GENETIC MANIPULATION OF INHIBITORY RECEPTOR SIGNALLING IN NK CELLS

BY

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DEDICATIONS

I dedicate my thesis to my father, mother, brother and sister. My father and mother work very hard to ensure that I receive the highest education possible. In addition, their wisdom and life experiences continue to help me navigate my life and career. My brother challenges me to do my best and my sister consistently cheers me up during my hardest times. It would have been impossible for me to achieve what I have achieved so far without my family's constant support and encouragement.

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List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome	**BCR**	B Cell Receptor
BM	Bone Marrow	**CEBP**	CCAAT/enhancer binding protein
CFSE	Carboxyfluorescein Succinimidyl Ester	**DAP**	DNAX-Activating Protein
DAG	Diacylglycerol	**DNA**	Deoxyribonucleic Acid
EGFP	Enhanced Green Fluorescent Protein	**Erk**	Extracellular Signal-Regulated Kinase
FBS	Fetal Bovine Serum	**Flt3L**	FMS-Like Tyrosine Kinase 3 Ligand
HIV	Human Immunodeficiency Virus	**HSC**	Hematopoietic Stem Cell
KIR	Killer Cell Ig-Like Receptor	**KO**	Knockout
Id	Inhibitor of DNA Binding	**IFN**	Interferon
IL	Interleukin	**IMDM**	Isocove's Modified Dulbecco's Medium
iNK	Immature NK	**IP_3**	Inositol 1,4,5-Triphosphate
IRF	Interferon Regulatory Factor	**IS**	Immunological Synapse
ITAM	Immunoreceptor Tyrosine-Based Activation Motif	**ITIM**	Immunoreceptor Tyrosine-Based Inhibitory Motif
LAK	IL-2 Activated NK	**LAMP-1**	Lysosomal-Associated Membrane Protein-1
LAT	Linker For Activation	**Lck**	Lymphocyte-Specific Protein-Tyrosine
LTR	Long Terminal Repeat	**MHC**	Major Histocompatibility

me	Motheaten	***me^v***	Motheaten Viable
MEF	Myeloid Elf Factor	**MIP**	Macrophage Inflammatory Protein
MOI	Multiplicity of Infection	**NK**	Natural Killer
NKP	NK Precursor	**PKC**	Protein Kinase C
PLC	Phospholipase C	**PIP_2**	Phosphatidylinositol 4,5-Bisphosphate
PSG	Penicillin, Streptomycin and Glutamine	**PTK**	Protein Tyrosine Kinase
RANTES	Regulated on Activation, Normal T Expressed and Secreted	**RCMV**	Rat Cytomegalovirus
RCTL	RCMV C-Type Lectin-Like	**RNA**	Ribonucleic Acid
RPMI	Rosewell Park Memorial Institute	**SCF**	Stem Cell Factor
7-AAD	7-Amino Actinomycin D	**SHP**	Src Homology 2 Domain-Containing Protein Phosphatase
SIN	Self-Inactivating Lentiviral Vector	**SLP-76**	Src Homology 2-Containing Leukocyte Phosphoprotein of 76 kDa
Syk	Spleen Tyrosine Kinase	**T-bet**	T-Cell Specific T-Box
TRC	The RNAi Consortium	**TCR**	T Cell Receptor
TNF	Tumour Necrosis Factor	**TRAIL**	TNF-Related Apoptosis-Inducing Ligand
VSV-G	Vesicular Stomatitus Vesicular Protein	**WRE**	Woodchuck Hepatitis Virus Post-Transcriptional Regulatory Element
ZAP70	Zeta Chain Associated Protein of 70 kDa		

Abstract

Natural killer (NK) cells are lymphocytes that provide an important line of defense against invading microorganisms, viruses and transformed cells. They express two groups of receptors on the cell surface, activation and inhibitory. The status of NK activity depends on the balance of signals generated from both groups of receptors. Inhibitory receptors recognize self-MHC and non-MHC molecules giving them an important role in the prevention of NK-mediated autoimmunity. They signal through immunoreceptor tyrosine based inhibitory motifs (ITIM), and recruit phosphatases such as SHP-1 to inhibit NK cell activation. Although well understood in mature NK cells, the role of NK receptor signalling in differentiation and acquisition of self-tolerance is currently unclear. The development of an efficient gene transfer system for genetically modifying primary mouse NK progenitors (NKPs) and mature NK cells will facilitate these studies.

In our first study, we demonstrated that human immunodeficiency virus type 1-based lentiviral vectors support an average of 40% transduction efficiency on primary NK cells. We further demonstrated efficient gene transfer into differentiating NK cells derived from the lentiviral-transduced mouse hematopoietic progenitor cells.

We then used this platform to test the **hypothesis that manipulating the balance of signals between activation and inhibitory receptors at the NK cell level will affect NK differentiation, acquisition of self-tolerance and NK-target interactions.** In these studies, we used our established lentiviral transduction protocol to either over-express the NK inhibitory receptor NKR-P1B or deliver shRNA sequences targeting the SHP-1 gene into mouse IL-2 activated NK cells.

We showed that NKR-P1B over-expression resulted in decreased cytotoxicity and cytokine production against tumour target cells expressing Ocil, the ligand for NKR-P1B. This study demonstrated the ability to alter NK-target interactions by manipulating NK receptor expression.

In addition, we demonstrated that SHP-1 shutdown in activated NK cells resulted in impaired IL-2-induced cellular proliferation, normal cytotoxicity towards YAC-1 targets, increased frequency of apoptosis and necrosis and increased spontaneous degranulation in culture. This study demonstrated an essential role of SHP-1 in mature NK, and suggested that SHP-1 may play a role in regulating self-tolerance and autoimmunity in mature NK.

CHAPTER 1. INTRODUCTION

1.1. Natural Killer cells

Natural killer (NK) cells represent a small population (up to 15%) of lymphocytes found in the blood and peripheral lymphoid organs, including the liver, peritoneal cavity and placenta.[1] They are part of the innate immune system and provide an important line of defense against many types of microorganisms, viruses and tumors when activated.[2,3] NK cell activation can occur either via direct NK-target interactions or by cytokines: Interleukin (IL)-2, IL-12, IL-15, IL-18, IL-21 and type I Interferons (IFN)α/β that are produced by other immune cells.[4-6]

Activated NK cells can perform two functions. One of their main functions is to mediate cytotoxicity against target cells by releasing perforin and granzymes contained within their lytic granules. NK cell cytotoxicity begins with the complex formation of an immunological synapse (IS) between the effector and the target cell.[7] The formation of an IS allows transport of the lytic granules to the surface of the NK cell where the perforin and granzymes are delivered to the target cell to mediate cytotoxicity. The function of perforin is to allow granzymes to enter into the target cell. However, the mechanism of perforin mediated delivery of granzymes into the target cell is currently under heavy debate.[8-11,11-15] The original model suggests that pore formation on the target cell membrane by perforin will allow the granzymes to enter the cell via the newly formed pores.[8,10] Data, however, suggests that perforin may not be necessary for the endocytosis of granzymes.[11-13] A newly proposed model suggests that perforin does not act on the plasma membrane. Instead, it may act on the endosomal membrane. Granzymes are endocytosed by the target cells. Once inside the cytosol, perforin located on the cell

membrane form pores in the endosomes releasing the granzymes.[11,14] It remains unclear, however, the mode in which perforin gains access to the endosomes in the cytosol. A third model proposes that perforin initially acts to form pores in the membrane. This will trigger a membrane repair response in which internal vesicles donate their membranes to patch the holes. Perforin and granzymes are both endocytosed into the cytosol via giant endosomes. Once in the cytosol, perforin acts to form pores in the endosomes, which will in turn release granzymes to kill target cells.[15] Granzymes A and B are the most abundantly produced granzymes in NK cells.[16-18] Upon entrance into the target cell cytosol, the granzymes act to direct the cell towards apoptotic cell death.[19] In all three models, the process of perforin and granzyme release (termed degranulation) is accompanied by the transport of the lysosome-associated membrane protein-1 (LAMP-1, CD107a) to the NK cell surface.[20] Anti-CD107a antibodies readily detect CD107a surface expression, thereby making it possible to identify NK cells that have mediated cytotoxic activity.

Besides perforin and granzyme-mediated target cell death, NK cells are also able to initiate Fas and Tumour Necrosis Factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated cytotoxicity. NK cells express Fas-ligand (Fas-L) and TRAIL that bind to Fas (CD95, Apo-1) and TRAIL-receptors, respectively, expressed on target cells. Fas is a widely expressed glycosylated cell surface molecule belonging to the TNF-Receptor family involved in mediating death signals.[21] During NK-target cell interactions, Fas-L bind to Fas resulting in Fas trimerization and activation of caspases leading to target cell apoptosis. TRAIL-induced apoptosis involves disruption of the

mitochondrial membrane potential, cytochrome c release, caspase activation and induction of DNA fragmentation.[22]

In addition to cytotoxicity, NK cells are also capable of secreting cytokines and chemokines that function to activate other immune cells during an infection. For example, NK cells rapidly produce IFN-γ and TNF-α in response to bacterial infections.[23-26] These cytokines act to amplify host innate and adaptive immune responses by maturing and activating dendritic cells, macrophages and T cells.[27] In addition, NK cells are known to produce chemokines such as Regulated on Activation, Normal T Expressed and Secreted (RANTES), Macrophage Inflammatory Protein (MIP)-1α and MIP-1β. These chemokines function to suppress viral replication.[28-30]

1.2. Natural Killer cell receptor expression

NK cells primarily circulate between the blood and peripheral lymphoid organs, constantly interacting with other cells in the host and surveying for any abnormal changes in the surface expression of "self" markers (for example, Major Histocompatibility (MHC) Class I or non-MHC Ocil), "foreign" molecules (for example, viral products) and stress-induced molecules (for example, MHC Class I related genes (MIC) A and B). They survey target cells by employing their activation and inhibitory receptors that are non-clonally expressed on the cell surface.[31-37]

The human MHC Class I receptors are monomeric type 1 glycoproteins containing either 2 or 3 extracellular Ig-like domains and they have been referred to as killer cell Ig-like receptors (KIRs). In contrast, the mouse MHC Class I receptors are members of the Ly49 superfamily. The Ly49 proteins are expressed as disulfide-linked homodimers, and individual Ly49s are found on subsets of the total NK-cell population. The Ly49 MHC Class I receptor gene family is located on chromosome 6 in a region designated as the NK gene complex.[38,39]

1.2.1. Major Histocompatibility (MHC) Class I inhibitory receptors

MHC Class I receptors are one type of NK cell inhibitory receptors that specifically recognize MHC Class I molecules. KIRs and Ly49 receptors represent a large class of MHC Class I receptors in human and murine NK cells, respectively. MHC Class I receptor heterodimers of CD94 and NK group 2A (NKG2A), NKG2C or NKG2E are expressed in both human and mouse NK cells.[40,41] There are different members in each receptor family that recognize different allelic determinants on MHC Class I

molecules. It is important to note that the number of KIR, Ly49 or CD94-NKG2 receptors that are expressed varies between different individuals and mouse strains. The reason for this receptor distribution is to form different NK cell clones. Different clones will be able to react to transformed or virus infected cells that down-regulate different MHC Class I allelic determinants.[42]

1.2.2. Non-MHC inhibitory receptors

Besides MHC Class I proteins acting as ligands for inhibitory receptors, non-MHC Class I molecules may also bind to another class of inhibitory receptors expressed by NK cells known as non-MHC inhibitory receptors. This class of receptors also play a vital role in NK cell self-tolerance. For example, the ligand for the non-MHC NK cell inhibitory receptor NKR-P1B has been identified to be the osteoclast inhibitory lectin (Ocil/Clr-b) expressed by all host hematopoietic cells.[33,43,44] The NKR-P1B receptor is a homodimeric type II transmembrane C-type lectin-like protein conserved among rodents, humans and other mammals. This conservation across different species suggests that NKR-P1B may play an important role in innate immunity.[45,46] This notion is further supported by studies that show down-regulation of Clr-b on many tumor cells.[45,46] In addition, Voigt et al characterized a rat cytomegalovirus (RCMV) C-type lectin-like (RCTL) gene product with homology to Clr-b. They concluded that cells infected with RCMV would undergo down-regulation of Clr-b followed by rapid up-regulation of RCTL. This NKR-P1B-RCTL interaction prevents NK cell cytotoxicity towards RCTL infected cells. These findings reveal the important role of NKR-P1B and non-MHC inhibitory receptors in NK cell self-tolerance and immunity to infection.

1.2.3. Activation receptors

The ligands for activation receptors, in general are either "self" cellular proteins up-regulated upon infection or "non-self" viral proteins. For example, the activation receptor NKG2D recognizes MHC Class I related molecules that are often induced by cellular distress in humans and mice.[1,47-54] Activation receptors, NKp46 and Ly49H on the other hand, recognize viral proteins such as influenza hemagglutinin and murine cytomegalovirus m157, respectively.[55,56]

Whereas the NKR-P1B receptor is inhibitory, another member of the NKR-P1 family, NKR-P1C (NK1.1) is activating. Experiments involving the cross-linking of NKR-P1C receptors with antibodies induced redirected lysis by NK cells against otherwise resistant targets.[31,57] Although the ligand for NKR-P1C is currently unknown, cross-linking experiments show that this receptor is activating in NK cells.

1.3. The Missing Self Hypothesis

Based on the observation that NK cells preferentially kill targets lacking MHC Class I molecules on the cell surface, Ljunggren and Karre proposed that NK cells recognize and eliminate cells that lack Class I.[38,58] Inhibitory receptors were immediately speculated to exist during initial NK cell studies because they were observed to preferentially kill targets that lacked MHC Class I expression. It was originally believed that any cell lacking MHC Class I expression was automatically prone to NK cell activity. This belief falls apart when analyzing NK cell activity against erythrocytes, which in humans do not express MHC Class I. In addition, host cells expressing low levels of MHC Class I are spared NK cell activity.[59]

The discovery of NK activation receptors helps to explain the mechanisms employed by NK cells to recognize potential targets. Why are some cells that do not express or express low levels of MHC Class I spared while other cells that express normal levels of MHC Class I attacked? It appears that NK cells do not rely specifically on inhibitory or activation receptors alone. NK cell activity is determined by a balance of signals generated from engagement of both inhibitory and activation receptors.[60-63]

Normal cells predominately express ligands for inhibitory receptors leading to attenuation of NK cell activity.[64] Infected cells on the other hand may down-regulate inhibitory self-ligands in an attempt to evade detection by the host adaptive immune system.[65] In many infection scenarios, down-regulation of inhibitory self-ligands in combination with up-regulation of activating ligands leads to an activation of NK cell activity. In other infection scenarios, however, viruses and tumours have evolved mechanisms to evade NK cell detection. For example, HIV-1 infected cells maintain self-

MHC expression (Human Leukocyte Antigen – C) and fail to express any activation ligands.[66] Therefore, it is obvious that infected cells evade NK cell activity by skewing the balance towards inhibition.

1.4. Natural Killer cell receptor signalling

1.4.1. Activation receptor signalling

Both NK cell inhibitory and activation receptor signalling pathways are similar to signalling pathways found in T and B lymphocytes.[38,59,67] NK activation receptors in particular signal through pathways similar to T and B cell antigen receptors (TCR and BCR). There are many complex pathways involved in NK cell receptor signalling. For simplicity, I have chosen to discuss only a few selected signalling molecules that directly relate to my studies.

Signalling through T Cell Receptor (TCR) CD3ζ, FcεRIγ and DNAX-Activating Protein of 12 kDa (DAP12)

Similar to TCRs and BCRs, NK activation receptors themselves do not contain cytoplasmic signalling domains but instead, they signal through small adaptor proteins. For example, NKp46 signals through TCR CD3ζ and FcεRIγ while mouse NKG2D and activating Ly49s can signal through DAP12.[68-73] The cytoplasmic tail of these adaptor proteins contains a single Immunoreceptor Tyrosine-based Activation Motif (ITAM). An ITAM (consensus sequence YxxI/Lx(6-8)YxxI/L) contains two phosphorylatable tyrosines that are configured precisely for the binding of the dual Src Homology 2 (SH2) domains of the Spleen Tyrosine Kinase (Syk)/Zeta Chain Associated Protein of 70 kDa (ZAP70) kinases.[74]

Lymphocyte-Specific Protein-Tyrosine Kinase (Lck) and Fyn

The phosphorylation of the dual tyrosine residues on ITAM is accomplished by the actions of Src family protein tyrosine kinases (PTKs), Lck and Fyn.[75] Lck and Fyn

are 56 and 59 kDa proteins, respectively. Structurally, they both contain an SH3 domain, an SH2 domain, a catalytic tyrosine kinase domain and a C-terminal negative regulatory domain. Lck and Fyn are regulated by their C-terminal negative regulatory domain. When the cell is in a normal resting state, this domain is not phosphorylated. It is believed that Lck and Fyn transphosphorylate their activation loop tyrosines upon receptor engagement and subsequent clustering of the ITAMs. It is the phosphorylation of the activation loop that triggers Lck and Fyn to phosphorylate the SH2 domains on ITAMs.

Spleen Tyrosine Kinase (Syk) and Zeta Chain Associated Protein of 70 kDa (ZAP70)

Syk and ZAP70 are next recruited as a result of ITAM phosphorylation. Syk and ZAP70 are 72 and 70 kDa proteins, respectively.[76] Structurally, they both contain an N-terminal SH2, a COOH-terminal SH2 and a catalytic tyrosine kinase domain. Upon binding to phosphorylated ITAM via the SH2 domains, Syk and ZAP70 are themselves phosphorylated and activated. However, there is current evidence that suggests different requirements for Syk and ZAP70 activation.[77-80] ZAP70 activation also requires Lck and Fyn kinase activity. Syk on the other hand, has been shown to initiate killing in the absence of Src family PTK costimulation.[79] In addition, evidence suggests that Syk, but not ZAP70, can itself phosphorylate the two tyrosine residues in the ITAM in a Src family PTK-independent manner.[78,80,81]

Although both Syk and ZAP70 kinases are expressed in NK cells,[82,83] studies on Ly49D/DAP12 engagement show that Syk, but not ZAP70, was phosphorylated.[83] A dominant negative form of Syk but not ZAP70 was able to suppress Ly49D signalling

and a pharmacological inhibitor of Syk activity, showed inhibited NK cytotoxicity.[84] Taken together, these studies suggest that mainly Syk is responsible for mediating further downstream events during Ly49D/DAP12 receptor signalling.

Linker For Activation (LAT) and Src Homology 2-Containing Leukocyte Phosphoprotein of 76 kDa (SLP-76)

Upon activation Syk/ZAP70 acts to recruit downstream adaptors such as LAT and SLP-76.[85-87] LAT and SLP-76 are 36-38 kDa and 76 kDa proteins, respectively.[88,89] LAT contains an SH3 domain, an SH2 domain and nine to ten tyrosine phosphorylation motifs[90] while SLP-76 contains an SH3 domain, an SH2 domain, and three tyrosine phosphorylation motifs.[88] These proteins however, do not contain any intrinsic catalytic activity. Their role is to act as an adaptor, integrating signals resulting from receptor engagement.[91,92] LAT and SLP-76 interact with Syk/ZAP70 via their SH2 domains and become activated when Syk/ZAP70 phosphorylates their tyrosine motifs. Activated LAT and SLP-76 come together at the membrane and acts to recruit the second line of effector/adaptor molecules involved in NK signalling.

Phospholipase C (PLC)-γ1 and PLC-γ2

The second line of effector/adaptor molecules includes PLC-γ1 and PLC-γ2. PLC-γ1 and PLC-γ2 have one SH3 and two SH2 domains sandwiched between two catalytic domains.[93] They interact with LAT and SLP-76 via their SH3 and SH2 domains. The exact mechanism in which PLC becomes phosphorylated is not known but it has been shown to involve Syk/ZAP70 kinase activity. Activated PLC functions to catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG)

and inositol 1,4,5-triphosphate (IP_3). IP_3 promotes Ca^{+2} mobilization and DAG along with mobilized Ca^{+2} activates Protein Kinase C (PKC).

Extracellular Signal-Regulated Kinase 1 and 2 (Erk1/2)

DAG and activated PKC goes on to activate Erk1/2 but the mechanism of Erk1/2 activation in NK cells is currently unclear.[38] Erk1/2 is a member of the Mitogen Activated Protein Kinases (MAPKs) family with a molecular weight of 41-45 kDa.[83] Activated Erk1/2 is believed to activate transcription factors responsible for NK cell cytotoxicity and cytokine production. However, this is also completely unknown.[38]

Signalling through DNAX-Activating Protein of 10 kDa (DAP10)

Although the majority of activating NK receptors utilize small adaptor proteins and ITAM to transmit activating signals, NK obtained from ZAP70-/- and Syk-/- mice are able still able to kill certain tumour cell lines. This suggests the possible existence of activating receptors that transmit signals independent of ZAP70 and Syk. NKG2D is an activating receptor that signals independent of ZAP70 and Syk. The expression of NKG2D on the NK cell surface requires a transmembrane-anchored adaptor protein named DAP10. Recall that mouse NKG2D expression may also utilize DAP12.[94-96] The cytoplasmic domain of DAP10 contains a YxxM motif that upon phosphorylation binds to the p85 subunit of phosphatidylinositol-3 kinase (PI3K). Activated PI3K is also capable of activating PLC. Therefore, the TCR CD3ζ, FcεRIγ, DAP10 and DAP12 signalling pathways ultimately converge to activate PLC followed by Erk1/2 activation.

1.4.2. Inhibitory receptor signalling

Unlike activation receptors that rely on adaptor proteins for signalling, inhibitory receptors express signalling domains in their cytoplasmic tails. KIRs, Ly49s, CD94-NKG2s and non-MHC inhibitory receptors all contain an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in their cytoplasmic portion.[38,97-100] The ITIM (consensus sequence V/IxYxxL/V) contains one tyrosine residue that is phosphorylated by activated Lck upon receptor engagement and ITIM clustering.

Src Homology 2 Domain-Containing Protein Phosphatase (SHP)-1

SHP-1 is recruited to the phosphorylated ITIM and functions to inhibit NK activity.[101-107] Although the exact role and targets of SHP-1 in murine NK are unclear, work in human KIR suggests mechanisms similar to T cell signalling. After recruitment to phosphorylated ITIM, activated SHP-1 works to dephosphorylate LAT and PLCγ in NK cells.[108] SHP-1 may also target the dephosphorylation of SLP-76, ZAP-70 and Syk.[91,109,110] The general consensus is that SHP-1 functions to inactivate early signalling molecules involved in NK activation.

The importance of SHP-1 in the inhibition of NK activity has been demonstrated. Experiments involving the over-expression of a catalytically inactive dominant negative form of SHP-1 (dnSHP-1) in human and murine NK cells resulted in decreased MHC-Class I mediated inhibition of cytotoxicity.[103,111] In addition, motheaten (*me*) and motheaten viable (me^v) mice that show complete or partial SHP-1 enzymatic activity loss display diminished ability for Ly49A to inhibit NK activity.[112] In addition, these mice contain hyperphosphorylated Src family kinases, ITAMs and LAT.[113,114] Taken together,

these studies suggest that SHP-1 is a very important molecule in NK cell inhibition. Disruption of SHP-1 enzymatic activity ultimately results in disruption of all inhibitory signalling in NK cells.

However, results from dnSHP-1 or SHP-1 knockout (KO) mice suffered from a couple of caveats. For example, wild SHP-1 enzymatic activity may still exist in dnSHP-1 NK cells. As a result, the competition between wild type and dnSHP-1 might produce unwanted side effects in the NK cells. Also, NK cells isolated from SHP-1 KO mice may be suffering from secondary effects due to over-activated macrophages/monocytes.[115,116] Therefore, it remains to be determined whether the effects associated with a loss of SHP-1 function in the dnSHP-1 and SHP-1 KO NK cells represented true functions of SHP-1 enzymatic activity or simply a summation of secondary effects. In addition, studying the mature NK cells from these animals cannot distinguish whether the observed "abnormality" is associated with a defect in SHP-1 signalling at the differentiation state or mature NK cell or both.

