THE ROLE OF SHIP IN NATURAL KILLER CELL LICENSING

by

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Abstract

Natural Killer (NK) cells comprise a unique subset of lymphocytes with innate ability to detect and kill abnormal cells. Inhibitory Ly49 receptors expressed on NK cells recognize major histocompatibility complex class I (MHC I) molecules and inhibit NK cell killing of normal cells that express high amounts of MHC I. In addition to maintaining self-tolerance of NK cells, the Ly49:MHC I interaction is required for the functional maturation of NK cells by a process termed "NK cell licensing." NK cells from MHC I-deficient β2 microglobulin (β2m)-KO mice are not cytotoxic as they are not licensed. Our goal was to determine the mechanism through which NK cells of MHC-I deficient mice are kept hyporesponsive to NK-sensitive targets. We compared unlicensed NK cells from β2m-KO mice with licensed wild type (WT) NK cells in each step of NK cell cytotoxicity. We found that LFA-1 mediated adhesion to target and polarization of actin and talin are functional in these cells, yet unlicensed NK cells fail to polarize cytotoxic granules when bound to the prototypic NK target YAC-1, or beads coated with combinations of ligands for NK stimulatory receptors, namely the LFA-1 ligand ICAM-1, NKG2D ligand H60, and 2B4 ligand CD48. Thus, NKG2D and 2B4 mediated signalling seemed impaired in unlicensed NK cells. Ly49 receptors, upon tyrosine phosphorylation, are thought to recruit both Src homology 2-containing phosphatase 1 (SHP1) and Src homology 2 domain-containing inositol-5′-phosphatase (SHIP), whereas NK cell licensing is known to be SHP1-independent. Therefore, we tested whether SHIP is involved in NK cell licensing. In both WT and β2m-KO NK cells, antibody mediated cross-linking of inhibitory Ly49 receptors induced recruitment of SHIP rather than SHP1. When NK cells bound to MHC I-deficient YAC-1, SHIP was enriched at the contact site of unlicensed, but not in licensed, NK cells. These

results suggest that during the process of NK cell licensing, Ly49C/I recruits SHIP and prevents SHIP's association with activating NK cell receptors allowing stimulatory signalling and cytotoxicity to occur. These findings provide insight into the mechanism of NK cell licensing and signalling generated by the Ly49: MHC I interaction.

Preface

Mice were housed in the BC Cancer Research Centre Animal Research Centre. All animal use was approved by the animal care committee of the University of British Columbia (Animal Care Protocol # A09-0994**)**, and animals were maintained and euthanized under humane conditions in accordance with the guidelines of the Canadian Council on Animal Care.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
BM	bone marrow
BSA	bovine serum albumin
CD2AP	CD2 associated protein
CFDA-SE	carboxyfluorescein diacetate, succinimidyl ester
CTL	cytotoxic T lymphocyte
DAP10	DNAX-activating protein of 10 kDa
DAP12	DNAX-activating protein of 12 kDa
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNAM-1	DNAX accessory molecule-1
EBV	Epstein Barr virus
EDTA	ethylenediamine tetraacetic acid
ERK	extracellular signal-related kinase
FACS	fluorescence activated cell sorting
FasL	Fas ligand
FCS	fetal calf serum
Grb2	growth factor receptor-bound protein 2
HBSS	Hank's balanced salt solution
ICAM-1	intercellular adhesion molecule-1
IL	interleukin

IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
KIR	killer immunoglobulin-like receptors
KO	Knockout
LFA-1	leukocyte function associated antigen-1
mAb	monoclonal antibody
MAPK	MAP kinase kinase
MHC	major histocompatibility complex
MCMV	mouse cytomegalovirus
MTOC	microtubule organizing centre
MULT-1	mouse ULBP like transcript-1
NKG2D	natural killer group 2D
NK	natural killer
PBS	phosphate buffered saline
PH	pleckstrin homology
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol (4,5) bisphosphate
PIP_3	phosphatidylinositol (3,4,5) trisphosphate
PLCγ	phospholipase C γ
PLL	poly L lysine
PMA	phorbol 12-myristate 13-acetate
poly I:C	polyinosinic-polycytidylic acid

PTEN	phosphatase and tensin homologue
RAE-1	retinoic acid early inducible-1
RT-PCR	reverse transcriptase polymerase chain reaction
SAP	slam-associated protein
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH3	Src homology 3
SHIP	Src homology 2 domain-containing inositol-5′-phosphatase
SHP	Src homology 2 domain-containing phosphatase
dsRNA	double stranded ribonucleic acid
sIC-1	soluble ICAM-1
SLAM	signaling lymphocyte activation molecule
TCR	T cell receptor
TNF	tumour necrosis factor
TRAIL	tumour necrosis factor apoptosis inducing ligand
ULBP1	UL-16 binding protein-1
WT	wild-type
ZAP70	ζ-associated protein of 70-kDA

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Chapter 1: Introduction

1.1 Natural Killer cells

Natural Killer (NK) cells comprise a unique subset of lymphocytes with the innate ability to detect and kill virally-infected and tumorigenic cells. Following development in the bone marrow, mature NK cells circulate in peripheral blood, lymphatics, and tissues, constantly scanning cell-surface molecules to identify abnormal cells. NK cells belong to the innate arm of the immune system as they express germ line encoded, non antigen-specific receptors, and can mediate cytotoxicity without prior sensitization. They are also an important link between innate and adaptive immunity as they secrete chemokines and cytokines, such as IFN-γ, GM-CSF, and TNF-α, that regulate antigen-specific responses from B and T cells. Tumor and virally-infected cells often avoid killing by cytotoxic T lymphocytes (CTLs), the other major cytotoxic lymphocytes, by down-regulating major histocompatibility complex class I (MHC I) molecules. This, however, makes them susceptible to NK cell killing. In addition, abnormal cells express a multitude of ligands recognized by NK cell stimulatory receptors, making them the perfect targets [1]. There are two major pathways by which NK cells mediate apoptosis of their target: (1) the granule exocytosis pathway and (2) the death receptor pathway, which involves engagement of Fas ligand (FasL) or TNF on the NK cell with Fas (CD95), or TNFR-related apoptosis inducing lingand (TRAIL) on the target cell, resulting in caspase-dependent apoptosis [2].

The most common pathway is granule exocytosis. The apoptosis-inducing granules are specialized secretory lysosomes, and their major cytotoxic components are granzymes, a type of serine protease, and perforin. Although human NK cells contain pre-formed granules and

thus have some level of basal activation, naïve murine NK cells are not cytotoxic as they lack granzyme B and perforin until primed or activated by a foreign agent [3]. Upon target recognition, secretory lysosomes are exocytosed by the NK cell and their cytotoxic contents are released. Perforin assists in the entry of granzymes into the cytosol of target cells, where they cleave various targets (e.g. caspases) resulting in apoptosis [4, 5]. The most common granzymes are granzymes A and B. Granzyme A causes slower, caspase-independent apoptosis, whereas granzyme B causes rapid, caspase-dependent cell death [6]. Each NK cell kills on average four target cells, terminating its cytotoxic activity due to diminished perforin and granzyme B levels [7].

NK cell-mediated cytotoxicity is highly regulated by the balance of stimulatory and inhibitory receptors on the NK cell surface, which structurally belong to either the C-type lectin-like family or the immunoglobulin (Ig) superfamily of receptors. As mentioned earlier, abnormal cells express multiple stimulatory ligands that activate NK cells, and very few inhibitory MHC I ligands. This is why they are efficiently killed by NK cells. Normal cells are spared because they express little to no stimulatory ligands and numerous inhibitory MHC I molecules [8, 9]. MHC I molecules are the classical ligand for NK cell inhibitory receptors. Once their count becomes scarce, NK cell cytotoxicity can proceed unconstrained and trigger cell lysis, a phenomenon referred to as "missing-self recognition" [10].

The actual killing of target cells by NK cells is a rapid, multi-step process that can take place within twenty minutes. The first step is the binding of NK cells to targets, mediated by the interaction of LFA-1 integrins on NK cell surface with ICAM-1 molecules on target cells. This induces polarization of the actin cytoskeleton (step 2). In our laboratory, we have previously shown that step 2 occurs in a talin1-dependent manner, and talin is also polarized

towards the target cell [11]. This is followed by translocation of the lytic granules, containing perforin and granzymes, to the interface between the NK cell and its target (step 3). And finally, the fusion of granules with the cell membrane and the release of perforin and granzymes towards the bound target cause apoptosis of target cells (step 4, **Fig.1.1)** [12]. The complex formed between an NK cell and a sensitive target is often termed the "lytic immune synapse". LFA-1, actin, and talin form a ring that provides stability to the immune synapse and acts as the scaffold for the assembly of stimulatory and inhibitory signaling complexes. It also provides a route for delivery of lytic granules to the target [13].

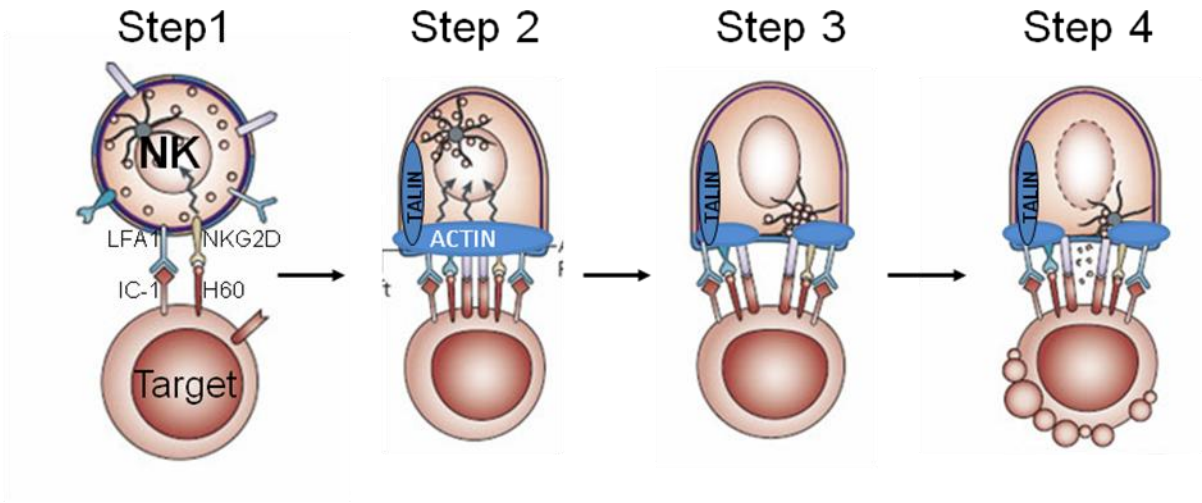


Figure 1.1 Sequential steps to NK cell cytotoxicity. The killing of target cells by NK cells is a multi-step process involving: 1) adhesion to target cells mediated by LFA-1 molecules, 2) polarization of the actin cytoskeleton mediated by talin and actin polymerization machinery, 3) redistribution of lytic granules and 4) release of their contents towards target cells triggered by ligation of the stimulatory receptors. Image adapted and modified from *Nature Reviews Immunology* [14].

1.2 Stimulatory receptors

As opposed to B and T cells, NK cells do not rely on a single dominant stimulatory receptor to initiate effector functions. Instead, they cooperatively use an array of stimulatory receptors that act in additive or synergistic manners to elicit a response. Hence, they are often referred

to as ‘costimulatory’ or ‘coactivating’ [15]. Following conjugate formation via the LFA-1: ICAM-1 interaction between an NK cell and its target, NK cell activating receptors bind their ligands on target cells. In contrast with inhibitory receptors, the majority of stimulatory receptors on NK cells lack intrinsic signalling domains and, instead, rely on transmembrane adapter proteins to transmit their signals. While some adapters (e.g. FcεRIγ, CD3ζ and DNAX adapter protein (DAP)-12) contain an immunoreceptor tyrosine-based activating motif (ITAM) with the YxxL/Ix[6–8]YxxL/I sequence, others do not (e.g. DAP10) [16].

1.2.1. NKGD2

NKG2D is an activating receptor and member of the C-type lectin-like family of receptors. It is expressed on NK cells, activated CD8+ T cells, and macrophages. It recognizes cell surface ligands distantly related to MHC I molecules. These comprise MULT1, H60 and Rae1 in mice, and MHC I heavy chain-related proteins and UL-16-binding proteins (ULBPs) in humans [17, 18]. DNA damage response can also induce expression of NKG2D ligands to alert NK cells of potentially damaged cells [19]. Murine NKG2D exists in two splice variants: the ‘long’ (NKG2D-L) isoform that associates only with the DAP10 adaptor protein, and a ‘short’ (NKG2D-S) isoform that is detected only after NK cell activation and can associate with either DAP10 or DAP12 [20, 21]. Although NKG2D has been presented as a primary activating receptor on NK cells, some studies suggest that it may co-stimulate rather than directly activate effector functions. For instance, NKG2D ligation alone was shown to be insufficient for degranulation of NK cells [22]. Also, stimulation of mouse lymphokine-activated NK cells (LAKs) by anti-NKG2D mAb alone does not induce cytokine release, but

co-stimulation via CD16, NK1.1, or Ly-49D in addition to NKG2D enables cytokine production [23].

1.2.2. 2B4

Another major stimulatory NK cell receptor is 2B4, also referred to as CD244. It belongs to the SLAM family of receptors that includes CRACC, SLAM, CD84 and Ly-9 in mice [24]. 2B4 is expressed on all immature and mature mouse and human NK cells, γδ T cells and on a large subset of $CD8^+$ T cells. The ligand for 2B4 is its relative CD48, a glycosyl-phosphatidyl inositol (GPI)-anchored cell surface glycoprotein. CD48 also interacts with the CD2 receptor, however the affinity of 2B4 for CD48 is 5 to 10 times higher than that of CD2, making the 2B4-CD48 interaction the predominant one in NK cells [25].

Originally, 2B4 was identified as a stimulatory cell surface receptor on murine NK cells. Antibody (Ab)-cross linking of 2B4 triggered cell-mediated cytotoxicity and cytokine secretion by NK cells [26]. Transfecting mouse cell lines with human CD48 enhanced their susceptibility to lysis by human NK cells, while adding blocking Ab for 2B4 or CD48 diminished the killing of CD48-expressing targets [27].

2B4, SLAM, and others of the CD2 sub-family contain multiple cytoplasmic tyrosine-based motifs (TxYxxI/V) called immunoreceptor tyrosine-based switch motifs (ITSMs). These motifs have the unique capacity to 'switch' between activating and inhibitory signal transduction. Upon 2B4 receptor activation, its four ITSMs are phosphorylated. This recruits SLAM-associated protein (SAP), which interacts with all four ITSMs through its SH2 domain [28]. The third phosphorylated ITSM can also bind inhibitory phosphatases SHP-1, SHP-2, and SHIP [29]. However, SAP prevents the binding of these negative regulators to 2B4,

by interacting with them itself. SAP also recruits the SH3 containing Src family kinase Fyn, which mediates 2B4 signal transduction leading to PLCγ activation, calcium mobilization, and cytotoxicity [30, 31]. Loss of function mutation of the SAP gene causes inherited X-linked lymphoproliferative disease (XLP), which manifests itself by the inability to control B-cell proliferation following Epstein-Barr virus (EBV) infection [32]. It was shown that XLP patients exhibit severe SLAM signalling defects, as well as impaired 2B4 receptor-mediated cytolysis of EBV-infected B cells [33]. One possible mechanism for this is: in the absence of SAP, inhibitory phosphatases SHP-1, SHP-2, and SHIP bind 2B4 and abrogate its activating signals [29].

Similar to NKG2D, recent studies with human NK cells suggest that 2B4 may act as a co-activating receptor rather than a primary stimulatory receptor. NKp46 co-expression with 2B4 was shown to exert synergistic effects on target killing [34]. NKG2D was likewise proposed as a coactivating receptor to 2B4 [31]. Despite this, there is affirmation that 2B4 can act as an inhibitory receptor in both humans and mice [35]. NK cells from 2B4-KO mice kill CD48-transfected target cells more efficiently than WT NK cells, suggesting that 2B4 might act as an inhibitory receptor [36]. The question of whether 2B4 stimulates or inhibits NK cells is perplexed by the expression of multiple SLAM family receptors on NK cells, as well as multiple members of the SAP family of signalling proteins that associate with SLAM family receptors [24]. It has been proposed that its first ITSM is sufficient to mediate stimulatory signalling, and its third, phosphatase-binding, ITSM mediates inhibitory functions [29].

