Roles of natural killer cell receptors and adaptors in antitumor and antivirus immunity

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Abstract

Natural killer (NK) cells are among the most important immune cells in antitumour immunity and antiviral immunity. NK cells can be activated by various surface receptors. There are, in general, two kinds of receptors controlling NK cell activation, namely activating receptors and inhibitory receptors. The activation of NK cells is primarily determined by the balance of activating and inhibitory signals. This thesis demonstrates the roles of various receptors and some of their adaptors in NK cell function. First, NKG2D and, to a lesser extent, DNAM-1 play activating roles in NK cytotoxicity towards tumours expressing their specific ligands. Second, we found that the integrin receptor LFA-1, but not Mac-1, is responsible for killing ICAM-1- and ICAM-2-expressing tumour cells. Third, upon MCMV infection, the interaction of SLAM family receptor 2B4 and its ligand CD48 stable the expansion of memory NK cells by inhibiting NK fratricide during the expansion phase. Finally, CD155-dependent surface degradation of DNAM-1 causes NK desensitization towards tumour cells. These findings provide fundamental knowledge to further our understanding of NK cell functions in antitumour and antiviral immunity.

Résumé

Les cellules tueuses naturelles (NK) comptent parmi les cellules immunitaires les plus importantes dans l'immunité antitumorale et l'immunité antivirale. Les cellules NK peuvent être activées par divers récepteurs de surface. Il existe, en général, deux types de récepteurs contrôlant l'activation des cellules NK, à savoir les récepteurs activateurs et les récepteurs inhibiteurs. L'activation des cellules NK est principalement déterminée par l'équilibre entre les signaux activateurs et inhibiteurs. Cette thèse démontre les rôles de divers récepteurs et de certains de leurs adaptateurs dans la fonction des cellules NK. Premièrement, NKG2D et, dans une moindre mesure, DNAM-1 jouent des rôles activateurs dans la cytotoxicité des NK envers les tumeurs qui expriment leurs ligands spécifiques. Deuxièmement, j'ai découvert que le récepteur d'intégrine LFA-1, mais pas Mac-1, est responsable de la destruction des cellules tumorales exprimant ICAM-1 et ICAM-2. Troisièmement, lors d'une infection par le MCMV, l'interaction du récepteur 2B4 de la famille SLAM et de son ligand CD48 favorise l'écurie des cellules NK à mémoire en inhibant le fratricide des NK pendant la phase d'expansion. Enfin, la dégradation de la surface du DNAM-1 par CD155 provoque la désensibilisation des NK aux cellules tumorales. Ces résultats apportent des connaissances fondamentales pour approfondir notre compréhension des fonctions des cellules NK dans l'immunité antitumorale et antivirale.

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List of abbreviations

Abl	Abelson tyrosine kinase
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen-presenting cell
ATCC	American Type Culture Collection
BCR	B cell receptor
Btk	Bruton's tyrosine kinase
CD3-ζ	CD3 ζ subunit
CHS	Contact hypersensitivity
CMV	Cytomegalovirus
Crk	Adaptor protein Crk
CT	Control
CTL	Cytotoxic T lymphocyte
CTV	CellTrace Violet
CX3CR1	C-X3-C motif chemokine receptor 1
CXCR6	CXC-chemokine receptor 6
DAP-10	DNAX-activating proteins of 10 kDa
DAP-12	DNAX activating protein of 12 kDa
DC	Dendritic cell
dKO	Double KO
DNAM-1	DNAX accessory molecule-1
DNFB	2,4-dinitro-1-fluorobenzene

EAT	Ewing sarcoma-associated transcript
EBV	Epstein-Barr virus
ERT	EAT-2-related transducer
FACS	Fluorescence-activated cell sorting
Fas-L	Fas ligand
FcR-γ	Fc receptor γ-chain
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GNLY	Granulysin
Grb2	Growth factor receptor-bound protein 2
GVHD	Graft-versus-host disease
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HTLV-1	Human T lymphotropic virus type 1
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM	Intercellular adhesion molecule
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IHWG	International Histocompatibility Working Group

IL	Interleukin
iNK	Immature NK
IP	Immunoprecipitation
IRE1α	Endoplasmic reticulum stress sensor inositol-requiring enzyme 1
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
KIR2DL4	Killer cell immunoglobulin like receptor, two Ig domains and long cytoplasmic tail 4
KLRG1	Killer cell lectin-like receptor G1
KO	Knockout
LAK	Lymphokine-activated killer
LAT	linker for activation of T cell
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen 1
LILR	Leukocyte immunoglobulin-like receptor
L-selectin	Selectin family of proteins L
Mac-1	Macrophage-1 antigen
MCMV	Mouse cytomegalovirus
M-CSF	Macrophage colony-stimulating factor
MEST	Mouse ear swelling test
MHC	Major histocompatibility complex
MICA/B	MHC class I chain-related protein A and B

mNK	Mature NK
NK	Natural killer
NK1.1	Natrual killer cell lectin-like receptor subfamily B, member 1
NKG2D	Natural killer group 2D
NKG2D-L	NKG2D long
NKG2D-S	NKG2D short
NKp30	Natural killer cell p30-related protein
NKp46	Natural killer cell p46-related protein
OXA	Oxazolone
p.i.	Post-infection
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
pfu	Plaque-forming unit
PILRβ	Paired immunoglobulin-like type 2 receptor β
PLC	Phospholipase C
PLGF	Placental growth factor
poly(I:C)	Polyinosinic-polycytidylic acid
PTK	Protein tyrosine kinase
PVR	Poliovirus receptor
RAET1	Retinoic acid early transcripts-1
RAG	Recombination-activating gene
SAP	SLAM-associated protein

SFR	SLAM family of receptors
SH2	SRC homology 2
SHIP	SH2 domain-containing inositol phosphatase
SHP	SH2 domain-containing protein tyrosine phosphatase
SLAM	Signalling lymphocytic activation molecule
SPF	Specific pathogen-free
Syk	Spleen tyrosine kinase
TCR	T cell receptor
T_H1	T helper 1
T_H2	T helper 2
TIGIT	T cell immunoglobulin and ITIM domain
tKO	Triple KO
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
ULBP	Unique long 16-binding protein
Vav	Vav Guanine Nucleotide Exchange Factor
VEGF	Vascular endothelial growth factor
VLA-4	Integrin very late antigen-4
WT	Wild type
XBP1	X-box-binding protein 1
XLP	X-linked lymphoproliferative
Zbtb32	Zinc finger and BTB domain containing 32

Contribution of Authors

Chapter 1: Rui Li summarized the background knowledge related to the studies. André Veillette corrected the writing.

Chapter 2: Rui Li planned and performed experiments, interpreted data and wrote the manuscript. Mingchao Zhong performed intraperitoneal injections for all animal experiments. Yan Lu performed a preliminary experiment. Silvia Vidal provided reagents, planned experiments and interpreted data. André Veillette planned experiments, interpreted data, wrote the manuscript and obtained funding.

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Chapter 4: Rui Li discussed and summarized the conclusions of the studies. André Veillette corrected the writing.

Contribution to original knowledge

Chapter 2 and Chapter 3 are original scholarships that make distinct contributions to the knowledge. In Chapter 2, our studies have given rise to new knowledge regarding NK cell receptors in the context of antitumour activity. Our findings provide insight into the redundant and complementary roles of DNAM-1, NKG2D and LFA-1 in NK antitumour activity. In Chapter 3, we are the first group to discover the novel inhibitory function of 2B4-CD48 in stable memory NK generation and also to find that SLAMF7 is important for memory NK cell expansion. Additionally, we discovered that in the absence of the Ly49H receptor, cytokines can still activate NK cells in MCMV infection. Overall, these original findings advance knowledge and could potentially aid future research, clinical therapy and diagnosis. More details about contribution to original knowledge are discussed in Chapter 4.

Chapter 1. General introduction

1.1. Immune system and immune responses

Immune system is the biological system that protects organisms from pathogens. It consists of immune organs, immune cells and immune molecules. The discovery of the immune system at the end of the nineteenth century stems from the pioneering work of several scientists, including Louis Pasteur, an important contributor to the germ theory of infectious diseases; Robert Koch, who conducted comprehensive studies on the etiology of infectious diseases; and Elie Metchnikoff, who discovered phagocytosis (1).

Many organs play roles in the immune system. These include a group of organs functioning as physical barriers, namely the skin and mucous membranes, which are supported by antibacterial enzymes, mucus, stomach acid and harmless bacteria that compete with invading pathogens. The lymphoid organs comprise the second group of organs and consist of the primary lymphoid organs such as bone marrow and thymus, which are the primary sites of lymphocyte production, and secondary lymphoid organs such as the spleen, lymph nodes, tonsils, and mucous layers on the tissues. The secondary lymphoid organs comprise the sites at which lymphocytes fight pathogens (1).

Immune cells are crucial members of the immune system. They include cells from two developmental lineages, the myeloid and lymphoid lineages. Both develop from multipotential hematopoietic stem cells. Primary myeloid cells include macrophages, monocytes, mast cells,

neutrophils, and myeloid dendritic cells (DCs) (2). Primary lymphoid cells consist of T cells, B cells, natural killer (NK) cells, and, perhaps, lymphoid DCs (3). The myeloid cells are all innate immune cells, while lymphoid cells have either innate or adaptive features. Some subsets of lymphoid cells can have both innate and adaptive characteristics (Figure 1.1).

Immune molecules, including antibodies, cytokines, and complements, are also important for immune responses. Antibodies are a group of proteins produced by B cells. They recognize and bind to specific antigens on bacteria, virus-infected cells, or tumour cells. Cytokines are small immunogenic proteins produced by immune cells that control the growth and activities of immune cells, tumour cells, and infected cells (4). The complement system comprises more than 30 immunogenic proteins circulating in the blood and tissue fluid that defend against pathogens via complement fragments that mediate opsonization, chemotaxis, and activation of immune cells through cytolysis (5).

1.1.1. Innate immunity

Innate immunity is the immune system's first procedure of defence against pathogens. It was first discovered by Elie Metchnikoff, who observed the phagocytic responses of immune cells in the presence of foreign pathogens (1). Innate immune responses are not pathogen-specific like adaptive immune responses. However, they develop rapidly during infection and are established within minutes of exposure to a new pathogen. These functions largely protect the body and inhibit

the expansion of pathogens such as rapidly proliferating bacteria and viruses. Innate immunity is built via multiple layers of protection, including epithelial surface boundaries, conserved antigen recognition, double-stranded viral RNA recognition, phagocytosis, cytotoxicity, and cytokines (6).

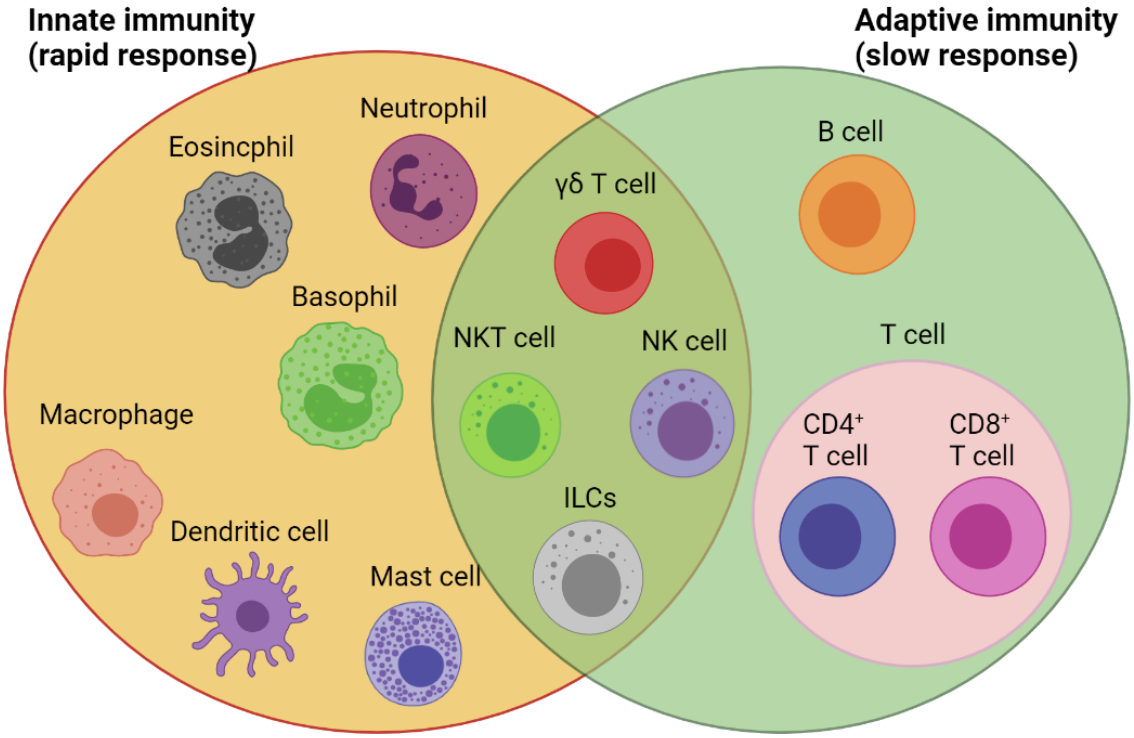

Figure 1.1 Innate immune cells and adaptive immune cells

Innate immune cells consist of macrophages, monocytes, mast cells, neutrophils, and DCs, and adaptive immune cells consist of B cells and T cells. NK cells, γδ T cells, NKT cells, and ILCs have both innate and adaptive features. The figure was created with BioRender.com

Among the innate immune responses, physical barriers are the first line of defence. The skin and mucosal surfaces form a barrier of the internal tissues and external environments. The mucosal surfaces are covered with mucus layers that protect against various external insults and contain a variety of substances that can eliminate pathogens or inhibit their expansion. Among the most important of these substances is defensin, a short, positively charged peptide with broad-range antimicrobial activity that can kill bacteria, fungi, parasites, and some types of viruses (6).

Although most invading pathogens are stopped by the epithelial barriers, some can get past, nevertheless. The next level of innate immune protection is offered by immune cells that recognize the general features of foreign pathogens. Upon recognizing the features of the invaders that are distinct from those of the host, the innate immune cells are activated and initiate immune responses, including inflammatory responses, cytotoxic responses, and phagocytosis. These responses can rapidly and efficiently eliminate invading pathogens without the need for previous encounters (6).

Depending on the sites and the types of stimuli, inflammatory responses can be initiated by macrophages, DCs, lymphocytes, neutrophils, plasma cells, and mast cells. Activation of these cells triggers inflammatory signalling pathways, subsequently producing cytokines (including chemokines) to eliminate pathogens. One of the earliest cytokines produced by activated macrophages and monocytes is tumour necrosis factor-alpha (TNF-α), which can stimulate changes in capillary shape to recruit more innate immune cells to the infection site. Additionally,

TNF-α can initiate other signals within seconds to prevent the spread of pathogens by inducing the death of infected cells (6-8).

After early cytokines are released into blood vessels, the surrounding immune cells are led to the infection site. Macrophages, DCs, neutrophils, and other phagocytic cells at the infection site ingest and digest pathogens and produce more cytokines that can mediate further eliminatory effects and modulate adaptive immune responses (6, 8). During phagocytosis, phagocytic cells, particularly DCs, also present antigens through their major histocompatibility complex (MHC), thus stimulating adaptive immunity by presenting antigens via MHC to T cells (9).

NK cells constitute another type of innate immune cell involved in defending against pathogens in the early stages of infection. Toll-like receptors (TLRs) expressed on NK cells can be activated by pathogen-associated molecular patterns (PAMPs) on microbial agents in the presence of cytokines produced by other immune cells, such as interleukin (IL)-12, IL-15, or IL-18. After activation, NK cells can produce various inflammatory cytokines, including interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α. Another important feature of activated NK cells is cytotoxicity, whereby NK cells release perforin and granzymes towards infected cells, subsequently eliminating pathogens by cellular lysis (10).

1.1.2. Adaptive immunity

Adaptive immunity has the distinct feature of immune memory. Although adaptive immunity takes days or weeks to be established upon encountering a pathogen, it subsequently provides protection against the same pathogen for years. Humans and animals develop long-lasting immune responses against many common pathogens after the first exposure. In humans and animals, the lymphoid organs contain immune cells at multiple developmental stages, i.e., naïve cells, effector cells, and memory cells. After being stimulated by novel antigens, a subset of naïve cells is activated, differentiates, and subsequently become effector cells. A second subset of naïve cells proliferates and differentiates into memory cells that do not engage in the first response but prepare the immune system for subsequent infection with the same antigen. Unlike effector cells, which only have a maximum life span of a few weeks, memory cells can survive for years or even lifelong. T cells and B cells are the immune cells that classically have the characteristic of memory, namely adaptive immune cells. Activated T cells are mainly responsible for cell-mediated immune responses, while activated B cells mediate humoral immune responses (6). These cells either directly attack pathogens or pathogen-infected cells, or produce antibodies to help phagocytic cells recognize pathogens and subsequently disrupt the infection. However, recent studies have elucidated that some types of NK cells also have adaptive features that provide long-term protection against previously encountered pathogens and other stimuli. The adaptive functions of NK cells are discussed in detail in the section 1.2.1.4.

T cells is a type of immune cells that develop from thymus and are among the most important cells responsible for adaptive immunity. Cytotoxic T cells ($CD8^+$) and helper T cells ($CD4^+$) are the two major types of T cells. Cytotoxic T cells play an important role on killing towards infected cells by releasing perforin, granzymes, and cytokines, and helper T cells assist the activation of cytotoxic T cells, B cells, NK cells, and macrophages by secreting a variety of cytokines and acting as on-site mediators. Both cytotoxic T cells and helper T cells bind MHC molecules via their T cell receptor (TCR). TCRs on cytotoxic T cells bind MHC class I (MHC I) molecules. In contrast, TCRs on helper T cells bind antigens embedded in MHC class II (MHC II) molecules. Cytotoxic T cells are responsible for the direct clearance of pathogens. They are essential for protection against intracellular infections such as viral infections that can avoid contact with circulating antibodies. Naïve $CD8^+$ T cells are activated and proliferate after their TCRs recognize a complementary antigen embedded in MHC I on antigen-presenting cells (APCs) with the help of stimulation to the coreceptor CD8 and cytokines secreted by helper T cells. After activation and proliferation, some naïve $CD8^+$ cells become memory cells, while others become activated cytotoxic T cells. These cell types can identify infected cells expressing previously encountered antigens. Once the specific antigen-expressing cells are identified, the T cells destroy them before the pathogen finishes its replication cycle and is released; thus, they can inhibit the progress of intracellular infections. Cytotoxic T cells can also cooperate with NK cells and other innate immune cells to eliminate early cancer cells. Upon activation, cytotoxic T cells produce perforin and granzymes that induce apoptosis of the infected cells. Activated T cells also produce cytokines,

such as interferons, that alter the expression of the ligands on infected cells to facilitate their detection by other immune cells and inhibit virus release (6, 11).

Helper T cells have important roles in adaptive immune responses against extracellular pathogens (such as bacteria) by identifying potential pathogens and sending activation signals to other immune cells. Based on the different functions, several types of T helper cells have been classified, T helper 1 (T_H1), T helper 2 (T_H2), T follicular helper cells (T_{FH}), T helper 17 (T_H17) and regulatory T cells (T_{REG}). After recognizing specific antigens embedded in the MHC II of APCs, T_H1 cells release cytokines to activate cytotoxic T cells and innate immune cells. In the meanwhile, T_H2 cells secrete IL-4, IL-5 and IL-13 which mediate protection against helminths, and could potentially cause allergies such as asthma. T_{FH} cells facilitate antibody production by interacting with B cells.

Upon stimulation by T_{FH} cells, naïve B cells differentiate and become plasma cells that secrete antibodies specific to the antigens recognized via B cell receptors (BCRs). When BCRs bind to an antigen presented by APCs, the corresponding B cells take up the antigen by receptor-mediated endocytosis. The antigenic proteins are degraded to peptides by the B cells and presented on their surface by MHC II. TCRs on T helper cells are activated following recognition of the specific antigens; they then express CD40L to bind the CD40 receptor on B cells and produce cytokines such as IL-4 and IL-21 to stimulate B cells (12). Subsequently, B cells are activated and begin proliferating, immunoglobulin class switching, somatic hypermutation and differentiation.

Ultimately, B cells differentiate into three groups, short-lived plasmablasts that responsible for antibody production, such as IgM production for quick responses to pathogens, long-lived plasma cells that need a longer time to develop but can secrete substantial amounts of antibodies, and memory B cells for future protection against pathogen reinfection (6, 12).

1.2. NK cells

NK cells were first discovered in the 1980s as lymphocytes with the ability to kill tumour cells (13-15). The word "natural" in their name describes their spontaneous cytolytic activity, which does not require priming or restriction by MHC-expressing target cells. A later study found that NK cells can also kill virus-infected cells, unhealthy cells and "missing-self" cells (cells that lack self MHC I molecules on their membrane) via cytotoxic molecules and pro-inflammatory cytokines (16). Apart from receptors of MHC I molecules, NK cells have groups of other receptors that can bind ligands expressed on the target cells. These receptors have either activating or inhibitory roles on NK cell functions.

Although NK cells are defined as innate immune cells based to their ability to mount a rapid response to pathogenic cells without antigen specificity, they have also been found to have adaptive-like immune features, including specific antigen recognition, clonal proliferation, and long-term memory (17, 18).

1.2.1. NK cell functions

NK cells were initially described as lymphocytes that could kill cancerous cells without prior activation. Unlike cytotoxic T cells, the cytotoxicity of NK cells does not depend on the single receptor-antigen interaction. Their activation and cytotoxicity depend on the balance of activating and inhibitory signalling combined with cytokines in the surrounding environment (Figure 1.2). Activated NK cells control virus-infected cells and malignant cells in several ways, including the induction of apoptosis through granule exocytosis. When NK cells detect unhealthy cells, they may release perforin, which generates pores on the membrane of target cells, and granzymes, which transmit through immune synapses, enter the targets through the membrane perforations and induce apoptosis intracellularly. NK cells also produce cytokines to modulate target cells, coordinate with other innate immune cells and stimulate adaptive immune responses. These cytokines include IFN-γ, GM-CSF, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), TNF-α, IL-5, IL-10, IL-13, and many types of chemokines. NK cells can also implement their cytolytic activity via death receptor pathways; Fas ligand (Fas-L) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) are two members of the tumour necrosis factor (TNF) superfamily expressed on some population of NK cells that are mainly involved in this process (19, 20).

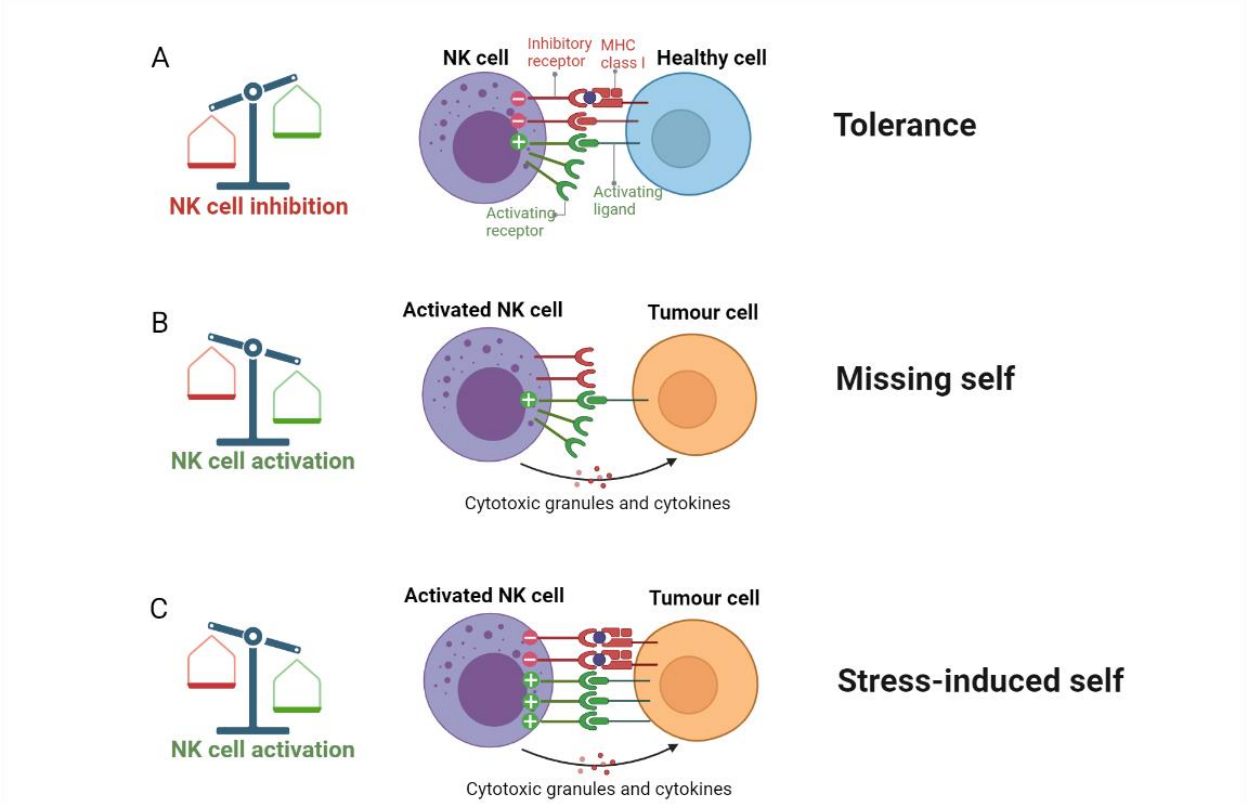

Figure 1.2 NK cell activating theory

A. On healthy cells, MHC I and other inhibitory ligands expression is high and activating ligands expression is low. When NK cells encounter them, NK cells receive an overall inhibitory signal. B. In tumour cells, MHC I and other inhibitory ligands expression is decreased. When NK cells encounter them, NK cells receive an overall activating signal. C. On stress-induced cells, the activating ligands expression is increased. When NK cells encounter them, NK cells receive an overall activating signal. The figure was created with BioRender.com

NK cells need to be activated before execute the effector activities. Apart from receptor-ligand associations, their activation also depends on cytokine stimulation. NK cell survival, proliferation, cytotoxicity and cytokine production require the presence of cytokines such as IL-12, IL-15, IL-18, IL-21, IFN-α and IFN-β. Many of these cytokines are produced by DCs (19, 21).

1.2.1.1. NK cell antitumour functions

NK cells' antitumour functions have been widely studied in mice and humans (22). In animal models, mice with reduced NK cell numbers or functional NK cell deficiency and mice treated with NK cell-depleting antibodies showed greater susceptibility to tumours (23). In human studies, the NK cells' natural cytotoxic ability negatively correlated with cancer incidence among patients. Additionally, a higher amount of NK cells infiltrating the tumours was corelated with a higher survival rate (23).

Like in T cells, granule exocytosis is the major process by which NK cells kill tumour cells. Researchers found that perforin-deficient NK cells could not kill YAC-1 (mouse thymoma cell line) or RMA-S (a lymphoma mutant cell line with decreased MHC I expression) targets in *in vitro* experiments (24, 25). *In vivo* studies showed that perforin-deficient mice were more susceptible to chemical-induced fibrosarcomas, which are controlled by NK cells rather than cytotoxic T lymphocytes (CTLs) (26). The role of granzymes in NK cell-mediated granule exocytosis remains controversial. Some studies have reported that granzyme A and granzyme B are necessary for NK

cells to rapidly induce the apoptosis of target cells (27, 28). On the other hand, another group has found that granzymes are dispensable in NK cell killing *in vitro* and *in vivo* (29). These conflicting results could be due to difference in experimental models used, and it is likely that compensatory pathways exist. However, they seem to prove that granzymes are important in at least some contexts. Further experiments need to be done to figure out the compensatory pathways of granzymes.

NK cell-mediated cytotoxicity also occurs via the death receptor pathway. Fas-L and TRAIL express on NK cells, which have been found to play roles in the killing of malignant cells that express the corresponding Fas and TRAIL receptors (22). *In vitro* studies showed that mouse and human NK cells with Fas-L expression could induce the death of Fas-expressing target cells (30, 31). In *in vivo* studies, both Fas and TRAIL were shown to be involved in protecting against tumours (23, 32).

Upon activation, NK cells can secrete IFN-γ, a multifunctional cytokine critical for cancer control. IFN-γ can directly influence tumour cells and tumour environments through measures including the upregulation of antigen expression on tumour cells, inhibition of tumour cell proliferation and inhibition of angiogenesis in tumour tissue. At the same time, IFN-γ can activate other innate and adaptive immune cells to coordinate antitumour activity and induce regulatory T cell death (23, 33). NK cell is a type of major IFN-γ-producing immune cells. The importance of IFN-γ in tumour

control was observed in IFN-γ-deficient mice, which spontaneously developed multiple malignancies including lymphomas and carcinomas (34). Additionally, some studies found that IFN-γ could induce the expression of TRAIL by NK cells, indicating that IFN-γ secretion can also promote the death receptor pathway (32). Apart from IFN-γ and the other cytokines mentioned above, activated NK cells also secrete chemokines to modulate and attract other innate and adaptive immune cells; these include CCL2, CCL3, CCL4, CCL5, XCL1, and CXCL8 (18).

1.2.1.2. NK cell antiviral functions

The antiviral function of NK cells was initially discovered in the 1980s. More and more evidence demonstrates the contribution of NK cells in controlling infections of different viruses, including cytomegalovirus (CMV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), herpes simplex virus (HSV), dengue virus, yellow fever virus, arenavirus lymphocytic choriomeningitis virus (LCMV), the paramyxovirus Sendai, the flavivirus Kunjin and Ebola virus (19).

Some viruses have also been reported to interfere with NK cell functions to escape from NK cell-mediated viral clearance. For instance, HCV-infected cells express envelope glycoprotein E2 that binds CD81, which prevents NK cells from recognizing HCV and decreases cytotoxicity and IFN-γ secretion. Similarly, the activating receptors' expression was found to be decreased in the NK cells of HIV patients. In general, NK cells are indispensable for the immune system to control viral invasion. Another important feature of NK cells is virus-specific memory, which protects the host

from viral infection and is discussed further in the section 1.2.1.4.

1.2.1.3. NK cell regulatory functions

NK cells regulate other immune cells not only in the context of infection or cancer but also in the healthy state by maintaining the balance of the immune system. They can act as regulatory cells to regulate T cells, B cells, DCs, and endothelial cells.

NK cells monitor DCs in the blood and lymphoid tissues. To maintain DC homeostasis, NK cells can eliminate immature DCs, but this could also potentially decrease the efficacy of DC-based vaccination. NK cell-mediated cytotoxicity can also lyse target cells into small fragments to improve antigen uptake by DCs and other APCs. Subsequently, this process can induce T cells to generate robust antigen-specific adaptive immune responses (35, 36). In allogeneic organ transplantation, host NK cells can kill donor-derived DCs to suppress alloreactive T cell development, thus promoting transplant tolerance (37). NK cells also play roles in some anti-inflammatory treatments. They can promote DC maturation by secreting IFN-γ and TNFs. Mature DCs produce IL-12, which subsequently activates NK cells. During the treatment of arthritis with TLR9 agonists, the interaction of NK cells and DCs promoted activation and IFN-γ secretion of NK cell, thus preventing neutrophil recruitment in the joint (36, 38).

NK cells can also directly influence T cells and B cells in inflammation and autoimmunity. NK

cells secrete IFN-γ to promote the priming of T_H1 cells. They also kill activated $CD4^+$ T cells under antibody blockade of CD94-NKG2A or reduced MHC I expression to prevent $CD4^+$ T cell-dependent autoimmunity (39). Some studies also showed that NK cells could control B cell-dependent autoimmunity by suppressing autoreactive B lymphocytes (36, 40, 41).