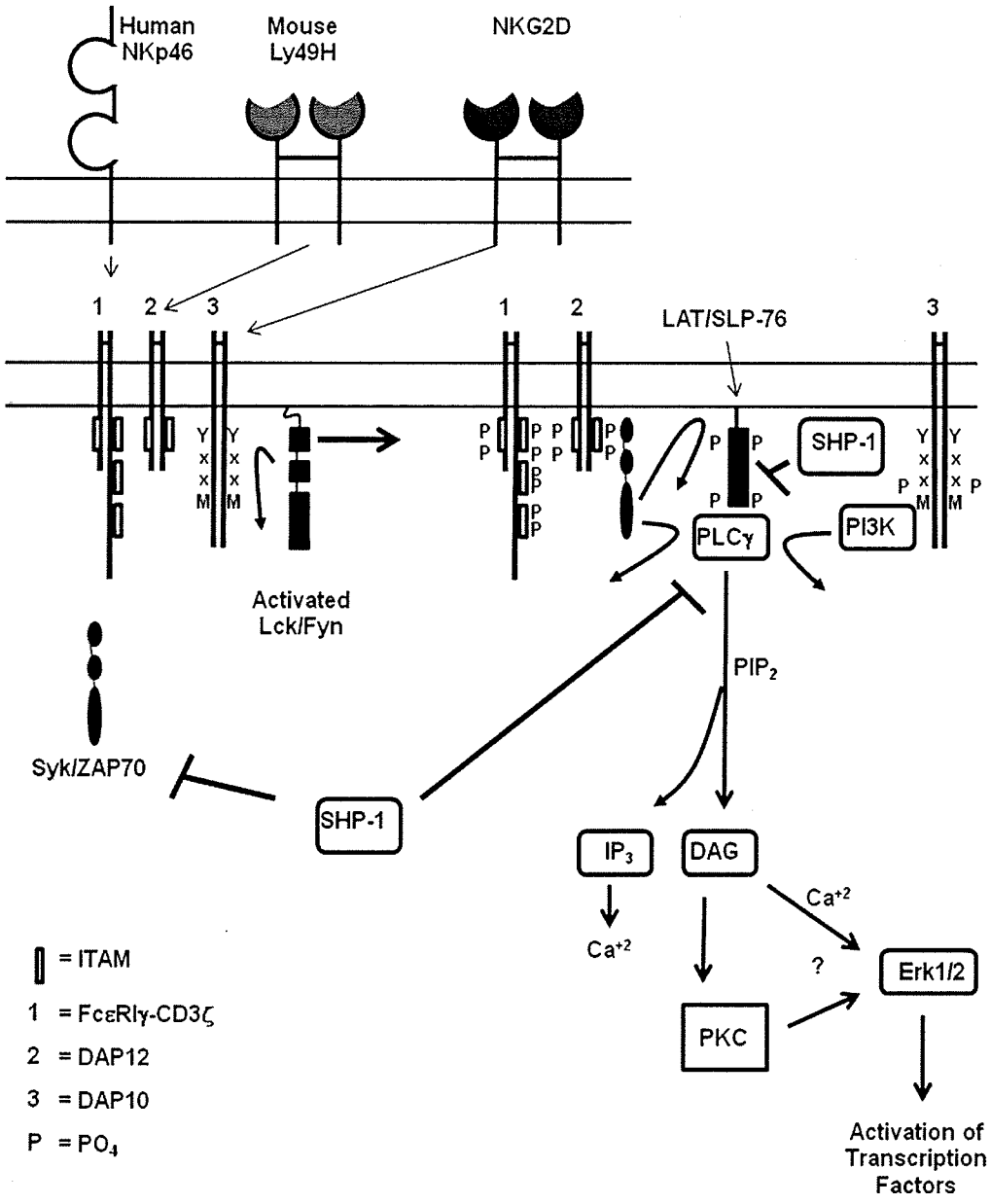

Diagram 1. Pathways involved in NK receptor signalling. Activation receptor engagement results in ITAM phosphorylation by Lck/Fyn. Syk/ZAP70 is recruited to the phosphorylated ITAM and is itself phosphorylated. This is followed by recruitment of LAT/SLP-76 and PLCγ. Syk/ZAP70 kinase activity phosphorylates PLCγ. Activated PLCγ hydrolyze PIP_2 into IP_3 and DAG. IP_3 causes Ca^{+2} mobilization while DAG activates PKC. Ca^{+2}, PKC and DAG activate Erk1/2. Activated Erk1/2 goes on to activate transcription factors required for NK cytotoxicity and cytokine production. Inhibitory receptor engagement results in phosphorylation of ITIM and activation of SHP-1. Activated SHP-1 phosphatase activity works to inactive Syk/ZAP70, LAT/SLP-76 and PLCγ. This results in attenuation of NK activity.

1.5. NK differentiation and acquisition of Self-Tolerance

1.5.1. NK differentiation

NK cells are derived from hematopoietic stem cells (HSCs) in the bone marrow (BM) and acquire different maturation states as they develop.[117,118] There are six stages of NK cell development, termed stages A to F.[119] Each developmental stage is characterized by requirements for additional growth factors, expression of new cell surface molecules and activation of essential transcription factors.

HSCs enter into stage A of NK development in the presence of IL-7, stem cell factor (SCF) and FMS-like tyrosine kinase 3 ligand (Flt3L).[120] Cells in stage A of development are known as NK precursors (NKPs). Cells at the NKP stage are lineage negative (Lin^-) and express only CD122 (IL-2Rβ).[121] Transcription factors activated at stage A include Ikaros, Inhibitor of DNA Binding (Id)2, Ets-1 and PU.1. These transcription factors are essential in the generation of normal NK numbers *in vivo*. In particular, Id2 and Ikaros are responsible for inducing expression of CD122 required for IL-15 responses.[122,123]

Cells in stage B and stage C of NK development are termed immature NKs (iNKs). NKPs need IL-15 in order to progress to stage B.[120] At stage B, iNK cells express NK1.1 and CD94-NKG2. These iNK however, remain poorly cytotoxic. Interaction of stage B iNKs with stromal cells move them into Stage C of development.[120] Stage C iNKs are defined by the expression of Ly49 receptors. Transcription factors activated at Stage C include Gata-3, T-cell Specific T-box (T-bet) and Interferon Regulatory Factor (IRF)-2. These transcription factors appear to be important in NK cell maturation in the BM and migration to the periphery.[119]

When development enters into stage D, the cells are termed mature NK (mNK) and this is characterized by the expression of integrin CD49b (DX5). As NK cells begin to mature functionally and enter stage E of development, integrin CD11b is up-regulated. The final stage of NK development, stage F is characterized by the up-regulation of sialoglycoprotein CD43. Transcription factors myeloid elf factor (MEF), microphthalmia-associated transcription factor (MITF) and CCAAT/enhancer binding protein (CEBP)-γ appear to be important at stage F. Deficiencies in MEF and CEBP-γ resulted in a decrease of both lysis of sensitive target cells and IFN-γ secretion suggesting their importance in NK cytotoxicity and cytokine production. Indeed, MEF interacts with the perforin gene promoter, providing a mechanism to explain the cytolytic defect.[124]

NK cells that successfully go through all six stages of development are exported to peripheral sites via the blood where they can mediate cytotoxicity and cytokine production after stimulation.[125]

Taken together, some aspects of NK development are well known. For example, we know the growth factors required, surface receptors expressed and transcription factors activated at each stage. However, the roles played by each receptor at various stages of development are still unclear. Besides CD122 that is required for IL-15 responses, are the other receptors required for NK differentiation? More importantly, the role of these receptors in NK cell acquisition of self-tolerance is currently unknown. In particular, what are the consequences of altering the normal receptor profile by either over-expressing or down-regulating particular receptors? These questions remain to be answered.

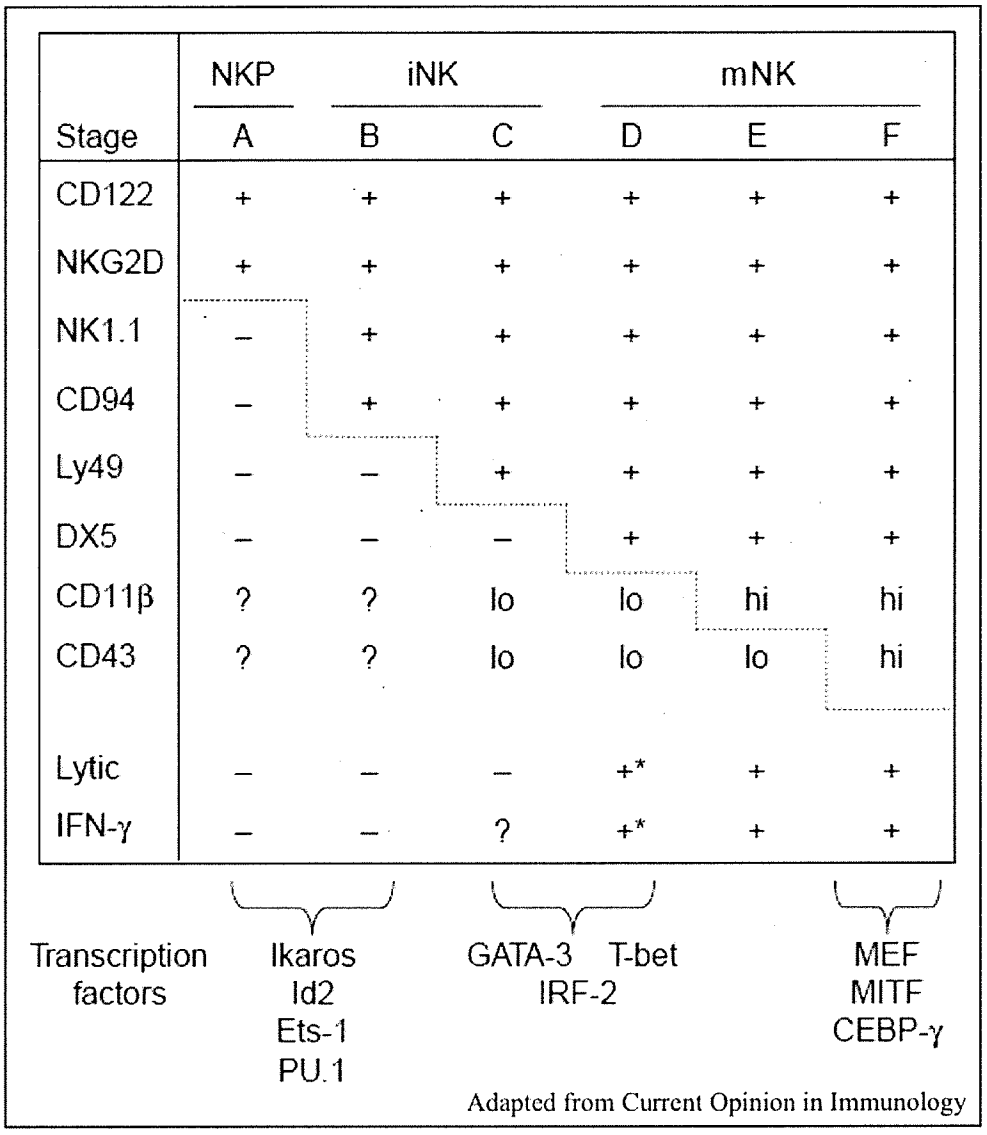

Diagram 2. A model for murine NK cell development via transcription factor regulation. The earliest NK precursors (NKPs) differentiate via immature NKs (iNKs) to mature NKs (mNKs). Markers characterizing each stage are listed. Note that the iNK stage is subdivided on the basis of Ly49 expression, and that the mNK stage is subdivided on the basis of differential expression of CD11b and CD43. Immature NK cells are nonlytic, whereas mNKs are lytic. Transcription factors controlling the different stages are indicated. +, expressed; -, not expressed; hi and lo, indicate expression levels; ?, not known. Figure adapted from Vosshenrich C, Samson-Villeger S and Di Santo J. **Current Opinion in Immunology** 2005, **17**:151-158.

1.5.2. Current models of NK Self-Tolerance

Two models have been proposed to explain how NK cells acquire self-tolerance during differentiation. However, the underlying molecular mechanism remains unknown.

"At Least One Model"

The "At Least One Model," predicts that NK cells only need as few as one receptor to develop self-tolerance. Just as long as the inhibitory signalling from that one receptor is enough to override the summation of activating signals, self-tolerance will be achieved.[126] A study involving the panel of human NK cell clones from two donors with different HLA haplotypes supported this model. Each clone expressed one or more receptors for the donor's HLA Class I molecules.[127]

"Arming Model"

The "Arming Model," predicts that NK cells become self-tolerant by engaging their inhibitory receptors with MHC Class I during differentiation. The main idea of this model is that self-tolerance requires the ability to mediate inhibitory signalling. This model is supported by studies involving MHC Class I-deficient mice. The NK cells isolated from these mice were abundant in numbers and non-self reactive. However, these cells showed reduced cytotoxicity and cytokine production against tumour cell targets.[128-130] The authors suggested that NK cells could achieve self-tolerance by one of two methods. The first method is to express inhibitory receptors for self-MHC. The second method, as seen in MHC Class I-deficient mice, is to become functionally incompetent.

Because they are not fully functional, there is no need for NK cells to express any inhibitory receptors.[2]

"Disarming Model"

The "Disarming Model," is very similar to the "Arming Model" in that they both acknowledge the need for inhibitory signalling in order for the NK cells to become self-tolerant. The "Disarming Model," however, also recognizes the need for activation signals. The model proposes that during differentiation, the activation receptors are constantly exposed to their respective ligands that are expressed at low levels on self-cells. This persistent stimulation forces NK cells to enter a hyporesponsive state.[37,131-133] NK cells can break this hyporesponsive state by engaging their inhibitory receptors with MHC Class I. The main idea of the "Disarming Model" is that self-tolerance is achieved by balancing inhibitory signals with activation signals. Just as long as they are in balance during differentiation, NK cells become self-tolerant.[134]

1.6. Limitations in the studies of Natural Killer cell function and differentiation

1.6.1. Problems associated with studying NK function

As mentioned earlier, there are many questions that remain to be answered regarding NK differentiation, acquisition of self-tolerance and NK-target interactions. This is in part due to lack of experimental systems that allow us to differentially study a protein's function(s) in primary NK progenitors, resting and activated primary NK cells. The major challenge in genetically engineering primary NK cells is to introduce stable genetic modification of NK cells efficiently under minimal *in vitro* manipulation, and without any impairment in cellular viability, phenotype or functions. To escalate the problem, NK cells are relatively resistant to exogenous gene transfer. Presently, there are limited reported successes in the transduction of genetic materials into primary IL-2 activated NK (LAK) cells.[135-144] Vaccinia vectors,[136,138] chimeric adenoviral vectors[145] or plasmid electroporation[142,146] have been used to introduce transgene(s) into these LAK cells. However, given the transient nature of these viral infections and plasmid electroporation, these techniques are limited to short-term experiments.[136,138,142,146] Retroviral vectors offer a complementary strategy to the existing methods because of their ability to integrate into host genome for stable gene expression.[147-149] Transduction of human LAK cells with retroviral vectors have been described.[135,143] However, the apparent reliance on NK cell activation (such as IL-2, IL-15) and repeated infections during retroviral vector transduction might not be desirable in studying the molecular events involved in NK cell activation or tolerance induction during NK development because of the pleiotropic effects of IL-2 receptor signalling.[150]

An additional limitation in studying NK cell function and differentiation is the apparent functional redundancy revealed in the existing KO models. A number of KO mice that are deficient in key signal transducers (for example, Lck) show a dramatic phenotype in T cell function but not in NK cells.[151] It may therefore be necessary to knockout multiple genes at the same time to reveal the role of a particular signalling pathway in NK cell differentiation and function. In addition, NK cells isolated from KO mice represent a final endpoint of the direct and indirect effects of the inactivation of the target genes that occur during the mouse development. To further complicate these limitations, available knockout mice models are sometimes lethal. For example, motheaten SHP-1 knockout (*me*) and SHP-1 inactive (me^v) mice suffer from chronic infection and die between 1 to 3 months of age.[111] NK cells isolated from these *me* and me^v mice show pleiotropic changes, possibly due to over-activated myeloid cells.

1.7. Human Immunodeficiency Virus (HIV)-1 based Lentiviral vectors

An ideal gene delivery system for primary NK cells should confer both efficient gene delivery and a stable phenotype without affecting cell immunophenotype or functions (cytokine production and cytotoxicity). Efficient gene delivery involves the successful import of the gene into the cell nuclei and a stable phenotype requires the delivered gene to incorporate into the host genome. These two criteria allow the vector to persist and remain active in the modified cells and their progeny.

The replication cycle of HIV-1 makes its vector an ideal candidate for use as a gene delivery system for primary NK cells. Similar to retroviral vectors, replication-incompetent human immunodeficiency virus type-1 (HIV-1) based lentiviral vectors have the ability to integrate into the host genome.[152-154] Lentiviral vectors, however, are advantageous over retroviral vectors because lentiviral vectors are able to transduce non-dividing cells[154-156] while retroviral vectors require active proliferation of the target cells for integration.[152-157] Therefore, the ability of lentiviral vectors to integrate into the host genome without the need for active cell proliferation renders them an ideal gene therapy vector for efficient and stable gene delivery into progenitor, resting and activated NK cells.

Molecular biology of HIV-1

The HIV-1 virion is round in shape with a diameter of approximately 100 nm. The viral core contains two copies of 9-kilobase single-stranded Ribonucleic Acid (RNA) and RNA-dependent Deoxyribonucleic Acid (DNA) polymerase (reverse transcriptase).[158] Surrounding the viral core is a bilayer envelope containing

glycoproteins that are encoded by the viral gene, *env*. *Env* encodes a precursor protein, gp160 that is cleaved into glycoproteins gp120 and gp41.[159] In addition to structural genes, the accessory genes: Transcriptional Transactivator (*tat*), Regulator of Virus Protein Expression (*rev*), Negative Factor (*nef*), Viral Protein R (*vpr*), Virion Infectivity Factor (*vif*) and Viral Protein U (*vpu*) are also expressed by HIV-1.[158] These accessory genes play important roles in the viral life cycle. For example, *tat* up-regulates all viral proteins while *rev* facilitates export of viral mRNAs to the cytoplasm. *Nef* and *vif* are both known to enhance viral infectivity while *vpu* enhances viral particle release from infected cells. Lastly, *vpr* enhances nuclear import of viral genomic material.

Human Immunodeficiency Virus-1 replication cycle

HIV-1 infects target cells by fusing its viral envelope with the target cell membrane. This is accomplished by the binding of HIV-1 gp120 glycoproteins with target cell CD4 surface receptor and chemokine receptors CCR5 and CXCR4.[158] These receptors are expressed on a subset of helper T lymphocytes and on monocytes/macrophages. After fusion of the viral envelope with the target cell membrane, the viral core enters the cell cytoplasm. Once in the cytoplasm, the two single-stranded RNAs are reverse transcribed into double-stranded DNA. The double-stranded DNA is then transported into the nucleus where it is integrated into the host genome. The integrated viral DNA will persist for the entire remaining lifespan of the infected cell. In addition, any daughter cells resulting from cell division will also contain a copy of the viral genome. New HIV-1 virions are produced when the viral DNA is

transcribed into single-stranded RNA and packaged into the viral core and bilayer envelope. The virions are then released to infect other cells.

Safety and efficiency of Lentiviral vectors

Because of the pathogenicity of the parental virus, the safety of using HIV-1 based lentiviral vectors for a gene therapy application is a major concern.[160] As a result, many measures have been developed to make HIV-1 based lentiviral vectors safe for gene therapy. For example, viral components were segregated into three separate plasmids and the sequence overlap between them was minimized to reduce the possibility of recombination.[161,162] Another measure taken was the pseudotyping of the HIV-1 vector envelope (*env*) gene with a vesicular stomatitus vesicular protein (VSV-G). This involves expressing the VSV-G vector separate from the gene transfer vector construct. Besides a wider host range, VSV-G pseudotyping eliminates the possibility of generating contaminating replication-competent virus through recombination.[162-165]

Another measure taken to improve the safety of HIV-1 based lentiviral vectors is the elimination of all accessory genes (*tat*, *rev*, *nef*, *vpr*, *vif* and *vpu*) that are not essential for cell transduction from a packaging construct.[160,163] Deletion of these genes greatly improves safety because accessory genes have been associated with a majority of HIV pathologies. For example, *vpr* is known to cause cell cycle arrest and apoptosis. In addition, *vpr* is suspected to be a cause of T-cell dysfunction in acquired immunodeficiency syndrome (AIDS) patients.[166]

To further improve the system, transcriptional elements of HIV-1 were removed from the vector. The 3' long terminal repeat (LTR) is required for the production of full-

length vector RNA in transduced cells. Therefore, deletions in the 3' LTR of the self-inactivating lentiviral vectors (SIN) lead to the abolishment of the promoter activity of the HIV-1 LTR. As a result, recombination, insertional oncogenesis and unwanted vector mobilization in the transduced cells is further eliminated.[160]

Besides improving the safety of HIV-1 based lentiviral vectors for use in gene therapy applications, progress has also been made in the improvement in the efficiency and stability of transgene expression in the transduced cells. For example, the incorporation of a central polypurine tract enhanced the import of the vector into the nuclei of transduced cells.[167]

Safe, efficient and stable lentiviral vector mediated gene delivery will aid to demonstrate the effects of manipulating the balance in NK signalling between engagement of activation and inhibitory receptors at both the NK differentiation and mature NK levels.

HYPOTHESIS AND AIMS

Rationale

Multiple activation and inhibitory receptors are expressed on mature NK cells and their progenitors. It remains unclear how NK cell receptor signalling influences NK differentiation and acquisition of self-tolerance. The ability to manipulate NK signalling pathways will allow us to study the role of these molecules in defining the NK cell receptor repertoire and the acquisition of target recognition specificity.

Global hypothesis

The balance of signals between activation and inhibitory receptors will affect NK differentiation, acquisition of self-tolerance and NK-target interactions. The same receptor signalling may have different outcomes depending on the differentiation status of NK cells.

Specific aims

1. To develop a protocol for efficient lentiviral-mediated *ex vivo* gene delivery into resting/non-proliferating and activated NK cells.
2. To develop a protocol for efficient lentiviral-mediated *ex vivo* gene delivery into NK progenitors.
    i. To drive transduced NK progenitors to differentiation into NK cells using an *in vitro* cytokine cocktail.
    ii. To differentiate transduced NK progenitors into NK cells by adoptive transfer into RAG2-/-γc-/- immunodeficient mice.

3. To determine if over-expressing an NK cell inhibitory receptor, NKR-P1B in mature BALB/c IL-2 activated (LAK) NK cells would skew the balance of receptor signalling leading to an altered NK-target cell specificity.
4. To determine if silencing an inhibitory signalling molecule, SHP-1 in mature C57BL/6 LAK NK cells would dampen the inhibitory signalling pathway resulting in an altered NK-target cell specificity.

CHAPTER 2. MATERIALS AND METHODS

Cell lines

293T cells used for virus production and titration were cultured in Iscove's modified Dulbecco's medium (IMDM) (HyClone, Logan, Utah) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin/glutamate (PSG) (Gibco, Grand Island, NY). BWZ and BWZ-Ocil cell lines were cultured in IMDM supplemented with 10% FBS, 1% PSG and 1.6 mM 2-mercaptoethanol (2-ME) (Sigma, St. Louis, MO). YAC-1, P815 and EL-4 cell lines were cultured in Rosewell Park Memorial Institute (RPMI) 1640 (HyClone) supplemented with 10% FBS, 1% PSG, and 1.6 mM/l 2-ME. Daudi cell line was cultured in RPMI 1640 supplemented with 10% FBS and 1% PSG.

Natural Killer (NK) cells purification, activation and expansion *in vitro*

C57BL/6 and BALB/c mice were purchased from Animal Care Services (The University of Manitoba). RAG2-/- and RAG2-/-γc-/- immunodeficient mice were kindly provided by Hergen Spits and Kees Weijer (University Medical Center, Utrecht, The Netherlands). The University of Manitoba's Review Board has approved all animal studies. Splenocytes were obtained from homogenized spleen by gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Sweden) according to the manufacturer's protocol. Next, NK cells were purified by one of two protocols. 1) Splenocytes were purified by complement depletion of T and NKT lymphocytes by adding Anti-Mouse CD-90 (Thy 1.2) Monoclonal Antibody and Low-Tox-M Rabbit Complement. Cell debris from complement depletion was removed using Lympholyte-M Separation Medium.

