Role of SLAM family receptors in immune cells

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Abstract

Signaling lymphocytic activation molecule (SLAM) family receptors (SFRs) are transmembrane receptors that are widely expressed on immune cells. They include six members: SLAM, 2B4, SLAMF7, SLAMF6, CD84 and Ly-9. Except 2B4, which binds to CD48, all SFRs are homotypic receptors that recognize the same receptor as a ligand. Through their immunoreceptor tyrosine-based switch motifs, SFRs can interact with SLAM-associated protein (SAP) adaptors and transduce activating signals. However, they are also capable of mediating inhibitory signals by associating with phosphatases. Thus, SFRs can be either activating or inhibitory in immune cells, depending on the specific conditions. My projects focus on understanding the role of SFRs in immune cells, in particular natural killer (NK) cells and multiple myeloma (MM) cells.

NK cells are innate lymphocytes with great cytotoxic capacity against tumor cells and virus-infected cells. They also play critical roles in immune regulation. My first project is to study the role of SFRs in NK cells, which express all SFRs except SLAM. By generating a mouse line lacking the entire ~400 kilobase *Slam* locus, I demonstrated that SFRs are primarily inhibitory in NK cells. Analyses of individual SLAM knock-out mice indicated that the inhibitory effect of SFRs is solely due to 2B4, with no appreciable impact from other SFRs. Furthermore, the inhibitory function of SFRs is enhanced by IL-12 but suppressed by type I interferon (IFN) via regulation of the expression level of SAP adaptors by cytokines. Finally, SFRs inhibit NK cell function, in part by preventing the induction of activated forms of LFA-1.

MM is an incurable disease due to the malignancy of plasma cells. One SLAM family receptor, SLAMF7, is highly expressed on MM cells, and a monoclonal antibody against SLAMF7 (elotuzumab) has been approved to treat patients with MM. My second project is to study the inhibitory role of SLAMF7 in NK cells and MM cells. Firstly, I showed that, in NK

cells, SLAMF7-mediated inhibition requires the tyrosine 261 of SLAMF7, Src family kinases and Src homology region 2 domain (SH2)-containing inositol phosphatase-1 (SHIP-1). Surprisingly, however, SLAMF7 is unable to mediate signaling in MM cells. Further analysis revealed that most MM cells lack CD45, which is required for Src family kinase activation. Hence, the mechanism responsible for SLAMF7-mediated inhibition involves Src family kinases, SHIP-1 and CD45, which is defective in MM cells.

In summary, these new findings demonstrate that SLAM family receptors play key roles in immune cells.

Résumé

Les récepteurs de la famille SLAM (Signaling Lymphocytic Activation Molecule) (SLAM family receptors, SFR) sont des récepteurs transmembranaires abondamment exprimés sur les cellules du système immunitaire. Cette famille comprend six membres: SLAM, 2B4, SLAMF7, SLAMF6, CD84 et Ly-9. À l`exception de 2B4 qui se lie à CD48, tous les autres SFRs sont des récepteurs homotypiques qui reconnaissent un récepteur identique comme ligand. Par l'intermédiaire de leurs motifs ITSM (Immunoreceptor Tyrosine-based Switch Motifs), les SFRs peuvent interagir avec les protéines adaptatrices de la famille SAP (SLAM-Associated Protein) et transduire des signaux activateurs. Cependant, ils ont également la capacité d'induire des signaux inhibiteurs en s'associant à des phosphatases. Ainsi, les SFRs peuvent avoir une fonction activatrice ou inhibitrice dans les cellules immunitaires, dépendant de conditions spécifiques. Mes recherches portent sur le rôle des SFRs dans les cellules du système immunitaire, notamment les cellules NK (Natural Killer) et les cellules de myélome multiple (MM).

Les cellules NK sont des lymphocytes innés ayant une capacité cytotoxique considérable contre les cellules tumorales ou des cellules infectées par des virus. Ils jouent également un rôle essentiel dans la régulation immunitaire. Mon premier projet de recherche consiste à étudier le rôle des SFRs dans les cellules NK, qui expriment tous les SFRs sauf SLAM. En générant une lignée de souris dont on a éliminé une région de ~400 kilobases de *slam*, j'ai démontré que les SFRs jouent principalement un rôle inhibiteur dans les cellules NK. Des analyses de souris déficientes pour l'un ou l'autre des récepteurs de la famille SLAM ont permis de montrer que l'effet inhibiteur des SFRs est due uniquement à 2B4, sans contribution appréciable des autres SFRs. En outre, la fonction inhibitrice des SFRs est renforcée par l'interleukine-12 mais inhibée par l'interféron de type I. Cette fonction est dépendante du niveau d'expression des protéines

adaptatrices de la famille SAP, contrôlé par les cytokines. Enfin, les SFRs inhibent la fonction des cellules NK, au moins en partie, en empêchant la génération de formes activées de LFA-1.

Le myélome multiple (MM) est une maladie incurable caractérisée par la malignité des plasmocytes. L'un des récepteurs de la famille SLAM, SLAMF7, est abondamment exprimé sur les cellules de MM. De plus, un anticorps monoclonal contre SLAMF7 (elotuzumab) a été approuvé pour traiter les patients atteints de MM. Mon deuxième projet de recherche consiste à étudier le rôle inhibiteur de SLAMF7 dans les cellules NK et les cellules de MM. Tout d'abord, j'ai démontré que, dans les cellules NK, l`inhibition induite par SLAMF7 nécessite la tyrosine 261 de SLAMF7, les kinases de la famille Src et SHIP (SH2-containing Inositol Phosphatase-1). Par contre, SLAMF7 est incapable d'induire la signalisation des cellules de MM. Une analyse plus approfondie a montré que la plupart des cellules de MM n'expriment pas CD45, qui est nécessaire pour l'activation des kinases de la famille Src. Par conséquent, le mécanisme responsable de l'induction de l'inhibition par SLAMF7 implique les kinases de la famille Src, SHIP-1 et CD45.

L'ensemble de ces résultats démontrent le rôle essentiel des récepteurs de la famille SLAM dans les cellules du système immunitaire.

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ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cell
BCR	B-cell receptor
BMSC	Bone marrow stromal cell
CLP	Common lymphoid progenitor
CRACC	CD2-like receptor activating cytotoxic cell
CRISPR	Clustered regularly interspaced short palindromic repeats
CS1	CD2 subset 1
Csk	C-terminal Src kinase
CTL	Cytotoxic lymphocytes
DC	Dendritic cell
dKO	Double knock-out
DN	Double negative
DNAM-1	DNAX Accessory Molecule-1
DP	Double positive
E:T	Effector-to-target
EAT-2	Ewing's sarcoma-associated transcript-2
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ERT	EAT-2-related transducer
ES	Embryonic stem
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FV	Friend retrovirus
GC	Germinal center
GFP	Green fluorescent protein
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus

HIV	Human immunodeficiency virus
HPC	Hematopoietic progenitor cell
HTLV-1	Human T-lymphotropic virus type 1
IAV	Influenza A virus
Id	Inhibitors of DNA binding
iEL	Intraepithelial lymphocyte
IFN	Interferon
IFNAR	Interferon-α/β receptor
ILC	Innate lymphoid cell
iNK	Immature NK
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITK	IL-2-inducible T cell kinase
ITSM	Immunoreceptor tyrosine-based switch motif
KD	Knock-down
KIR	Killer cell immunoglobulin-like receptor
KLRG-1	Killer cell lectin-like receptor subfamily G member 1
KO	Knock-out
LAK	Lymphokine-activated killer
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Leukocyte function-associated antigen 1
LILRB1	Leukocyte immunoglobulin-like receptor subfamily B member 1
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAC-1	Macrophage-1 antigen
MCMV	Murine cytomegalovirus
mDC	Myeloid dendritic cell
MDSC	Myeloid-derived suppressor cell
MGUS	Monoclonal gammopathies of undetermined significance

MHC	Major histocompatibility complex
MHV-68	Murine gammaherpesvirus-68
MM	Multiple myeloma
mNK	Mature NK
MTOC	Microtubule-organizing center
Mϕ	Macrophage
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKP	NK cell precursor
NO	Nitric oxide
ORF	Open reading frame
pDC	plasmacytoid dendritic cell
PIPKI	Type I phosphatidylinositol 4-phosphate 5-kinase
PLC	Phospholipase C
PMA	Phorbol myristate acetate
polyI:C	Polyriboinosinic-polyribocytidilic acid
PTP	Protein tyrosine phosphatase
Pyk2	Protein tyrosine kinase 2
RAE1	retinoic acid early inducible 1
ROS	Reactive oxygen species
SAP	SLAM-associated protein
SCF	Stem-cell factor
SFR	SLAM family receptor
SH2	Src homology region 2
SHIP-1	SH2-containing inositol phosphatase-1
SHP	SH2-containing phosphatase
siRNA	Small interfering RNA
SLAM	Signaling lymphocytic activation molecule
SMAC	Supramolecular activation cluster
SP	Single positive

TCR	T-cell receptor
TF	Transcription factors
T_{FH}	Follicular T cell
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TRAIL	TNF-related apoptosis-induced ligand
ULBP1	UL16 binding protein 1
VLA-4	Very late antigens-4
WASp	Wiskott-Aldrich syndrome protein
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis protein
XLP	X-linked lymphoproliferative disease
β2M	β2-microglubulin

Preface and contribution of authors

The following is a manuscript-based thesis, which contains four chapters.

Chapter 1 is an introduction that reviews the literature in immunology and summarizes the historical and current knowledge related to my work.

Chapter 2 is the original work that I carried out and was published in Journal of Experimental Medicine. I am the first author in this publication. In chapter 2, I firstly evaluated the overall role of SLAM family receptors (SFRs) in NK cells and uncovered the mechanism in SFR-mediated signaling. I performed all the work in this chapter, excluding the experiment shown in Figure 2.6. I planned experiments, interpreted data and prepared the manuscript. This project was conducted in collaboration with Dr. Stephen N. Waggoner in Cincinnati Children's Hospital Medical Center. Dr. Ming-Chao Zhong and Ms. Shaohua Zhang generated the SLAM family receptor (SFR) knock-out (KO) mice. Dr. Yan Lu generated the Gm10521 KO mice. Dr. Stacey A. Cranert and Ms. Sarah E. Mahl generated the results shown in Figure 2.6. Dr. Jun Chen, Mr. Rui Li, Dr. Ning Wu and Dr. Dominique Davidson provided informative advice and shared reagents. Dr. Stephen N. Waggoner planned experiments, interpreted data and provided funding. My supervisor, Dr. André Veillette guided the project, planned experiments, generated reagents, helped write the manuscript and provided funding.

Chapter 3 is another original work that I undertook in my PhD study, and it was published in Molecular and Cellular Biology. I am the first author in this publication. In chapter 3, I firstly studied the role of SLAMF7 in multiple myeloma cells and discovered the signaling mechanism involved in SLAMF7-mediated inhibition. I performed all the work in this chapter. I planned experiments, interpreted data and prepared the manuscript. This project was conducted in collaboration with Bristol-Myers Squibb. Dr. Mario-Ernesto Cruz-Munoz and Dr. Ning Wu

provided informative advice and shared reagents. Dr. Michael Robbins provided reagents, interpreted data and provided funding. My supervisor, Dr. André Veillette, guided the project, planned experiments, generated reagents, helped write the manuscript and provided funding.

Chapter 4 is a summary of my PhD work detailed in chapter 2 and chapter 3. In chapter 4, I summarized my original findings, proposed the possible explanations and discussed the future perspectives.

Chapter 2 and Chapter 3 are original scholarships that make distinct contributions to the knowledge. The work described in Chapter 2 uncovered the inhibitory effect of SFRs on NK cell function, elucidated how SFRs are regulated by cytokines and discovered how SFRs inhibit NK cell activation. It is the first work to thoroughly evaluate the overall role of SFRs on NK cells, and helps to improve our understanding about SFRs function in immune cell regulation. The work described in Chapter 3 studied the role of SLAMF7 on multiple myeloma cells. It is the first publication to figure out the signaling effect of elotuzumab (targeting SLAMF7) on multiple myeloma cells. For the first time, we showed that SLAMF7-mediated signaling is impaired in multiple myeloma cells. This work provides an important insight into the action of SLAMF7 on multiple myeloma treatment.

Chapter 1: General introduction

1.1. Innate and adaptive immunity

The immune system consists of a variety of effector cells and molecules to protect the body against disease. Pathogens such as viruses, bacteria, fungi or parasites, as well as self-transformed cells, must be cleared by the immune system to achieve a healthy state of the individual. Once the pathogens break through the physical or chemical barrier of the skin or mucosa, the immune system must react properly and efficiently. Firstly, immune cells must detect the presence of pathogens, which is termed immunological recognition. After encountering pathogens, immune cells are activated and act to effectively eliminate the pathogen. Simultaneously, the immune cells must be regulated and prevented from becoming over-reactive. Proper immune regulation must be achieved after the pathogens are eliminated. Following pathogen clearance, immunological memory is acquired, especially by adaptive immune cells. Consequently, subsequent appearance of the same pathogens will trigger those memory cells to proliferate rapidly and initiate a stronger pathogen clearance response. During the immune response, both innate and adaptive immune cells are critical for host defense. The innate immune response is initiated rapidly (in hours) upon exposure to pathogen, while the adaptive immune response requires days to develop. However, the adaptive immune response eliminates pathogens more efficiently than the innate immune response[1].