NK cells also interact with endothelial cells, under both normal and pathological circumstances. Several receptors expressed on NK cells can recognize ligands on endothelial cells, including integrin very late antigen-4 (VLA-4), selectin family of proteins L (L-selectin), and C-X3-C motif chemokine receptor 1 (CX3CR1). These allow NK cells to regulate blood vessels in different ways. On the one hand, NK cells can promote angiogenesis during pregnancy. Enriched uterine NK cells can secrete vascular endothelial growth factor (VEGF), placental growth factor (PLGF), and granulysin (GNLY) to promote vascular development in endometrial tissue in pregnancy (42). On the other hand, NK cells may attack the vasculature during viral infection and organ transplantation. In a human CMV infection study, scientists found that NK cells were responsible for the pathogenesis of vascular injury (43). Additionally, during organ transplantation, recipient NK cells may attack donor endothelial tissues if their inhibitory receptors are unable to recognize the donor's MHC I molecules (44).

1.2.1.4. Memory features of NK cells in antitumour, antiviral, and regulatory functions

NK cells were defined as innate immune cells involved in quick responses in host defence to

pathogen invasion. NK cells can recognize pathogens' general "non-self" features, get activated and eliminate them without the need for priming. However, evidence indicates that some subpopulations of NK cells also can remember specific invading antigens for months and years. Upon re-stimulation by the same antigens, these subsets are activated and expand rapidly and abundantly (45). These "memory" processes are alike to those of conventional adaptive immune cells, T cells and B cells, which are described as adaptive features. Scientists named these NK cell populations as"memory NK cells" or "memory-like NK cells" to highlight these adaptive features (17). So far, several kinds of pathogens or chemicals have been found able to stimulate NK cells to generate memory. Based on the substance used for the initial stimulus, memory NK cells can be categorized into three types: hapten-specific memory NK cells, cytokine-induced memory NK cells and virus-specific memory NK cells (46).

Hapten-specific memory NK cells are the first type of memory NK cells to gain scientists' attention. Haptens are small chemical compounds that penetrate skin surfaces and form immunogenic hapten-carrier complex antigens by binding and modifying epidermal proteins. 2,4-dinitro-1-fluorobenzene (DNFB) and oxazolone (OXA) are two haptens that can stimulate contact hypersensitivity (CHS) responses in areas such as the skin on the ears, which can be quantitively analyzed by measuring the swelling thickness. Previous studies have shown that these two haptens can activate the adaptive immune system, be recognized by adaptive cells and generate CHS responses during further re-stimulation. In 2006, a group of researchers found that mice lacking

conventional adaptive immune cells (T and B cells) could still have CHS response when re-stimulated by the same hapten used for initial stimulation but not other haptens (45). Further deletion of NK cells via knockout of the *IL2RG* gene would abolish the CHS response during re-stimulation. Adoptive transfer experiments transferring donor NK cells from specific hapten-sensitized mice to naïve recipients showed that the hapten-specific sensitized NK cells could respond to re-stimulation by the same hapten even more than four months after the initial sensitization. Several later studies also confirmed this hapten-specific memory-like feature of NK cells. Additionally, they reported that the hapten-specific memory feature is found in the liver NK cells but not in the NK cells of other organs. Moreover, experiments using blocking antibodies or knockout mice showed that natural killer group 2D (NKG2D), CD18, P-selectin, E-selectin, and CXC-chemokine receptor 6 (CXCR6) may be involved in the generation of hapten-specific NK memory. Cytokines such as IL-12, IFN-γ, and IFN-α also play essential roles in memory formation (45, 47, 48). However, further study is required to understand most of the mechanisms by which hapten-specific NK cell memory is formed. A better comprehension of this process can help to potentially improve treatments for hapten-related allergies.

Cytokine-induced memory NK cells are found in both mice and humans. Studies showed that *in vitro* in advance stimulation of mouse NK cells with various cytokines, including IL-12, IL-15 and IL-18, could enhance the IFN-γ production ability of NK cells when encounter with tumour cells or cytokines, and that this enhancement could last longer than four months following adoptive

transfer the cells into naïve mice (49). This long-term NK cell function-enhancing effect does not need the involvement of any specific antigen. Such cells are categorized as memory NK cells because they can "remember" their cytokine-mediated activation history and continue to maintain a more sensitive state than naïve NK cells. These findings led to the idea of generating human cytokine-induced memory NK cells to improve NK cell immunotherapy against cancer. For instance, a phase I clinical trial using cytokines pre-activated NK cells to treat acute myeloid leukemia is ongoing. However, much research remains to be done to make the cytokine-induced memory NK cells a safe and efficient treatment for broader use (46, 50).

Virus-specific memory NK cells form another type of memory NK cells discovered in the last decade. Scientists found that a mouse $Ly49H^+$ NK cell population could generate memory responses against mouse cytomegalovirus (MCMV) infection (51). Unlike hapten-induced NK cell memory, which is only limited to liver NK cells, this MCMV-specific memory NK cell subset is distributed systemically. It can be found in many different organs, including blood, spleen, lung, liver, lymph node, bone marrow, and other lymphoid tissues. Upon initial MCMV infection, the $Ly49H^+$ NK subsets recognize the m157 viral ligand of infected cells via its Ly49H receptor, are activated and expand rapidly (Figure 1.3). After eliminating the viruses, the $Ly49H^+$ NK cells become reduced in number throughout the contraction period. The remaining $Ly49H^+$ cells survive for months and years, and they react much faster and generate much more vigorous effector functions upon reinfection with MCMV but not other viruses.

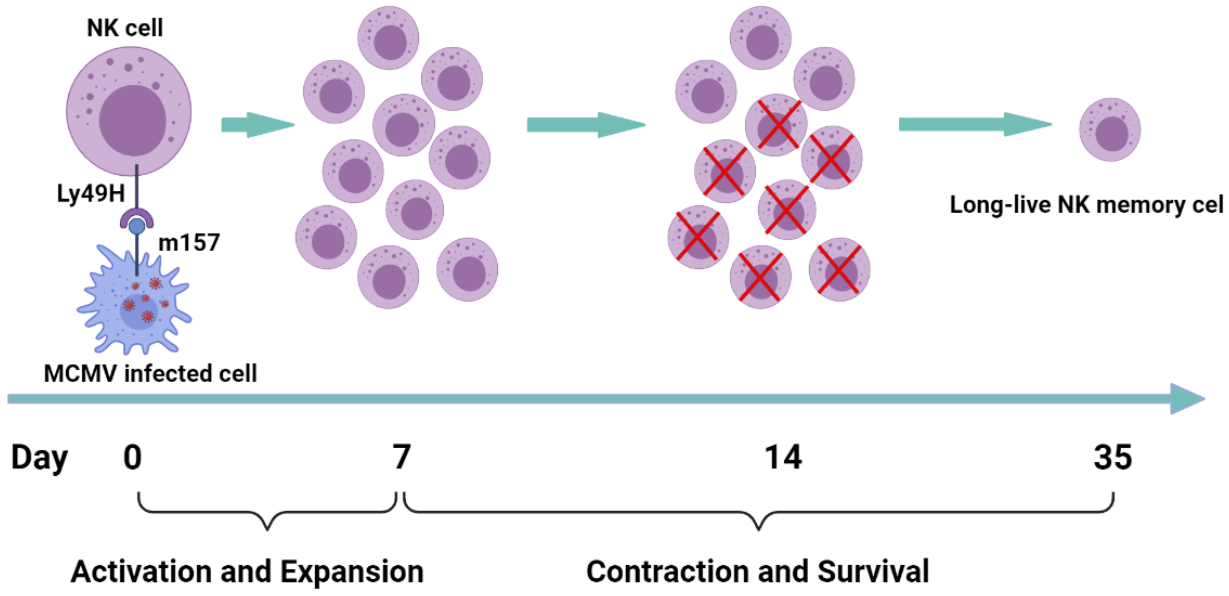

Figure 1.3 MCMV-specific memory NK cell generation

Upon initial MCMV infection, the Ly49H$^+$ NK subsets recognize the m157 viral ligand of infected cells via its Ly49H receptor, are activated and expand rapidly (day 0 to day 7). After eliminating the viruses, the Ly49H$^+$ NK cells reduce in number throughout the contraction period (day 7 to day 28). The Ly49H$^+$ cells remaining after contraction survive for months and years (after day 35). The figure was created with BioRender.com

Several mechanisms are involved in the generation of MCMV-specific memory NK cells. Studies have found that the NK cell activating receptor DNAX accessory molecule-1 (DNAM-1), recombination-activating genes (RAGs), transcription factor Zbtb32, and cytokines IL-12, IL-15

and IFN-α are necessary for the generation of MCMV-specific memory NK cells (52-55). In humans, a CD94/NKG2C$^+$ subset of NK cells has caught scientists' attention. During initial human cytomegalovirus (HCMV) infection, this subset expands faster than other NK cells and persists as memory NK cells. In some HCMV-infected cases, the CD94/NKG2C$^+$ subset can account for more than 70% of the entire NK populations. Studies on HCMV-seropositive individuals found that these memory NK cells have the memory-like character of mouse memory NK cells (56, 57). In clinical procedures, such as hematopoietic stem cell transfusion, the CD94/NKG2C$^+$ NK cells of recipients of stem cells from an HCMV-seropositive donor displayed better effector functions against HCMV upon reinfection than those who received transplants from HCMV-seronegative donors. The ligand HLA-E39 expressed on infected cells is responsible for the triggering of CD94/NKG2C and the rapid expansion of the CD94/NKG2C$^+$ subset of NK cells. Another population of human NK cells, the high-affinity IgE receptor subunit-γ (FcRγ)-deficient NK cells, was recently found to have CD94/NKG2C-independent memory features. During HCMV infection, FcRγ-deficient NK cells subsets facilitate enhanced antibody-dependent cellular cytotoxicity (ADCC) towards infected target cells coated with HCMV-specific antibodies (58, 59). Virus-specific memory NK cells were also discovered in infection with other viruses, such as influenza viruses, HSV-2 and, HCV (46, 60, 61). In the future, more detailed mechanistic studies will help understand the characters and functions of the memory NK cells. Moreover, understanding the operating mechanisms of variety of memory NK cells will allow the development of potential treatments for several diseases, such as allergies, infections, cancers, and graft-versus-host disease

(GVHD).

1.2.2. NK cell receptors, adaptors, and signalling

To avoid pathological damage to tissues, NK cells have complicated and robust mechanisms to control cytotoxicity and cytokine production (36). In recent years, efforts have been made to study the NK cells' mechanisms on identify healthy or "self" cells and unhealthy or "non-self" cells. Many scientists in the field agree on the concept of "dynamic equilibrium". In this hypothesis, NK cell activation is controlled by various activating and inhibitory receptors on its cell membrane (62). The integration of inhibitory and activating pathways upon receiving signals from neighbouring cells controls the balance and regulation of NK cell activities and decides whether they will kill nearby targets.

1.2.2.1. Activating receptors and signalling

Activating receptors on NK cells can recognize specific ligands on potential target cells that may be either unhealthy cells or immune cells undergoing activation. Many activating receptors have been reported, of which several types have been studied extensively, including the immunoreceptor tyrosine-based activation motif (ITAM)-bearing NK cell receptor complexes, NKG2D receptor and its adaptor complexes, signalling lymphocytic activation molecule (SLAM) family receptors, and adhesion receptors.

ITAM adaptors contain one to three ITAM motifs (YxxL/Ix6-8YxxL/I). ITAM-bearing NK cell receptor complexes typically consist of membrane receptors associated with ITAM adaptors, including Fc receptor γ-chain (FcRγ), DNAX activating protein of 12 kDa (DAP-12), and CD3 ζ subunit (CD3-ζ). ITAM adaptors contain a negatively charged transmembrane aspartic acid residue that can bind the corresponding receptors, typically containing a positively charged lysine or arginine at the same place of the transmembrane region, to form complexes. Depending on the maturation, differentiation and activation status of the cells, they may use different downstream signalling pathways. In mouse NK cells, FcRγ can couple with CD16, Killer cell lectin-like receptor subfamily B, member 1 (NK1.1 or NKRp1c) and, Natural killer cell p46-related protein (NKp46); DAP-12 can couple with activating Ly49s (including Ly49D, Ly49H, and Ly49P), CD94/NKG2C heterodimer, NKG2D-S (the short form of NKG2D), and paired immunoglobulin-like type 2 receptor β (PILRβ); and CD3-ζ can couple with NKp46. In human NK cells, FcRγ can couple with NKp46, Natural killer cell p30-related protein (NKp30), CD16 and killer cell immunoglobulin like receptor, two Ig domains and long cytoplasmic tail 4 (KIR2DL4); DAP-12 can couple with some activating KIRs including KIR2DS2, KIR2DS4 and KIR3DS1, CD94-NKG2C, and NKp44; and CD3-ζ can couple with NKp46, NKp30, and CD16. Upon receiving activating signals from binding ligands on target cells, these receptors activate Src family protein tyrosine kinases (PTKs) that phosphorylate ITAMs; the phosphorylated ITAMs subsequently recruit and activate Syk family kinases Syk and ZAP70 (63). Activated Syk family kinases can mediate downstream signals involving phospholipase C-γ (PLC-γ) and Vav Guanine Nucleotide

Exchange Factor (Vav), thus inducing cytoskeletal reorganization and, as a result, leading to cell polarization and cytotoxic granule secretion. Interestingly, in Syk/ZAP70 double-deficient mice and FcRγ/DAP-12/CD3-ζ triple-deficient mice, NK cell cytotoxicity was only partially impaired (64, 65). Another study that used CD45-deficient mouse NK cells, which abolished the enzymatic function of Src family kinases, reported no influence on cytotoxicity but found that cytokine production was largely impaired (66, 67).

CD16 (FcγRIII) is a transmembrane protein that couples with FcRγ and CD3ζ in its transmembrane domain to transduce activating signals. CD16 allows NK cells to bind with target cells by an indirect mechanism. During the existence of the IgG antibodies, CD16 can bind the Fc domain of the IgG antibodies. Simultaneously, the antigens expressed on tumour cells or infected cells bind to the Fab domain of the specific antibodies. This process could induce ADCC in the context of antitumour and antivirus activities. Upon binding of CD16 with the Fc domains of the IgG antibodies, NK cells will be activated, and cytotoxicity towards target cells will be induced subsequently. The ADCC effect exists on NK cells and other types of immune cells, which express CD16 or other Fc receptors. However, NK cells are the most critical effector of ADCC. Many antibody treatments against cancers are partially dependent on the effect of ADCC on NK cells (68-70).

NKG2D is an important activating receptor expressed on several types of immune cells including

NK cells and several subsets of T cells. It not only pairs with DAP-12 but also with DNAX activating protein of 10 kDa（DAP-10, containing a YxxM motif). In mice, NKG2D has two isoforms, NKG2D long (NKG2D-L), generally expressed in non-activated NK cells, and NKG2D short (NKG2D-S), generally expressed in activated NK cells due to the alternative splicing of *KLRK1* genes. NKG2D-L has 13 additional amino acids in the cytoplasmic region, while the other sequences are the same as in NKG2D-S. The extra amino acids physically prevent binding to DAP-12. NKG2D-S can pair with both DAP-10 and DAP-12, while NKG2D-L can only pair with DAP-10. However, only NKG2D-L expressed in human NK cells, pairing with only DAP-10. NKG2D genes have limited polymorphism in both mice and humans. However, they can recognize a wide range of ligands. For instance, in humans, NKG2D can bind with at least eight ligands, including MICs, RAET1s. In mice, its ligands include Rae1s, MULT1, H60s. Upon recognizing these antigens, NKG2D activates the adaptors DAP-10 or DAP-12. However, the downstream effects of DAP-10 and DAP-12 activation differ significantly. DAP-12 contains the ITAM motif and therefore recruits Syk and ZAP70 tyrosine kinases and couples to Vav2 and Vav3. In contrast, DAP-10 has a YINM motif, also known as immunoreceptor tyrosine tail (ITT) motif, that enables binding and activation of Vav1 (62, 67, 71). Many tumour cells express different NKG2D ligands. In mice, resting NK cells can be activated efficiently by *in vitro* NKG2D cross-linking (72) and kill hematopoietic cells expressing RAET1 ligands without prior priming (71, 73). In humans, upon ligand binding to NKG2D, NK cells primed by IL-2 or IL-15 can initiate cytotoxicity and cytokine production (71).

DNAM-1 (CD226) is an activating receptor expressed on multiple immune cells, including NK cells, $CD8^+$ T cells, $CD4^+$ T cells, and some types of myeloid cells. DNAM-1 belongs to the immunoglobulin (Ig) superfamily but its cytoplasmic domain is different from that of most other Ig superfamily members. CD155 and CD112 have been identified as the ligands of DNAM-1, which are expressed on many transformed cells and virus-infected cells (74). Inhibitory receptors T cell immunoglobulin and ITIM domain (TIGIT) and CD96 share these ligands with DNAM-1 and can compete with DNAM-1 for ligand binding, thus limit the DNAM-1 activating function (75, 76). DNAM-1-mediated signals are necessary for NK cell activation. The signals are transduced by the phosphorylation of tyrosine Y319 in the ITT-like motif of the intracellular region of DNAM-1. Phosphorylated Y319 and asparagine N321 then coordinate together to bind the Grb2 adaptor, subsequently activating exchange factor Vav1, phosphatidylinositol 3'-kinase (PI3'K), and PLC-γ1. Through these signals, the activation of DNAM-1 enhances NK cell cytotoxicity by promoting actin polymerization and the polarization of lytic granules towards target cells (77).

Integrins are cell adhesion molecules that control adhesion and interactions between cells. Integrin receptors are heterodimeric receptors that consist of α and β chains. There are 18 α chains and eight β chains that assemble 24 heterodimeric receptors with different binding specificities and signalling pathways (78). Integrins play important roles in cell trafficking, cell migration, immune synapse formation and receptor co-stimulation (79). In NK cells, β2 integrins play predominant

roles among all integrins. There are four β2 integrins: lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18), macrophage-1 antigen (Mac-1; CD11b/CD18), integrin alpha X (CD11c/CD18) and, integrin αDβ2 (CD11d/CD18). All of them are expressed in mouse and human NK cells. Among these integrins, LFA-1 is reported to have important roles in NK cell functions. Apart from its function in NK cell-target cell adhesion, LFA-1 is also an essential part of the immune synapses between NK cells and target cells. Some studies have also reported that LFA-1 is important for NK cell activation as a co-stimulatory receptor (80). Upon binding with a receptor such as intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, activated LFA-1 also triggers granule polarization in human NK cells (81), activating exchange factor Vav1 and kinase Pyk2 signalling, which lead to actin polymerization (80, 82).

1.2.2.2. Inhibitory receptors and signalling

NK cells express various inhibitory receptors on their cell membranes and regulate cell activation and functions. The primary inhibitory NK cell receptors include inhibitory KIRs in humans, inhibitory Ly49s (Ly49a, Ly49c, Ly49e, Ly49f, Ly49g and Ly49i) in mice, and leukocyte immunoglobulin-like receptors (LILRs) and CD94/NKG2A in both species. Unlike ITAM-coupled receptors, which are heteromeric, inhibitory receptors are monomeric and contain immunoreceptor tyrosine-based inhibition motifs (ITIMs), (I/L/V/S)XYXX(L/V), in their cytoplasmic domains. When inhibitory receptors bind with their ligands on nearby cells, the tyrosine residue of ITIM is phosphorylated and subsequently recruits and activates the Src homology 2 (SH2) domain-

containing protein tyrosine phosphatase (SHP)-1, SHP-2 or SH2 domain-containing inositol phosphatase (SHIP)-1 (83-85). These activated phosphatases inhibit NK cell functions by dephosphorylating the molecules triggered by activating receptors, such as the exchange factor Vav1, linker for activation of T cells (LAT), PLCγ1/2 and PI3,4,5P3 which are important substrates for several activating signalling pathways. Inhibitory signalling through these processes can inhibit NK cell functions such as Ca^{2+} influx, degranulation, cytokine production and proliferation. Recent studies also found that not only dephosphorylation but also phosphorylation has a significant role in inhibitory signalling. During the interaction of specific inhibitory receptors with MHCI molecules, the adaptor protein Crk binds and is phosphorylated by Abelson tyrosine kinase (Abl), a process essential for separating Crk from the Cbl-Crk-C3G complex and subsequently diminishing the corresponding activating signal pathway. A dynamic balance is set between the activating and inhibitory signals and determines various NK cell functions (62, 67, 85).

1.3. SLAM family receptors, their ligands and SLAM-associated protein (SAP) family adaptors

The SLAM family of receptors is a group of receptors found to have important and complex roles in the immune system. It includes SLAM (also called SLAMF1 or CD150), Ly-9 (SLAMF3 or CD229), 2B4 (SLAMF4 or CD244), CD84 (SLAMF5), SLAMF6 (Ly108 in mice and NTB-A in humans) and SLAMF7 (CRACC or CD319). Most SLAM family receptors are self-ligands. The only exception is 2B4, whose ligand is CD48 (also known as SLAMF2). CD48 is not categorized

in the SLAM family due to structural and functional differences. The SLAM family receptors are expressed only on hematopoietic cells, including many types of immune cells, and generally, each immune cell expresses several members of the SLAM family of receptors (86, 87). The SLAM family receptors play many important roles in modulating immune cells due to their ability to interact with downstream SH2 domain-containing adaptors (86, 88-90).

1.3.1. SLAM family receptors and their ligands

SLAM family receptors consist of a transmembrane domain and a cytoplasmic domain. The cytoplasmic domain contains immunoreceptor tyrosine-based switch motifs (ITSMs; TIpYXXV/I) that allow the SLAM family receptors to bind with adaptors containing SH2 domains. The SAP family of adaptors is the primary adaptors bound by SLAM family receptors (91). SAP family adaptors include three members, namely SAP, Ewing sarcoma-associated transcript (EAT)-2, and EAT-2-related transducer (ERT). SAP and EAT-2 are expressed in both mice and humans, whereas ERT is only expressed in mice (87).

Experiments in which SLAM family receptors were triggered by specific anti-receptor antibodies or ectopic ligands expressed on non-hematopoietic cells were thought as the evidences of their activating roles (92). However, it was observed that they could become inhibitory under some conditions. The initial discovery of the inhibitory functions of SLAM family receptors was in a immunodeficiency disease called X-linked lymphoproliferative (XLP) disease in human. Due to

the mutations in the *SH2D1A* gene, NK cells in XLP patients do not express functional SAP protein, thus causing the immunodeficiency. Similar findings were also observed in SAP knockout mice (88, 92).

1.3.2. Adaptors associated with SLAM family receptors

Although SLAM family receptors mainly bind SAP family adaptors, they can also bind several other molecules, namely the phosphatases SHP-1, SHP-2, and SHIP-1. In all cases, the binding is mediated by the ITSM motifs on the SLAM family receptors and the SH2 domains on the phosphatases.

SAP expression can be detected in both T cells and NK cells, whereas EAT-2 and ERT are expressed only in NK cells. However, the expression of these SAP adaptors is not detectable in B cells, macrophages or DCs (86, 87). Studies have shown that SAP adaptors are important for activating NK cells and $CD8^+$ T cells, which are triggered by the association of SLAM family receptors and their ligands on hematopoietic targets (93, 94). This function has also been confirmed by *in vitro* experiments using non-hematopoietic targets ectopically expressing the ligands of SLAM family receptors (93).

However, in the cells lacking expression of SAP family adaptors, the function of SLAM family receptors is reversed. They exhibit super-inhibitory phenotypes, suppressing the activation of NK

cells and $CD8^+$ T cells when encountering SLAM-expressing hematopoietic target cells. Additionally, a lack of SAP adaptors in NK cells and T cells causes immune diseases. Recent studies have found that SAP-deficient humans and mice have impaired NK cell activation, $CD8^+$ T cell activation, NK/T cell development and $CD4^+$ follicular T helper (Tfh) cell-dependent germinal centre reactions and antibody responses. These defects are presumably because of the reversion of SLAM family receptors into inhibitory receptors(86, 92, 93).

Several studies have been conducted to shed light on SAP functions in the past few decades. SAP was found to play two roles in controlling NK cell and $CD8^+$ T cell activation. First, SAP recruits and activates the Src-related protein tyrosine kinase Fyn. SAP has an arginine (R78)-based motif (RFFRKVKN) that binds the SH3 domain of Fyn. This interaction subsequently activates the exchange factor Vav1, thus enhancing the conjugate formation activities of effector cells towards target cells (95, 96). The Fyn-dependent mechanism was initially found in NK cells and is predicted to occur in many other lymphocytes expressing SAP. Second, SAP can compete with inhibitory phosphatases and prevent the interaction of SLAM family receptors with the inhibitory effectors. The inhibitory molecules involved in this competition include SHIP-1 for 2B4 and SLAMF7 in NK cells and SHP-1 for SLAMF6 in T cells (86, 97, 98).

Similarly, EAT-2 expression in NK cells can also activate cells in two ways. First, EAT-2 has been reported to promote activating signals via its phosphorylated tyrosine-based motif (Y127). This

motif can associate and activate PLC-γ, calcium fluxes, and extracellular signal-regulated kinases (Erk). These signals can promote NK cell cytotoxicity towards targets. Second, similar to SAP, EAT-2 can compete with inhibitory phosphatases and prevent the interaction of SLAM family receptors to inhibitory receptors (97, 99).

Recent studies found that in HCMV infection, epigenetic changes of the EAT-2 gene occurred in a subset of NK cells in some patients. As a result, these NK cells lost EAT-2 expression (59, 86). These EAT-2-deficient NK cell subsets expressed NKG2C and had little or no expression of the Syk kinase and FcRγ. These cells exhibited a decreased killing against tumour cells, believed to be due to the absence of EAT-2 causing diminished activation of SLAM family receptors. However, unexpectedly, these subsets had an enhanced ability to kill antibody-coated cells by ADCC. They seemed to be memory-like NK cells. Extensive further studies are nessessary to understand the enhanced ADCC effect and the generation of memory-like features in these EAT-2-deficient NK cells.

The ITSM motif allows SLAM family receptors to switch their roles even in the presence of SAP family adaptors. 2B4 is one of the SLAM family receptors that can be either activating or inhibitory when associated with SAP. It was reported based on *in vitro* and *in vivo* experiments that NK cells from 2B4-deficient mice showed augmented activation responses against targets, including hematopoietic tumour cells and activated T cells triggered by LCMV infection (100, 101).

Moreover, upon LCMV infection, more rapid proliferation of virus-specific memory $CD8^+$ T cells was seen in 2B4-deficient mice than in wild-type mice. Furthermore, in an anti-CD3 antibody-induced intestinal inflammation experiment, upon stimulation, 2B4-deficient mice had more cytotoxic $CD8\alpha\beta^+$ intra-epithelial lymphocyte expansion than wild-type mice (102). In contrast to the inhibitory roles of 2B4 in mice, the activating function of 2B4 was reported in several human studies (103-106).

In sum, these findings suggest that 2B4 can be either activating or inhibitory. The relative expression level of SAP family adaptors compared to inhibitory phosphatases SHP-1, SHP-2, and SHIP-1 may determine the function of 2B4 on a specific condition. The expression level of CD48 on target cells may be another factor that determines 2B4 signals. Lower CD48 interaction with 2B4 may induce inhibitory signals, whereas higher levels of CD48 interaction with 2B4 may achieve activating signalling (86, 87). Nevertheless, future studies are needed to investigate the detail mechanism of the dual function of 2B4.

1.4. Rationale

The fundamental mechanisms implicated in NK cell functions are regulated by receptor-ligand interactions. However, the diversity of interactions between receptors and adaptors and between receptors and ligands is complex and not well understood. NKG2D, DNAM-1 and 2B4 have been studied *in vitro* and *in vivo* to shed light on their functions in tumours and infectious diseases.

However, their complementary and redundant roles, and interactions with other receptors, are largely unknown. Additionally, the integrin receptors, LFA-1 and Mac-1, have been found to have signalling functions in immune cells such as macrophages and DCs, but their signalling functions in NK cells are not well studied. Thus, there are gaps in our understanding of the complex roles of NK cells and single molecules as well as their intermolecular interactions.

Although much research has focused on the mechanistic and functional study of various immune therapies and diagnostics, few mechanisms have been elucidated given their complexity. For instance, many immune therapies use a common unified strategy for all patients, but the treatment outcomes are largely diverse. This is due to individual differences in ligand expression, immune cell composition and characteristics, tumour site accessibility and cytokine availability among patients. Thus, more studies on different aspects of NK cells and their receptors are needed.

Chapter 2. Relative contribution of various activating natural killer cell receptors to natural cytotoxicity

Preface

The manuscript presented in Chapter 2 is prepared to submit to journals. It described our study on the function of natural killer cell receptors on natural cytotoxicity. The study was focused on the antitumour function of natural killer cells.

2.1. Abstract

Natural killer (NK) cells are among the most important immune cells in antitumour immunity. Many efforts have been made to study the functions of the various activating NK cell receptors in antitumour immunity. However, most studies have been focused on the functions of single receptors. In this study, we showed that several activating NK cell receptors functionally complement each other to achieve high cytotoxic activity against various tumour targets. First, we found that natural killer group 2 member D (NKG2D) and DNAX accessory molecule-1 (DNAM-1) have complementary roles in NK cell killing. However, NKG2D is functionally more important than DNAM-1. Second, the integrin lymphocyte function-associated antigen 1 (LFA-1) has an important role in NKG2D- and DNAM-1-independent cytotoxicity. LFA-1-dependent killing is mediated partially through the immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor Fc receptor γ-chain (FcRγ). Third, we observed that LFA-1, but not 2B4, is critical for killing of hematopoietic target cells by human NK cells. These results highlight the relative contributions of various activating NK cell receptors to natural cytotoxicity in mice and humans.

2.2. Introduction

NK cells are among the most important immune cells in antitumour immunity (1-4). They are activated by various monomorphic surface receptors (5-7). As described in Chapter 1, there are two kinds of receptors controlling NK cell activation—activating receptors and inhibitory receptors (8). The activation of NK cells is controlled by the balance of the multiple activating and inhibitory signals. Many efforts have been made to understand which receptors are responsible for activating the NK cells that kill tumours (9-11). However, most studies conducted thus far have been focused on the functions of single receptors. How these receptors coordinate and complement each other is unknown. Putting pieces of evidence together is necessary to understand the mechanisms by which NK cells mediate antitumour activity for future therapeutic purposes.

Natural killer group 2 member D (NKG2D) is a primary activating receptor of NK cells and the binding of its ligands stimulates the cytotoxicity and cytokine production (12, 13). NKG2D can bind many major histocompatibility complex (MHC) class I-like ligands, including the MHC class I chain-related protein A and B (MICA/B) and retinoic acid early transcripts-1 (RAET1)/unique long 16 (UL-16)-binding proteins (ULBP) family proteins that are frequently induced or upregulated on tumour cells (14, 15). NKG2D does not contain signalling elements in its cytoplasmic domain. In mice, its signalling mainly depends on the adaptors DNAX-activating protein of 10 kDa (DAP-10) and 12 kDa (DAP-12) (16, 17). In humans, only DAP-10 is associated

with NKG2D. DAP-10 contains a YXXM tyrosine-based motif that binds and activates the p85 subunit of phosphatidylinositol 3'-kinase (PI3'-K) and growth factor receptor-bound protein 2 (Grb2), thus enabling NKG2D to transduce downstream signals and inducing NK cell activation (6, 17).

DNAX accessory molecule-1 (DNAM-1) is another primary activating receptor expressed on the NK cell surface. DNAM-1 can recognize the ligands poliovirus receptor (PVR/CD155) and nectin-2 (CD112), which are broadly expressed by hematopoietic cells, epithelial cells and endothelial cells and can be upregulated on tumour cells (18-22). DNAM-1 has a signal-transducing motif in its intracellular domain for signal transduction that is named immunoreceptor tyrosine tail (ITT) motif. Our laboratory's previous studies discovered that DNAM-1 can triggers activating signals in NK cells. These signals are initiated by the phosphorylation of tyrosine Y319 but not S326 in the ITT motif of DNAM-1. They also require asparagine at position 321 (N321), to cooperates with phosphorylated Y319 to interact with the adaptor Grb2. The interaction of DNAM-1 with Grb2 subsequently activates the guanine nucleotide exchange factor Vav-1 (Vav-1), PI 3'-K and phospholipase C (PLC)-γ1. These signals promote actin polymerization and the polarization of lytic granules towards target cells to enhance NK cell cytotoxicity (23).