Antibody, complement proteins and Lympholyte-M were all used according to manufacturer's protocol (Cedarlane, Hornby, ON). Next, NK cells were purified with EasySep Mouse panNK Positive Selection Kit (StemCell Technologies, Vancouver, BC) with slight modifications to their protocol to improve yield. The time allowed for the centrifuge tube to remain in the magnet was increased from 5 minutes to 10 minutes. 2) NK cells were enriched with EasySep Mouse NK Enrichment Kit (StemCell Technologies) according to manufacturer's protocol. In experiments that required proliferating or activated cells, purified NK cells were incubated in 24 well culture plates at a concentration of 1 x 10^6 cells/ml/well. Cells were incubated in supplemented RPMI 1640 (10% FBS, 1% PSG, 1.6 mM 2-ME) containing 1000 units/ml of IL-2 (except indicated otherwise) (obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health, contributed by M. Gatley, Hoffman-LaRoche, Nutley, NJ) at 37°C and 5% CO_2.

Isolation of Progenitor Stem cell (PSC) and NK differentiation

Bone marrow cells were obtained from C57BL/6 or RAG2-/- mice. Progenitor stem cells were enriched with EasySep Mouse Progenitor Stem Cell Negative Selection kit (StemCell Technologies). Transduction was conducted immediately after enrichment of progenitor stem cells using the spin protocol. 0.3 x 10^6 PSCs were placed into each well of a 48 well culture plate (BD Falcon, Franklin Lakes, NJ). After transduction, virus-containing supernatant was removed. PSCs differentiation into NK cells was conducted by one of two methods. 1) The transduced progenitor stem cells were cultured in NK

differentiation conditioned medium[168] (RPMI 1640 supplemented with 10% FBS, 1% PSG, 1.6 mM 2-ME, 0.5 ng/ml of mIL-7, 30 ng/ml of Stem Cell Factor (SCF), and 50 ng/ml Flt3L. On day 3 after transduction, 0.5 ml of fresh media was added. On day 5, cells were pelleted, old media was removed, and cultured in 0.5 ml of complete RPMI media containing 30 ng/ml of IL-15 (All cytokines were purchased from Peprotech Inc, Rocky Hill, NJ). On day 8, 0.5 ml of fresh media was added. On day 10, old media was replaced with fresh media containing 30 ng/ml of IL-15 and 1000 U/ml of IL-2. On day 14, differentiated NK cells were surface stained for NK markers and analyzed immediately in flow cytometry. 2) The transduced PSCs (0.7×10^6), were injected intravenously into RAG2-/-γc-/- mice[169] that had received a total body irradiation of 200 Rad. Peripheral blood was obtained 4 weeks after the adoptive transfer. Cells were surface stained for NK markers and analyzed immediately in flow cytometry. $EGFP^+$ NK cells were identified in $CD3^-NK1.1^+$ populations.

Construction of vectors

pRRL-PGK-EGFP-SIN18[160], PGK-C in short (appendix figure i), was derived from pRRL-PGK-EGFP[170] and pHR-CMV-LacZ-SIN18. To generate pHR-CMV-LacZ-SIN18, A *Kpn*I-*Xba*I fragment containing the polypurine tract and the 3' LTR was excised from a pHR plasmid and subcloned into the corresponding sites of pUC18. This plasmid was digested completely with *Eco*RV and partially with *Pvu*II and self-ligated. A plasmid carrying a 400-nucleotide-long deletion of U3 was recovered. An *Xho*I linker was inserted in the *Eco*RI site of the deletion plasmid, and an *Xho*I-*Xba*I fragment was cloned back into the pHR-CMV-LacZ plasmid digested with the corresponding enzymes. pRRL-

PGK-EGFP-SIN18 was generated by replacing the 590-bp *Eco*RI-*Afl*II fragment from pRRL-PGK-GFP with the 200-bp *Eco*RI-*Afl*II fragment from pHR-CMV-lacZ-SIN-18 in a four-part ligation with a 2.95-kb *Afl*II fragment, a 2.8-kb *Afl*II-*Bam*HI fragment, and a 760-bp *Bam*HI-*Eco*RI fragment from pRRL-PGK-GFP.

FUGW[171] (appendix figure i) was based on a self-inactivating (SIN) vector that was derived from pHR-MCS.[153] pHR-MCS was constructed by replacing a 420-bp *Bam*HI-*Xho*I fragment of pHR with a fragment of multiple cloning sites containing *Bam*HI, *Eco*47III, *Bst*XI, *Sac*II, *Pst*I, *Eco*RI, *Cla*I, *Xba*I, *Hpa*I, and *Xho*I sites. PCR amplification products of pHR-MCS, a *Bsp*EI-*Apa*I (8 to +180) fragment, were cloned into pcDNA3.1/Zeo(+) (Invitrogen) between the *Mlu*I and *Apa*I sites to generate pHIV-SIN (numbers in parentheses indicate positions relative to the transcriptional start site). FUGW was generated from pHR-MCS by incorporating a human ubiquitin-C internal promoter to drive the GFP reporter gene. The woodchuck hepatitis virus post-transcriptional regulatory element (WRE) was inserted downstream of GFP to increase the level of transcription. To increase the titre of the virus, the human immunodeficiency virus-1 (HIV-1) flap element was inserted between the 5' LTR and the human ubiquitin-C internal promoter.

TRIP-GFP[167] (appendix figure i) was derived from pHR-CMV-LacZ. The EGFP gene replaced LacZ reporter gene. EGFP gene was amplified by PCR using Pfu polymerase from pEGFP-N1 plasmid, adding *Bam*HI and *Xho*I restriction sites in 5′ and 3′, respectively. PCR primer were as follows: *Bam* GFP, 5′ CC GGATCC CCA CCG GTC GCC ACC 3′; *Xho* GFP, 5′ CC CTCGAG CTA GAG TCG CGG CCG 3′. pHR-GFP vector was constructed by cloning back this PCR fragment into *Bam*HI and *Xho*I

sites of pHR-CMV-LacZ, replacing the LacZ ORF by EGFP. A 178 bp fragment of pLAI3 (4793 to 4971), encompassing Cppt and CTS, was amplified by PCR. *Nar*I restriction sites were added in 5′ of the primer in the aim to insert this fragment into the unique *Cla*I site of pHR-GFP: *Nar* TRIP^{+}, 5′ GTC GTC GGCGCC GAATTC ACA AAT GGC AGT ATT CAT CC 3′; *Nar* TRIP^{-}, 5′ GTC GTC GGCGCC CCA AAG TGG ATC TCT GCT GTC C 3′. Insertion of this flap sequence in the correct orientation gave rise to the TRIP-GFP plasmid vector.

pSIN-18-cppt-RhMLV-E,[172] Cppt-2E in short (appendix figure i), was derived from pRRL-PGK-EGFP-SIN18 and pCS-Rh-MLV-E (which was derived from pCS-Rh-MLVΔ-E). To make pCS-MLVΔ-E, pCSCG[153] was digested with *Eco*RI, filled in with Klenow fragments, and digested with *Nhe*I to liberate a 500-bp fragment that contains the CMV promoter. The resulting 8-kb fragment was then ligated to an ~950-bp fragment of pBS-MLV-EM. The latter fragment was prepared by digesting pBS-MLV-EM with *Bst*XI, filling in with Klenow fragments, and digesting with *Spe*I. To generate pCS-Rh-MLV-E, two intermediate constructs (pCS-CMV-IRES-EGFP and pCS-Rh-MLVΔ-IRES-EGFP) were made. For pCS-CMV-IRES-EGFP, pSRαIRES-EGFP was digested with *Bgl*II, filled in with Klenow fragments, and digested with *Eco*RI to liberate an ~1.4-kb fragment that contained the IRES-EGFP gene sequence. This 1.4-kb fragment was then cloned into the *Hpa*I and *Eco*RI sites of pCS.[173] For pCS-Rh-MLVΔ-IRES-EGFP, pCS-Rh-MLVΔ–E was digested with *Xho*I, filled in with Klenow fragments, and then digested with *Bam*HI to liberate an ~900-bp fragment that contained the Rh-MLV LTR. This DNA fragment was ligated to the *Eco*47III and *Bam*HI sites of the pCS-IRES-EGFP vector. pCS-Rh-MLV-E was then constructed by cloning a ~1.7-kb fragment of pBS-

MSV-EM (*Spe*I digested, filled in with Klenow fragments, and *Xho*I digested) into the *Hpa*I and *Xho*I sites of pCS-Rh-MLV-Δ-IRES-EGFP. To generate pSIN-18-cppt-RhMLV-E, an ~600-bp fragment (which contains the housekeeping phosphoglycerate kinase [PGK] promoter) of pRRL-PGK-EGFP-SIN18 was liberated by *Eco*RV and *Age*I. The resulting vector fragment was then ligated to the ~1.4kb fragment of CS-Rh-MLV-E (*Sac*II digested, blunted by T4 polymerase, and *Age*I digested) that contains the Rh-MLV promoter and the partial *gag* sequence of MoMLV.

Cppt-DC-SIGN (appendix figure i) was derived from SIN18-cppt-RhMLV-E by digestion with restriction enzymes *Bam*H1 and *Sal*I, removal of the GFP reporter gene and replacing with the human DC-SIGN gene.

The TRC shRNA library[174,175] (appendix figure i) was produced with pLKO.1 containing a 1.9-kb stuffer sequence in place of the shRNA cassette. The pLKO.1 plasmid was doubly digested by *Eco*RI and *Age*I to release the 1.9-kb stuffer fragment. DNA fragments were then separated using a 1% agarose gel. The 6-kb *Eco*RI/*Age*I band was excised and DNA was extracted and eluted. Mixed sense and antisense hairpin oligonucleotides were obtained from IDT DNA Technology in a 96-well plate format. Oligonucleotide pairs were annealed with the prepped vector.

Virus production, titration and transduction protocols

VSV-G pseudotyped lentiviral vectors were prepared, concentrated, and titered as described previously.[172] Briefly, on the day before transfection, 17.5 x 10^6 293T cells were plated on a T-175 flask (BD Falcon) using 40 ml of supplemented IMDM media. On the day of transfection, the old media was removed and replaced with 25 ml fresh

supplemented media containing 100 μl of 10 mM chloroquine (Sigma). The function of chloroquine is to prevent 293T cell lysozymes from degrading plasmid DNA. Next, 5 μg of pVSV-G, 12.5 μg of p8.2Δvpr and 12.5 μg of the lentiviral vector was added in a 50 ml centrifuge tube (BD Falcon). Cell culture grade water (Hyclone) was added to the tube to a final volume of 977 μl. The tube was vortex vigorously and incubated on ice for 10 minutes. Next, 133 μl of 2 M $CaCl_2$ (Sigma) was added to the tube drop-by-drop with vigorous vortexing after every few drops. The tube was incubated on ice for 5 minutes. After that, 1110 μl of 2 x HEPES buffered saline (1% HEPES w/v, 1.6% NaCl w/v, 0.72% 0.25 M Na_2HPO_4 v/v and 1% v/v 1 M KCl (all from Sigma) in cell culture grade water) was added to the tube drop-by-drop with vigorous vortexing after every few drops. The tube was incubated on ice for 20 minutes. Next, the transfection mixture was added to the roof of the T-175 flask and mixed with the media. The flask was turned right-side up and returned to the incubator for 8 hours. After that, the transfection mixture was removed by aspiration and 40 ml of CS supplemented IMDM media (IMDM, 10% CS and 1% PSG) was added. The viral supernatant was collected in a 50 ml centrifuge tube 3 days post-transfection, centrifuged at 1200 RPM for 5 minutes and filtered through a 0.22-μm-pore-size filter. To concentrate the lentivirus, the supernatant was ultracentrifuged at 17,000 RPM for 1.5 hours at 4°C. After that, the supernatant was poured out and the viral pellet was resuspended with the 200 μl drop left in the tube plus 100 μl of fresh CS supplemented IMDM. Next, the tube was left at 4°C overnight and vortexed vigorously before freezing the viral supernatant at -70°C until needed.

Virus titration with 293T cells was completed by plating 5 x 10^4 cells per well of a 24-well dish the day before titration. On the day of titration the concentrated viral

supernatant was diluted to 0.1x (0.3 μl virus in 299.7 μl supplemented IMDM). Next, 2.4 μl of 1 mg/ml Polybrene (final concentration = 8 μg/ml) (Sigma) was added and the whole mixture was vortexed well. After that, the old media was removed from the well and 250 μl of the viral mixture was added. The plate was returned to the incubator for 2 hours before the viral mixture was removed and replaced with 1 ml of fresh supplemented IMDM. The cells were harvested 3 days post-transduction and analyzed in flow cytometry. The titre was calculated with the formula, titre = (1 x 10^5 cells) x (% $EGFP^+$ cells) x (4) x (dilution factor).

Transduction of resting, IL-2 activated NK (2.0 x 10^5 cells), or bone marrow progenitor cells was carried out using either the 'spin" or "no-spin" protocol as described below. In the "no-spin" protocol, cells were pelleted at 2000 RPM for 5 minutes at room temperature in 1.5 ml screw cap tubes and resuspended in 0.25 ml of viral supernatant (at various MOI) in the presence of 8 μg/ml of Polybrene (Sigma). Cells were incubated for 2 hours at 37°C and 5% CO_2. In the "spin" protocol, cell suspensions were placed in 48 well culture plates and centrifuged at 2000 RPM for 1 hour at room temperature. For experiments involving two rounds of transduction, this was conducted 24 hours apart. For both "no-spin" and "spin" protocols, virus-containing supernatant was removed at the end of transduction. Transduced NK cells were cultured in 0.5 ml of supplemented RPMI media containing 1000 units/ml of IL-2 (unless otherwise indicated) for 3 days before analysis. NK cells transduced with TRC vectors were incubate in IL-2 supplemented medium with 24 μg/ml of puromycin (Calbiochem, San Diego, CA) for 24 hours. The puromycin containing medium was removed and replaced with fresh IL-2 supplemented medium for 3 days before analysis. In the experiment involving the Reverse

Transcriptase inhibitor AZT (Sigma), 0.2 mM AZT (final concentration) was added to the NK culture overnight, before transduction and post-transduction. The same concentration of AZT was used in the transduction culture. Transduced NK cells were cultured for 3 days post-transduction before analysis by flow cytometry.

Interferon-γ production

LAK NK cells (1 x 10^6 cells) were incubated in 5 ml round-bottom tubes (BD Falcon, Franklin Hills, NJ) overnight in 300 μl of supplemented RPMI 1640 containing 1000 units/ml IL-2, 10 ng/ml PMA, 0.5 μg/ml ionomycin, and 10 μg/ml Brefeldin A (BFA) (all from Sigma). Brefeldin A prevents the exocytosis of cytokine-containing vesicles, therefore, allows detection of cytokine production intracellularly following stimulation.[20] IFN-γ production was analyzed by intracellular staining and flow cytometry.

Flow cytometry

Anti-NK1.1 PE, anti-NK1.1 APC, Biotinated anti-NKG2A/C/E, Biotinated anti-Ly49 C+I, Biotinated anti-Ly49 F, Strepavidin PECy5, and anti-IFN-γ PE were all purchased from eBioscience (San Diego, CA). DX5 PE was purchased from BD Bioscience (San Diego, CA). Anit-human DC-SIGN PE was purchased from R&D Systems (Minneapolis, MN). Biotinated anti-Ocil mAb was a generous gift from Dr. James R. Carlyle (University of Toronto). Non-specific binding of antibodies was prevented by addition of Fc Blocker (eBioscience) followed by 10 minutes of incubation on ice. The primary antibody was then added followed by 30 minutes of incubation on

ice. The samples were then washed with 1x PBS supplemented with 2% fetal calf serum (FCS) (HyClone) and 0.2% sodium azide (Sigma). If a secondary antibody was required, it was added after the wash, incubated on ice for 30 minutes and washed once again with supplemented 1x PBS. If no intracellular staining was needed, cells were fixed with 2% paraformaldehyde (Sigma). If cells were intracellularly stained, 4% paraformaldehyde was used to fix the cells. After fixing, cells were permeablized with saponin (Sigma). The intracellular antibody was then added and incubated on ice for 30 minutes. The cells were then washed with supplemented 1x PBS. Data acquisition was performed on a FACSCalibur (BD) using Cell Quest software (BD). A minimum of 15 000 cells were acquired for each sample. For cell cycle/division analysis, 0.5 x 10^6 cells were pelleted and resuspended in 0.5 ml of DNA staining solution (0.1% w/v sodium citrate, 0.3% v/v Triton-x 100, 0.002% w/v Ribonuclease A (all 3 from Sigma) and 0.01% w/v propidium iodide (Calbiochem)). Cell suspension was incubated in the dark on ice for 30 minutes before analysis using Guava Easycyte Mini (Guava Technologies, Hayward, CA). For cell viability analysis, 0.1 x 10^6 cells were pelleted and resuspended in 40 µl of 1x binding buffer (HyClone), 1 µl of Annexin V-PE (BioVision, Mountain View, CA) and 2 µl of 7-amino actinomycin D (7-AAD) (Sigma, St. Louis, MO). Cell suspension was incubated in the dark at room temperature for 30 minutes before analysis using Guava Easycyte Mini (Guava Technologies). Data was analysed using FACS Express 2 software (De Novo Software, ON). Cell sorting was done using Beckman Coulter sorter. Cells were stained with anti-CD3 antibody before sorting. Two populations were obtained from the sorting, $CD3^-EGFP^-$ and $CD3^-EGFP^+$.

^{51}Chromium release cytotoxicity assay

NK cytotoxicity was accessed in a standard 4-hour ^{51}Cr-release assay against YAC-1, Daudi, P815, BWZ, or BWZ-Ocil. Each killing condition was done in triplicates in 96-well V-bottomed plates (Nunc, Roskilde, Denmark). Target cells were incubated with 100 μCi (GE Healthcare Bio-Sciences, Quebec) for 1 hour at 37°C and 5% CO_2. Target cells were washed 2 times and incubated for 10 minutes in RPMI 1640 supplemented with 10% FCS. 2000 target cells in 100 μl of supplemented RPMI 1640 was added along with 100 μl of effector cells (at the appropriate effector:target ratio, 1:1 to 10:1) into each well. Plates were incubated at 37°C and 5% CO_2 for 4 hours. Cells were centrifuged at 500 RPM for 5 minutes before 40 μl of the supernatant was counted in a liquid scintillation and luminescence counter (Trilux 1450 Microbeta). Percentage of target cells killed was calculated using the formula, % Killing = [(experimental release – spontaneous release)/(total release – spontaneous release)] x 100.

***In vivo* survival analysis of transduced NK cells**

Freshly isolated C57BL/6 NK cells were transduced with the Cppt-2E lentiviral vector at MOI = 40 IU/cell using the "spin" protocol. The transduced cells (1-2x10^6), were injected intravenously into RAG2-/-γc-/- mice[176] that had received a total body irradiation of 200 Rad. Peripheral blood and splenocytes were obtained 2 weeks after the adoptive transfer. Cells were surface stained for NK markers and analyzed immediately in flow cytometry. EGFP$^+$ NK cells were identified in CD3$^-$NK1.1$^+$ populations. Splenocytes were cultured in IL-2 for 5 days before flow cytometry analysis.

CD107a degranulation assay[177]

Effector and target cells (0.2×10^6 each, 1:1 ratio) were mixed in 5 ml centrifuge tubes to a final volume of 0.2 ml. 1 μl of anti-CD107a-PE (BD, San Diego, CA) antibody was added, tubes were mixed and incubated at 37°C and 5% CO_2 for one hour. 6 μg of monensin (Sigma) (at 2 mg/ml in methanol) was added, tubes were mixed and incubated at 37°C and 5% CO_2 for an additional 4 hours before analysis in flow cytometry. Monensin was added to prevent the acidification of endocytic vesicles, therefore, avoiding the degradation of reinternalized surface CD107a proteins and allowing for the visualization of CD107a following stimulation.[20] If additional antibody staining was required, cells were stained before analysis.

Western blots

EL-4 cells (2.0×10^6) were washed twice with PBS before lysis with 40 μl of 0.5% Triton-X lysis buffer (with 10 mmol/l Tris Base and 150 mmol/l NaCl) (all three from Sigma) for 30 minutes on ice with vortexing every 10 minutes. Following lysis, cell debris was pelleted at 13,000 RPM for 10 minutes at room temperature. The pellet was discarded and the supernatant was harvested and placed on ice. The protein concentration was immediately measured using the Bradford Protein Assay following manufacturer's protocol (Biorad, Mississauga, ON). Each protein sample was then adjusted to a concentration of 2 μg/μl (total volume of 50 μl) with 1x PBS. 50 μl of 2x reducing buffer (contains sodium dodecyl sulfate (Biorad) and 2-ME) was added to each protein sample. Prepared samples were boiled at 100°C for 3 minutes and centrifuged at 13,000 RPM for 1 minute at room temperature. 20 μl of each sample was loaded onto 10%

polyacrylamide gels (Biorad). The gel was run at 90 – 110 V until the proteins were completely resolved. The proteins were transferred onto charged polyvinylidene difluoride membranes (Biorad) at 27 V overnight at 4°C. Membranes were blocked with 10% non-fat milk in 1x PBS and 0.1% Tween 20 (Fisher Scientific, Ottawa, ON) (PBST) for 1 hour at room temperature. The blocked membrane was incubated with primary rabbit anti-SHP-1 antibody (Upstate/Cedarlane) diluted in PBST (final concentration of 1 μg/ml) overnight at 4°C. The membranes were washed 3 times for 10 minutes each with PBST. Donkey anti-rabbit HRP (Biolegend, San Diego, CA) secondary antibody (1/2000 dilution) was added to the membrane for 1 hour at room temperature. The membranes were washed 4 times for 30 minutes each with PBST. Bands were visualized by ECL Plus/Advance Western Blotting Detection System (Amersham, Piscataway, NJ) according to manufacturer's protocol. X-ray film (Kodak, Rochester, NY) was developed using the Mini Medical Series film developer (AFP Imaging).

Carboxyfluorescein Succinimidyl Ester (CFSE) labelling

NK cells (0.75×10^6) were centrifuged at 1500 RPM for 10 minutes at room temperature and the supernatant was removed. Cells were resuspended in 5 ml of 1x PBS. 5 ml of 3 μmol/l CFSE (Molecular Probe) was added to the cells and incubated at room temperature for 7 minutes. 5 ml of FBS was added and mixed very gently. Cells were centrifuged at 1500 RPM for 10 minutes at room temperature. Cell pellet was resuspended in 10 ml of supplemented RPMI 1640 medium and again centrifuged at 1500 RPM for 10 minutes at room temperature. Cell pellet was then resuspended in 1 ml

of IL-2 (1000 units/ml) supplemented RPMI 1640 medium and transferred into a 24 well plate and cultured at 37°C and 5% CO_2.

The RNAi consortium (TRC) Lentiviral shRNA library[174]

TRC Lentiviral shRNA library is a collaborative effort based at the Broad Institute of MIT and Harvard, and includes six MIT and Harvard associated research institutions and five international life sciences organizations. TRC will have lentiviral shRNA libraries targeting 15,000 human and 15,000 mouse annotated genes with a goal of validating each shRNA in multiple cell lines.

Statistical analysis

Data were analyzed statistically by a two-tailed Student's t-test. A p value of < 0.05 was considered statistically significant.

CHAPTER 3. RESULTS

3.1. Lentiviral vectors mediate stable and efficient gene delivery into primary murine Natural Killer cells

3.1.1. VSV-G pseudotyped lentiviral vectors allow stable, efficient gene transfer and expression in primary murine NK cells

We used VSV-G pseudotyped, replication-incompetent lentiviral vectors that expressed an EGFP reporter gene (Cppt-2E or FUGW) to evaluate the efficiency of lentiviral transduction in murine IL-2 activated NK (LAK) cells. The percentage of cells that are transduced and the level of transgene expression define the efficiency of gene transfer. $CD3^{-}NK1.1^{+}$ NK cells purified from splenocytes of C57BL/6 mice were cultured in IL-2 (purity >95%) before transduction (Fig. 1A). These LAK cells were transduced at different MOI (4, 20 and 40 IU/cell) using a "no-spin" protocol that has been used successfully in efficient transduction of primary primate T cells.[172] The transduced cells were analyzed for EGFP expression 3 days post-transduction. In this single-step transduction protocol of primary LAK cells, we demonstrated a transduction efficiency of 26% of the $CD3^{-}NK1.1^{+}$ cells, at an MOI of 20 IU/cell (Fig. 1B). No pseudo-transduction was observed because EGFP expression in these transduced cells was suppressed in the presence of the reverse transcriptase inhibitor, AZT (Fig. 1B). Lentiviral vectors confer stable EGFP gene expression in this proliferating, transduced primary LAK culture *in vitro* for over two weeks (Fig. 1C).

