcription factor GATA3 (72, 292). However, GATA3 is expressed in all lineages of ILCs, including NK cells, during their development (67), suggesting its role in NK cell development. In chapter 2, we investigated the role of GATA3 at the late stages of NK cell development, by generating an NK cell-specific *Gata3*-deficient (*NKp46-Cre-Gata3^{fl/fl}*) mouse model where GATA3 deletion occurs upon acquisition of NKp46 expression and following the differentiation of NK cells into the immature NK cell stage. We were able to successfully delete GATA3 only in NK cells of *NKp46-Cre-Gata3^{fl/fl}* mice. Indeed, I demonstrated that GATA3 was abrogated at the genomic and protein level. This deletion of GATA3 was specific to NK cells because T cells, which normally express GATA3, maintained expression of GATA3 protein in *NKp46-Cre-Gata3^{fl/fl}* mice at levels similar to that of control littermates. Other cells such as B cells do not normally express GATA3, and accordingly, B cells from both *NKp46-Cre-Gata3^{fl/fl}* mice and control littermates lacked GATA3 expression, although GATA3 was not deleted at the genomic levels in B cells of *NKp46-Cre-Gata3^{fl/fl}* mice. Taking together, we were able to successfully generate a model of NK cell-specific *Gata3*-deficient mouse.

The maturation of murine NK cells can be dissected into four subsets based on the expression of CD27 and CD11b (18, 19). In a previous study, CD27 SP, DP, and CD11b SP subsets of NK cells were sorted, and their gene expression was analysed by microarray. The less mature CD27 SP subset of NK cells showed enhanced expression of transcripts involved in the cell cycle (such as *Myc* and *Myb*) compared with their CD11b SP counterparts (18). However, increased expression of transcripts involved in cytotoxicity (such as perforin,

granzyme B, and Fas ligand) were found in CD11b SP cells as compared with CD27 SP cells. Notably, DN and CD27 SP cells showed increased rates of proliferation as compared with DP and CD11b SP cells, both at steady-state and during homeostatic proliferation (18). In another study, the authors compared the functional differences between DP and CD11b SP cells, and found that DP cells produce more cytokines and are more cytotoxic than CD11b SP cells (19). In mice, little is known about the response of different NK cell subsets during infections. In humans, CD56^{bright} are thought to be immature NK cells, and the CD56^{dim} are thought to be mature NK cells. CD56^{bright} NK cells produce more cytokines than CD56^{dim} NK cells, whereas CD56^{dim} NK cells are more cytotoxic than CD56^{bright} NK cells (371).

In the absence of GATA3, I demonstrated that NK cells displayed an immature phenotype, characterized by a defect in the ability of NK cells to differentiate beyond the CD27+CD11b- stage of development. As a result, these NK cells failed to egress from the bone marrow to reach peripheral organs, as the frequencies of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice were increased in the bone marrow but decreased in the spleen and liver. This phenotype could be explained by the role of the chemokine receptor CXCR4 in modulating NK cell retention in the bone marrow (31). Less mature NK cells express higher levels of CXCR4 allowing their retention in the bone marrow, where the ligand CXCL12 is abundant. Therefore, as NK cells mature, they lose expression of CXCR4 and are able to exit the bone marrow. Indeed, NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice had higher expression of the chemokine receptor CXCR4 and showed enhanced ability to migrate towards a high gradient of CXCL12 *in vitro*, which was blunted by using the CXCR4 antagonist AMD3100. In addition, *in vivo* treatment of *NKp46-Cre-Gata3^{fl/fl}* mice with AMD3100 allowed the NK cells to mobilize from the bone marrow parenchyma to the sinusoids leading to increased NK cells

in the blood, suggesting the role of GATA3 in promoting NK cells release from the bone marrow through CXCR4 downregulation.

Due to the lack of a suitable antibody to detect S1P5 receptor by flow cytometry, we could not measure the expression of S1P5 on NK cells. Since NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice are immature, they should express low levels of S1P5 as compared to NK cells from wild type mice or control littermates, because the ligand S1P is known to specifically attract mature NK cells to the blood sinusoids and allow NK cells to exit the bone marrow (31). Moreover, NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice had decreased expression of the adhesion molecules CD11b and CD62L, which may also play a role in bone marrow egress and redistribution into peripheral organs. For instance, CD62L is known to be important for the homing of immune cells into peripheral lymph nodes (372).

The loss of GATA3 in NK cells was associated with a reduction in the mRNA levels of key transcription factors, including *E4bp4*, *Id2*, and *T-bet*, which have been shown to be important for NK cell differentiation. In addition, NK cell-specific deletion of GATA3 led to decreased expression of the surface receptors CD62L, CD11b and KLRG1. It remains unknown which of these differences are associated with a shift in the prominence of various NK cell subsets or due to direct interaction of GATA3 with specific target genes. Future studies looking at the ability of GATA3 to bind the promoter of these target genes should be tested using ChIP assay to better understand how GATA3 regulates NK cell differentiation at the molecular level. However, due to the low proportion of NK cells in the mouse spleen, it is challenging to obtain sufficient numbers of purified NK cells to perform ChIP assay.

In the liver, there are two different subsets of NK cells, the CD49a-CD49b+, and CD49a+CD49b- populations. The CD49a-CD49b+ subset is the more abundant population of conventional NK cells that derive from the bone marrow. However, the CD49a+CD49b-

subset of NK cells is believed to originate from hepatic hematopoietic progenitor cells (288). Therefore, these liver-resident NK cells may follow a different developmental pathway. In this report, I found that GATA3 is required for the development of liver-resident NK cells, as the frequencies of CD49a⁺CD49b⁻ NK cells were reduced in the livers of *NKp46-Cre-Gata3^{fl/fl}* mice. These results indicate that GATA3 is required to maintain homeostasis of both conventional and tissue-resident NK cells within the periphery. Consistent with these data, GATA3 has also been shown to be essential for the development of thymus-derived NK cells (16). It is possible that GATA3 is also important for the development and maintenance of other tissue-resident NK cells, such as those found in the uterus.

A previous report showed that GATA3 was required for the production of IFN γ by NK cells, however the authors had tested this function using only a limited number of stimuli (72). Since several mechanisms of NK cell activation can induce IFN γ , I investigated the role of GATA3 in enabling this effector function upon the stimulation with various stimuli, including through engagement of activating receptors and using the stimulatory cytokines IL-12 and IL-18 which are potent inducers of IFN γ . I found that GATA3 was less important for IFN γ production through activating receptors but was required when NK cells were stimulated by cytokines.

NK cells play an essential role in the control of MCMV infection *in vivo*. Therefore, we tested whether *NKp46-Cre-Gata3^{fl/fl}* mice would be more susceptible to MCMV infection as a result of the defect in NK cell maturation. To our surprise, these mice were quite resistant to MCMV infection. Despite the immature phenotype of NK cells in *NKp46-Cre-Gata3^{fl/fl}* mice and their paucity in peripheral organs, these NK cells were able to expand at a higher rate and reach proportions similar or even higher than those of NK cells from the control littermates. In addition, the cytotoxicity of *Gata3*-deficient NK cells was intact. Taken

together, in the absence of GATA3, NK cells are able to exhibit an effective immune response for the control of MCMV infection.

During the acute phase of MCMV infection, there are diverse molecules such as cytokines and chemokines, both host-derived and virus-derived, that may contribute to inflammation. These extrinsic factors may potentially dictate the immune response. It is possible that in *NKp46-Cre-Gata3^{fl/fl}* mice, the increased proliferation of NK cells is due to the activation by excessive extrinsic factors. Thus, to exclude the potential differences in the inflammatory conditions that may occur during MCMV infection *in vivo*, I compared the proliferative capability of NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice and control mice *ex vivo* by stimulating the cells with different concentrations of IL-2 or IL-15. Using this approach, I was able to determine that the enhanced proliferative potential of *Gata3*-deficient NK cells was cell-intrinsic. These results are consistent with another report demonstrating that immature NK cells exhibit higher homeostatic proliferation than their mature counterparts (18). Interestingly, the intrinsic proliferative potential of immature NK cells was associated with an enhanced ability to upregulate CD25, known to increase the sensitivity of cells to stimulation via IL-2.

Previous reports have demonstrated the ability of NK cells to produce IL-10 during MCMV infection (99, 333). However, the complete kinetics of IL-10 production by NK cells was never clearly demonstrated throughout the course of MCMV infection. In chapter 3, we were able to demonstrate that NK cells and T cells participate in a coordinated regulatory response, where NK cells are major producers of IL-10 early during acute MCMV infection, while T cells exhibit a delayed response, resulting in a division of labor to sustain appropriate levels of IL-10. Interestingly, the proportion of liver-resident NK cells was diminished during

MCMV infection, and these cells were able to produce IL-10 but at lower levels than bone marrow-derived conventional NK cells. Therefore, the low frequencies of liver-resident NK cells in combination with their reduced capacity of producing IL-10 suggests that they may play a minor regulatory role during MCMV infection.