Integrins play roles in adhesion between cells and cell adhesion to the extracellular matrix (24, 25). They are heterodimeric receptor complexes that contain an α chain and a β chain. Unlike typical

signalling receptors that can only transduce signals from the extracellular to the intracellular environment, integrins can perform two-way signalling, i.e., inside-out and outside-in signalling (26). When cells are stimulated by growth factors, cytokines or other activating agents, intracellular activators such as talin and kindlins are activated and transduce activating signals to integrins by binding to their cytoplasmic tails. This process activates the integrins and leads to conformational changes in their extracellular domains, increasing their binding affinity for their ligands. Alternatively, when the integrins' extracellular domain binds their ligands like intercellular adhesion molecule (ICAM)-1 and ICAM-2, activating signals are transduced to the intracellular environment through either an ITAM-dependent pathway or ITAM-independent pathway (27-32). In both cases, this can activate Syk kinase-dependent pathways and subsequent downstream signalling. In NK cells, activated integrins such as β2 integrins are necessary for firm adhesion to targets and effective killing (33).

2B4, also known as signalling lymphocyte activation molecule family 4 (SLAMF4) or CD244, is a heterotypic SLAM family receptor (SFR) expressed on NK cells and several other immune cells. The ligand of 2B4 is CD48 (SLAMF2). 2B4 associates with the SLAM-associated protein (SAP) family of adaptors, including SAP, Ewing's sarcoma-activated transcript-2 (EAT-2) and, in mice but not humans, EAT-2-related transducer (ERT). These adaptors all contain a Src homology 2 (SH2) domain (34, 35). Our previous study in mice showed that 2B4 play an inhibitory role in mouse NK cells, it can inhibit LFA-1 from activation through recruitment of the lipid phosphatase

SHIP-1 (36). However, we still do not fully know how 2B4 via SHIP-1 influences the function and signals of LFA-1, and whether 2B4 is also inhibitory in human NK cells.

In this study, we investigated multiple mouse and human tumour cell lines, including hematopoietic and non-hematopoietic cells. We used these cell lines as targets to study the roles of various activating NK cell receptors in cytotoxicity. We also used mice lacking one or more NK cell activating receptors, receptor-blocking antibodies and targets lacking ligands for specific receptors. We found that NKG2D and DNAM-1 were crucial for the killing of target cells expressing their ligands. NKG2D was important for killing of NKG2D ligand-bearing targets regardless of whether DNAM-1 ligands were expressed or not. Conversely, DNAM-1 was necessary only when NK cells lacked NKG2D or when target cells did not express NKG2D ligands. We also noted that NK cells lacking both DNAM-1 and NKG2D, or when their corresponding ligands were missing on targets, maintained killing capacity towards certain targets. The integrin receptor LFA-1 was largely responsible for this DNAM-1- and NKG2D-independent killing. Finally, we demonstrated that the activating function of LFA-1 was partially mediated through the ITAM-bearing adaptor FcRγ. This study provides a basic understanding of the complementary and redundant roles of primary NK cell activating receptors in natural cytotoxicity toward tumour cells.

2.3. Results

2.3.1. Characterization of mouse tumour cells with diverse expression patterns of ligands for activating NK cell receptors

To study the functions of various activating NK cell receptors in cytotoxicity against different types of tumours, we used multiple mouse tumour cell lines that expressed different patterns of ligands for activating NK cell receptors (Figure 2.1). We checked the expression of SFR ligands SLAMF1, CD48 (SLAMF2), SLAMF3 (Ly-9), SLAMF5 (CD84), SLAMF6 (Ly108) and SLAMF7 (CRACC; total NKG2D ligands (using a soluble NKG2D-Fc fusion protein as probe); DNAM-1 ligands CD155 and CD112; and integrin ligands ICAM-1 and ICAM-2.

The targets included lymphoma cell lines RMA-S and EL-4; thymoma cell line YAC-1; neuroblastoma cell line Neuro-2a; melanoma cell line B16 (F10); and rectal carcinoma cell line CMT-93. In particular, the hematopoietic cells RMA-S, EL-4 and YAC-1 all expressed SFR ligands, as expected (Figure 2.1A). They displayed high levels of SLAMF1, CD48 and CD84. YAC-1 cells, but not RMA-S or EL-4 cells, also expressed high levels of NKG2D ligands and DNAM-1 ligand CD155. All of them also expressed integrin ligands ICAM-1 and ICAM-2 at high levels. In contrast, the non-hematopoietic cells Neuro-2a, B16 and CMT-93 did not express SFR ligands (Figure 2.1B). However, they all expressed NKG2D ligands and the DNAM-1 ligand CD155. Neuro-2a cells also expressed high levels of the integrin ligands ICAM-1 and ICAM-2,

while B16 and CMT-93 cells had little or no integrin ligand expression.

Thus, in our panel of mouse target cells, hematopoietic target cells universally expressed ligands for SFRs and integrins, but only one expressed ligands for NKG2D and DNAM-1. Non-hematopoietic target cells consistently expressed ligands for NKG2D and/or DNAM-1. One also expressed ligands for integrins. However, none expressed ligands for SFRs. This diversity in ligand expression among target cells suggested that they trigger NK cell activation through different NK cell receptors.

2.3.2. NKG2D and, to a lesser extent, DNAM-1 have important roles in mouse NK cell cytotoxicity

We first tested the roles of NKG2D and DNAM-1. To this end, NKG2D and DNAM-1 double-knockout mice (dKO) were generated, by breeding NKG2D and DNAM-1 single KO mice until homozygous mice lacking both NKG2D and DNAM-1 were obtained (Figure 2.2A).

Previous studies showed that loss of NKG2D alone or DNAM-1 alone had little or no impact on NK cell development, except for moderately decreased expression of Ly49s on NK cells from NKG2D KO mice (23, 37). We found DNAM-1 NKG2D dKO NK cells also had normal development, although they exhibited slightly decreased expression on some Ly49s (Ly49G2, Ly49C/I/F/H, Ly49A/D), which is similar as previously reported in NKG2D KO mice. Other than

that, the receptors expressed on dKO NK cells were normal (data not shown).

The cytotoxicity mediated by splenic NK cells from wild-type (WT), NKG2D KO, DNAM-1 KO or dKO mice towards the various mouse targets detailed above was then tested, using a chromium-51 release assay. In comparison to WT NK cells, NKG2D KO NK cells showed reduced cytotoxicity towards YAC-1, Neuro-2a and CMT-93. No defect was seen towards RMA-S and B16, which lacked or expressed low levels of NKG2D ligands. These features were seen whether NK cells were activated *in vitro* with IL-2 (Figure 2.2B and C) or primed *in vivo* with polyinosinic-polycytidylic acid (poly(I:C)) (Figure 2.2D and E).

Compared to WT NK cells, DNAM-1 KO NK cells showed decreased killing only towards B16 cells, as we reported (23). Lack of DNAM-1 had no effect on killing of RMA-S, YAC-1, Neuro-2a and CMT-93, even if some of these targets expressed DNAM-1 ligands, such as YAC-1, Neuro-2a and CMT-93. Nonetheless, compared to NKG2D KO NK cells, NKG2D DNAM-1 dKO NK cells showed a further decrease in the killing of YAC-1, Neuro-2a and CMT-93. The impact of DNAM-1 deficiency on killing of B16 was also moderately accentuated (Figure 2.2B-E).

These results suggested that NKG2D, and to a lesser extent DNAM-1, were required for killing of targets bearing their ligands. For tumour cells bearing ligands for both NKG2D and DNAM-1, NKG2D seemed to play a more critical role, compared to DNAM-1.

2.3.3. LFA-1, but not Mac-1, is the critical activating receptor for killing of tumour cells expressing ICAM-1 and ICAM-2

From the cytotoxicity assays performed using NKG2D DNAM-1 dKO NK cells, we found that these two primary receptors were not responsible for NK cell cytotoxicity towards RMA-S cells. Additionally, NK cells lacking NKG2D and DNAM-1, in particular IL-2-activated NK cells, still had a partial capacity to kill most targets (Figure 2.2). Thus, we tried to identify additional NK cell receptors that enabled killing of these targets.

We already reported that SFRs were not critical for NK cell cytotoxicity (36). However, we found that the integrin LFA-1 (CD11a/CD18) was necessary for NK cell-mediated killing of some tumour targets. Thus, we decided to test whether LFA-1 was broadly implicated in NK cell-mediated cytotoxicity towards the targets studied herein. LFA-1 KO NK cells, as well as NK cells lacking the related integrin Mac-1 (CD11b/CD18), were evaluated.

First, we analyzed the impact of loss of LFA-1 or Mac-1 on NK cell development and differentiation. Compared to WT mice, LFA-1 KO mice or Mac-1 KO mice generally displayed normal NK cell numbers and cell surface markers in spleen or bone marrow (Figure 2.3 and Figure 2.5). The most notable exception was a loss of expression of LFA-1 or Mac-1 on LFA-1 KO and Mac-1 KO NK cells, respectively. There was also a slightly increased number (less than 2-fold) of

NK cells, in addition to small alterations in the expression of Ly49D, NKG2A/C/E, NKp46 and KLRG1, on NK cells of LFA-1 KO mice, compared to WT mice.

Compared to WT NK cells, LFA-1 KO NK cells showed significantly decreased killing of RMA-S, EL-4 and YAC-1, all hematopoietic cells expressing high levels of the LFA-1 ligands ICAM-1 and ICAM-2. Killing of RMA-S or EL-4 was completely abolished in the absence of LFA-1. Similar results were obtained whether NK cells were propagated *in vitro* in the presence of IL-15, IL-15 + IL-12 or IL-15 + IFN-b (Figure 2.4A and C). However, lack of LFA-1 had little or no effect on killing of the non-hematopoietic cell lines Neuro-2a, B16 and CMT-93. The only exception was Neuro-2a, which expressed high levels of ICAM-2 and showed modestly reduced cytotoxicity by LFA-1 KO NK cells (Figure 2.4B and C).

Unlike LFA-1 KO NK cells, Mac-1 KO NK cells displayed only minimal defects in cytotoxicity. Most strikingly, killing of RMA-S, EL-4 and YAC-1 were not affected. Nonetheless, cytotoxicity towards B16 and CMT-93, but not Neuro-2a, was slightly reduced (Figure 2.6).

Hence, in combination, these results showed that LFA-1, but not Mac-1, was critical for killing of RMA-S, EL-4 and YAC-1, which all expressed high levels of ICAM-1 and ICAM-2 and are hematopoietic. However, LFA-1 had little or no impact on killing of B16 and CMT-93, which expressed little or no ICAM-1 and ICAM-2. A partial role of LFA-1 was noted towards killing of

Neuro-2a that correlated with higher expression levels of ICAM-2 on these targets. Mac-1 was partially required for cytotoxicity towards B16 and CMT-93.

2.3.4. LFA-1-dependent cytotoxicity requires expression of ICAM-1 and ICAM-2 on targets

To confirm that ICAM-1 and ICAM-2 expressed on targets were necessary for LFA-1-dependent killing, we generated RMA-S and EL-4 cells lacking ICAM-1 alone (ICAM-1 KO), or ICAM-1 and ICAM-2 (ICAM-1,2 dKO), using CRISPR-Cas-mediated genome editing. Target cells transfected with an empty plasmid were used as control (CT). Polyclonal population of target cells lacking these ligands were used for experimentation (Figure 2.7A and B).

Using WT NK cells as effector cells, we observed that lack of ICAM-1 alone on RMA-S or EL-4 resulted only in a modest defect in NK cell cytotoxicity. However, loss of both ICAM-1 and ICAM-2 led to the total loss of NK cell cytotoxicity (Figure 2.7C–F). Similar findings were made with NK cells propagated in the presence of IL-15, IL-15 + IL-12 or IL-15 + IFN-b. This phenotype was analogous to that observed with LFA-1 KO NK cells.

We previously reported that loss of SFRs, in particular 2B4, on NK cells resulted in augmented killing of hematopoietic tumour cells through an LFA-1-dependent mechanism. To determine if ICAM-1and ICAM-2 were triggering LFA-1 in this setting, similar experiments were conducted using NK cells from mice lacking all SFRs (SFR KO mice). As reported, compared to WT NK

cells, SFR KO NK cells exhibited augmented killing towards RMA-S or EL-4. This effect was fully abolished when ICAM-1,2 dKO target cells were used (Figure 2.7G-J).

Thus, ICAM-1 and ICAM-2 were the ligands triggering LFA-1 during killing by WT NK cells. They were also responsible for the augmented cytotoxicity by SFR KO NK cells.

2.3.5. LFA-1 is coupled in part to FcRγ to promote natural cytotoxicity

The mechanism by which LFA-1 promotes actin polarization leading to granule polarization and cytotoxicity implicates the associated β2 subunit CD18, as well as the kinase Syk and Pyk2 (24, 38). However, the effectors that are immediately downstream of LFA-1, and that are suppressed by 2B4, are poorly understood.

We recently reported that, in macrophages, the CD18-coupled integrin Mac-1 promotes phagocytosis by coupling to the ITAM-containing subunits FcRγ and DAP-12. To identify if similar ITAM-containing subunits were immediate effectors of LFA-1 in NK cells, we immunoprecipitated LFA-1 from IL-2-activated WT mouse NK cells and identified potential associated proteins using mass spectrometry. LFA-1 KO NK cells were used as the negative control. Compared to anti-LFA-1 immunoprecipitates obtained from LFA-1 KO NK cells, those generated from WT NK cells displayed several additional proteins (data not shown). These included not only LFA-1, but also some other integrins (integrin α1, α5, β1, β3), SLAMF7, Ly-9, NK1.1, Ly49D,

CD44 and CD69. In particular, they also contained FcRγ, but not DAP-12 or the other ITAM-bearing subunit CD3ζ that are also expressed in NK cells (Figure 2.8A–C). 2B4 was also present. Likewise, LFA-1 and 2B4 were detected in anti-FcRγ immunoprecipitated generated from WT NK cells, but not from FcRγ KO NK cells. Moreover, LFA-1 and FcRγ were noted in anti-2B4 immunoprecipitates obtained from WT NK cells, but not from SFR KO NK cells. We also compared the proteins recovered by immunoprecipitation of LFA-1, FcRγ and 2B4. LFA-1, 2B4 and FcRγ were the only known NK cell activation-related proteins that were present in all three types of immunoprecipitates (Figure 2.8D).

Therefore, LFA-1, as well as its regulator 2B4, were co-immunoprecipitated with FcRγ in mouse NK cells.

2.3.6. FcRγ is partially required for LFA-1-dependent killing

To investigate the potential involvement of FcRγ in LFA-1-dependent cytotoxicity, we performed experiments using commercially-available FcRγ KO mice, which were in a mixed 129-C57BL/6 (B6) background. FcRγ KO mice displayed normal NK cell, T cell and B cell numbers and percentages in spleen (Figure 2.9A), and a normal NK cell percentage in bone marrow (Figure 2.9B). The mice also had normal NK cell development in spleen and bone marrow (Figure 2.9C). As expected, NK cells from FcRγ KO mice had abolished surface expression of the FcRγ-associated receptors NKp46 and CD16 (Figure 2.9D and E). Since the commercial FcRγ KO mice

were generated using 129 background ES cells and the CD244-encoding gene is close to the FcRγ-encoding gene on chromosome 1, 2B4 expressed on FcRγ KO NK cells was the 129 form and could not be detected by the antibody specific to B6 form of 2B4. However, other receptors had little or no alterations of expression on FcRγ KO NK cells (Figure 2.9D and E).

In cytotoxicity assays, FcRγ KO NK cells showed a partial defect in killing of the hematopoietic targets RMA-S and EL-4, compared to WT NK cells (Figure 2.10A and C). Interestingly, this defect was observed in NK cells stimulated with IL-15 or IL-15 + IFN-β, but was rescued by treatment with IL-15 + IL-12. FcRγ-deficient NK cells had normal killing of YAC-1 cells (Figure 2.10A and C), and of the non-hematopoietic cells Neuro-2a, B16 and CMT-93 (Figure 2.10 B and D).

To confirm that the partially decreased killing capacity of FcRγ KO NK cells was not due to the partial 129 background of these FcRγ KO mice, we also generated our own pure B6 background FcRγ KO mouse (named FcRγ KO (B6)), using the CRISPR-Cas-9 KO system. The absence of FcRγ protein expression in KO mice was confirmed by immunoblotting of NK cell lysates (Figure 2.11A). NK cell, T cell and B cell percentages in spleen, as well as NK cell development and differentiation, were comparable to those of WT mice (Figure 2.11B and C). The expression of NK cell receptors, including 2B4, was comparable with that of WT B6 NK cells in both spleen and bone marrow, except for absent expression of NKp46 and CD16 (Figure 2.11D and E). As was

the case of mixed background FcRγ KO NK cells, the NK cells from FcRγ KO (B6) mice showed defective killing of RMA-S and EL-4 cells, but not of other targets (Figure 2.12A-D), and the defect was rescued by IL-12.

Thus, the ability of NK cells to kill LFA-1-dependent target cells was partially dependent on FcRγ.

2.3.7. LFA-1, but not 2B4, is critical for killing of hematopoietic target cells by human NK cells

To assess whether the ability of human NK cells to kill hematopoietic target cells also required LFA-1, similar experiments were performed using various human tumour cell targets and human peripheral blood-derived NK cells. The human target cells analyzed were the B cell lymphoblastoid cell line 721.221, B cell lymphoma cell line Daudi, chronic myeloid leukemia cell line K562 and cervical carcinoma cell line HeLa. 721.221 and Daudi cells expressed ICAM-1, ICAM-2 and SFR ligands, but not NKG2D or DNAM-1 ligands (Figure 2.13B). In contrast, K562 and HeLa cells expressed ICAM-1, ICAM-2, NKG2D ligands and DNAM-1 ligands, but not SFR ligands (Figure 2.13C).

Human peripheral blood-derived NK cells were acquired from healthy human volunteers. These cells showed high expression of CD11a, the α chain of LFA-1, and CD18, the β chain of LFA-1 (Figure 2.13A). Small amounts of CD11b, but not CD11c, were also noted. To address the roles of

the CD11 integrins and CD18 in killing, cytotoxicity assays using blocking antibodies against these integrins were conducted. Antibody blockade of either CD11a or CD18, but not of CD11b or CD11c, dramatically decreased NK cell killing towards hematopoietic cells 721.221, Daudi and K562 (Figure 2.14A and B). A much smaller impact was seen towards HeLa cells. This result suggested that LFA-1 was needed for the ability of human NK cells to mediate cytotoxicity towards tumour cells, in particular hematopoietic cells.

Human peripheral blood-derived NK cells also express 2B4 and its adaptor SAP (Figure 2.13A). Previous studies using anti-2B4 antibody stimulation experiments or overexpression of the 2B4 ligand CD48 on target cells suggested that, unlike in the mouse where 2B4 is inhibitory, 2B4 is an activating NK cell receptor in humans. To study genetically the role of 2B4 engagement in human NK cell cytotoxicity, we generated 721.221 variants lacking CD48, using CRISPR-Cas-mediated genome editing. The absence of CD48 expression on 721.221 cells was confirmed by flow cytometry (Figure 2.14C).

In degranulation assays monitoring CD107a exposure, we found that CD48 KO 721.221 triggered an enhanced, rather than a diminished, ability of human NK cells to degranulate, compared to control 721.221 cells (Figure 2.14D). The effect was small, but highly reproducible between donors. It was also accompanied by augmented killing (Figure 2.14E).

Thus, LFA-1, but not 2B4, was critical for killing of hematopoietic target cells by human NK cells.

2.4. Discussion

In this chapter, we demonstrated that three primary NK cell receptors contributed to the activation of NK cell cytotoxicity, namely NKG2D, DNAM-1 and LFA-1, and that their contribution varied depending on the targets, largely relating to the ligand expression pattern on target cells.

In particular, using NK cells from mice lacking NKG2D, DNAM-1 or both, we found that NKG2D was critical for killing of target cells expressing NKG2D ligands (Figure 2.15A). This was true both for hematopoietic and for non-hematopoietic cell targets. The function of NKG2D could not be fully replaced by DNAM-1, in cases of target cells expressing ligands for both receptors. This feature may relate to qualitative differences between the signals triggered by NKG2D and DNAM-1. Although both receptors can couple to PI 3' kinase via the ITT motif of NKG2D-associated DAP-10 or the DNAM-1-intrinsic ITT motif, NKG2D can also couple to the ITAM-bearing subunit DAP-12, which triggers a more diverse type of signals involving the Syk and Btk protein tyrosine kinases .

Nonetheless, the defect caused by loss of DNAM-1 in NK cells accentuated the defect caused by loss of NKG2D, implying that DNAM-1 partially compensated for NKG2D when targets expressed ligands for both receptors and expression of NKG2D was eliminated. This partial compensation may relate to the shared coupling of these two receptors to PI 3' kinase via the ITT motifs.

Our data with NK cells devoid of NKG2D and DNAM-1 showed that, in the absence of engagement of NKG2D and DNAM-1, killing of the target cells studied was largely dependent of the integrin LFA-1 (Figure 2.15B). This was especially true for hematopoietic target cells, which more frequently expressed the LFA-1 ligands ICAM-1 and ICAM-2, compared to non-hematopoietic target cells. This notion was supported by analyses of LFA-1 KO NK cells or mutant target cells lacking ICAM-1 and ICAM-2. It was also consistent with our analyses of human blood-derived NK cells treated with blocking anti-LFA-1 antibodies. In contrast to LFA-1, the related integrin Mac-1 (CD11b/CD18) had little or no impact on NK cell killing.

Although LFA-1 is known to trigger signals via CD18 that lead to granule polarization in NK cells, the immediate effectors of CD18 are not well understood. Considering the data that the related integrin Mac-1 is coupled to ITAM-bearing subunits required for its function in macrophages and neutrophils (31, 39), we assessed if LFA-1 could be co-immunoprecipitated with ITAM-containing chains in NK cells. We observed that LFA-1 was co-immunoprecipitated with FcRγ in NK cells, and vice-versa. Moreover, analyses of two different FcRγ KO mice showed that NK cell-mediated killing of LFA-1-dependent targets RMA-S and EL-4 was partially compromised by FcRγ deficiency. Hence, we propose that LFA-1 signalling leading to cytotoxicity implicated in part FcRγ.

LFA-1 and FcRγ were also co-immunoprecipitated with 2B4, which we previously reported suppresses NK cell activation by inhibiting the activating function of LFA-1 via the lipid phosphatase SHIP-1. Hence, it is possible that 2B4-recruited SHIP-1 diminished the function of LFA-1 at least in part by inhibiting signalling via FcRγ. ITAM signalling is known to trigger activation of Btk family protein tyrosine kinases, which are documented targets of SHIP-1-mediated inhibition.

As pointed out earlier, published studies have reported that two ITAM-bearing subunits, FcRγ and DAP-12, transduce activating signals for Mac-1; this has been demonstrated in macrophages and neutrophils (31, 39, 40). However, the situation of LFA-1 in NK cell cytotoxicity seems different, since LFA-1-associated proteins in our mass spectrometry studies contained FcRγ, but not DAP-12. Additionally, our unpublished results have suggested that DAP-12-deficient mice have no defect in killing of RMA-S and EL-4 cells. Moreover, we have generated FcRγ-DAP-12 dKO mice, and NK cells from these mice do not show a further decrease in killing of RMA-S or EL-4 cells, compared to FcRγ single KO NK cells (unpublished results). These data are consistent with those of a study by another group, in which NK cells from FcRγ and CD3ζ dKO mice or FcRγ, CD3ζ and DAP-12 triple KO mice showed a partial defect in cytotoxicity towards RMA-S, while DAP-12 KO NK cells had no defect (41). Thus, we assume that ITAM-independent signalling exists within the LFA-1-mediated signalling pathway. Indeed, some studies found that integrin receptors could transduce signals through ITAM-independent pathways by directly binding Syk through

CD18 (29, 30, 42). This may explain why NK cells lacking ITAMs can still exhibit partial LFA-1-dependent cytotoxicity. In the future, it will be important to find out which pathway is responsible for this ITAM-independent LFA-1 signalling.

We found that the decreased cytotoxicity of FcRγ KO NK cells could be rescued via pre-stimulation with IL-12. This indicates that the expression or function of activating receptors or their downstream effectors changed under IL-12 treatment, rendering NK cells less dependent on FcRγ signalling. Further investigation of the molecular changes that resulted in the rescued cytotoxicity of FcRγ-deficient NK cells will be necessary to understand better the downstream signalling effectors of LFA-1.

Previous studies have strongly argued that, unlike in mouse NK cells, 2B4 is an activating receptor in human NK cells. However, this conclusion was based on experiments using anti-2B4 antibodies or target cells ectopically expressing the ligand of 2B4, CD48. No studies using human blood-derived NK cells lacking 2B4 or target cells rendered deficient in CD48 were done. Herein, we found that loss of CD48 on the target cell line 721.221 resulted in an increase, albeit modest, rather than a decrease, in degranulation and killing by human blood-derived NK cells. Hence, these data suggested that, like in mice, 2B4 may be primarily an inhibitory receptor in humans. Generation of *bona fide* 2B4 KO NK human blood-derived NK cells using CRISPR-Cas will aid in validating or not this possibility.

The findings in this Chapter unveil the multiplicity and plasticity of primary activating NK cell receptors and their corresponding pathways, explain the complementary, redundant and coordinating roles of activating receptors in NK cell activation, and provide new insights for future therapeutic interventions against human cancer.

2.5. Materials and methods

2.5.1. Mice

Commercial FcRγ KO (Fcer1g−/−; named FcRγ KO) mice containing 129 background alleles (B6.129P2-Fcer1gtm1Rav N12) were created by targeting the Fcer1g gene in E14 ES cells and injecting the targeted cells into C57BL/6 blastocysts. The mice were obtained from Taconics (Hudson, New York, USA). Our pure B6 background FcRγ-deficient mice (named FcRγ KO (B6)) were generated via CRISPR/Cas9-mediated genome editing using three guide RNA mixtures with the following sequences: 5′-GTCGACAGTAGAGTAGGGTA-3′, 5′-CTTGAGTCGACAGTAGAGTA-3′ and 5′-GCCGCAGCTCTGCTATATCC-3′. RNA was injected into fertilized oocytes of C57BL/6J mice. FcRγ-deficient mice were then screened for reduced CD16 expression on splenocyte NK cells, followed by PCR amplification and sequencing of the Fcer1g locus. Immunoblotting was performed to confirm that the FcRγ protein was absent in the NK cells. Mice lacking LFA-1 (CD11a; Itgal−/−), or Mac-1 (CD11b; Itgam−/−) were obtained from the Jackson Laboratory. Mice lacking DNAM-1 (Cd226−/−), or NKG2D (Klrk1−/−) were kindly provided by M. Colonna (Washington University, St. Louis, MO), and D. Raulet (University of California, Berkeley, Berkeley, CA), respectively. DNAM-1/NKG2D double-knockout mice were produced by crossing DNAM-1 knockout mice with NKG2D knockout mice until homozygosity was achieved. All mice were maintained in the C57BL/6J background and kept in specific pathogen-free (SPF) animal facilities. Sex- and age-matched mice between 7 and 16

weeks of age were used for experiments. WT C57BL/6 littermates were used as controls whenever possible. Animal experiments were approved by the Animal Care Committee of the Institut de recherches cliniques de Montréal and performed as defined by the Canadian Council of Animal Care.

2.5.2. Cells

To prepare poly(I:C)-activated NK cells, mice were given intraperitoneal (i.p.) injections of 250 µg poly(I:C) (Sigma-Aldrich) 36 hours before sacrifice. Splenocyte NK cells were purified by positive NK selection using the EasySep™ Mouse CD49b Positive Selection Kit (Catalog No. 18755, STEMCELL Technologies). For the preparation of IL-2-activated LAK cells, positively selected NK cells were cultured in 1,000 U/ml mouse IL-2 (Catalog No. 210-12, PeproTech) for 4 days before functional assays were performed. To prepare NK cells activated by IL-15, IL-15 + IL-12 and IL-15 + IFN-β, freshly isolated splenocytes were processed with negative NK selection using the EasySep™ Mouse NK Cell Isolation Kit (Catalog No. 19855, STEMCELL Technologies). The purified cells were then cultured for 3 days in IL-15 (100 ng/ml; Catalog No. 210-15, PeproTech) with or without the addition of IL-12 (50 ng/ml; Catalog No. 210-12; Peprotech) or IFN-β (2000 U/ml; Catalog No. 12405-1, PBL Assay Science) for overnight stimulation before functional assays were performed.

Human peripheral blood-derived NK cells were acquired from the peripheral blood mononuclear

cells (PBMCs) of healthy volunteers. Red blood cells were removed using Ficoll-Paque Plus (Catalog No. 17-1440-02, GE Healthcare). The PBMCs were then cultured in complete RPMI containing 200 U/ml human IL-2 (Catalog No. 200-02, PeproTech) at 37 °C with 5% CO_2 for 7 days to induce the expansion of NK cells and stimulated overnight with 50 ng/ml of human IL-12 to activate NK cells.

Mouse and human tumour cell lines including B16F10, CMT-93, YAC-1, Neuro-2a, EL4, K562, Hela and Daudi were purchased from the American Type Culture Collection (ATCC). RMA-S was generously provided by the Chambers lab (Karolinska Institute, Sweden). 721.221 was acquired from the International Histocompatibility Working Group (IHWG). Knockout cells were generated by CRISPR-Cas-mediated gene editing using the plasmid pSpCas9(BB)-2A-GFP. K562 cells ectopically expressing CD48 or control were obtained by the transfection of the PFB vector with or without the CD48 gene. YTS-Flag-tagged mouse 2B4 (YTS-Flag-m2B4) and YTS-GFP were generated by our former lab member H. Guo using the PFB vector.

2.5.3. Antibodies

For flow cytometry, the following antibodies and reagents against mouse proteins were purchased from ThermoFisher Scientific: anti-CD27 (LG.7F9), anti-CD122 (TM-b1), anti-Ly49G2 (4D11), anti-Ly49C/I/F/H (14B11), anti-KLRG1 (2F1), anti-NKG2A/C/E (20d5) and anti-Granzyme B (GB11). The following antibodies were purchased from BioLegend: anti-SLAMF1 (TC15-

12F12.2), anti-Ly-9 (Ly9ab3), anti-SLAMF6 (330-AJ), anti-CD84 (mCD84.7), anti-SLAMF7 (4G2), anti-CD3 (145-2C11), anti-NK1.1 (PK136), anti-TCR-β (H57-597), anti-CD11b (M1/70), anti-CD107a (1D4B), anti-DNAM-1 (10E5), anti-NKG2D (CX5), anti-CD11a (M17/4), anti-CD11c (N418), anti-CD18 (M18/2), anti-2B4 (m2B4 (B6) 458.1), anti-CD48 (HM48-1), anti-NKp46 (29A1.4), anti-CD49b (DX5), anti-CD16/32 (93), anti-NKP46 (29A1.4), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD2 (RM2-5), anti-Ly49D (4E5), anti-CD69 (H1.2F3), anti-KI67 (16A8), anti-BCL-2 (BCL/10C4), anti-IFN-γ (XMG1.2), anti-ICAM-1 (YN1/1.7.4), anti-ICAM-2 (3C4 (MIC2/4)), anti-CD155 (TX56), rat IgG1 κ isotype control antibody (RTK2071), Armenian hamster IgG isotype control antibody (HTK888), rat IgG2a κ isotype control (RTK2758), rat IgG2b κ isotype control (RTK4530) and mouse IgG1 κ isotype control antibody (MOPC-21). Anti-CD112 (502-57) was purchased from Abnova. Recombinant mouse NKG2D-Fc chimera protein (Catalog No. 139-NK-050) was purchased from R&D Systems. Anti-CD16/32 (2.4G2) and anti-CD25 (7G7) hybridoma supernatant were generated in A.Veillette's laboratory.

Antibodies against human CD3 (APA1/1), human CD56 (5.1H11), human CD16 (3G8), human SLAMF6 (NT-7), human SLAMF7 (162.1), human SLAMF1 (A12 (7D4)), human CD84 (CD84.1.21), human Ly-9 (HLy-9.1.25), human 2B4 (C1.7), human CD48 (BJ40), human CD11a (HI111), human CD11b (ICRF44), human CD11c (3.9), human CD18 (TS1/18), human CD107a (H4A3), human ICAM-1 (HA58), human ICAM-2 (CBR-IC2/2), human CD155 (TX24), human CD112 (TX31) and matched isotype controls were purchased from BioLegend. Recombinant

human NKG2D-Fc chimera protein (Catalog No. 129-NK-050) was purchased from R&D Systems. Before each staining, human cells were incubated for 30 minutes with Human TruStain FcX™ (Fc Receptor Blocking Solution; Catalog No. 422302, BioLegend) containing staining buffer to block the Fc receptors.