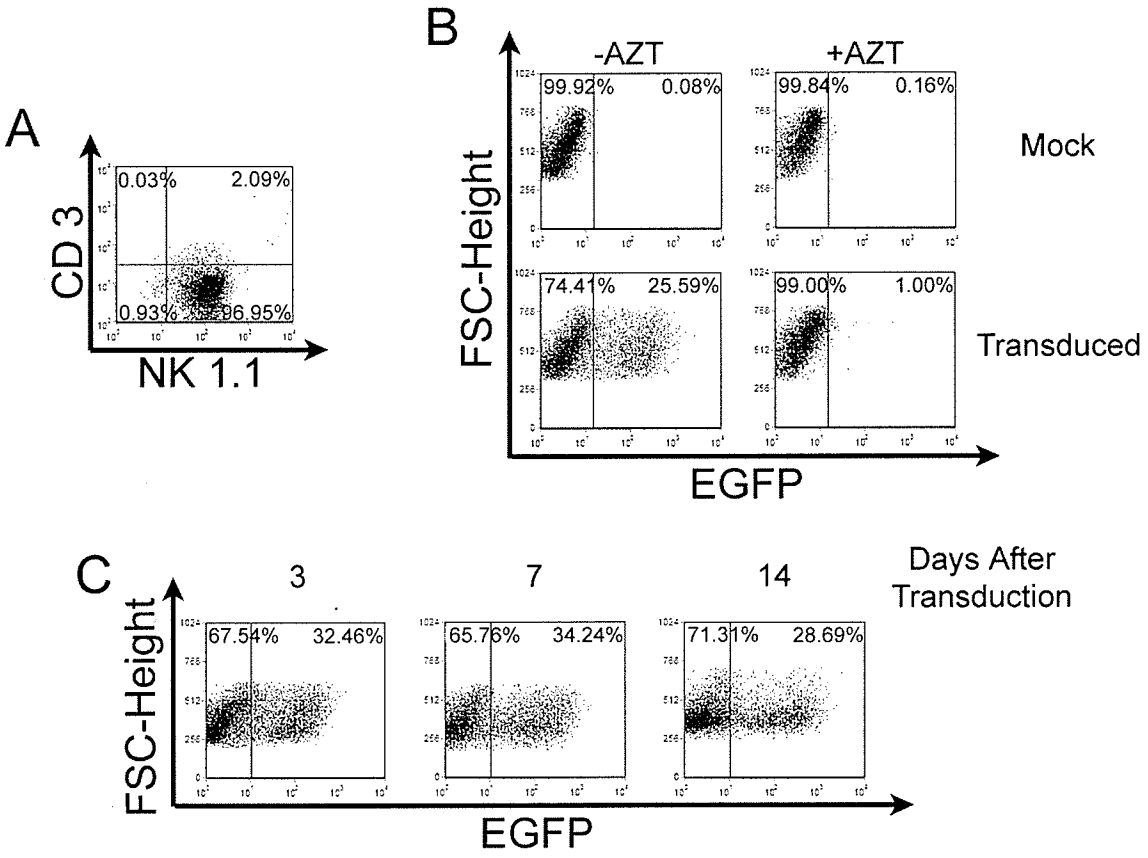

Figure 1. VSV-G pseudotyped lentiviral vectors allow efficient gene transfer into purified IL-2 activated primary murine NK cells (LAK). (A) NK cells were purified from splenocytes of C57BL/6 mice and cultured as LAK cells, as described in the Materials and Methods. Purity of the LAK cells was analyzed by surface staining with anti-NK1.1-PE and anti-CD3-PECy5 in flow cytometry. Quadrants were set according to its respective isotype control. (B) Efficient transduction of primary LAK cells. Day 5 LAK cells were transduced with the EGFP-expressing lentiviral vectors (Cppt-2E, MOI 20 IU/cell) under the "no-spin" protocol (Materials and Methods). They were cultured in the presence or absence of AZT for 3 days before flow cytometry analysis. EGFP expression in the $CD3^{-}NK1.1^{+}$ gated population was shown. (C) Stable EGFP expression in the lentiviral transduced LAK cells was maintained *in vitro*. Day 5 LAK cells were transduced (Cppt-2E, MOI 40 IU/cell) and maintained for over a two-week period. Transduced LAK cells were harvested 3, 7 and 14 days post-transduction and analyzed for their EGFP expression in flow cytometry. Data shown are representative of 3 experiments.

We next examined whether the transduction efficiency of primary LAK cells could be enhanced by a centrifugation-based virus inoculation protocol ("spin" protocol) that is commonly used in retroviral transduction.[135,141,143] Parallel experiments were set up to compare the transduction efficiency of LAK cells, using the "no-spin" and "spin" protocols at different MOIs. At an MOI of 20 IU/cell, an average "spin" transduction supported higher transduction efficiency than that of "no-spin", at a range of 31.5%-43.3% versus 21.7%-26.7% respectively ($p < 0.05$). The biggest difference was observed at a lower MOI (4 IU/cell) – an average of 26.6% (ranges 19.0%-30.4%) and 13.8% (ranges 11.7%-16.9%) under the "spin" and "no-spin" protocol, respectively ($p < 0.05$) (Fig. 2A). We therefore concluded that the "spin" protocol is more efficient in transducing primary LAK cells at lower MOI, when compared to that obtained in the "no-spin" protocol. We used the "spin" protocol in all the subsequent transduction experiments. We further demonstrated that the transduction protocol could be used to express surface receptors (eg. DC-SIGN) (Fig. 2B). Transduction efficiency can be further augmented by incubating the transduced LAK cells in additional IL-12 ($p < 0.0001$) (Fig. 2B) or by an additional "spin" transduction ($p < 0.0001$) (Fig. 2C).

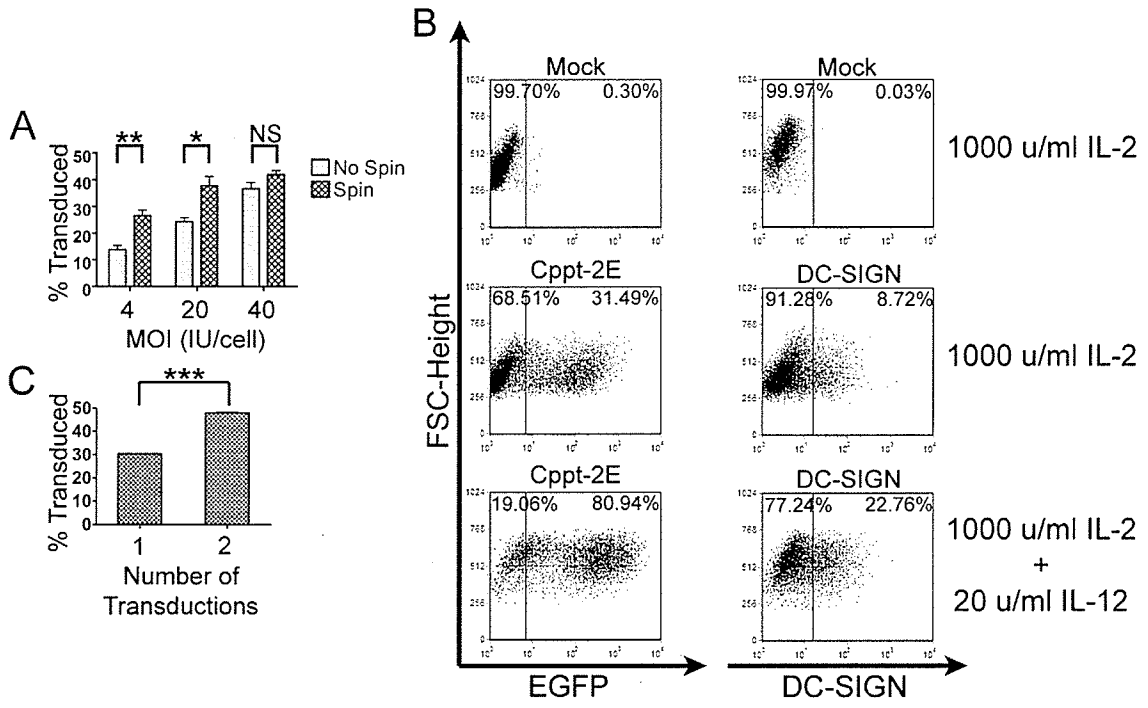

Figure 2. Lentiviral vectors are capable of delivering genes other than EGFP. (A) A single-step centrifugation-based transduction protocol supports a higher transduction efficiency of LAK cells at low MOI. Day 5 LAK Cells were transduced with the EGFP-expressing, FUGW lentiviral vectors under the "spin" or "no-spin" protocol at different MOIs (40, 20, and 4 IU/cell). Transduced LAK cells were analyzed for EGFP expression 3 days post-transduction. EGFP expression in the $CD3^{-}NK1.1^{+}$ gated population was shown. (B) Efficient delivery of DC-SIGN. Day 5 LAK cells were transduced with the DC-SIGN-expressing, Cppt-DC-SIGN lentiviral vectors under the spin protocol at MOI = 40 IU/cell. DC-SIGN expression was analyzed 3 days post-transduction. (C) Two rounds of transduction on LAK cells increases gene delivery efficiency compared to single round of transduction. Day 5 LAK cells were transduced twice, 24 hours apart with Cppt-2E lentiviral vectors under the "spin" protocol at MOI = 20 IU/cell. Data shown are representative of 3 experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

The choice of internal promoters used in lentiviral vectors has a profound effect on the level of transgene expression in transduced primate T cells and hematopoietic stem cells.[172] A murine leukemia virus long terminal repeat viral promoter (RhMLV) from rhesus macaques lymphoma is shown to support higher level of transgene expression in human T lymphocytes when compared to that of an internal cytomegalovirus (CMV) immediate/early promoter.[172] An endogenous human ubiquitin-C promoter (Ubi-C) has been used in a transgenic mouse model *in vivo* to provide reliable expression in various tissues.[171] We, therefore, used lentiviral vectors FUGW and Cppt2E, which carried the internal Ubi-C and RhMLV promoters, respectively, to examine EGFP reporter gene expression in the transduced LAK cells. We found that both vectors supported transduction and expression of EGFP reporter genes in the primary LAK cells. Optimal transduction efficiencies of 40% and 33% using the FUGW and Cppt-2E vectors, respectively, were achieved at an MOI of 40 (Fig. 2, 3A). However, the internal viral RhMLV promoter allowed approximately 3-fold higher level of EGFP expression in transduced cells when compared to that of the Ubi-C promoter (MFI of 120 versus 40) ($p < 0.0001$) (Fig. 3A). We further compared the activities of other commonly used promoters (eg. CMV or PGK) in expressing EGFP reporter gene in the transduced LAK cells at low MOI. We demonstrated that their promoter activities in descending order: RhMLV (MFI 216), PGK and Ubi-C (MFI ~85), and CMV (MFI 45) ($p < 0.0001$) (Fig. 3B).

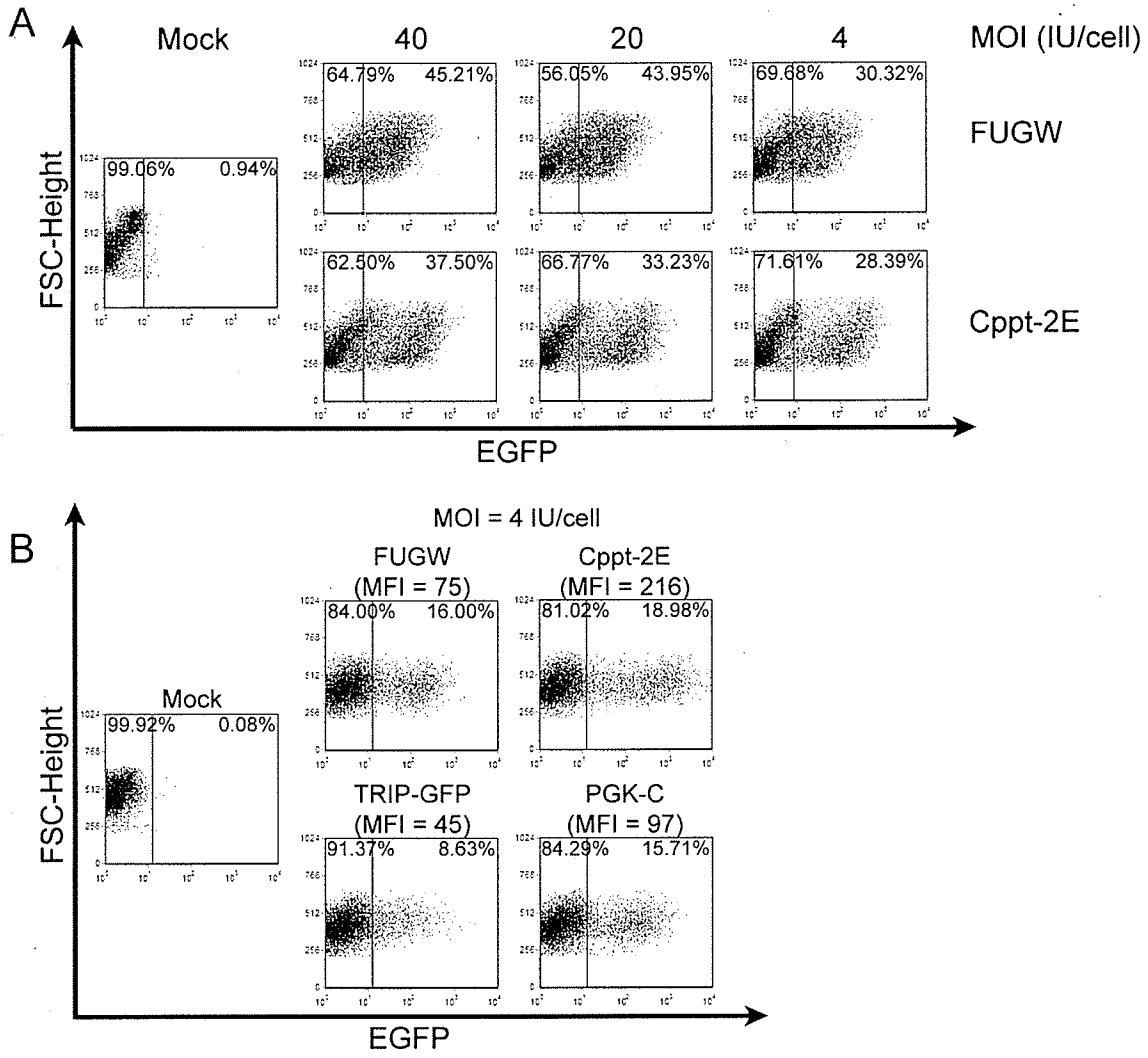

Figure 3. The choice of internal promoter controls the level of transgene expression in the transduced LAK cells. ($p < 0.0001$) (A) Day 5 LAK cells were transduced with either Cppt-2E or FUGW vectors at different MOIs (40, 20, 4 IU/cell) using the "spin" protocol as described. They were analyzed for EGFP expression 3 days post-transduction. EGFP expression in the $CD3^{-}NK1.1^{+}$ gated population was shown. (B) Day 5 LAK cells were transduced with Cppt-2E, FUGW, TRIP-GFP or PGK-C vectors at MOI = 4 IU/cell using the "spin" protocol as described. Data shown are representative of 3 experiments.

3.1.2. Lentiviral transduction does not impair NK cell functions

Identification of multigene families of NK receptors in mice and humans revealed the polyclonal nature of mature NK cell populations with respect to their NK receptor expression and distribution.[42] Upon activation, these NK cells produce chemokines/cytokines (such as IFN-γ) and trigger cytolytic activities towards target cells.[118] We, therefore, used these parameters to examine whether our single-step transduction effects NK phenotype and/or functions.

Surface expression of NK receptors NKG2A/C/E, Ly49C+I, Ly49F, NKG2D and NK1.1 on $EGFP^+$ transduced LAK cells were analyzed to determine whether transduced cells exhibit different NK receptor phenotypes. When compared to the control LAK cells (mock-transduced and $EGFP^-$ LAK cells in the transduced culture), we observed no difference in the surface expression of the panel of NK receptors examined in the $EGFP^+$ LAK cells (Fig. 4A). The transduction process and the transgene expression of EGFP had no impact on surface expression of these commonly studied NK receptors. In addition, the data indicate that this VSV-G pseudotyped lentiviral vector system was not biased towards a particular subset(s) when used in transduction of polyclonal LAK cells.

Induction of IFN-γ production was used as a measure to examine whether cytokine production in lentiviral transduced LAK cells was affected. LAK cells were harvested, stimulated with PMA and ionomycin (potent inducers of IFN-γ production)[178] overnight and stained for intracellular IFN-γ production. Non-stimulated $EGFP^+$, mock and $EGFP^-$ LAK cells were used to measure basal levels of IFN-γ production. The percentage of IFN-γ^+ cells and MFI of IFN-γ production in the $EGFP^+$ transduced LAK cells were compared to the mock-transduced and the $EGFP^-$ LAK cells in the transduced

culture. No basal level of IFN-γ production was detected in all three LAK NK populations (data not shown). Approximately 80% of $NK1.1^+$ NK cells were IFN-γ-positive in all three groups of LAK cells analyzed (Fig. 4B). No significant impairment in IFN-γ induction in the transduced cells was observed meaning that lentiviral transduction does not effect normal NK cell cytokine production.

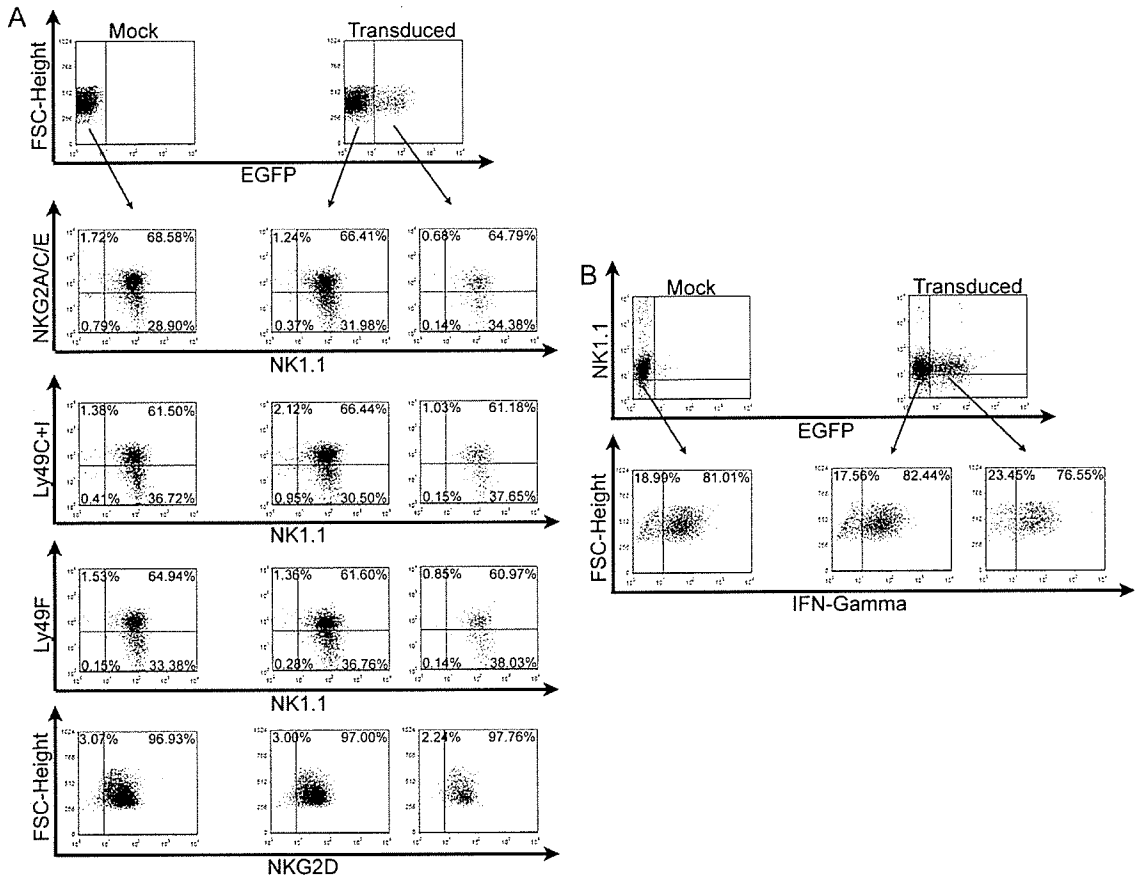

Figure 4. Lentiviral transduction does not impair surface NK receptor expression or IFN-γ production. (A) Mock-transduced and EGFP-transduced (FUGW, MOI 40 IU/cell) LAK cells were analyzed for their surface expression of NKG2A/C/E, Ly49C+I, Ly49F, NKG2D, and NK1.1. (B) Lentiviral transduced LAK cells produced IFN-γ upon stimulation. LAK cells transduced with FUGW (MOI 40 IU/cell) were stimulated in ionomycin and PMA overnight to induce IFN-γ production. The cells were intracellularly stained for IFN-γ and analyzed in flow cytometry. All quadrants were set according to its respective isotype control. Data shown are representative of 2 experiments.

Next, NK cell-mediated cytotoxicity was examined in the transduced LAK cells. Mock-transduced and lentiviral-EGFP-transduced LAK cells were harvested on day 7 post-transduction. We used FACSorting to isolate the $EGFP^+$ and $EGFP^-$ cells of the transduced LAK culture (>95% purity). These purified LAK cells, together with the unsorted mock-transduced cells, were used as effectors in a standard ^{51}Cr release cytotoxicity assay. The prototypic NK-sensitive cell line, YAC-1, was used as NK targets. A relatively NK-resistant Daudi target cell line was used in the same assay to demonstrate specificity of NK target recognition. No significant impairment of cytolytic activities in the sorted $EGFP^+$-transduced LAK cells (Fig. 5) was observed when compared to the sorted $EGFP^-$ LAK or unsorted mock-transduced LAK effectors. These lentiviral transduced cells exhibited efficient YAC-1 killing of 49% at an E:T ratio of 1:1. The percentage of YAC-1 killing was increased as we increased the E:T ratios (up to 80% at an E:T ratio of 10:1). It remained to be determined why the sorted $EGFP^-$ control LAK cells (from the transduced culture) consistently exhibited a slightly higher cytotoxicity when compared to the unsorted mock-transduced LAK control. However, there was no significant difference in the YAC-1 killing when we compared the cytolytic activity of the sorted $EGFP^+$ and $EGFP^-$ LAK cells (Fig. 5A). In addition, target specificities towards YAC-1 and Daudi targets were maintained in all three LAK cell populations (Fig. 5B).