Since many cell types can produce IL-10, the use of neutralizing antibodies against IL-10 or blocking antibodies targeting the IL-10 receptor limits our understanding of the relative contribution of specific cell types in regulating the immune response through IL-10. Such limitations also arise with the use of IL-10-deficient mice. Furthermore, IL-10-deficient mice develop spontaneous inflammatory bowel disease, making them an inadequate model for the study of IL-10 during infection. Therefore, to dissect the role of NK cell-derived IL-10, we generated a mouse model in which NK cells specifically lack IL-10 (*NKp46-Cre-Il10^{fl/fl}*). Effectively, this mouse model generated NK cells that failed to produce IL-10.

Notably, the loss of IL-10 in NK cells from *NKp46-Cre-Il10^{fl/fl}* mice did not affect their proportions in the periphery. More importantly, their maturation level and expression of activating and inhibitory receptors were normal, suggesting that they are intrinsically similar to NK cells from control littermates. Since T cell-derived IL-10 is required to maintain gut homeostasis as evidenced by the spontaneous inflammation that occurs in the colon of *CD4-Cre-Il10^{fl/fl}* mice, we tested whether NK cell-derived IL-10 may also contribute in regulating inflammation in the gut at the steady-state. However, six months old *NKp46-Cre-Il10^{fl/fl}* mice did not show any signs of spontaneous inflammation. Taken together, we showed that *NKp46-Cre-Il10^{fl/fl}* mouse is an adequate model to test the role of NK cell-derived IL-10 during MCMV infection.

Considering that NK cells are major producers of IL-10 during MCMV infection, it was intriguing to investigate the role of NK cell-derived IL-10 in regulating immune responses

during infection and how it would impact disease manifestation. However, in immunocompetent hosts, NK cell-derived IL-10 did not influence the outcome of the disease since both *NKp46-Cre-Il10^{fl/fl}* and *Il10^{fl/fl}* mice showed comparable body weight change, similar viral burden in their spleens and livers, and similar degree of liver damage based on the concentrations of liver enzyme in the serum. In fact, the systemic levels of IL-10 were only moderately reduced in *NKp46-Cre-Il10^{fl/fl}* mice, while the levels of pro-inflammatory cytokines were comparable with those in the control mice. Additionally, NK cell-derived IL-10 had little impact in regulating the T cell response in immunocompetent mice.

The regulation of immune response during persistent infection is critical and needs to be fine-tuned to prevent immune-mediated tissue damage while allowing the clearance of the pathogen. Thus, the role of NK cell-derived IL-10 may be more significant during sustained infection. To test this hypothesis, I infected perforin-deficient mice with MCMV as a model of sustained viral infection. Using this model, I studied the kinetics of IL-10 production by NK cells. Interestingly, while NK cells in immunocompetent host produce acute levels of IL-10, in perforin-deficient mice, NK cells produced more sustained levels of IL-10, suggesting a demanding requirement for regulatory signals mediated by IL-10 during persistent infections. Perforin-deficient mice have been shown to have a higher viral burden compared to wild type mice (99), presumably as a consequence of the inability of NK cells to kill the infected cells via a perforin-dependent manner. However, whether the NK cells from perforin-deficient mice produce more sustained levels of IL-10 because of the high viral titer is unclear. One possible explanation for the increased levels of IL-10 is that NK cells from perforin-deficient mice undergo extensive proliferation. Consistently, mice that lack both perforin and Ly49H expression have comparable viral burdens with that in mice lacking perforin alone, except that

in the absence of Ly49H, NK cells fail to proliferate and are poor producers of IL-10, suggesting that antigen-specific NK cell expansion is critical for IL-10 production.

Since NK cells from perforin-deficient mice produce IL-10 for a longer duration throughout the course of MCMV infection, determining the regulatory role of this sustained level of IL-10 was more promising. Interestingly, although MCMV-infected perforin-deficient mice suffered more bodyweight loss in the absence of NK cell-derived IL-10, no difference in the viral burden was observed in their spleens and livers, as compared with their control littermates. Instead, this increased body weight loss was attributed to increased liver damage and could be due to increased immunopathology since T cells from *PKO-NKp46-Cre-Il10^{fl/fl}* mice were hyperactivated as compared to T cells from *PKO-Il10^{fl/fl}* mice. Taken together, these data suggest that NK cell-derived IL-10 reduces tissue injury by regulating the adaptive immune response.

In this study, we have shown that IL-10 can regulate T cell activation during persistent viral infection. Although the role of IL-10 on antitumor responses has been controversial, numerous lines of evidence suggest that IL-10 can suppress tumor growth. For example, tumor cells that produce more IL-10 induce enhanced tumor regression and show reduced tumor-infiltrating macrophages, and increased T cell cytotoxic function (373-375). Other studies have found that systemic delivery of IL-10 induces tumor regression (376, 377). The pegylated form of IL-10 has been used to allow sustained levels of serum IL-10 and was shown to induce higher cytotoxicity, proliferation and IFN γ production by T cells in tumor-bearing mice (373). Although there are a few preclinical trials being conducted using IL-10 cytokine or anti-IL-10 immunoglobulin for the treatment of cancer and autoimmune diseases, none of these treatments has been approved yet (378). A pegylated version of the human IL-10 is currently

in preclinical trial and has shown some promising results as a monotherapy, with 4 out 15 patients with renal cell cancer responding to the treatment (379, 380).

Concluding remarks

NK cells are innate immune cells that play an essential role in controlling viral infections. Thus, understanding how they develop from the bone marrow will provide valuable insight into diseases caused by NK cell deficiencies. In chapter 2, I showed that GATA3 is important for the terminal maturation of NK cells (Figure 4.1). More specifically, GATA3 is required for the transition of the CD27⁺CD11b⁻ subset into the CD27⁺CD11b⁺ subset. As a consequence, *Gata3*-deficient NK cells are retained in the bone marrow, and fewer cells are able to egress and reach peripheral organs such as the spleen (Figure 4.1). Surprisingly, GATA3 deletion in NK cells is not required for the proliferation and cytotoxic functions of NK cells during MCMV infection. In fact, in the absence of GATA3, NK cells proliferate robustly, at a higher rate than wild type NK cells (Figure 4.1). In addition, their cytotoxic function is equal to that of wild type NK cells.

These results will inspire us to discover deleterious mutations in the *Gata3* gene within the human population that could lead to defects in NK cell development. Methods to reintroduce functional GATA3 protein through gene editing or via gene overexpression could be used to restore the maturation of NK cells in patients with mutations in *Gata3* gene. Such manipulations could be performed *ex vivo* on expanded autologous NK cells or hematopoietic stem cells to be used for adoptive transfer. In addition, patients who suffer from a decreased number of peripheral NK cells due to defect in maturation could be treated with a CXCR4 antagonist *in vivo* to promote the egress of immature NK cell from the bone marrow to the periphery where they can fight infections and cancer.

Several studies are aiming to improve the *ex vivo* expansion of NK cells for cancer immunotherapy (381-383). Since I showed that *Gata3*-deficient NK cells exhibit enhanced