1.2.3. NKp46

NKp46 (NCR1) is a member of the natural cytotoxicity receptor (NCR) group on NK cells, alongside NKp44 and NKp30. While all three are expressed in humans, only NKp46 is expressed in mice [37]. NCRs associate with non-MHC I ligands on abnormal cells, and transduce their signals with the help of ITAM-containing adapter proteins. NKp46 was first identified by the Morretta group and shown to be expressed on all human NK cells, regardless of activation state [38]. Ab cross-linking induces NK cell cytotoxicity, calcium ion release, and cytokine production by resting and cultured NK cells [38]. NKp46 is described as a major activating receptor and a highly specific marker of NK cells. It belongs to the Ig-superfamily of surface receptors, and presumably interacts with the ITAM-containing CD3ζ adapter protein to mediate signalling. Although NKp46 can trigger cytolysis of different tumorigenic cells, the actual tumor ligand(s) for it have not been identified [39]. Among viral ligands, NKp46 has been found to associate with haemagglutinin of influenza virus and haemagglutinin–neuraminidase of parainfluenza virus [40]. However, the mechanism of interaction between NKp46 and its viral ligands remains to be deciphered.

1.2.4. Other stimulatory receptors

A number of other activating receptors have been identified in human and mouse NK cells. A group of them specifically bind to MHC I or MHC I-like molecules on target cells; these include NKG2C, NKG2E, human KIR2DS and KIR3DS, as well as mouse Ly49D, Ly49H, Ly49P and Ly49W. While KIR2DS is involved in attenuating viral spreading and AIDS progression in HIV positive individuals, donor KIR2DS expression reduces relapse after haplo-identical transplantation for acute leukemia [41]. NK cells from C57BL/6 mice express

activating Ly49D receptors that recognize H-2D^{d} molecules, and are important for rejecting bone marrow cell (BMC) allografts *in vivo* and lysing allogeneic lymphoblasts *in vitro* [42]. The ligand for the Ly49H receptor has been identified to be the murine cytomegalovirus-encoded MHC I-like protein m157 (MCMV) and it mediates resistance to the virus [43].

As mentioned earlier, human NK cells express natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46. NKp44 mediates signalling through the DAP-12 adapter protein, while NKp30 and NKp46 act in complex with CD3ζ and/or FcRγ adapters [16]. *In vitro* assays with human NK cells revealed that both NKp46 and NKp44 bind the haemagglutinins of Influenza virus [40, 44]. And recently, a murine homologue of NKp46 has been described; however the ligand for this receptor remains to be identified [45].

While the NK1.1 alloantigen has extensively been used as NK and NKT cell marker in C57BL/6 mice, further analysis of NK cells from diverse mouse strains confirmed that the NK1.1 epitope is shared by two alternative splicing products of the *NKR-P1*gene [46]. NKR-P1 (CD161) is a member of the family of type II transmembrane C-type lectin-like receptors in NK cells, giving rise to two receptors with entirely opposing functions. NKR-P1B is the inhibitory form of this receptor and NKR-P1C is the triggering form. NKR-P1C ligation induces cytotoxicity, cytokine production, increased calcium ion flux, and enhanced kinase activity [46]. Recent studies show that signalling through NKR-P1C requires association with FcRγ adapter protein, as NK cells from FcRγ-deficient mice are not cytotoxicic nor do they produce IFN-γ upon NKR-P1 cross-linking [47]. Nonetheless, elaborate molecular mechanisms of NKR-P1signalling have not been determined yet.

NK cells also participate in Ab-dependent cell-mediated cytotoxicity (ADCC) via the CD16 activating receptor, which is a multi-subunit immune recognition complex, also referred to as FcγRIIIA. CD16 is a low affinity receptor for IgG that, in humans, associates with hetero- or homodimers of CD3ζ and FcRγ adapter molecules, and with homodimers of FcRγ in mice [16, 48].

1.3. Inhibitory receptors

Polymorphic MHC I molecules are the classical ligands for NK cell inhibitory receptors, such as the human killer Ig-like receptors (KIRs), the analogous murine Ly49 receptors, CD94/NKG2A receptors in both humans and mice, and the leukocyte Ig-like receptor B1 (LILRB1) [9]. Structurally, LILRB1s and KIRs are monomeric type I glycoproteins of the immunoglobulin superfamily, while CD94-NKG2A and Ly49 receptors are type II glycoproteins with a C-type lectin-like domain. Despite the disparate extracellular domains, these receptors share a common signalling motif in their cytoplasmic domain. It is the immunoreceptor tyrosine-based inhibitory motif (ITIM) of the (I/L/V/S)XYXX(L/V) sequence, where X designates any amino acid, and slashes separate distinct amino acids that may occupy a given position [1]. The interaction between NK inhibitory receptors and MHC I generates a dominant signal that abrogates NK cell effector functions.

1.3.1. Ly49

In humans, killer-cell immunoglobulin-like receptors **(**KIRs) are a major family of NK cell receptors recognizing MHC I molecules. In mice, the analogous function is exhibited by the Ly49 receptor family, which includes both inhibitory and activating receptors that signal

through either ITIMs or kinase-activated adapter proteins, respectively [49]. Structurally, these are homodimeric disulfide-linked type II transmembrane glycoproteins, with each chain composed of a C-type lectin-like domain (CTLD) connected to the cell membrane by an α-helix stem [50].

The mouse Ly49 family of NK cell receptors includes at least 23 members, from Ly49A through W. Although not all have been characterized, it is known that distinct Ly49 members can discriminate between different MHC I molecules (e.g. H-2D, H-2K, H-2L). MHC I is highly polymorphic, and a particular Ly49 receptor only recognizes a limited repertoire of MHC I molecules [51]. For instance, Ly49A specifically binds H-2D^{d} and H-2D^{k} [46] whereas Ly49C binds H-2K^{b} and H-2D^{b} [52, 53]. A target that does not display the specific MHC I molecule recognized by the available Ly49 receptors on a given subset of NK cells will endure lysis. The same fate awaits if the target additionally over-expresses specific ligands for NK cell activating receptors.

The expression of Ly49 receptors is stochastic and each receptor can be expressed by a distinct but overlapping subset of NK cells. Thus, each NK cell usually co-expresses two or more different Ly49 receptors [54, 55]. In vivo, NK cells attack allogeneic and MHC I-deficient cells (e.g. tumour cells), but not self-cells. When the latter down-modulate their MHC I as a result of mutation, infection, or transformation, they become sensitive to autologous NK cells. This is the principal idea behind the 'missing self' hypothesis [10]. Signalling through inhibitory Ly49 receptors involves ITIM phosphorylation. In contrast to human KIRs each containing two cytoplasmic ITIMs, inhibitory Ly49s have a single cytoplasmic ITIM [56]. This is probably why Ly49 homodimerization is essential for performing their inhibitory function.

An elaborate description of signalling through the ITIM follows in the next section of this thesis.

1.3.2. NKG2A

The CD94/NKG2A receptor is expressed in both humans and mice, and is a disulfide-linked heterodimer consisting of two subunits encoded by the *CD94* and *NKG2A* genes. It is expressed on approximately fifty percent of adult mouse NK cells and nearly all fetal and neonatal mouse NK cells, as well as on some $CD8^+$ T cells [57]. The CD94 subunit of this receptor has a short cytoplasmic domain with no signalling function, while the NKG2A subunit contains two ITIMs. Upon ligand binding, these ITIMs become phosphorylated and recruit SHP-1 or SHP-2 phosphatases [58]. Ultimately, inhibitory signaling through the CD94/NKG2A receptor prevents actin-dependent recruitment of stimulatory receptors to the contact site between an NK cell and its target, evading its lysis [59].

In humans, the MHC I ligand recognized by the CD94/NKG2A receptor complex is HLA-E, and in mice it is the homologous $Qa1^b$ molecule. The peptide binding sites of HLA-E and $Qa1^b$ are occupied by peptides derived from MHC I molecules. Without these peptides, HLA-E and $Qa1^b$ are not expressed on cell surface, but are degraded in the cytoplasm of host cells [60]. This serves as a surveillance mechanism for NK cells expressing CD94/NKG2A, enabling them to monitor global MHC I expression levels on target cells [58].

1.3.3. Other inhibitory receptors

Some NK cells express inhibitory receptors that bind ligands other than MHC I. Examples include the lectin-like receptor KLRG1 which, in humans and mice, binds cadherins and

assesses the loss of tissue integrity [61, 62]. NKR-P1, also a lectin-like receptor, binds to LLT1 in humans and Clrb/Ocil in mice [63-65]. LAIR-1 interacts with collagen and plays a role in control activation in the extracellular matrix [66]. Siglec-7 (CD328) and Siglec-9 (CD329), bind sialic acid residues on target cells. NK cells also have orphan inhibitory receptors, such as IRp60 (CD300a), for which the ligands remain unidentified [67, 68].

1.4. Intracellular signals

1.4.1. Stimulatory

In order for NK cells to mount a response, engagement of primary or, most often, of a combination of coactivating receptors must produce a stimulatory threshold strong enough to counteract the input from inhibitory receptors. Attaining this threshold induces assembly of multi-subunit receptor complexes, with specialized adapter proteins that transduce signals into the cell via distinct signaling pathways. Three major activating pathways have been identified in both mouse and human NK cells: signalling through immunoreceptor tyrosine-based activation motif (ITAM)-bearing NK receptor complexes, DAP10-associated NKG2D receptor complexes, and the 2B4 (CD244) receptor system [1].

DNAX-activating protein of 12 kDa (DAP12) is an example of a specialized adapter that signals through the ITAM in its cytoplasmic domain. In mice, DAP12 associates with stimulatory NK receptors CD16, NKG2-S, Ly49D and Ly49H [20]. This association activates Src family protein tyrosine kinases (PTKs), which phosphorylate the ITAM on tandem tyrosine residues, thereby recruiting additional tandem SH2 PTKs of the Syk family, Syk and ZAP-70 [69]. In turn, various transmembrane and cytosolic adapters come into play and activate a number of signalling cascades involving molecules like phosphoinositide 3-kinase

(PI3K), phospholipase C-γ (PLCγ), Vav1, Rho, Ras, and extracellular signal-related kinase (ERK). Moreover, ITAM-receptor signalling also increases intracellular calcium ion levels, induces actin polarization, release of lytic granules, and transcription of cytokine genes [1]. Other examples of ITAM-containing adapters include FcεRIγ and CD3ζ, which bind NKp46, CD16 & NK1.1, respectively [47, 70].

A different adapter protein implicated in NK cell stimulatory signalling is DAP10. The DAP10 stimulatory pathway is independent of Syk-family protein kinases and mediates signals distinct from DAP12. In place of an ITAM, it contains a YINM sequence in its cytoplasmic domain. This sequence can bind either the p85 subunit of PI3K, or the growth factor receptor bound protein 2 (Grb2). Depending on the binding partner, DAP10 downstream signalling can take different routes. DAP10 binding of p85 activates PI3K and induces production of phosphatidylinositol-3,4,5-triphosphate (PIP_3) at the immune synapse. Recruitment of Grb2 results in phosphorylation of SLP-76, PLCγ2, and Vav1 – the Rac-Cdc42 family exchange factor. Grb2 can also indirectly interact with DAP10 at the synapse via increased PIP_3 levels, in complex with Sos1 and Vav1. Interestingly, it was found that intact binding sites for both p85 and Grb2 are required for full calcium release and cytotoxicity to occur [71]. Activation signals passing through DAP10 seem sufficient to induce cytotoxicity, but not cytokine secretion, against specific tumor cell lines [20], whereas ITAM dependent signalling through DAP12 can trigger both cytotoxicity and cytokine secretion in NK cells [72]. Despite their differences, signaling through either DAP10 or DAP12 eventually leads to a common cytotoxicity pathway involving Rac activation, subsequent activation of mitogen-activated protein kinase (MAPK) and ERK by means of p21-activated kinase (PAK) and mitogen-activated or extracellular signal-regulated protein kinase kinase (MEK), leading

to polarization and release of cytotoxic granules [69, 73]. To some extent, DAP10 and DAP12 complement one another during NK cell activation. Studies with resting NK cells from DAP10-deficient (*Hcst*$^{-/-}$) mice reveal under-expression of NKG2D receptors, until *in vitro* or *in vivo* stimulation. This suggests compensation by DAP12 dependent expression of NKG2D-S [74]. Still, NKG2D-dependent killing of tumorigenic targets is impaired in these mice, and so is their ability to reject mismatched BM grafts [74, 75]. NK cells from DAP12-deficient mice also show diminished killing of tumor cells, as well as a diminished and non-functional repertoire of Ly49 activating receptors [76, 77]. It was also recently shown that both adapters are necessary for optimal killing of mouse cytomegalovirus (MCMV) by NK cells, as this is a Ly49H-dependent process, a stimulatory receptor from which signalling employs both DAP10 and DAP12. Double deletion of DAP10 and DAP12 annihilates expression of Ly49H and resistance to MCMV infection [78].

1.4.2. Inhibitory

As oppose to stimulatory signalling in NK cells, inhibitory signals occur through one major pathway involving immunoreceptor tyrosine-based inhibition motifs (ITIMs) contained within the cytoplasmic tails of NK inhibitory receptors. Upon receptor engagement, its ITIM tyrosine residue(s) is (are) phosphorylated, most likely by a Src family kinase. This results in recruitment of the Src homology 2 containing phosphatase-1 (SHP-1), SHP-2, or the Src homology2-containing inositol phosphatase-1 (SHIP-1). Inhibitory receptors recruit these phosphatases to the contact site between an NK cell and its target, where they dephosphorylate substrates of tyrosine kinases involved in NK cell activation, thereby blocking it at an early stage [1]. For instance, a direct substrate of the SHP-1 phosphatase is

Vav1 – a major regulator of actin organization [79]. SHP-1 dephosphorylates Vav1 and prevents polarization of the actin cytoskeleton, opposing NK cell activation [59, 80]. It is important to note that these inhibition events are transient and do not prevent the same NK cell from becoming activated upon encounter with an abnormal target [1].

The ITIM was initially discovered in the cytoplasmic tail of FcγRIIb receptor, which mediates B cell inhibitory signalling. Phosphorylated ITIM of FcγRIIb can bind both SHP-1 and SHIP phosphatase, but only SHIP is necessary for B-cell inhibition [81]. Studies with human and mouse NK cells expressing KIR/FcγRIIb chimeric receptors and dominant negative mutants of SHIP and SHP-1 revealed that SHP-1 is indispensible for KIR-mediated inhibition, while SHIP is required for inhibition through FcγRIIb [82]. In NK cells, Ly49 and NKG2A are thought to predominantly associate with SHP-1 or SHP-2, while KLRG1 associates with SHIP-1 [58, 83]. The reasons for this differential association are undetermined. While SHP-1 and SHP-2 carry out their inhibitory role by removing tyrosine phosphates, SHIP-1 converts phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) to $PI(3,4)P_2$ by removing the 5′ phosphate [84, 85]. This blocks activation of serine/threonine kinase Akt. Engagement of ADCC-mediating receptor CD16 on NK cells also recruits SHIP, which interacts with the CD3ζ adapter protein and acts as a negative regulator of NK function [86].

Wang *et al.* found that SHIP in mouse NK cells associates with Ly49A and Ly49C inhibitory receptors, and homozygous SHIP-KO mice present an alternate Ly49 repertoire with excessive amounts of Ly49A and Ly49C/I receptor [87]. Both of these have been proposed to play a role in NK cell licensing, a topic discussed in the next section of the thesis. In addition, SHIP-KO mice are not able to reject MHC I mismatched bone marrow (BM) grafts, nor kill tumorigenic or virally infected targets [87].

As mentioned earlier, the third cytoplasmic ITSM of the 2B4 receptor attracts negative regulators SHP-1, SHP-2 and SHIP-1, likely enabling it to transduce inhibitory signals [29]. In compliance with studies identifying 2B4 as an inhibitory receptor on NK cells, it was shown that lack of response of SHIP-KO NK cells to various targets is due to predominant inhibition through 2B4 [88]. Removing 2B4 restores cytolytic capacity to SHIP-KO NK cells, as seen with double (2B4 and SHIP) knock-out animals [89]. As it turns out, in the absence of SHIP, 2B4 expression is up-regulated and recruitment of SHP-1 to this inhibitory receptor increases 10 to 16 fold, as opposed to WT NK cells where inhibition is mostly mediated by SHIP. Blocking SHP-1 function with sodium orthovanadate alleviates its inhibitory effects and re-establishes killing capacity to SHIP-KO NK cells [90].