1.1.1. Innate immunity

After pathogens breach the physical and chemical barrier of the skin and mucosa, they first encounter and activate innate immune cells, inducing an inflammatory response. Most innate cells are myeloid cells, including macrophages, granulocytes (neutrophils, basophils and eosinophils), dendritic cells (DCs) and mast cells[1].

Macrophages reside in almost all tissues. They have different names in different organs (e.g., Kupffer cells in the liver, microglia in the brain and spinal cord, etc.). After recognizing pathogens, macrophages engulf and kill them via “phagocytosis”. During this process, the pathogens are initially surrounded by the cell membrane and are subsequently internalized into the cells within the phagosome. The phagosome has a low pH to destroy the pathogens. Furthermore, the lysosome, which contains enzymes and proteins, can fuse with the phagosome, damaging the pathogens[2]. Upon activation, macrophages secrete pro-inflammatory chemokines or cytokines to attract and activate other immune cells to the site of infection and initiate further immune response. Macrophages are responsible not only for engulfing pathogens but also for clearing dead cells and cell debris in the body[3].

DCs are phagocytic cells that extend finger-like dendrites around the cells. They have a great capacity to capture and engulf pathogens. The purpose of this process is not to clear the foreign microorganisms, but to present antigens to T cells to trigger adaptive immune responses. Thus, DCs are known as antigen-presenting cells (APCs) and bridge the innate and adaptive immune systems. Macrophages can also act as APCs[4].

Neutrophils are the most important and the most numerous innate immune cells. They are also phagocytic cells with a relatively short life span (only a few days). Unlike macrophages, neutrophils are not present in healthy tissues. However, they are abundant in the blood. Approximately 40% to 75% of white blood cells are neutrophils. In response to inflammation induced by pathogens, the blood vessel diameter increases, leading to slower blood flow at the site of inflammation. Moreover, the expression of cell adhesion molecules on the endothelial cells of blood vessels is upregulated, helping circulating neutrophils to extravasate at the site of inflammation. There, they recognize, take up and destroy pathogens by phagocytosis[1, 5].

The functions of basophils, eosinophils and mast cells are less well studied. These cells might play important roles in defense against parasites. Clinically, they are also involved in allergic responses[1].

In addition to innate immune cells with a myeloid origin, NK cells are of great importance in host defense against pathogens. They originate from common lymphoid progenitors (CLPs), which generate T cells, B cells and NK cells. NK cells possess great cytotoxic ability against tumor cells, virus-infected cells and transformed cells. They destroy target cells by releasing perforin and granzymes or by binding to death receptors via FAS ligand or TNF-related apoptosis-induced ligand (TRAIL)[6]. We will discuss the development and function of NK cells in details in Section 1.2. (natural killer cells).

Innate lymphoid cells (ILCs) are innate immune cells from the lymphoid lineage, which differ from T cells, B cells and NK cells. They are recently defined immune cells with important role in protective immunity, regulating inflammation and homeostasis[7]. Three groups of ILCs have been classified, namely ILC1, ILC2 and ILC3. They produce different cytokines and require different transcription factors for their development. ILC1 express some critical NK cell markers, such as NK1.1 in C57BL/6 background mice, and they produce type 1 cytokines, notably IFN-γ and TNF-α. However, unlike NK cells, they have low cytotoxic capacities[8]. ILC2 secrete type 2 cytokines such as IL-4, IL-5, IL-9 and IL-13. These cells participate in responses to helminth infection and in the development of allergic lung inflammation. ILC3 express the NK cell marker NKp46 and produce IL-22, and they play a critical role in mucosal tissues[9, 10]. ILCs are often found on mucosal surfaces, as well as in the liver and spleen. They are considered tissue-resident cells. Thus, depending on the tissue type, they may have different functions via the secretion of immunoregulatory cytokines[11].

1.1.2. Adaptive immunity

When pathogens overwhelm the protection of the innate immune system, the adaptive immune response is triggered. Overlapping with the innate immune response, the adaptive immune response exerts an effect at a later time, typically after days rather than hours. Innate immunity serves as the trigger and helper for adaptive immunity. APCs (e.g., DCs) help to activate adaptive immune cells, and inflammatory cytokines or chemokines secreted by innate immune cells (e.g., macrophages, neutrophils, NK cells) help to recruit adaptive immune cells to the site of infection. Cell-mediated immunity and humoral immunity generate an efficient adaptive immune response. The former is mediated by T cells, while the latter is performed by B cells. Despite their different functions, both cell-mediated immunity and humoral immunity play critical roles in host defense[1].

1.1.2.1. Cell-mediated immunity

T cells (or T lymphocytes) express antigen-specific T-cell receptors (TCRs), which are composed of two different protein chains. Most T cells are αβ T cells, which express TCRs consisting of alpha (α) and beta (β) chains that are responsible for recognizing peptides loaded by major histocompatibility complex (MHC) molecules. Some T cells express TCRs consisting of gamma (γ) and delta (δ) chains and are termed γδ T cells. Unlike αβ T cells, γδ T cells recognize protein antigens that do not seem to require antigen processing, and some even recognize lipid antigens[12, 13]. γδ T cells possess characteristics of both innate and adaptive immune cells. On the one hand, they react rapidly (in hours) to a variety of foreign antigens. On the other hand, they rearrange TCR genes. These cells are highly abundant in the gut mucosa, and they play

important roles against infections, as well as tumor cells[12].

αβ T cells are involved in cell-mediated immunity. They are divided into two subsets, namely CD4 T cells and CD8 T cells, depending on the expression of CD4 or CD8 co-receptors on the surface. αβ T cells originate from common lymphoid progenitors (CLPs) in the thymus and require positive selection and negative selection during maturation.

Positive selection enables T cells to acquire self-MHC restriction, while negative selection allows them to be self-tolerant. During positive selection, T cells interact with thymic cortical epithelial cells, and only those whose TCRs interact with self-peptide:self-MHC complexes can survive and mature. In the thymus, αβ T cells undergo stages simplified as double negative (DN)-double positive (DP)-single positive (SP). At the time of maturation, they become either CD4 T cells or CD8 T cells[1, 14].

During development, T cells that react strongly with self-antigens will die via apoptosis. This process is termed negative selection. Negative selection is mostly driven by bone marrow-derived dendritic cells and macrophages. Self-reactive T cells will die via apoptosis during negative selection[1, 14].

When T cells complete their development in the thymus, they will circulate in the bloodstream and migrate to peripheral lymphoid organs such as the spleen or lymph nodes (LNs). In the peripheral lymphoid organs, naïve T cells can sample peptide:MHC complexes on APCs (e.g., DCs, macrophages and B cells). When a pathogen invades the body, APCs take up the pathogen and present peptide:MHC I to CD8 T cells or peptide:MHC II to CD4 T cells. By encountering those pathogen-activated APCs, T cells will be primed by three types of signals: signal 1, signal 2 and signal 3. Signal 1 involves the interaction of the TCR and co-receptor CD4 or CD8 on T cells and the peptide:MHC complex on APCs. On its own, signal 1 is not sufficient

to stimulate naïve T cells to proliferate and differentiate into effector T cells. It requires the aid of signal 2 (primarily involved in T cell survival and proliferation) and signal 3 (primarily involved in T cell differentiation). Signal 2 is provided by co-stimulatory molecules on T cells, including CD28 that binds to B7 molecules (B7.1, B7.2) on APCs. It can also be mediated by other molecules such as ICOS and members of the TNF receptor family. Signal 3 is mainly provided by cytokine receptors, and it directs naïve T cells to differentiate into different subtypes. For example, CD4 T cells can differentiate into Th1 (by IL-12 and IFN-γ), Th2 (by IL-4), Th17 (by TGF-b and IL-6), Tr1/Th3 (by IL-10) or Treg cells (by TGF-b)[1, 15].

When T cells are primed and activated by APCs, CD8 T cells and CD4 T cells function differently in response to threats. CD8 T cells will differentiate into cytotoxic lymphocytes (CTLs) and clear the target cells either by inducing programmed cell death of target cells via Fas-Fas ligand interactions or by direct killing of target cells via releasing perforin and granzymes. Additionally, CTLs can secrete cytokines such as IFN-γ, TNF-α and LT-α to contribute to host defense[16].

After activation, CD4 T cells can undergo polarization into different subsets (Th1, Th2, Th17, Treg, etc.) and combat threats by secreting cytokines. They serve as helper cells for CTLs, B cells or other immune cells. Similar to CTLs, Th1 cells express Fas ligand and CD40 ligand, and they release IFN-γ, GM-CSF and TNF-α. They are specialized to activate macrophages during infection. Th2 cells favor the promotion of immune responses against parasites and also induce allergic responses. These cells secrete IL-4, IL-5 and IL-13, and they express CD40 ligand, which activates B cells. Th17 cells are specialized to promote inflammatory responses by recruiting neutrophils through the release of cytokines such as IL-17A, IL-17F and IL-6. Treg cells function to inhibit immune response by secreting IL-10 and TGF-β[17]. Thus, different

CD4 T cell subsets are specialized to provide different functions.

During viral or bacterial infection, the infected cells must be eliminated to prevent the replication and invasion of pathogens. CTLs are critical for the destruction of these infected cells. Moreover, effector CD4 T cells serve as helper cells to enhance both cell-mediated immunity and humoral immunity[16].

1.1.2.2. Humoral immunity

Pathogens can either multiply in the extracellular spaces of the body or be transmitted from one infected cell to another, which in both cases expose them in the extracellular fluids. Humoral immunity can protect the body against pathogen that is exposed in the extracellular space. It is mediated by antibody that is secreted by plasma cells. B cells express surface immunoglobulins termed B-cell receptors (BCRs), and they are able to give rise to plasma cells after activation.

B cell development occurs in bone marrow. Differentiated from CLPs, B cells go through several stages to reach maturity as follows: early pro B cell - late pro-B cell - large pre-B cell - small pre-B cell - immature B cell - mature B cell. In early and later pro-B cell stages, the heavy chain genes are rearranged, while in large and small pre-B cell stages, the light chain genes are rearranged. In the end, immature B cells express surface IgM[1]. The interaction of B cells and bone marrow stromal cells (BMSCs) is essential for B cell development. BMSCs not only provide help to maintain the B cell lineage by expressing stem-cell factor (SCF) and by secreting IL-7 but also trigger negative selection of immature B cells[18]. Immature B cells that react with self-antigens presented by BMSCs have several possible fates. One possibility is that the self-reactive immature B cells will go through apoptosis or clonal deletion. Alternately, they could produce a new BCR via further gene rearrangement. If not, they could become

immunologically tolerant or induce a permanent state of unresponsiveness known as anergy[1]. After negative selection, mature B cells migrate to peripheral lymphoid organs for activation.

After antigen recognition, BCRs not only trigger signals inside cells but also deliver antigen to intracellular sites where the antigen is degraded and presented by the MHC II complex. The peptide:MHC II complex can be recognized by CD4 helper T cells that are already activated by the same antigen[1].

When antigen-specific B cells enter secondary lymphoid organs such as the spleen or LNs via the bloodstream, they are trapped in the T-cell zone and interact with antigen-specific T cells. Subsequently, both antigen-specific T cells and B cells migrate to the border of the T cell and B cell zone and start to form a germinal center. The interaction of the peptide:MHC complex (on B cells) and TCR (on T cells) triggers T cells to generate membrane-bound molecules (such as CD40 ligand) and secreted molecules (such as IL-4) that can activate B cells. In the germinal center, antigen-specific B cells proliferate and undergo somatic hypermutation, affinity maturation and class switching, finally differentiating into antigen-producing plasmablasts or memory B cells. Plasmablasts begin to secrete antibody. After several rounds of proliferation, they either die or become plasma cells[1, 19].

Antibody produced by plasma cells is the most important factor in humoral immunity, and it contributes to the immune response in three different ways. On the one hand, antibody can neutralize pathogens and prevent their entry into cells. On the second hand, it can opsonize pathogens and trigger phagocytosis by phagocytes (or antibody-dependent cell-mediated cytotoxicity by NK cells). Finally, it can activate complement, which enhances opsonization and lyses pathogens[20].

1.2. Natural killer cells

NK cells are innate lymphocytes with cytotoxic capacity. They are one of the first lines of immune defenses against pathogens, tumor cells, virus-infected cells and transformed cells. Unlike T cells and B cells, NK cells do not have antigen-specific receptors, such as TCRs or BCRs. However, they express other receptors that regulate NK cell development and effector functions.

1.2.1. NK cell receptors

Different types of receptors are expressed on NK cells and contribute to NK cell functions. These receptors include activating receptors, inhibitory receptors, cytokine receptors, chemokine receptors and adhesion receptors[21]. The outcome of NK cell action relies on the integration of signals that are mediated by these receptors.

1.2.1.1. Activating receptors

Activating receptors are a group of receptors that mediate activating signals in response to ligand engagement. Depending on the distinct signaling complexes, they can be classified into three groups: immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors, DAP10-associated receptors and SLAM family receptors[21, 22]. They use different signaling adaptors in their cytoplasmic domains.