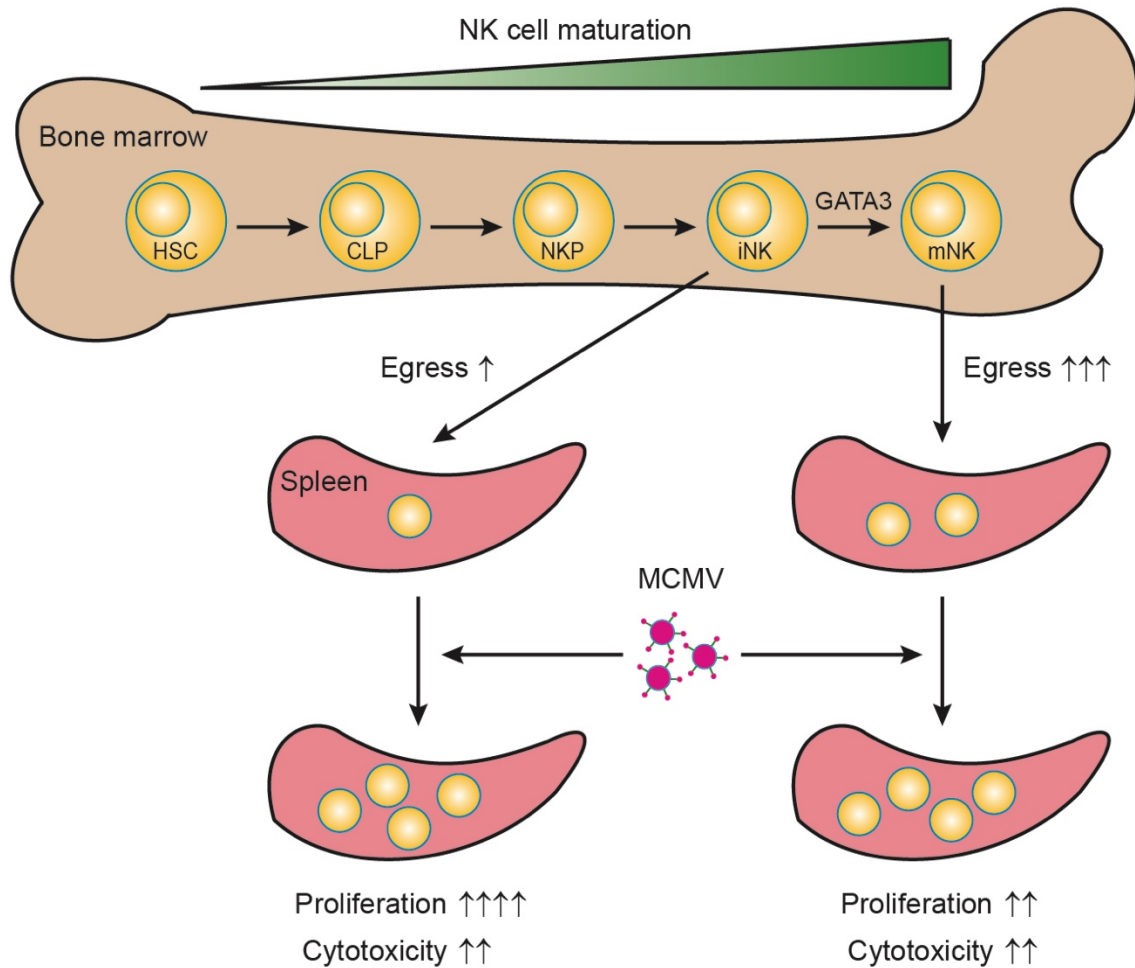


Figure 4.1. GATA3 is essential for the terminal maturation of NK cells. The developmental program of NK cells, starting from hematopoietic stem cells (HSCs) mainly occurs in the bone marrow. The transition of immature NK (iNK) cells into mature NK (mNK) cells is mediated by GATA3. iNK cells are less efficient than mNK cells at exiting the bone marrow. As a consequence, mNK cells are better than iNK at seeding the peripheral organs such as the spleen and liver. Interestingly, upon challenge with murine cytomegalovirus (MCMV), the iNK cells in the periphery are able to expand extensively and reach numbers equivalent to those of mNK cells. This enhanced proliferative potential of iNK cells is cell-intrinsic. In addition, the cytotoxic functions of iNK cells are equivalent to those of mNK cells during MCMV infection.

intrinsic proliferation, pharmacological inhibition of GATA3 in NK cells might be beneficial for ex vivo expansion of NK cells where the higher proliferative activity of NK cells is desirable. In addition, current therapies using NK cells for adoptive transfer require the pre-activation of NK cells with IL-12/15/18 (148), which induces high expression of IL-2R α (CD25) and increases the sensitivity of NK cells to IL-2, which is normally found at low concentrations *in vivo* (384, 385). Since I have shown the higher expression of IL-2R α on NK cells from *NKp46-Cre-Gata3^{fl/fl}* mice, I envision that inhibition of GATA3 can induce IL-2R α expression on NK cells. Taken together, these findings will advance our understanding of essential transcription factors that control the differentiation and functions of NK cells and will prompt us to target downstream proteins in ways to improve NK cell-based therapies to fight microbial infections and cancers.

The pro-inflammatory functions of NK cells during infections are well documented in the literature. Upon recognition of an MCMV-infected cell, NK cells are activated and will execute their main effector functions, including killing the infected cell, producing the pro-inflammatory cytokine IFN γ , and proliferating (Figure 4.2). Few studies have investigated the regulatory functions of NK cells. Previously, an interesting report has shown that the proliferation of NK cells induces chromatin remodeling at the *Il10* locus from a closed chromatin state to an open chromatin state, leading to enhanced IL-10 production (208). Thus, as NK cells proliferate, they acquire the ability to produce the anti-inflammatory cytokine IL-10 and can contribute in regulating inflammation. In chapter 3, we demonstrated that NK cells are major producers of IL-10 during the early phase of MCMV infection. Moreover, during sustained MCMV infection, I showed that NK cell-derived IL-10 inhibits the production of

In *Perforin*-deficient mice

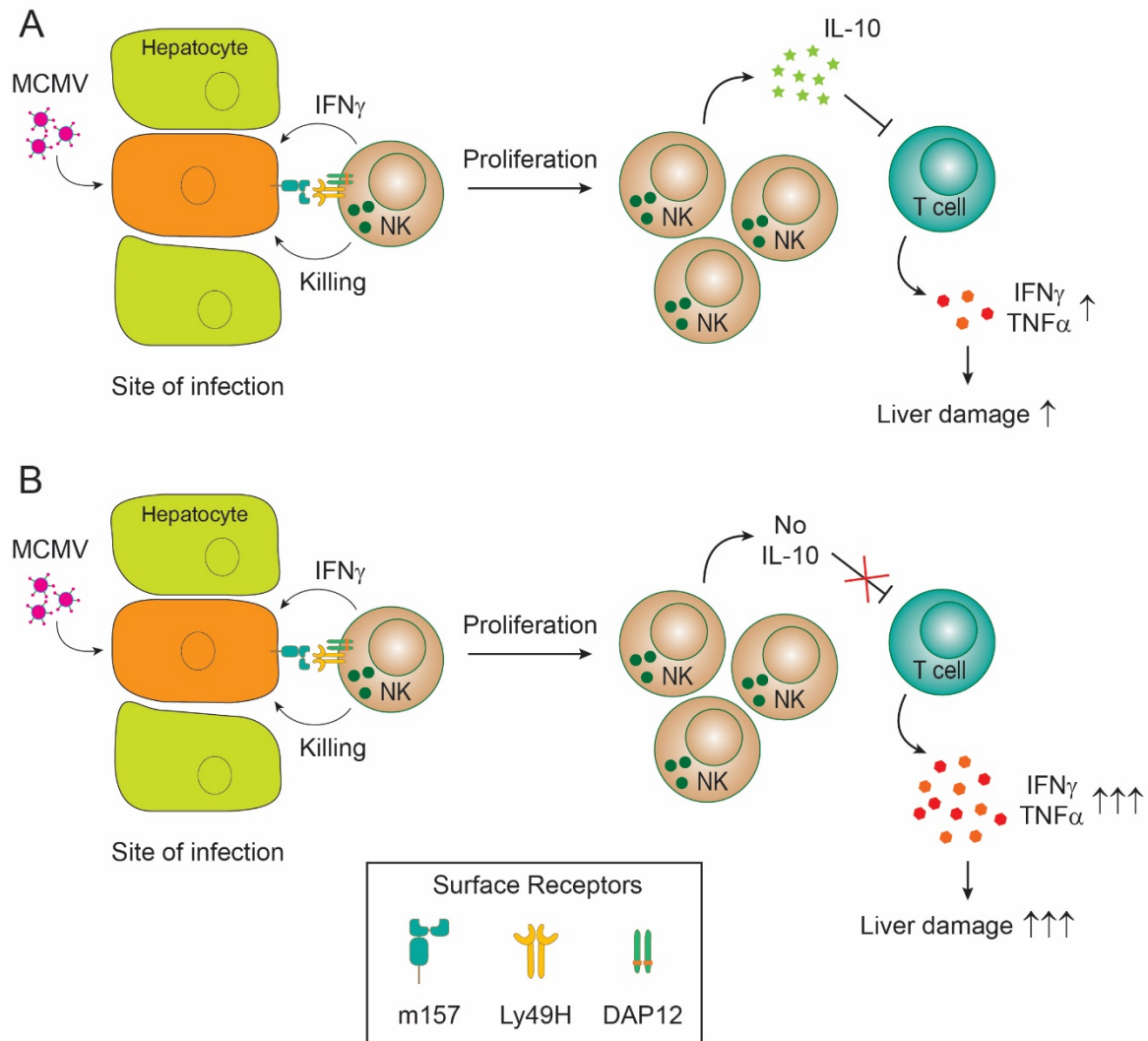


Figure 4.2. NK cell-derived IL-10 plays a key role in regulating T cell response during sustained murine cytomegalovirus (MCMV) infection. MCMV-infected cells, for example hepatocytes, will express activating ligands on their surface, such as the viral protein m157. m157 is an MHC class I homolog that is recognized by NK cell activating receptor Ly49H. Upon engagement of Ly49H receptor, NK cells will exhibit their effector functions, including the early production of pro-inflammatory cytokine IFN γ , the killing of infected cells, and cellular division. **(A)** The proliferation of NK cells is associated with production of the anti-inflammatory cytokine IL-10 which regulates the function of T cells. In particular, IL-10 reduces the production of IFN γ and TNF α by T cells at later stages of infection. **(B)** In the absence of NK cell-derived IL-10 during sustained MCMV infection, T cells will secrete exaggerated levels of IFN γ and TNF α cytokines leading to increased liver damage.

pro-inflammatory cytokines such as IFN γ and TNF α by T cells and prevents liver damage (Figure 4.2A). Thus, *Il10*-deficient NK cells will fail to limit the production of pro-inflammatory cytokines by T cells, which would lead to increased liver damage (Figure 4.2B).