1.5. NK cell licensing

The interaction between inhibitory Ly49 receptors and MHC I is important for the functional maturation of NK cells by a process termed NK cell "licensing" [91]. Given the stochastic expression of inhibitory ligands specific for MHC I, it is evident that some NK cells do not go through licensing, and are thus functionally impaired as they are unable to induce cytotoxicity. In fact, NK cells from MHC I-deficient mutant mice are not cytotoxic because they are not licensed. Mice homozygous for the deleted β2m gene, (β2m-KO) are deficient for MHC class Ia and Ib expression. Although abundant in number, NK cells from β2m-KO mice show low capacity to reject allogenic bone marrow transplants, and low cytotoxicity against tumor cells [92]. Since the functional impairment is apparent but not absolute, β2m-KO NK cells are deemed "hyporesponsive". Moreover, studies have revealed that β2m-KO NK cells express the same cell surface markers as WT NK cells. For instance, they express

CD11b, DX5, Ly49C/I, and other Ly49 receptors [93]. This implies that the functional impairment arising from the lack of Ly49C/I-MHC I interaction alters intracellular pathways that, in-turn, prevent cytotoxicity.

The NK cell licensing model was initially described by the Yokoyama group, who noticed the differential killing capacity and IFNγ production between WT and β2m-KO upon NK1.1 cross-linking [91]. Focusing on Ly49A and Ly49C, due to readily available Ab's and extended research, they found that in order for NK cells to develop functionally (i.e. be able to lyse targets and produce interferon), these must interact with specific host MHC I molecules via their inhibitory Ly49 receptors. Furthermore, they found that licensing is mediated through the Ly49 ITIM. Gene transfer experiments revealed intact Ly49A, but not cytoplasmic-domain deleted Ly49A, renders NK cells functional upon ligand binding. However, this is different from ITIM-mediated inhibitory signalling, because licensing is observed in SHP-1 deficient NK cells [91]. If ITIM-mediated NK cell licensing does not require SHP-1, it potentially involves other phosphatases (e.g. SHIP). The Yokoyama group chose the term 'licensing' as opposed to 'education' in order to avoid confusion with events occurring during T-cell development.

Another group led by D. Raulet has described a subset of NK cells that do not express MHC I-specific receptors [93]. These represent about 10 to 13 percent of NK cells in normal B6 mice, and lack expression of Ly49A, Ly49C, or NKG2A receptors altogether. Similar to NK cells from β2m-KO mice, this newly described subset exhibits diminished levels of cytotoxicity toward prototypic targets (e.g. YAC-1), and reduced IFN-γ production in response to cross-linking of stimulatory receptors [93]. This reinforces the idea that MHC I-Ly49 interaction is necessary for NK functional maturation and acquisition of effector functions.

The next question addressed by both the Yokoyama and Raulet groups inquired whether licensing is a reversible process and whether responsiveness of an NK cell be altered according to its environment. Alternatively, they asked whether licensing is set once during development and remains unchanged throughout NK adult-hood, irrespective of surroundings. To test this, peripheral NK cells from MHC I deficient mice were transferred into WT MHC I competent hosts. A gain of function was observed as they began to produce IFNγ and degranulate at similar levels as WT NK cells in response to the standard NK cell target YAC-1. Plus, affirmative of the licensing hypothesis, only the cells expressing Ly49C could gain function after being transferred into hosts expressing specific MHC I ligand for Ly49C, while Ly49C negative cells remained unlicensed [94]. Hence, licensing is not solely restricted to NK cell development in the BM. Similar results were observed by the Raulet group, who additionally reported that hyporesponsive cells did not divide post transfer, indicative of their initial mature state [95]. In a similar manner, when WT NK cells were introduced into MHC I deficient hosts, they lost their capacity to produce IFNγ in response to NKG2D and NK1.1 stimulation, and were not able to reject mismatched grafts [95].

It is important to note that licensing does not apply to cultured NK cells, as stimulation with potent cytokines (e.g. interleukin-2) over time reverses their hyposensitive state and renders them cytotoxic [93, 96]. Moreover, contrary to aforementioned studies describing the functional impairment of NK cells from MHC I deficient mice, a group led by L. Lanier showed that "unlicensed" NK cells from β2m-KO mice are the primary fighters against MCMV infection *in vivo* [78]. Their depletion makes mice susceptible to MCMV, whereas transfer of unlicensed NK cells into WT hosts is more protective than transfer of licensed NK cells into β2m-KO hosts [78]. Nonetheless, MCMV studies might be looking at a completely distinct phenomenon,

as cytotoxicity against this virus is mediated by Ly49H, which is present in β2m-KO mice [97]. Lanier's study also showed that SHP-1 signalling limited proliferation of licensed, but not unlicensed NK cells during infection [78], confirming the idea that ITIM-mediated NK cell licensing relies on a different phosphatase. The precise mechanism by which the interaction between MHC I and inhibitory Ly49 mediates licensing of NK cells remains a major question to be resolved in the field of NK cell biology.

1.6 Thesis objectives and hypothesis

The goal of my MSc project was to determine the mechanism through which NK cells of MHC I deficient mice are kept hyporesponsive to otherwise NK-sensitive targets. The objective of this thesis was to elucidate the major molecules involved in inhibiting NKG2D mediated cytotoxicity in β2m-KO NK cells. I hypothesized that in licensed NK cells, the binding of MHC I to inhibitory Ly49C/I receptor causes phosphorylation of its ITIM and recruitment of SHIP. This prevents SHIP from localizing to the NKG2D-mediated stimulatory synapse, allowing cytotoxicity. In unlicensed NK cells, Ly49 ITIM does not associate with SHIP and SHIP localizes to the NKG2D-mediated stimulatory synapse, where it inhibits cytotoxicity. I tested this hypothesis in Chapter 3 by comparing unlicensed NK cells from β2m-KO mice with wild type (WT) B6 mouse NK cells.

Chapter 2: Materials and Methods

2.1 Tissue culture

2.1.1. Mice

C57BL/6 (B6) mice and β2microglobulin (β2m)-deficient B6.129P2-B2mtm1Unc/J mice (β2m-KO mice hereafter) were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in the Animal Resource Centre (ARC) of the BC Cancer Research Centre (BCCRC). WT and SHIP-deficient BALB/c mice were generously donated by Gerry Krystal's laboratory (Vancouver, Canada) [98].

2.1.2. Murine splenocyte preparation

Splenocytes were isolated from two to six month old mice. Single cell suspensions were generated by grinding spleens with the blunt end of a 3ml syringe and passing them through a 70 µm strainer onto a 10 cm Petri dish (BD Falcon). Cells were washed with RPMI 1640 media supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, 5 x 10^{-5} M β-mercaptoethanol (StemCell Technologies), resuspended in 0.8% ammonium chloride (StemCell Technologies) to lyse red blood cells, washed again and resuspended in media. Red blood cells were not lysed for NK cell enrichment.

2.1.3. Generation of poly I:C primed *ex-vivo* NK cells

One to three B6 and/or β2m-KO mice were subcutaneously injected with 200 µl of 1 mg/ml Polyinosinic-polycytodylic acid (poly I:C) (Sigma-Aldrich, St.Louis, MO). 24 hours post-

injection, splenocytes were extracted as described in **2.1.2.**, and NK cells isolated using EasySep® NK cell negative selection kit (StemCell Technologies).

2.1.4. YAC-1 culture

Murine lymphoid cell line YAC-1, originally from American Type Culture Collection (ATCC, Rockland, MD) was cultured in RPMI 1640 media supplemented with 10% FCS, penicillin, streptomycin, 5 x 10^{-5} M β-mercaptoethanol (StemCell Technologies).

2.2. Antibodies and commercial reagents

2.2.1. Primary antibodies

Table 2.2.1. Primary antibodies

Antibody/ Probe	Clone	Species	Monoclonal/Polyclonal	Conjugate	Company/ cat #	Use/ Dilution
β tubulin	KMX-1	Ms	monoclonal	purified	CH 3408	C 1:50
CD3	145-2C11	ar hm	monoclonal	PE	BD 553063	F 0.1 µg/ml
CD27	LG.3A10	ar hm	monoclonal	PE	BD 558754	F 0.1 µg/ml
Granzyme B		Rb	polyclonal	purified	AB 4059	C 1:50
Granzyme B	16G6	Rat	monoclonal	FITC	B 11-8822-80	F 0.1 µg/ml
IgG2a	R19-15	Rat	monoclonal	FITC	BD 553390	F 0.1 µg/ml
FcR	2.4G2	Rat	monoclonal	hyb sup	ATCC HB197	F 100 µl per 4 x 10^6 cells
Ly49C/I	5E6		monoclonal	FITC	BD 553276	C, F 0.1 µg/ml
Ly49C/I	5E6		monoclonal	purified	PM 01621D	C 0.2 µg/ml
LFA-1	TIB213		monoclonal	purified	ATCC	C 1: 50
Mac1 (CD11b)	TIB128		monoclonal	FITC	ATCC	F 0.1 µg/ml
NK1.1		Ms	monoclonal	PerCP	BD	F 0.1 µg/ml
NKp46	29A1.4		monoclonal	APC	BL 137608	F 0.1 µg/ml
Phalloidin	------	------	------	Alexa 647	MP A-22287	C 1:50
Phalloidin	------	------	------	rhodamine	MP R415	C 1:100
SHIP	P1C1	Ms	monoclonal	purified	SC-8425	C 1:30

Antibody/ Probe	Clone	Species	Monoclonal/Polyclonal	Conjugate	Company/ cat #	Use/ Dilution
SHP-1	PhosphoY536	Rb	polyclonal	purified	AB 51171	C 1:100
Talin	8D4	Ms	monoclonal	purified	S 3287	C 1:100
7AAD	-	-	-	fluorescent DNA dye	S 9400	F 1:1000

Legend: SG Stressgen; SC Santa Cruz; AB Abcam; CH Chemicon; UP Upstate; S Sigma; B eBioscience; ML Millipore; BD BD Bioscience; MP Invitrogen Molecular Probes; ATCC American Tissue Culture Collection; BL BioLegend; ms mouse; gt goat; rb rabbit; ar hm Armenian hamster. C confocal; W western blotting; F FACS; cytotoxicity assay; Ad adhesion assay; FITC fluorescein isothiocyanate; PE phycoerythrin; APC allophycocyanin

2.2.2. Secondary antibodies

Table 2.2.2. Secondary antibodies

Antibody	Animal	Conjugate	company/cat#	use/dil'n
mouse IgG	donkey	Alexa Fluor 488	MP A21202	C 10 µg/ml
mouse IgG	goat	Alexa Fluor 568	MP A11031	C 10 µg/ml
goat IgG	donkey	Alexa Fluor 488	MP A11055	C 10 µg/ml
goat IgG	rabbit	Alexa Fluor 488	MP A11078	C 10 µg/ml
goat IgG	donkey	Alexa Fluor 568	MP A11057	C 10 µg/ml
rabbit IgG	goat	Alexa Flour 488	MP A11008	C 10 µg/ml
rabbit IgG	donkey	Alexa Fluor 488	MP A21206	C 10 µg/ml
rabbit IgG	donkey	Alexa Fluor 647	MP A31573	C 10 µg/ml
mouse IgG	-	Streptavidin 488	MP S-32354	C 10 µg/ml
mouse IgG	donkey	Alexa Fluor 647	MP A31571	C 10 µg/ml

Legend: MP Invitrogen Molecular Probes; C confocal.

2.2.3. Reagents

Murine soluble recombinant ICAM-1 (sICAM-1) [99] was from StemCell Technologies (Vancouver BC, Canada). Calcein-AM and carboxy-fluorescein diacetate succinimidyl ester (CF-DA SE) were from Invitrogen Molecular Probes (Eugene, OR). Polybead® polystyrene 10 micron microspheres were from Polysciences (Warrington, PA). Bovine serum albumin

(BSA), saponin, polyinosinic-polycytodylic acid (poly I:C), and poly-L-lysine (PLL) were from Sigma-Aldrich (St.Louis, MO). BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit with BD GolgiPlug™ was from BD Biosciences.

2.3. CD48 and H60-Fc fusion proteins

CD48-Fc fusion protein was prepared by Boxiang Jiang of our laboratory. CD48 cDNA was cloned by RT-PCR from YAC-1 cells using forward primer 5'ACGAATTCAGTATTATGTGCTTCATAAAACAGGGATG 3' and reverse primer 5'ACGGATCCACTTACCTGTAGATCTGGCTAGATCACAAGGTAGAG 3', subcloned into pBluescript and sequenced. The cDNA encoding the extracellular domain of CD48 was PCR-amplified, sequenced and subcloned into pIG vector [10]. The CD48-Fc/pIG construct was then transfected into 293T cell line and the fusion protein secreted into the media was purified by protein A affinity chromatography. The purified protein was analyzed and quantified by Western Blot and Coomassie Blue staining. H60 cDNA was cloned by Ryan Russell by RT-PCR from BALB/c mouse splenocytes, subcloned into pBluescript and sequenced. The cDNA encoding the extracellular domain of H60 was PCR-amplified, sequenced and subcloned into the pIG vector. H60-Fc fusion protein was produced and purified as described for CD48-Fc fusion protein.

2.4. ICAM-1 adhesion assay

Splenocytes were isolated from poly I:C injected B6 and β2m-KO mice, and NK cells generated using EasySep® NK cell negative selection kit (StemCell Technologies). Cells were resuspended in Hank's balanced salt solution (HBSS) at a concentration of 10^6/ml and

labelled with 1 µg/ml Calcein-AM for 30 minutes at 37°. Cells were washed twice with HBSS 2% FCS, resuspended at 10^6/ml in 500 µl volumes, and aliquoted into 3 Eppendorf tubes. For specificity control, NK cells in one of the tubes were incubated with 1.5 µg/ml of anti-LFA1 antibody (TIB213) for 10 minutes at 37°. Meanwhile, a flat-bottomed 96 well immunoassay plate (NUNC Maxisorp) was coated with soluble sICAM-1 (10 µg/ml in 0.1 M sodium bicarbonate buffer pH 8.0) for 60 minutes at room temperature. An extra set of control wells was left uncoated with sICAM-1. The plate was washed three times with phosphate buffered saline (PBS) and blocked with 0.5 mg/ml heat inactivated bovine serum albumin (BSA) for 30 minutes at room temperature. Following blocking, the plate was washed 3 times with PBS and cells were distributed onto the plate at a concentration of 10^5/100 µl per well. The initial fluorescence intensity was read by CytoFluor 2300 (Millipore, Bedford, MA). Plates were washed five times with 100 µl pre-warmed HBSS 2% FCS to remove non-adherent cells, and fluorescence intensity was read again to assess percent adhesion of NK cells bound to sICAM-1 coated wells, and that of specificity controls, cells plated on wells coated with 0.5 mg/ml BSA, or cells pre-incubated with anti-LFA-1. The percent adhesion was calculated as the ratio of the post-wash to pre-wash fluorescence values, minus background fluorescence values.

2.5. Confocal microscopy

2.5.1. Binding of NK cells to beads

Polystyrene beads (Polybead® polystyrene 10 micron microspheres, Polysciences Inc., Warrington, PA) were mixed at 1:1 ratio with 20 µg/ml of recombinant sICAM-1, H60-Fc, CD48-Fc, or 0.005% poly-L-lysine (PLL), diluted in Adhesion Buffer (20 mM Tris-HCl, 150

mM NaCl, pH 8.2). The mixture was incubated for 1 hr at room temperature, with occasional agitation to prevent settling. Beads were washed 3 times with 100 µl RPMI 10% FCS media and resuspended in 150 µl media. Coverslips were soaked in PLL (Sigma-Aldrich, St.Louis, MO) over-night on a 10 cm Petri dish, washed three times with PBS and left to dry against the side of the dish. Poly I:C primed *ex-vivo* NK cells were generated (**2.1.3**.) and resuspended in the same media as beads at 1.25×10^6/ml. Cells (2.5×10^5 in 200 µl) were gently mixed with 25 µl (10^5) beads in an Eppendorf tube and incubated at 37º for 5 minutes. Dry PLL coated coverslips were placed into a 12 well plate. Cell-bead conjugates were gently resuspended by flicking the tubes, carefully pipetted onto the coverslips, and incubated at 37º for 15 minutes. Coverslips were fixed with 4% formaldehyde, and samples were permeabilized with Hank's Saponin solution (HBSS containing 2% FBS, 5 mM Ethylenediaminetetraacetate (EDTA), 0.5% Saponin).