ITAM is defined by the sequence (D/E)xxYxxL/I$x_{(6-8)}$YxxL/I, where D is aspartic acid, E is glutamic acid, Y is tyrosine, L is leucine, I is isoleucine, x represents any amino acid and $x_{(6-8)}$ denotes any 6-8 amino acids. There are three ITAM-bearing adaptors that are constitutively expressed in mature NK cells: FcεRI-γ, CD3-ζ and DAP12. They are type I transmembrane

proteins with very short extracellular domains. In the cytoplasmic region, FcεRI-γ and DAP12 have a single ITAM, whereas CD3-ζ contains three. FcεRI-γ, CD3-ζ and DAP12 can form homodimers, while in some cases FcεRI-γ and CD3-ζ can form heterodimers[22]. CD16, NKp46 and human NKp30 interact with FcεRI-γ and CD3-ζ to transduce activating signals, while human NKp44, mouse NKG2D, CD94/NKG2C, human KIR-S and mouse activating Ly49 bind to DAP12 to mediate activating signals[21]. Upon stimulation, the tyrosine in ITAM is phosphorylated by Src family kinases (e.g., Fyn, Src, Yes, Lck, Lyn and Fgr in NK cells) and recruits Syk and ZAP-70 to mediate downstream signals involving PLC-γ and Vav[23-25]. Activation of ITAM-associated receptors induces cytoskeleton reorganization, leading to the actin polarization and release of perforin and granzymes[22].

NKG2D and mouse activating Ly49 receptors can associate with DAP10 to mediate activating signals. Human NKG2D binds only to DAP10, whereas mouse NKG2D interacts with either DAP10 or DAP12, depending on its isoforms. In mice, NKG2D has long (NKG2D-L) and short (NKG2D-S) isoforms. NKG2D-L only pairs with DAP10, while NKG2D-S pairs with DAP10 or DAP12[26, 27]. DAP10 is able to recruit either p85 or Grb2 through its YINM motif. Src family kinases are required for DAP10 phosphorylation, but Syk family kinases or LAT are not necessary. DAP10 phosphorylation induces activation of SLP-76, Vav-1 and PLC-γ2, in a Grb2-dependent manner[28]. The events downstream of PI3K and Grb2 in NKG2D signaling are not completely elucidated, but evidence has shown that Jak2, Stat5, Akt, MEK1/2 and Erk are involved in this process[29].

SLAM family receptors (SFRs) mediate activating signals via SAP family adaptors. They contain the immunoreceptor tyrosine-based switch motif (ITSM) in their cytoplasmic domain, and they recruit SAP family adaptors following stimulation. The ITSM is defined by the

sequence TIYxx(V/I), where T is threonine, I is isoleucine, Y is tyrosine, V is valine, and x denotes any amino acid. The activating signals mediated by SAP family adaptors will be discussed in Section 1.3.3 (SLAM family receptor signaling in NK cells).

1.2.1.2. Inhibitory receptors

NK cells express a group of inhibitory receptors that recognize MHC class I molecules (MHC I), which prevent their auto-reactivity. These receptors include killer cell immunoglobulin-like receptors (KIR) in humans, inhibitory Ly49 receptors in mice and CD94/NKG2A in both species. They all contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tails, which recruit phosphatases to suppress effector responses[30]. ITIM has a conserved S/I/V/LxYxxI/V/L sequence, where S is serine, I is isoleucine, V is valine, L is leucine, Y is tyrosine, and x denotes any amino acid. Upon ligand engagement, the tyrosine in ITIM is phosphorylated by Src-family kinases and further recruits phosphatases such as Src homology region 2 domain (SH2)-containing phosphatase-1 (SHP-1), SH2-containing phosphatase-2 (SHP-2) or SH2-containing inositol phosphatase-1 (SHIP-1)[31]. The phosphatase can dephosphorylate the protein substrates linked to activating signals. For the inhibitory KIRs, SHP-1 suppresses activating signals by dephosphorylating the guanine nucleotide exchange factor Vav1[32]. The recognition of MHC class I molecules helps NK cells distinguish abnormal or foreign cells from normal cells, thus, they are of great importance for tumor rejection.

Other inhibitory receptors expressed on NK cells include T cell immunoreceptor with Ig and ITIM domains (TIGIT), killer cell lectin-like receptor subfamily G member 1 (KLRG-1) and leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1). TIGIT inhibits NK cell activation by competing with CD155, the ligand of DNAM-1, and by directly inducing

inhibitory signals[33, 34]. KLRG-1 binds to cadherin on target cells and inhibits NK cell cytotoxicity[35]. LILRB1 also bears ITIMs and recognizes MHC I to mediate inhibitory signals on human NK cells[36]. Additionally, SLAM family receptors also have the ability to trigger inhibitory signals. In their cytoplasmic domain, ITSMs not only recruit SAP family adaptors for positive signals but also bind to phosphatases such as SHP-1, SHP-2 and SHIP-1 to mediate negative signals. Different SLAM family receptors can preferentially interact with different phosphatases. For example, SHIP-1 is the major molecule involved in 2B4-mediated inhibition, while SHP-1 is responsible for SLAMF6-mediated education[37, 38].

1.2.1.3. Cytokine receptors

Cytokines are critical for NK cell development and activation. Various cytokine receptors are expressed on NK cells, including interleukin 1 receptor (IL-1R), IL-2R, IL-12R, IL-15R, IL-18R, IL-21R and interferon-α/β receptor (IFNAR), some of which share the same receptor subunit.

IL-15 is the most important cytokine in NK cell development. IL-15R is composed of IL-15R alpha, beta (CD122) and gamma (common gamma chain, γ_c) units. It shares the same beta and gamma units with IL-2R. In humans, IL-15 induces the generation of $CD3^-CD56^+$ NK cells from $CD34^+$ hematopoietic progenitor cells (HPCs) *in vitro*[39]. IL-15-deficient mice fail to develop NK cells because IL-15 is required for NK cell homeostasis and survival *in vivo*[40, 41]. DC is one type of cell that produces IL-15, and the expression of IL-15R alpha on DCs is required to trans-present IL-15 to NK cells[42].

IL-2 is commonly used to activate NK cells *in vitro*. By binding to IL-2R (composed of IL-2R alpha (CD25), beta (CD122) and gamma (γ_c) chains), it activates NK cells via the MAPK

kinase (MKK)/ERK pathway[43]. NK cells cultured with IL-2 are termed lymphokine-activated killer (LAK) cells, and they have great cytotoxic capacity and cytokine secretion ability.

IL-12, IL-18 and type I IFN are cytokines that are known to potently activate NK cells. They are produced by activated DCs and are involved in NK-DC crosstalk, leading to NK cell activation. IL-12 can be induced in myeloid DCs (mDCs) by different stimuli, including actinobacillus[44], lipopolysaccharide (LPS)[45, 46] and polyriboinosinic-polyribocytidilic acid (polyI:C)[47]. IL-12 induces IFN-γ production by NK cells, which is synergized by IL-18[48]. Type I IFN is largely produced by plasmacytoid DCs (pDCs) and activates the JAK/STAT signaling pathway in response to ligation with interferon-α/β receptor (IFNAR)[49, 50]. In humans, type I IFN is excessively secreted by pDCs during viral infection, while IL-12 is largely induced by mDCs in response to stimuli such as bacterial infection[51, 52]. Although both type I IFN and IL-12 effectively activate NK cells, considering the type of infections involved, NK cells might behave differently. We will discuss the effects of cytokines on NK cells in Chapter 2.

1.2.1.4. Chemokine receptors

NK cells express various chemokine receptors for migration and activation. In humans, $CD56^{dim}CD16^{+}$ NK cells express CX3CR1, CXCR1, CXCR2, CXCR3 and CXCR4. They migrate rapidly in response to CXCL12 (also known as stromal cell-derived factor 1, SDF1) and CXC3L1 (also known as fractalkine in humans and neurotactin in mice)[53, 54]. Additionally, $CD56^{bright}CD16^{-}$ NK cells express CXCR3, CXCR4, CCR5 and CCR7. They migrate vigorously in response to CCL19, CCL21, CXCL10, CXCL11 and CXCL12[53-55].

Chemokines not only trigger NK cell migration but also enhance NK cell activity. For example, CCL2, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL10, and CXC3L1

can enhance NK cell cytotoxicity[56, 57]; CCL19 and CCL21 can stimulate the proliferation of $CD56^{dim}CD16^{+}$ NK cells[58, 59].

Upon activation, NK cells can also secrete cytokines or chemokines to recruit other effector cells and to enable immunosurveillance. They produce CCL3, CCL4 and CCL5 to attract granulocytes and macrophages to the site of infection[55], as well as to inhibit HIV replication *in vitro*[60].

1.2.1.5. Adhesion receptors

Integrins are transmembrane receptors that mediate cell-cell interactions or cell-extracellular matrix (ECM) interactions. They are heterodimers composed of the α (alpha) and β (beta) subunits. In mammals, 18 different α units have been identified, such as CD49a (ITGA1), CD49b (ITGA2), CD11a (ITGAL), CD11b (ITGAM), CD11c (ITGAX) and CD51 (ITGAV), and 8 different β units have been reported, including CD29 (ITGB1), CD18 (ITGB2), and CD61 (ITGB3). With the different combinations, ~24 unique integrins can be obtained[61].

NK cells express several integrins, in particular those composed of β1 and β2 subunits. They include the leukocyte function-associated antigen 1 (LFA-1, αLβ2), the macrophage-1 antigen (MAC-1, αMβ2) and the very late antigen-4 (VLA-4, α4β1). LFA-1 plays important roles in NK cell activation. It is not only an adhesion molecule that is critical for immunological synapse formation[62], but also a receptor that is capable of mediating activating signals in NK cells[63].

LFA-1 is composed of CD11a (αL) and CD18 (β2) and is widely expressed on hematopoietic cells such as T cells, B cells, NK cells, macrophages and neutrophils. It binds to ICAM-1 and ICAM-2[63]. LFA-1 is structurally regulated and has three distinct conformations,

namely inactive, intermediate and active conformations, with increasing affinity for its ligand. As indicated in Figure 1.1, in the inactive state, LFA-1 forms a closed conformation, with its headpiece bending and closely approaching the plasma membrane, leading to a low affinity for its ligand. When NK cells encounter target cells or stimuli, they generate “inside-out” signals, resulting in a conformation change in LFA-1. During this process, LFA-1 extends its headpiece toward the ligand and forms an intermediate stage. After swinging out of its hybrid domain, LFA-1 acquires a high affinity for its ligand and forms an active conformation[64].

When NK cells encounter hematopoietic target cells, an early “inside-out” signal causes LFA-1 to switch its conformation from an inactive to an active form. As a result, it binds tightly to its ligands, ICAM-1 and ICAM-2, and triggers activating “outside-in” signals. Stimulation of LFA-1 leads to the phosphorylation of protein tyrosine kinase 2 (Pyk2), which is required for the association with the scaffold protein paxillin and the formation of the microtubule-organizing center (MTOC)[65]. By binding to the cytoskeletal protein talin, LFA-1 recruits type I phosphatidylinositol 4-phosphate 5-kinase (PIPKI), Wiskott-Aldrich Syndrome protein (WASp) and the Arp2/3 complex, which leads to actin polarization[66]. Additionally, LFA-1 engagement induces the phosphorylation of several activating molecules, such as PLC-γ and Syk[67]. Thus, LFA-1 activates NK cells by modulating the cytoskeleton and by transducing positive signals. In humans, several monoclonal antibodies (mAb24, 327C) have been used to detect the active form of LFA-1[68]. It is an effective tool for monitoring LFA-1 activation and will be discussed in Chapter 2.

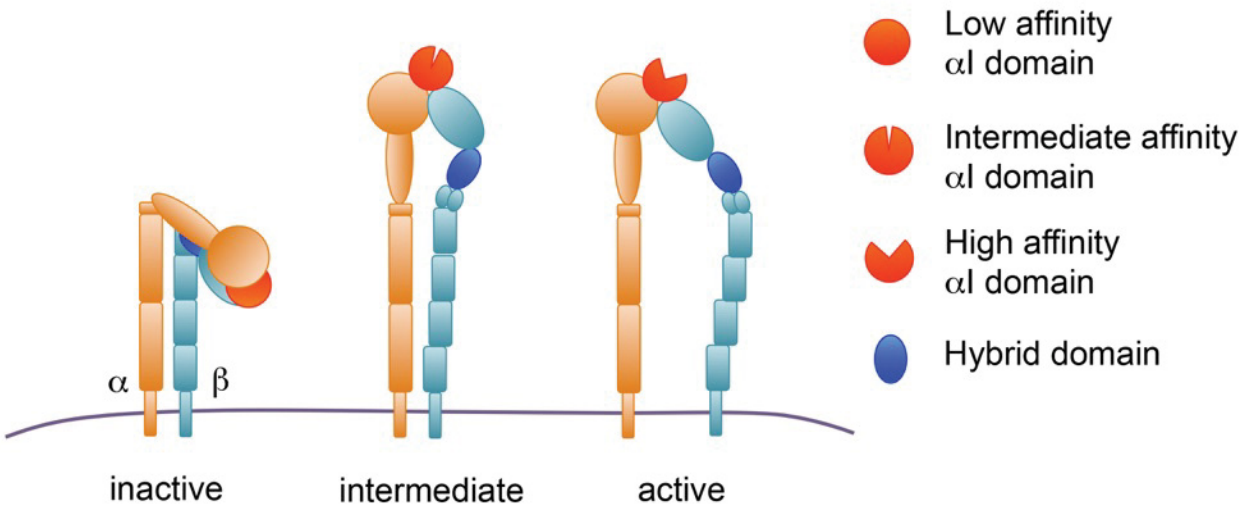

Figure 1.1. Conformations of LFA-1. LFA-1 has three conformations (inactive, intermediate and active). In the inactive conformation, LFA-1 bends its headpiece with minimum accessibility to the ligand. Upon activation, LFA-1 extends its headpiece to the ligand and forms an intermediate conformation. After swinging out of its hybrid domain, LFA-1 acquires a high affinity for its ligand in an active conformation.