2.5.4. Cytotoxicity assays

Chromium-51 release assays were used to determine the cytotoxicity of NK cells. The target cells were labelled for 1 hour with chromium-51 at 37 °C. Then, 3,000 target cells were incubated with effector cells at a ratio of 1:25, 1:10, 1:5 or 1:1 for 6 hours for poly(I:C)-activated NK cells or 4 hours for IL-2-activated NK cells at 37 °C with 5% CO_2. Then, 50% of the supernatant was harvested and chromium-51 release was measured using a gamma counter. Killing efficiency was determined with the following formula: (experimental release−average of minimal release)/(average of maximal release−average of minimal release) × 100%. Cytotoxicity assays using IL-15 with or without IL-12 and IFN-β-activated NK cells were performed using the same protocol as for IL-2-activated NK cells except that 3,000 labelled target cells were incubated with effector cells at a ratio of 1:10, 1:5 or 1:1 for 4 hours at 37 °C with 5% CO_2. Human peripheral blood-derived NK cell cytotoxicity was measured in the same manner as IL-2-activated mouse NK cytotoxicity except for the use of human NK cells and human target cells. For blocking experiments, 5 mg/ml of purified anti-human (h) CD11a, anti-hCD11b, anti-hCD11c or anti-hCD18 antibodies or isotype controls were added during the incubation of NK cells with targets at

effector/target (E/T) ratios of 25:1 or 5:1.

2.5.5. Degranulation assay

Degranulation assays were performed using FACS analysis by measuring the CD107a expression on the surface of human peripheral blood-derived NK cells after incubating them with target cells at an E/T ratio of 1:1 for 4 hours.

2.5.6. Conjugate Assay

For the conjugate assay, NK cells were stained using the CellTrace™ Violet (CTV) Cell Proliferation Kit (ThermoFisher Scientific; Catalog No. C34557) and targets were stained using the CellTrace™ CFSE Cell Proliferation Kit (ThermoFisher Scientific; Catalog No. C34554) followed by co-incubation for 15 minutes at 37 °C with 5% CO_2. Then, CTV and CFSE double-positive conjugates were measured by flow cytometry.

2.5.7. Immunoprecipitation, immunoblots and mass spectrometry

Immunoprecipitation and immunoblot assays were performed as described previously (39). Mass spectrometry was performed by the Proteomics Core Facility at the Institut de recherches cliniques de Montréal. Protein database searching was achieved with Mascot 2.5 (Matrix Science) and data analysis was conducted using Scaffold (version 3.6).

2.5.8. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously (39). Briefly, YTS-GFP or YTS-Flag-m2B4 were incubated with the same number of K562-puro or K562-CD48 for 30 minutes to form conjugates. The cells were then loaded on poly-d-lysine-coated coverslips. Fixing, blocking and staining were conducted on coverslips. The slices were processed and analyzed using an LSM 710 Confocal Microscope (Zeiss).

2.5.9. Statistical analysis

The GraphPad Prism software (version 9) was used for unpaired or paired Student's t-tests (two-tailed). P values < 0.05 were considered statistically significant.

Figure legends

Figure 2.1 Mouse tumour cells ligands expression pattern

(A) Expression of various ligands on hematopoietic mouse tumour cell lines, including SFR ligands, NKG2D ligands, DNAM-1 ligands and integrin ligands; filled curves, isotype controls. n = 3. (B) Same as A except showing the expression of various ligands on non-hematopoietic mouse tumour cell lines. n = 3.

Figure 2.2 Roles of NKG2D and DNAM-1 in NK antitumour immunity

(A) Expression of NKG2D and DNAM-1 on WT, NKG2D KO, DNAM-1 KO and dKO mouse NK cells. $n \geq 3$. (B) NK cytotoxicity of IL-2-stimulated NK cells from WT, DNAM-1 KO, NKG2D KO, and dKO mice against hematopoietic (RMA-S and YAC-1) and non-hematopoietic (Neuro-2a, B16 and CMT-93) target cells at the indicated effector/target (E/T) ratio. (C) Statistics of results at the 25:1 E/T ratio from independent experiments. $n \geq 3$. (D) and (E) Same as B and C, except using poly(I:C)-stimulated NK cells as the effector cells. $n = 3$. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.3 LFA-1 KO mouse NK cell development and receptor characterization

(A) Percentages and absolute numbers of NK cells ($CD3^-/NK1.1^+$), T cells ($CD3^+/NK1.1^-$) and B cells ($CD3^-/NK1.1^-$) in the spleen of WT and LFA-1 KO mice. n = 3. (B) Percentages of NK cells in the bone marrow of WT and LFA-1 KO mice. n = 3. (C) CD11b and CD27 were stained to check the maturation status of WT and LFA-1 KO NK cells. Stage 1 (S1), $CD11b^-/CD27^-$; Stage 2 (S2), $CD11b^-/CD27^+$; Stage 3 (S3), $CD11b^+/CD27^+$; Stage 4 (S4), $CD11b^+/CD27^-$. The left panel shows a representative analysis and the right panel shows the corresponding statistics. n = 3. (D) Expression of various NK cell receptors on WT and LFA-1 KO splenic NK cells; filled curves, isotype controls. n = 3. (E) Statistics of D. n = 3. (F) and (G) Same as D and E except showing the analysis for bone marrow NK cells. n = 3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.4 LFA-1 promotes NK cell cytotoxicity towards hematopoietic target cells

(A) NK cytotoxicity of NK cells from WT and LFA-1 KO mice stimulated with IL-15, IL-15 + IL-12 or IL-15 + IFN-β towards hematopoietic target cells (RMA-S, EL-4 and YAC-1) at the indicated effector/target (E/T) ratio. n = 4. (B) Same as A except that non-hematopoietic Neuro-2a, B16 and CMT-93 cells were used as targets. n = 4. (C) Statistics of results at the 10:1 E/T ratio from A and B. n = 4. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.5 Mac-1 KO mouse NK cell development and receptor characterization

(A) Percentages and absolute numbers of NK cells ($CD3^{-}/NK1.1^{+}$), T cells ($CD3^{+}/NK1.1^{-}$) and B cells ($CD3^{-}/NK1.1^{-}$) in the spleen of WT and Mac-1 KO mice. n = 3. (B) Percentages of NK cells in the bone marrow of WT and Mac-1 KO mice. n = 3. (C) CD122 and CD49b were stained to check the maturation status of WT and Mac-1 NK cells in the spleen and bone marrow. mNK, $CD122^{+}/C49b^{+}$; iNK, non-double-positive cells. The panels show the mNK and iNK percentages. n = 3. (D) Expression of various NK cell receptors on WT and Mac-1 KO splenic NK cells; filled curves, isotype controls. n = 3. (E) Same as D except showing the analysis for bone marrow NK cells. n=3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.6 Mac-1 has no effect on NK cell cytotoxicity towards ICAMs-expressing target cells

(A) NK cytotoxicity of NK cells from WT and Mac-1 KO mice stimulated with IL-15, IL-15 + IL-12 or IL-15 + IFN-β towards hematopoietic target cells (RMA-S, EL-4 and YAC-1) at the indicated effector/target (E/T) ratio. $n \geq 3$. (B) Same as A except that non-hematopoietic Neuro-2a, B16 and CMT-93 cells were used as targets. $n \geq 5$. (C) Statistics of results at the 10:1 E/T ratio from A. $n \geq 3$. (D) Statistics of results at the 10:1 E/T ratio from B. $n \geq 5$. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.7 LFA-1 ligands ICAM-1 and ICAM-2 are important for LFA-1-dependent killing

(A) Expression of ICAM-1 and ICAM-2 on RMA-S CT, RMA-S ICAM-1 KO and RMA-S ICAM1,2 dKO cells; filled curves, isotype controls. n = 3. (B) Expression of ICAM-1 and ICAM-2 on EL-4 CT, EL-4 ICAM-1 KO and EL-4 ICAM1,2 dKO cells; filled curves, isotype controls. n = 3. (C) NK cytotoxicity of WT and LFA-1 KO NK cells against RMA-S CT, RMA-S ICAM-1 KO and RMA-S ICAM-1,2 KO after various cytokine stimulations. n = 3. (D) Same as C except that EL-4 CT, EL-4 ICAM-1 KO and EL-4 ICAM-1,2 KO were used as targets. n = 3. (E) Statistics of results at the 10:1 E/T ratio from C. n = 3. (F) Statistics of results at the 10:1 E/T ratio from D. n = 3. (G) Same as C except that WT and SFR KO were used as effector cells and RMA-S CT and RMA-S ICAM-1,2 KO were used as targets. (H) Same as G except that EL-4 CT and EL-4 ICAM-1,2 KO were used as targets. (I) Statistics of results at the 10:1 E/T ratio from G. n = 3. (J) Statistics of results at the 10:1 E/T ratio from H. n = 3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.8 Associations between LFA-1, 2B4 and FcRγ

(A) Mass spectrometric analysis of anti-LFA-1 immunoprecipitates from WT or LFA-1 KO NK cells. The means of the normalized total ion current (TIC) for the potential interactors are shown. n = 3. (B) Same as A except that 2B4 was immunoprecipitated from WT or SFR KO NK cells. n = 3. (C) Same as B except that FcRγ was immunoprecipitated from WT or FcRγ KO mice. n = 3. (D) The Venn diagram shows the number of proteins that existed in two or three immunoprecipitates.

Figure 2.9 FcRγ KO mouse NK cell development and receptor characterization

(A) Percentages and absolute numbers of NK cells ($CD3^-/NK1.1^+$), T cells ($CD3^+/NK1.1^-$) and B cells ($CD3^-/NK1.1^-$) in the spleen of WT and FcRγ KO mice. n = 3. (B) Percentages of NK cells in the bone marrow of WT and FcRγ KO mice. n = 3. (C) CD11b and CD27 were stained to check the maturation status of WT and FcRγ KO NK cells. Stage 1 (S1), $CD11b^-/CD27^-$; Stage 2 (S2), $CD11b^-/CD27^+$; Stage 3 (S3), $CD11b^+/CD27^+$; Stage 4 (S4), $CD11b^+/CD27^-$. The left panel shows a representative analysis and the right panel shows the corresponding statistics. n = 3. (D) Expression of various NK cell receptors on WT and FcRγ KO splenic NK cells; filled curves, isotype controls. n = 3. (E) Same as D except showing the analysis for bone marrow NK cells. n = 3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.10 FcRγ is important for LFA-1-dependent killing

(A) NK cytotoxicity of NK cells from WT and FcRγ KO mice stimulated with IL-15, IL-15 + IL-12 or IL-15 + IFN-β towards hematopoietic target cells (RMA-S, EL-4 and YAC-1) at the indicated effector/target (E/T) ratio. n = 3. (B) Same as A except that non-hematopoietic Neuro-2a, B16 and CMT-93 cells were used as targets. n = 3. (C) Statistics of results at the 10:1 E/T ratio from A. n = 3. (D) Statistics of results at the 10:1 E/T ratio from B. n = 3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.11 Charcterization of FcRγ KO (B6) mice

(A) Immunoblotting shows the FcR-γ protein expression in WT and FcRγ KO (B6) mice; β-actin served as a control. n > 3. (B) Percentages of NK cells ($CD3^-/NK1.1^+$), T cells ($CD3^+/NK1.1^-$) and B cells ($CD3^-/NK1.1^-$) in the spleen of WT and FcRγ KO (B6) mice. n = 3. (C) CD11b and CD27 were stained to check the maturation status of WT and FcRγ KO NK cells. Stage 1 (S1), $CD11b^-/CD27^-$; Stage 2 (S2), $CD11b^-/CD27^+$; Stage 3 (S3), $CD11b^+/CD27^+$; Stage 4 (S4), $CD11b^+/CD27^-$. The left panel shows a representative analysis, and the right panel shows the corresponding statistics. n = 3. (D) Expression of various NK cell receptors on WT and FcRγ KO (B6) splenic NK cells; filled curves, isotype controls. n = 3. (E) Same as D except showing the analysis for bone marrow NK cells. n = 3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.12 FcRγ KO (B6) NK cells showed the same defect on LFA-1-dependent killing as was the case of mixed background FcRγ KO NK cells

(A) NK cytotoxicity of NK cells from WT and FcRγ KO (B6) mice stimulated with IL-15, IL-15 + IL-12 or IL-15 + IFN-β towards hematopoietic target cells (RMA-S, EL-4 and YAC-1) at the indicated effector/target (E/T) ratio. n = 3. (B) Same as A except that non-hematopoietic Neuro-2a, B16 and CMT-93 cells were used as targets. n = 3. (C) Statistics of results at the 10:1 E/T ratio from A. n = 3. (D) Statistics of results at the 10:1 E/T ratio from B. n = 3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$; ns (not significant) $P > 0.05$.

Figure 2.13 Charcterization of human peripheral blood NK cells and human tumour cell lines

(A) Expression of receptors and SAP adaptor on human peripheral blood NK cells; filled curves, isotype controls. n = 3. (B) Expression of various ligands on SFR^+ human tumour cell lines, including SFR ligands, NKG2D ligands, DNAM-1 ligands and integrin ligands; filled curves, isotype controls. n = 3. (C) Same as B except showing the expression of various receptors/ligands on SFR^- human tumour cell lines. n = 3.

Figure 2.14 LFA-1 but not 2B4 is critical for killing in human peripheral blood NK cells

(A) Human NK cell cytotoxicity towards SFR^+ targets 721.221 and Daudi in the presence of integrin-blocking antibodies or isotype controls. The graph shows the statistics of three independent experiments. (B) Same as A except using two SFR^- targets, K562 and Hela. (C) Expression of CD48 on 721.221 WT control (left panel, black), 721.221 CD48 KO (left panel, red). $n \geq 3$. (D) A degranulation assay was conducted using human NK cells incubated with 721.221 WT or 721.221 CD48 KO cells at a 1:1 ratio. Human NK cells incubated without targets served as an experimental control. Degranulation was determined by surface staining of CD107a. Isotype IgG served as the staining control. The left panel shows a representative result and the right panel shows the statistics. $n = 5$. (E) Cytotoxicity of human NK cells against 721.221 WT or 721.221 CD48 KO at various E/T ratios. The left panel shows a representative result and the right panel shows the statistics in 10:1 E/T ratio. $n = 5$.

Figure 2.15 The role of NK cell receptors in antitumour immunity

(A) NKG2D- and DNAM-1-dependent cytotoxicity. NKG2D and, to a lesser extent DNAM-1, are crucial for killing the targets expressing their specific ligands. (B) LFA-1-dependent cytotoxicity. LFA-1 are important for NKG2D- and DNAM-1-independent NK cytotoxicity. LFA-1 activates NK cells by engaging with ICAMs expressing on targets and transducing activating signalling partially through FcR-γ and its downstream components. The activation of LFA-1 is partially inhibited by 2B4-CD48 inhibitory signalling.

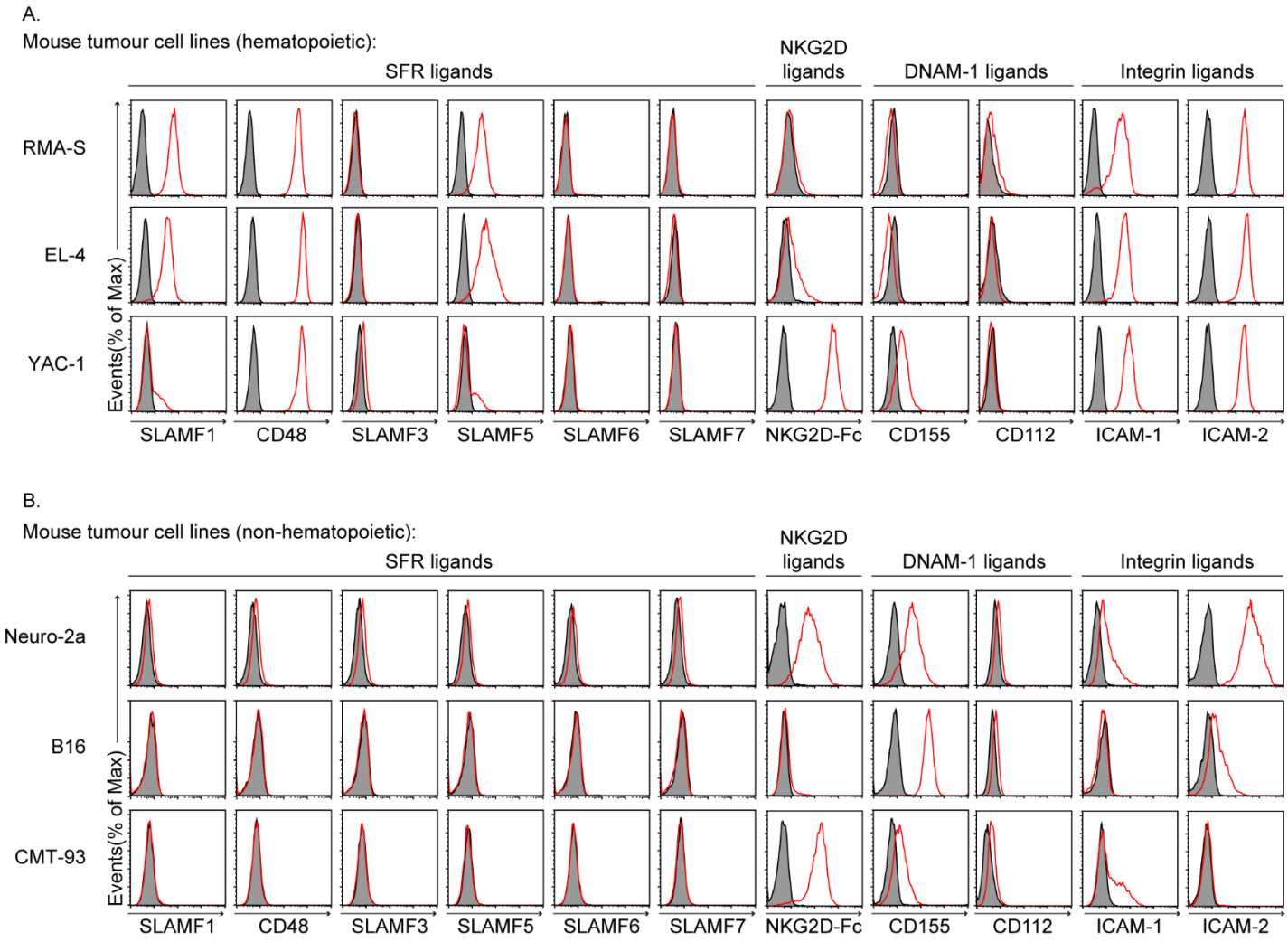

Figure 2.1 Mouse tumour cells ligands expression pattern

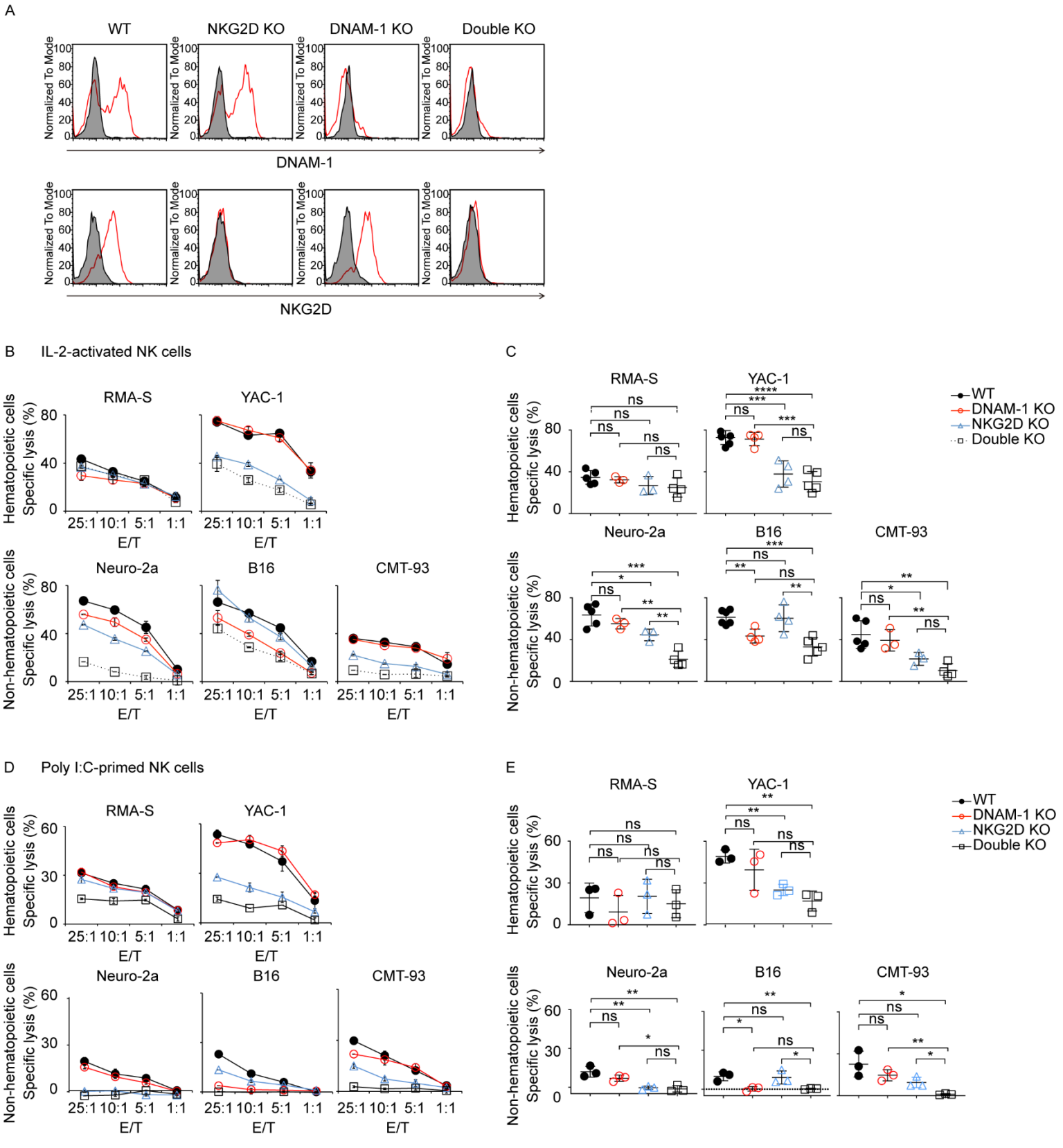

Figure 2.2 Roles of NKG2D and DNAM-1 in NK antitumour immunity

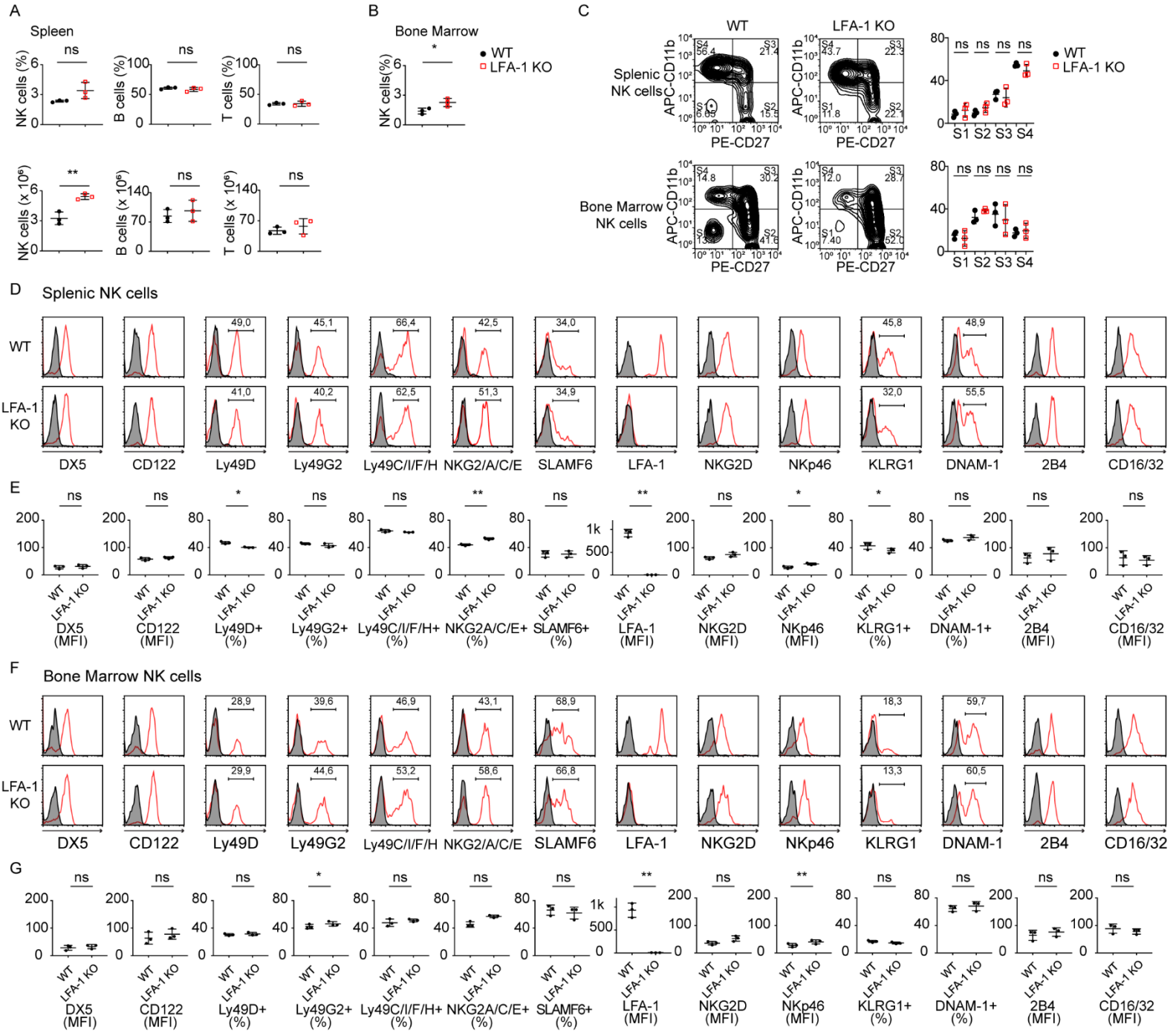

Figure 2.3 LFA-1 KO mouse NK cell development and receptor characterization

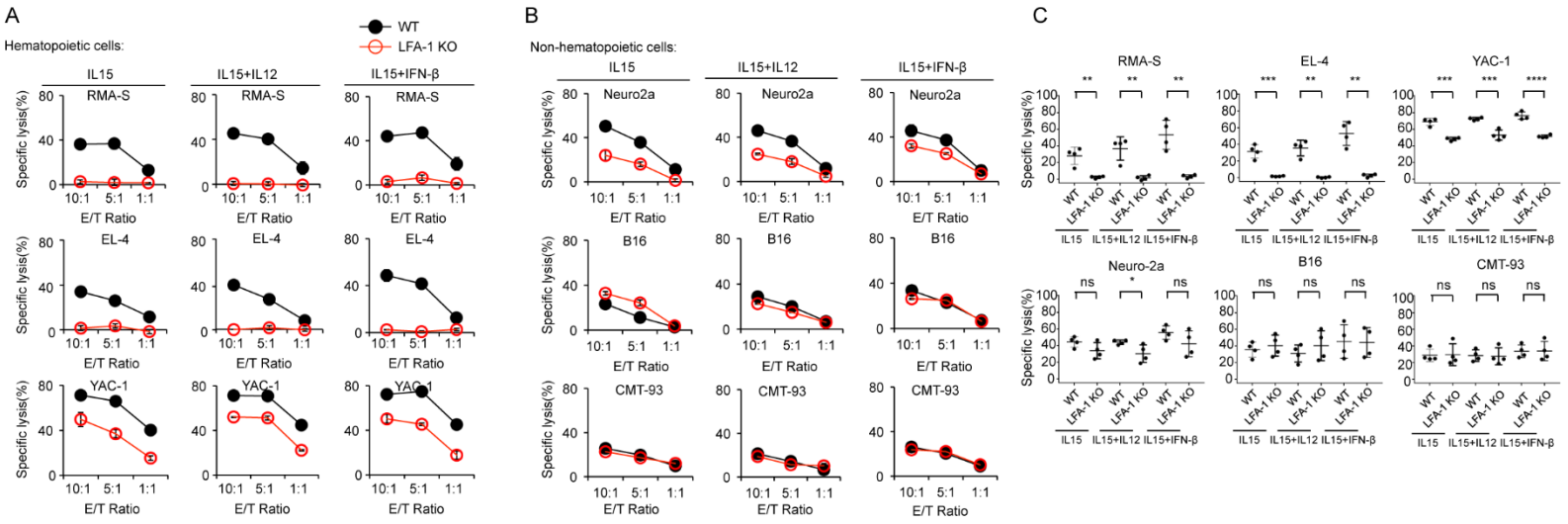

Figure 2.4 LFA-1 promotes NK cell cytotoxicity towards hematopoietic target cells

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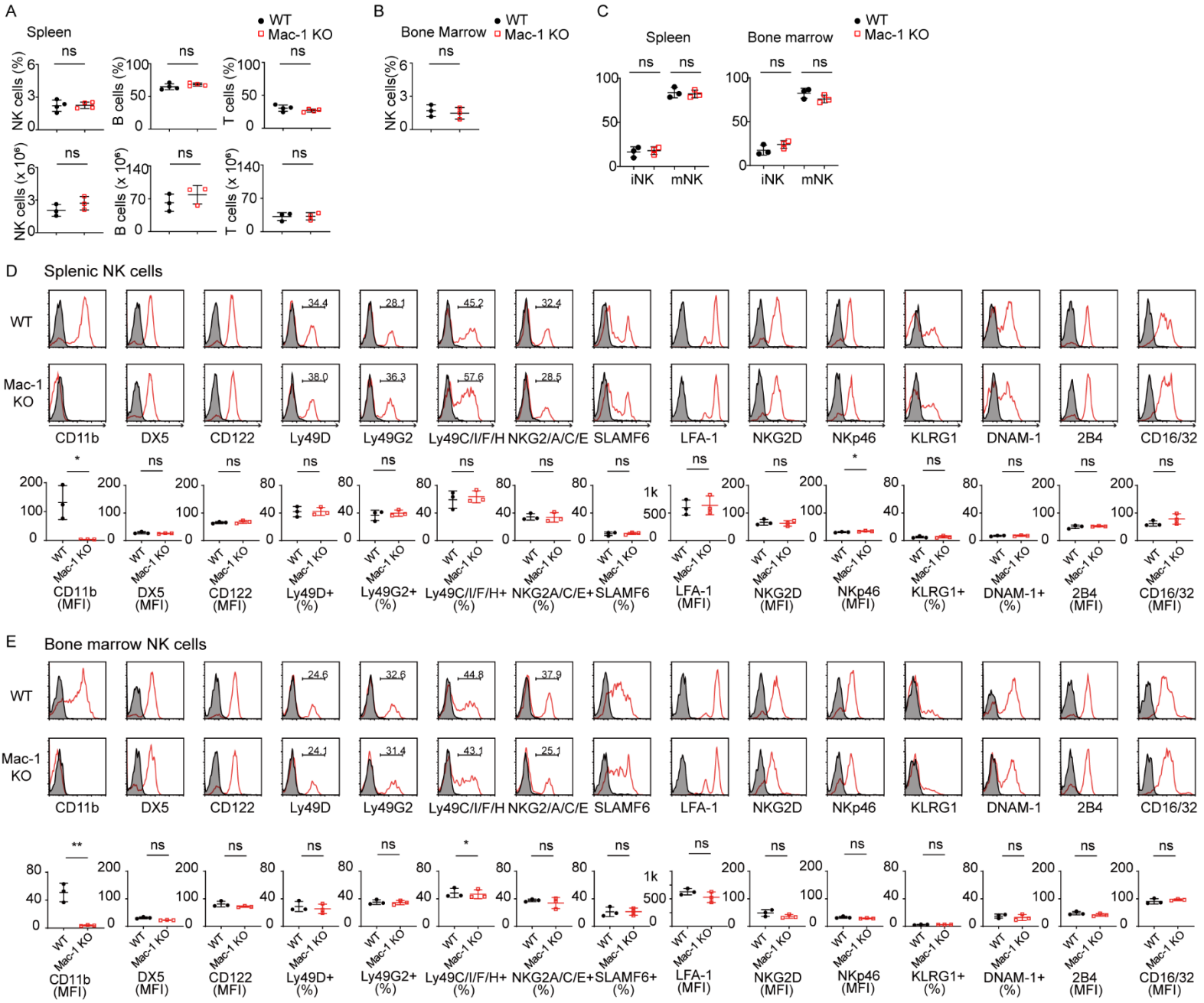