2.5.2. Binding of NK cells to YAC-1

YAC-1 were cultured in RPMI 1640 media supplemented with 10% FCS, 5×10^{-5} M β-mercaptoethanol and penicillin/streptomycin, at 37° for at least five days prior to use. Cells were washed with the same media and resuspended at 10^7/ml. Coverslips were soaked in PLL (Sigma-Aldrich, St.Louis, MO) over-night on a 10 cm Petri dish, washed three times with PBS and left to dry against the side of the dish. Poly I:C primed *ex-vivo* NK cells were generated (**2.1.3**.) and resuspended in RPMI 1640 10% FCS at 10^7/ml. NK cells (10^6 in 100 µl) were gently mixed with YAC-1 (10^6 in 100 µl) in an Eppendorf tube and incubated at 37º for 5 minutes. Dry PLL coated coverslips were placed into a 12 well plate. NK-YAC-1 conjugates were gently resuspended by flicking the tubes, carefully pipetted onto the

coverslips, and incubated at 37º for an additional 15 minutes. Cells on coverslips were fixed with 4% formaldehyde, and samples were permeabilized with Hank's saponin solution (HBSS, 2% FCS, 5 mM EDTA 0.5% saponin).

2.5.3. Staining for confocal microscopy

Actin, Talin, Granzyme B, β-tubulin, SHIP, and SHP1 were stained by incubating cover slips with the appropriate dilution of primary antibody in 300 µl HBSS-Saponin buffer on a slow shake for 1 h, at room temperature. Coverslips were washed 3 times with the same buffer, and incubated with the corresponding Alexa Fluor conjugated secondary antibody at 10 µg/ml for 1 h at room temperature. Finally, the slips were washed twice with HBSS and mounted on slides using Vectashield Hard Set Antifade Mounting Medium (Vector Laboratories, Inc., Burlingame, CA).

2.5.4. Acquisition and analysis of confocal images

Cells, cell-bead, or NK-YAC-1 conjugates were analyzed using a Nikon C1-si confocal microscope with a 100× objective lens, zoom 4 (BC Cancer Agency, Vancouver). To avoid bleed-through, images were collected using sequential scanning. Alexa Fluor 488 was excited at 488 nm and the emission filter was HQ 515/30. Alexa Fluor 568 and rhodamine were excited at 568 nm and the emission filter was HQ 600/50. Z-stacks were collected using 0.30 µm Z steps and reconstructed into 3D images using Volocity software (Improvision, Lexington, MA). For quantification of fluorescence intensity at the site of binding, the sum intensity in the channel of interest was determined using Volocity software for the area of contact between a cell and a target bead or YAC-1, and compared to the sum fluorescent

intensity of the whole same cell. For quantification of co-localization between Ly49C/I and SHIP or SHP-1, the overlap coefficient (R) values out of 1.00 were computed by Volocity software. All values were exported to Microsoft Excel or GraphPad software for statistic analysis. Averages, standard deviation and T-test values were calculated.

2.6. Ly-49 cross-linking experiments

2.6.1. Induction of Ly49 capping

Splenocytes were harvested from B6 and β2m-KO mice previously injected with poly I:C, and NK cells were isolated using EasySep® NK cell negative selection kit (StemCell Technologies).

Cells were resuspended in cold HBSS with 2% FCS and aliquoted in equal amounts into three 5 ml polystyrene tubes. After spinning and discarding the supernatant, cells remained in 100 µl volume and were incubated with 20 µg/ml of anti-Ly49C/I Ab (5E6) at 4°C for 30 minutes. NK cells were then transferred to the cold room and washed twice with cold PBS 2% FCS. To induce capping, cells were incubated with AlexaFluor-568 conjugated anti-mouse IgG at 37°C for 30 minutes, whilst un-capped controls were kept at 4°C. Cells underwent two final washes with PBS 2% FCS.

2.6.2. Confocal analysis of SHIP or SHP-1 localization with cross-linked Ly49C/I

Once cross-linked with anti-Ly49C/I antibody (**2.6.1.)**, NK cells were transferred onto PLL-coated coverslips (**2.5.1**.) and incubated at 37ºC for 5 minutes to allow binding. Cells were fixed with 4% formaldehyde for 15 minutes on ice, washed twice in PBS 2% FCS and permeabilized with HBSS saponin solution for 5 minutes at room temperature. Biotinylated

anti-SHIP antibody was added at a 1:30 dilution or anti-SHP-1 was added at a 1:100 dilution with slow shaking for one hour at room temperature. Following washing, Alexa Fluor-488 conjugated donkey anti-mouse or anti-rabbit antibody was added for detection of SHIP and SHP-1, respectively. Cells were further analyzed by confocal microscopy and Volocity software, as described in **2.5.4.**

2.7. Cytotoxicity assays

YAC-1 cells were harvested, resuspended at 2×10^6/ml in PBS containing 1 µM carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen Molecular Probes), and incubated for 15 minutes at 37°C. Cells were washed thrice with RPMI 1640 media supplemented with 15% FCS, and resuspended at 10^5/ml cells in regular RPMI 1640 media (10% FCS). Poly I:C activated *ex-vivo* NK cells were isolated as described in **2.1.3.** and resuspended in the same media as targets. CFSE labelled YAC-1 (10^4) cells were mixed at varying ratios with NK cells in a 96 well round-bottomed tissue culture plate. After 4 h, cells were washed and resuspended in 300 µl of 1 mg/ml 7-amino-actinomycin D (7AAD), and analyzed by flow cytometry. The level of cytotoxicity was determined as the percentage of $CFSE^+$ $7AAD^+$ cells out of all $CFSE^+$ cells, minus the percent spontaneous release ($CFSE^+$ $7AAD^+$ target cells incubated without effectors).

2.8. Flow cytometry

Single cell suspension of splenocytes were prepared **(2.1.2.),** counted, and Fc receptors were blocked for 10 minutes on ice with 100 µl 2.4G2 hybridoma supernatant per 4×10^6 cells. Cells were washed once and resuspended in 50-100 µl of PBS 2% FCS. Antibody was added

at appropriate concentrations and cells were incubated for 30 minutes at 4°. Cells were washed twice with PBS 2% FCS and, where required, secondary antibody was added and cells were incubated for another 30 minutes at 4°. Following two PBS 2% FCS washes, cells were resuspended in PBS 2% FCS containing 5 µg/ml propidium iodide and analyzed by flow cytometry using FACSCalibur (BD). Results were further interpreted on CellQuest Pro and FlowJo software. For intracellular staining with granzyme B and granzyme B isotype control antibody, cells were first fixed and permeabilized using Cytofix/CytopermTM Plus kit (BD Biosciences). Antibodies were added to cells and incubated for 30 minutes at 4°C. Cells were washed twice and resuspended in PBS, 2% FCS prior to flow cytometric analysis.

Chapter 3: Results

3.1. Unlicensed β2m-KO NK cells do not kill the prototypic target YAC-1

The first essential experiment to be performed was to assess whether unlicensed NK cells of β2m-KO mice are indeed non-cytotoxic. Thus, we carried out a series of cytotoxicity assays with varying effector to target ratios, using the standard NK cell target YAC-1. NK cells isolated from un-primed WT and β2m-KO mice did not kill YAC-1 cells (data not shown). Therefore, the mice were injected with poly I:C prior to NK cell isolation and cytotoxicity assays. As expected, while WT NK cells efficiently killed YAC-1 in a proportional effector to target manner, β2m-KO NK cells had very low cytotoxicity against YAC-1. The minimal level of cytotoxicity observed for unlicensed NK cells (triangles) in **Figure 3.1.** may be due to the fact that β2m-KO mice express a low level of MHC I, which might have allowed for the licensing (and hence, cytotoxicity) of a small portion of NK cells.

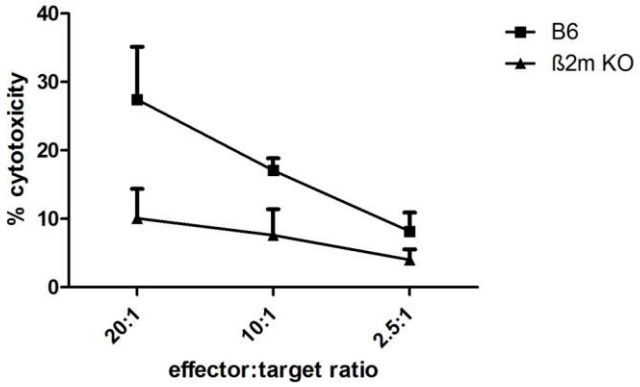
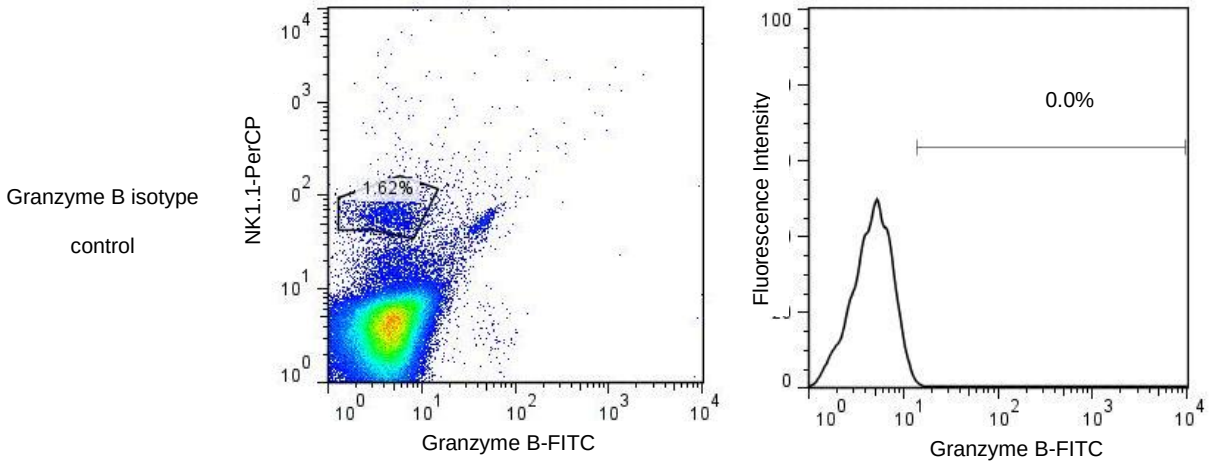


Figure 3.1 β2m-KO NK cells do not kill the prototypic NK target YAC-1. Cytotoxicity against YAC-1 target cells was analyzed by FACS. YAC-1 targets were labeled with CFDA-SE and incubated for 4 hours with WT (square) or β2m-KO (triangle) NK cells. Following incubation, cells were washed and analyzed by flow cytometry as described in Materials and Methods. Results are representative of at least 3 independent experiments, each done in triplicate. Error bars indicate ± SD.

3.2. Unlicensed NK cells from poly I:C primed β2m-KO mice contain similar amounts of Granzyme B as WT NK cells

In order to verify that unlicensed NK cells actually do have killing potential, we decided to confirm the presence of cytotoxic granules – essential components of NK cell cytotoxicity [22]. Hence, we performed intracellular staining for granzyme B, which is contained in cytotoxic granules, in NK cells isolated from poly I:C primed β2m-KO mice, and compared it to the Granzyme B content in WT NK cells. An isotype control antibody was used as negative control in both cases. It was found that unlicensed NK cells contain comparable amounts of Granzyme B to licensed NK cells (**Fig.3.2**). This finding implies that they have potential for cytotoxicity.



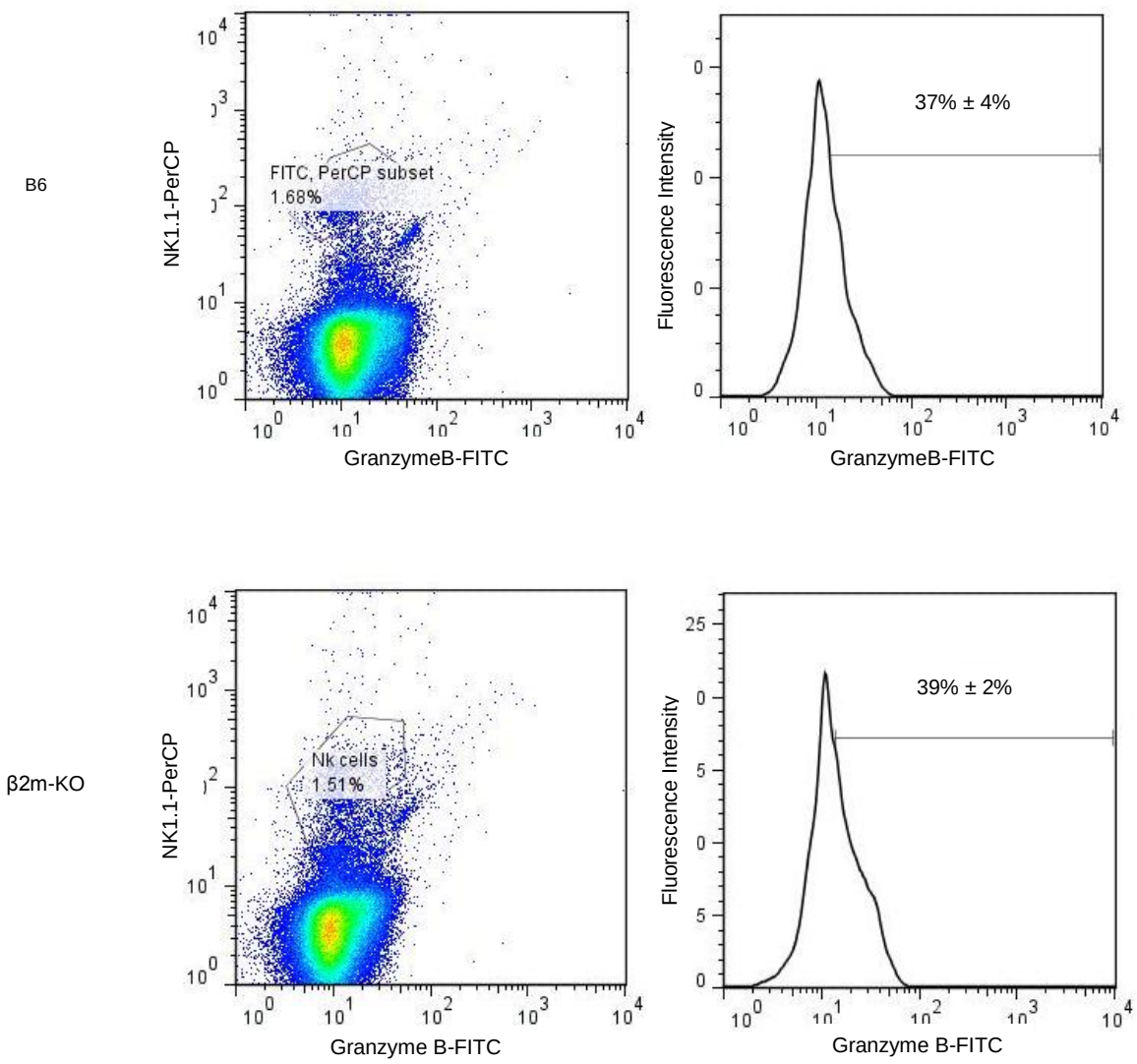


Figure 3.2 β2m-KO NK cells from poly I:C primed mice contain granzyme B amounts comparable with WT NK cells. Freshly isolated WT and β2m-KO NK cells were analyzed by FACS for the presence of granzyme B. Compared to isotype control, each histogram represents mean percentage (± SD) of NK cells positive for granzyme B from three independent experiments.