These findings will help us identify chronically-infected patients that suffer from tissue injury due to defects in IL-10 production or exaggerated levels of pro-inflammatory cytokines such as IFN γ and TNF α . Exogenous delivery of IL-10 or inhibitors of pro-inflammatory cytokines could be used to reduce tissue damage in these patients. Another option could be to generate ex vivo expanded autologous NK cells with enhanced production of IL-10 via genetic engineering, which could be used for the adoptive transfer to prevent tissue damage. Therefore, these studies provide us with better insight into the functions of regulatory NK cells and highlight the potential benefit of using IL-10 as a treatment to prevent tissue injury during persistent viral infections.

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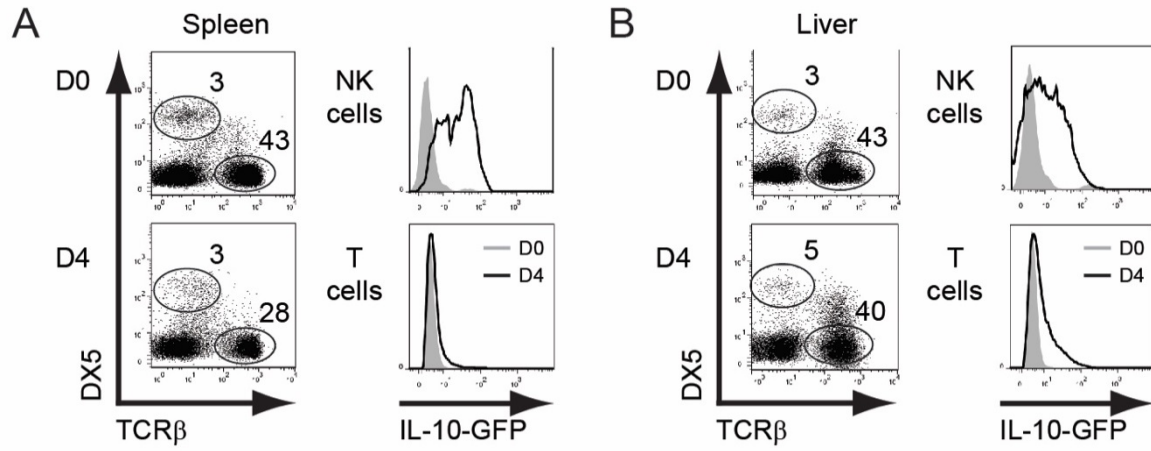
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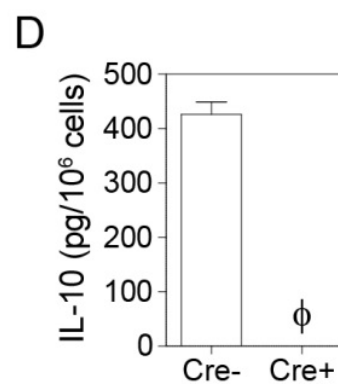
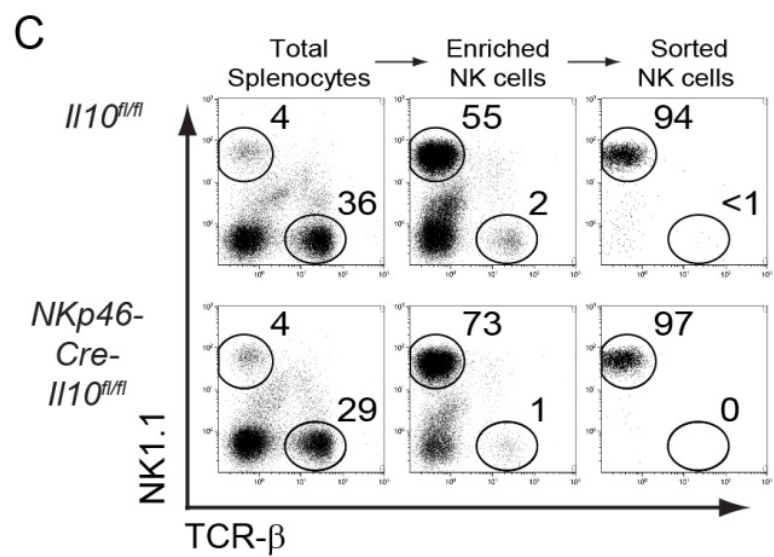
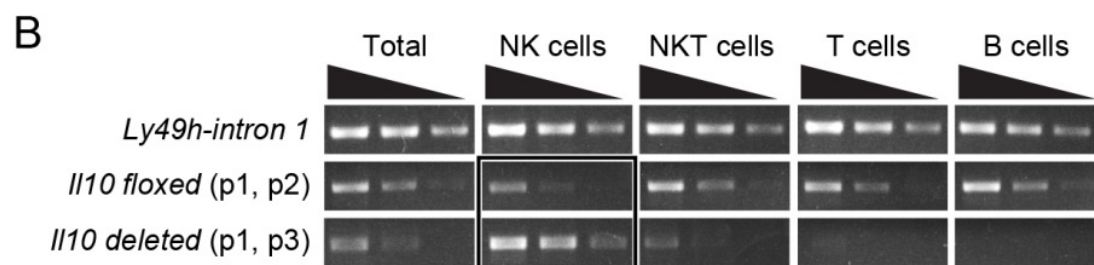
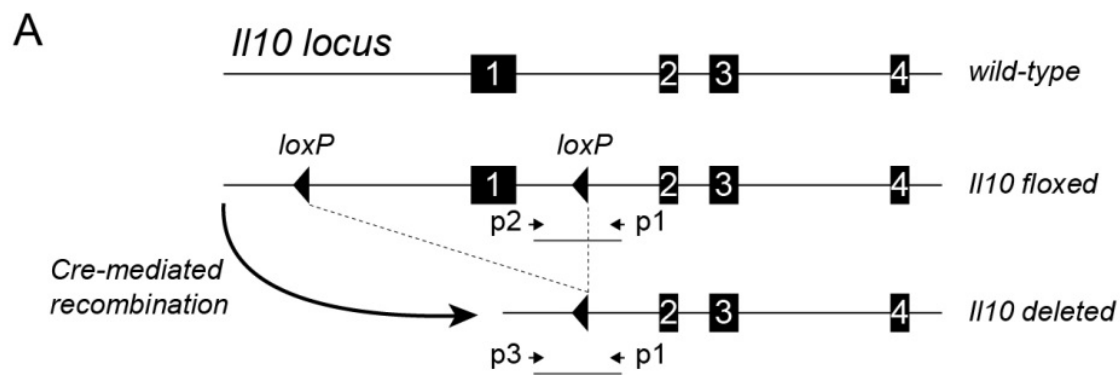
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Appendix