Figure 2.5 Mac-1 KO mouse NK cell development and receptor characterization

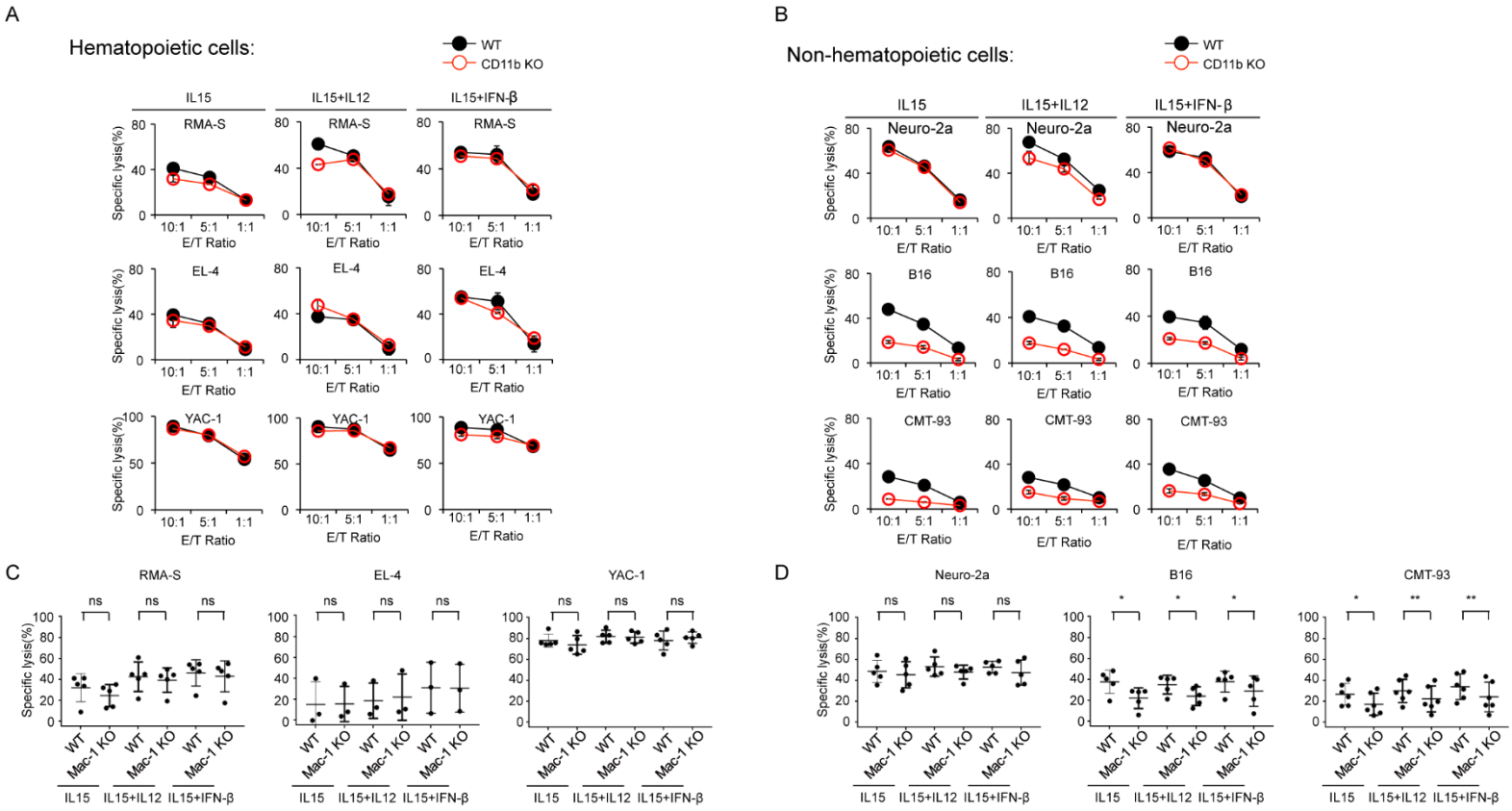

Figure 2.6 Mac-1 has no effect on NK cell cytotoxicity towards ICAMs-expressing target cells

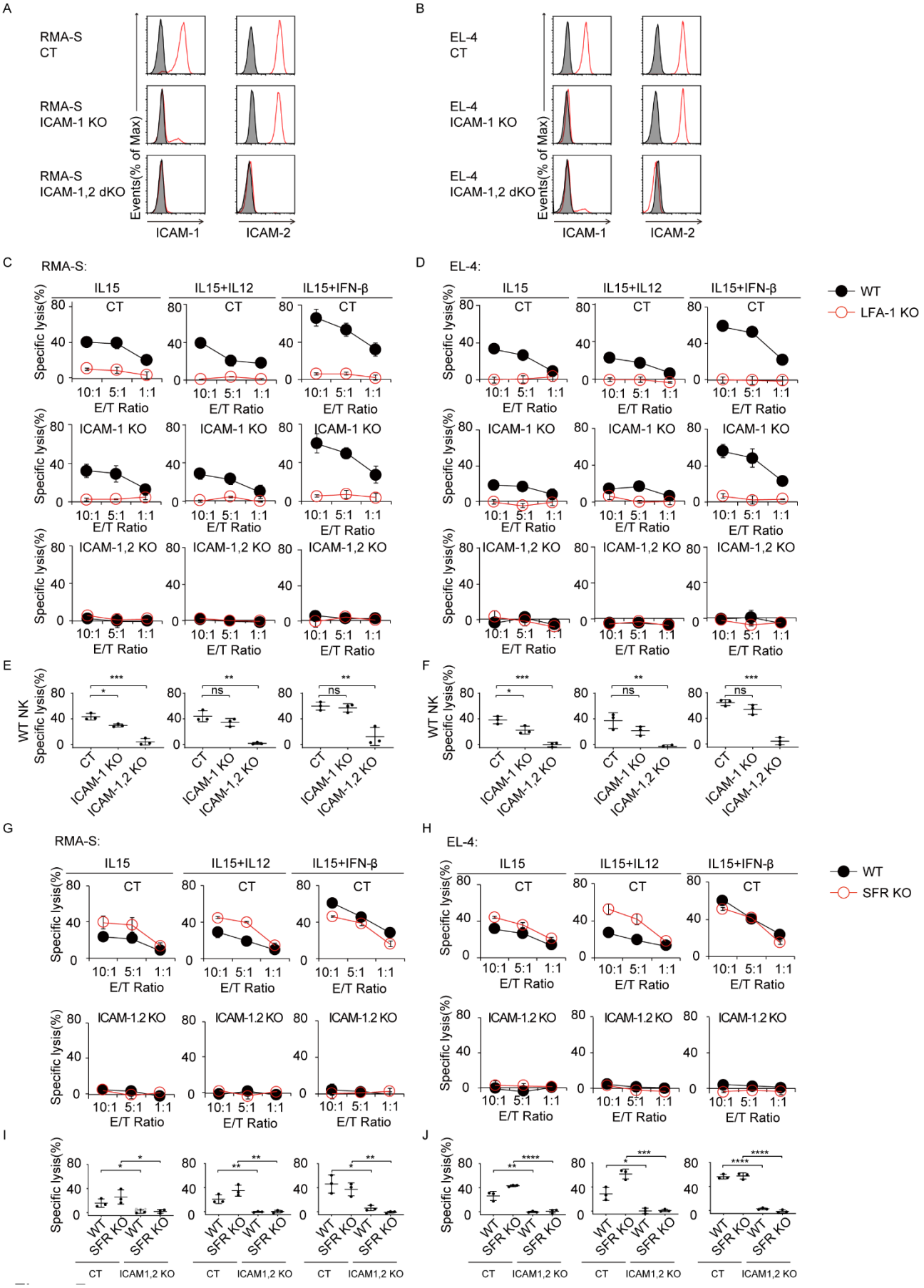

Figure 2.7 LFA-1 ligands ICAM-1 and ICAM-2 are important for LFA-1-dependent killing

A

IP:LFA-1

Identified Proteins	WT	LFA-1 KO
LFA-1	3.68E+09	0
FcRγ	8.52E+06	0
2B4	1.62E+06	0

B

IP:2B4

Identified Proteins	WT	SFR KO
2B4	2.52E+06	0
FcRγ	7.27E+04	0
LFA-1	5.47E+04	0

C

IP:FcRγ

Identified Proteins	WT	FcRγ KO
FcRγ	3.35E+06	0
LFA-1	2.84E+06	0
2B4	2.72E+05	0

D

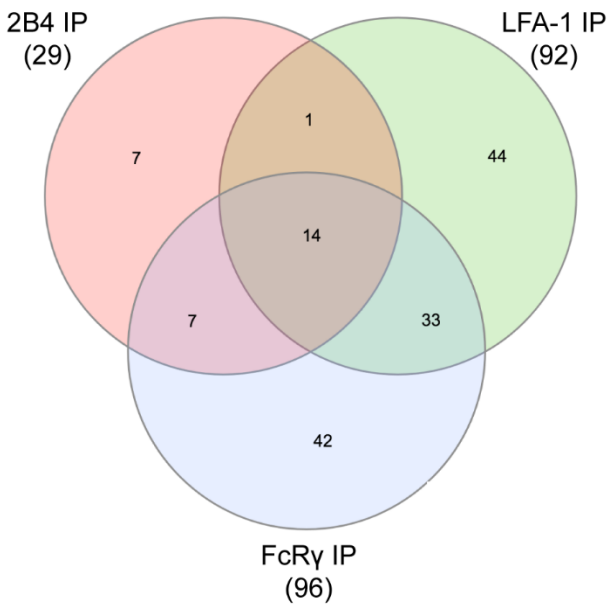

Figure 2.8 Associations between LFA-1, 2B4 and FcRγ

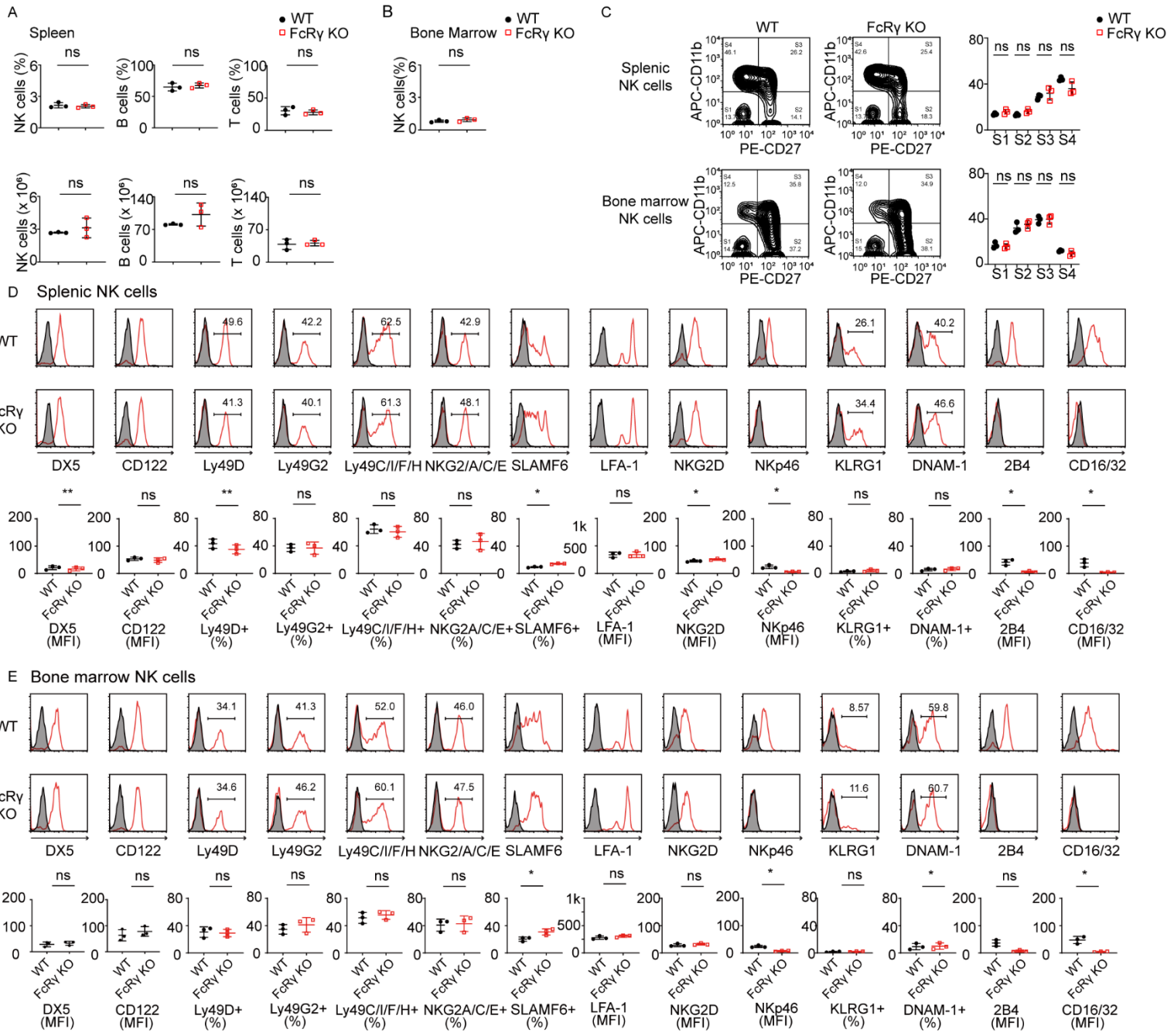

Figure 2.9 FcRγ KO mouse NK cell development and receptor characterization

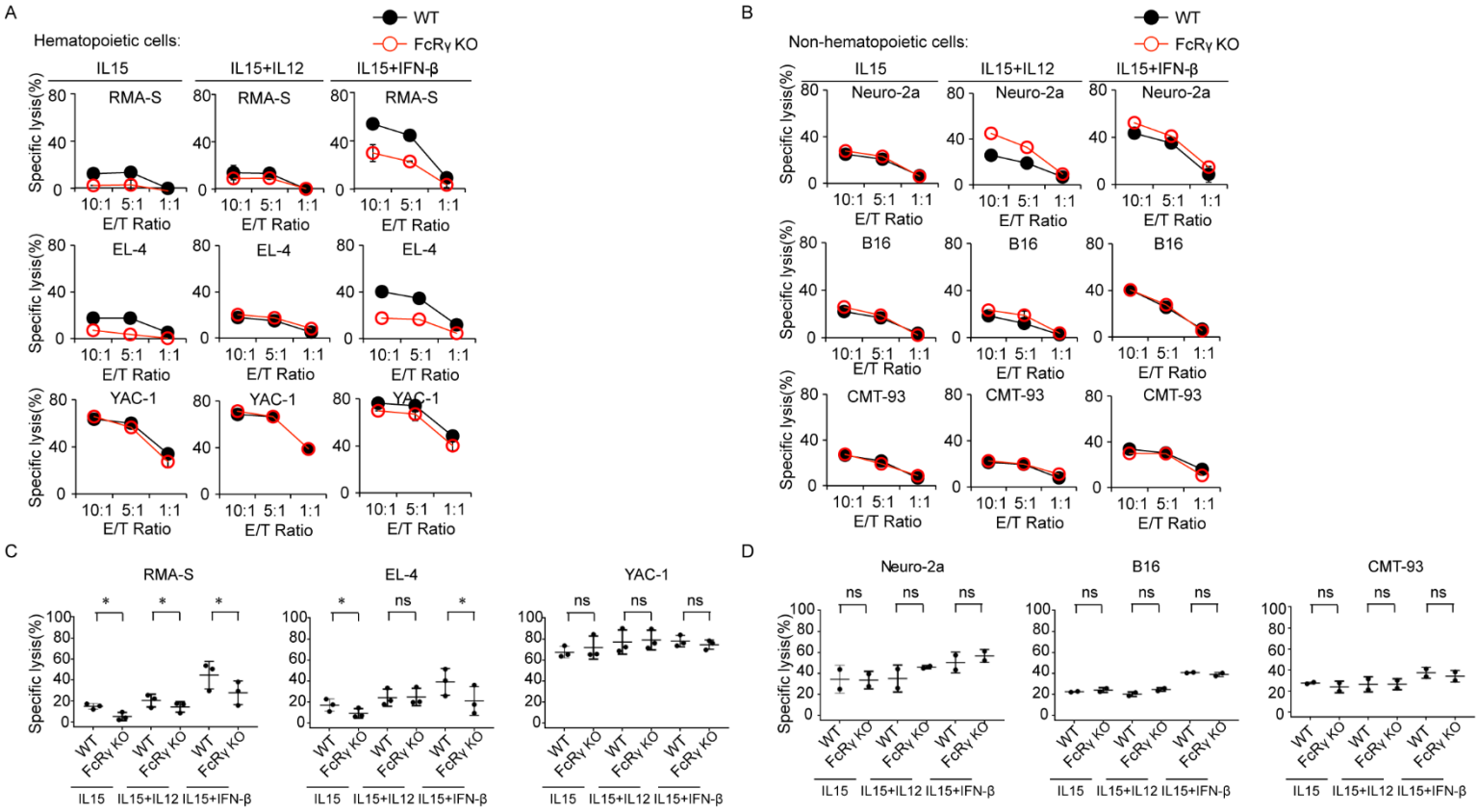

Figure 2.10 FcRγ is important for LFA-1-dependent killing

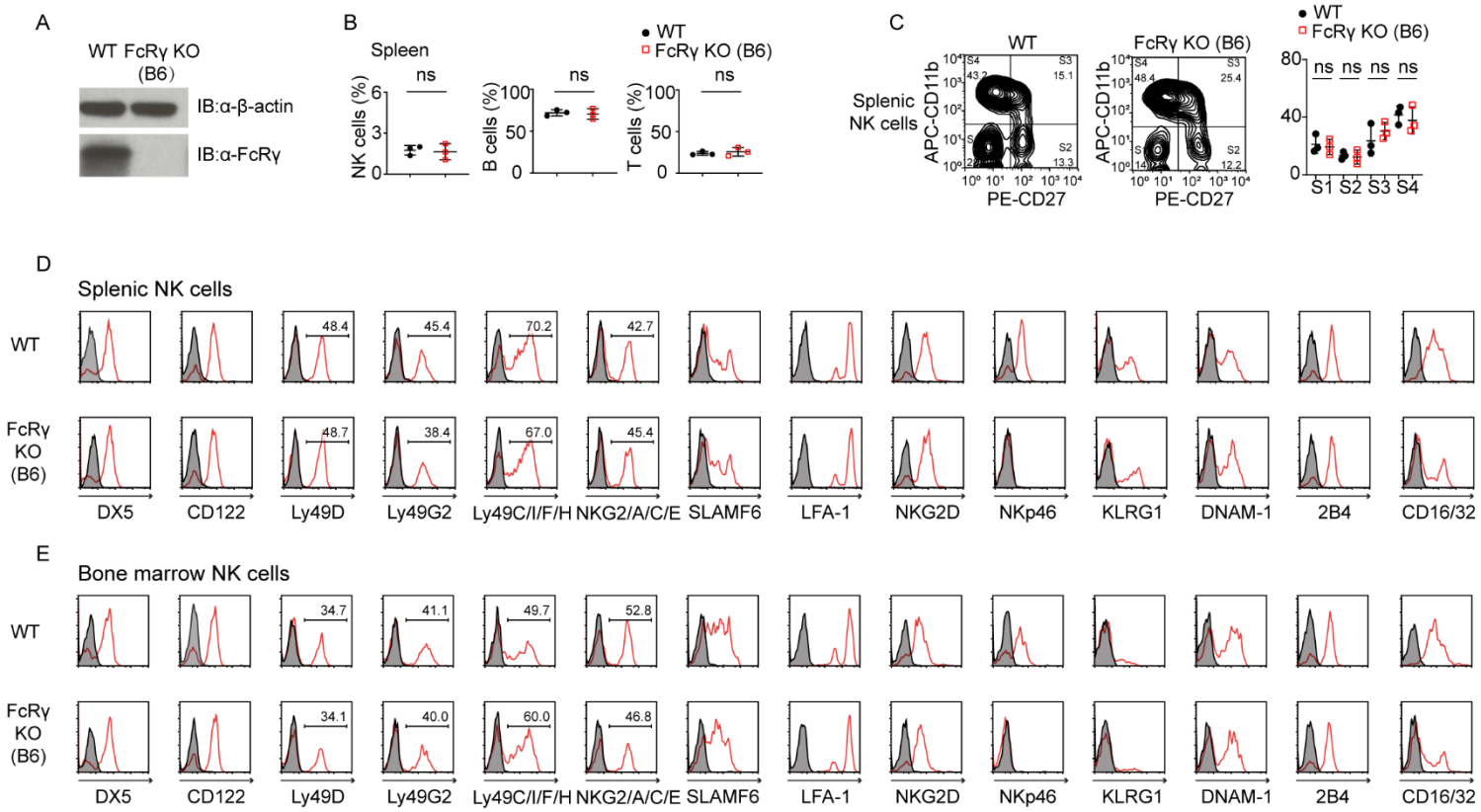

Figure 2.11 Charcterization of FcRγ KO (B6) mice

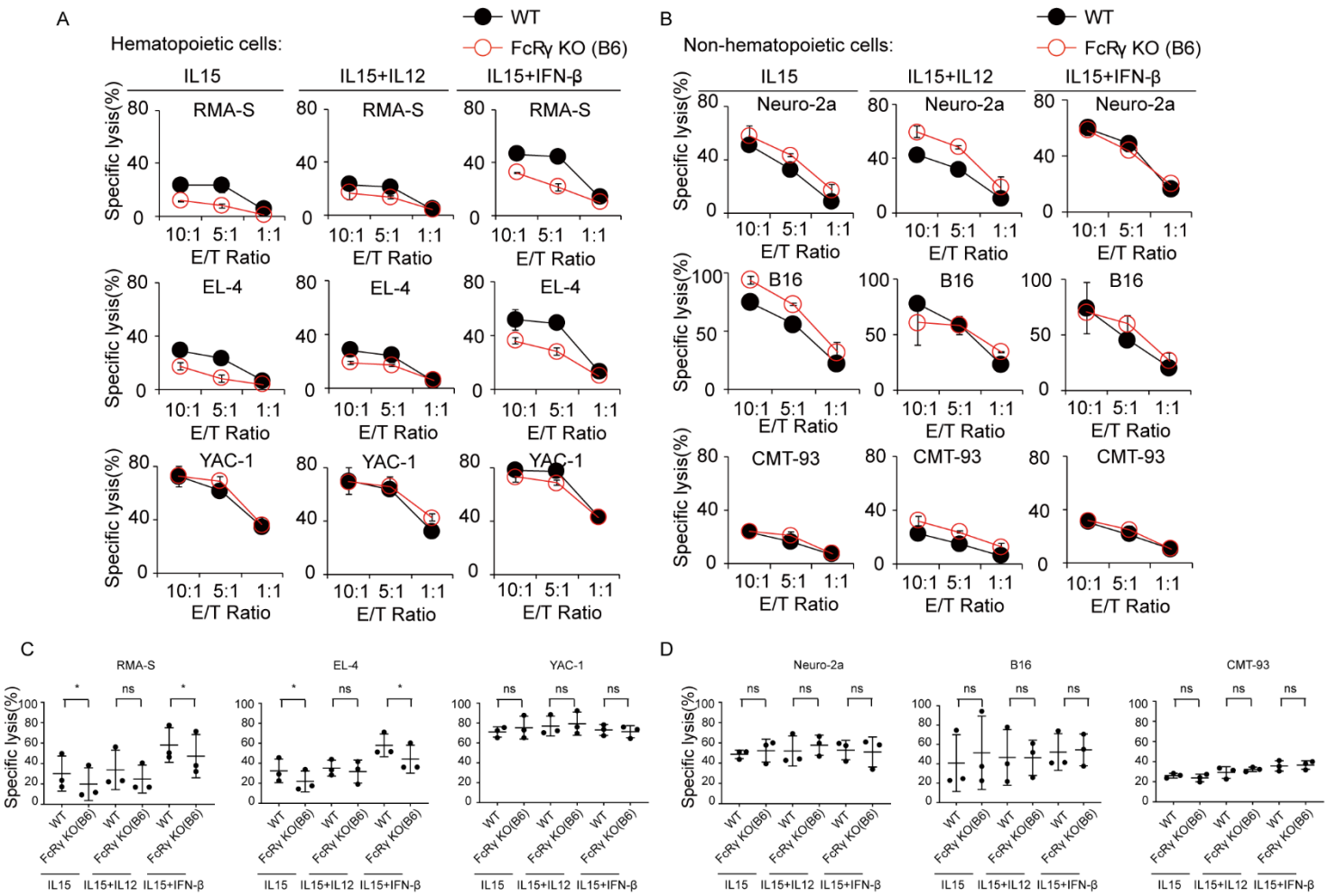

Figure 2.12 FcRγ KO (B6) NK cells showed the same defect on LFA-1-dependent killing as was the case of mixed background FcRγ KO NK cells

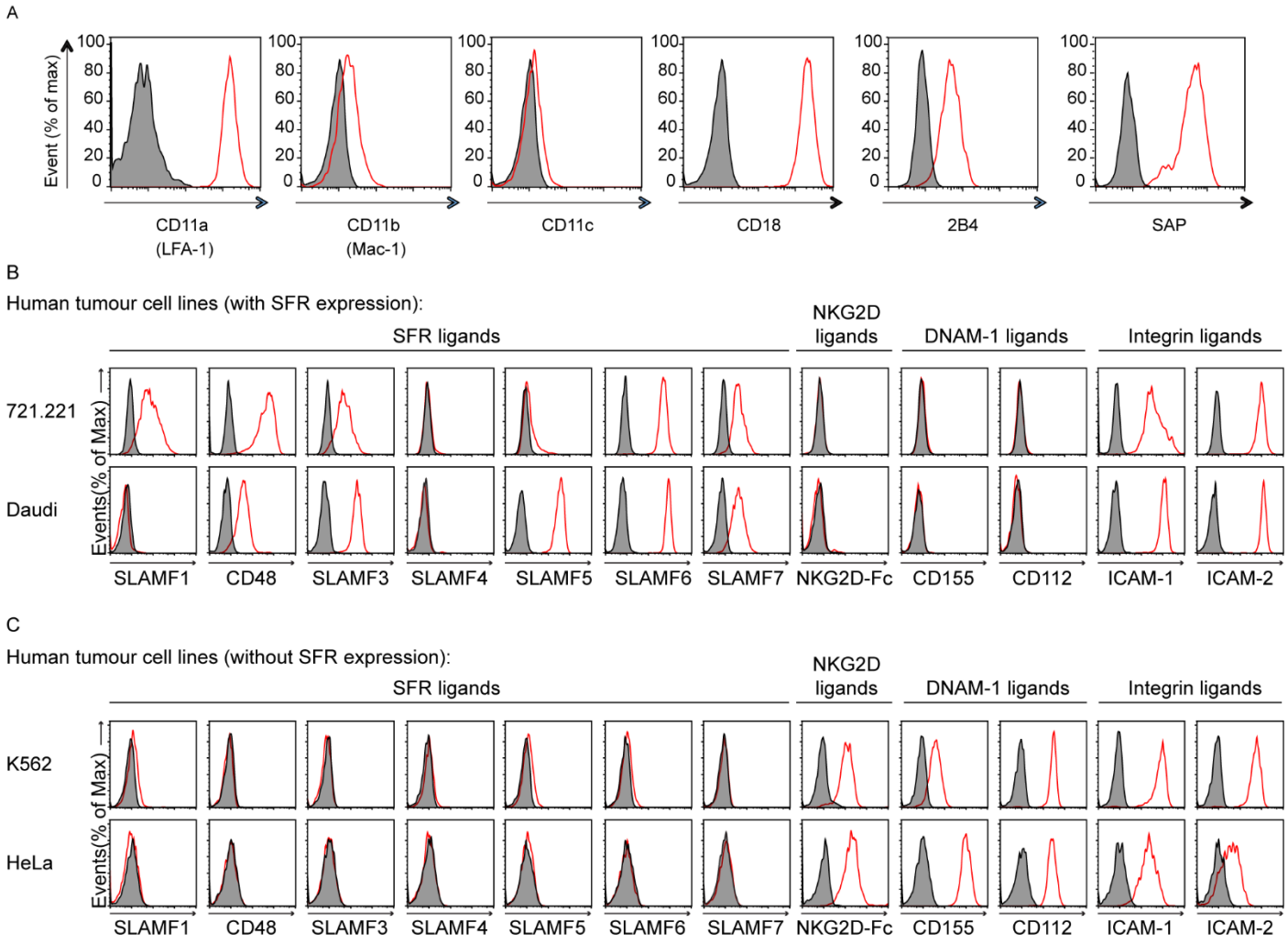

Figure 2.13 Characterization of human peripheral blood NK cells and human tumour cell lines

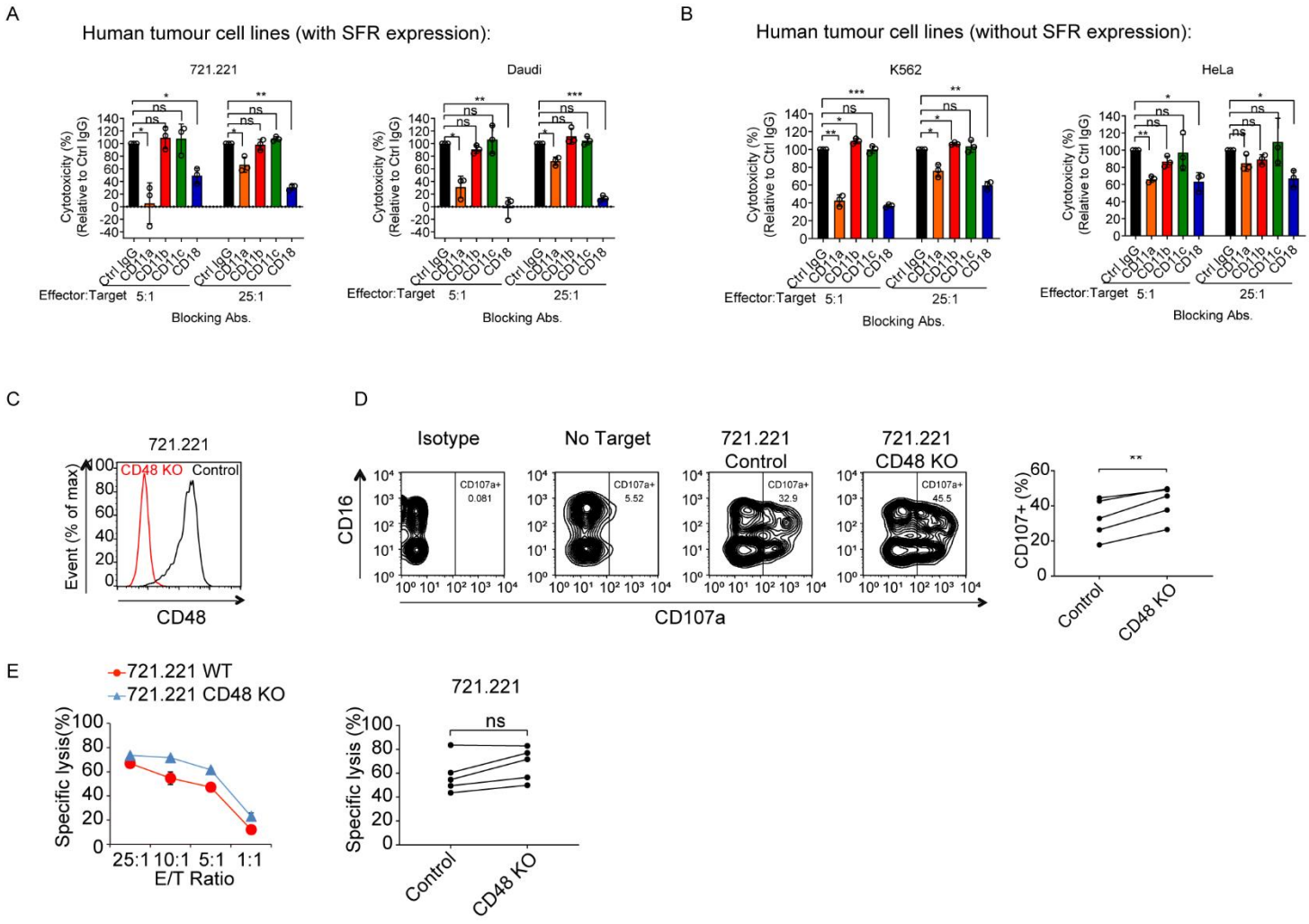

Figure 2.14 LFA-1 but not 2B4 is critical for killing in human peripheral blood NK cells

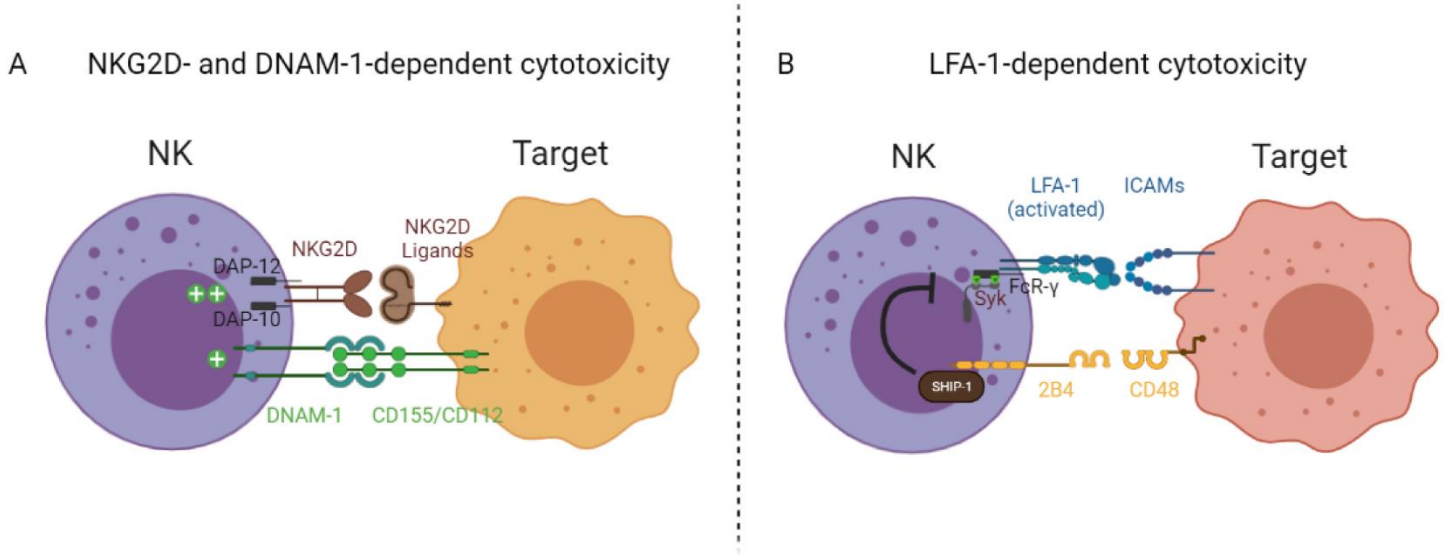

Figure 2.15 The role of NK cell receptors in antitumour immunity

2.6. References

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Chapter 3. The role of SLAM family receptors in stable memory NK cell generation

Preface

The manuscript presented in Chapter 3 is also prepared to submit to journals. It described our study on the functions of SLAM family receptors on memory natural killer cell generation. The study was focused on the antivirus function of natural killer cells.