3.3. LFA-1 mediated adhesion is intact in unlicensed NK cells of β2m-KO mice

Having confirmed the lack of cytotoxicity in β2m-KO NK cells, we proceeded with a step-by-step comparison between unlicensed and licensed NK cells to deduce the step defective in unlicensed NK cells of β2m-KO mice. The first step in NK cell cytotoxicity is adhesion to

target cells. This is thought to be primarily mediated by the integrin LFA-1 on NK cells and ICAM-1 on target cells [11, 13]. Therefore, β2m-KO and WT NK cells were tested for their ability to adhere to purified immobilized sICAM-1 **(2.4)**. Both WT and β2m-KO NK cells bound to ICAM-1 coated flat plastic surface to comparable levels, and neither bound to surfaces not coated with ICAM-1 **(Fig.3.3).** Further, binding of WT and β2m-KO NK cells to ICAM-1 coated wells was reduced if LFA-1 was blocked with anti-LFA-1 antibody (**Fig.3.3**). These results suggest that the LFA-1:ICAM-1 interaction is intact in unlicensed NK cells, and would therefore allow for efficient adhesion to targets.

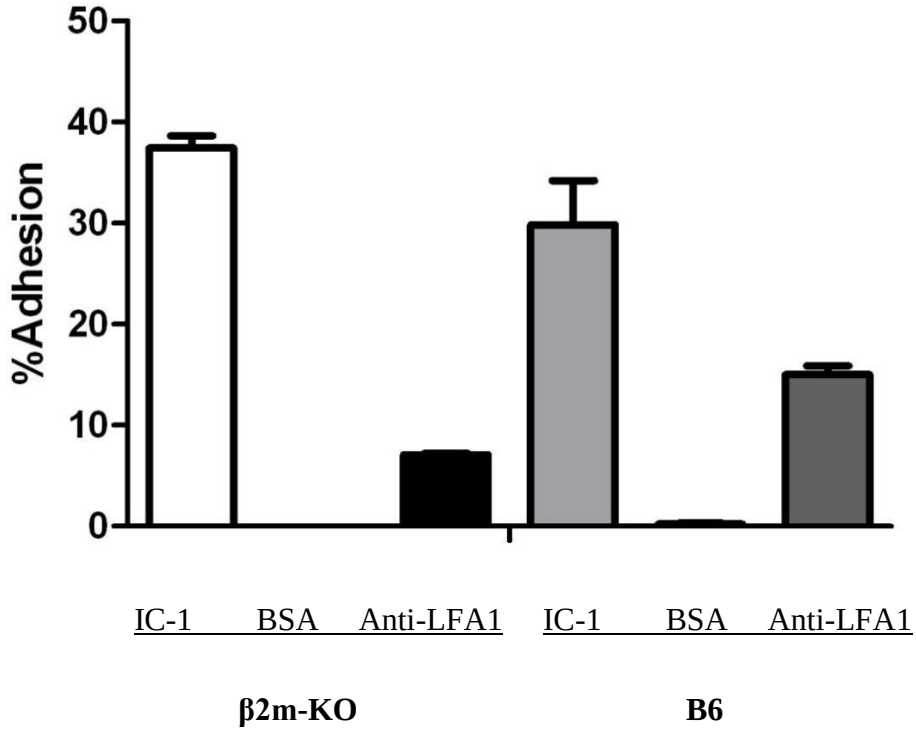


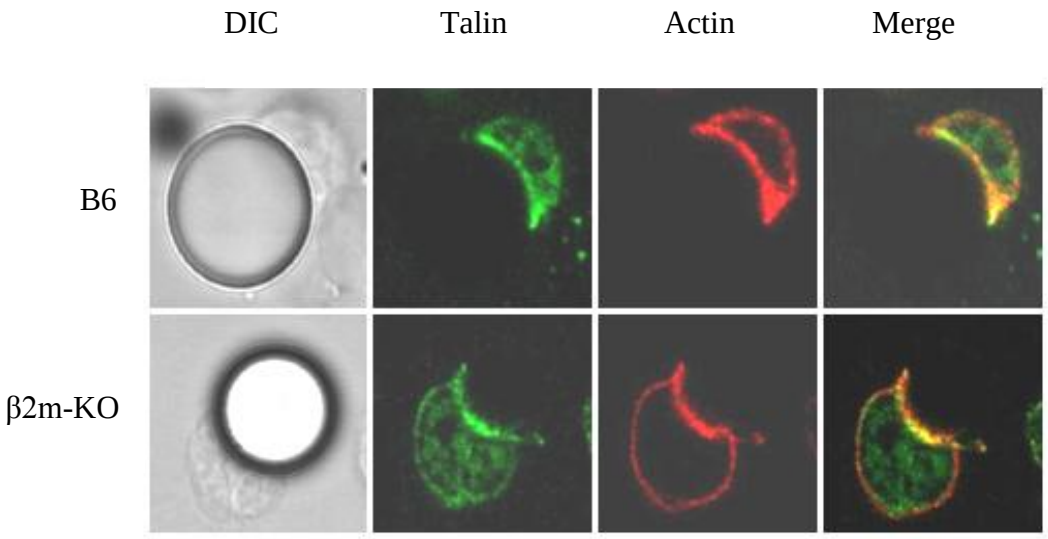
Figure 3.3 β2m-KO NK cells from poly-IC primed mice adhere to purified and immobilized ICAM-1 at similar levels as WT NK cells. NK cells purified from poly I:C injected mice were stained with Calcein-AM, and allowed to bind to 96 well plates coated with recombinant ICAM-1 or BSA control. In some wells, anti-LFA1 blocking antibody was added. Initial fluorescence was read by cytofluor, then the plate was washed to remove non-adherent cells. Remaining fluorescence was read and cell adhesion calculated as a ratio of remaining to initial fluorescence. The results depict one of three experiments performed in triplicate and show the mean values ± SD.

3.4. The LFA-1/ICAM-1 interaction induces efficient polarization of talin and actin in β2m-KO NK cells

The second step of NK cell cytotoxicity involves polarization of talin and actin cytoskeleton towards the contact site between an NK cell and its target. Previous studies in our laboratory have shown that ICAM-1 binding to LFA-1 induces talin-dependent intracellular signalling events that lead to actin polymerization at the site of LFA-1 ligation [11]. For this study, I used a model system devised in our laboratory, in which cell-size (10 μm diameter) polystyrene beads coated with ligand(s) of choice were used in place of target cells. This allowed for visualization of signalling events generated by individual receptor-ligand interactions. I incubated poly I:C-primed WT and β2m-KO NK cells with plastic beads coated with ICAM-1 and examined the distribution of F-actin and talin in NK cells by confocal microscopy. Twenty images per condition were collected from one experiment, using the same instrument settings. In all of the collected WT and β2m-KO NK cells, actin and talin accumulated at the site of contact between the cell and ICAM-1-coated bead (**Fig.3.4A**.)

Quantitative analysis of 5 WT and 8 β2m-KO confocal images confirmed that binding of LFA-1 to ICAM-1 resulted in significant localization of actin and talin in WT as well as in β2m-KO NK cells (**Fig 3.4B**.) These results indicate that the LFA-1:ICAM-1 interaction induces the polarization of actin and talin to the contact site in both WT and β2m-KO NK cells to the same level.

A



B

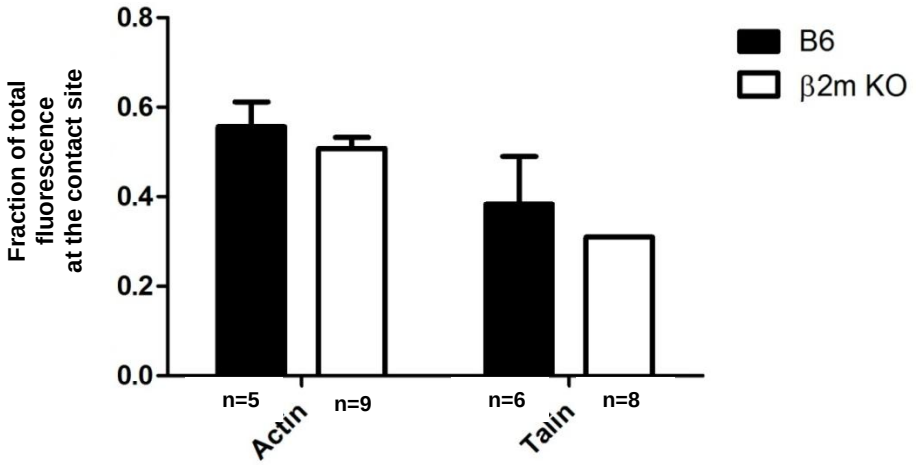


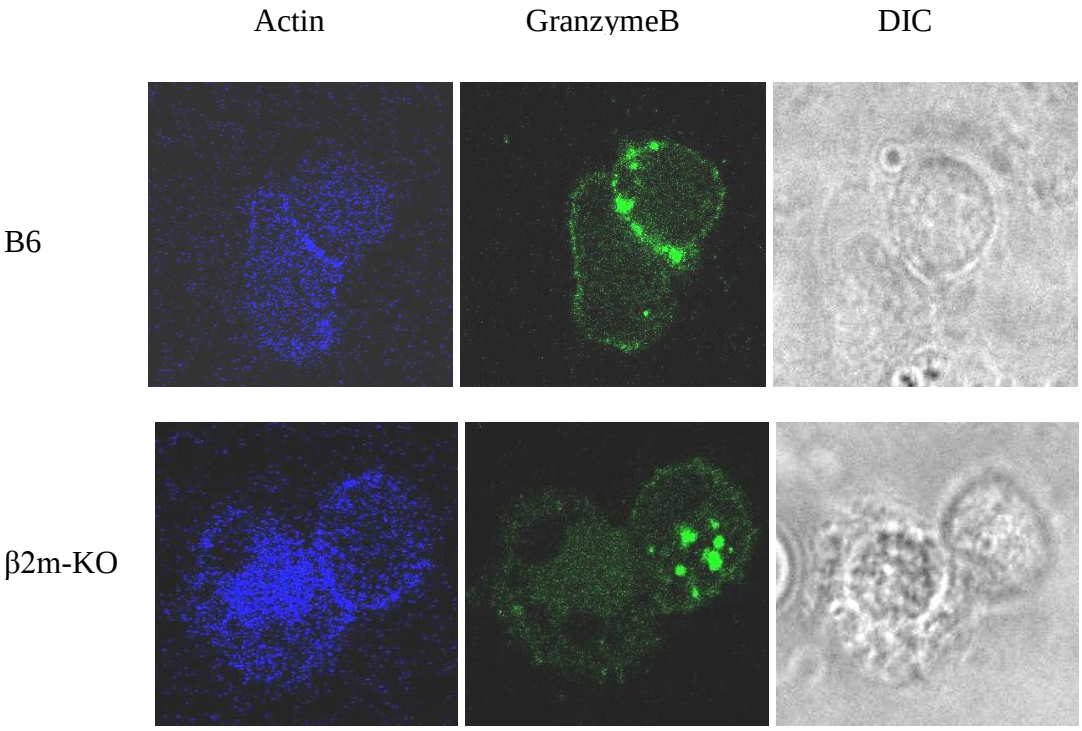
Figure 3.4 WT and β2m-KO NK cells efficiently polarize their actin and talin cytoskeleton once bound to polystyrene beads coated with LFA-1 ligand ICAM-1. To allow binding, poly I:C primed *ex-vivo* NK cells were incubated with 10 μm polystyrene beads previously coated with recombinant soluble ICAM-1, at 37°C for 20 minutes. The formed conjugates were fixed, permeabilized, stained for actin and talin, and analyzed by confocal microscopy. The above images depict mid sections of cell-bead complexes. Confocal images from **(A)** were further quantified for the distribution of actin and talin (**B**) using Volocity software (Improvision). The ratio of the contact intensity over non-contact

site intensity was calculated for n=5 to n=9 cells per condition and results represented as means ± SD.

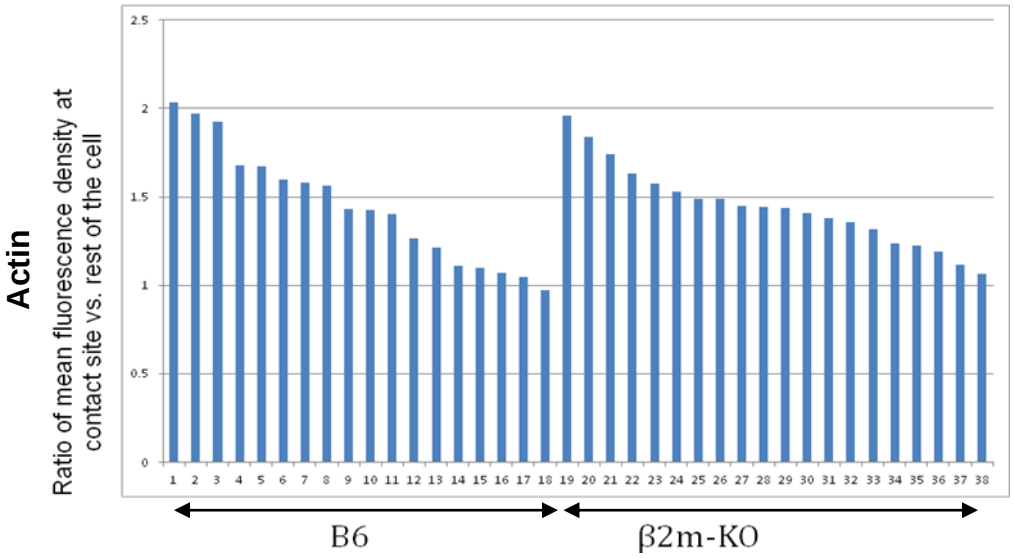
3.5. Polarization of lytic granules is defective in unlicensed NK cells bound to YAC-1

To assess the subsequent step in NK cell cytotoxicity, namely polarization of lytic granules towards the contact site between an NK cell and its target, we decided to use the prototypic NK target YAC-1. Freshly isolated NK cells from poly I:C injected WT and β2m-KO mice were incubated with YAC-1 to allow binding. Following incubation, the formed conjugates were analyzed by confocal microscopy for localization of F-actin and granzyme B. WT NK cells bound to YAC-1 and polarized both actin and granzyme B towards the target (**Fig.3.5**), whereas unlicensed NK cells could only polarize actin but not granzyme B (**Fig.3.5**). This experiment was conducted three times with consistent results. Analysis of 18 B6 and 20 β2m-KO images using Volocity software further confirmed our observations. These results suggest that the polarization of lytic granules, an essential step for NK cell mediated cytotoxicity, is defective in unlicensed NK cells of β2m-KO mice.