1.2.2. Development of NK cells

Like T cells and B cells, NK cells are derived from common lymphoid progenitors (CLPs). Following development, they undergo four stages: CLP, NK cell precursor (NKP), immature NK (iNK) cell and mature NK (mNK) cell, as shown in Figure 1.2. Briefly, two major processes are required. The first is the commitment of CLP to the NK cell lineage (CLP to NKP), which is termed NK cell commitment. The second is phenotypic and functional NK cell maturation (from NKP to iNK to mNK).

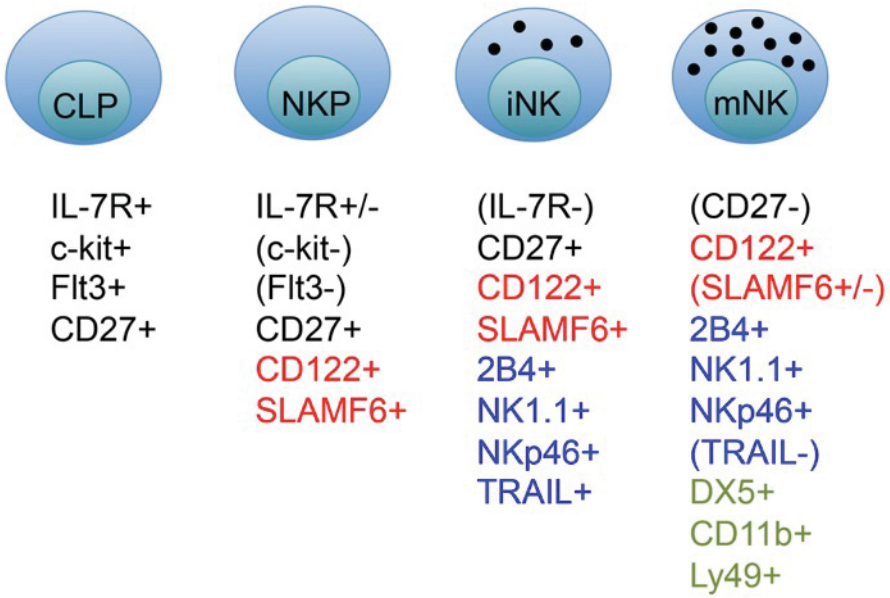

Figure 1.2. Stages of NK cell development. Following development, NK cells undergo four stages: common lymphoid progenitor (CLP), NK cell precursor (NKP), immature NK (iNK) and mature NK (mNK). The surface markers are shown below. CD122 and SLAMF6 are first expressed in NKP; 2B4, NK1,1, NKp46 and TRAIL are first expressed on iNK; DX5, CD11b and Ly49 are first expressed on mNK. The markers in parentheses indicate that they are downregulated from earlier stages.

1.2.2.1. NK cell commitment (CLP - NKP)

The CLP lacks common lineage markers such as CD3, CD4, CD8, CD19, NK1.1, Ter119 and Gr-1, but it expresses IL-7R, CD27, c-kit (CD117), Sca-1 and Flt-3[69]. To develop into a NKP, it acquires IL-15R expression but downregulates IL-7R[70], reflecting a shift from IL-7 dependence (CLP) to IL-15 dependence (NKP).

NKP is characterized as Lin^{-} $CD122^{+}$ $NK1.1^{-}$ $DX5^{-}$. It is present not only in bone marrow but also in the fetal thymus, spleen, liver and lymph node[70, 71]. The interaction between CLP and stromal cells is critical to generate NKP, and cytokine signals via c-kit, Flt-3 or γc-dependent

receptors are important for NK cell commitment[72-74].

The generation of NKP involves a complicated transcriptional network involving a variety of transcription factors (TFs). Nfil3 (E4BP4)-deficient mice do not have NK cells because of defects in the generation of NKP from CLP[75, 76]. PU.1-deficient mice have decreased numbers of NKP in chimeric mice[77]. Ets-1 regulates NK cell development by promoting Id-2 and T-bet expression[78]. E-box proteins (E2A, E2-2, HEB) and inhibitors of DNA binding (Id) proteins counter-regulate NK cell fate. Overexpression of Id-2 favors NK cell development by inhibiting the generation of T cells and B cells, and Id-2 mutant mice lack NK cells[79, 80]. Furthermore, some transcription factors regulate NK cell development by promoting IL-15R expression. These include T-bet, Eomes, Runx3 and Cbfβ[81, 82]. IL-15 signals through STAT5, thus STAT5-deficient mice lack NK cells[83]. IL-15 also affects NK cell commitment by driving Nfil-3 expression through PDK1 and mTOR signaling[84].

1.2.2.2. NK cell maturation (NKP - iNK - mNK)

NK cell maturation is a complicated and continuous process. From NKP to iNK, NK cells lose the expression of IL-7R but gain the expression of NK1.1, NKp46 and TRAIL. From iNK to mNK, NK cells lose CD27 and TRAIL expression but express DX5, CD11b and Ly49[85, 86]. In mice, mNK cells are characterized as non-B and non-T cells expressing CD122, NK1.1, DX5, NKG2D and CD16. mNK cells exhibit variable expression of Ly49 receptors and CD94/NKG2A/C. iNK cells represent an intermediate stage between NKP and mNK, thus any NK cell that lacks one or more mNK cell markers is broadly considered an iNK cell[87].

Transcription factors, including Gata-3, IRF-2, T-bet and eomes, are important for NK cell maturation. Eomes is required for Ly49 expression and is critical for the transition from iNK to

mNK[88]. Gata-3 is necessary for the expression of CD11b and Ly49 receptors, and Gata-3-deficiency results in poor IFN-γ production with no alteration of NK cell cytotoxicity[89]. T-bet-deficient and IRF-2-deficient NK cells display a phenotype similar to Gata-3-deficient NK cells, with abnormal expression of CD11b but normal cytotoxicity. Studies suggest that these three TFs might be involved in the same TF activation cascade (Gata-3 to IRF-2 to T-bet)[90, 91], potentially explaining the similar phenotypes of the single deficient mice in NK cell maturation.

Several TFs are involved in the effector function of mature NK cells, including MEF, MITF and CEBP-γ. Deficiencies in these TFs do not alter NK cell development and maturation, but they decrease the expression level of perforin and granzymes in NK cells, leading to reduced cytotoxicity and cytokine production[92, 93].

With the identification of new cell types by different research groups, it should be noted that some "so-called" iNK cells might belong to other cell types. For example, in the liver of C57BL/6 mice, there are two groups of $CD3^-$ $NK1.1^+$ NK cells based on staining with DX5. People used to refer to the $DX5^+$ population as mNK and the $DX5^-$ population as iNK[94]. However, hepatic $CD3^-$ $NK1.1^+$ $DX5^-$ cells are now defined as ILC1 and exhibit memory-like properties[8, 95, 96].

NK cell development undergoes a sequential process as follows: CLP-NKP-iNK-mNK. However, where these stages occur remains unclear. NKPs and iNKs are found not only in the bone marrow but also in the spleen, liver and lymph nodes. It is possible that these NKPs are circulated to the spleen, liver and lymph nodes from bone marrow, or that they are generated in *situ* in those organs[87]. It is also possible that thymic NK cells originated from the thymus rather than the bone marrow[97].

1.2.3. NK cell education

The NK cell response is determined by integrated signals mediated by various receptors that are stimulated during encounters with target cells. However, prior to meeting the target cells, NK cells must be "educated" by neighboring cells to acquire sufficient cytotoxic capacity.

1.2.3.1. Concept of "missing-self"

In 1990, Karre and colleagues first introduced the "missing-self" hypothesis. They proposed that NK cells provide immune surveillance against cells that downregulate MHC I[98]. Because all nucleated cells express MHC I, abnormal cells with MHC I downregulation are recognized as "non-self" cells and attacked by NK cells. Those "non-self" cells are often associated with cellular transformation or viral infection. Therefore, NK cells serve as guardian cells to detect these pathological changes[30]. NK cells must be educated through binding with MHC I by the inhibitory receptors (KIR in humans, inhibitory Ly49 in mice and CD94/NKG2A in both species). Education is a process for NK cells to recognize self-cells and in the meantime enables NK cells to have cytotoxic potentials against non-self cells. Based on the "missing-self" hypothesis, target cells without MHC I expression are more susceptible by educated NK cells. NK cell education is not only important for self-tolerance, but also critical for immunosurveillance.

The original "missing-self" hypothesis emphasizes the importance of NK cell inhibitory receptors that recognize MHC I. However, it cannot explain the cytotoxic ability of NK cells toward target cells in certain conditions. For example, human erythrocytes lack MHC I expression, but peripheral blood NK cells do not attack them[30]. This phenomenon may be due

to a lack of ligands that are capable of engaging activating receptors on NK cells. In another case, some tumor cells trigger sufficient NK cell activation, even when the inhibitory receptors for MHC I are ligated[99, 100]. Therefore, the hypothesis has been further revised as "NK cells detect and attack abnormal cells that lack MHC I or overexpress ligands for activating NK cell receptors"[30]. The revised "missing-self" hypothesis is detailed in Figure 1.3. To summarize, the activation of NK cells is dependent on the integration of signals involved in the recognition of target cells.

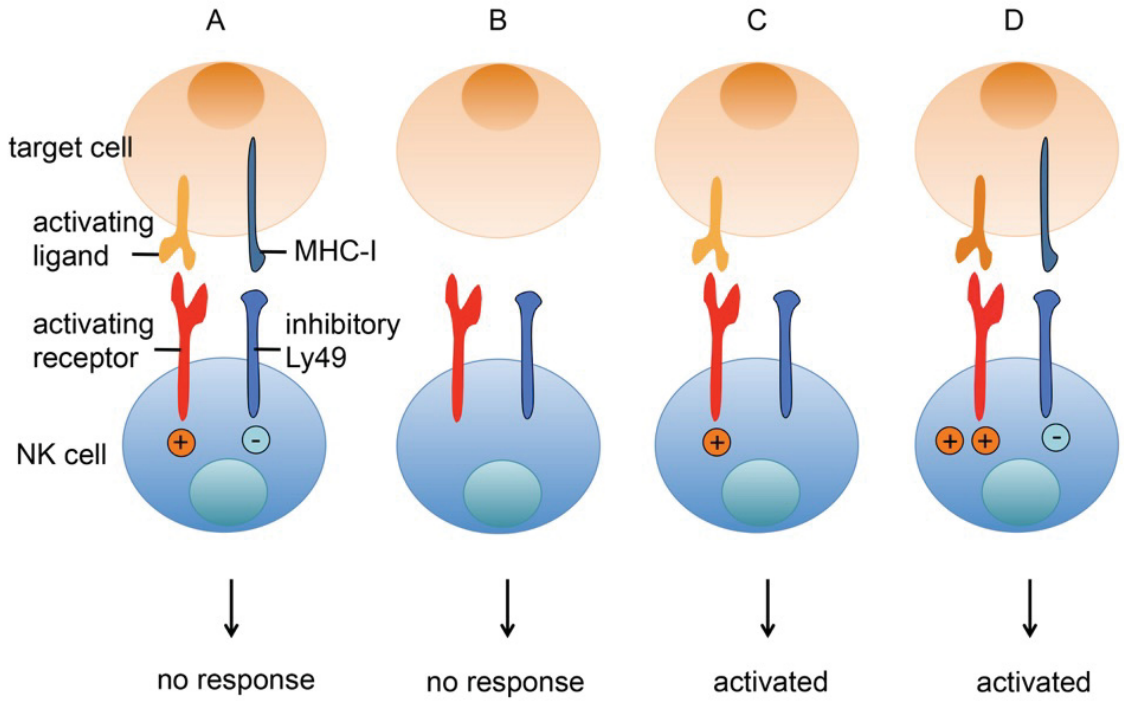

Figure 1.3. Revised "missing-self" hypothesis. NK cells respond to target cells that lack MHC I or overexpress ligands for activating NK cell receptors. (A) Target cells that express activating ligands and MHC I do not trigger NK cell activation. (B) Target cells missing MHC I do not activate NK cells in the absence of activating ligands. (C) Target cells that lose MHC I and express activating ligands trigger sufficient signals to activate NK cells. (D) Target cells that overexpress activating ligands but express MHC I are able to activate NK cells.

1.2.3.2. MHC I-dependent education

Based on the "missing-self" hypothesis, NK cells that lack the inhibitory MHC I-specific receptors should have enhanced cytotoxic activities in comparison to those from wild-type mice. However, in contrast, those NK cells are hyporesponsive. This phenomenon underlies NK cell education, which involves both MHC I-dependent and MHC I-independent mechanisms.