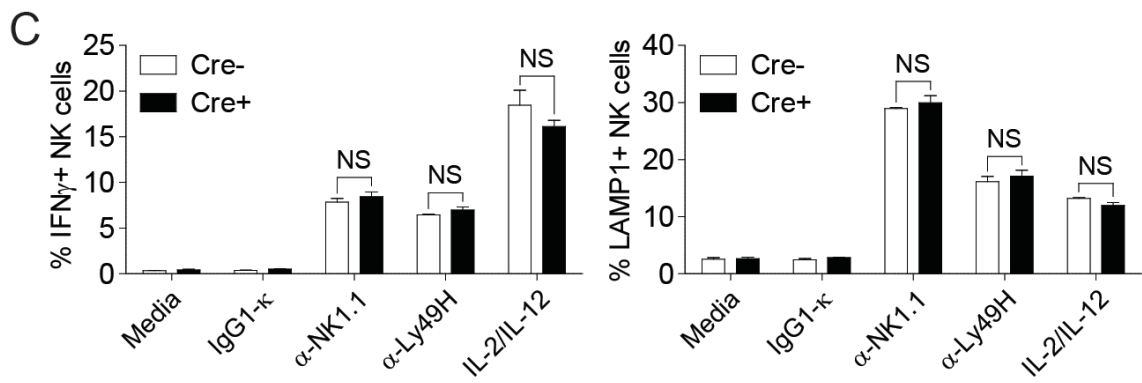
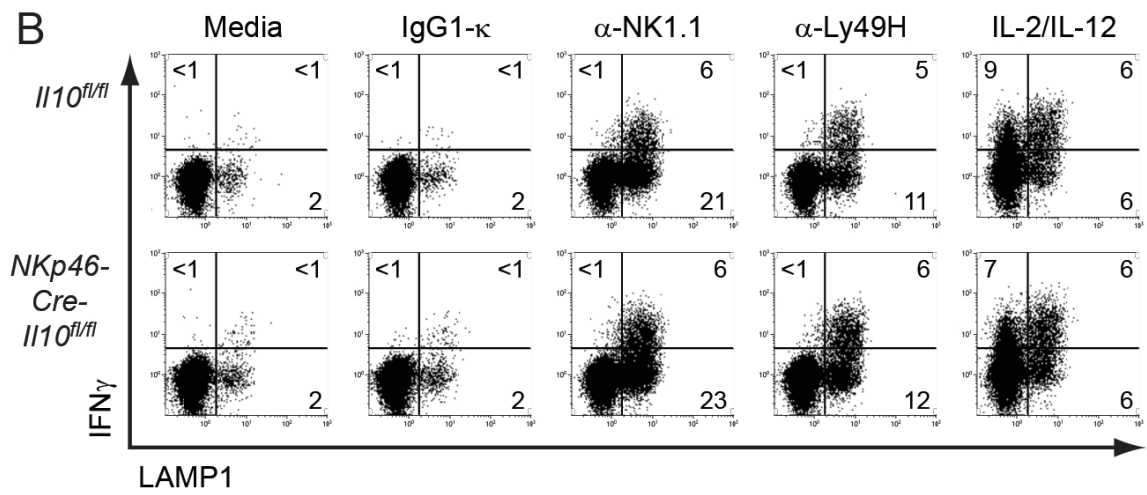
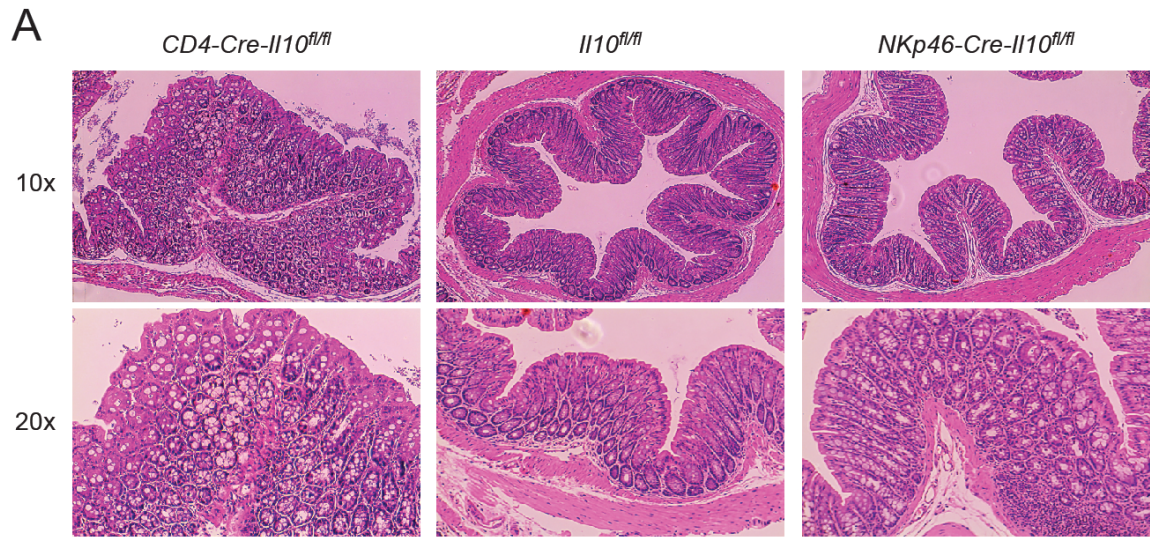


Supplementary Figure 1. NK cells are major producers of IL-10 during the early stages of MCMV infection. IL-10-GFP mice were either uninfected or challenged with MCMV i.p. and the splenic and hepatic lymphocytes were analyzed on the indicated days. The overlay plots depict IL-10-GFP expression in NK cells and T cells in the spleens (**A**) and livers (**B**) of uninfected (D0) and infected (D4) mice (two experiments).

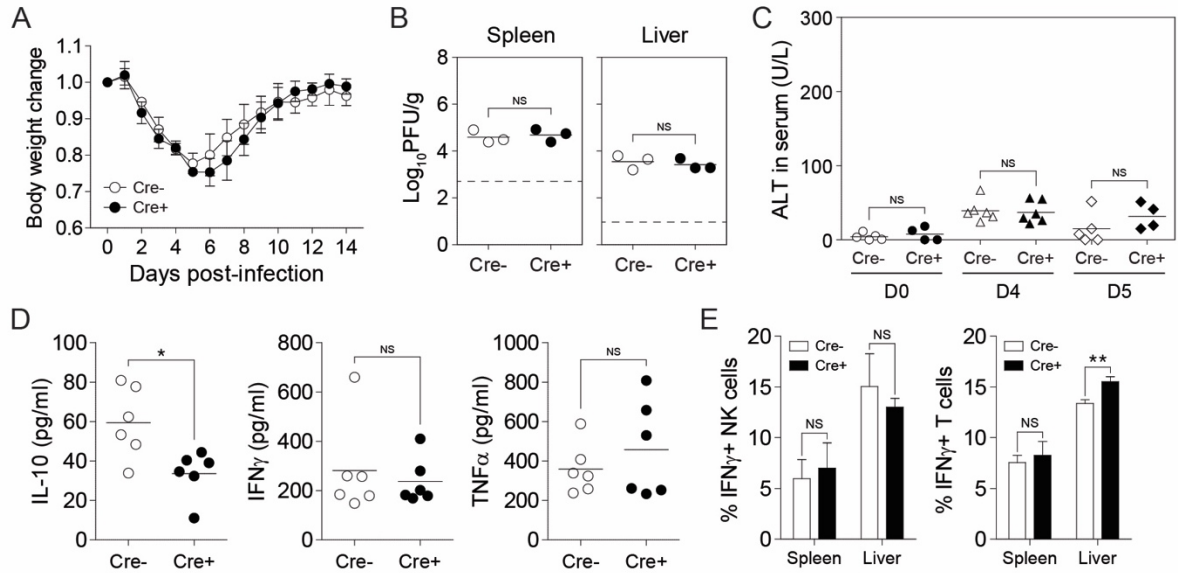


Supplementary Figure 2. Deletion of *Il10* gene (exon 1) occurs predominantly in NK cells.