A


Actin
GranzymeB
DIC
B6
β2m-KO

B


Actin
Ratio of mean fluorescence density at contact site vs. rest of the cell
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
B6
β2m-KO

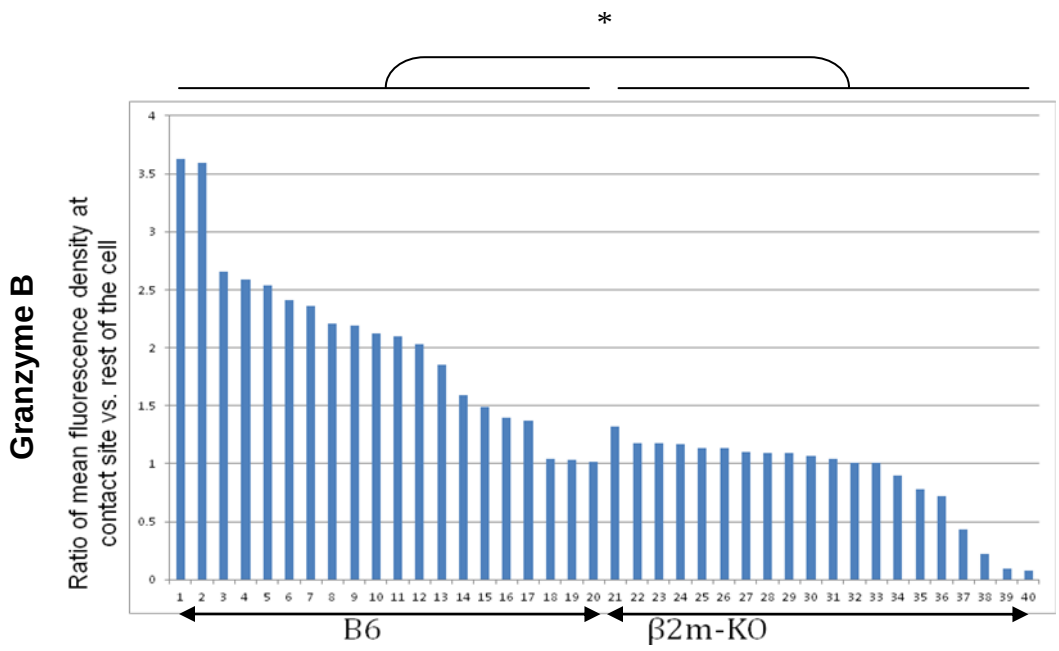
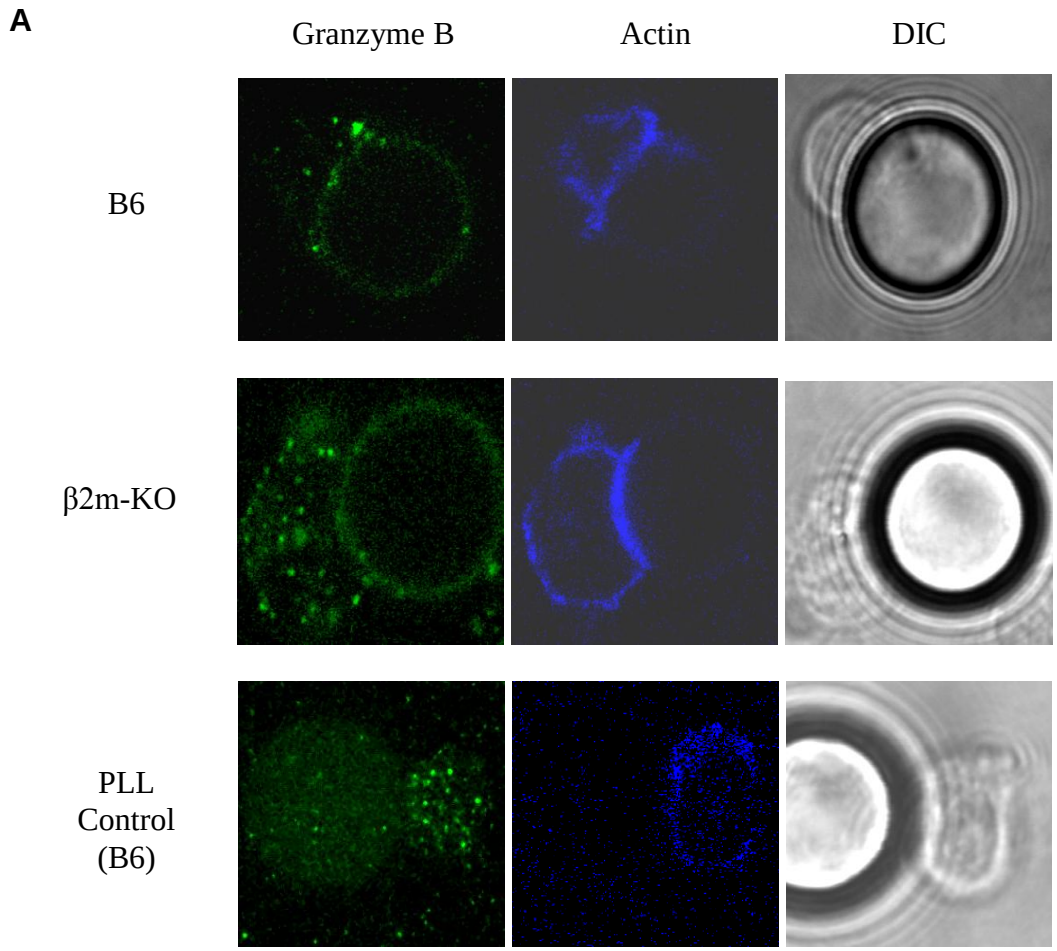


Figure 3.5 Actin polarizes but lytic granules do not polarize in β2m-KO NK cells bound to YAC-1 targets. To allow binding, poly I:C activated *ex-vivo* NK cells were incubated with YAC-1 at 37°C for 20 minutes. The formed conjugates were fixed, permeabilized, stained for actin and granzyme B, and analyzed by confocal microscopy. The images in **(A)** depict mid sections of NK-YAC-1 complexes, which were further quantified for the distribution of actin (**B**) and granzyme B (**C**) using Volocity software (Improvision). The ratio of the contact intensity over non-contact site intensity was calculated for n=18 to n=20 cells per condition and results represent exact values for each n. * indicates p= 3.37×10^{-6}

3.6. Unlicensed NK cells fail to polarize granzymes towards beads coated with ICAM-1, and the NKG2D ligand H60

To confirm the results obtained in the previous experiment, we made use of the bead-target system once again. *Ex-vivo* NK cells from poly I:C injected B6 and β2m-KO mice were allowed to bind beads coated with ICAM-1 + H60. Beads coated with PLL, which efficiently bound to cells but did not ligate specific receptors, were used as negative control. Confocal analysis revealed that WT NK cells efficiently polarize their actin cytoskeleton and lytic granules to the contact site between cells and beads coated with ICAM-1 + H60 (**Fig.3.6A**). β2m-KO NK cells fail to polarize lytic granules, although they efficiently re-

distribute their actin cytoskeleton towards the bead (**Fig. 3.6A**). Neither WT nor KO NK cells redistributed actin or granzyme B, when bound to control beads coated with PLL (**Fig. 3.6A**). To quantify the level of protein accumulation observed with confocal microscopy, we measured the fluorescence density at the point of contact between the cell and the bead, and compared it to the fluorescence density of the rest of the cell. We analyzed 16 to 20 cells per condition from one experiment, using the same instrument settings (**Fig. 3.6B**). These results confirmed our previous finding that unlicensed NK cells fail to polarize lytic granules towards their targets.



B

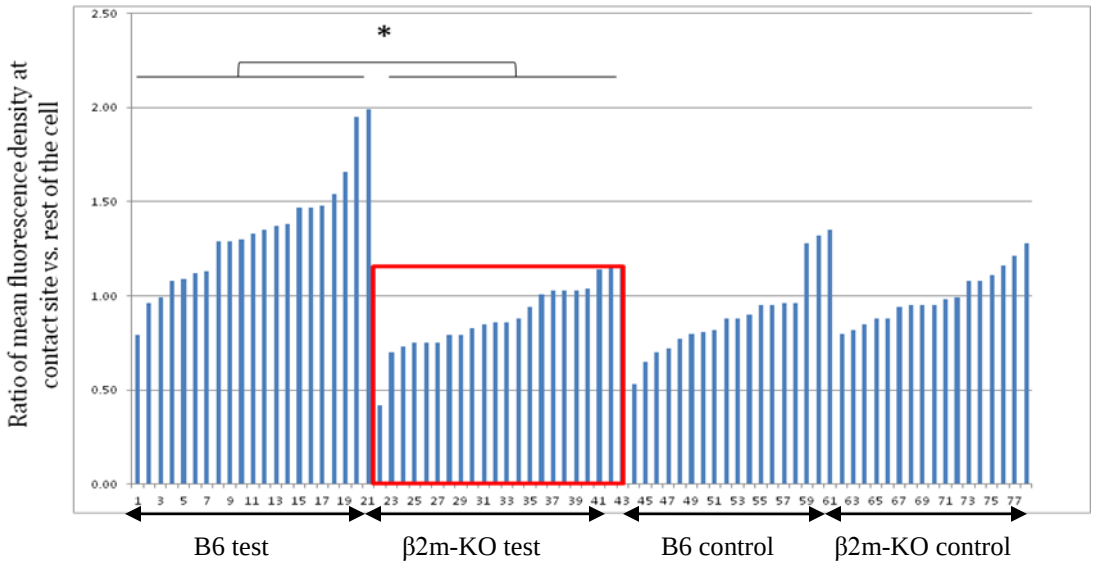


Figure 3.6 Actin polarizes but lytic granules do not polarize in β2m-KO NK cells bound to beads coated with ICAM-1 and NKG2D ligand H60. To allow binding, poly I:C primed *ex-vivo* NK cells were incubated with 10 μm polystyrene beads previously coated with ICAM-1 and H60, or with poly-L-lysine for 20 minutes at 37°C. The formed conjugates were fixed, permeabilized, stained for GranzymeB, and analyzed by confocal microscopy. The images in **(A)** depict mid sections of cell-bead complexes, which were further quantified for the distribution of granzyme B (**B**) using Volocity software. The ratio of the contact intensity over non-contact site intensity was calculated for n=20 cells per condition and results represent exact values for each n. * indicates $p= 1.17 \times 10^{-5}$

3.7 Unlicensed NK cells fail to polarize granzymes towards beads coated with ICAM-1, NKG2D ligand H60, and additional stimulatory ligand CD48

Findings from the previous experiment (**3.6**.) indicate that NKG2D ligation, combined with LFA-1 ligation, induces lytic granule polarization in licensed, but not in unlicensed NK cells. However, this does not entirely explain why unlicensed NK cells do not kill YAC-1, as we have shown in **3.5.** It is known that NKG2D has moderate effect on its own to fully activate NK cytotoxicity [22], and co-stimulation by additional signals is likely required for YAC-1 killing. The most probable receptor that may cooperate with NKG2D is 2B4, which interacts with CD48 ligands [23]. Therefore, we carried out the same confocal experiment as in **3.6**,

using ICAM-1, H60, and CD48 coated beads as targets. Quantification of confocal microscopy (**Fig.3.7.)** revealed that unlicensed NK cells are still impaired in polarizing lytic granules in the presence of NKG2D and additional 2B4 co-stimulatory signaling.

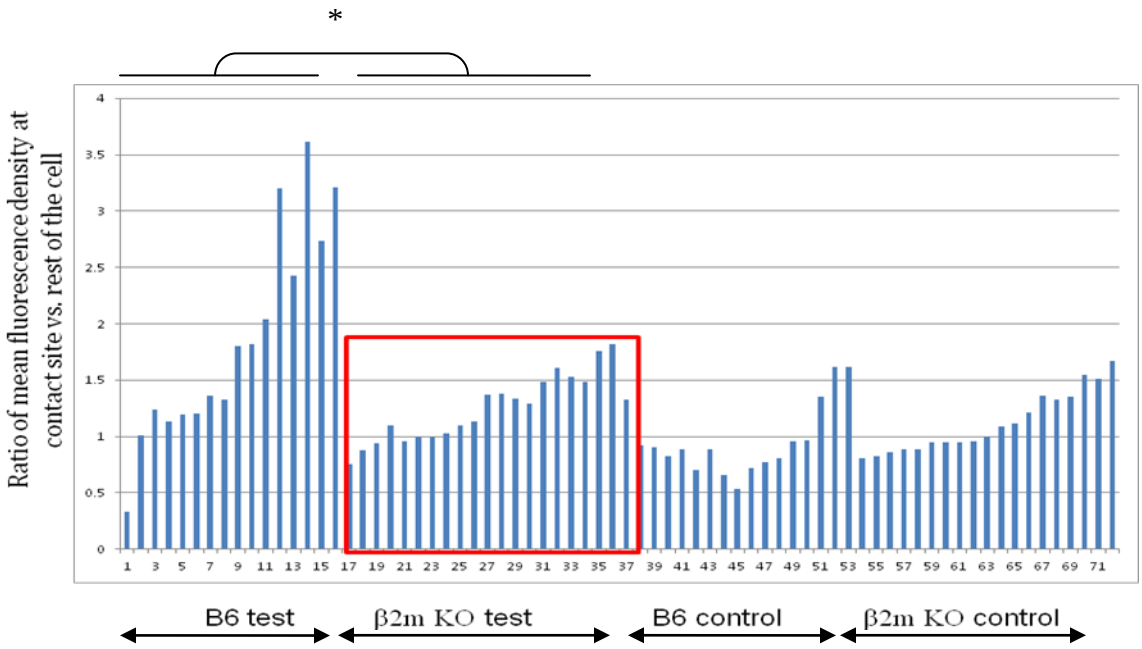


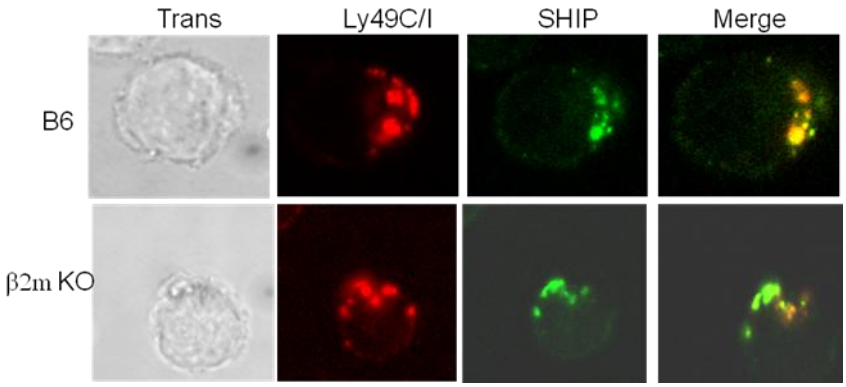
Figure 3.7 In the presence of additional stimulation from CD48, ligand for 2B4 stimulatory receptor on NK cells, unlicensed NK cells are still deficient in granule polarization. The experiment described in Figure 3.6 was repeated with target beads coated with ICAM-1, H60, and additional stimulatory CD48 ligands. The above graph shows quantification of granzyme B distribution, analyzed by Volocity. The ratio of the fluorescence intensity at the contact site over that at non-contact site was calculated (16 to 20 cells per condition) and results represent exact values for each n. * indicates $p= 1.8 \times 10^{-3}$

3.8. Ab mediated cross-linking of inhibitory Ly49C/I receptors induces recruitment of SHIP rather than SHP-1 phosphatase in both licensed and unlicensed NK cells

The above results show that NKG2D signalling and NKG2D, together with 2B4 activation, signals leading to lytic granule polarization are impaired in unlicensed NK cells. SHIP and SHP-1 are two well characterized negative regulators of cell signaling known to interact with the ITIMs of Ly49 inhibitory receptors on NK cells [1]. We decided to test if either SHP-1 or

SHIP were involved in inhibiting NK cell mediated cytotoxicity. First, we set out to decipher where these phosphatases localize once Ly49C/I interacts with its ligand, mimicking the Ly49C/I-MHC-I interaction key to NK cell licensing. Hence, we performed a co-capping experiment. Poly I:C primed NK cells were isolated from B6 and β2m-KO mice, and capping of Ly49C/I inhibitory receptors was induced by cross-linking them with anti-Ly49C/I mAb (5E6), followed by incubation with secondary AlexaFluor conjugated antibody at 37ºC. Uncapped controls were incubated with secondary antibody at 4ºC. The cells were fixed, permeabilized, stained for SHP-1 and SHIP, and analyzed by confocal microscopy. Antibody cross-linking induced strong colocalization between Ly49C/I and SHIP in both licensed and unlicensed NK cells (**Fig. 3.8A**). Not much colocalization was seen with SHP-1 and Ly49C/I in either cell type (**Fig.3.8B**). These observations were consistent throughout three trials of this experiment. Twenty cells were collected from one trial and analyzed using the same instrument settings. To quantify the level of colocalization between proteins, we used the overlap coefficient function of Volocity software, which produces an overlap value out of 1.00 (**Fig.3.8C**). Results suggest that SHIP, rather than SHP-1 phosphatase, colocalizes with Ab cross-linked Ly49C/I receptors of both licensed and unlicensed NK cells.