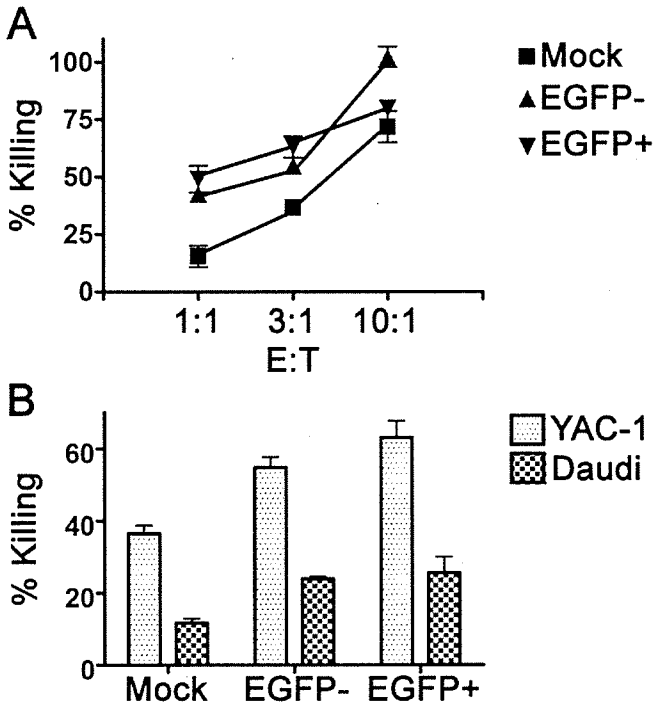

Figure 5. NK cell-mediated cytotoxicity and target specificity are not altered by lentiviral transduction. LAK cells were transduced by the Cppt-2E vector at an MOI of 20 IU/cell using the "spin" protocol. Mock-transduced and the lentiviral transduced LAK cells were cultured for 7 days before cell sorting. The $EGFP^+$ or $EGFP^-$ NK cells that were $CD3^-$ were sorted and used as effector cells in a 4-hour ^{51}Cr release assay. Unsorted mock-transduced LAK were used as effectors for comparison. YAC-1 (A, B) and Daudi cells (B) were used as target cells in the assay. Data shown are representative of 2 experiments.

To examine whether lentiviral transduction has any effect on cellular viability, transduced LAK cells were stained with Annexin V to measure apoptosis. We observed that $EGFP^+$ NK cells did not show increased frequencies of apoptosis (3-5%) when compared to the mock-transduced or $EGFP^-$ untransduced controls (data not shown). More importantly, we demonstrated *in vivo* persistence of $EGFP^+$ NK cells in the peripheral blood and spleen of the recipient RAG2-/-γc-/- immunodeficient mice 2-3 weeks after we had adoptively transferred with the lentiviral transduced NK cells into these animals (Fig. 6).

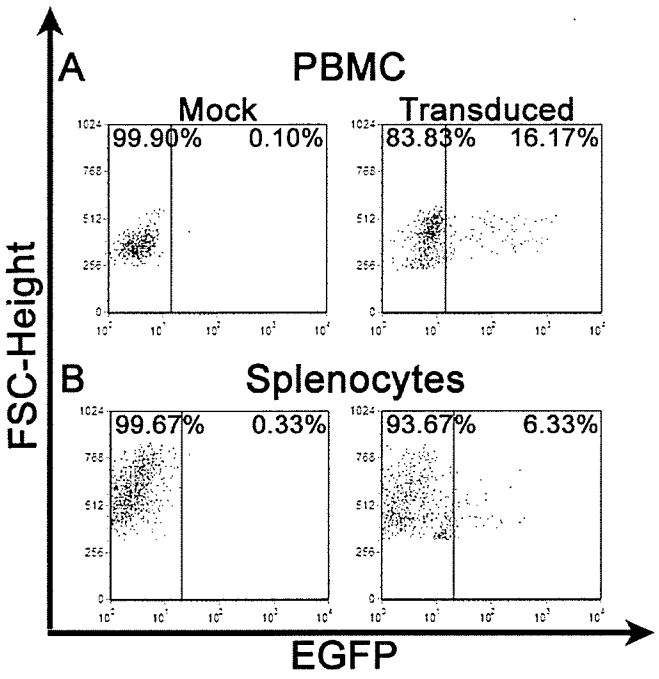

Figure 6. Transduced NK cells persist *in vivo*. Freshly isolated NK cells were transduced with the Cppt-2E lentiviral vector at MOI = 40 IU/cell using the "spin" protocol, cultured in IL-2 for 6 days. The transduced NK cells were i.v. injected into irradiated RAG2-/-γc-/- recipient mice. Peripheral blood and splenocytes were collected on day 15 after the adoptive transfer. EGFP expression of the $CD3^{-}NK1.1^{+}$ gated population in peripheral blood (A), and splenocytes after 5 days *in vitro* culture in IL-2 (B) was analysed. Splenocytes were cultured in IL-2 to induce NK cell proliferation, activation and transgene expression.

3.1.3. Lentiviral vectors are able to transduce resting NK and Progenitor cells without prior cytokine activation

Previously published work used only IL-2 activated NK cells in their gene transfer studies.[135,136,141,143,144] A unique feature of an HIV-1 based lentiviral vector is its ability to enter the nucleus without the need to activate cells prior to transduction. We examined whether lentiviral vectors supported efficient gene transfer into resting/non-proliferating NK cells. *Ex vivo* NK cells were isolated (purity over 86%) (Fig 7A), and confirmed to be at its resting state by cell cycle analysis (Fig. 7B). They were transduced by an EGFP-expressing lentiviral vector without prior IL-2 stimulation. Transduction efficiency was evaluated by measuring the percentage of $EGFP^+$ cells in IL-2 containing culture medium at day 5 post-transduction *in vitro*. We demonstrated efficient transduction of these *ex vivo* NK cells, 41.9% (range 36.0%-49.3%) at an MOI of 40 IU/cell. At lower MOI, we were still able to obtain transduction efficiencies of 33.6% (range 32.0%-35.3%) and 15.4% (range 14.2%-17.2%) at MOI of 20 and 4 IU/cell, respectively (Fig. 7C).

High levels of exogenous IL-2 are necessary, and routinely used in maintaining NK cultures *in vitro*. However, NK cell activation by high levels of exogenous IL-2 may not be desirable given the pleiotropic effects of IL-2 receptor signalling. Therefore, we examined the concentration requirement of IL-2 on transgene expression in these transduced *ex vivo* NK cells. Briefly, freshly isolated NK cells were transduced with the FUGW lentiviral vectors without prior IL-2 activation, and cultured in complete medium supplemented with different concentrations of IL-2 (100, 200, 500 and 1000 U/ml) *in vitro*. At day 3 post-transduction, the effect of exogenous IL-2 on EGFP expression was analyzed by flow cytometry. EGFP expression ranging from 21% - 48% was observed in

all IL-2 culture conditions (Fig. 7D). As reported previously, we found that maintenance of primary NK cells *in vitro* required high concentrations of IL-2 (500 - 1000 U/ml).[135,141,143,144] At lower IL-2 concentrations, fewer activated NK cells and lower cellular proliferation/viability were observed (data not shown). The lowest percentage of EGFP-expressing cells (about 21%) was observed in the transduced NK culture that was maintained at the lowest IL-2 concentration (100 U/ml) tested in the 3-day post-transduction period. Since we observed different levels of EGFP expression in the NK cells (that were all freshly isolated and transduced in the absence of prior IL-2 activation) under different IL-2 culture conditions, we concluded that IL-2 concentration determines the activation status of NK cells thereby determining the level of transgene expression in these cells. IL-2 is not required for lentiviral transduction because no IL-2 was added during the transduction procedure and excess viral particles were removed before the addition of various IL-2 concentrations in the culture. We also examined the effects of different cytokine combinations, commonly used in NK studies, in augmenting transgene expression in the transduced resting NK cells. We found that IL-2 was better than IL-15 alone in supporting the transduced resting NK cells ($p < 0.05$) (Fig. 7E). A combination of IL-2/IL12 or IL12/IL15 supported a 3-fold increase in transduction efficiency when compared to IL-2 alone ($p < 0.0001$) (Fig. 7E).

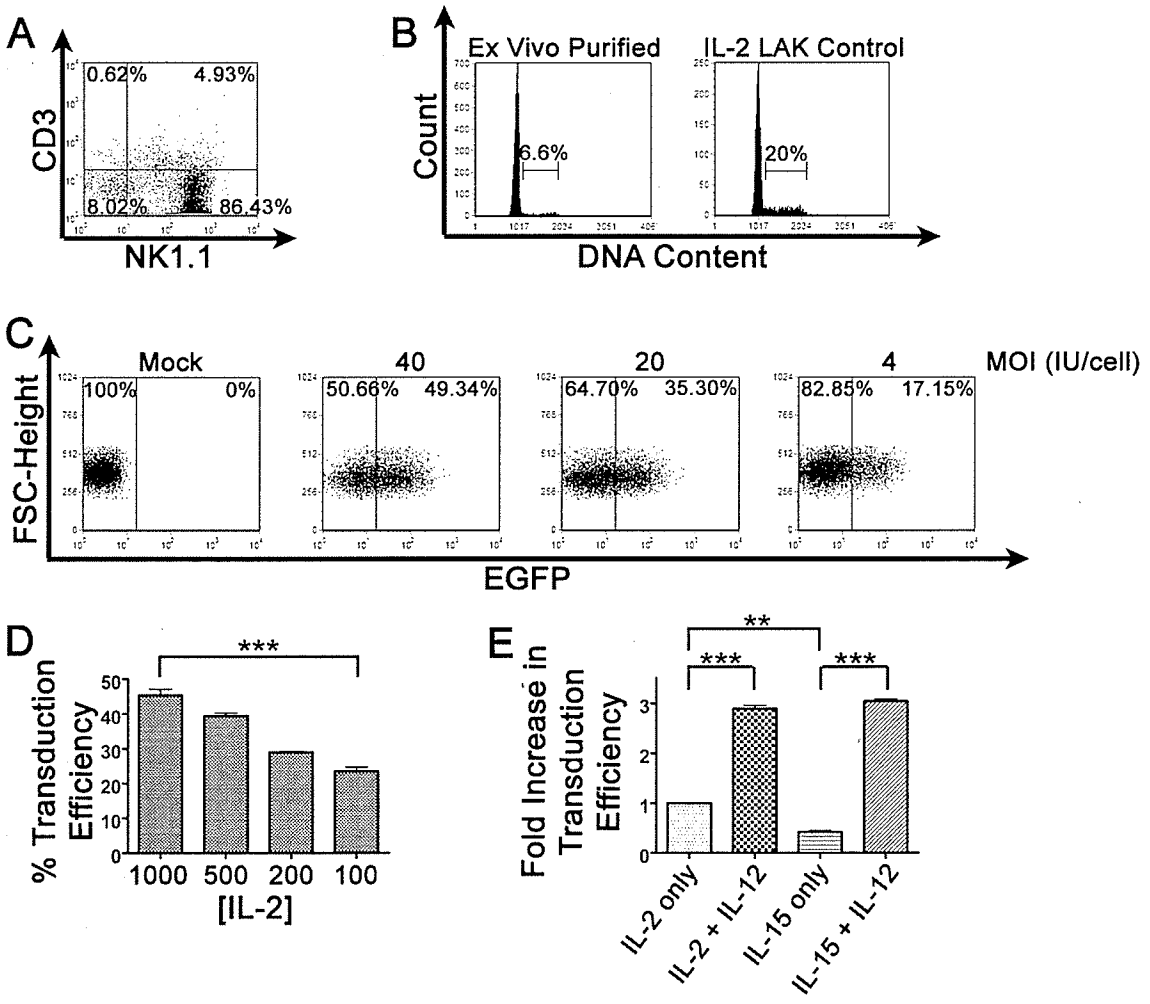

Figure 7. Lentiviral vectors transduce freshly purified *ex vivo* NK cells without prior IL-2 activation. *Ex vivo* NK cells were purified as described previously. (A) Purity of the NK cells was analyzed by surface staining with anti-NK1.1-PE and anti-CD3-PECy5 in flow cytometry. (B) Cell cycle/division analysis on freshly purified and day 5 LAK NK cells. Cells were stained with DNA staining solution and analyzed in flow cytometry. (C) Purified $CD3^{-}NK1.1^{+}$ NK cells were transduced, in the absence of IL-2, with the FUGW vectors at different MOI using the spin protocol. They were cultured in IL-2 containing medium for 5 days before analysis for EGFP expression. EGFP expression in the $CD3^{-}$ $NK1.1^{+}$ gated population was shown. Data shown are representative of 3 experiments. (D) IL-2 augmented transgene expression in the *ex vivo* purified NK cells that were transduced without prior IL-2 activation. *Ex vivo* $CD3^{-}NK1.1^{+}$ enriched NK cells were transduced with FUGW (MOI: 40 IU/cell). They were cultured in RPMI medium supplemented with different concentrations of IL-2 (as indicated). EGFP expression was analyzed in flow cytometry 3 days post-transduction. (E) Activation of transduced resting NK by IL-2 lead to higher EGFP expression compared to IL-15. NK cells were transduced with Cppt-2E (MOI = 40 IU/cell) and incubated in their respective cytokines. EGFP expression was analyzed in flow cytometry 5 days post-transduction. Data is representative of 3 experiments. **, $p < 0.01$; ***, $p < 0.0001$.

We next examined whether lentiviral vectors were able to transduce murine hematopoietic progenitors and maintain stable transgene expression during NK differentiation *in vitro*[179] and *in vivo*. We used an EGFP-expressing lentiviral vector to transduce enriched hematopoietic stem cells from C57BL/6 or RAG2-/- mice without prior cytokine stimulation. After the single-step lentiviral transduction, these progenitors were differentiated (mock or transduced) into NK cells. Developing NK cells were evaluated by surface expression of the serological NK marker NK1.1. Using an *in vitro* NK differentiation culture condition, we found that over 20% of $NK1.1^+$ cells derived from the progenitor cells expressed the EGFP reporter gene (Fig 8A). Using an *in vivo* NK differentiation protocol where transduced progenitors were adoptively transferred into irradiated RAG2-/-γc-/- immunodeficient mice, we found that over 6% of $NK1.1^+$ cells expressed the EGFP reporter gene (Fig. 8B). In addition, we found that starting with progenitors from T and B cell deficient mice (RAG2-/-) can dramatically increase the adoptively transferred NK cell population while eliminating the $CD3^+$ T cells. Taken together, we concluded that lentiviral vectors were capable of transducing *ex vivo* NK cells and NK progenitor cells without the need of prior cytokine stimulations.

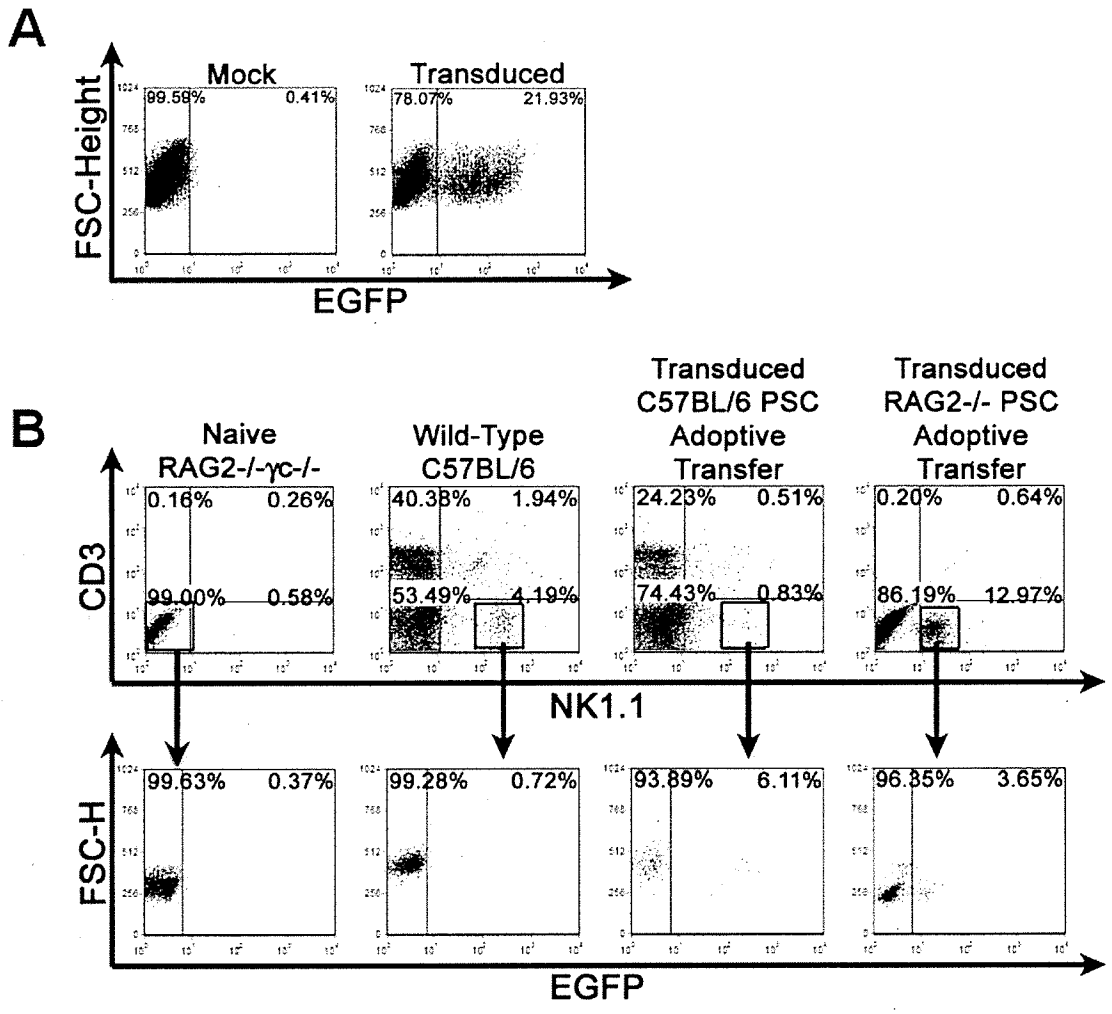

Figure 8. Efficient gene transfer in differentiating NK cells that were derived from the lentiviral-transduced murine hematopoietic progenitor cells *in vitro*. Bone marrow cells of C57BL/6 or RAG2-/- mice were isolated and enriched for progenitor stem cells in using EasySep Mouse Progenitor Stem Cell Negative Selection kit (StemCell Technologies). They were transduced with Cppt-2E (MOI: 40 IU/cell) in the absence of cytokines under the spin protocol. (A) After transduction, they were cultured in supplemented RPMI containing a cocktail of cytokines that support NK differentiation *in vitro*. EGFP expression and NK differentiation was analyzed by flow cytometry with staining with anti-NK1.1-PE and anti-CD3-PECy5. Data is representative of 3 experiments. (B) After transduction, they were adoptively transferred by i.v. injection into irradiated RAG2-/-γc-/- recipient mice. Peripheral blood was collected on day 30 after the adoptive transfer. EGFP expression of the $CD3^{-}NK1.1^{+}$ gated population in peripheral blood was analyzed in flow cytometry.

3.2. Over-expression of inhibitory receptor NKR-P1B in mature NK Cells resulted in an altered NK-target cell specificity

The outcome of an NK-target interaction is determined by the interplay of signals generated from inhibitory and activation receptors.[60,62,63,180] Therefore, we wanted to evaluate whether manipulating the balance of signals generated by the simultaneous engagement of activating and inhibitory receptors will alter NK-target cell specificity. We used our established primary NK lentiviral transduction protocol to over-express an NK inhibitory receptor, NKR-P1B in BALB/c LAK. NKR-P1B is a homodimeric type II transmembrane C-type lectin-like inhibitory receptor that binds Ocil/Clr-b expressed by all host hematopoietic cells.[33,43] NKR-P1B is recognized by anti-NK1.1 antibodies. We used BALB/c LAK because they do not express NK1.1. In addition, BALB/c NK cells are speculated to express an endogenous receptor for Ocil that is not recognized by the anti-NK1.1 antibody (Carlyle, J.R., unpublished data). Therefore, it allows us to use anti-NK1.1 antibodies to detect NKR-P1B over-expression in BALB/c LAK.

3.2.1. Over-expression of NKR-P1B in murine IL-2-activated (LAK) NK cells

In our previous work, we demonstrated that lentiviral vectors mediate stable and efficient gene delivery into primary murine NK cells using an enhanced green fluorescent protein as a reporter gene.[181] Employing the established transduction protocol, we wanted to evaluate the efficiency of over-expressing an inhibitory receptor NKR-P1B[33] in BALB/c LAK cells. Day 5 LAK cells were transduced twice on consecutive days (as described in materials and methods) by the spin protocol with Cppt-NKR-P1B lentiviral vector containing virus at an MOI = 40 IU/cell. The transduced cells were analyzed for

NKR-P1B expression 3 days post-transduction. In this two round transduction protocol of primary BALB/c LAK cells, we demonstrated a transduction efficiency of over 7% (Fig. 9). I initially transduced Day 5 LAK cells only once, however, the transduction efficiency was very low, ~2% (data not shown). From here on in, mock represents NK cells that were never introduced to lentiviral virus, NKR-P1B$^-$ represents NK cells that encountered virus but did not undergo transduction and NKR-P1B$^+$ represents NK cells that underwent transduction and expressed the NKR-P1B receptor.

Because only a proportion of the transduced cells were expressing the NKR-P1B receptor, we were working with a non-homogenous cell population. Therefore, flow cytometry was an advantageous technique to use for analysis because it allowed us to separate cell populations by employing regional gates. The CD107a degranulation assay and intracellular staining for TNF-α were chosen over the ^{51}Cr release cytotoxicity assay and ELISA respectively, because the former techniques allowed us to separate cell populations by regional gating.

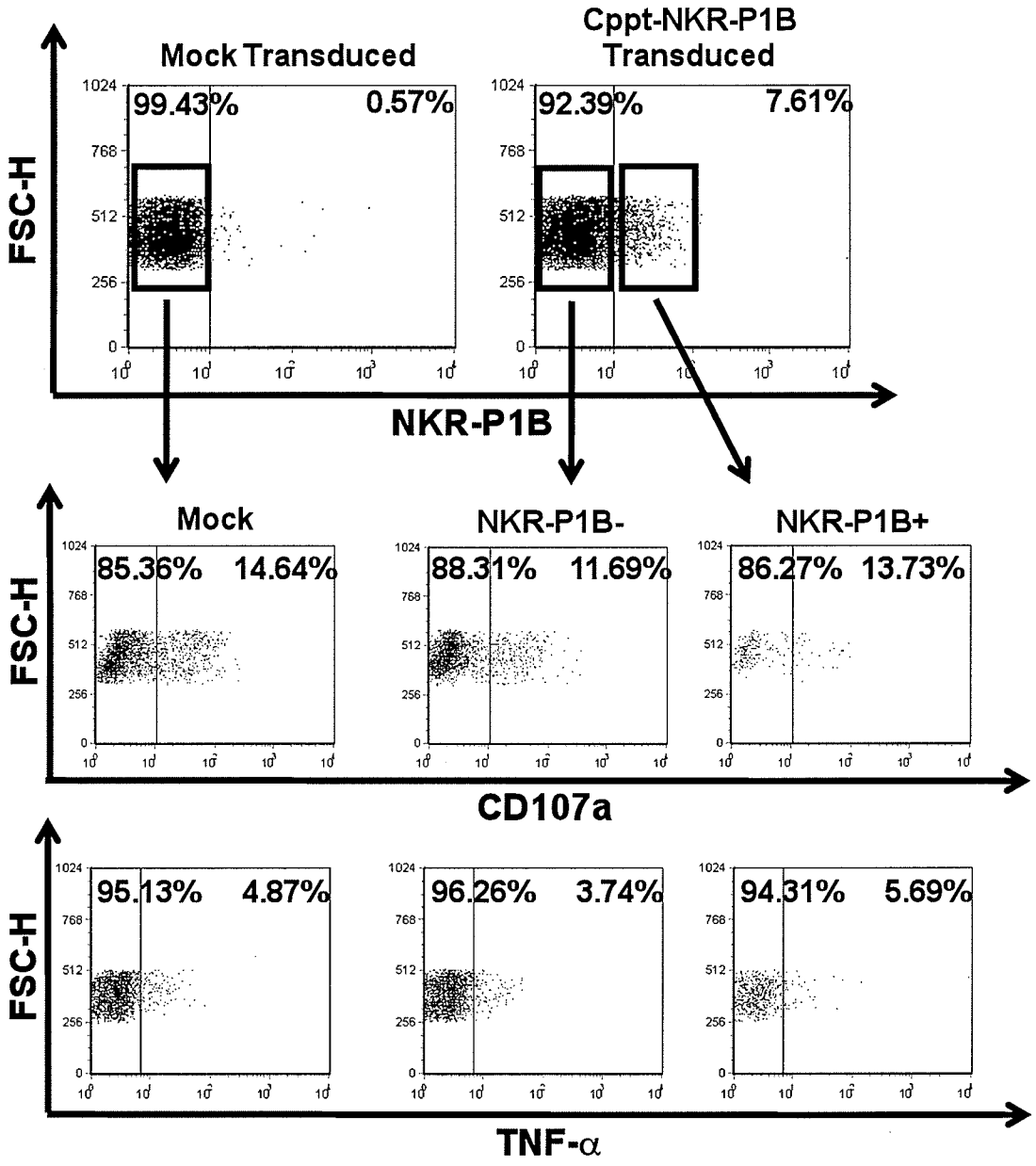

Figure 9. Lentiviral-mediated over-expression of NKR-P1B in BALB/c IL-2-activated LAK cells. Day 5 LAK cells were transduced on 2 consecutive days with Cppt-NKR-P1B vectors at MOI = 40 IU/cell. Cells were centrifuged in 48-well plates at 2000 RPM for 2 hours at room temperature. NKR-P1B surface expression was analyzed 3 days post-transduction by staining with anti-NK1.1-APC antibody in flow cytometry. Functional analysis was conducted by gating on each respective LAK population after incubation with YAC-1 target cells, staining with anti-CD107a PE or anti-TNF-α FITC and analysis in flow cytometry. Data is representative of 3 experiments.