The classical concept of NK cell education focuses on the importance of self MHC I recognition between NK cells and neighboring cells. In C57BL/6 mice, several NK cell inhibitory receptors are involved in MHC I-dependent education. Ly49C and Ly49I recognize the classical MHC I, H-2K^{b}[101]. CD94/NKG2A recognizes H-2D^{b} presented on the non-classical MHC I, Qa-1[102]. Ly49A recognizes the non-classical MHC-1^{b} molecule, H2-M3[103]. The acquisition of these inhibitory receptors is stochastic, thus NK cells can express no or several such receptors[104]. In fact, in wild-type mice, approximately 15% of NK cells lack inhibitory receptors that are specific for self MHC I, and these NK cells are self-tolerant and hyporesponsive, even with sufficient activating receptor expression[105].

The use of genetically modified mice provides further evidence and insight into NK cell education. β2-microglubulin (β2M) KO mice are deficient of MHC I. NK cells from β2M KO mice are hyporesponsive and are unable to reject grafts from β2M KO mice, unlike NK cells from wild-type mice[106]. NK cells from *Kb*$^{-/-}$ *Db*$^{-/-}$ mice respond poorly to anti-NK1.1 stimulation, whereas the NK cell response is rescued in *H2-D*^{d} transgenic *Kb*$^{-/-}$ *Db*$^{-/-}$ mice[107]. The concept of NK cell education has also been confirmed in humans[108]. Three models of NK cell education have been proposed and are termed "Arming", "Disarming" and "Tuning", as indicated in Figure 1.4[109, 110].

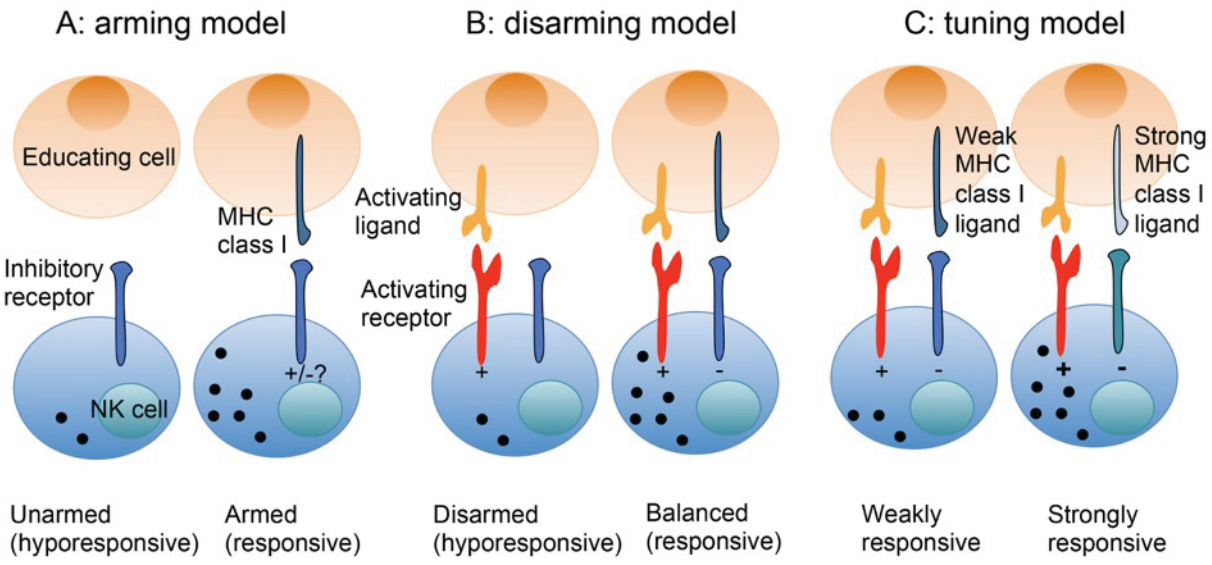

Figure 1.4. Models for NK cell education. (A) In the arming model, NK cells must receive inhibitory signals by engaging MHC I on educating cells to acquire a higher state of responsiveness. In the absence of such signals, NK cells are hyporesponsive. (B) In the disarming model, NK cells are activated by default. In the absence of inhibitory receptors, NK cells are persistently stimulated and become anergic. The presence of inhibitory receptors balances the activating signals and allows NK cells to be responsive. (C) In the tuning model, the NK cell response is dynamic and dependent on the strength of the inhibitory signals. Weak engagement of inhibitory receptors leads to a weak NK cell response, while strong engagement of inhibitory receptors results in a strong NK cell response.

Arming: The arming model proposes that NK cells must receive inhibitory signals by engaging MHC I on neighboring cells to acquire a higher state of responsiveness. In the absence of such signals, NK cells become hyporesponsive. This model emphasizes the critical role of inhibitory signals mediated by MHC I engagement to license NK cells[31, 107, 111].

Disarming: The disarming model highlights the importance of activating signals in NK cell education. In the default state, NK cells have the property of high responsiveness. However, in

the absence of inhibitory signal engagement with MHC I on neighboring cells, NK cells are persistently stimulated by activating signals. To avoid becoming auto-reactive, anergy is induced, and NK cells become hyporesponsive. In this model, the absence of inhibitory signals disarms the NK cells from being highly responsive[112].

Tuning: The arming and disarming models represent two extremes to explain NK cell education. The former highlights the importance of inhibitory signals, while the latter stresses the significance of activating signals. The tuning model incorporates both concepts and assumes that the NK cell response is quantitative rather than simply qualitative. The tuning model proposes that the NK cell response is determined by the integration of both activating and inhibitory signals. Strong engagement of inhibitory receptors leads to weak stimulation of NK cells; thus NK cells are hyperresponsive. Weak engagement of inhibitory receptors results in strong stimulation of NK cells; therefore NK cells are hyporesponsive. Intermediate stimulation contributes to an intermediate NK cell response[104, 113, 114].

1.2.3.3. MHC I-independent education

The tuning model extends our knowledge regarding NK cell education because it addresses the importance of both activating receptors and inhibitory receptors in the NK cell response prior to encountering target cells. Indeed, a recent study from our laboratory suggests that NK cell education can also be regulated via an MHC I-independent pathway. In that study, we showed that a SLAM family receptor, SLAMF6, renders NK cells hyperresponsive to nonhematopoietic cells in the absence of SAP. Phosphatase SHP-1 is involved in SLAMF6-mediated education, and the presence of SAP diminishes this effect[38]. Another recent study also suggests that SLAM family receptors are critical for NK cell education since SFR-deficient NK cells display

enhanced cytotoxicity toward hematopoietic cells[115]. We will discuss the phenotype of SFR-deficient NK cells in Chapter 2.

Other molecules involved in MHC I-independent education have been discussed, including 2B4, CD48, NKR-P1B, Clr-b, TIGIT and CD155[116]. Although some of these effects may not be strictly due to "education", they provide insight into our understanding of NK cell education and suggest that NK cell education can be either MHC I-dependent or MHC I-independent.

1.2.4. Functions of NK cells

NK cells have potent cytotoxicity to eliminate tumor cells, virus-infected cells or self-transformed cells. They not only serve as the guardian cells to protect the body from foreign dangers, but also regulate the immune response by interacting and communicating with other immune cells. NK cell contribute to the immune response either by mediating direct killing toward target cells or by secreting cytokines that affect other immune cells.

1.2.4.1. NK cells against tumor cells

Tumor cells are a type of target cell that can be killed efficiently by NK cells *in vitro* or *in vivo*. Due to metabolic stress such as malignant transformation or viral infection, tumor cells often downregulate surface MHC I expression, which protects them from being recognized by adaptive immune cells. However, changes in the expression level of MHC I render tumor cells susceptible to NK cells.

Additionally, tumor cells often upregulate ligands that bind to the activating receptors of NK cells. For example, the stress ligand retinoic acid early inducible 1 (RAE1) is induced in transformed cells by the DNA damage pathway[117]. UL16 binding protein 1 (ULBP1) is

upregulated during human cytomegalovirus (HCMV) infection[118]. NKG2D on NK cells recognizes its ligands, RAE1 and ULBP1, and triggers NK cell activation. Furthermore, NKG2D also mediates NK cell recognition and cytotoxicity against polyomavirus-induced tumors[119]. NK cells can prevent the metastasis of B16F10.9 melanoma (B16) and Lewis lung carcinoma (D122) *in vivo*, in part due to NKp46-mediated recognition, even though the ligands of NKp46 on tumor cells are unknown[120]. NK cells can also recognize and kill human and mouse melanoma cells *in vitro* and *in vivo* via natural cytotoxicity receptors (NCRs) and DNAM-1[121].

Tumor cells trigger NK cell cytotoxicity by enhancing activating receptor function, suppressing inhibitory receptor function or both. As a result, they are destroyed and cleared by NK cells.

1.2.4.2. NK cells against viral infection

NK cells have essential roles in the control of viral infection in humans and in mice. During murine cytomegalovirus (MCMV) infection, the NK cell activating receptor Ly49H is responsible for genetic resistance to MCMV by recognizing the viral m157 protein[122, 123]. The Ly49H-m157 interaction triggers activating signals on NK cells and leads to NK cell degranulation and cytokine production[122, 124, 125]. Thus, MCMV can be efficiently controlled in Ly49H-positive mouse strains such as C57BL/6, but not in Ly49H negative strains such as BALB/c[126-128].

In chronic hepatitis B virus (HBV) infection, NK cell frequencies are elevated in liver from patients compared with healthy donors[129, 130]. NK cells from HBV-infected patients increase the expression level of activating receptors such as NKG2C, NKp30 and NKp46 and decrease

the expression level of the inhibitory receptor NKG2A[129, 131]. This phenomenon correlates with enhanced NK cell cytotoxicity in chronic HBV infection, but with suppressed secretion of TNF-α and IFN-γ[130, 132].

NK cells also detect virus-infected cells via different mechanisms. Genetic resistance to influenza A virus (IAV) is mediated, in part, by NKp46, which recognizes IAV hemagglutinin[133]. Hence, NKp46 KO mice have higher sensitivity to IAV infection[134]. To control ectromelia virus, NKG2D and CD94/NKG2E contribute, in part, to the activation of NK cells[135, 136]. NK cells also recognize erythroid cells that are infected by friend retrovirus (FV) via the NKG2D-RAE-1 interaction[137]. During human immunodeficiency virus (HIV) infection, the virus escapes the NK cell-mediated response by suppressing NKG2D ligand expression on infected cells and by stimulating inhibitory receptors on NK cells[138, 139].

1.2.4.3. NK cells in immune regulation

In addition to their direct cytotoxicity against tumor cells and virus-infected cells, NK cells regulate the immune system during health and in pathological conditions. On the one hand, NK cells secrete cytokines, such as IFN-γ, TNF-α, MIP-1α, MIP-1β and RANTES, to amplify the immune response. On the other hand, NK cells lyse unwanted or over-activated cells to maintain a healthy state of the immune system[140, 141]. Both ways contribute to immune cell regulation.

NK cells can induce DC maturation by secreting IFN-γ, which leads to secretion of IL-12 by DCs[45]. Furthermore, IFN-γ secreted by NK cells and IL-12 released by DCs can promote CD4 T cell differentiation into Th1 cells that enhance anti-viral immunity[142]. In humans, both immature and mature DCs can stimulate the proliferation and activation of resting NK cells to control tumor invasion. However, activated NK cells are able to kill immature DCs via their

activating receptor NKp30 or TRAIL, but unable to kill mature DCs[143, 144]. The crosstalk between NK cells and DCs is important to limit the supply of DCs, which modulates the adaptive immune response[143, 145].

In the case of LCMV infection, NK cells do not exhibit direct antiviral effects; the virus titer remains the same in the spleen during the first three days of infection in the presence or absence of NK cells[146]. Nonetheless, NK cells contribute to immune regulation by controlling virus-specific T cells. During infection of LCMV clone 13 at a low virus dose (5×10^4 p.f.u.), virus-specific T cells can rapidly eliminate the virus and protect the host; at an intermediate virus dose (2×10^5 p.f.u.), a sufficient functional T cell response is triggered, and severe immune pathology is induced, leading to host death; at a high virus dose (2×10^6 p.f.u.), virus-specific T cells are exhausted, resulting in persistent virus infection. The reaction against LCMV is dependent on the dose of the virus, and NK cells act as rheostats by controlling anti-virus T cells, as shown in Figure 1.5. At a high virus dose, the absence of NK cells leads to insufficient control of functional T cells, ultimately triggering lethal immune pathology and host death. At an intermediate virus dose, the lack of NK cells helps prevent the lethal immunopathology due to the increase in virus-specific T cell numbers, which can clear the virus more efficiently. As a result, after the rapid clearance of virus, T cells acquire an exhausted phenotype and produce few cytokines, leading to a reduced or no immune pathology[147]. Interestingly, 2B4 on NK cells serves as an inhibitory receptor in the LCMV infection model[148]. Moreover, type I IFN protects activated T cells from being killed by NK cells[149, 150]. We will discuss the role of 2B4 and type I IFN in NK cell activation in detail in Chapter 2.