A



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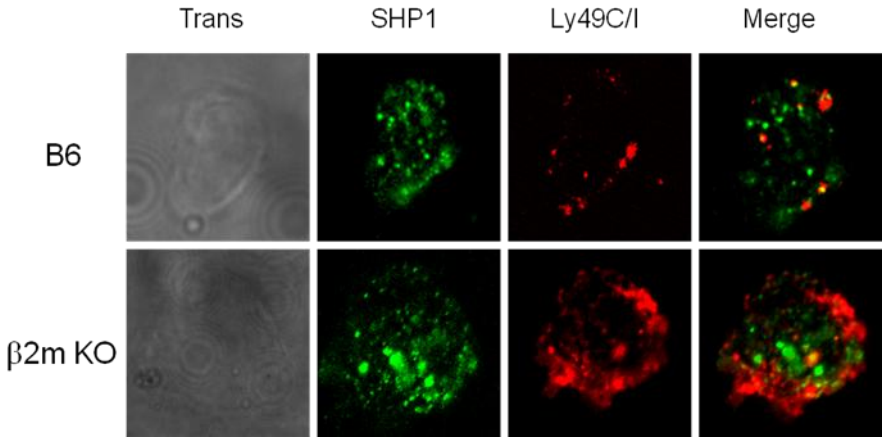

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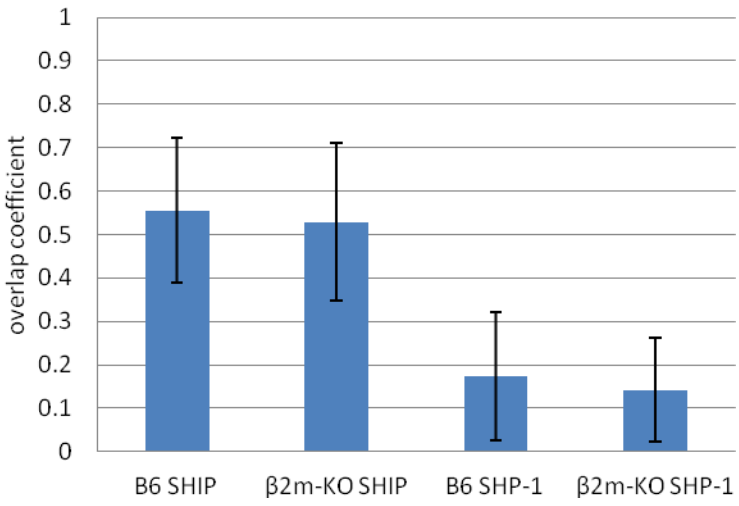
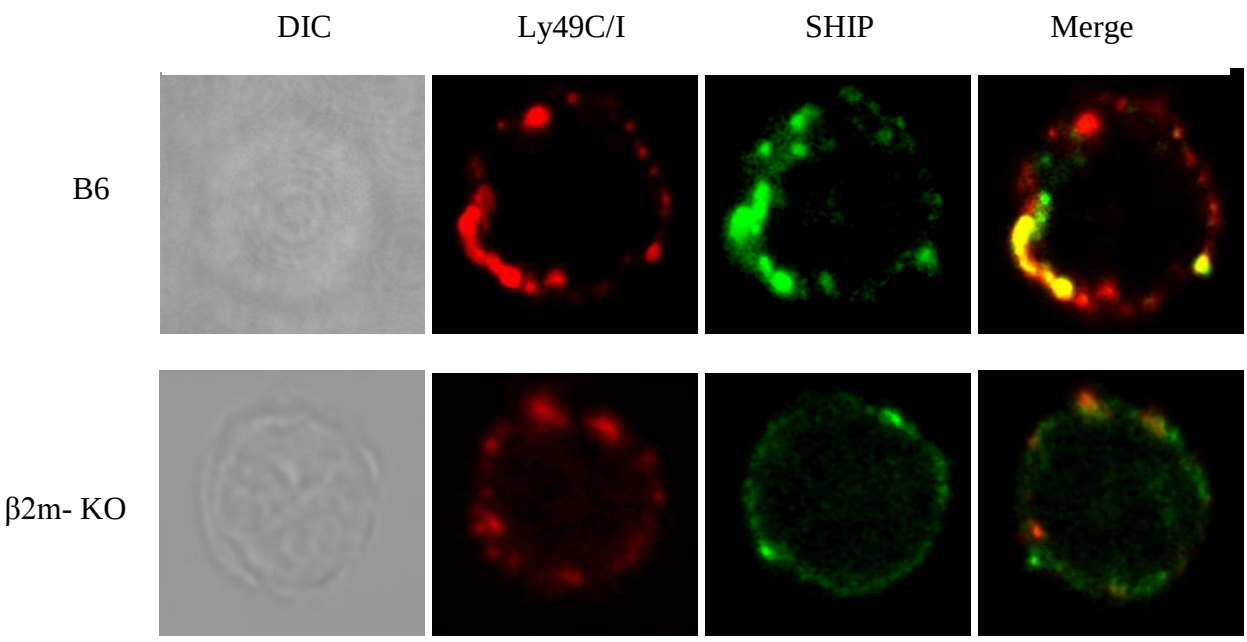


Figure 3.8 In B6 and β2m-KO NK cells from poly I:C primed mice, Ab mediated cross-linking of inhibitory Ly49C/I receptors induces recruitment of SHIP rather than SHP-1 phosphatase. Poly I:C primed *ex-vivo* NK cells were incubated at 37ºC with anti-Ly49C/I to induce capping. Cells were further fixed, permeabilized, stained for SHIP (**A**) or SHP-1 (**B**), and analyzed by confocal microscopy**.** Fluorescence was quantified using Volocity and overlap coefficients (i.e. colocalization) were deduced for Ly49C/I and SHIP or SHP-1, respectively **(C).** Results are representative of mean coefficient overlap values ± SD, for 20 cells per condition.

To determine the localization of SHIP in WT and β2m-KO NK cells without Ly49C/I cross-linking, Ly49C/I capping was prevented by incubating cells at 4ºC with secondary AlexaFluor-568 conjugated anti-mouse IgG, as oppose to incubating at 37ºC. In WT NK cells, SHIP still colocalized with Ly49C/I (**Fig.3.9A**). However, in β2m-KO NK cells there was no sign of such colocalization (**Fig.3.9A**). Quantification of confocal microscopy further confirmed this observation (**Fig.3.9B**). The results suggest that the interaction between Ly49C/I and SHIP pre-exists prior to Ly49C/I cross-linking in WT NK cells, whereas in unlicensed NK cells of β2m-KO mice this interaction is only induced post Ly49C/I capping presumably because it mimics ligation of Ly49C/I by MHC I.

A



B

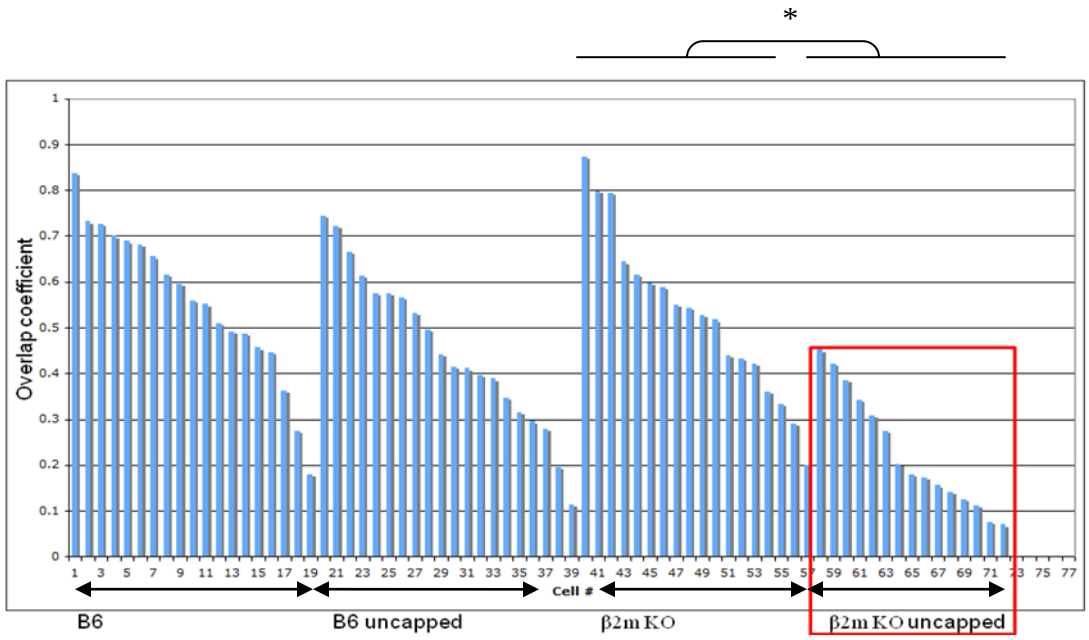


Figure 3.9 Uncapped Ly49C/I colocalizes with SHIP in WT, but not in β2m-KO NK cells. Poly I:C primed *ex-vivo* NK cells were incubated at 4ºC with anti-Ly49C/I and at 4ºC with secondary AlexaFluor-568 conjugated anti-mouse IgG to prevent capping, as controls. Cells were fixed, permeabilized, stained for SHIP, and analyzed by confocal microscopy **(A).** Fluorescence was quantified using Volocity, and overlap coefficients (i.e. colocalization) between Ly49C/I and SHIP were deduced for capped and uncapped specimens **(B).** Results represent overlap coefficient for each cell (n) ranging from n=18 to n=20 per condition. * indicates $p= 1.46 \times 10^{-7}$

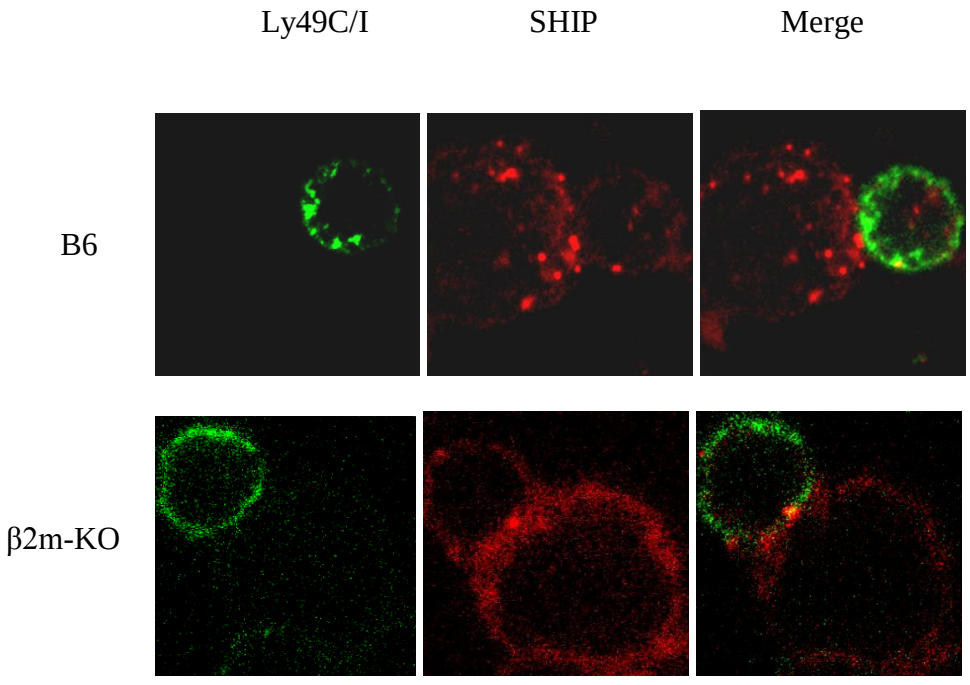
3.9. SHIP localizes to the contact site of unlicensed NK cells bound to YAC-1 targets

In order to visualize the distribution of Ly49C/I and SHIP in a real NK cell-target scenario, we incubated NK cells from poly I:C injected WT or β2m-KO mice with YAC-1 cells. Following incubation, the formed conjugates were fixed, permeabilized, stained, and analyzed by confocal microscopy for the localization of SHIP and Ly49C/I. We observed that in WT NK cells adhered to YAC-1 targets, SHIP localized away from the contact area between the NK cell and YAC-1 (**Fig.3.10A**).

In contrast, in β2m-KO NK cells, SHIP accumulated directly at the immunological synapse (**Fig.3.10A**). To quantify the level of protein accumulation detected by confocal microscopy, we calculated the ratio of SHIP fluorescence density at the contact site as compared to the fluorescence density in the remainder of the cell. This ratio was calculated for 30 licensed and 40 unlicensed NK cells (**Fig.3.10B**), and quantitative results emphasize the fact that SHIP is more abundant at the contact site of unlicensed NK cells, but not of licensed NK cells bound to YAC-1 targets.

We further analyzed confocal images and quantified colocalization between Ly49C/I and SHIP in licensed versus unlicensed NK cells bound to YAC-1 targets. Overlap coefficients (R), as computed by Volocity software, revealed similar levels of overlap between Ly49C/I and SHIP in WT and β2m-KO NK cells (**Fig.3.10C**).

A



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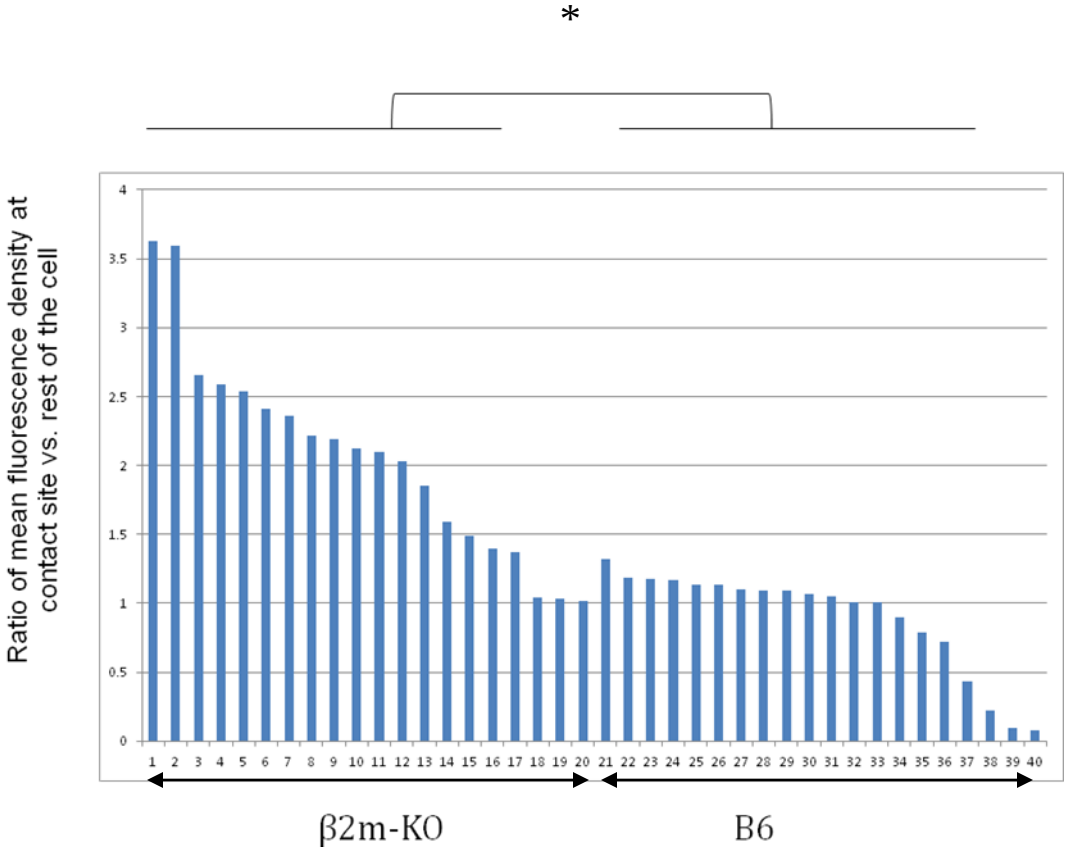
*
Ratio of mean fluorescence density at contact site vs. rest of the cell
β2m-KO
B6

C

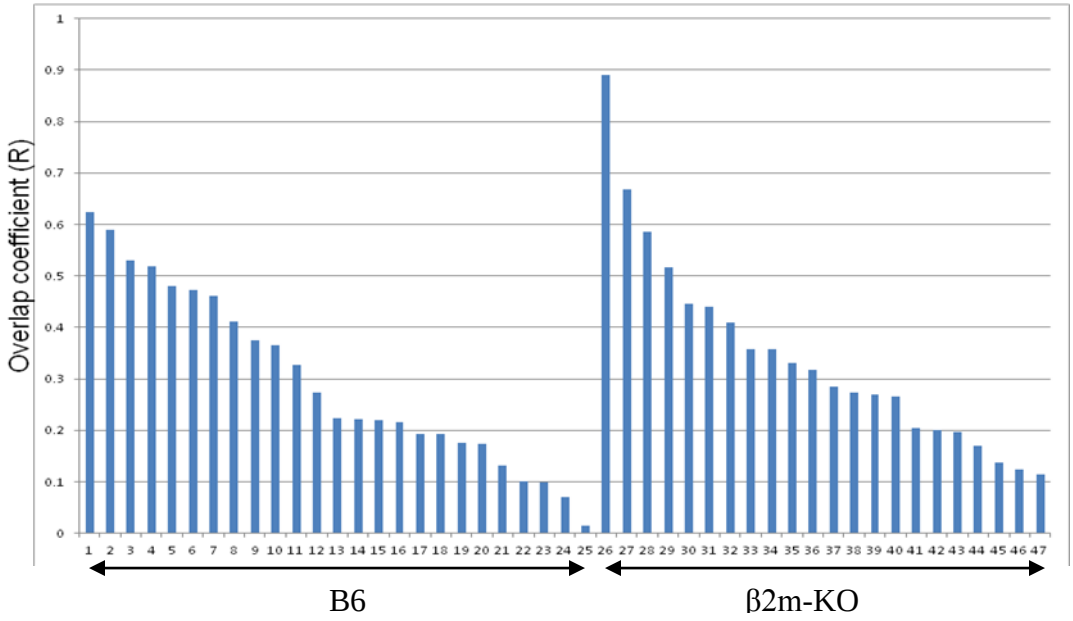
Overlap coefficient (R)
B6
β2m-KO

Figure 3.10 SHIP localizes away from the contact site in WT cells, but is abundant at the contact site of β2m-KO NK cells bound to YAC-1 targets. To allow binding, poly I:C activated *ex-vivo* NK cells were incubated with YAC-1 for 20 minutes at 37°C. The formed conjugates were fixed, permeabilized, stained for L49C/I and SHIP, and analyzed by confocal microscopy. The above images **(A)** depict mid sections of NK-YAC-1 complexes. Images were further analyzed for the distribution of SHIP and Ly49C/I usingVolocity. **(B)** The ratio of SHIP fluorescence density at the contact site versus rest of the cell for every cell at unlicensed (n=40) and WT (n=30) NK cells. * indicates $p= 6.6 \times 10^{-3}$. **(C)** The overlap coefficient (i.e. colocalization) of Ly49C/I and SHIP in WT (n=25) and β2m-KO (n=22) NK cells.