3.2.2. Establishment of CD107a degranulation assay for flow cytometry analysis of NK cytolytic activity

NK cell cytotoxicity is one of the possible outcomes resulting from interactions with virus infected or transformed cells.[182,183] Cytotoxic lysis of the target cells occurs when NK cells release their cytotoxic granules containing perforin and granzymes.[177] The perforin and granzymes act on the target cells causing them to undergo apoptotic cell death.[184,185]

The most common protocol to measure NK-mediated target cell lysis is a standard ^{51}Cr release cytotoxicity assay. This protocol involves incubating NK cells with ^{51}Cr labeled target cells. ^{51}Cr is released into the supernatant as the target cells undergo lysis. The percentage of specific lysis is determined by measuring the amount of ^{51}Cr that has been released by the target cells.

An alternative protocol to assess NK cell mediated cytotoxicity is to measure effector cell degranulation. Lining the membrane of cytotoxic granules is the lysosomal-associated membrane protein-1 (LAMP-1 or CD107a).[186,187] CD107a has been recently identified as a marker of degranulation on human $CD8^+$ cytotoxic T and NK lymphocytes because its expression has been shown to be associated with perforin and granzyme release upon antigen stimulation and target interactions.[20,188] Although CD107a expression has been studied quite extensively on human NK cells, its surface expression on mouse NK cells has not been as intensely studied. We therefore tested CD107a surface expression on mouse NK cells and its correlation with target cell ^{51}Cr release using the well documented NK targets.

Briefly, Day 5 BALB/c LAK cells were used as effectors in a standard 4-hour ^{51}Cr release cytotoxicity assay or CD107a degranulation assay. The degranulation assay

involved incubating effectors with target cells in media containing anti-CD107a antibody and monensin for 5 and 4 hours, respectively. The NK-sensitive YAC-1 and NK-resistant P815 cell lines were used to demonstrate NK-target specificities. To further demonstrate NK-target specificities, NK-sensitive BWZ and NK-resistant BWZ-Ocil (expresses Ocil, a ligand for the NKR-P1B inhibitory receptor) sister cell lines were included in both cytotoxicity assays.

We demonstrated that NK-target specificities were displayed in both target cell ^{51}Cr release and effector cell CD107a expression (Fig. 10). There was a direct correlation in the sensitivity of YAC-1 and resistance of P815 toward NK lysis between ^{51}Cr release from the target cells and CD107a expression on the effector NK cells ($p < 0.01$). Similarly, BWZ displayed increased sensitivity to NK lysis compared to BWZ-Ocil in both ^{51}Cr release and CD107a expression ($p < 0.05$).

Taken together, these data demonstrated that CD107a is a marker of mouse NK cytotoxic activity. Also, CD107a expression directly correlates with ^{51}Cr release as demonstrated by target specificities for NK-sensitive and –resistant targets.

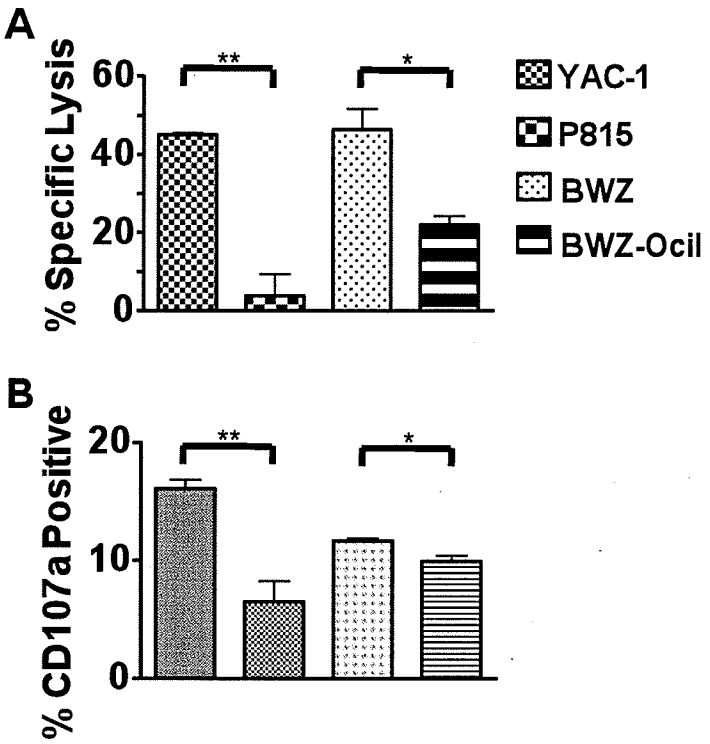

Figure 10. NK CD107a degranulation directly correlates with target cell ^{51}Cr release during NK cell-mediated cytotoxicity. (A) Day 5 IL-2-activated NK (LAK) cells were used as effectors at a 1:1 ratio in a 4-hour ^{51}Cr release assay. (B) Day 5 LAK cells were used as effectors in a CD107a degranulation assay. Briefly, LAK cells were co-cultured with target cells at a 1:1 ratio in 5 ml centrifuge tubes. Tubes were incubated at 37°C for 5 and 4 hours in the presence of anti-CD107a-PE antibody and monensin, respectively. Analysis was conducted in flow cytometry. Data is representative of 3 experiments. *, $p < 0.05$; **, $p < 0.01$.

3.2.3. Over-expression of NKR-P1B resulted in decreased NK cell activity against Ocil-expressing target cells

NKR-P1B is an inhibitory receptor normally expressed on NK cells. Therefore, we hypothesized that BALB/c LAK cells over-expressing NKR-P1B should show decreased cytotoxicity against Ocil-expressing target cells. To evaluate our hypothesis, we used sister cell lines BWZ and BWZ-Ocil as targets. Because BWZ-Ocil expresses the ligand for NKR-P1B, it should be killed less compared to BWZ. In addition, we used NK-sensitive YAC-1 and NK-resistant P815 target cells to evaluate whether target specificity was maintained in all three NK populations.

Briefly, LAK cells were incubated with target cells at a 1:1 ratio in the presence of anti-CD107a antibody and monensin for 5 and 4 hours, respectively. The percentage of LAK cells that underwent degranulation was determined in flow cytometry. Around 15% of the cells in all three populations underwent degranulation against YAC-1 compared to about 5% of the cells undergoing degranulation against P815 (Fig. 11A). The fold difference in CD107a degranulation seen between the NK-sensitive target YAC-1 and NK-resistant target P815 were similar (around 3-fold, differences were not significant) in all three NK cell populations (Fig 11B). I next compared the NK degranulation against BWZ and BWZ-Ocil (Fig 11C). All three NK populations showed decreased degranulation against BWZ-Ocil compared to BWZ. However, the NKR-P1B$^+$ population showed a significantly greater fold decrease in degranulation compared to mock and NKR-P1B$^-$ ($p < 0.001$ and $p < 0.01$ respectively) (Fig. 11D). The degranulation difference for the mock and NKR-P1B$^-$ populations was about 1.5-fold while the difference for the NKR-P1B$^+$ population was about 2.5-fold.

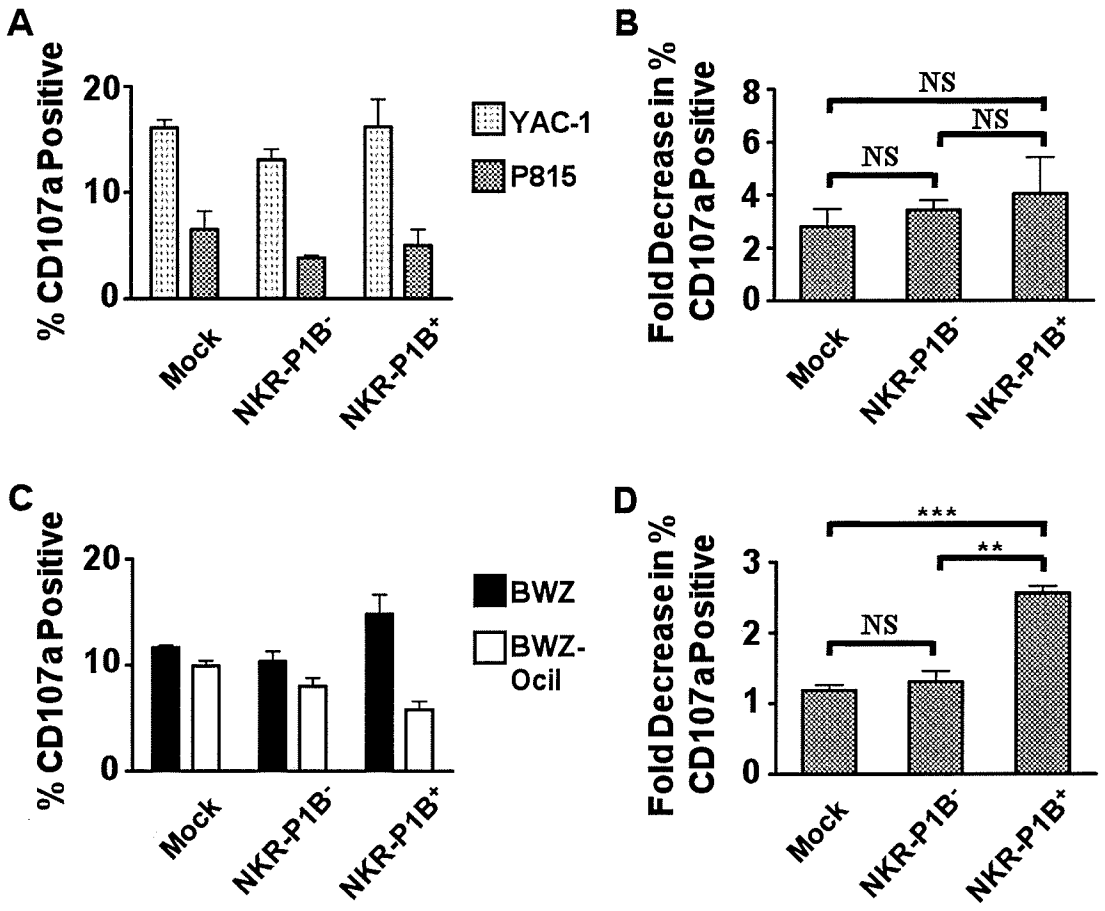

Figure 11. NKR-P1B over-expression resulted in decreased CD107a degranulation against Ocil-expressing targets. Day 5 IL-2-activated NK (LAK) cells were transduced on 2 consecutive days with Cppt-NKR-P1B vectors at MOI = 40 IU/cell. Cells were centrifuged in 48-well plates at 2000 RPM for 2 hours at room temperature. The mock-transduced and lentiviral-transduced LAK cells were cultured for 3 days before they were used as effectors in a CD107a degranulation assay. Briefly, LAK cells were co-cultured with target cells at a 1:1 ratio in 5 ml centrifuge tubes. Tubes were incubated at 37°C for 5 and 4 hours in the presence of anti-CD107a-PE antibody and monensin, respectively. Analysis was conducted in flow cytometry. (A) and (B) YAC-1 and P815 (C) and (D) BWZ and BWZ-Ocil were used as target cells. Fold decrease was calculated by: (% CD107a Positive, YAC-1)/(% CD107a Positive, P815) or (% CD107a Positive, BWZ)/(% CD107a Positive, BWZ-Ocil). Data is representative of 3 experiments. NS, non-significant; **, $p < 0.01$; ***, $p < 0.0001$.

In addition to cytotoxicity, NK cytokine production is also dependent on the balance of receptor signalling.[118] Therefore, we hypothesized that NKR-P1B inhibitory signalling is capable of decreasing NK cytokine production against Ocil expressing target cells. Again, we used sister cell lines BWZ and BWZ-Ocil as the non-Ocil-expressing and Ocil-expressing target cells, respectively. NK-sensitive YAC-1 and NK-resistant P815 were used to evaluate target specificity in all three NK populations.

Briefly, LAK cells and target cells were incubated at a 1:1 ratio overnight in the presence of brefeldin A. TNF-α expression was analyzed by intracellular staining in flow cytometry. Target specificity was maintained in all three NK populations because around 4% of the cells in each population underwent TNF-α production against YAC-1 compared to around 0.6% of the cells producing TNF-α against P815 (Fig 12A). In addition, the fold difference in TNF-α production seen between YAC-1 and P815 were comparable (around 6.5-fold, differences were not statistically significant) in all three NK cell populations (Fig 12B). When comparing TNF-α production against BWZ and BWZ-Ocil, the mock and NKR-P1B$^-$ populations produced 1.5-fold less TNF-α against BWZ-Ocil compared to BWZ. NKR-P1B$^+$ NK cells showed a 2-fold decrease in TNF-α production against BWZ-Ocil compared to BWZ (Fig 12C and D). Therefore, NKR-P1B$^+$ NK cells showed a significantly larger fold decrease in TNF-α expression compared to mock and NKR-P1B$^-$ NK cells ($p < 0.05$). As a result, NKR-P1B inhibitory signalling is also capable of decreasing TNF-α production upon binding to Ocil.

Therefore, the CD107a degranulation and TNF-α production studies showed that the NKR-P1B receptor, when over-expressed in BALB/c LAK cells was fully capable of inhibitory signalling. Upon binding to Ocil, NKR-P1B signaling was capable of skewing

the NK activity outcome towards decreased cytotoxicity and TNF-α production against the target cell. However, target specificities against non-Ocil expressing targets were not affected as seen in YAC-1 versus P815 cells. Taken together, these results suggest the feasibility of directly manipulating NK cell receptor expression leading to an altered degree of NK target recognition.

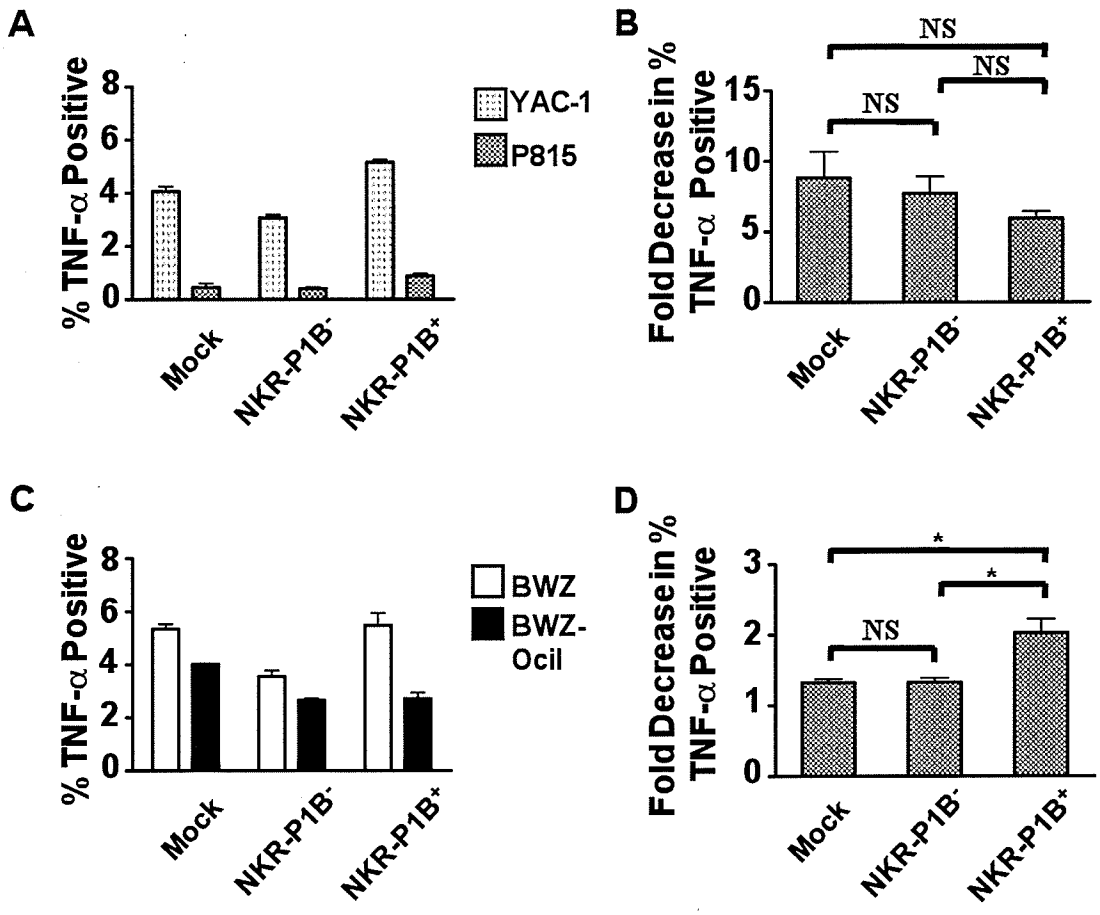

Figure 12. NKR-P1B over-expression resulted in decreased TNF-α expression against Ocil-expressing targets. Day 5 IL-2-activated NK (LAK) cells were transduced on 2 consecutive days with Cppt-NKR-P1B vectors at MOI = 40 IU/cell. Cells were centrifuged in 48-well plates at 2000 RPM for 2 hours at room temperature. The mock-transduced and lentiviral-transduced LAK cells were cultured for 3 days before they were used as effectors in a cytokine production assay. Briefly, LAK cells were co-cultured with target cells at a 1:1 ratio in 5 ml centrifuge tubes overnight with brefeldin A. Cells were extracellular stained with anti-NK1.1-APC and intracellular stained with anti-TNF-α-PE. Analysis was conducted in flow cytometry. (A) and (B) YAC-1 and P815 (C) and (D) BWZ and BWZ-Ocil were used as target cells. Fold decrease was calculated by: (% TNF-α Positive, YAC-1)/(% TNF-α Positive, P815) or (% TNF-α Positive, BWZ)/(% TNF-α Positive, BWZ-Ocil). Data is representative of 3 experiments. NS, non-significant; *, $p < 0.05$.

3.3. Stable shutdown of SHP-1 phosphatase in mature murine NK resulted in loss of cell viability and impaired NK functions

To reiterate, the status of NK cell activity is dependent on the signals generated from the simultaneous engagement of activation and inhibitory receptors.[60,62,63,180] Because NK inhibitory receptors signal through SHP-1, we wanted to evaluate the functional effects of disrupting the inhibitory signalling pathways by using our current transduction protocol to introduce short hairpin RNA (shRNA) sequences against SHP-1 into primary C57BL/6 LAK cells. We used the shRNA approach because it allowed us to silence SHP-1 in wild type mature NK cells.

3.3.1 TRC clone 28966 mediated efficient SHP-1 shutdown in EL-4 and primary murine IL-2-activated NK cells

To identify shRNA sequences targeting the mouse SHP-1 gene, we searched The RNAi Consortium (TRC) lentiviral shRNA library from Open Biosystems.[174] TRC lentiviral vectors contain a puromycin selection marker that allows us to select for transduced cells. The search returned five constructs available for mouse SHP-1 silencing, TRC clones 28964-68. To identify the clone that mediated most efficient SHP-1 shutdown, we used a murine T lymphoma cell line, EL-4 that has been shown to express SHP-1.[189] EL-4 cells were used to screen each clone because of their high transduction efficiency and fast rate of proliferation. Briefly, we transduced EL-4 cells with each clone, selected for transduced cells with the puromycin marker and assayed for efficiency of SHP-1 shutdown. TRC vector containing shRNA targeting the EGFP gene (designated as shEGFP) was used as a negative control for SHP-1 down-regulation. Analysis by Western Blot and confirmation by intracellular staining in flow cytometry

revealed that TRC clone 28966 mediated the most efficient SHP-1 silencing in EL-4 cells (~85% down-regulation) (Fig 13A and B). Clone 28968 is second with about 60% down-regulation and 28965 yielded no shutdown.

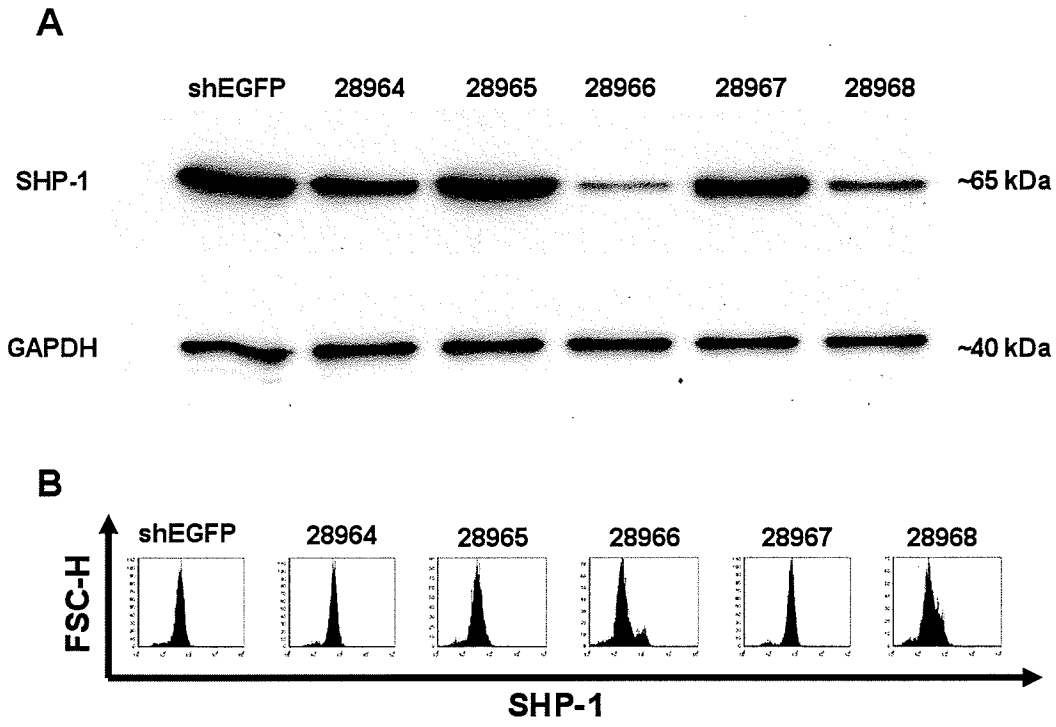

Figure 13. TRC clone 28966 mediated most efficient SHP-1 shutdown in EL-4 cells. EL-4 cells were transduced on two consecutive days by the "spin protocol," with TRC lentiviral vectors and incubated for 3 days post-transduction. Transduced cells were puromycin selected for 24 hours followed by 3 days incubation. Cells were assayed for SHP-1 expression by (A) western blot and (B) intracellular staining with primary rabbit anti-SHP-1 and secondary anti-rabbit Alexa Fluor 488 antibodies in flow cytometry. Data is representative of 2 experiments.

After identifying TRC clone 28966 as the most efficient shRNA sequence for SHP-1 shutdown in EL-4 cells, we wanted to test its efficiency in mature C57BL/6 LAK cells. We transduced LAK cells by the "spin" protocol at MOI = 40 IU/cell (2 rounds in order to increase transduction efficiency), puromycin selected and assayed for SHP-1 protein expression by intracellular staining in flow cytometry. Mock and shEGFP transduced NK cells were used as negative controls for SHP-1 down-regulation. In all of the following experiments, mock NK cells were never subjected to puromycin selection because they were never transduced with TRC vectors. As shown in figure 10, clone 28966 mediated about 85% SHP-1 down-regulation compared to mock and shEGFP (Fig. 14). Therefore, we have identified that clone 28966 mediated efficient SHP-1 shutdown in mouse LAK cells.