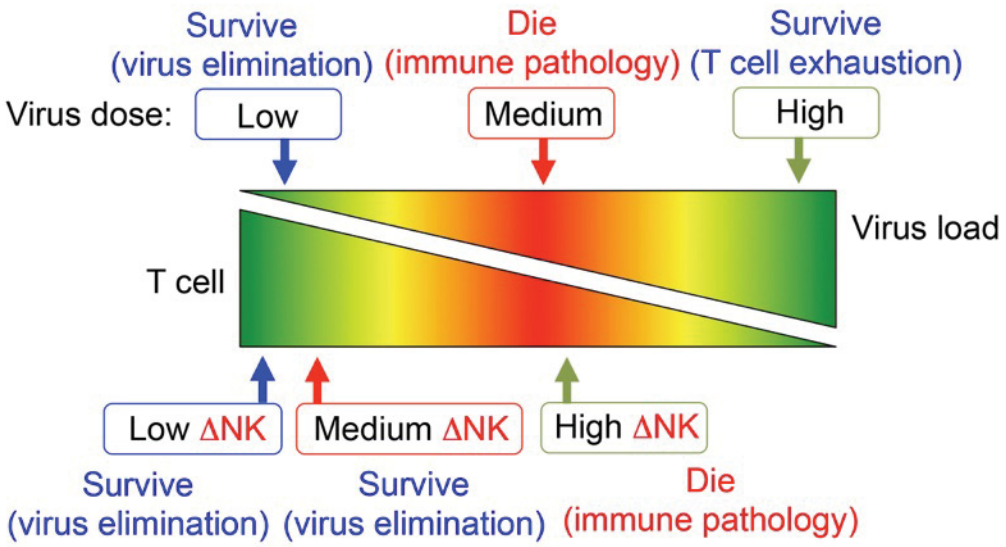

Figure 1.5. Pathogenesis of LCMV infection at different virus doses. During LCMV infection, virus-specific T cells are responsible for the control of virus. (Top) At a low virus dose, T cells clear the virus rapidly; at a medium dose, T cells induce a lethal immune pathology; at a high dose, T cells are exhausted, leading to chronic viral infection. (Bottom) NK cells control activated T cells by direct lysis. Depletion of NK cells (Δ NK) leads to a stronger T cell response, which changes the dose of virus to induce immune pathology. The red area indicates the zone of severe immune pathology.

1.3. SLAM family receptors and SAP family adaptors

1.3.1. SLAM family receptors

The signaling lymphocyte activation molecule (SLAM) receptors include 6 transmembrane receptors, namely SLAM (SLAMF1, CD150), 2B4 (SLAMF4, CD244), Ly-9 (SLAMF3, CD229), CD84 (SLAMF5), SLAMF6 (Ly108, NTB-A, CD352) and SLAMF7 (CRACC, CS1, CD319). Exclusively expressed on hematopoietic cells, SLAM family receptors (SFRs) are homotypic receptors, except 2B4, which binds to CD48. SFRs recognize the same receptor as a ligand expressed either on another cell (*trans* interaction) or on the same cell (*cis* interaction). SFRs are type I glycoproteins with one Ig variable (V)-like domain and one C2 domain in the

extracellular regions, except Ly-9, which contains four Ig-like domains[151]. In their intracellular region, SFRs bear one or more immunoreceptor tyrosine-based switch motifs (ITSMs). The expression patterns of SLAM family receptors and phenotypes of single knock-out mice are indicated in Table 1.1.

Table 1.1. SLAM family receptors and CD48.

Receptor	Expression pattern	Altered function of indicated immune cells in knock-out mice	Number of ITSMs	Interaction with	
				SAP	EAT-2
SLAMF1 (SLAM, CD150)	T, B, DC, Mϕ, plat	CD4 T, GC T_{FH}, Mϕ, plat, NKT	2	+	+
SLAMF3 (Ly-9, CD229)	T, B, NK, NKT, DC, Mϕ	CD4 T, innate-like CD8 T	1	+	+
SLAMF4 (2B4, CD244)	NK, CD8 T, γδ T, DC, Mϕ, baso, eos	NK	3	+	+
SLAMF5 (CD84)	T, B, NK, NKT, DC, Mϕ, gran, plat, mast, eos	T, B (GC), NKT	2	+	+
SLAMF6 (Ly108, NTB-A, CD352)	T, B, NK, NKT, DC, neutro, eos	T, B, NK, NKT neutro,	2	+	+
SLAMF7 (CRACC, CD319, CS1)	T, B, NK, NKT, Mϕ, DC, PC	NK, Mϕ	1	-	+
SLAMF2 (CD48)	T, B, NK, NKT, Mϕ, DC, gran, mast	T			

ITSM, immunoreceptor tyrosine-based switch motif; T, T cell; B, B cell; NKT, natural killer-T cell; T_{FH}, follicular helper T cell; NK, natural killer cell; DC, dendritic cell; Mϕ, macrophage; plat, platelet; baso, basophil; eos, eosinophil; mast, mast cell; gran, granulocyte; neutro, neutrophil; PC, plasma cell; GC, germinal center.

The *SLAM gene locus* is located on chromosome 1, which encodes not only the six SFR

members but also CD48 (the ligand of 2B4) and a short non-conserved open reading frame (ORF) of unknown function named *Gm10521*.

1.3.1.1. SLAMF1 (SLAM, CD150)

SLAM is expressed on thymocytes, B cells, DCs, activated T cells, germinal center (GC) follicular T cells (T_{FH}), macrophages and platelets [152-156]. NK cells do not express SLAM. Upon TCR engagement, CD4 T cells from SLAM KO mice produce less IL-4 but slightly more IFN-γ compared with CD4 T cells from wild-type mice[157]. GC T_{FH} cells also produce less IL-4 in SLAM KO mice[158]. In response to lipopolysaccharide (LPS), macrophages from SLAM KO mice produce less IL-12, TNF-α, IL-6 and nitric oxide (NO)[157]. Moreover, SLAM-deficient macrophages display impaired phagosome maturation and inefficient killing of bacteria[155]. During platelet aggregation, SLAM is phosphorylated and associated with the adaptor SAP. Additionally, SLAM-deficient mice display some defects in platelet aggregation and arterial thrombotic processes[154].

1.3.1.2. SLAMF3 (Ly-9, CD229)

Ly-9 is expressed on T cells, NKT cells, B cells, NK cells, macrophages and DCs. Ly-9-deficient T cells have a mild Th2 defect and produce less IL-4 following TCR stimulation. They also proliferate poorly and produce little IL-2 after TCR stimulation[159]. Ly-9 is localized in the immunological synapse between T cells and B cells, suggesting that it might participate in the regulation of T-B interactions[160]. In NK cells, Ly-9 serves as an activating receptor in the presence of SAP, since overexpression of Ly-9 on the melanoma cell line B16 enhances wild-type NK cell cytotoxicity. In contrast, the absence of SAP renders Ly-9 inhibitory[161].

Ly-9 is an alloantigenic maker with two distinguished alleles (Ly-9.1 and Ly-9.2). The Ly9.2 allele is expressed in C57BL/6 and related strains (C58, C57L. and C57BL/10), whereas the Ly9.1 allele is expressed in most other mouse strains, such as A/J, BALBc, C3H and 129 [162, 163]. Sequencing of the Ly-9 gene in C57BL/6 and BALBc revealed 9 discrepancies between these two stains, four of which were in the first Ig-like domain, which might affect the ligand-binding ability[164].

1.3.1.3. SLAMF4 (2B4, CD244) and SLAMF2 (CD48)

2B4 is expressed on NK cells, activated CD8 T cells, γδ T cells, CD8 intraepithelial lymphocytes (iELs), monocytes, basophils and eosinophils [165]. It recognizes CD48 as a ligand, which is expressed widely on hematopoietic cells.

The role of 2B4 on CD8 T cells is still controversial. On the one hand, the 2B4/SAP pathway provides activating signals for CTL lytic activity against EBV-positive target cells[166]. Co-expression of 2B4 and CD160 on CTL is correlated with high levels of perforin and granzyme B, suggesting increased lytic activity[167]. On the other hand, blockade of 2B4/CD48 interactions enhances human T-lymphotropic virus type 1 (HTLV-1)-specific CD8 T cell function toward HTLV-1-infected cells[168]. Exhausted T cells express high levels of 2B4, as well as other inhibitory receptors such as PD-1, CTLA-4, LAG-3, TIM-3 and CD160[169].

In human and mouse NK cells, stimulation of 2B4 either by antibody crosslinking or by ectopic expression of CD48 on nonhematopoietic target cells results in NK cell activation, involving signaling pathways that are mediated by SAP and EAT-2[161, 170, 171]. Paradoxically, however, NK cells from 2B4 KO mice display increased cytotoxicity toward CD48-positive target cells compared with NK cells from wild-type mice[148, 172]. Hence, the

functional role of 2B4 (activating or inhibitory) in NK cells is still not fully understood and will be examined in Chapter 2.

In humans, two 2B4 isoforms, h2B4-A and h2B4-B, have been identified. These two isoforms differ in a small portion of the extracellular domain. Compared with h2B4-B, h2B4-A has a stronger affinity for CD48 and induces greater cytotoxicity and intracellular calcium release when crosslinked with CD48. The expression level of both isoforms varies in different immune cells, indicating distinct function of 2B4 in those cells [173, 174].

Most mouse strains, including 129, SJL, NZB, DBA, BALBc, C3H, CBA and A/J, have four copies of the 2B4 gene, which can be detected by C9.1 monoclonal antibody (mAb) (CD244.1). However, 2B4 from C57BL/6 mice can be detected by anti-CD244.2 antibody but not C9.1 mAb [175].

1.3.1.4. SLAMF5 (CD84)

CD84 is expressed on T cells, B cells, NK cells, NKT cells, monocytes, DCs, eosinophils, neutrophils and platelets. CD84, as well as Ly108, is critical for prolonged T cell-B cell interactions and germinal center formation via the SAP signaling pathway. Hence, CD84 KO mice have fewer T_{FH} cells, impaired T-B cell adhesion and impaired GC formation[176]. In human B cells, CD84 is upregulated on a subset of memory B cells and is capable of transducing signals through SAP and/or EAT-2, suggesting that CD84 might act as an important receptor in B cell memory[177]. In wild-type NK cells, CD84 seems to have less important functions compared with other SLAM family receptors. However, it acquires an inhibitory effect in the absence of SAP family adaptors[161].

1.3.1.5. SLAMF6 (Ly108, NTB-A)

SLAMF6 is expressed on T cells, B cells, NK cells, DCs, NKT cells, eosinophils and neutrophils. Human SLAMF6 is also termed NTB-A, whereas mouse SLAMF6 is often denoted Ly108. SLAMF6, as well as SLAMF1 and SLAMF5, is required for NKT cell development[178, 179]. SLAMF6 KO CD4 T cells produce less IL-4 than wild-type CD4 T cells in response to leishmania mexicana infection[180]. Neutrophils from SLAMF6 KO mice produce more IL-12, IL-6 and TNF-α, whereas they produce less reactive oxygen species (ROS) and promote less bacterial killing[180]. SLAMF6 is highly expressed in NKP but is downregulated during NK cell development. The role of SLAMF6 in NK cell education is discussed in Section 1.2.3.3. (MHC I-independent education).

In mice, SLAMF6 has at least three isoforms: Ly108-1, Ly108-2 and Ly108-H1. These isoforms contain identical extracellular regions with distinct sequences in their cytoplasmic tails. Ly108-1 has two ITSMs with one additional tyrosine in the cytoplasmic tail and is involved in the pathogenesis of lupus, whereas Ly108-2 bears two ITSMs with two additional tyrosines and contributes to the sensitization of T cells and B cells to apoptosis. Ly108-H1 contains only one ITSM and suppresses *Sle1b*-associated lupus disease by inhibiting T cell proliferation[151, 181, 182].

1.3.1.6. SLAMF7 (CRACC, CS1, CD319)

SLAMF7 is expressed on NK cells, B cells, DCs, plasma cells and activated T cells. Unlike other SLAM family receptors, SLAMF7 is only associated with EAT-2 but not SAP.

In T cells that lack EAT-2, engagement of SLAMF7 inhibits cell proliferation and cytokine production after antigen stimulation[183]. In B cells, antibody stimulation against SLAMF7

enhances B cell proliferation as well as mRNA expression of several growth-promoting cytokines, suggesting that SLAMF7 might promote B cell growth[184]. In NK cells, SLAMF7 mediates activating signals via EAT-2, involving PLC-γ signaling and calcium influx. Therefore, NK cells from SLAMF7 KO mice show reduced killing of SLAMF7-positive target cells compared with NK cells from wild-type mice[183, 185].

In humans, due to alternative splicing, two isoforms of SLAMF7 have been identified: SLAMF7-long (SLAMF7-L) and SLAMF7-short (SLAMF7-S). SLAMF7-L contains tyrosines (Y261, Y281) that are critical for binding to EAT-2, and it is capable of mediating activating signals in NK cells. In contrast, SLAMF7-S does not contain the tyrosines and is not functional. The mechanism underlying SLAMF7-mediated signaling will be discussed in Chapter 3.

1.3.2. SAP family adaptors

SLAM family receptors bear one or more ITSMs in their intracellular regions. Upon phosphorylation, they can bind to SLAM-associated protein (SAP) family adaptors. The presence of SAP family adaptors is critical for SLAM family receptor-mediated activation in hematopoietic cells.