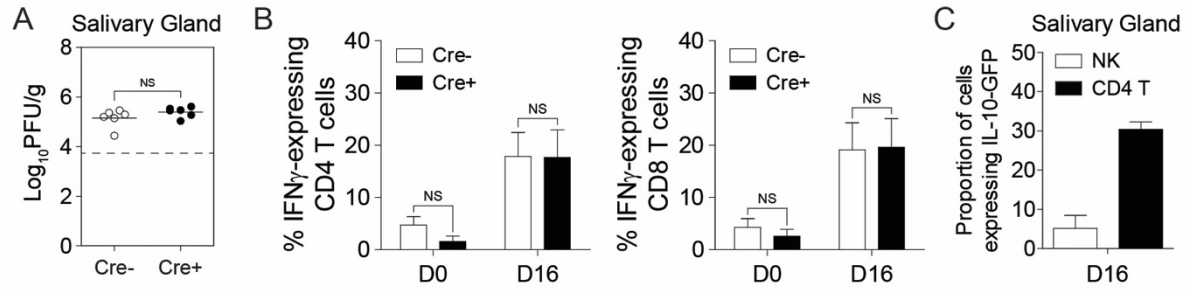
Genomic deletion of *Il10* and protein expression of IL-10 in *NKp46-Cre-Il10^{fl/fl}* mice and control littermates. **(A)** In *Il10^{fl/fl}* mice, exon 1 of *Il10* gene is flanked with *loxP* sites. Crossing *Il10^{fl/fl}* mice with *NKp46-Cre* mice allows for Cre-mediated recombination to occur, leading to exon 1 deletion. The schematic diagram shows the location of gene-specific primers used to determine Cre-mediated recombination in *NKp46-Cre-Il10^{fl/fl}* mice. **(B)** Sorted NK, NKT, T, and B cells from *NKp46-Cre-Il10^{fl/fl}* mice were genotyped to determine the presence of *Il10*-floxed allele and *Il10*-deleted allele. Semiquantitative PCR was performed on 5-fold serial dilutions of genomic DNA (50ng, 10ng, and 2ng) from the indicated cell populations (two experiments). **(C)** NK cells purity following magnetic cell enrichment and flow sorting. Flow cytometric analysis shows the purity of NK cells from *Il10^{fl/fl}* (Cre-) and *NKp46-Cre-Il10^{fl/fl}* (Cre+) mice after NK cell enrichment followed by sorting. Data are from one experiment representative of two independent experiments (*two experiments*). **(D)** Highly purified NK cells from *Il10^{fl/fl}* (Cre-) and *NKp46-Cre-Il10^{fl/fl}* (Cre+) mice were stimulated with IL-2/IL-12 for 18 hours. The IL-10 production in conditioned media was analyzed by CBA (n = 3; two experiments). Data represent mean + SD. ns: non-significant. ϕ : not detected.



Supplementary Figure 3. No signs of spontaneous inflammation observed in *NKp46-Cre-Il10^{fl/fl}* mice. (A) Colon histology of 6 months old *CD4-Cre-Il10^{fl/fl}*, *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice is performed (n = 3; two experiments). Tissues are fixed in 10% formaldehyde and paraffin-embedded. Histological sections are shown at the indicated magnification. (B) Representative plots of intracellular IFN γ and LAMP1 expression on NK cells from *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice upon stimulation with the indicated antibodies or cytokines. (C) Percentage of IFN γ -producing and LAMP1-expressing NK cells from *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice upon stimulation with the indicated antibodies or cytokines (n = 2; two experiments). Data represent mean + SD. ns: non-significant.



Supplementary Figure 4. NK cell-derived IL-10 is dispensable during acute MCMV infection. *Il10^{fl/fl}* (Cre-) and *NKp46-Cre-Il10^{fl/fl}* (Cre+) mice were either uninfected or given 12,000 PFU or 50,000 PFU MCMV i.p. for the following analyses: **(A)** Body weight change of infected mice relative to their initial body weight at day 0 post-infection (n = 3; two experiments). **(B)** Viral titer in the spleen of *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice at day 5 post-infection (n = 3; two experiments). **(C)** Serum levels of ALT enzyme at the indicated days post-infection (three experiments; n = 5 for D0 Cre-, n = 4 for D0 Cre+, n = 6 for D4 Cre-, n = 6 for D4 Cre+, n = 5 for D5 Cre-, n = 4 for D5 Cre+). **(D)** Serum levels of IL-10, IFN γ , and TNF α were measured at day 4 post-infection (n = 6; two experiments). **(E)** NK cells and T cells from the spleens and livers of *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice at day 5 post-infection were stained for IFN γ production (n = 3; two experiments). Data represent mean + SD. ns: non-significant; *p < 0.05; **p < 0.01.



Supplementary Figure 5. NK cell derived IL-10 is not important to control viral persistence in the salivary gland during MCMV infection. *Il10^{fl/fl}* (Cre-) and *NKp46-Cre-Il10^{fl/fl}* (Cre+) mice were either uninfected or given 50,000 PFU MCMV i.p. **(A)** Viral titer in the salivary glands of *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice at day 16 post-infection. **(B)** CD4 T cells and CD8 T cells from the salivary glands of *Il10^{fl/fl}* and *NKp46-Cre-Il10^{fl/fl}* mice at day 16 post-infection were stained for IFN γ production. **(C)** Proportions of NK cells and CD4 T cells from the salivary glands of IL-10-GFP mice infected with 5,000 PFU MCMV i.p. at day 16 post-infection. For A, data are from one experiment representative of three independent experiments, with six mice per group. For B, data are pooled from three independent experiments, with four to thirteen mice per group. For C, data are from one experiment representative of two independent experiments, with two mice per group. Data represent mean + SD. ns: non significant.

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(option in Microbiology and Immunology)
University of Ottawa, Ottawa, Ontario, Canada
Overall GPA of 8.3/10 *Magna Cum Laude*

Publications

1. **Alaa Kassim Ali**, Amandeep Kaur Komal, Saeedah Musaed Almutairi, and Seung-Hwan Lee. (2019) Natural killer cell-derived IL-10 prevents liver damage during sustained murine cytomegalovirus infection. *Front Immunol.* **10**: 2688.
2. Soonho Kweon, Minh-Trang Thi Phan, Sejong Chun, HongBi Yu, Jinho Kim, Seokho Kim, Jaemin Lee, **Alaa Kassim Ali**, Seung-Hwan Lee, Sang-ki Kim, Junsang Doh, Duck Cho. (2019) Expansion of human NK cells using K562 cells expressing OX40 ligand and short exposure to IL-21. *Front Immunol.* **10**: 879.
3. Saeedah Musaed Almutairi*, **Alaa Kassim Ali***, William He, Doo-Seok Yang, Peyman Ghorbani, Lisheng Wang, Morgan D Fullerton and Seung-Hwan Lee. (2019) Interleukin-18 up-regulates amino acid transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. *J Biol Chem.* **294**: 4644-4655. *Equal contribution (Article was selected to appear in a special virtual issue on "Immunometabolism" at *JBC* as part of the exciting advances in immunometabolism in recent years)

Publications (continued)

4. Minh-Trang Phan, Sejong Chun, Sun-Hee Kim, **Alaa Kassim Ali**, Seung-Hwan Lee, Seokho Kim, Soo-Hyun Kim and Duck Cho. (2017) Natural Killer Cell Subsets and Receptor Expression in Peripheral Blood Mononuclear Cells of a Healthy Korean Population: Reference Range, Influence of Age and Sex, and Correlation between NK Cell Receptors and Cytotoxicity. *Hum Immunol.* **78**: 103-112.
5. **Alaa Kassim Ali**, Jun Seok Oh, Eric Vivier, Meinrad Busslinger and Seung-Hwan Lee. (2016) NK cell-specific Gata3 ablation identifies the NK cell maturation program required for bone marrow exit and control of proliferation. *J Immunol.* **196**: 1753-1767.
6. Jun S. Oh, **Alaa K. Ali**, Sungjin Kim, Daniel J. Corsi, Curtis L. Cooper and Seung-Hwan Lee. (2016) NK cells lacking FcεRIγ are associated with reduced liver damage in hepatitis C virus infection. *Eur J Immunol.* **46**: 1020-1029.
7. **Alaa Kassim Ali**, Neethi Nandagopal and Seung-Hwan Lee. (2015) IL-15-PI3K-AKT-mTOR: a critical pathway in the life journey of natural killer cells. *Front. Immunol.* **6**: 355.
8. Neethi Nandagopal, **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. (2014) The Critical Role of IL-15–PI3K–mTOR Pathway in Natural Killer Cell Effector Functions. *Front Immunol.* **5**: 187. (Article was selected as one of the most relevant pieces of research published across the various specialties)

Oral presentations

1. Elucidating the role of Natural Killer cell derived IL-10 on T cell activation during a sustained viral infection. At the BMI Seminar Day 2019, University of Ottawa, Ottawa, Ontario, February 2019. (**Won 3rd Place**)
2. Elucidating the mechanism of Natural Killer cell functional switch to IL-10 producers. At the Work in Progress (WIP) Seminar, University of Ottawa, Ottawa, Ontario, March 2017.
3. Elucidating the mechanism of Natural Killer cell functional switch to IL-10 producers. At the BMI Seminar Day 2017, University of Ottawa, Ottawa, Ontario, March 2017. (**Won 1st Place**)
4. Investigation of the inflammatory milieu inducing natural killer cell maturation during virus infection and tumorigenesis. At the 5th annual symposium on Cytokines in Inflammation, Ageing, Cancer and Obesity, Eastman, Quebec, Canada, May 2016. (**Selected for a short talk, Won 2nd place for Oral presentation**)

Oral presentations (continued)

5. Investigation of the inflammatory milieu inducing natural killer cell maturation during virus infection and tumorigenesis. At the 29th Annual Spring Meeting of the Canadian Society for Immunology, Ottawa, Ontario, Canada, April 2016. **(Selected for a short talk in this national conference)**
6. Natural killer cell-specific Gata3 ablation identifies the maturation program required for bone marrow exit and control of proliferation. At the Work in Progress Seminar, University of Ottawa, Ottawa, Ontario, November 2015.
7. GATA-3 regulates the NK cell maturation program required for bone marrow exit and proliferation. At the 15th Meeting of the Society for Natural Immunity, Montebello, Quebec, Canada, May 2015. **(Selected for a short talk in this international conference, Won Travel Award)**
8. GATA-3 regulates the NK cell maturation program required for bone marrow exit and proliferation. At the BMI Seminar Day 2015, University of Ottawa, Ottawa, Ontario, March 2015. **(Won 2nd Place).**
9. Gave a talk at the OHRI-uOttawa Immunology Journal club in November 2014. Presented an article entitled: The adaptor TRAF3 restrains the lineage determination of thymic regulatory T cells by modulating signaling via the receptor for IL-2.
10. The Role of GATA-3 in Natural Killer Cell Development. At the Work in Progress Seminar, University of Ottawa, Ottawa, Ontario, October 2014.