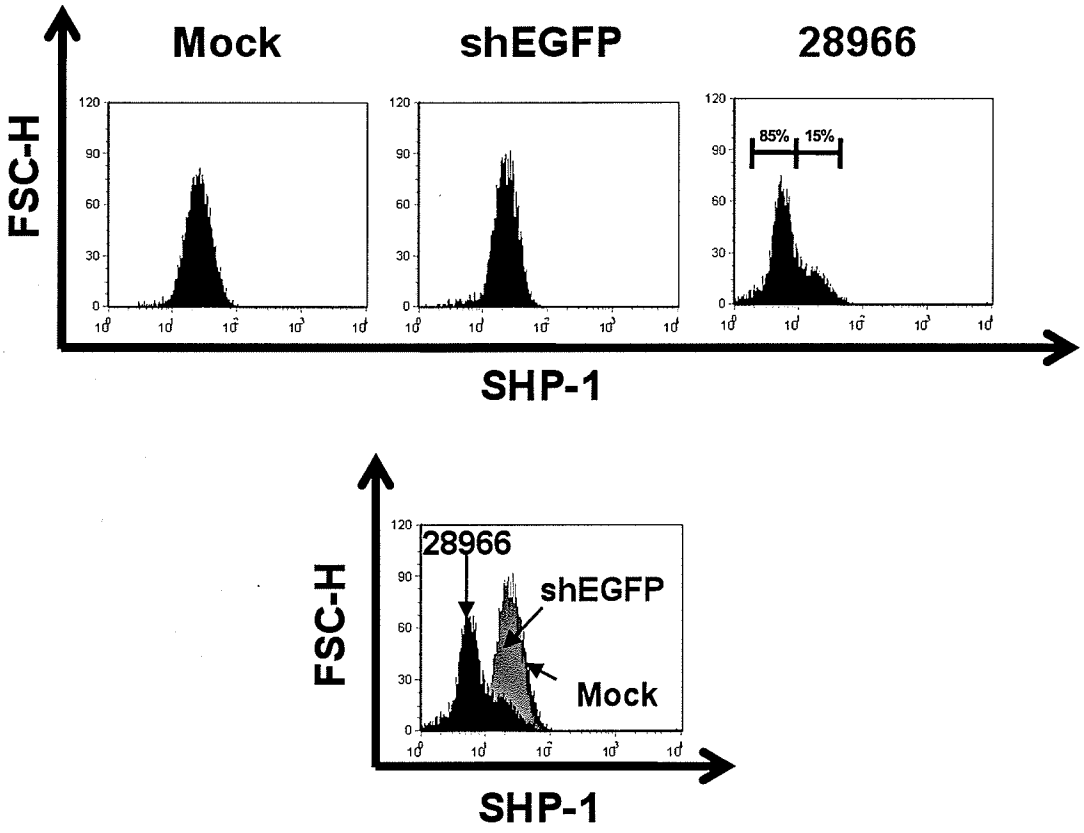

Figure 14. TRC clone 28966 mediated efficient SHP-1 shutdown in LAK cells. Purified C57BL/6 NK cells were incubated in IL-2 supplemented media for 5 days. IL-2 activated cells were transduced on two consecutive days with TRC lentiviral vectors and incubated for 3 days post-transduction. Transduced cells were puromycin selected for 24 hours followed by 3 days incubation. Cells were assayed for SHP-1 expression by intracellular staining with primary rabbit anti-SHP-1 and secondary anti-rabbit Alexa Fluor 488 antibodies in flow cytometry. Data is representative of 3 experiments.

3.3.2 SHP-1 shutdown impaired IL-2 induced NK cell proliferation

Although shEGFP control transduced and clone 28966 transduced NK cells were subjected to identical experimental protocols and IL-2 supplemented culture conditions, clone 28966 transduced NK cells consistently yielded a lower cell number (data not shown). Therefore, we suspected that the observed lower cell number from clone 28966 transduced NK cells may be due to a lower rate of proliferation compared to mock and shEGFP transduced NK cells. Mock LAK cells were used as a control for normal IL-2 induced proliferation while shEGFP transduced LAK cells were used as a control for normal IL-2 induced proliferation after transduction and puromycin selection. To test this speculation, we labelled mock, shEGFP and clone 28966 transduced NK cells post-puromycin selection with carboxyfluorescein succinimidyl ester (CFSE) and cultured the cells in IL-2 supplemented media. we harvested the cells 7 days after CFSE labelling, stained with anti-CD11b antibody and analyzed in flow cytometry. Upon analysis of CD11b expression, clone 28966 transduced NK cells showed a lower mean fluorescent intensity (MFI) of 30.6 compared to 66.5 and 54.0 MFI in the mock and shEGFP transduced NK cultures, respectively (Fig. 15A). Higher CD11b MFI corresponds with higher rates of cellular proliferation (data not shown). CFSE dilution analysis revealed that clone 28966 transduced NK cells showed a higher MFI of 486 compared to 65 and 151 for mock and EGFP transduced NK cells, respectively (Fig. 15B). Each round of cellular proliferation reduces the MFI in half because the CFSE is partitioned equally to each daughter cell.[190] Therefore, a higher CFSE MFI corresponds with fewer rounds of proliferation. The combination of lower CD11b and higher CFSE MFI in clone 28966

transduced NK cells showed that IL-2 induced cellular proliferation was impaired in SHP-1 down-regulated NK cells.

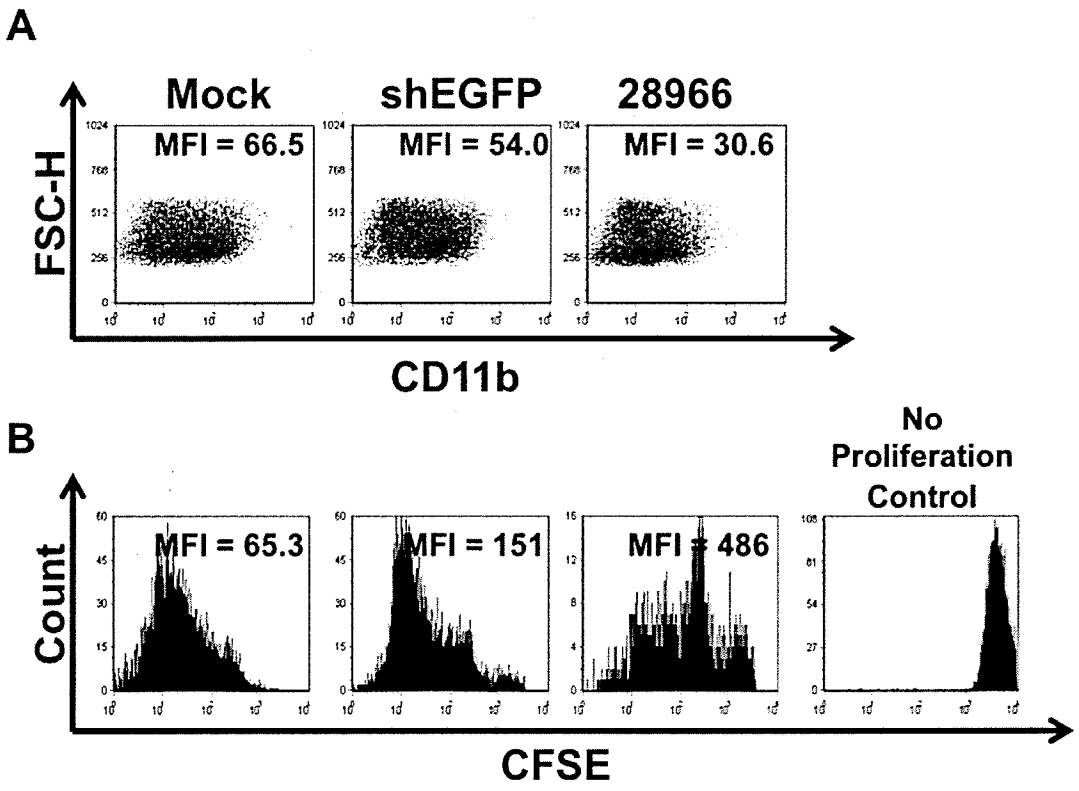

Figure 15. SHP-1 shutdown impaired IL-2-induced NK cell proliferation. Purified NK cells were incubated in IL-2 supplemented media for 5 days. IL-2 activated cells were transduced on two consecutive days with TRC lentiviral vectors and incubated for 3 days post-transduction. Transduced cells were puromycin selected for 24 hours followed by CFSE labelling. CFSE labelled cells were stained with anti-CD11b-PE antibody and analyzed in flow cytometry, 7 days post-puromycin selection.

3.3.3 SHP-1 shutdown in activated NK cells resulted in normal cytotoxicity towards YAC-1 targets

SHP-1 shutdown in NK cells resulted in decreased IL-2 induced cellular proliferation. Therefore we tested whether SHP-1 shutdown in NK cells will disrupt normal NK cytotoxic activity. To test this, clone 28966 transduced and puromycin selected LAK cells were used as effectors in a standard ^{51}Cr release cytotoxicity assay with YAC-1 as the target cells. YAC-1 is used in NK cytotoxicity assays as NK-sensitive target cells. Mock LAK cells were used as a control for normal cytotoxicity while shEGFP transduced LAK cells were used as a control for normal cytotoxicity after lentiviral transduction and puromycin selection. Contrary to our expectation, the differences in YAC-1 killing at a 1:1 ratio from mock, shEGFP and clone 28966 transduced NK cells were statistically non-significantly (Fig. 16). The percentage of specific lysis of YAC-1 observed from all three NK populations was around 65%.

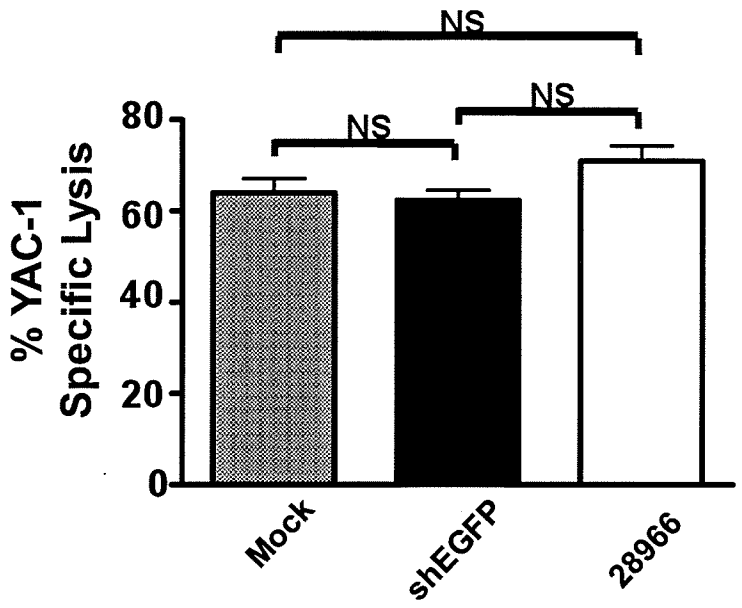

Figure 16. Down-regulation of SHP-1 protein expression in activated NK cells resulted in normal cytotoxicity towards YAC-1 targets. Purified NK cells were incubated in IL-2 supplemented media for 5 days. IL-2 activated cells were transduced on two consecutive days with TRC lentiviral vectors and incubated for 3 days post-transduction. Transduced cells were puromycin selected for 24 hours followed by 3 days incubation. Cells were then used as effectors (1:1 ratio) in a standard 4-hour ^{51}Cr cytotoxicity assay. NS, non-significant.

3.3.4. SHP-1 shutdown in mature NK cells resulted in loss of cell viability

SHP-1 shutdown in mature NK cells resulted in decreased IL-2-induced cellular proliferation. These observations prompted us to suspect that perhaps the observed results were linked to a loss of cell viability. We, therefore, assayed for cellular apoptosis and necrosis. We used mock LAK cells as a control for normal apoptosis and necrosis and shEGFP LAK cells as a control for normal apoptosis and necrosis after transduction and puromycin selection. Briefly, we stained mock, EGFP transduced and clone 28966 transduced NK cells with Annexin V and 7-amino actinomycin D (7-AAD) in flow cytometry. Clone 28966 transduced NK cells showed a statistically significant increase in both cellular apoptosis ($p < 0.01$) and necrosis ($p < 0.05$) compared to mock and EGFP transduced NK cells. 31.1% of clone 28966 transduced NK cells were undergoing apoptosis compared to 13.6% of mock and 19.3% of EGFP transduced NK cells (Fig. 17). 10.6% of clone 28966 transduced NK cells were undergoing necrosis compared to 4.3% of mock transduced and 5.6% of EGFP transduced NK cells.

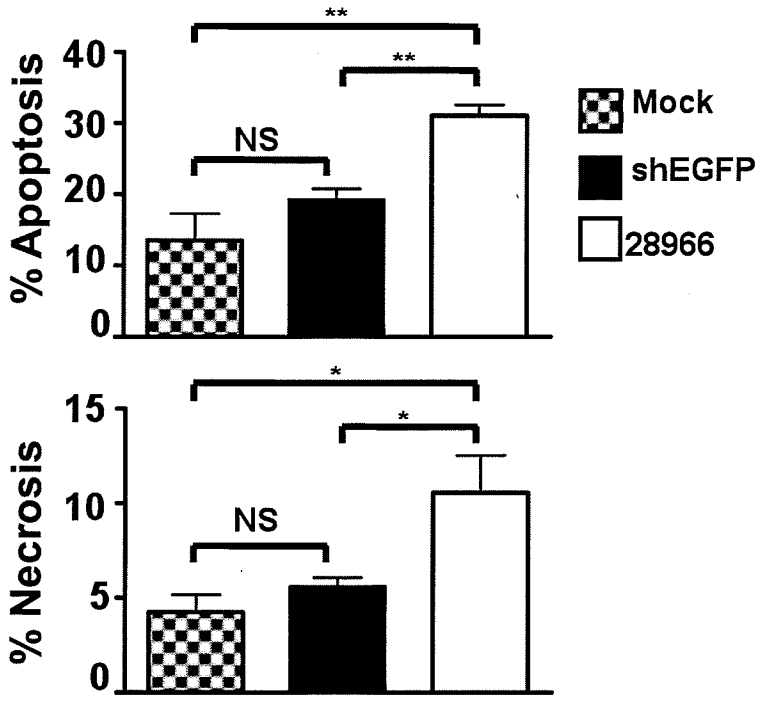

Figure 17. SHP-1 shutdown in mature activated NK cells resulted in loss of cell viability. Purified NK cells were incubated in IL-2 supplemented media for 5 days. IL-2 activated cells were transduced on two consecutive days with TRC lentiviral vectors and incubated for 3 days post-transduction. Transduced cells were puromycin selected for 24 hours followed by 3 days incubation. Cells were then assayed for apoptosis and necrosis by staining with Annevin V-PE and 7-amino actinomycin D (7-AAD) in flow cytometry. Data is representative of 3 experiments. NS, non-significant; *, $p < 0.05$; **, $p < 0.01$.

3.3.5. SHP-1 shutdown in mature NK cells resulted in increased spontaneous degranulation

The balance of signals generated from simultaneous ligand interaction with inhibitory and activating receptors function to control NK-mediated self-nonself discrimination.[44] Normal cells predominantly express ligands for inhibitory receptors. This skews the balance of receptor signalling towards attenuation of NK cell activity leading to self-tolerance.[37,64] We hypothesized that by silencing SHP-1, the signals generated from inhibitory receptor-ligand interactions are disrupted. As a result, inhibitory signals no longer predominate and NK self-tolerance is broken. To determine if SHP-1 down-regulated NK cells remained self-tolerant, we conducted CD107a degranulation assays on each NK culture. Mock LAK cells were used as a control for normal degranulation and shEGFP LAK cells were used as a control for normal degranulation after transduction and puromycin selection. Briefly, we evaluated NK cell CD107a degranulation on days 1, 2, 3 and 7 after puromycin selection by incubating mock, shEGFP transduced and clone 28966 transduced NK cells with anti-CD107a antibody and monensin for 5 and 4 hours, respectively, before analysis in flow cytometry. If SHP-1 silenced NK cells mediate cytotoxic activity against each other in culture due to a loss in self-tolerance, we would be able to detect increased CD107a degranulation. On day 1 after puromycin selection, 5.9% of clone 28966 transduced NK cells showed degranulation activity compared to 2.7% and 2.9% degranulation from mock and shEGFP transduced NK cells, respectively (Fig. 18). The differences, however, were not statistically significant. On day 2 after puromycin selection, 3.7% of clone 28966 transduced NK cells showed degranulation activity compared to 1.0% and 1.4% degranulation from mock and shEGFP transduced NK cells, respectively. The differences

were statistically significant ($p < 0.05$). On day 3 after puromycin selection, clone 28966 transduced NK cells continued to show a significant increase in CD107a degranulation compared to mock and shEGFP transduced NK cells ($p < 0.0001$). Eight point three percent (8.3%) of clone 28966 transduced NK cells showed degranulation activity compared to 2.5% and 2.4% degranulation from mock and shEGFP transduced NK cells, respectively. The statistically significant increase in degranulation ($p < 0.0001$) continued to day 7 where 6.9% of clone 28966 transduced NK cells showed degranulation activity compared to 1.9% and 2.1% degranulation from mock and shEGFP transduced NK cells, respectively. These results showed that down-regulation of SHP-1 caused NK cells to undergo increased spontaneous degranulation suggesting that SHP-1 plays a role in NK-mediated self-tolerance and prevention of autoimmunity. In addition, the enhanced degranulation and activation may lead to NK cell cycle arrest. As a result, a proportion of the arrested cells may start to undergo cell death, therefore, explaining the loss of cell viability and impaired IL-2 induced cellular proliferation.

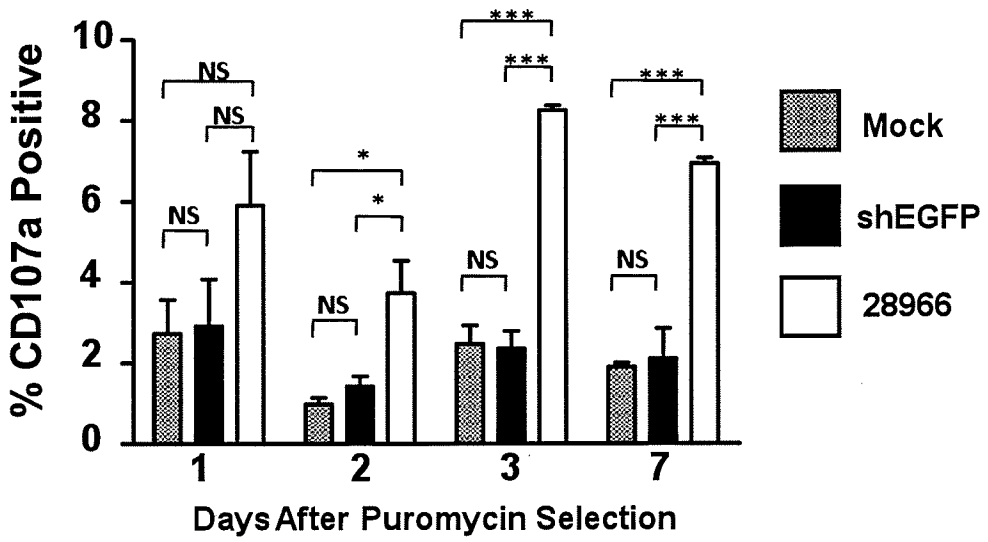

Figure 18. SHP-1 shutdown in activated NK cells resulted in increased spontaneous degranulation. Purified NK cells were incubated in IL-2 supplemented media for 5 days. IL-2 activated cells were transduced on two consecutive days with TRC lentiviral vectors and incubated for 3 days post-transduction. Transduced cells were puromycin selected for 24 hours followed by 1, 2, 3 or 7 days incubation. Cells were then used in a CD107a degranulation assay. Briefly, cells were incubated with anti-CD107a-PE antibody and monensin in 5 ml centrifuge tubes for 5 and 4 hours, respectively, at 37°C before analysis in flow cytometry. Data is representative of 3 experiments. NS, non-significant; *, $p < 0.05$; ***, $p < 0.0001$.

CHAPTER 4. DISCUSSION

Multiple activation and inhibitory receptors are expressed on the surface of mature NK cells and their progenitors. The underlying role of NK cell receptor signalling in NK differentiation and acquisition of self-tolerance remains unclear. We are interested in studying the cellular factors and molecular signalling mechanisms involved in NK differentiation and target recognition. However, the current lack of an experimental system that will allow us to differentially study a protein's function(s) in primary NK progenitors, resting and activated primary NK cells presents a limitation in these studies.

Genetic modification of primary NK cells has been a major challenge because they are relatively resistant to exogenous gene transfer. The use of adenoviral vectors as a vehicle for gene delivery into NK cells is ineffective because NK cells do not express the appropriate receptors required for transduction.[191] Non-viral based gene transfer methods such as transfection and particle mediated gene gun delivery showed gene delivery that is of transient nature.[192-194] Stable and efficient gene delivery into primary human IL-2-activated NK (LAK) cells have been reported using retroviral and lentiviral vectors.[135,143,144] It remains to be examined whether lentiviral vectors support efficient transduction of primary mouse NK cells, especially the freshly isolated, unstimulated NK (progenitors) cells. Therefore, I examined the ability of lentiviral vectors to introduce stable genetic modification of primary mouse NK cells under minimal *in vitro* manipulation (such as cytokine activation and viral vectors exposure). I focused on a single-step transduction protocol that involved 1-2 hours of viral vector incubation (Fig. 2). I evaluated the use of a centrifugation-based virus inoculation method in achieving efficient NK transduction at lower virus concentration (MOI: 20 and 4 IU/cell) (Fig. 2). I

also examined the need of IL-2 activation in lentiviral vector-mediated transduction (Fig. 7). My data collectively demonstrated that lentiviral vectors in a 1-hour centrifugation-based inoculation stably transduced 30-40% of primary NK cells. The spin protocol yields a higher transduction efficiency of the NK cells at lower MOI when compared to that obtained in the "no-spin" 2-hour incubation protocol. I demonstrated that IL-2 augmented transgene expression in the transduced NK cells *in vitro*. However, it is not required for efficient lentiviral transduction of primary NK cells. To my knowledge, I provided the first demonstration that lentiviral vectors are able to transduce resting NK and differentiating NK cells derived from the transduced hematopoietic progenitor cells without the need of prior cytokine activation. These data are of high relevance to genetic manipulation of human and mouse NK cells. First, in the studies of NK cell activation and differentiation, it is now possible to modify *ex vivo* purified NK (progenitor) cells to harbour the genetic changes before cytokine-induced activation in culture conditions *in vitro*. Second, the ability to repopulate genetically modified NK cells in the immunodeficient animals (such as RAG2-/-γc-/- mice)[142,146,195] will allow the evaluation of human and mouse NK cell differentiation or gene therapy *in vivo*.

My current study highlights the flexibility of the use of lentiviral vectors in genetic manipulation of primary NK cells. First, I demonstrated that VSV-G pseudotyped lentiviral vector transduction did not alter surface expression of the NK receptors (NKG2A/C/E, Ly49C+I, Ly49F, NKG2D and NK1.1) that are commonly used in defining murine NK subsets (Fig. 4A). It thus suggests that VSV-G pseudotyped lentiviral vector transduction is not biased against particular NK subset(s) and is suitable for transduction of polyclonal NK cells. With the recent advances in engineering of other

viral envelope proteins for lentiviral vector pseudotyping, targeting (and genetic engineering) a specific NK subset both *in vitro* and *in vivo* is possible.[196-198] Second, I studied EGFP reporter gene expression under the control of different internal promoters (RhMLV and Ubi-C promoter) in transduced NK cells and found that the viral internal RhMLV promoter allowed 3-fold higher expression (MFI) of EGFP in the transduced cells compared to that of the Ubi-C promoter (Fig. 3). Thus, the level of transgene expression in the modified NK cells, if desired, can be regulated by the choice of internal promoters (such as Ubi-C, RhMLV, CMV) used in the lentiviral vectors. Third, I showed that lentiviral transduction did not effect normal NK cell function, such as, cytotoxicity and cytokine production. It remains to be determined, however, why the sorted $EGFP^-$ control LAK cells (from the transduced culture) consistently exhibited a slightly higher cytotoxicity when compared to the unsorted mock-transduced LAK control. I speculate that the LAK cells are somehow activated by the cell sorting process. Another possibility is that exposure to lentiviral vectors has some activating effects on the cells. Lastly, I used here an EGFP reporter gene to demonstrate the ability of the lentiviral vector system to over-express the transgene of interest in primary NK cells. My supervisor and others have also developed similar lentiviral vector systems to deliver short hairpin RNA to down-regulate target gene expression via RNA interference.[199-201] Therefore, this current transduction protocol can be used to introduce a target gene-specific shRNA sequence(s) into primary NK cells to down-regulate its protein expression. The ability to over-express or down-regulate genes of interest will allow me to study a protein's function(s) in NK differentiation, acquisition of self-tolerance and NK-target interactions.