3.1. Abstract

Natural killer (NK) cells are innate immune cells that were recently found to have adaptive immune features. Upon stimulation by mouse cytomegalovirus (MCMV) infection, a Ly49H$^+$ subset of NK cells exhibits enhanced expansion and long-lived memory features. They were identified as memory NK cells. The signalling lymphocytic activation molecule (SLAM) family of receptors (SFRs) and their ligands play important roles in activating or inhibitory signalling of NK cells and other cells, in both innate and adaptive immune responses. Here, we found that the SLAM family receptor 2B4 and its ligand CD48 were necessary for stable memory NK cell generation. We propose that this function may be executed through the protection of NK cells from being killed by adjacent immune cells by 2B4-CD48-mediated inhibitory signalling. Thus, our study revealed a novel role of the SLAM family receptor 2B4 and its ligand CD48 in stable memory NK cell formation.

3.2. Introduction

Natural killer (NK) cells are innate immune cells that facilitate rapid recipient defences in response to pathogens. NK cells can recognize cellular changes induced by pathogens, and can be activated and eliminate the pathogens without the need for priming. However, recent evidence indicates that some subsets of NK cells also remember the specific antigenic features of invaders for months and years (1-7). Upon re-stimulation by the same antigens, these subsets are activated and expand rapidly and abundantly. These processes are very similar to the adaptive features of T cells and B cells. These subsets were named memory NK cells or memory-like NK cells to highlight these features (2). So far, several types of stimuli have been found able to stimulate NK cells to generate memory. Based on the substance involved in the stimulus, memory NK cells can be categorized into three types: hapten-specific memory NK cells, cytokine-induced memory-like NK cells and virus-specific memory NK cells (8).

As described in Chapter 1, mouse cytomegalovirus (MCMV)-specific memory NK cells are one of the virus-specific memory NK cells discovered in the last two decades. Upon initial MCMV infection, the Ly49H$^+$ NK cells recognize the m157 viral ligand expressed on infected cells via the Ly49H receptor, are activated and expand rapidly. After eliminating the virus, the Ly49H$^+$ NK cells become reduced in number through the contraction period. The Ly49H$^+$ cells remaining after contraction survive for months and years, and they react much faster and generate much more vital

effector functions upon re-infection with MCMV, but not other pathogens.

The signalling lymphocytic activation molecule (SLAM) family of receptors (SFRs) is a group of receptors recently found to have important and complex roles in the immune system (9). SFRs mediate activating or inhibitory effects during NK cell activation. At least in mice, the SLAM family receptor 2B4 (also known as SLAMF4 or CD244) and its ligand (CD48) inhibit NK cell activation by preventing the generation of activated forms of the activating receptor lymphocyte function-associated antigen 1 (LFA-1) (10). This inhibition plays a dual role, either beneficial or deleterious, in recipient health. On the one hand, it can coordinate with other inhibition signals to prevent NK cells, and possibly other immune cells, from killing surrounding normal cells in the steady-state. On the other hand, it decreases cytotoxicity and cytokine production, thus hindering the killing of harmful cells such as tumour cells and immunopathogenic activated cells (10). Whether SFRs have a role in the generation and maintenance of memory NK cells is still unknown.

In this study, we found that SFRs played an essential role in the stable generation of MCMV-specific memory NK cells. In the adoptive transfer model that transfers Ly49H$^+$ NK cells into Ly49H-deficient mice, a lack of 2B4 expression in the recipient immune cells or a lack of its ligand CD48 on the donor NK cells dramatically impaired stable NK cell expansion in response to MCMV infection and significantly decreased MCMV-specific memory NK cell distribution in the blood and organs. The findings showed a critical inhibitory role of 2B4-CD48 in the stable

generation of memory NK cells, possibly by inhibiting NK cell to be killed by surrounding immune cells during NK cell activation.

3.3. Results

3.3.1. NK cells lacking all SFRs fail to expand stably after MCMV infection

To study whether SFRs were implicated in MCMV-specific memory NK cell expansion and contraction, we used a well-established adoptive transfer competition model described elsewhere (11-13). Enriched splenic NK cells from wild-type (WT) mice and knockout (KO) mice lacking one or more SFRs were mixed. Adjustments were made to ensure that the mixture contained equal numbers of WT and KO Ly49H$^+$ NK cells. Cell mixtures were then administered by intravenous (i.v.) injection into Ly49H-deficient recipient (recipient) mice, 1 day before infection (day −1) (Figure 3.1A). An intraperitoneal (i.p.) injection of MCMV followed the next day (day 0). Each week post-infection (p.i.), the proportions of WT and KO Ly49H$^+$ NK cells in the blood of recipient mice were measured by flow cytometry. They were monitored at least until day 35, when long-lived memory NK cells had formed.

The first experiment was performed using a mixture of WT and SFR KO donor NK cells. In blood, WT cells showed robust expansion on day 7 p.i. However, SFR KO Ly49H$^+$ NK cells showed severely impaired expansion, with a significantly lower percentage (reduced by ~95%) than WT cells 7 days p.i. (day 7; Figure 3.1B, left). The defect in the number of SFR KO Ly49H$^+$ NK cells did not recover in the following weeks (Figure 3.1B, right). Splenic NK cells were also isolated on day 35. Splenic SFR KO Ly49H$^+$ NK cells showed the same defect as that observed for blood

NK cells (Figure 3.1C).

To determine which SFR(s) was(were) promoting stable expansion of memory NK cells, we performed experiments with NK cells from mice lacking either SLAMF1, SLAMF5, and SLAMF6 SFRs together as a triple knockout (SLAMF1,5,6 tKO), individual SFRs (2B4 KO, SLAMF6 KO and SLAMF7 KO) or the 2B4 ligand CD48 (CD48 KO) (Figure 3.1D-H). We also tested CD2 KO mice since CD2 can compete with 2B4 for CD48 binding (Figure 3.1I). In some experiments, cells from B6.SJL mice expressing CD45.1 were used as WT controls to distinguish them in flow cytometric assays from KO cells, which expressed CD45.2.

CD48 KO Ly49H$^+$ NK cells displayed the most significant defect in expansion (reduced by ~80-90%, compared to WT) that was very similar to the defect observed with SFR KO NK cells (Figure 3.1F). SLAMF1,5,6 tKO, 2B4 KO, SLAMF6 KO and CD2 KO displayed mild or no differences compared to WT (Figure 3.1D, E, G, I). In fact, as pointed out in the Discussion, SLAMF1,5,6 tKO NK cells seemingly displayed augmented memory NK cell generation. Interestingly, SLAMF7 KO NK cells also showed a significantly decreased proportion (reduced by ~50%) of Ly49H$^+$ NK cells, but to a lesser extent than SFR KO and CD48 KO NK cells (Figure 3.1H).

Hence, CD48 and, to a lesser extent, SLAMF7, but not other SFRs, had critical roles in stable expansion of MCMV-specific memory NK cells. Given these results, we focused the rest of our

study on CD48. The role of SLAMF7 will be studied in the future.

3.3.2. NK cell development and differentiation are normal in CD48 KO mice

To exclude the possibility that loss of CD48 affected NK cell development or differentiation, we evaluated the NK cell development and repertoire of cell surface markers in uninfected CD48 KO mice.

Many NK cell receptors are expressed differentially at the various stages of NK cell development and differentiation (14). We analyzed NKG2D, NKG2A/C/E, DNAM-1 and NKp46, which start being expressed in immature NK (iNK) cells. We also analyzed DX5 (CD49b), Ly49 receptors and KLRG1, which begin to be expressed in mature NK (mNK) cells. Integrin receptor LFA-1, activation marker CD69 and SFRs were also examined. Furthermore, we studied functional NK cell maturation using differential surface expression of CD27 and CD11b (Mac-1), which define stage 1 ($CD27^{-}CD11b^{-}$), stage 2 ($CD27^{+}CD11b^{-}$), stage 3 ($CD27^{+}CD11b^{+}$) and stage 4 ($CD27^{-}CD11b^{+}$) NK cells (14). These studies showed that the receptor repertoire and the development of CD48 KO NK cells were normal, compared to WT NK cells (Figure 3.2A and B).

Thus, the decreased stable expansion of CD48 KO $Ly49H^{+}$ NK cells in response to MCMV infection was not due to changes of NK cell repertoire, differentiation or development.

3.3.3. CD48, but not CD2, is crucial for stable expansion of memory NK cells using CD45.1 and CD45.2 as markers

Recently, researchers found that cell surface proteins can transfer from one cell to another in a process called trogocytosis (15-18). To confirm that the greater percentage of CD48-positive NK cells in the mixing experiments was not due to transfer of the CD48 protein from WT NK cells to CD48 KO NK cells, we used B6.SJL mice as WT mice to distinguish more clearly the WT and the CD48 KO NK cells. WT and CD48 KO NK cells were distinguished by staining with anti-CD45.1, which is expressed on WT B6.SJL-derived NK cells. These studies showed the same defect as the previous experiments with regular B6 mice and CD48 KO mice (Figure 3.2C). In contrast, no defect was seen when WT B6.SJL-derived NK cells were transferred with CD2 KO NK cells (Figure 3.2D).

Thus, the decreased number of $Ly49H^+$ NK cells derived from CD48 KO NK cells was not due to trogocytosis.

3.3.4. The defect in stable expansion of CD48 NK cells in response to MCMV infection is seen in all tissues

MCMV-specific NK cells are reported not to be organ-specific and to be distributed in many lymphoid organs (1, 8). To determine if loss of CD48 affected the tissue distribution of MCMV-specific memory NK cells, experiments were performed using WT and CD48 KO cells as detailed

in Figure 3.1A, except that various tissues were analyzed on day 7 (Figure 3.3A) and day 56 (Figure 3.3B) after MCMV infection.

Ly49H$^+$ donor NK cells were detected in spleen, liver, lung, lymph nodes and bone marrow. As was the case for blood (Figure 3.1F), CD48 KO Ly49H$^+$ NK cells had a significantly decreased proportion compared with WT Ly49H$^+$ NK cells in all the mentioned organs (Figure 3.3A and B).

Hence, the impaired stable expansion of memory NK cells due to a lack of CD48 was not limited to the blood and occurred in all organs.

3.3.5. Promotion of stable expansion of memory NK cells by CD48 is due to a 2B4-dependent inhibitory signal sent to recipient cells

CD48 is a glycoprotein without the cytoplasmic domain, and studies have shown that CD48 functions primarily as a ligand (9, 10). Thus, it was of interest to test whether CD48-dependent memory NK cell expansion was due to cell-intrinsic or cell-extrinsic effects related to its ligand function. CD48 is a ligand for 2B4 and CD2. 2B4 is expressed on NK cells, macrophages, dendritic cells (DCs) and a small subset of T cells, while CD2 is expressed on NK cells and T cells (19). However, the findings of Figure 3.1 showed that loss of CD48, but not 2B4 or CD2, on transferred Ly49H$^+$ NK cells resulted in compromised expansion on memory NK cells (Figure 3.1E, F, I). Similar results were obtained when transferred Ly49H$^+$ NK cells simultaneously lacked 2B4 and

CD2 (Figure 3.4A).

To address if CD48 on Ly49H$^+$ NK cells interacted with 2B4, CD2 or both on recipient immune cells, similar experiments were conducted using WT Ly49H$^+$ NK cells and CD48 KO Ly49H+ NK cells as donor cells, except that they were transferred into Ly49H KO recipients also lacking 2B4, CD2 or both (Figure 3.4B).

When 2B4-Ly49H dKO or 2B4-CD2-Ly49H tKO mice were used as recipients, the differences in the percentages of expanded MCMV-specific memory NK cells between WT and CD48 KO Ly49H$^+$ transferred NK cells disappeared (Figure 3.4C and E). However, when CD2-Ly49H dKO were used as recipients, the reduced expansion of CD48 KO Ly49H$^+$ NK cells, compared to WT Ly49H$^+$ NK cells, persisted (Figure 3.4D).

In keeping with a role of 2B4 on recipient cells, we observed that, upon MCMV infection, 2B4 expression was increased both on donor and on recipient NK cells (Figure 3.4F). However, CD48 expression was elevated on Ly49H$^+$ donor NK cells, but not on Ly49H$^-$ recipient NK cells. CD2 expression was unchanged on all NK cells.

Thus, during MCMV infection, CD48 on Ly49H$^+$ NK cells played a role as the ligand for 2B4 on Ly49H$^-$ recipient cells. This effect was associated with an increased expression of 2B4 on recipient

NK cells. It is known that activated NK cells (10, 13, 20), T cells (21, 22), macrophages (23) and DCs (24) can kill or phagocytose other immune cells. Also, some of these eliminating processes are inhibited by 2B4-CD48 signalling, at least in NK cells (10, 20, 25). We propose that the promotion of stable memory NK cell expansion by CD48 was due to a 2B4-CD48 inhibitory signal sent from CD48 on Ly49H$^+$ NK cells to 2B4 on recipient immune cells, such as recipient NK cells, T cells, macrophages or DCs.

3.3.6. Compromised stable memory NK cell generation in the absence of 2B4-CD48 interaction is infection-dependent but LFA-1-independent

To ascertain if the relative loss of CD48 KO NK cells *in vivo* was triggered by the MCMV infection or was an intrinsic feature of transferred CD48 KO NK cells, we transferred WT/CD48 mixed Ly49H$^+$ NK cells into Ly49H KO recipients without subsequent MCMV infection. We examined the donor NK cells 8 days post cell transfer. Without MCMV stimulation, the NK cells did not expand, and the WT and CD48 KO Ly49H$^+$ percentages were identical (Figure 3.5A). This result indicated that MCMV infection was necessary for the impact of 2B4-CD48 on NK cell expansion.

Although recipient NK cells lacked the MCMV-specific receptor Ly49H, they may still be activated by the cytokines produced by other cells such as macrophages and dendritic cells during MCMV infection (26). To examine whether MCMV infection activated the Ly49H$^-$ recipient NK cells, we tested the expression of activating markers KLRG1 and CD69 after infection. Recipient

NK cells exhibited higher levels of CD69 and KLRG1 than uninfected controls on day 4 and/or day 7 post-infection (Figure 3.5B and C). Interestingly, in contrast to Ly49H$^+$ donor NK cells whose maturation was significantly increased upon MCMV infection as judged by increased proportions of CD27$^-$CD11b$^+$cells, the development of recipient NK cells changed much less after MCMV infection (Figure 3.5D).

Our previous studies found that the inhibitory effect of 2B4 was achieved through inhibition of LFA-1 (10). Thus, it was of interest to check whether loss of memory NK cells in the absence of the 2B4-CD48 interaction was due to compromised LFA-1 function. To this end, we analysed if injection of blocking anti-LFA-1 antibodies influenced the expansion of WT Ly49H$^+$ NK cells during MCMV infection of mice given WT/CD48 mixed donor cells (Figure 3.5E and F). The blocking anti-LFA-1 antibodies did not reduce the greater expansion of WT NK cells in comparison to CD48 KO NK cells.

Therefore, the maintenance advantage provided by expression of CD48 on Ly49H$^+$ NK cells was seen only during infection. Moreover, although recipient NK cells lacked Ly49H in these assays, these cells displayed evidence of activation during MCMV infection, presumably due to cytokines produced during infection. Finally, the less marked accumulation of CD48 KO NK cells during infection did not seem to be dependent on LFA-1. Presumably, other activating receptors were involved, such as NKG2D or DNAM-1.

3.3.7. CD48-deficient NK cells show decreased proliferation and increased death during the memory NK cell expansion phase

A reduction in stable generation of memory NK cells can be due to reduced proliferation, increased death or both. We tested NK cell proliferation by injecting Cell Trace Violet (CTV)-labelled WT/CD48 KO mixed donor cells into the recipients. CD48 KO Ly49H$^+$ NK cells showed less dilution of CTV and lower expression of proliferation marker Ki67 on day 4, indicating slower NK cell proliferation (Figure 3.6A–C). Surprisingly, expression of the activation marker KLRG1 was lower on CD48 KO cells than on WT cells, suggesting that the remaining CD48 KO cells were less activated (Figure 3.6E). However, expression of Ki67 and KLRG1 on CD48 KO cells recovered to the same level as on WT cells by day 7 (Figure 3.6D and F), implying only a transient defect in activation. CD48 KO NK cells also had fewer mature NK cells at stages 3 and 4 and more immature NK cells at stages 1 and 2, on both day 4 and day 7 (Figure 3.6G and H).

We also tested cell death. Compared to WT NK cells, CD48 KO NK cells displayed a mild, albeit not statistically significant, enhancement of activated caspase expression on day 4 that reverted to normal by day 7 (Figure 3.7A and B).

Hence, compared to WT NK cells, CD48 KO NK cells displayed a transient defect in activation and proliferation, as well as perhaps increased cell death, in response to MCMV infection. These

studies are currently being completed through additional experimentation.

3.4. Discussion

NK cells were considered innate immune cells responsible for rapidly eliminating virus-infected cells and tumour cells (27). However, in the last decade, more and more evidence indicates that NK cells can also be involved in adaptive immunity (28-30). These NK cells with adaptive immune features are called memory (or memory-like) NK cells. Memory NK cells have been categorized into three groups based on their routes of activation, namely cytokine-induced memory NK cells, hapten-induced memory NK cells and virus-specific memory NK cells (8), which differ in certain aspects. For instance, hapten-induced memory NK cells are limited to the liver (31, 32), while some virus-specific memory NK cells have been found in many primary organs (1, 8).

Several aspects of MCMV-specific NK cells have been studied, including the expansion phase, contraction phase and memory phase, and functional analyses of MCMV-specific NK cells have been carried out. First, MCMV-specific memory NK cells are mostly derived from a KLRG1-negative progenitor cell population that can be eroded by excessive interleukin (IL)-15 (33). Second, to activate signalling through the Ly49H receptor, its ligand m157 and its adaptor expressed in NK cells, DAP-12, are essential to induce the formation of memory NK cells (1). Third, several other factors are important for the proliferation of MCMV-specific memory NK cells, such as cytokines IL-12, IL-15, IL-18 and type I interferons (IFNs) (13, 34-36); DNAM-1 signalling (7); recombination-activating genes (RAGs) (12); the transcription factor zinc finger

and BTB domain-containing 32 (Zbtb32) (11); microRNA-155 (37); endoplasmic reticulum stress sensor inositol-requiring enzyme 1 (IRE1α) and its substrate, the transcription factor X-box-binding protein 1 (XBP1) (38); and cell-intrinsic adrenergic signalling and Rsad2/Viperin (39). Fourth, after eliminating the virus, the optimal contraction status of memory NK cells is controlled by the proapoptotic factor Bim (40) and BNIP3- and BNIP3L-mediated mitophagy (41). Finally, in the memory phase, memory NK cells can be reactivated and expanded by Ly49H-m157 engagement during re-infection with MCMV and exhibit a stronger response against MCMV infection (1). However, this response diminishes when the memory NK cells encounter other pathogens such as influenza virus or *Listeria* (42). In this study, we discovered the role of 2B4 and its ligand CD48 in MCMV-specific memory NK cell expansion and maintenance. This study adds to the understanding of memory NK cells.

At the beginning of the study, we used a well-established competition adoptive transfer mouse model (7, 11), in which we mixed various KO NK cells with the same number of WT NK cells and transferred them into Ly49H KO mice followed by a low dose of MCMV infection. The promoting or inhibitory function of the designated molecules was evaluated by comparing the proliferated Ly49H$^+$ MCMV-specific NK cell numbers. By using SFR KO NK cells mixed with WT NK cells as donors, we discovered that SFRs had a significant role in promoting memory NK cell expansion. Compared to WT NK cells, SFR KO NK cells showed a dramatic decrease in Ly49H$^+$ NK cell numbers. Further studies using individual SFR KO mice or KOs of the SFR-

related molecules CD48 and CD2 revealed that CD48 on donor NK cells was largely responsible for SFR-dependent memory NK cell expansion. Later, by using 2B4-Ly49H dKO, we found that a lack of 2B4, but not CD2, on recipient cells results in decreased Ly49H$^+$ NK cell expansion upon MCMV infection even for WT donor NK cells. Both 2B4 and CD2 are ligands of CD48. Thus, 2B4 but not CD2 on the recipient cells and CD48 on donor cells were primarily responsible for SFR-dependent memory NK cell expansion. Among the cytotoxic and phagocytic cells, 2B4 is expressed on NK cells, macrophages, DCs and a subset of T cells. Thus, we suspect that the promoting effect was due to 2B4-CD48 interaction between donor NK cells and recipient NK cells or other recipient immune cells expressing 2B4, such as macrophages, DCs or T cells.

From previous studies, we know that, in mice, the 2B4-CD48 interaction provides inhibitory effects towards *in vitro* cytotoxicity against tumour cells and activated CD4$^+$ T cells and *in vivo* regulation of T cells during LCMV infection (10, 20, 43-45). Additionally, another group found that NK cells can kill other NK cells by fratricide killing in an NKG2D-dependent manner upon MCMV infection, when mice lacking type 1 interferons (13). In this light, we hypothesize that the SFR- or 2B4-CD48-dependent promotion of stable Ly49H$^+$ NK cell expansion was through the inhibitory function of 2B4 that prevented recipient immune cells from killing or phagocytosing donor NK cells (Figure 3.8). Indeed, a study showed that 2B4-CD48 binding could inhibit NK cell fratricide killing *in vitro* and *in vivo* (20). We plan to test further this hypothesis in *in vitro* experiments using various WT or 2B4 KO cytotoxic and phagocytic immune cells as effectors and

WT or CD48 KO NK cells as targets.

We also observed a transient defect in activation and proliferation of CD48 KO NK cells in response to MCMV infection. Given the limited ability of CD48 to trigger signals, we presumed that this is not due to any direct impact of CD48 on activation or proliferation. Perhaps, it is because host NK cells preferentially killed the subset of more activated CD48 KO NK cells, which possibly expressed higher levels of ligands for activating receptors. Moreover, less mature NK cells may have lower levels of ligands for activating receptors. These cells could mature after a few days when fratricide killing stopped because the virus was eliminated and the inflammatory environment diminished. This possibility may explain why the surviving CD48 KO NK cells transiently had lower Ki67, KLRG1, proliferation and maturation on day 4 that mostly recovered by day 7.

Because of time constraints, some key experiments remain to be performed. For instance, to find out which recipient immune cells are responsible for the observed effect, we need to verify further our hypothesis by using RAG-IL-2Rg dKO mice as recipients, which are lacking NK, B and T cells. Also, we will need to deplete the individual immune cells on recipient mice by depleting antibodies. If the killing is NK cell-dependent or T cell-dependent, the differences in phenotype between WT and CD48 KO donors should be eliminated in RAG-IL-2Rg dKO mice. If the phenotype is macrophage- or DC-dependent, it should be eliminated by their specific depleting

antibodies. Additionally, in contrast to our previous observations on the role of 2B4 in killing tumour cells in an LFA-1-dependent manner, we found that 2B4-mediated inhibition upon MCMV infection was independent of LFA-1. Thus, it is important to determine which activating receptor(s) mediate(s) this activity. NKG2D is one of the candidate receptors we would like to investigate in future experiments because a recent study discovered that NKG2D was the main activating receptor for killing during MCMV infection (13). Furthermore, more repeats will be needed for some experiments to ascertain statistical significance.

We noticed that the recipient NK cells lacking the MCMV-specific receptor Ly49H could also be activated during MCMV infection, which might be due to the secretion of cytokines by cells such as DCs and macrophages. The activated recipient Ly49H KO NK cells differed in some respects from the activated Ly49H$^+$ donor NK cells. For instance, the Ly49H KO NK cells were less mature than the activated Ly49H$^+$ donor NK cells. Their expression levels of CD69 and KLRG1 also seemed different. In natural MCMV infection, the virus mainly infects DCs (46). However, some studies have suggested that i.p. injection of MCMV can also infect macrophages (47-49). In the future, it will be important to determine which innate immune cells are mainly infected and which cytokines are produced by these cells to activate Ly49H$^-$ NK cells. Our study is consistent with another study, which also found that Ly49H$^-$ NK cells can be activated during MCMV infection (42).

A few questions remain to be solved regarding the functions of SFRs in MCMV-specific memory NK cells. First, we found that a lack of 2B4 on donor immune cells did not lead to enhanced killing of memory NK cells because the total Ly49H$^+$ proportion in the blood did not change (data not shown). Instead, we observed a small but statistically significantly increased proportion of 2B4 KO Ly49H$^+$ NK cells compared to WT Ly49H$^+$ NK cells, which may indicate that NK cells from mice born with a 2B4 deficiency adapted to live with surrounding cells lacking 2B4 and developed an alternate inhibitory pathway to avoid being killed. Second, SLAMF7 KO donor NK cells showed significantly decreased numbers of memory of NK cells upon MCMV infection. It will be interesting to find out whether this is due to SLAMF7 having a direct role in activating memory NK cells or an inhibitory role in protecting memory NK cells from being killed by others and what downstream signalling is involved therein. Third, we observed an increased proportion of MCMV-specific memory NK cells in SLAMF1,5,6 tKO donors, while SLAMF6 KO donor NK cells showed a slightly decreased proportion of memory NKs. This discrepancy between SLAMF1,5,6 tKOs and SLAMF6 KOs suggests that SLAMF1, SLAMF5 or both have an inhibitory effect on memory NK cell expansion. Moreover, their effect can neutralize and mask the minor activating role of SLAMF6. Further study using SLAMF1 and SLAMF5 single KO mice will be necessary to uncover the individual roles of these SFRs.

2B4 is an SFR containing an immunoreceptor tyrosine-based switch motif (ITSM). Depend on the environment, 2B4 can be either inhibitory or activating depending on the environment (50). 2B4-

deficient mice showed enhanced virus-specific $CD8^+$ T cell function in chronic LCMV infection (43, 44). Additionally, our previous studies found that mouse cells lacking 2B4 showed enhanced killing towards hematopoietic targets, including activated $CD4^+$ T cells and macrophages *in vitro* and LCMV-specific activated $CD8^+$ T cells *in vivo* (10). In humans, anti-2B4 antibodies improved the function of $CD8^+$ T cells or $CD4^+$ T cells in response to infections such as hepatitis C virus (HCV) (51), human T lymphotropic virus type 1 (HTLV-1) (52) and tuberculosis (53). In this study, we discovered a new role of 2B4 and its ligand CD48 in promoting stable expansion of memory NK cells during MCMV infection, by inhibiting killing or phagocytosis executed by adjacent immune cells.

Further study will be necessary to elucidate the role of SFRs in the expansion of hapten-induced memory NK cells and cytokine-induced NK cells in more detail and verify their effects in human NK cell models. Researchers have found that NK cells from humans infected with human CMV (HCMV) and cytokine-induced human NK cells, can become long-lived memory features and have therapeutic potential (8). So, it will be interesting to see whether SFRs also have a role in HCMV-infected NK cells and cytokine-induced human memory NK cells. These studies will provide fundamental knowledge informing potential improvements in NK cell therapeutics against infections and perhaps cancer.

3.5. Materials and methods

3.5.1. Mice

Mice lacking all SFRs (SFR KO), SLAMF1, 5, and 6 (SLAMF1,5,6 tKO), individual SFRs (including 2B4 KO, SLAMF7 KO and, SLAMF6 KO), the ligand CD48 (CD48 KO), or CD2 (CD2 KO) have been described elsewhere (10, 50, 54-56). Ly49H KO mice were generated and generously provided by the laboratory of Dr. Silvia Vidal at McGill University, Montreal, Canada (57). B6.SJL (CD45.1) mice and RAG-1 KO mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). 2B4-Ly49H dKO and CD2-Ly49H dKO were generated by breeding the 2B4 KO or CD2 KO mice with Ly49H KO mice until double-deficient homozygosity was achieved. The 2B4-CD2-Ly49H tKO mice were generated by first breeding 2B4 KO mice with CD2 KO mice to generate 2B4-CD2 dKO mice and then breeding 2B4-CD2 dKO mice with Ly49H KO mice until triple-deficient homozygosity was achieved. All mice were maintained in the C57BL/6J background. All mice were kept in specific pathogen-free (SPF) animal facilities. Sex- and age-matched mice between 7 and 16 weeks of age were used for experiments. Littermates were used whenever possible, except for experiments involving B6.SJL mice. Animal experiments were approved by the Animal Care Committee of the Institut de recherches cliniques de Montréal and performed as defined by the Canadian Council of Animal Care.