1.3.2.1. Structure and expression of SAP family adaptors

There are three members of SAP family adaptors: SAP, Ewing's sarcoma-associated transcript-2 (EAT-2) and EAT-2-related transducer (ERT). The gene encoding SAP, *SH2D1A*, is located on the X chromosome, whereas *SH2D1B1* (encoding EAT-2) and *SH2D1B2* (encoding ERT) are located on chromosome 1. In humans, the ERT-encoding gene is a pseudogene. SAP consists almost entirely of a single Src homology 2 (SH2) domain, while EAT-2 and ERT are composed

of a single SH2 domain and a short carboxy-terminal tail. SAP is expressed in T cells, NK cells, NKT cells, eosinophils and platelets[152, 154, 165, 186-188]. EAT-2 is expressed in NK cells, DCs, macrophages, CD8 T cells and platelets[189-191]. ERT is only found in mouse NK cells[189, 191, 192]. The expression patterns of SAP family adaptors and phenotypes of single knock-out mice are shown in Table 1.2.

Table 1.2. SAP family adaptors.

Adaptor	Expression pattern	Altered function of indicated immune cells in knock-out mice	Chromosomal localization	Downstream effector
SAP (SH2D1A)	T, (?B), NK, NKT, eos, plat	CD8, Th2, T_{FH}, B, NK, NKT, plat	X	Fyn
EAT-2 (SH2D1B1)	CD8 T, (?B), NK, DC, Mϕ	NK	1	PLC-γ
ERT (SH2D1B2)	mouse NK	NK	1	?

T, T cell; Th2, type 2 helper T cell; T_{FH}, follicular helper T cell; B, B cell; NK, natural killer cell; NKT, natural killer-T cell; DC, dendritic cell; Mϕ, macrophage; plat, platelet; eos, eosinophil.

1.3.2.2. XLP and SAP

SH2D1A, which encodes SAP, is frequently mutated in X-linked lymphoproliferative disease (XLP). XLP is an inherited disorder characterized by lymphohistiocytosis, hypogammaglobulinemia and lymphomas. Due to inactivating mutations of SAP, XLP patients display a fatal response to Epstein-Barr virus (EBV) infection. Thus, they fail to clear EBV-infected B cells and display a massive expansion of lymphocytes, which can infiltrate the liver, bone marrow or other organs[186, 193-195].

SH2D1A is mutated in ~60% of cases of XLP[196]. The phenotype of XLP patients is largely recreated in SAP-deficient mice. SAP-deficient mice display impaired NKT cell development and have very few NKT cells[188, 197]. The CD4 T cells from SAP-deficient mice have reduced Th2 cytokine production, and the follicular T_{FH} cells are defective, leading to severely compromised germinal center (GC) formation. Although it is unclear whether B cells express small amounts of SAP, SAP-deficient B cells seem to have both T cell-dependent and T cell-independent defects, resulting in reduced antibody production[198-200]. SAP-deficient mice display vigorous CD8 T cell proliferation and infiltration in the liver and lung after murine gammaherpesvirus-68 (MHV-68) infection (a virus homologous to EBV)[201, 202]. This phenomenon resembles the large expansion of reactive CD8 T cells in XLP patients with EBV infection. SAP-deficient NK cells have reduced cytotoxicity and cytokine production, similar to NK cells from XLP patients[37, 161, 203]. Hence, XLP patients often suffer from EBV infection due to an impaired immune system.

In addition to *SH2D1A*, mutations have been identified in two other genes, encoding XIAP (X-linked inhibitor of apoptosis protein) and ITK (IL-2-inducible T cell kinase), in XLP-like disease with fatal infectious mononucleosis.

The gene encoding XIAP is located on the X chromosome in both humans and mice. T lymphocytes with XIAP deficiency show enhanced apoptosis after engagement with various receptors, including the TCR, the death receptor CD95 and the TNF-associated apoptosis-inducing ligand receptor (TRAIL-R). Similar to SAP-deficient patients, XIAP-deficient patients also have impaired NKT cell development and frequently suffer from EBV infection, implying that NKT cells might play a key role in the immune response to EBV[204, 205].

The gene encoding ITK is located on chromosome 5 in humans and on chromosome 11 in mice. A homozygous missense mutation of ITK (R335W) have been reported in 2 girls from a consanguineous family. They have no NKT cells and display massive proliferation of EBV-infected B cells[206].

1.3.3. SLAM family receptor signaling in NK cells

SAP family adaptors activate NK cell signaling in two major ways. Firstly, SAP family adaptors serve as natural blockers and prevent the coupling of SLAM family receptors to inhibitory molecules, such as SHIP-1, SHP-1, SHP-2 and Csk. Alternatively, they mediate activating signals, which lead to different activating effects between SAP and EAT-2[151, 196].

Several lines of evidence support the natural blocker model. SAP family adaptors and inhibitory molecules such as SHIP-1, SHP-1, SHP-2 and Csk all contain the SH2 domain, which is principally capable of binding to phosphorylated tyrosines in SFRs[152]. The competition of binding to phosphorylated tyrosines is supported by biochemical studies using lysates from transfected cells, although it has not been substantiated in normal immune cells[170]. In a study investigating 2B4 stimulation, SHIP-1 phosphorylation was enhanced in SAP family adaptor KO NK cells compared with wild-type NK cells[37]. This finding suggests that SAP family adaptors do inhibit the function of SHIP-1, albeit it could either be due to direct competition or to indirect suppression of SHIP-1 function via activating signals.

The activating signals mediated by SAP and EAT-2 are not identical. As shown in Figure 1.6, SAP couples the Src family protein tyrosine kinase Fyn to SLAM family receptors via arginine (R78). Fyn phosphorylates the exchange factor Vav-1. As a consequence, SAP stabilizes conjugate formation of NK cells and target cells to enhance NK cell cytotoxicity[37,

161]. EAT-2 activates NK cells via its carboxy-terminal region, which consists of one tyrosine (Y127) in humans and two tyrosines (Y120, Y127) in mice. After phosphorylation of its tyrosine(s), EAT-2 interacts with the N-terminal SH2 domain of PLC-γ and subsequently triggers signals involving calcium influx and Erk activation. Therefore, EAT-2 accelerates the polarization of cytotoxic granules toward target cells[191]. SAP and EAT-2 trigger two distinct activating effects. Hence, they are both required for NK cell activation. ERT is only expressed in cytokine-primed NK cells, e.g., lymphokine-activated killer (LAK) cells, and its function remains undetermined.

It should be noted that, in wild-type NK cells, 2B4 stimulation induces some phosphorylation of SHIP-1, indicating that inhibitory signals exist even in the presence of SAP family adaptors[37]. In another case, mouse SLAMF7 bears two critical tyrosines: Y261 and Y281. Y281 is responsible for SLAMF7-mediated activation, whereas Y261 is critical for its inhibitory function. Both Y261 and Y281 are phosphorylated following SLAMF7 stimulation, suggesting that activating signals and inhibitory signals co-exist in wild-type NK cells[183, 207]. The presence of SAP family adaptors does not completely suppress the inhibitory signals. The outcome of SFR-mediated signaling is dependent on the integration of both.

In mice, SLAM family receptors (SFRs) on wild-type NK cells that express both SAP and EAT-2 enhance NK cell cytotoxicity toward B16 melanoma cells transfected with SFRs compared with B16 non-transfectants[161]. When SFRs are stimulated by antibody-crosslinking, SFRs induce activating signals involving the SAP-Fyn-Vav1 pathway or the EAT-2-PLC-γ-Erk pathway[37, 191]. In both systems, SFRs are extensively stimulated, either by ectopic expression of SFRs as ligands on nonhematopoietic cells or by crosslinking with antibody. In these conditions, SFRs behave as activating receptors. Nevertheless, whether SFRs behave the same

when stimulated with SFRs on normal hematopoietic cells is still unknown. This issue will be discussed in Chapter 2.

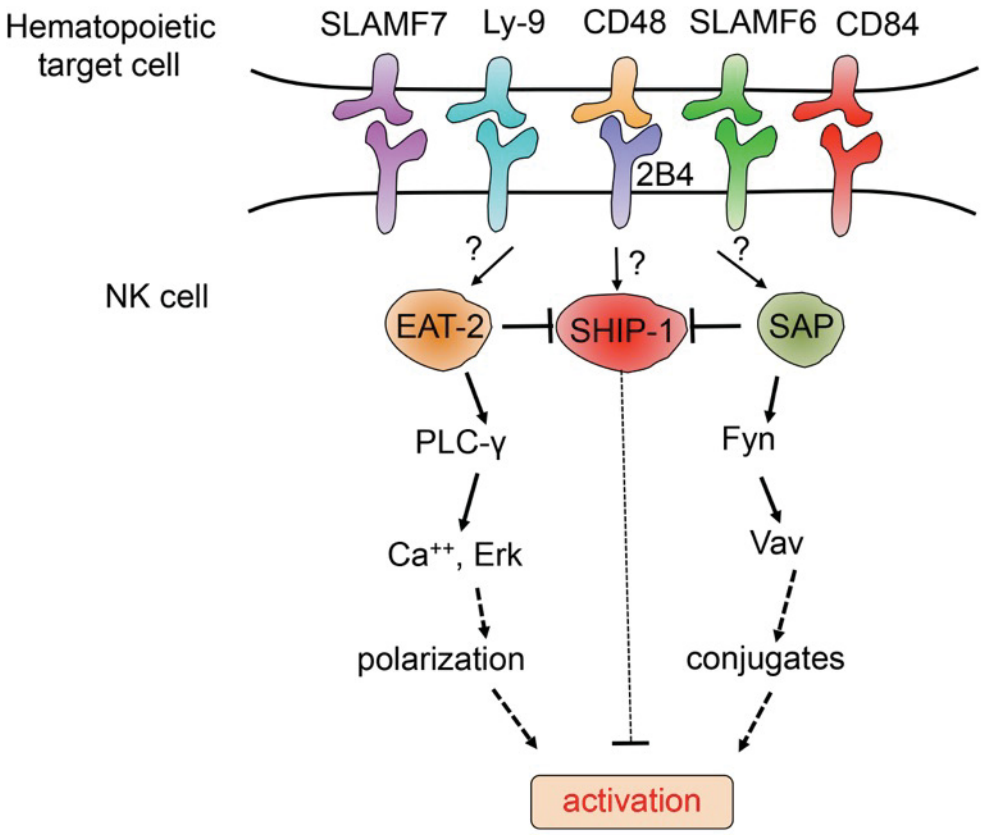

Figure 1.6. Signaling of SLAM family receptors in NK cells. SLAM family receptors activate NK cells via SAP family adaptors. Considering 2B4 as an example, upon engagement to CD48, 2B4 binds to SAP and EAT-2. SAP recruits Fyn and activates Vav, promoting conjugate formation. EAT-2 activates PLC- γ , induces calcium influx and mediates Erk activation, leading to granule polarization. Additionally, SAP and EAT-2 serve as natural blockers that inhibit the recruitment of negative effectors such as SHIP-1.

1.4. Multiple myeloma cells and SLAMF7

1.4.1. Multiple myeloma and treatments

Multiple myeloma (MM) is a malignancy of plasma cells and is characterized by a large accumulation of abnormal plasma cells in bone marrow. In most cases, MM is associated with abundant production of a monoclonal antibody termed paraprotein in the blood. MM remains an incurable disease, and MM patients often suffer from bone pain, bone fractures, anemia and renal disease. Each year, there are more than 100,000 new cases of MM worldwide, and newly diagnosed patients can survive an average of 7 to 8 years with currently available treatments[208, 209].

MM is a disease associated with abnormal plasma cells that are derived from B lymphocytes. Due to oncogenic mutation or chromosomal translocation, plasma cells bypass cell cycle control and extensively proliferate, producing a large amount of paraproteins[210]. Although currently incurable, MM can be treated with the available therapeutic modalities. The treatment of MM is mainly to decrease and limit the clonal plasma cell population. There are currently two new therapeutic modalities. One is to use the proteasome inhibitor bortezomib, which prevents the degradation of proteins including pro-apoptotic factors and leads to programmed cell death[211, 212]. The other is to use immunomodulatory drugs such as thalidomide and lenalidomide, albeit the mechanism is not fully understood[213, 214]. Even though the treatments can prolong the lifetime of MM patients, they cannot completely cure the disease. In the end, MM patients will suffer from relapse with progressive disease and die.

1.4.2. Effect of elotuzumab (anti-SLAMF7) against MM

New therapeutic targets are needed to treat MM. Elotuzumab (Empliciti), a humanized monoclonal antibody against SLAMF7, has been approved by the FDA for the treatment of MM in combination with lenalidomide and dexamethasone[215, 216]. It is one of the only two

FDA-approved monoclonal antibodies used to treat MM. The other one is daratumumab, which targets CD38 overexpressed on MM cells[217].

Low expression levels of SLAMF7 have been observed on resting mature B cells. However, following activation, the expression level of SLAMF7 is upregulated[184]. Plasma cells express high levels of SLAMF7, which is absent on most malignant hematopoietic cells[218]. Interestingly, however, it is highly expressed on cells in MM and in other diseases associated with abnormal plasma cells such as plasmacytomas, smoldering myeloma and monoclonal gammopathies of undetermined significance (MGUS)[218, 219].