In my second study, I used the lentiviral-mediated gene delivery protocol to test the hypothesis that over-expression of the inhibitory receptor, NKR-P1B on primary mouse LAK cells will skew the balance of NK receptor signalling towards inhibition. I showed that NKR-P1B over-expression resulted in decreased NK cytotoxicity and TNF-α production upon interaction with target cells expressing Ocil, the ligand for NKR-P1B.[43]

CD107a degranulation assays demonstrated that NK cytotoxic activity can be altered by over-expressing NKR-P1B. The target specificities for NK-sensitive YAC-1 and NK-resistant P815 target cells were maintained even though I over-expressed NKR-P1B on the effector NK cells (Fig. 11A). However, NKR-P1B over-expression resulted in decreased CD107a degranulation against BWZ-Ocil cells compared to parental BWZ cells (2.6 fold decrease) (Fig. 11B and C). Although mock and NKR-P1B$^-$ NK cells also exhibited decreases in CD107a degranulation against BWZ-Ocil cells (1.2 and 1.3 fold decreases, respectively), the decrease from NKR-P1B$^+$ NK cells was significantly higher ($p < 0.0001$ and $p < 0.01$, respectively).

In addition to cytotoxicity, TNF-α production can also be altered by NKR-P1B over-expression. The specificities of TNF-α production against YAC-1 and P815 were maintained in NKR-P1B expressing NK cells (Fig. 12A). However, when comparing TNF-α production against BWZ and BWZ-Ocil, NKR-P1B$^+$ cells produced 2.0 fold less TNF-α against BWZ-Ocil. This difference was significantly higher than the 1.3 fold decrease observed from mock and NKR-P1B$^-$ NK cells (figures 12B and C).

It is very interesting to observe differences in both cytotoxicity and cytokine production between BWZ and BWZ-Ocil in the mock and NKR-P1B$^-$ NK cell

populations. Results should be similar since mock and NKR-P1B$^-$ NK cells are not expressing the NKR-P1B receptor. One possible explanation is that from genomic studies, BALB/c NK cells are believed to express an endogenous receptor for Ocil. It has not yet been identified because of a lack in antibodies able to detect receptor expression.

It is well documented that the outcome of an NK-target interaction is determined by the balance of signals generated by simultaneous engagement of activation and inhibitory receptors.[117] However, most of these studies focused on the manipulation of NK receptor ligands on target tumour cell lines *in vitro*.[202,203] Unlike these past studies, direct manipulation of NK receptor expression will enable us to enhance NK target recognition. My work demonstrated our ability to over-express an NK receptor on mouse LAK cells to skew the balance of receptor signalling between inhibitory and activating receptors, leading to an altered NK-target interaction. Direct manipulation of NK receptor expression will provide a novel approach to exploring the therapeutic potential of NK cells. In addition, it is possible to construct chimeric receptors that define new NK target specificities. This will recruit the NK-mediated arm of immunity to control microorganisms, viruses or tumours that are currently able to evade NK cytotoxicity.[204-207]

NKR-P1B along with other NK cell inhibitory receptors mediate their signalling through the recruitment and activation of Src Homology 2 Domain-Containing Tyrosine Phosphatase-1 (SHP-1) that goes on to attenuate activation molecules such as LAT, PLCγ, SLP-76, ZAP70 and Syk.[91,108-110] Therefore, an alternative to manipulating the balance of receptor signalling besides over-expressing receptors is to silence molecules involved in receptor signalling. For example, silencing SHP-1, a molecule involved in

effector inhibition,[33,106,112] may disrupt NK cell inhibitory receptor signalling and skew the balance towards activation of NK cell activity. However, SHP-1 is not specific to NK receptors because it is involved in other signalling pathways. Therefore, to address receptor signalling, I can cross-link and engage specific NK inhibitory receptors (such as Ly49C and I) with antibodies to test whether down-regulation of SHP-1 protein expression disrupts NK inhibitory receptor signalling function.

For my third study, I demonstrated the effects of down-regulating SHP-1 protein expression in mature LAK cells. SHP-1 shutdown in mature LAK cells resulted in impaired IL-2-induced cellular proliferation, normal cytotoxicity against YAC-1 target cells, loss of cell viability and increased spontaneous degranulation.

Previous studies have demonstrated the effects of disrupting SHP-1 enzymatic activity on NK cell function. Over-expression of a catalytically inactive, dominant-negative form of SHP-1 (dnSHP-1) in human and mouse NK cells resulted in decreased MHC-Class I mediated inhibition of cytotoxicity.[103,111] In addition, NK cells isolated from dnSHP-1 or SHP-1 knockout mice (motheaten, *me* and motheaten viable, *me*^{v} mice show complete and partial loss of enzymatic activity, respectively) were less cytotoxic against target cells compared to wild-type mice.[111,115,208] However, results from dnSHP-1 or SHP-1 KO mice suffered from a couple of caveats. For example, wild type SHP-1 enzymatic activity may still exist in dnSHP-1 NK cells. There may be unknown side effects from competition between wild type SHP-1 and dnSHP-1 in the NK cells. Also, NK cells isolated from SHP-1 KO mice may be suffering from secondary effects due to over-activated macrophages/monocytes.[115,116] Therefore, it remained to be determined whether the effects associated with a loss of SHP-1 function in the transgenic mice

represented a summation of direct NK defects in its differentiation pathway, and/or a defect in mature NK cell function.

I began by showing that TRC clone 28966 mediated about 85% SHP-1 shutdown in mature LAK NK cells. Despite the fact that the SHP-1 shutdown is not 100% (which is rarely observed in shRNA-mediated RNA interference), I found that SHP-1 "knocked down" NK cells have a lower cell number compared to mock or shEGFP transduced NK cells. This suggested to me that cellular proliferation may be affected by SHP-1 shutdown. When I investigated NK cell proliferation by CFSE and CD11b staining, the results showed that SHP-1 down-regulated NK cells were impaired in IL-2 induced proliferation (Fig. 15). This impaired proliferation immediately informed me that SHP-1 shutdown had profound effects on NK cells. I subsequently studied whether NK cell cytotoxicity was affected by down-regulation of SHP-1. The NK-sensitive cell line, YAC-1 was used as target cells. SHP-1 shutdown in mature NK cells resulted in normal cytotoxicity towards YAC-1 target cells (Fig.16).

The observation of impaired proliferation prompted me to suspect that down-regulation of SHP-1 was affecting NK cell viability. I therefore analyzed NK cell apoptosis and necrosis. Analysis of cell viability by Annexin V and 7-amino actinomycin D (7-AAD) revealed that SHP-1 shutdown resulted in NK cells that showed increased apoptosis and necrosis compared to mock- and shEGFP-transduced NK cells (Fig. 17). This raised the question of why does down-regulation of SHP-1 in NK cells result in a loss in cell viability?

It is well known that NK cells discriminate between self and non-self by engaging its inhibitory receptors with MHC Class I expressed on target cells.[37,64] Inhibitory signals

will predominate upon MHC Class I ligation thereby preventing NK-mediated autoimmunity. I, therefore, reasoned that if inhibitory receptor signalling was disrupted by SHP-1 down-regulation, NK cells may no longer be self-tolerant. CD107a degranulation analysis revealed that NK cells with down-regulated SHP-1 expression underwent increased spontaneous degranulation (Fig. 18). This suggested that down-regulating SHP-1 resulted in NK cells that were mediating cytotoxicity against each other. Increased NK activity against each other may help to explain the increased apoptosis and necrosis. The results obtained in this study revealed a very important role for SHP-1 in mature NK cells. SHP-1 plays a role in transmitting the inhibitory signals generated by binding of self ligands leading to NK cell self-tolerance and prevention of autoimmunity.

Future Directions

My current work focused on manipulating the balance of receptor signalling (the NKR-P1B inhibitory receptor and SHP-1 phosphatase) in mature NK cells to study the effects of altering receptor signalling on NK-target interactions. Future work will study the role of these molecules in NK differentiation. Building on the results from over-expressing NKR-P1B in mature BALB/c LAK, I plan to study the effects of manipulating the balance of receptor signalling on NK differentiation and acquisition of self-tolerance by over-expressing NKR-P1B on NK progenitor cells. NKR-P1B-Ocil engagement will skew the balance of receptor signalling towards inhibitory on the NK progenitor cell as it progresses through the stages of NK differentiation and acquisition of self-tolerance. I plan to transduce wild type BALB/c NK progenitors with Cppt-NKR-P1B lentiviral

vectors followed by differentiation by both *in vitro* culture conditions and *in vivo* reconstitution in RAG2-/-γc-/- immunodeficient mice. I have demonstrated the ability to differentiate NK progenitors into mature NK cells both *in vivo* and *in vitro* (figure 8 and appendix figure ii). Briefly, *in vitro* NK differentiation involves initial culturing of hematopoietic stem cells (HSCs) in IL-7, SCF, and Flt3L. This allows HSCs to differentiate into NK precursors (NKPs). Further culturing in IL-15 allows the NKPs to progress into immature NKs (iNKs). Interaction of iNKs with OP9 stromal cells allow them to mature into fully functional NK cells. The mature NK cell phenotype and functions will be compared to wild type non-transduced cells. Results obtained in this study will demonstrate the effects of increasing inhibitory signalling, NKR-P1B in particular, on NK differentiation and acquisition of self-tolerance. In addition, these results will be compared to the results obtained from over-expressing NKR-P1B in mature NK cells. This will allow for the comparison of effects arising from NKR-P1B over-expression at the progenitor NK and mature NK cell level.

We will study NK differentiation in the absence of the ligand (Ocil) of the NKR-P1B inhibitory receptor *in vitro*. I can use the lentiviral transduction protocol to introduce shRNA sequences against Ocil into OP9 stromal cells. We will test whether down-regulation of Ocil expression in OP9 stromal cells will decrease inhibitory signalling during the course of NK differentiation. I have already demonstrated that TRC clone 65883 is able to mediate about 95% Ocil down-regulation in OP9 (appendix figure iii). I plan on differentiating enriched wild type BALB/c NK progenitors by *in vitro* culture conditions. I will compare the phenotype and function of NK cells differentiated on Ocil-down-regulated OP9 cells versus NK cells differentiated on wild type OP9 cells. The

results obtained in this study will complement the results obtained from over-expressing NKR-P1B in NK progenitors. Manipulating NKR-P1B expression on NK progenitors will provide insights from the perspective of manipulating the NK receptors while Ocil down-regulation will provide insights from the perspective of manipulating the receptor ligands.

Lastly, I plan to study the role of SHP-1 in NK differentiation and acquisition of self-tolerance. Because SHP-1 is a crucial molecule involved in inhibitory receptor signalling, down-regulating SHP-1 in NK progenitors will disrupt signalling from NKR-P1B and other inhibitory receptors during differentiation and acquisition of self-tolerance. I plan on transducing wild type C57BL/6 NK progenitors with shRNA targeting the SHP-1 gene (TRC clone 28966). Non-transduced and shEGFP transduced progenitors will be used as controls. Transduced NK progenitors will be differentiated by both *in vitro* culture conditions and *in vivo* reconstitution in RAG2-/-γc-/- immunodeficient mice. The mature NK cell phenotype and functions will be compared to non-transduced and shEGFP transduced NK cells. The results obtained this study will serve three functions. First, it will show the role of SHP-1 in NK differentiation and acquisition of self-tolerance. Second, the results will be compared with the results obtained from studies involving SHP-1 knockout (*me* and *me*^{v}) mice. My protocol however, has an advantage over studies involving SHP-1 knockout mice because I will use wild type NK progenitor cells. These wild type progenitor cells have not been exposed to the chronic inflammatory environment as seen in *me* and *me*^{v} mice. As a result, any secondary effects from over-activated macrophages/monocytes are avoided.

Lastly, the results will allow me to compare the effects of SHP-1 down-regulation at the progenitor NK and mature NK cell level.

In summary, I have established the lentiviral transduction platform to over-express receptors or silence signalling molecules at the progenitor stem cell and mature NK cell levels. To address my global hypothesis that the balance of signals between activation and inhibitory receptors will effect NK differentiation, acquisition of self tolerance and NK-target interactions, I used a receptor-signalling pair (NKR-P1B-Ocil and SHP-1) to manipulate mature NK-target cell recognition. By over-expressing NKR-P1B in mature NK cells and skewing the NK activity outcome towards decreased cytotoxicity and TNF-α production against the target cell, I proved that it is possible to directly manipulate NK cell receptor expression leading to an altered degree of NK target recognition.

My work will setup a novel platform for us to study a protein's function(s) in NK differentiation and the molecular mechanism underlying acquisition of NK cell self-tolerance.

Future work will study this receptor pair at the NK progenitor level. These data, collectively will allow us to formally address our global hypothesis that induction of the same receptor signalling at different states of NK cells will produce different functional outcomes in NK differentiation and target recognition.

Appendix

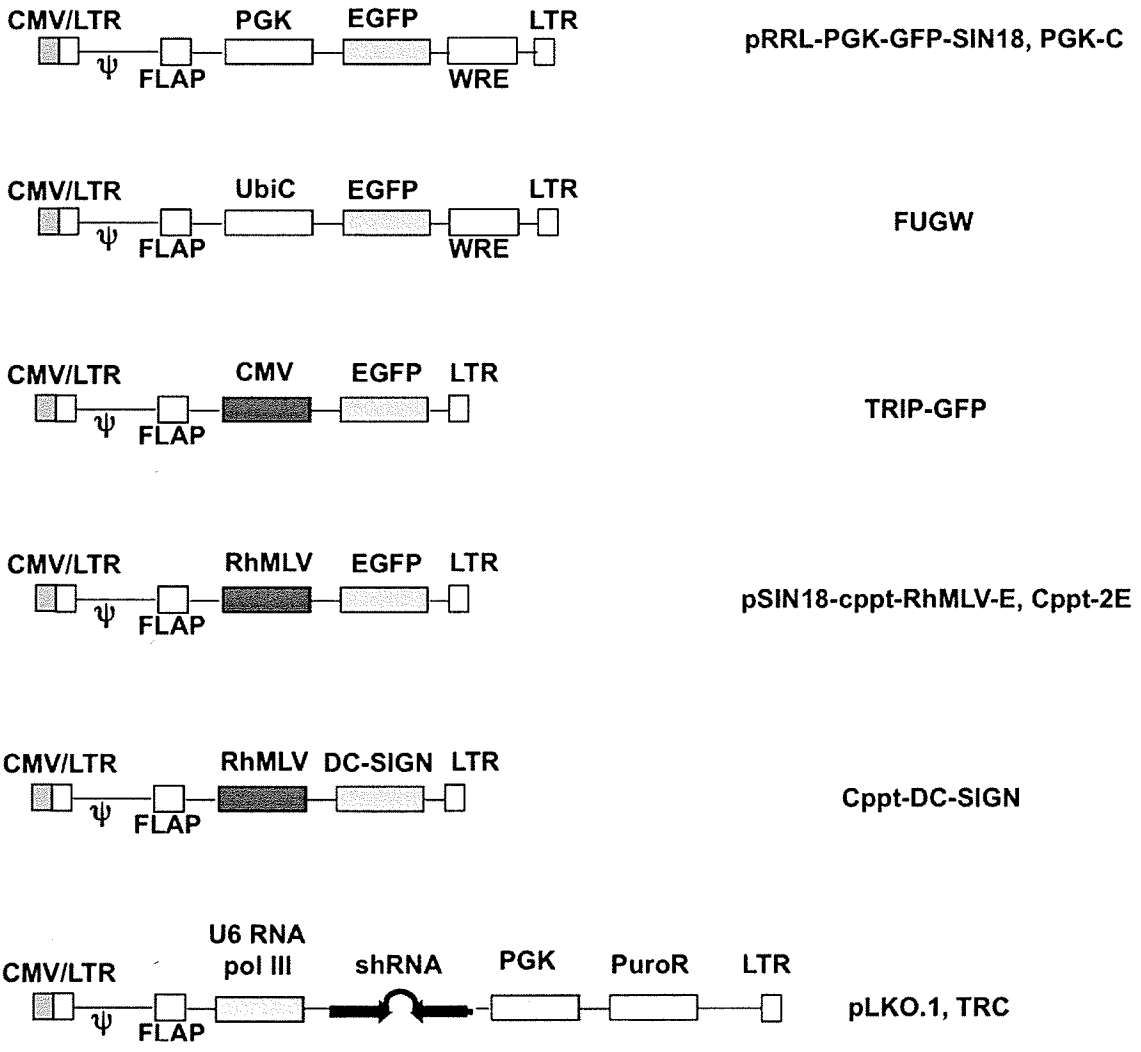

Figure i. Schematic representation of vector genomes.

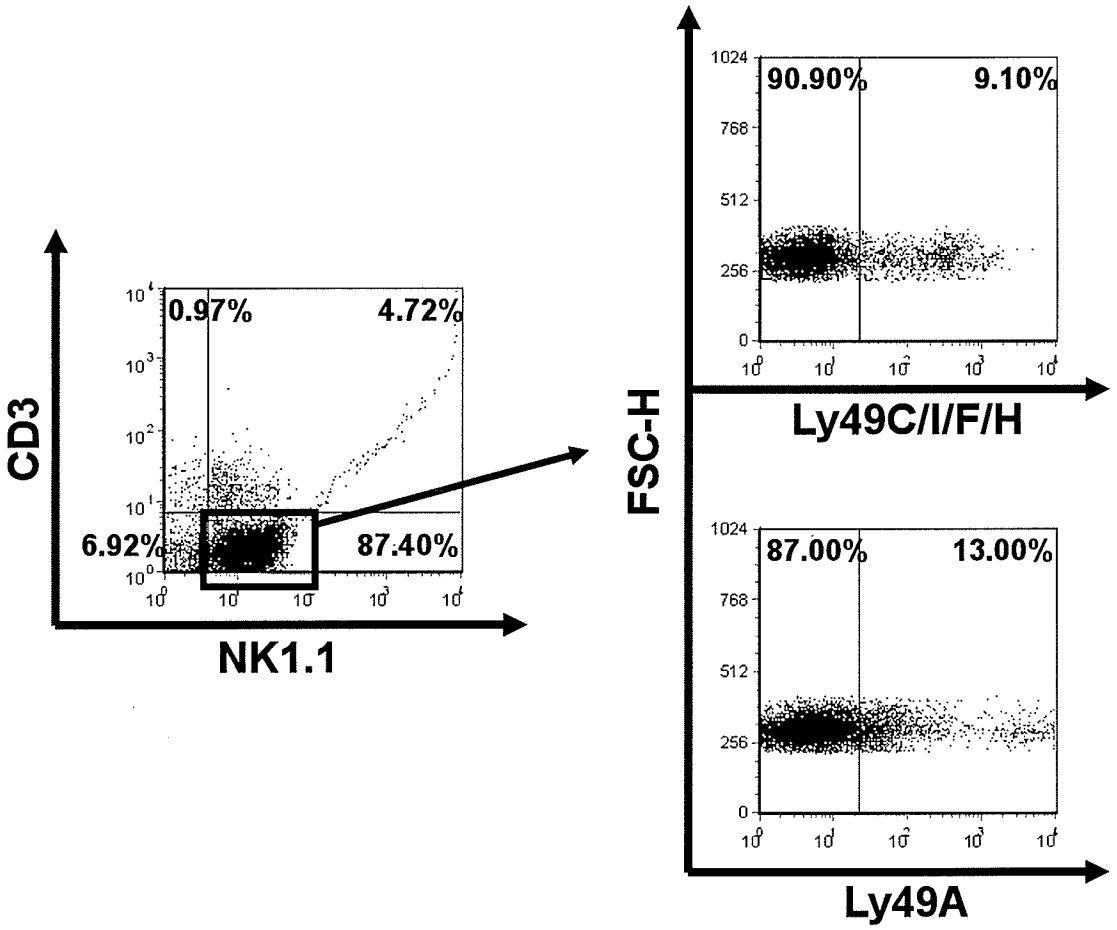

Figure ii. Mature NK cells differentiated *in vitro* from hematopoietic stem cells. Hematopoietic stem cells were enriched from C57BL/6 bone marrow cells using StemCell EasySep Mouse Progenitor Stem Cell Negative Selection kit. Cells were incubated in RPMI 1640 supplemented with 0.5 ng/ml of mIL-7, 30 ng/ml of SCF and 50 ng/ml of Flt3L for 5 days. Old media was removed and replaced with RPMI 1640 supplemented with 30 ng/ml of IL-15 for 5 days. Lastly, cells were incubated with OP9 stromal cells for 5 days. Cell surface receptors were analyzed by staining with CD3-PECy5, NK1.1-PE, Ly49C/I/F/H-APC and Ly49A-APC in flow cytometry.

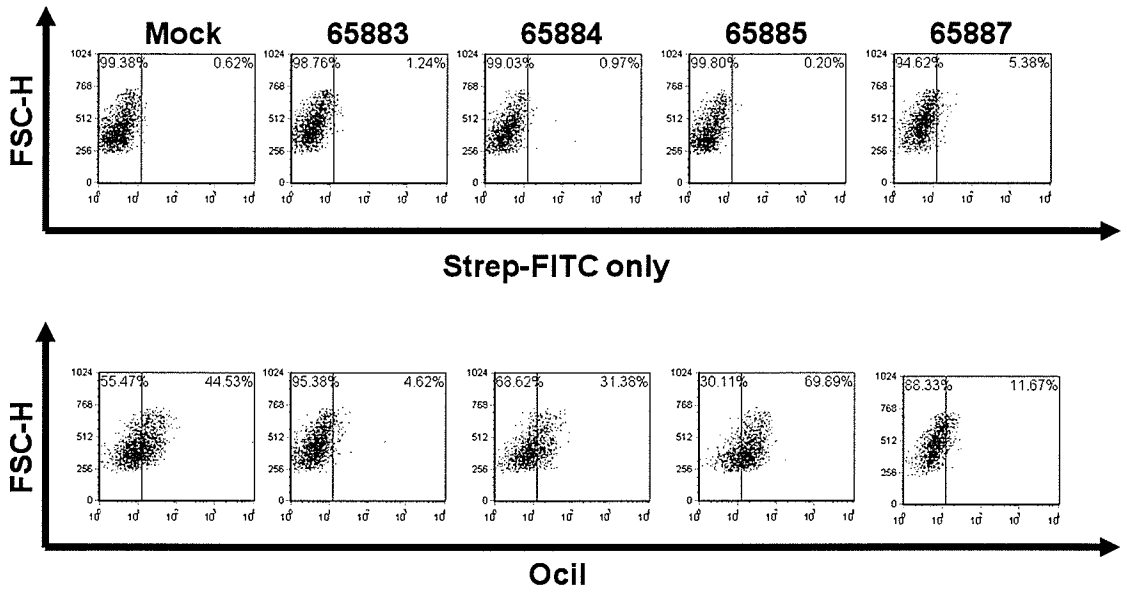

Figure iii. TRC clone 65883 mediated efficient Ocil shutdown in OP9 stromal cells. OP9 cells were transduced with TRC lentiviral vectors and incubated for 3 days post-transduction. Transduced cell were puromycin (64 μg/ml) selected for 72 hours followed by 3 days incubation. Cells were assayed for Ocil expression by staining with anti-Ocil-biotin and streptavidin-FITC antibodies in flow cytometry.

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