3.5.2. NK cell enrichment and adoptive transfer

NK cells were enriched from mouse splenocytes using the EasySep™ Mouse NK Cell Isolation Kit (Catalog No. 19855) and EasySep™ magnet (Catalog No. 18000) from STEMCELL Technologies. Depending on batch variation, enriched NK cells had purities ranging from 60% to 95%. Additionally, the percentage of the Ly49H$^+$ subset in total enriched NK cells varied slightly from mouse to mouse. Thus, in the competition experiments, the Ly49H$^+$ NK cell percentage in each enriched donor cell population was determined by fluorescence-activated cell sorting (FACS) and the total number of donor cells injected was individually adjusted to ensure that equal numbers of Ly49H$^+$ NK cells were used. Enriched cell mixtures containing a total of 1.2×10^5 Ly49H$^+$ NK cells equally taken from two donors were injected. In the non-competition experiments, WT (or B6.SJL, depending on the experiment) enriched cells containing 1.2×10^5 Ly49H$^+$ NK cells were injected. To check NK cell proliferation after MCMV infection, NK cells were prelabelled with the CellTrace™ Violet (CTV) Cell Proliferation Kit (Invitrogen, Catalog No. C34557) according to the manufacturer's instructions before injection. In all cell transfer experiments followed by an MCMV challenge, the cells were mixed in PBS and administered to recipients by i.v. injection one day before MCMV infection.

3.5.3. Virus infections

MCMV (Smith strain) was purchased from The Centre for Phenogenomics, Montreal. For the adoptive transfer experiments, mice were infected with 7.5×10^2 plaque-forming units (pfu)

MCMV by i.p. injection one day after cell transfer. MCMV infection experiments were performed in a biosafety level 2 laboratory.

3.5.4. Antibodies

For flow cytometry, the following antibodies and reagents were purchased from ThermoFisher Scientific: anti-CD27 (LG.7F9), anti-CD122 (TM-b1), anti-Ly49G2 (4D11), anti-Ly49C/I/F/H (14B11), anti-KLRG1 (2F1), anti-NKG2A/C/E (20d5), anti-Granzyme B (GB11) and the CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit. The following antibodies were purchased from BioLegend: anti-SLAM (TC15-12F12.2), anti-Ly-9 (Ly9ab3), anti-SLAMF6 (330-AJ), anti-CD84 (mCD84.7), anti-SLAMF7 (4G2), anti-CD3 (145-2C11), anti-NK1.1 (PK136), anti-TCR-β (H57-597), anti-CD11b (M1/70), anti-CD107a (1D4B), anti-DNAM-1 (10E5), anti-NKG2D (CX5), anti-CD11a (M17/4), anti-CD11c (N418), anti-CD18 (M18/2), anti-2B4 (m2B4 (B6) 458.1), anti-CD48 (HM48-1), anti-NKp46 (29A1.4), anti-CD49b (DX5), anti-CD16/32 (93), anti-NKP46 (29A1.4), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD2 (RM2-5), anti-Ly49D (4E5), anti-CD69 (H1.2F3), anti-KI67 (16A8), anti-BCL-2 (BCL/10C4), anti-IFN-γ (XMG1.2), rat IgG1 κ isotype control antibody (RTK2071), Armenian hamster IgG isotype control antibody (HTK888), rat IgG2a κ isotype control (RTK2758), rat IgG2b κ isotype control (RTK4530) and mouse IgG1 κ isotype control antibody (MOPC-21). Anti-CD16/32 (2.4G2) and anti-CD25 (7G7) hybridoma supernatant were generated in A.V.'s laboratory. For caspase staining, the CaspGLOW™ Fluorescein Active Caspase Staining Kit was purchased from BioVision

(Catalog No. K180-100).

3.5.5. Flow cytometry

Before antibody staining, all cells were blocked for 30 minutes with anti-CD16/32 (2.4G2) and anti-CD25 (7G7) hybridoma supernatant mixture unless specified to avoid false staining due to the binding of Fc receptors to the Fc portion of the antibodies. After blocking, cells were stained with antibodies or the respective isotype controls on ice for 30 minutes. The cells were then washed with phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) and analyzed. Cells isolated from MCMV-infected mice were fixed using the Fixation/Permeabilization Kit (BD Biosciences) before being taken out from the biosafety level 2 laboratory to avoid MCMV contamination. For intracellular staining, the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific, Catalog No. 00-5523-00) was used for permeabilization as per the manufacturer's instructions. Caspase staining was performed using the CaspGLOW™ Fluorescein Active Caspase Staining Kit (BioVision, Catalog No. K180-100) as per the manufacturer's instructions. The stained cells were analyzed using a CyAn™ ADP flow cytometer (Beckman Coulter).

3.5.6. Statistical analysis

GraphPad Prism software (version 9) was used for unpaired or paired Student's t-tests (two-tailed). P values < 0.05 were considered statistically significant.

Figure legends

Figure 3.1 SFR KOs show decreased MCMV-specific memory NK expansion.

(A) Schematic diagram of the competition adoptive transfer experiments used in this study. Equal numbers of WT and various KO donor Ly49H$^+$ NK cells were mixed in a 1:1 ratio and i.v. injected into Ly49H KO recipient mice on Day −1 (1 day before virus infection). 7.5 x 10^2 pfu MCMV was i.p. injected into recipients on Day 0. (B) Equal numbers of Ly49H$^+$ NK cells from WT and SFR KO donors were injected into Ly49H KO recipients one day before MCMV infection. The proportion of WT versus SFR KO NK cells in the Ly49H$^+$ population in the blood was analyzed. CD48 was used as a marker to distinguish WT (CD48$^+$) and SFR KO (CD48$^-$) cells. The left panel shows a representative flow cytometric analysis of the proportion of WT and SFR KO Ly49H$^+$ NK cells in blood on Day 7; the right panel shows the statistics for the cell proportions on Days 7, 14, 21 and 28. n = 3. (C) Data were acquired from the same mice as in B. The left panel shows a representative result for the proportion of WT and SFR KO Ly49H$^+$ NK cells in splenocytes on Day 35; the right panel shows the corresponding statistics. n = 3. (D) Equal numbers of Ly49H$^+$ NK cells from WT and SLAM1, 5, 6 tKO donors were injected into Ly49H KO recipients one day before MCMV infection. The proportion of WT versus SLAM1, 5, 6 tKO cells in the Ly49H$^+$ NK cell population in blood was analyzed. The left panel shows a representative flow cytometric analysis of the proportion of WT and SLAM1, 5, 6 tKO Ly49H$^+$ NK cells in blood on Day 7; the middle panel shows the statistics for the cell proportions on Days 7, 14, 21 and 28; the right panel shows the proportion of WT and KO memory NK cells surviving long-term in blood on the

indicated date. n = 4. (E–I) The same experiments as in D are shown using different combinations of donor cells as indicated in the figures. (E) n = 8. (F) n = 11. (G) n = 4. (H) n = 6. (I) Left and middle panels, n = 7; right panel, n = 4. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, NS (not significant) $P > 0.05$.

Figure 3.2 CD48 KOs show decreased MCMV-specific memory NK expansion in the blood.

(A) Flow cytometric analyses of primary NK cell receptor repertoires in freshly isolated WT or CD48 KO splenocyte NK cells. Antibody staining results are depicted as red histograms and isotype controls as filled grey histograms. Results are representative of at least three experiments. (B) Flow cytometric analyses of primary NK cell receptor repertoires in freshly isolated WT or CD48 KO splenocyte NK cells. CD11b and CD27 were stained to check differences in maturation status between WT and CD48 KO NK cells. Stage 1 (S1), $CD11b^{-}/CD27^{-}$; Stage 2 (S2), $CD11b^{-}/CD27^{+}$; Stage 3 (S3), $CD11b^{+}/CD27^{+}$; Stage 4 (S4), $CD11b^{+}/CD27^{-}$. The left panel shows a representative analysis and the right panel shows the corresponding statistics. n = 3. (C) Equal numbers of $Ly49H^{+}$ NK cells from WT (B6.SJL) and CD48 KO donors were injected into Ly49H KO recipients one day before MCMV infection. The proportion of WT (B6.SJL) versus SFR KO NK cells in the $Ly49H^{+}$ NK cell population in blood was analyzed. CD45.1 was used as a marker to distinguish WT (B6.SJL; $CD45.1^{+}/CD45.2^{-}$) and CD48 KO ($CD45.1^{-}/CD45.2^{+}$) cells. The left panel shows a representative flow cytometric analysis of the proportion of WT and CD48 KO $Ly49H^{+}$ NK cells in blood on Day 7; the right panel shows the statistics for the cell proportions on Days 7, 14, 21 and 28. n = 7. (D) Same as C, but with WT (B6.SJL) and CD2 KOs as donor cells. n = 3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, NS (not significant) $P > 0.05$.

Figure 3.3 CD48 KOs show decreased MCMV-specific memory NK expansion in all tissues.

(A) Equal numbers of Ly49H$^+$ NK cells from WT and CD48 KO donors were injected into Ly49H KO recipients one day before MCMV infection. The proportion of WT versus CD48 KO NK cells in the Ly49H$^+$ population was analyzed in the organs specified in the graphs on Day 7. n = 2–5. (B) Same as E, but with analysis on Day 56 to evaluate long-lived cell proportions. n = 3–11. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, NS (not significant) $P > 0.05$.

Figure 3.4 2B4 but not CD2 is responsible for CD48-dependent memory NK cell expansion.

(A) Equal numbers of Ly49H$^+$ NK cells from WT and CD2-2B4 dKO donors were injected into Ly49H dKO recipients one day before MCMV infection. The proportion of WT versus CD2-2B4 dKO cells in the Ly49H$^+$ NK cell population in blood was analyzed. The left panel shows a representative flow cytometric analysis of the proportion of WT and CD2-2B4 dKO Ly49H$^+$ NK cells in blood on Day 7; the middle panel shows the statistics for the cell proportions on Days 7, 14, 21 and 28; the right panel shows the proportion of WT and KO memory NK cells surviving long-term in blood on the indicated date. n = 7. (B) Schematic diagram of the competition adoptive transfer experiments used in this study. Equal numbers of WT and CD48 KO donor Ly49H+ NK cells were mixed in a 1:1 ratio and i.v. injected into 2B4-Ly49H dKO, CD2-Ly49H dKO or 2B4-CD2-Ly49H tKO recipient mice on Day −1 (1 day before virus infection). 7.5 x 102 pfu MCMV was i.p. injected into recipients on Day 0. (C) Equal numbers of Ly49H$^+$ NK cells from WT and CD48 KO donors were injected into 2B4-Ly49H dKO recipients one day before MCMV infection. The proportion of WT versus CD48 KO cells in the Ly49H$^+$ NK cell population in blood was analyzed. The left panel shows a representative flow cytometric analysis of the proportion of WT and CD48 KO Ly49H$^+$ NK cells in blood on Day 7; the middle panel shows the statistics for the cell proportions on Days 7, 14, 21 and 28; the right panel shows WT and KO memory NK cell proportions surviving long-term in blood on the indicated date. n = 10. D) Same as C, but CD2-Ly49H dKO mice were used as recipients. n = 5. (E) Same as C, but 2B4-CD2-Ly49H tKO mice were used as recipients. n = 5. (F) Ly49H$^+$ NK cells from WT (B6.SJL) donors were injected into

Ly49H dKO recipients. Expression of CD48, 2B4 and CD2 was analyzed on Ly49H$^+$ NK cells (CD45.1$^+$/Ly49H$^+$) and recipient NK cells (CD45.1$^-$/Ly49H$^-$). The upper pannel shows a repersentitive representative flow cytometric analysis, the lower panel shows the statistics. The grey-filled line represents the isotype control, the black line represents uninfected control NK cells and the red line represents NK cells 7 days p.i. Non-infected, n=2. Infected, n = 4. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, NS (not significant) $P > 0.05$.

Figure 3.5 NK cell elimination inhibited by 2B4-CD48 interaction is MCMV-dependent but LFA-1-independent.

(A) Equal numbers of $Ly49H^+$ NK cells from WT and CD48 KO donors were injected into Ly49H KO recipients without MCMV infection on the second day. The proportion of WT versus CD48 KO NK cells in the $Ly49H^+$ NK cell population in blood was analyzed. The left panel shows a representative flow cytometric analysis of the proportion of WT and CD48 KO $Ly49H^+$ NK cells in blood on Day 7 (8 days after cell transfer; to unify the date with other experiments, Day 0 was defined as 1 day after cell transfer); the middle panel shows the statistics for the cell proportions on Days 7, 14, 21 and 28; the right panel shows the proportion of WT and CD48 KO memory NK cells surviving long-term in blood on the indicated date. n = 3. (B) Expression of activating markers KLRG1 and CD69 on recipient NK cells ($Ly49H^-$) on Day 4. Gray-filled line, isotype control; black line, uninfected NK cell control; red line, infected recipient NK cells. n > 3. (C) Expression of activating markers KLRG1 and CD69 on recipient NK cells ($Ly49H^-$; up) or WT donor cells ($Ly49H^+$; down) on Day 7. Gray-filled line, isotype control; black line, uninfected NK cell control; red line, infected NK cells. n = 3. (D) Development of $Ly49H^-$ recipient NK cells (up, right) and $Ly49H^+$ donor cells (down, right) on Day 7 p.i.; same-age, same-sex, uninfected $Ly49H^-$ NK cells (up, left) and $Ly49H^+$ NK cells (down, left) from the same WT mice served as the control. n = 4. (E) The same experiment as in Figure 3.1F, with the additional treatment of mice with 200 μg α-LFA-1 blocking antibody (right) or its isotype control rat IgG2a (left) by i.v. injection on Day −1 and Day 3 p.i. The figure shows the statistics for the percentage of WT and CD48 KO $Ly49H^+$

cells in blood on Day 7. n = 3. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, NS (not significant) $P > 0.05$.

Figure 3.6 Proliferation and maturation marker expression on WT and CD48 KO Ly49H+ donor NK cells.

Equal numbers of Ly49H+ NK cells from WT and CD48 KO donors were injected into Ly49H KO recipients on the day before MCMV infection. Flow cytometric analyses were performed using splenocyte NK cells on the indicated date. (A–B) In a separate experiment, WT and CD48 KO donor cells were stained with Celltrace Violet (CTV) before transfer into recipients. On Day 4 (A) and Day 5 (B), CTV dilution was analyzed on Ly49H+ splenocyte NK cells to assess cell division. n = 3. (C) Intracellular staining of KI67 on Day 4. From left to right, panels show the histogram overlay of expression of KI67 on WT and CD48 KOs, KI67+ percentage in WT L49h+ NK cells, KI67+ percentage in CD48 KO Ly49H+ NK cells, and the corresponding statistics. n = 6. (D) Same as C left panel, but showing the results on Day 7. n = 3. (E) The surface expression of KLRG1 was analyzed on Day 4. The left panel shows a representative graph depicting the overlay of KLRG1 expression on WT (blue) and CD48 KO (red) Ly49H+ cells. n = 4. (F) Same as the left panel of E, but on Day 7. n = 3. (G) NK maturation was measured on Day 4 in WT and CD48 KO Ly49H+ NK cells. The two panels on the left show a representative graph depicting WT and CD48 KO Ly49H+ NK cell development on Day 4. The right panel shows the corresponding statistics. n = 4. (H) Same as G, but analyzed on Day 7. n = 6. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, NS (not significant) $P > 0.05$.

Figure 3.7 Apoptosis marker expression on WT and CD48 KO Ly49H+ donor NK cells.

Similar to Figure 3.6, equal numbers of $Ly49H^+$ NK cells from WT and CD48 KO donors were injected into Ly49H KO recipients on the day before MCMV infection. Flow cytometric analyses were performed using splenocyte NK cells on the indicated date. (A) Flow cytometric analysis of $caspase^+$ cells in WT or CD48 KO $Ly49H^+$ NK cells on Day 4. The two panels on the left show representative results; the right panel shows the corresponding statistics. n = 4. (B) Same as A, but analyzed on Day 7. n = 4. Each symbol in this figure represents an individual mouse; error bars represent mean with s.d. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, NS (not significant) $P > 0.05$.

Figure 3.8 The role of 2B4-CD48 in memory NK cell generation

Schematic diagram of the finding in this study. (A) Upon MCMV infection, the virus-infected cells (macrophages or DCs) can activate NK cell and other immune cells by Ly49H-m157 binding or cytokine stimulation. Some activated immune cells acquired the ability to kill or phagocytose activated NK cells when their activating receptor recognize the ligand on the NK cells. However, this eliminating ability is inhibited by inhibitory signalling generated through 2B4 on the effector cells binding with CD48 on target NK cells. (B) In the absence of 2B4 on effector cells or CD48 on target NK cells, NK cell fratricide will be executed among MCMV-activated immune cells.

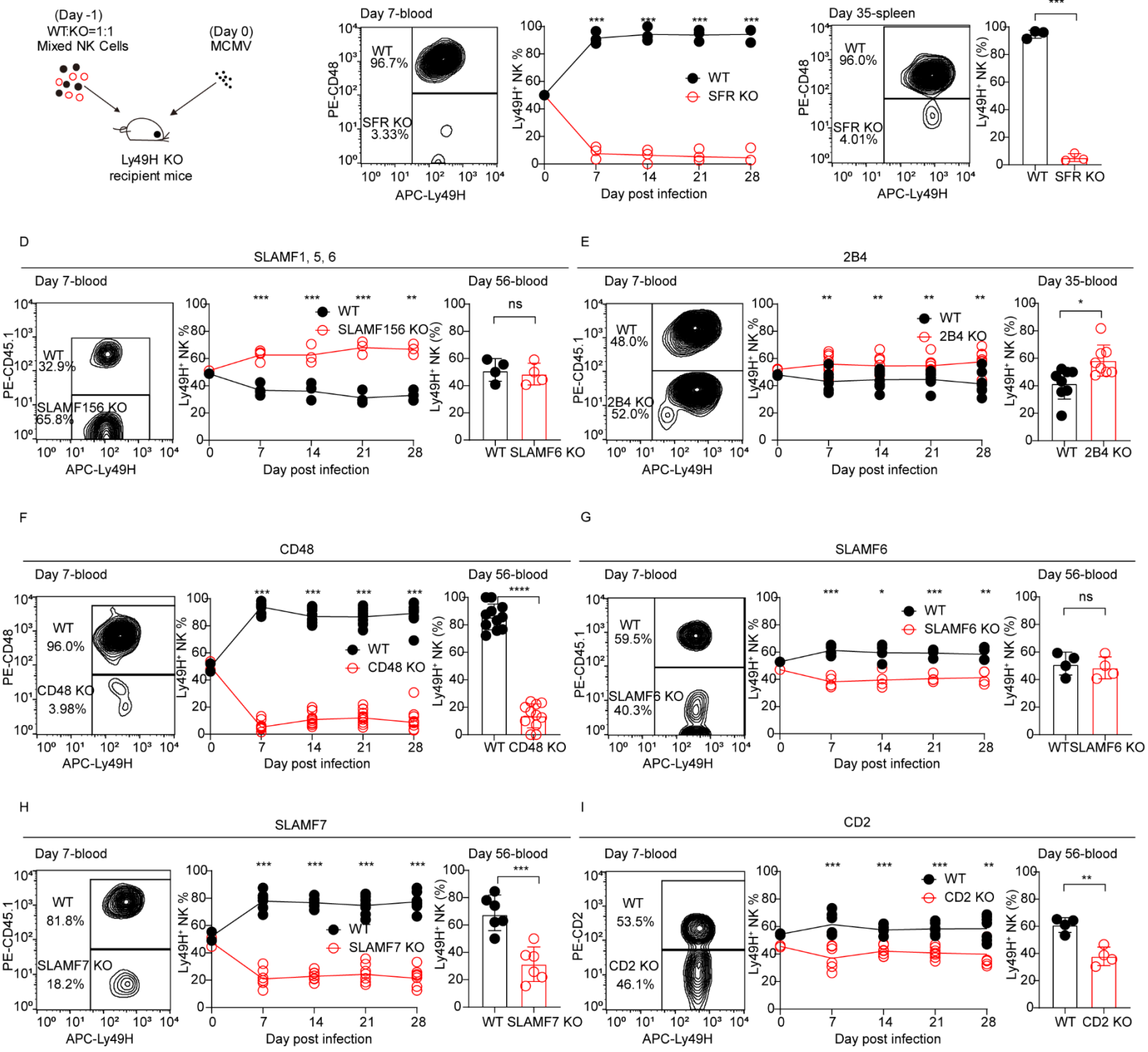

Figure 3.1 SFR KOs show decreased MCMV-specific memory NK expansion

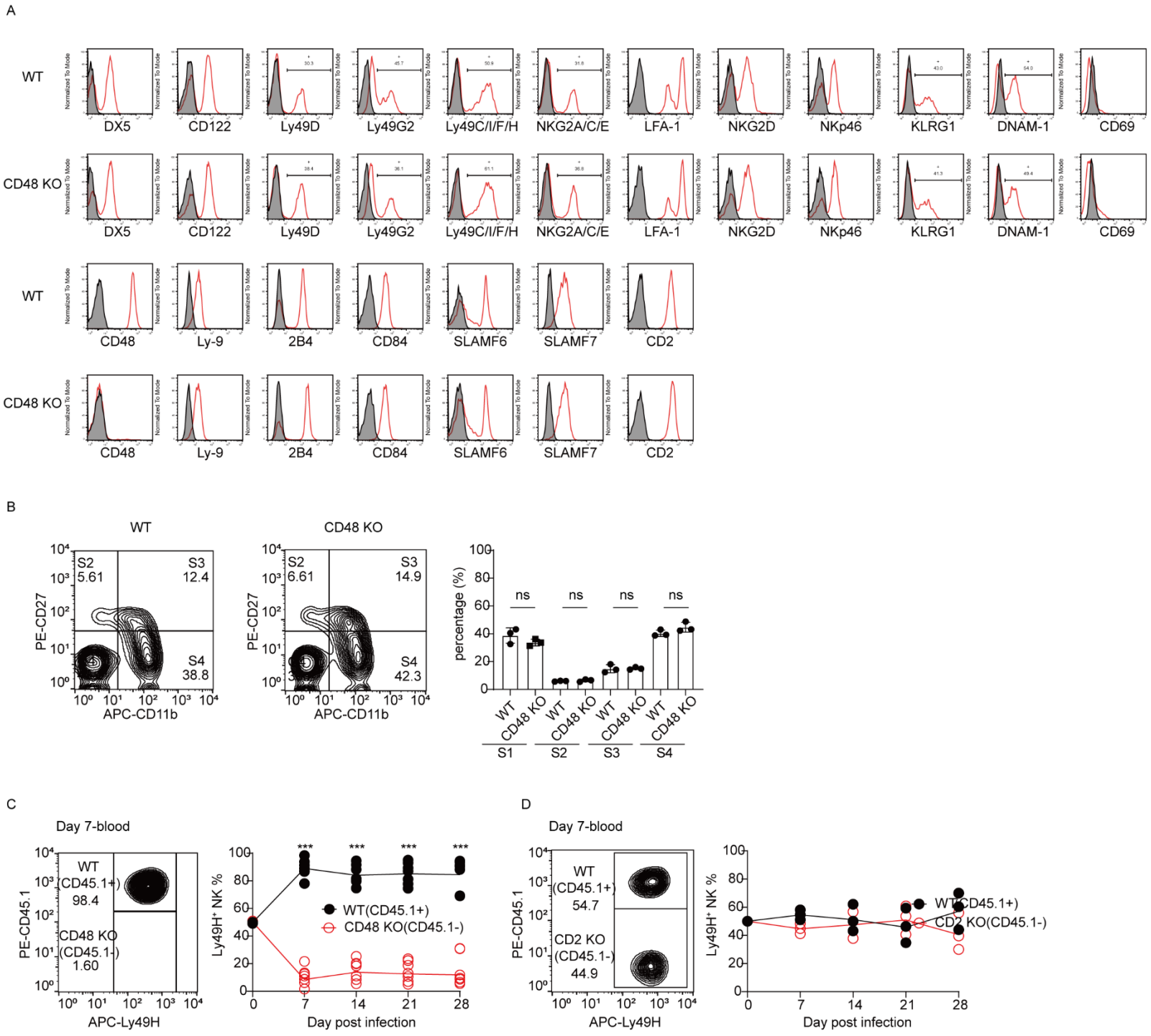

Figure 3.2 CD48 KOs show decreased MCMV-specific memory NK expansion in the blood

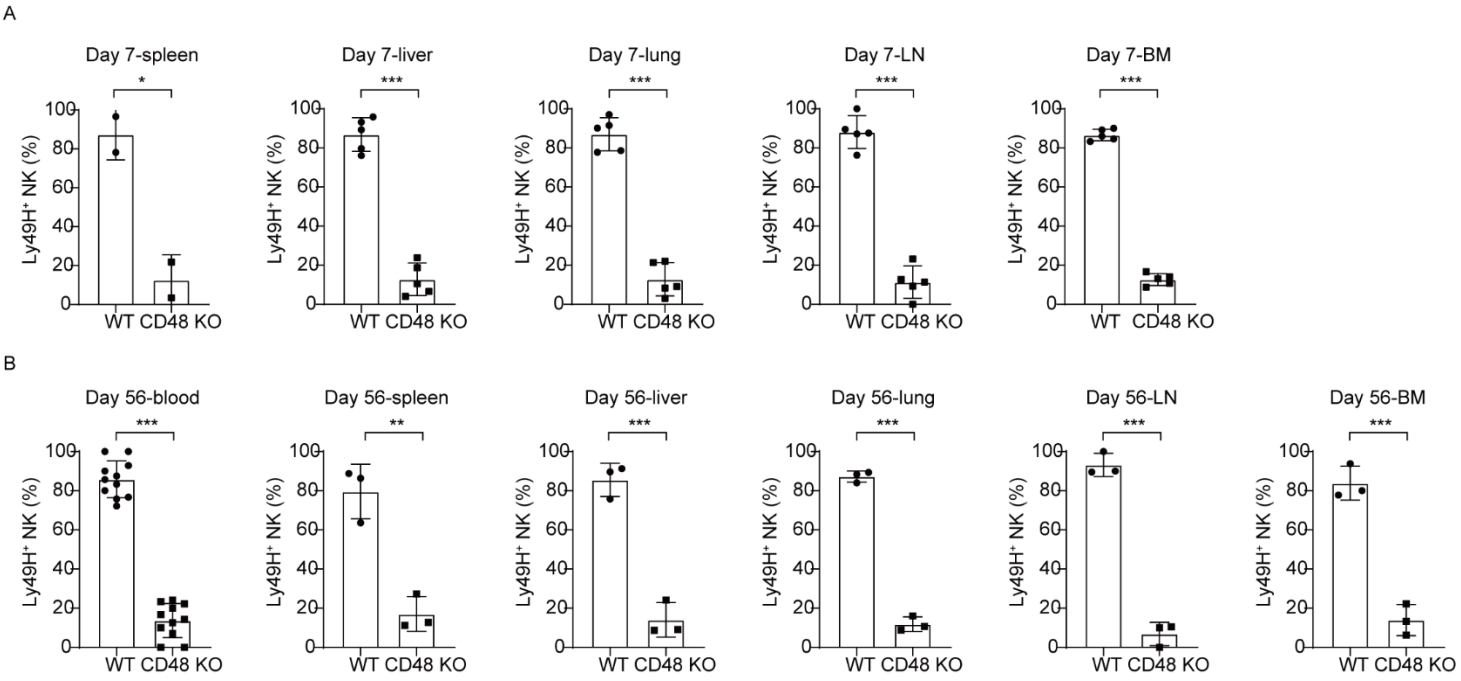

Figure 3.3 CD48 KOs show decreased MCMV-specific memory NK expansion in all tissues

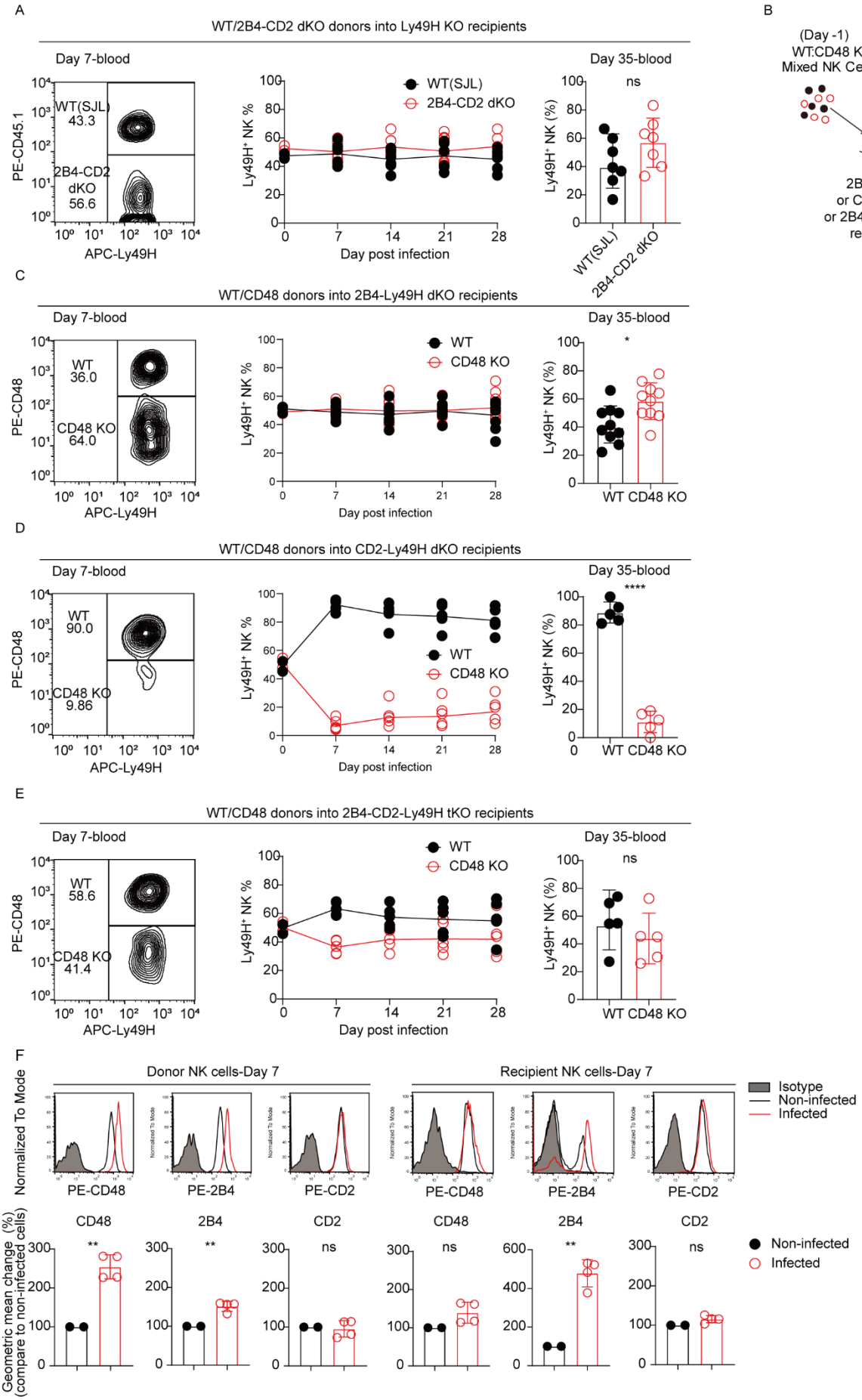

Figure 3.4 2B4 but not CD2 is responsible for CD48-dependent memory NK cell expansion