3.10. NK cells from SHIP-KO mice contain a greater subset of immature cells than WT

The above results suggest that SHIP localization to the target contact site may inhibit NK cell cytotoxicity and thus mediate hypo-responsiveness of unlicensed NK cells. It implies that SHIP-deficient NK cells would be hyper-responsive. However, SHIP-KO NK cells have been reported to have lower expression of Ly49 and are hypo-responsive [80, 82]. To solve this apparent contradiction, we analyzed NK cells from SHIP-KO mice and WT BALB/c mice. NK cells ($NKp46^{+}$) can be divided into subsets based on the expression of CD27 and Mac-1, namely the most immature $Mac\text{-}1^{lo}CD27^{-}$ subset, the less immature $Mac\text{-}1^{lo}CD27^{+}$ subset (which produces cytokines but is not very cytotoxic), mature cytotoxic $Mac\text{-}1^{hi}CD27^{+}$ subset, and the terminally differentiated $Mac\text{-}1^{hi}CD27^{-}$ subset [100]. Our analysis showed that most SHIP-KO mouse NK cells are $Mac1^{lo}CD27^{+}$, whereas the majority of NK cells from WT mice are $Mac1^{hi}CD27^{+}$ and $Mac1^{hi}CD27^{-}$ (**Fig. 3.11**). These results suggest that NK cells in SHIP-KO mice are unable to fully mature and hence are less cytotoxic than WT NK cells.

WT SHIP-KO

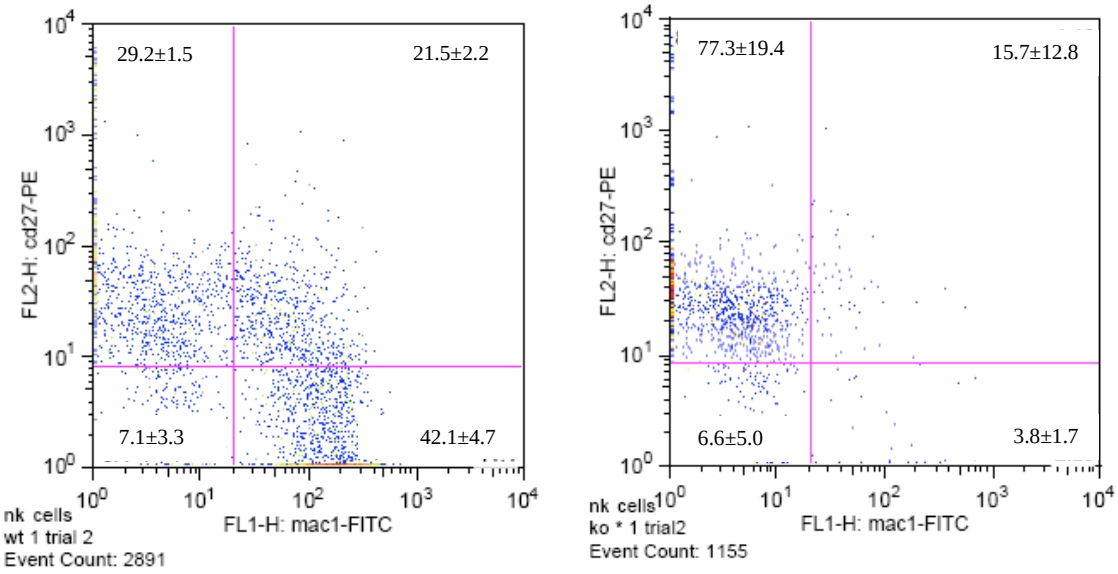

Figure 3.11. SHIP-KO NK cells differ phenotypically from WT, as they contain a significantly larger subset of immature NK cells. WT and SHIP-KO spleen cells were stained with NKp46-APC to distinguish NK cells, and with CD27-PE and Mac1-FITC. They were further analyzed by FACS for NK maturation phenotyping as defined by CD27/Mac1 profiling. Results are representative of at least three independent experiments, with mean percentages ± SD shown in each quadrant.

Chapter 4: Discussion

In order to determine the mechanism that mediates NK cell licensing, we have analyzed unlicensed NK cells from MHC I deficient β2m-KO mice and compared them with licensed NK cells from WT B6 mice. While the former are much less cytotoxic against the prototypic NK target YAC-1 than the latter, our analyses have found no difference between the two NK cell populations in granzyme B expression, adhesion to ICAM-1 and polarization of actin, all of which are critical for NK cell-mediated killing of target cells. Although others have reported that NK cells from β2m-KO mice are deficient in actin polarization [101], we have found that F-actin aggregates at the contact site of both licensed and unlicensed NK cells bound to YAC-1 cells or ICAM-1-coated beads. Instead, we have found that β2m-KO NK cells fail to polarize lytic granules upon binding to YAC-1 cells, whereas clear polarization of lytic granule toward bound target is seen about 46% of WT NK cells. It should be noted that not all NK cells in WT B6 mice are licensed. Only those expressing Ly49C/I, approximately 40% of NK cells in B6 mice [52], are thought to interact with self-MHC I (H-2K^b and D^b) and become licensed [91].

Polarization of lytic granules is crucial to NK cell cytotoxicity, and specific inhibition of granule polarization in NK cells disables target lysis [13, 102]. While the process of lytic granule polarization in NK cells is not well understood, previous studies in our laboratory have shown that co-ligation of LFA-1 and the stimulatory receptor NKG2D is sufficient for the induction of lytic granule polarization in NK cells [11]. NK cell killing of YAC-1 cells is thought to be mediated by NKG2D and 2B4 [23, 31, 72]. Therefore, it is likely that stimulatory signals generated by NKG2D and 2B4 might be impaired in β2m-KO NK cells. Indeed,

binding of beads coated with ligands for LFA-1 and NKG2D or those for LFA-1, NKG2D and 2B4 induces significant granule polarization toward the bound beads in WT NK cells, while β2m-KO NK cells fail to polarize lytic granules. Since LFA-1 function of β2m-KO NK cells is unaffected, as determined by adhesion to ICAM-1 and polarization of the actin cytoskeleton, these results suggest that stimulatory signals mediated by NKG2D and 2B4 are impaired in β2m-KO NK cells.

In naive mouse NK cells, NKG2D signalling occurs primarily through the DAP10 adapter protein. DAP10 contains an SH2 domain which, upon receptor ligation, interacts with the p85 subunit of PI3K, thereby recruiting it to the cell membrane and activating it [103]. PI3K recruitment and activation leads to downstream signaling cascade involving Rac1, PAK1, MEK, ERK, and is pivotal to NK cell cytotoxicity. Studies employing PI3K specific inhibitors show impairments in perforin and granzymeB polarization, and suppression of NK cell killing function [73]. The SH2 domain of DAP10 is a potential site of interaction with the inhibitory phosphatase SHIP [104]. SHIP is known to prevent PI3K recruitment to cell membranes. Signaling through DAP10 can also occur through the Grb2 adapter. In B cells, SHIP is recruited to the plasma membrane in complex with Grb2. Thus, regardless of the activation pathway taken by DAP10, SHIP has a potential inhibitory role in NKG2D/DAP10 mediated signaling.

It has been shown that the ITIM of self-MHC I reactive Ly49 is crucial for NK cell licensing, yet the SHP-1 phosphatase is not [91]. In addition to SHP-1, SHIP has also been reported to associate with Ly49 ITIM [87]. In fact we have found that antibody-mediated cross-linking of Ly49C/I induces SHIP, and not SHP-1 colocalization with Ly49C/I, in both licensed and

unlicensed NK cells. Moreover, analysis of SHIP distribution in NK cells without Ly49C/I cross-linking suggests that in WT NK cells, SHIP may associate with Ly49C/I prior to Ab-mediated cross-linking. While in β2m-KO NK cells, this association seems to be induced by Ly49C/I cross-linking. Consistent with our results, Wang *et al.* found that under normal physiological conditions, in B6 mice, SHIP is associated with Ly49C/I [87]. There has been no report in the literature of SHIP co-localizing with Ly49C/I in unlicensed NK cells upon Ab-mediated cross-linking.

As mentioned earlier, DAP10 acts in complex with NKG2D to transduce stimulatory signals in naïve NK cells. Confocal analysis has shown that SHIP localizes to the contact site between a β2m-KO NK cell and a YAC-1 target. On the contrary, when a WT NK cell is bound to a YAC-1, SHIP localizes away from the contact site. This suggests that SHIP is present at the immunological synapse of unlicensed NK cells upon encounter with target cells. It is possible that SHIP exhibits its inhibitory function at the synapse formed between unlicensed NK cells and their targets, thereby disrupting NKG2D signalling.

Following this study, I propose the following model for the role of SHIP in NK cell licensing

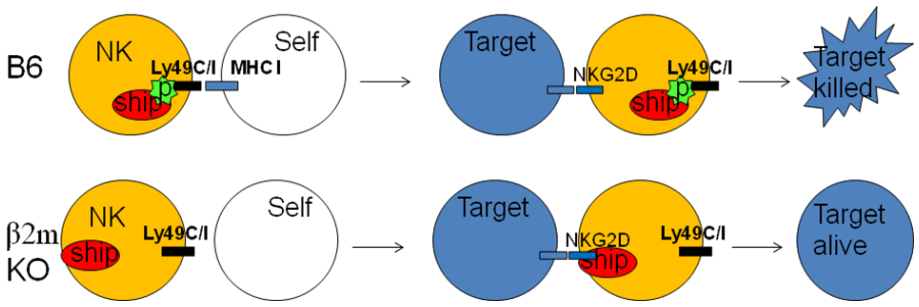

Fig. 4.1. Model for SHIP's role in NK cell licensing. In licensed NK cells, binding of MHC I to inhibitory Ly49C/I receptor causes ITIM phosphorylation and recruits SHIP. This prevents SHIP from localizing to the NKG2D-mediated stimulatory synapse, which enables cytotoxicity. In unlicensed NK cells, Ly49C/I ITIM is not phosphorylated as it does not bind to MHC I and does not associate with SHIP. Therefore, SHIP is free to localize to NKG2D-mediated stimulatory synapse, where it inhibits DAP10-mediated stimulatory signals.

This model is consistent with experiments performed by *Raulet et al.* and *Yokoyama et al.*, in which unlicensed mature NK cells become licensed and acquire lytic function when transplanted into a MHC-I-sufficient host [94]. In reciprocity, when WT NK cells are transferred into MHC-I deficient hosts, their licensing state is reversed and the capacity to kill is perished [95]. Both situations imply that licensing is flexible and is governed by the MHC-I environment.

Another phenomenon which can reset the MHC-I requirement for licensing is cytokine stimulation *in vitro*. Kim *et al.* showed that culturing hyporesponsive NK cells with IL-2 restores their lytic ability [91]. We observed similar effects with IL-15 stimulation (data not shown). Yokoyama *et al.* also reported IFNγ production by unlicensed NK cells cultured with IL-12 and IL-18 [53]. Recently, Orr *et al.* identified unlicensed NK cells a primary defense against MCMV infection [78]. However, MCMV resistance is mediated through the Ly49H activating receptor, which is expressed on unlicensed NK cells. Ly49H signals through the ITAM-containing DAP12 adapter protein, while DAP10 plays no significant role in this [105]. Moreover, Ly49H/DAP12 signalling seems to enhance NK cell sensitivity to IL-15 [106]. IL-15 is a pro-inflammatory cytokine, whose presence during MCMV infection may additionally contribute to the responsiveness of unlicensed NK cells.

The PI3K activation pathway in NK cells is downstream of NKG2D-DAP10 and, as we have indicated, may be inhibited by SHIP. The ITAM-PTK activation pathway is DAP10 independent, and hence unlikely to be affected by SHIP. In fact, the ITAM-PTK activation pathway dominates during MCMV infection and cytokine activation, which may be a reason why unlicensed NK cells become responsive.

If NK cell licensing is mediated by the sequestering of SHIP away from the NKG2D/DAP10 complex by ITIM phosphorylated Ly49C/I, then SHIP-KO NK cells are expected to be all licensed and hyper-responsive. However, the effects of SHIP-deficiency on NK cell functions are rather complex. SHIP-KO NK cells of the B6 background have been shown to be incapable of rejecting mismatched BM grafts and killing tumourigenic or virally-infected targets suggesting that SHIP-KO NK cells are hypo-responsive [87]. NK cells from SHIP-KO mice primed in vivo with poly I:C are also poor producers of IFNγ upon cross-linking of stimulatory receptors, including NKG2D [89]. SHIP-KO NK cells have also been shown to express lower levels of Ly49s than WT NK cells [89]. When they are expanded in culture in the presence of IL-2, they are less cytotoxic against the B6 lymphoid cell line RMA (MHC I^+) transfected with the NKG2D ligand RAE-1. On the other hand, NK cells from SHIP-KO mice of the B10.D2 background (H-2^d) display the same level of cytotoxicity as WT NK cells against target cells expressing H-2^d while their cytotoxicity is higher than WT NK cells against RAE1-transfected RMA (H-2^b) cells. It should be noted that the cytotoxicity of NK cells in these studies is analyzed following IL-2 stimulation, which is known to activate unlicensed NK cells. To clarify these contradicting results, we have analyzed NK cells from SHIP-KO and WT BALB/c mice for the expression of CD27 and Mac-1, which defines the maturation levels of NK cells [100]. We have found SHIP-KO NK cells to be significantly more immature than WT NK cells, which would explain the hyporesponsiveness of SHIP-KO NK cells and lower expression of Ly49s.

Altogether, my findings provide insight into the mechanism of NK cell licensing, and will hopefully initiate further studies on signalling generated by the interaction between inhibitory receptors and MHC I, which is essential for self versus non-self distinction. A natural

progression of this project would be to establish that the interaction between Ly49C/I and SHIP is essential to NK cell licensing. It would thus be relevant to verify the phosphorylation state of the Ly49C/I ITIM in licensed versus unlicensed NK cells, and to elucidate the mechanisms by which the Ly49C/I ITIM is phosphorylated. In addition, it is necessary to confirm the physical interaction between SHIP and components of the NKG2D activating complex, Grb2 and DAP10. Finally, it would be interesting to know whether SHIP inhibits both NKG2D and 2B4-mediated signaling, as its association with the latter has already been established [29].

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