Poster presentations

1. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Investigating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection. At the 8th Annual Symposium on Cytokines in Inflammation, Ageing, Cancer & Obesity, St. Camille, Quebec, May 2019.
2. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Investigating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection. At the 32th Annual Spring Meeting of the Canadian Society for Immunology, Banff, Alberta, April 2019.
3. Saeedah Almutairi, **Alaa Kassim Ali**, William He, Doo-Seok Yang, and Seung-Hwan Lee. IL-18 upregulates nutrient transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. At the 3th uOttawa Flow Cytometry Symposium, Nanoscale Flow Cytometry for Cancer, Infection and Disease, University of Ottawa, Ottawa, Ontario, May 2018.

Poster presentations (continued)

4. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Elucidating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection. At the 31th Annual Spring Meeting of the Canadian Society for Immunology, London, Ontario, June 2018.
5. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Elucidating the role of natural killer cell derived IL-10 in regulating T cell activation during a sustained viral infection. At the 7th Annual Symposium on Cytokines in Inflammation, Ageing, Cancer & Obesity, St. Camille, Quebec, May 2018.
6. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Investigating the role of natural killer cell derived IL-10 in regulating T cell activation during a sustained viral infection. At the BMI Scientific Symposium 2018, Montebello, Quebec, May 2018.
7. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Elucidating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection. At the BMI Poster Day 2018, University of Ottawa, Ottawa, Ontario, May 2018.
8. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Elucidating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection. At the 30th Annual Canadian Student Health Research Forum, Winnipeg, Manitoba, June 2017.
9. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Investigating the role of natural killer cell derived IL-10 in modulating T cell activation during a sustained viral infection. At the 6th Annual Symposium on Cytokines in Inflammation, Ageing, Cancer & Obesity, Eastman, Quebec, May 2017.
10. **Alaa Kassim Ali**, Amandeep Kaur Komal and Seung-Hwan Lee. Elucidating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection. At the 30th Annual Spring Meeting of the Canadian Society for Immunology, Banff, Alberta, April 2017.
11. Jun S. Oh, **Alaa Ali**, and Seung-Hwan Lee. Investigation of NK cell proportion and function during IFN-free direct-acting antivirals therapy of chronic HCV. At the 6th Canadian Symposium on Hepatitis C Virus (CSHCV), Banff, Canada, March 2017.
12. **Alaa Kassim Ali** and Seung-Hwan Lee. Investigation of the inflammatory milieu inducing natural killer cell maturation during virus infection and tumorigenesis. At the BMI Poster Day 2016, University of Ottawa, Ottawa, Ontario, May 2016.

Poster presentations (continued)

13. **Alaa Kassim Ali**, Jun Seok Oh, Eric Vivier, Meinrad Busslinger and Seung-Hwan Lee. Investigation of the inflammatory milieu inducing natural killer cell maturation during virus infection and tumorigenesis. At the 29th Annual Spring Meeting of the Canadian Society for Immunology, Ottawa, Ontario, April 2016. (**Won CSI Poster Award**)
14. Jun Seok Oh, **Alaa Kassim Ali**, Sungjin Kim, Daniel J. Corsi, Curtis L. Cooper and Seung-Hwan Lee. NK cells lacking FcεRIγ are associated with reduced liver damage in chronic HCV infection. At 5th Canadian Symposium on HCV, Montreal, Quebec, February 2016.
15. **Alaa Kassim Ali**, Eric Vivier, Meinrad Busslinger and Seung-Hwan Lee. Gata3 regulates the maturation program required for bone marrow exit and proliferation in Natural Killer cells. At the 15th Meeting of the Society for Natural Immunity, Montebello, Quebec, May 2015.
16. Jun Seok Oh, **Alaa Kassim Ali**, Curtis Cooper and Seung-Hwan Lee. Physical and functional characteristics, and maintenance of novel HCMV-associated ADCC-potent NK subset during chronic HCV infection. At the 15th Meeting of the Society for Natural Immunity, Montebello, Quebec, May 2015.
17. **Alaa Kassim Ali**, Amandeep Kaur Komal, and Seung-Hwan Lee. The Role of GATA-3 in Natural Killer Cell Development. At 27th Annual Meeting of the Canadian Society of Immunology, Quebec City, Quebec, March 2014.
18. Neethi Nandagopal, **Alaa Kassim Ali**, Amandeep Kaur Komal, and Seung-Hwan Lee. The Indispensable Role of IL-15-PI3K-mTOR Pathway in Natural Killer Cell Functions At the 27th Annual Meeting of the Canadian Society of Immunology, Quebec City, Quebec, March 2014.

Conferences

- May 3, 2019** 8th Annual Symposium on Cytokines in Inflammation, Ageing, Cancer and Obesity (CIACCO-2019) *St. Camille, Quebec, Canada*
- Presented a poster entitled: "Investigating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection"
 - Selected for a 2 minutes speed talk
- Apr 12, 2019 – Apr 15, 2019** 32th Annual Spring Meeting of the Canadian Society for Immunology (CSI 2019) *Banff, Alberta, Canada*
- Presented a poster entitled: "Investigating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection"

Conferences (continued)

- Oct 25, 2018** The 3th uOttawa Flow Cytometry Symposium, Nanoscale Flow Cytometry for Cancer, Infection and Disease *Ottawa, Ontario, Canada*
- Presented a poster entitled: "IL-18 upregulates nutrient transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells"
- June 1, 2018 – June 4, 2018** 31th Annual Spring Meeting of the Canadian Society for Immunology (CSI 2018) *London, Ontario, Canada*
- Presented a poster entitled: "Elucidating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection"
 - **Won an ImmunoPrecise Travel Award in the amount of \$1,000**
 - Presented a 1-minute slide
- May 25, 2018** 7th Annual Symposium on Cytokines in Inflammation, Ageing, Cancer and Obesity (CIACCO-2018) *St. Camille, Quebec, Canada*
- Presented a poster entitled: "Elucidating the role of natural killer cell derived IL-10 in regulating T cell activation during a sustained viral infection"
 - Selected for a 2 minutes speed talk
- May 17, 2018 – May 18, 2018** BMI Scientific Symposium 2018 *Montebello, Quebec, Canada*
- Presented a poster entitled: "Investigating the role of natural killer cell derived IL-10 in regulating T cell activation during a sustained viral infection"
- June 6, 2017 – June 9, 2017** 30th Annual Canadian Student Health Research Forum (CSHRF-2017) *Winnipeg, Manitoba, Canada*
- Nominated by the University of Ottawa as being among the top 5% of doctoral students, to participate at this conference
 - Presented a poster entitled: "Elucidating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection"
 - Attended the Gairdner Symposium (Talks were given by five Global Leaders in Health Research that are also Gairdner Laureates)
 - **Won a CIHR Travel Award in the amount of \$1,000**
- May 5, 2017** 6th Annual Symposium on Cytokines in Inflammation, Ageing, Cancer and Obesity (CIACCO-2017) *Eastman, Quebec, Canada*
- Presented a poster entitled: "Investigating the role of natural killer cell derived IL-10 in modulating T cell activation during a sustained viral infection"
 - **Chaired the third session on cancer and autoimmunity**; announced the student presentations, animated the question period and ensured that the time is maintained

Conferences (continued)