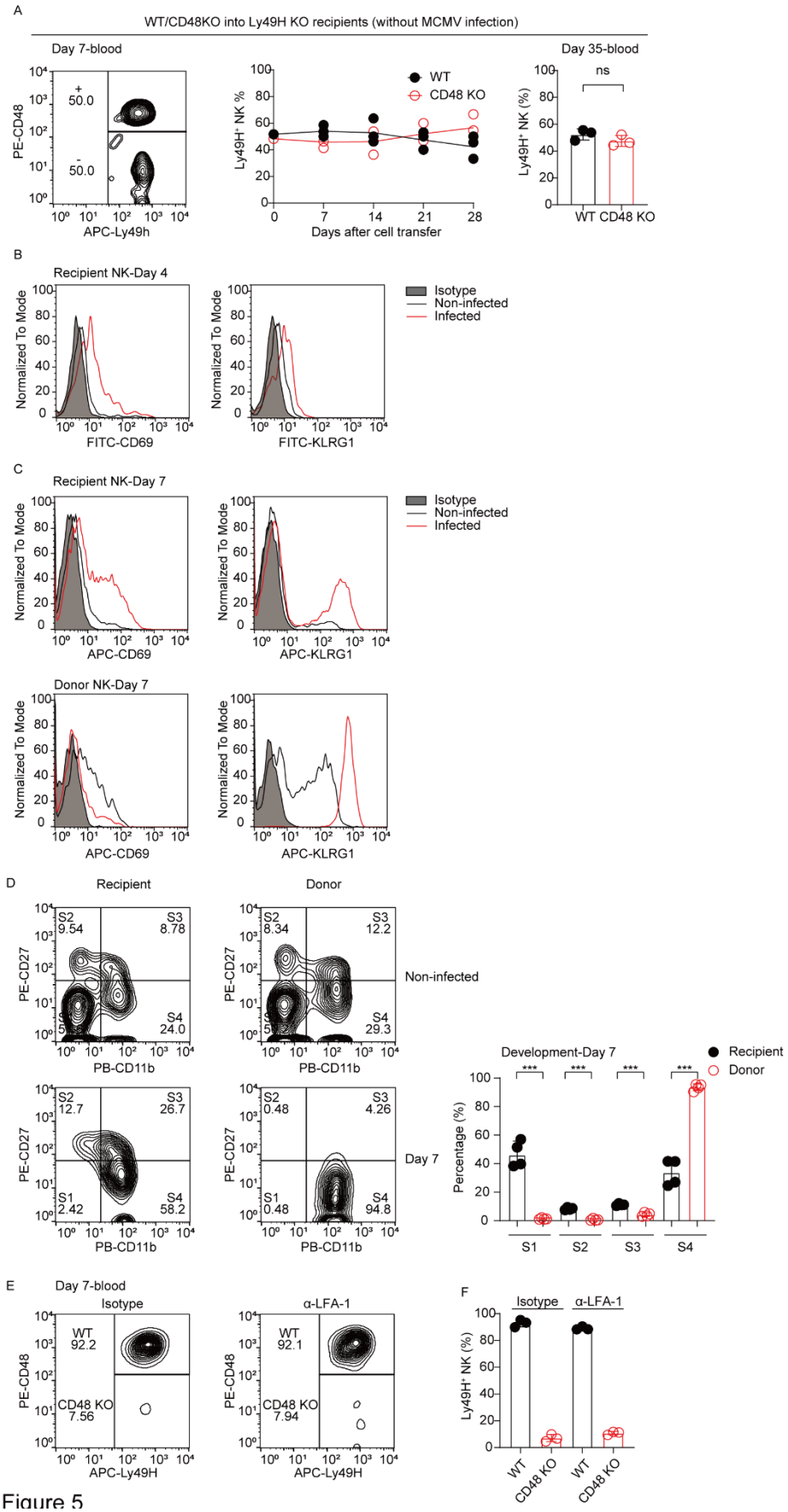

Figure 5

Figure 3.5 NK cell elimination inhibited by 2B4-CD48 interaction is MCMV-dependent but LFA-1-independent

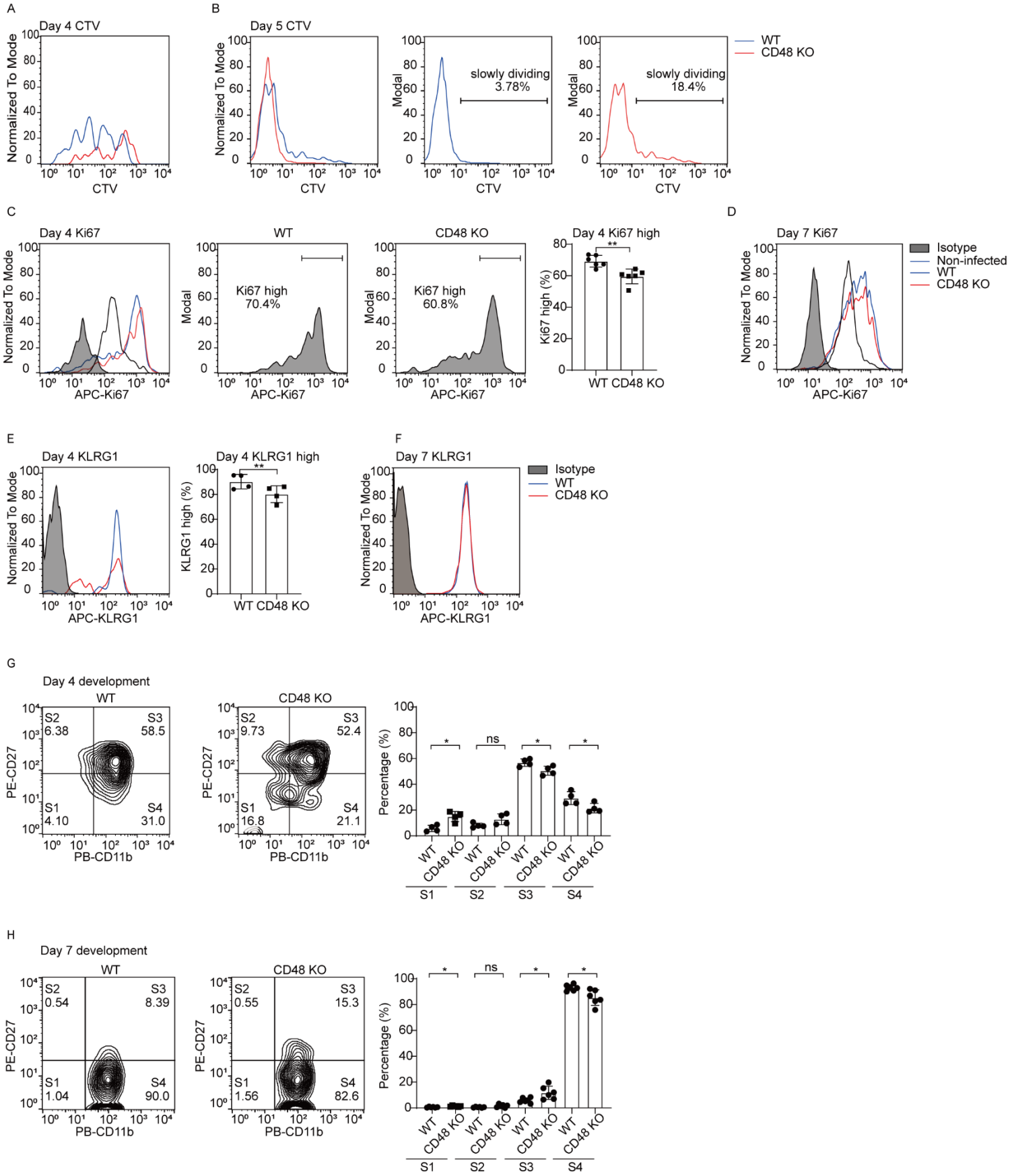

Figure 3.6 Proliferation and maturation marker expression on WT and CD48 KO Ly49H+ donor NK cells

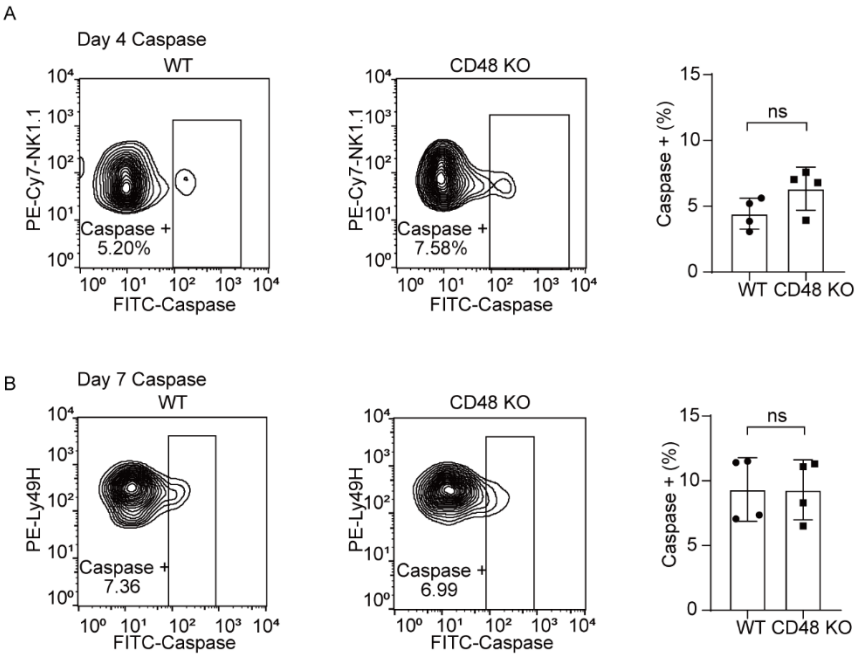

Figure 3.7 Apoptosis marker expression on WT and CD48 KO Ly49H+ donor NK cells

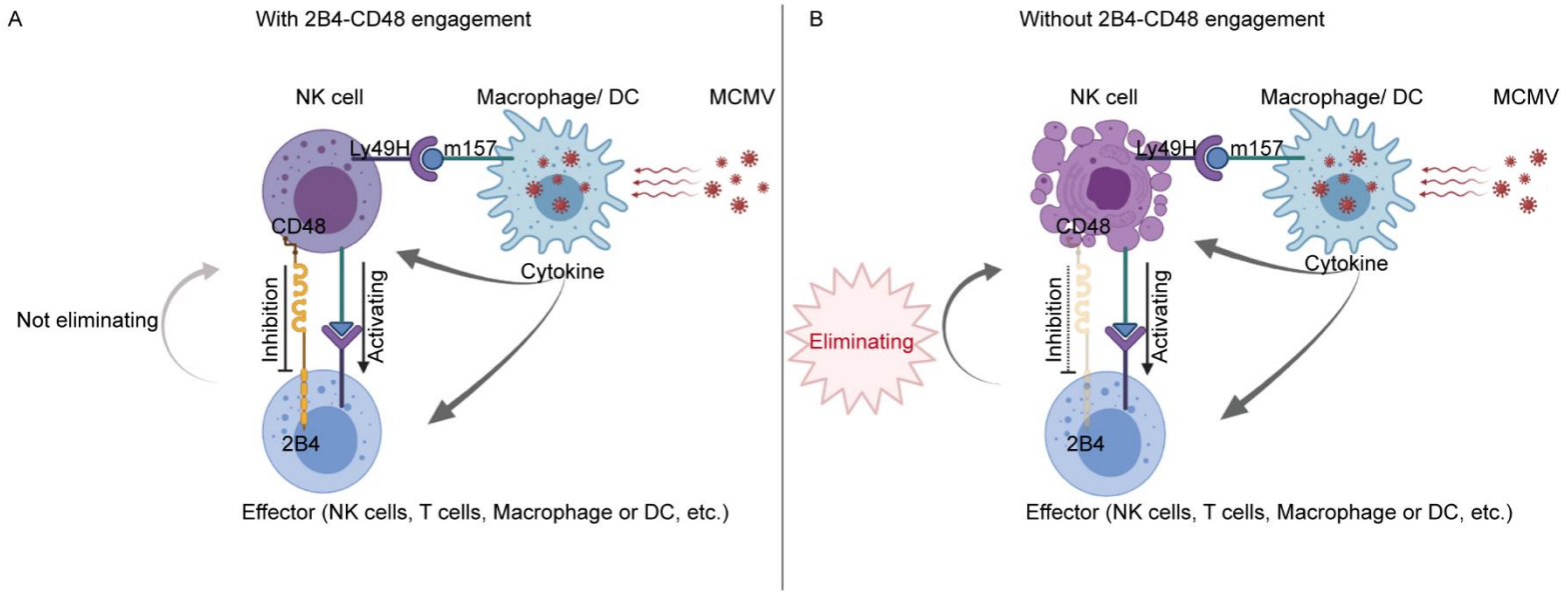

Figure 3.8 The role of 2B4-CD48 in memory NK cell generation

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Chapter 4. General discussion

Our studies focused on NK cell functions with regard to antitumour and antiviral activity. In this chapter, I discuss the rationale for these studies, summarize their findings, describe their contribution to original knowledge, point out their limitations, and describe work done in collaboration with another laboratory that was not part of my thesis but is relevant to the discussion of my thesis.

1. Summary of findings

Our studies focused on the role of primary receptors in NK cell antitumour and antiviral activity. Several findings were made. (1) We found that both NKG2D and DNAM-1 are important for NK cell cytotoxicity and elucidated the complementary roles of NKG2D to DNAM-1 as well as the redundant role of DNAM-1 to NKG2D in NK cell cytotoxicity towards tumour targets. (2) We also found that the integrin receptors, LFA-1, but not Mac-1, have essential roles in DNAM-1- and NKG2D-independent NK cell cytotoxicity towards different ICAMs-expressing tumour cells. (3) We confirmed that the ITAM adaptor, FcRγ, is associated with LFA-1 and essential for LFA-1-dependent cytotoxicity. (4) Additionally, we discovered that cytokines could influence the dependence of killing activity on specific receptors and adaptors and mask the function of some receptors. (5) We also revealed the role of 2B4-CD48 and other SFRs in memory NK cell generation. (6) We confirmed that the function of 2B4-CD48 in stabilizing memory NK cell generation is not limited to blood but also occurs in multiple organs. (7) We further confirmed that during MCMV infection, NK cells elevate the expression of CD48 and 2B4, presumably to avoid

being eliminated by adjacent cells. (8) Finally, we found that cytokines can activate NK cells in the absence of Ly49H-m157 interactions during MCMV infection.

2. Contribution to original knowledge

Our studies have given rise to new knowledge regarding NK cell receptors in the context of antitumour activity. We are the first group to create the DNAM-1-NKG2D dKO to study the complementary and redundant roles of these two receptors. We also created the first pure B6 background FcRγ KO mice to exclude the possible effect of the 129 background locus of SFRs in the previous version of FcRγ KO mice. Additionally, we are the first to show the interaction of FcRγ, LFA-1 and 2B4 by IP and mass spectrometry and to find that FcRγ is involved in LFA-1-dependent cytotoxicity. We also discovered that Mac-1 is not important for NK cell cytotoxicity against ICAMs-expressing tumour cells. We are the first group to find that not only ICAM-1 but also ICAM-2 is important for LFA-1-dependent killing. Additionally, our studies unveiled the inhibitory role of 2B4 in human NK cell cytotoxicity towards CD48-expressing target cells. We are the first group to discover the novel inhibitory function of 2B4-CD48 in stable memory NK generation and to find that SLAMF7 is important for memory NK cell expansion. Finally, we discovered that in the absence of the Ly49H receptor, cytokines can still activate NK cells in MCMV infection.

Our findings provide insight into the redundant and complementary roles of DNAM-1, NKG2D,

LFA-1 in NK antitumour activity. Previous antibody blockade studies showed that blocking both NKG2D and DNAM-1 could inhibit NK cell cytotoxicity towards targets expressing both NKG2D ligands and DNAM-1 ligands to a greater extent than blocking single receptors (107, 108). Here, we provide genetic evidence for the importance of NKG2D and DNAM-1 in cytotoxicity against tumour targets expressing the corresponding ligands, thereby potentially providing fundamental knowledge for tumour diagnosis and the prediction of treatment outcomes by analyzing ligand expression on patient tumour cells and receptor expression. This knowledge could also aid CAR-NK cell design. For instance, the signalling domains of DNAM-1, NKG2D and 2B4 have been used by different groups to design CAR-NK cells (109-111). Our cytotoxicity experiments showed that DNAM-1 and NKG2D signalling are important for triggering NK cell cytotoxicity. However, our experiments using different cytokine-stimulated mouse NK cells showed that the signalling function of ITSM-containing receptor 2B4 could be inhibitory. Additionally, in the cytotoxicity experiments using human peripheral blood NK cell killing of CD48-positive and CD48-negative 721.221 tumour cells, 2B4 seems also play an inhibitory role in NK cell cytotoxicity. Thus, although several groups using CAR-NK cells expressing 2B4 signalling domains have obtained promising results *in vitro* and *in vivo*, our results indicate that more studies are needed to understand the signal-switching machinery of 2B4 before it can be applied in human patients, especially in combination with different cytokine stimulation strategies.

Our determination of the critical role of 2B4-CD48 in stabilizing the expansion of MCMV-specific

memory NK cells gives a hint regarding the expansion of other types of memory NK cells, especially cytokine-induced human memory NK cells and HCMV-induced human memory NK cells, which have potential for future use in the treatment of cancers. Although we did not find 2B4-CD48 to be very important in cytotoxicity, 2B4-CD48 may be important for human NK cell expansion and survival. However, further experiments are needed to validate these hypotheses.

Overall, these original findings advance knowledge and could potentially aid future clinical therapy and diagnosis.

3. Study limitations

Due to time and resource limitations, the comprehensiveness of our studies is limited. More experiments are needed to test more possibilities. For instance, we only tested cytotoxicity using tumour cells as targets, while we did not test receptor functions in cytotoxicity towards normal immune cells such as T cells and macrophages, which will be helpful to draw a more comprehensive picture of the roles of these receptors. Although we confirmed the association of LFA-1, 2B4 and FcRγ by mass spectrometry, immunoprecipitation and immunofluorescence microscopy, these techniques have limitations. They do not distinguish whether the molecules are binding directly or indirectly. We also could not separately analyze the functions of LFA-1 with regard to conjugation and cytotoxicity. We noted the differences in killing activity in FcRγ KO and 2B4 KO NK cells cultured with different cytokines. To determine the reason for this discrimination,

we performed RNA sequencing on NK cells cultured with IL-15, IL-15 + IL-12 and IL-15 + IFN-b (data not shown). However, we did not find any significant change in the RNA expression related to this phenotype. We tested the function of LFA-1 on human peripheral blood NK cells by using blocking antibodies. However, genetic evidence obtained by knocking out LFA-1 on human NK cells or ligands on targets using CRISPR-Cas will be more convincing. In the study described in Chapter 2, we only tested the cytotoxicity effect but not IFN-γ production, which is the other important antitumour function of NK cells. Overall, although we obtained knowledge of several different receptors, in-depth mechanistic studies were insufficient for some candidates.

For MCMV experiments, we used adoptive transfer to compare the differences between WT and CD48 KO NK cells. To mimic better the physiological conditions of natural infection, we may need to directly infect the WT and CD48 KO mice and compare absolute memory NK cell numbers. Further studies are needed to resolve other questions, such as whether NK cells or other immune cells are the dominant cell type responsible for decreased memory NK cell expansion in CD48 KO mice. This could be determined via experiments using mice lacking NK, B and T cells (RAG-IL-2Rγ dKO) and using specific cell-depleting antibodies. Additionally, whether CD48 KO NK cells are eliminated by killing or phagocytosis and the responsible activating receptor need to be identified. Finally, due to time limitations, some experiments lacked enough repeats to obtain statistical information. This limitation will be addressed in the near future.

4. Other works done in collaboration with another group

Apart from the projects described in Chapters 2 and 3, I also conducted a study in collaboration with other researchers. Some of the results were published in the journal Immunity as an article titled "CD155 on Tumor Cells Drives Resistance to Immunotherapy by Inducing the Degradation of the Activating Receptor CD226 in $CD8^+$ T Cells" (112). In this study, we found that DNAM-1 expression on the mouse and human NK cells can be degraded upon the engagement by its ligand CD155 on target cells (Appendix 1). After 4 hours of coincubation of mouse NK cells with RMA-S cells ectopically expressing mouse CD155 ($mCD155^+$), we found that the surface expression of CD226 significantly decreased compared to NK cells co-incubated with $mCD155^-$ RMA-S targets. The decreased DNAM-1 expression could be partially rescued by the mutation of tyrosine Y319 in the DNAM-1 cytoplasmic domain (Appendix 1A and B). Additionally, co-incubation of human NK cell line YT-S expressing mouse DNAM-1 with $CD155^+$ RMA-S cells also resulted in reduced mDNAM-1 expression on the YT-S cell surface compared to co-incubation with $CD155^-$ RMA-S cells (Appendix 1C). YT-S cells expressing various mutated forms of mDNAM-1 (Y319F, S326A, Y322F, N321Q) showed that Y319 is important for the downregulation of mDNAM-1 on YT-S cells (Appendix 1D–F). Additionally, the PP2-mediated inhibition of Src kinases diminished the degradation effect and confirmed that Src is involved in the downregulation process (Appendix 1G). Finally, we performed immunoprecipitation and confirmed the association of DNAM-1 with AP-2, probably through binding to the AP-2-binding motif YxxØ (Ø represents a large hydrophobic residue) in the cytoplasmic domain of DNAM-1 (113), implying that the degradation

of DNAM-1 on NK cells is mediated by AP-2-dependent endocytosis (Appendix 1H). Together with our collaborators' observations regarding DNAM-1 degradation on T cells, these findings revealed a new mechanism wherein tumour cells evade immune cell recognition by downregulating the DNAM-1 receptor. Together with the findings described in Chapter 2, these results provide an explanation for cancer treatment failure and insights for developing new therapeutic strategies against cancers.

5. General overview

NK cells have gained increasing attention in scientific studies and therapeutic applications in recent years. Several NK cell functions have been revealed in different conditions and treatment approaches. Compared to T cell therapies, the safety of NK cell therapies is relatively high and the sources of NK cells are relatively easy to acquire. One of the major therapeutic applications of NK cells is their use as immune cell therapy against cancers. In clinical trials, NK cell therapy showed promising results in treating hematopoietic malignancies such as acute myeloid leukemia (AML) (114-116). Four primary sources of NK cells are used for cancer treatment, namely allogeneic NK cells, autologous NK cells, the NK-92 cell line or cord blood-expanded NK cells. They have been reported to treat hematopoietic cancers with a good record (117). In addition to their activity against hematopoietic cancers, NK cell treatments have fewer side effects such as cytokine release syndrome, neurotoxicity or graft-versus-host disease (GvHD) than T cell treatments.

Moreover, many studies are testing the use of optimized NK cells to treat solid tumours. The use of chimeric antigen receptor (CAR)-bearing NK cells (CAR-NK cells) expressing specific chimeric receptors to recognize specific antigens on tumour cells is one of the most-studied strategies to improve the efficacy of NK cell treatment, and is recognized as part of the second generation of cell therapy (114, 118, 119). This approach has several advantages over the original NK cell therapies and CAR-T cell therapies. First, NK cell treatments normally cause minimal or no cytokine release syndrome and neurotoxicity. Second, NK cells can be activated by multiple mechanisms, thus providing robust killing towards tumour cells. Third, CAR-NK cells have high feasibility to provide "off-the-shelf" treatments (120). During the fast development in recent years, CAR-NK cells containing the signalling domains of NKG2D, DNAM-1 and 2B4 have been developed and tested in preclinical studies (109-111). Additionally, many antibody-based immunotherapies are dependent on endogenous NK cell involvement in patients to achieve the best outcomes (121, 122). This process occurs through ADCC.

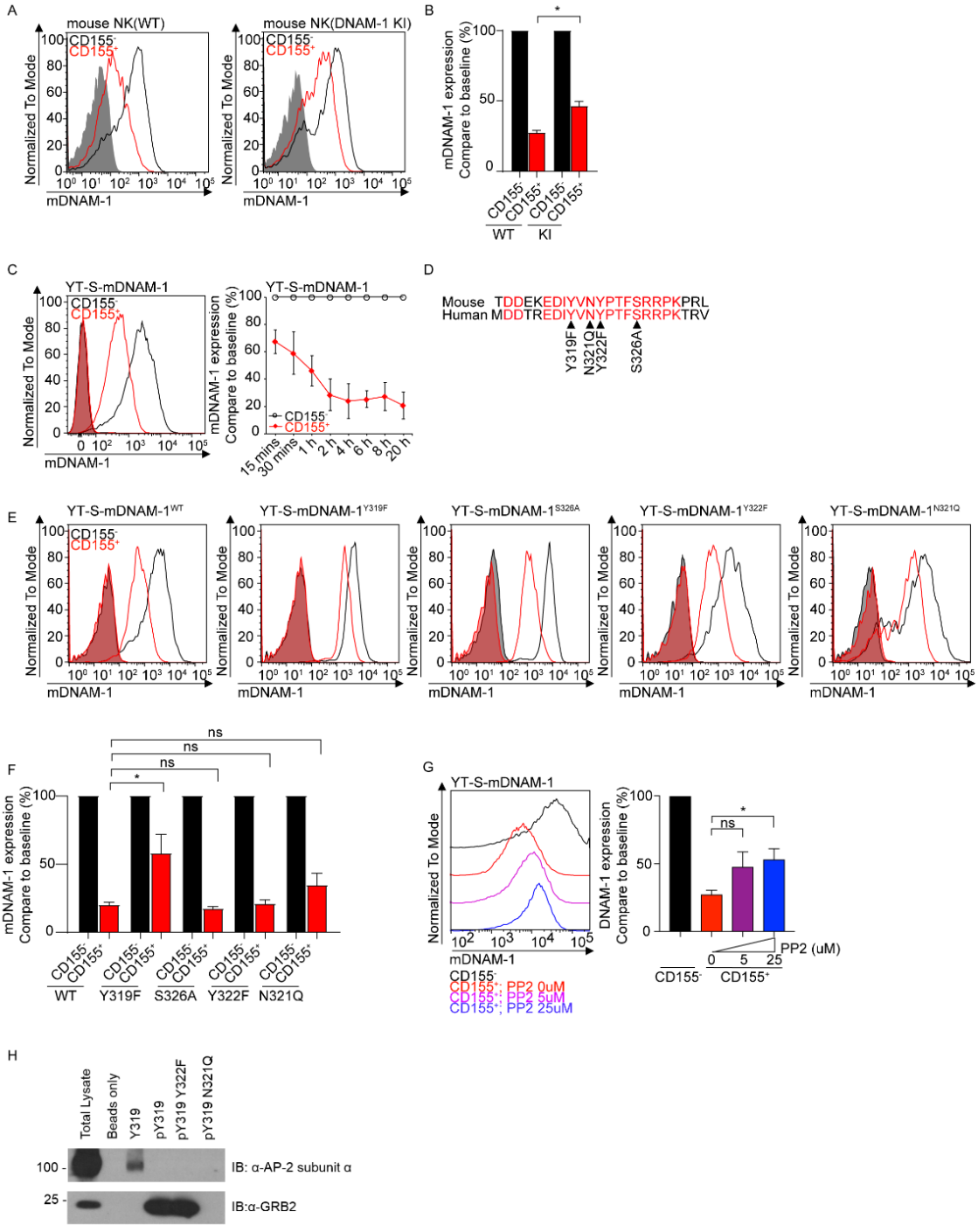
Notably, during the current coronavirus disease of 2019 (COVID-19) pandemic, stem-cell expanded NK cell infusions are also being used to promote the recovery of COVID-19 patients in several ongoing phase I clinical trials (123, 124), of which some have already shown promising outcomes (125). Furthermore, given the broad spectrum of NK cell activities with regard to regulating or killing activated immune cells, it can be predicted that NK cells may also be actively involved in regulating immune cells in other diseases such as auto-immunity or auto-inflammatory

disorders.

The versatility of NK cell functions suggests promising potential therapeutic applications in cancer and infectious diseases. However, many aspects of NK cell biology are still unknown. Comprehensive knowledge and an in-depth understanding of the associated mechanisms are needed to improve therapies and diagnostics, achieve the desired outcomes, and avoid potential side effects.

NK cells have low frequency in the peripheral blood and cord blood (about 10%-20%). *In vitro* expansion is necessary for acquiring enough cells to achieve the desired efficacy. However, with the current *in vitro* expansion methods, the expansion folds are limited. Therefore, studying the expansion of NK cells in different conditions is needed.

Appendix



Appendix figure 1

Figure Legend

Appendix figure 1. CD155-dependent DNAM-1 internalisation in NK cells via Y319, Src kinases and AP-2

(A) Surface expression of mouse (m) CD226 in WT (left) and CD226 KI (right) mouse NK cells was determined by flow cytometry after incubation with either mCD155- or mCD155+ RMA-S cells. Isotype controls are shown as filled histograms (n = 4, representative results of four experiments are shown). (B) Percentage of relative MFI of mCD226 compared to baseline are shown (n = 4, mean ± SEM, cumulative results are quantified from panel A). (C) Surface expression of mCD226 in YT-S-mCD226WT cells after incubation for 4 hours (left) with either mCD155- or mCD155+ RMA-S. (n = 6, representative results of six experiments are shown). Time course experiments are shown (right) (n = 2, mean ± SD, cumulative results of two experiments are shown). (D) Similarities of the CD226 amino acid sequence between human and mouse. Amino acids that are conserved are highlighted in red. The arrows indicate the positions of mutated sites created in various YT-S-mCD226 mutant cells. (E) Surface expression of mCD226 on YT-S expressing WT or mutated mCD226 are shown after incubation with either mCD155- or mCD155+ RMA-S. Isotype controls are shown as filled histograms. (n = 6 for WT, Y319F, N321Q; n=3 for Y319F, Y322F). (F) Relative MFI of mCD226 compared to baseline are shown (n = 6 for WT, Y319F, N321Q; n=3 for Y319F, Y322F, mean ± SEM, cumulative results are quantified from panel E). (G) PP2 inhibition of mCD226 internalisation in YT-S-mCD226WT after incubation with

mCD155+ RMA-S. (n = 3, mean ± SEM, representative results of three experiments are shown). (H) Four biotinylated peptides corresponding to the C-terminus of mouse CD226 were used for pull-down assay: Y319, peptide without phosphorylation; pY319, peptide with phosphorylation at tyrosine 319; pY319 Y322F, tyrosine 319 was phosphorylated and tyrosine 322 was mutated to phenylalanine; pY319 N321Q, tyrosine 319 was phosphorylated and asparagine 321 was mutated to glutamine. Peptides were coupled to avidin-agarose beads and used to capture binding proteins from lysates of mouse thymocytes. Bound proteins were probed by immunoblotting with anti-AP2 subunit alpha and anti-GRB2 antibodies. Total cell lysate (Total Lysate) and beads alone (Beads only) served as controls. The migration of molecular mass markers is indicated on the left. Representative of n = 3. Statistics: paired two-tailed Student's t-test (B, F and G); ns = $p > 0.05$, * = $p < 0.05$.

Methods for Appendix figure 1

Internalization assays in NK cells

Mouse NK cells and the human YT-S NK cell line were used for internalization assays. For mouse NK cells, IL-2 activated NK cells from either WT or CD226Y mice were co-cultured with equal number of RMA-S cells (expressing or not mouse (m) CD155) for 4 hours at 37°C. Internalization of CD226 was then determined by flow cytometry. For human YT-S NK cells, assays were done as described above, except using YT-S cells were used as effector cells. These YT-S cells ectopically expressed either WT mCD226 or various mutated of mCD226 (Y319F, S326A, Y322F, N321Q). For time course experiments, the culture times indicated in the figures were used for the co-culture. For PP2 inhibition, the cells were co-cultured in the presence of 0, 5 or 25 μM of PP2.

Peptide pull-down experiments

Biotinylated peptides encompassing the 19–amino acid C-terminal sequence of mouse DNAM-1, phosphorylated or not at Y319, were synthesized by the W.M. Keck Facility (Yale University). Variants of this peptide in which individual residues were mutated were also created. Peptide pull-down experiments was performed as previously described (Zhang et al., 2015; Pérez-Quintero et al., 2014). Peptides were dissolved in DMSO and coupled to Neutravidin agarose beads (Thermo Fisher Scientific). Beads were then incubated with C57BL/6 mouse thymocytes lysates for 90 min

at 4°C. They were extensively washed, and association with AP-2 or GRB2 was detected by immunoblotting.

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