- April 7, 2017 – April 10, 2017** 30th Annual Spring Meeting of the Canadian Society for Immunology (CSI 2017) *Banff, Alberta, Canada*
- Presented a poster entitled: "Elucidating the role of natural killer cell derived IL-10 on T cell activation during a sustained viral infection"
- Nov 28, 2016** 1st Montreal Symposium on Immunogenetics of Infectious and Inflammatory Diseases *Montreal, Quebec, Canada*
- May 20, 2016** 5th Annual Symposium on Cytokines in Inflammation, Ageing, Cancer and Obesity (CIACCO-2016) *Eastman, Quebec, Canada*
- Selected for a short talk
 - Gave a short talk entitled: "Investigation of the inflammatory milieu inducing natural killer cell maturation during virus infection and tumorigenesis"
 - **Won 2nd place for oral presentation (Awarded a \$75 prize)**
- April 1, 2016 – April 4, 2016** 29th Annual Spring Meeting of the Canadian Society for Immunology (CSI 2016) *Ottawa, Ontario, Canada*
- Selected for a short talk
 - Gave a short talk entitled: "Investigation of the inflammatory milieu inducing natural killer cell maturation during virus infection and tumorigenesis"
 - **Won CSI Poster Award in the amount of \$150**
- May 2, 2015 – May 6, 2015** 15th Meeting of the Society for Natural Immunity (NK2015) *Montebello, Quebec, Canada*
- Very important conference dedicated to studies on Natural Killer cells
 - Selected for a short talk
 - **Won a Travel Award in the amount of 500 USD**
 - Gave a short talk entitled: "GATA-3 regulates the NK cell maturation program required for bone marrow exit and proliferation"
 - Presented a poster
- March 6, 2014** 27th Annual Canadian Society for Immunology Conference (CSI 2014) *Quebec City, Quebec, Canada*
- Presented a poster entitled: "The Role of GATA-3 in Natural Killer Cell Development"

Teaching assistantship

2015 -2017
(3 Fall Semesters) **Teaching Assistant** for 4th year Immunology course (MIC4125)
University of Ottawa, Ottawa, Ontario, Canada
Proctored and marked midterm and/or final exams
Provided office hours for students who wish to see exam corrections

Volunteer work

May 5, 2017 6th annual symposium on Cytokines in Inflammation, Ageing, Cancer and Obesity (CIACCO-2017) *Eastman, Quebec, Canada*

- **Chaired the third session on cancer and autoimmunity**
- Announced the student presentations
- Animated the question period and ensured that the time is maintained

April 1, 2016 – April 4, 2016 29th Annual Spring Meeting of the Canadian Society for Immunology (CSI 2016) *Ottawa, Ontario, Canada*

- Received and guided registrants at the conference
- Gave out name tags and conference material
- Ensured that only registrants with meal tickets enter the dining area

Winter 2014 – Winter 2015 Assistant of Work in Progress (WIP) Seminar
University of Ottawa, Ottawa, Ontario, Canada

- Distributed weekly flyers of upcoming presentations
- Chaired the session and animated the question period

Employment

Summer 2011 **Interviewer**, Opinion Search Inc.
160 Elgin St. Suite 1800, Ottawa, Ontario, Canada
Conducted surveys in Canada and USA

2006-04 – 2008-09 **Produce Clerk**, Belfast Fruit Inc.
1010 Belfast Road, Ottawa, Ontario, Canada
Sorted and displayed fruits and vegetables
Helped the clients with the products and kept the store clean

Honours and awards

2019-04 BMO Financial Group Graduate Bursaries - \$4,000
2019-03 Won third place as a presenter at the BMI seminar Day 2019 at uOttawa
Awarded a prize in the amount of 50\$

Honours and awards (continued)

2019-01 – 2019-12	Tuition Fee Scholarship - University of Ottawa - \$2,358/semester
2018-06	Won an ImmunoPrecise Travel Award in the amount of \$1,000
2018-01 – 2018-12	Ontario Graduate Scholarship - Gov. of Ontario (2/3) and uOttawa (1/3) \$15,000
2017-06	Nominated by the University of Ottawa, as being among the top 5% of doctoral students, to participate at the CSHRF-2017 conference Won a CIHR Travel Award in the amount of \$1,000
2017-03	Won the Syed Sattar PhD Student Award in recognition of my contributions to research and publications - BMI department at the University of Ottawa Prize in the amount of 500\$
2017-03	Won first place as a presenter at the BMI seminar Day 2017 at uOttawa Awarded a prize in the amount of 100\$
2017-01 – 2017-12	QEII-GSST - Gov. of Ontario (2/3) and uOttawa (1/3) - \$15,000
2016-05	Selected for a short talk at the 5th annual symposium on Cytokines in Inflammation, Ageing, Cancer and Obesity (CIACCO-2016) and Won 2 nd place for oral presentation (Awarded a prize in the amount of 75\$)
2016-05 – 2018-12	Excellence Scholarship - University of Ottawa - \$2,358/semester
2016-05 – 2016-12	Ontario Graduate Scholarship - Gov. of Ontario (2/3) and uOttawa (1/3) \$10,000
2016-04	Selected for a short talk at the 29th Annual Spring Meeting of the Canadian Society for Immunology and received a CSI Poster Award (\$150)
2016-03	AAI Trainee Poster Award in the amount of \$300 (declined)
2015-12	Faculty of Graduate and Postdoctoral Studies Dean's Scholarship - \$1,500
2015-09 – 2016-04	Admission Scholarship - University of Ottawa - \$3,000/semester
2015-05	Selected for a short talk at the NK2015 international conference meeting Received a Travel Award in the amount of 500 USD
2015-03	Won second place as a presenter at the BMI seminar Day 2015 at uOttawa Awarded a prize in the amount of 75\$
2014-09 – 2015-08	QEII-GSST - Gov. of Ontario (2/3) and uOttawa (1/3) - \$15,000
2014-09 – 2015-08	Excellence Scholarship - University of Ottawa - \$7,650.51
2013-09 – 2014-08	Admission Scholarship - University of Ottawa - \$7,500
2012-09 – 2013-04	Admission Scholarship 3rd renewal - University of Ottawa - \$1,500
2011-09 – 2012-04	Admission Scholarship 2nd renewal - University of Ottawa - \$1,500
2009-09 – 2010-04	Queen Elizabeth II Scholarship - Gov. of Ontario - \$2,631
2009-09 – 2010-04	Bourse de la francophonie - University of Ottawa - \$2,500
2009-09 – 2010-04	Renewable Admission Scholarship - University of Ottawa - \$3,000

Leadership

- Winter 2018** Team captain for men's 7 vs 7 intramural soccer team
University of Ottawa, Ottawa, Ontario, Canada
- Recruited a total of 16 players
- Winter 2017** Team captain for men's 7 vs 7 intramural soccer team
University of Ottawa, Ottawa, Ontario, Canada
- Recruited a total of 17 players
- Summer 2016** Supervised and trained a summer student how to genotype mice
University of Ottawa, Ottawa, Ontario, Canada
- Trained the student to extract genomic DNA from mouse tail sample, perform a regular PCR, and run amplicons on agarose gel
 - Also trained the student to perform quantitative real-time PCR and to analyze the data
- Fall 2014 – Winter 2015** Supervised and trained a Bachelor student
University of Ottawa, Ottawa, Ontario, Canada
- Undergraduate Research Opportunity Program (UROP)
 - Trained the student to culture B16F10 melanoma tumor cells (which grow in the mouse lung), to harvest the lung and to stain the tumor colonies
- Fall 2013** Supervised and trained a Bachelor student
University of Ottawa, Ottawa, Ontario, Canada
- Undergraduate Research Opportunity Program (UROP)
 - Trained the student to perform Polymerase Chain Reaction (PCRs) and to use a genetic approach called “Marker-Assisted Congenic” to accelerate the production of a specific congenic mouse strain

Extracurricular commitment

- Summer 2018** Participated in the co-ed 7 vs 7 indoor soccer league
Ottawa Footy Sevens, Carleton University, Ottawa, Ontario, Canada
- Played for “Net Neutrality” team
 - Won first place in the tournament
- Fall 2017** Participated in the co-ed 7 vs 7 indoor soccer league
Ottawa Footy Sevens, Carleton University, Ottawa, Ontario, Canada
- Played as a striker for “Slow Lorises” team
 - Won second place in the tournament

Extracurricular commitment (continued)

- Summer 2017** Participated in the co-ed 6 vs 6 indoor volleyball league
Ottawa Volley Sixes, Carleton University, Ottawa, Ontario, Canada
- Played for “Block, Dig, Set & Miss” team
- Summer 2017** Participated in the co-ed 7 vs 7 outdoor soccer league
Ottawa Footy Sevens, Carleton University, Ottawa, Ontario, Canada
- Played as a striker for “CF Covfefe” team
- Summer 2016** Participated in the men’s 11 vs 11 outdoor soccer league
RA center, Ottawa, Ontario, Canada
- Played as a striker for “Chicadios” team