The action of elotuzumab against MM has been studied by several research groups, and different mechanisms have been proposed. The predominant effect of elotuzumab is to induce antibody-dependent cell-mediated cytotoxicity (ADCC) against MM cells. Elotuzumab binds to MM cells via its fragment antigen-binding (Fab) portion and interacts with NK cells via its fragment crystallizable (Fc) portion. This process triggers NK cell activation against MM cells[218, 220, 221]. In addition, elotuzumab may directly stimulate SLAMF7 expressed on NK cells and enhance NK cell cytotoxicity[222]. In addition, elotuzumab may have some effects on the MM microenvironment. Some bone marrow stromal cells (BMSCs) express SLAMF7 and interact with MM cells via the SLAMF7-SLAMF7 interaction. This process can promote MM cell survival and growth. By blocking this interaction, elotuzumab may prevent MM cell growth in the microenvironment[219]. Additionally, some SLAMF7 positive myeloid-derived suppressor cells (MDSCs), which suppress the anti-tumor response, are also present in bone marrow together with MM cells. By preventing the function of MDSCs by blocking SLAMF7, elotuzumab may also promote anti-tumor responses against MM cells[223].

Although various studies have investigated the anti-tumor effects of elotuzumab, the direct

cytostatic or cytotoxic effects on MM cells remain unknown[221]. Based on a study conducted in NK cells, SLAMF7 can be either activating or inhibitory, depending on the presence or absence of EAT-2[183]. MM cells do not seem to express EAT-2, indicating that SLAMF7 on MM cells may not be an activating receptor. The direct role of SLAMF7 on MM cells needs to be determined, which will be discussed in Chapter 3.

Chapter 2: Deletion of *Slam* locus in mice reveals inhibitory role of SLAM family in NK cell responses regulated by cytokines and LFA-1

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Deletion of *Slam* locus in mice reveals inhibitory role of SLAM family in NK cell responses regulated by cytokines and LFA-1

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ABSTRACT

SLAM family receptors (SFRs) can mediate either activating or inhibitory effects during NK cell activation. Herein, we addressed the global role, regulation and mechanism of action of the SLAM family in NK cells, by analyzing a mouse lacking the entire ~400 kilobase-*Slam* locus, which encodes all six SFRs and CD48, the ligand of SFR 2B4. This mouse displayed enhanced NK cell activation responses towards hematopoietic target cells. Analyses of mice lacking individual SFRs showed that the inhibitory function of the *Slam* locus was due solely to 2B4 and was not influenced positively or negatively by other SFRs. Differences in NK cell responses between recognition of targets expressing or lacking ligands for SFRs were enhanced by IL-12, but suppressed by type I IFN. Cytokines also changed the levels of SAP adaptors, which prevent the inhibitory function of SFRs. The enhanced activation responses of SFR-deficient NK cells were dependent on integrin LFA-1, but not on DNAM-1 or NKG2D. SFR-mediated inhibition prevented the generation of "activated" forms of LFA-1. Hence, the *Slam* locus has an overall inhibitory role during NK cell activation that is solely dependent on 2B4. This effect is influenced by cytokines and leads to suppression of LFA-1 activity.

INTRODUCTION

Natural killer (NK) cells play key roles in anti-tumor and anti-viral immunity, as well as in normal immune regulation, through their capacity to kill abnormal or activated cells, in particular hematopoietic cells [30, 224, 225]. Activation of NK cells is determined by the balance between stimulation of various activating and inhibitory receptors, by ligands that may or may not be present on potential target cells. This activation is also influenced by cues received from surrounding cells prior to encounters with targets, in particular other hematopoietic cells. This effect can take place during or after NK cell maturation, and is commonly termed NK cell education [224, 226-228].

SLAM family receptors (SFRs) include 6 transmembrane receptors named SLAM (SLAMF1, CD150), 2B4 (SLAMF4, CD244), Ly-9 (SLAMF3, CD229), CD84 (SLAMF5), SLAMF6 (Ly108, NTB-A) and SLAMF7 (CRACC, CS1) [151, 196, 229, 230]. They are expressed only on hematopoietic cells. All SFRs except 2B4 are homotypic receptors, i.e. they recognize as ligand another molecule of the same receptor expressed either on another cell (*trans* interaction) or, in some cases, on the same cell (*cis* interaction). 2B4 interacts with CD48 (SLAMF2), the expression of which is also restricted to hematopoietic cells. Although CD48 is related to SFRs, it is strictly speaking not a SFR, as, unlike SFRs, it is attached to the plasma membrane via a glycosylphosphatidylinositol moiety. Other receptors known as SLAMF8 and SLAMF9 are also not true members of the SLAM family, as they do not have as yet determined ligands and they significantly differ from SFRs in their cytoplasmic domain. All *bona fide* SFRs except SLAM are expressed on NK cells.

By way of "immunoreceptor tyrosine-based switch motifs" (ITSMs) located in their cytoplasmic domain, all SFRs associate with SAP adaptors [151, 196, 229, 230]. SAP adaptors

include SAP, EAT-2 and, in mice but not humans, ERT. They are composed almost exclusively of a Src homology 2 (SH2) domain. All SAP adaptors are expressed in NK cells. Through their ITSMs, SFRs can also associate with SH2 domain-bearing inhibitory molecules such as protein tyrosine phosphatases SHP-1 and SHP-2, and inositol phosphatase SHIP-1. When associated with SFRs, SAP adaptors prevent the interactions of SFRs with phosphatases.

SFRs and SAP adaptors have been clearly implicated in normal immune regulation and in immunological diseases [151, 196, 229-231]. The *SLAM* locus (*Slam* in mice), which encompasses the genes coding for all SFRs and CD48 on chromosome 1, is highly polymorphic in humans and mice. Some of these polymorphisms have been linked to autoimmune diseases [151, 196, 229-231]. In addition, the SAP-encoding gene (*SH2D1A*) is mutated and inactivated in a human primary immunodeficiency, X-linked lymphoproliferative (XLP) disease [232, 233]. We and others showed that loss of SAP or other SAP adaptors converts SFRs into "super-inhibitory" receptors, due to enhanced coupling of SFRs to inhibitory effectors [37, 161, 191, 234-236]. This alteration compromises activation of NK cells and T cells, leading to multiple immune cell defects, including reduced NK cell cytotoxicity in response to hematopoietic target cells. These defects likely underlie the pathophysiology of XLP disease.

Although SFRs are "super-inhibitory" in NK cells lacking SAP adaptors, there is much debate about the functions of SFRs in normal NK cells, which contain SAP adaptors [231]. This is due in part to the fact that mice lacking individual SFRs usually exhibit minor phenotypes, possibly due to redundancy between SFRs [151, 196, 229, 230]. However, it has been difficult to address the issue of redundancy by breeding together mice lacking individual SFRs, given that all genes encoding SFRs are located in the same gene locus. Nonetheless, there is currently evidence that SFRs can be either activating or inhibitory in NK cells containing SAP adaptors,

depending on the context [231].

For instance, it was reported that triggering of several SFRs on mouse or human NK cells, including 2B4, SLAMF7, SLAMF6 and Ly-9, using their ligands ectopically expressed on non-hematopoietic target cells or, in some cases, anti-SFR monoclonal antibodies (MAbs), results in enhanced NK cell activation [151, 196, 229, 230]. Although the physiological relevance of these modes of stimulation is unclear, these data suggested that SFRs are positive regulators of NK cell activation. Paradoxically, however, NK cells from mice lacking the SFR 2B4 displayed enhanced activation in response to hematopoietic target cells *in vitro* and *in vivo* [148, 172, 237]. Hence, at least one SFR, 2B4, is primarily an inhibitory receptor in mouse NK cells expressing SAP adaptors. The roles of the other SFRs in normal NK cells are largely unknown.

Several important issues regarding the role, regulation and mechanism of action of SFRs in normal NK cells remain elusive. As SFRs can be activating or inhibitory, whether the global function of the SLAM family in normal NK cells is activating, inhibitory or neutral needs to be ascertained. In addition, the impact of the individual SFRs other than 2B4 in this setting has to be elucidated. Likewise, the potential effects of host factors, such as the cytokine milieu, on the impact of engagement of SLAM receptors during target cell recognition should be assessed. And last, the activating NK cell receptors whose functions are either enhanced or suppressed by SLAM receptors in normal NK cells must be identified.

Herein, we addressed these various issues by creating and analyzing a new mouse lacking the entire 400 kilobase (kb)-*Slam* locus on chromosome 1. We found that *Slam* locus-deleted mice displayed enhanced NK cell activation in response to hematopoietic target cells. This effect was ascribed solely to the SLAM receptor 2B4 and was not influenced by the other SLAM

receptors. We also found that the impact of engagement of SFRs on NK cells by SFRs on targets was influenced by cytokines. Cytokines also controlled the levels of SAP adaptors, suggesting that they directly influenced SFR function. We also obtained evidence that SLAM receptors inhibited NK cell activation by interfering with the function of LFA-1, but not of other activating receptors such as DNAM-1 and NKG2D.

RESULTS

Generation of mouse lacking the entire *Slam* locus

To address the overall function of SFRs, a mouse lacking the entire 400 kb-*Slam* locus on chromosome 1 was generated. A gene deletion approach was favoured over multi-gene targeted mutations, as the genes encoding SFRs frequently undergo alternatively splicing that can create alternative protein products when point mutations or small deletions are created. In addition to the genes coding for all 6 SFRs and CD48, the ligand of 2B4, the *Slam* locus contains no other known genes with the exception of a short non-conserved open reading frame (ORF) of unknown function named *Gm10521* (www.ensembl.org). To address the possible impact of *Gm10521*, a *Gm10521*-disrupted mouse was also engineered (see The inhibitory effect of the *Slam* locus is mediated by 2B4 with no appreciable impact from other SFRs or *Gm10521* section).

Deletion of the *Slam* locus was achieved by sequential gene targeting in C57BL/6 embryonic stem (ES) cell, in which *loxP* sites were inserted at the two extremities of the locus, followed by Cre-mediated deletion of the locus (Figure 2.1A). Resulting mice lacked all SFRs, as well as CD48, on immune cells, including NK cells and T cells, compared to wild-type littermates (Figure 2.1B). No effect was seen on the expression levels of SAP adaptors (Figure 2.1C). Mice were viable and fertile (data not shown). They were extensively backcrossed (6-10 generations) to the C57BL/6 background prior to experimentation.

Compared to wild-type mice, mice lacking the *Slam* locus ("SFR knock-out (KO)" mice) had no alterations in NK cell numbers in spleen and bone marrow (Figure 2.1D,E). They also displayed little or no change in the total numbers of T cells and B cells in spleen (Figure 2.1D). However, SFR KO mice had a small alteration of NK cell maturation, particularly in spleen: e.g. an increased proportion of immature $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ NK cells, with decreased

proportions of mature $CD27^{-}CD11b^{+}$ NK cells (Figure 2.1F,G). SFR KO mice also had modest changes in NK cell repertoire (Figure 2.2A-D). Compared to wild-type mice, they displayed a small decrease (less than 5%) in the proportions of $Ly49A/D^{+}$, $Ly49C/I/F/H^{+}$ or $KLRG1^{+}$ NK cells (Figure 2.2A-D). Levels of NKG2D and DNAM-1 were also slightly increased (Figure 2.2A-D). These effects were seen in spleen and, to a lesser extent, in bone marrow.

Hence, deletion of the *Slam* locus in mice resulted in loss of all SFRs and CD48. It had no appreciable impact on NK cell development, although it had a small impact on NK cell maturation and repertoire.

NK cells from *Slam* locus-deleted mice display enhanced responses towards hematopoietic target cells *in vitro*

Activation of SFR KO NK cells in response to various target cells, either hematopoietic or non-hematopoietic, was examined. In parallel, we studied NK cells from mice lacking the SAP adaptor (*Sh2d1a*$^{-/-}$; SAP KO), or lacking the *Slam* locus in addition to SAP (SFR-SAP double (d) KO), in order to test the previously proposed idea that SAP KO NK cells have reduced cytotoxicity towards hematopoietic target cells because SFRs become super-inhibitory [37, 161, 191, 235]. Either IL-2-activated or IL-15-activated NK cells were used for these studies.

As reported previously [37, 161, 191], SAP KO NK cells exhibited compromised natural cytotoxicity towards hematopoietic target cells RMA (class I major histocompatibility complex (MHC)-positive lymphoma), RMA-S (class I MHC-negative variant of RMA) and YAC-1 (class I MHC-negative thymoma), when compared to wild-type NK cells (Figure 2.3A,B). No defect was seen towards non-hematopoietic target cells B16 and CMT-93. In striking contrast, SFR KO NK cells displayed augmented killing of hematopoietic target cells, in comparison to wild-type

NK cells. This effect was especially marked towards RMA and RMA-S, but was also seen to a smaller extent towards YAC-1. A small increase in cytotoxicity was also seen towards B16, but not CMT-93. SFR-SAP dKO NK cells also displayed enhanced responses towards these various targets, in a manner analogous to SFR KO NK cells.

All SFRs are self-ligands, with the exception of 2B4, which binds CD48 [151, 196, 229, 230]. Therefore, to ensure that the increased responses of SFR KO NK cells were not due to altered maturation or repertoire, similar experiments were conducted using NK cells, either wild-type or SFR KO, incubated with activated $CD4^+$ T cells or macrophages, which were either wild-type or SFR KO. In keeping with the results obtained with hematopoietic cell lines (Figure 2.3A,B), SFR KO NK cells had enhanced cytotoxicity towards wild-type $CD4^+$ T cells and macrophages, when compared to wild-type NK cells (Figure 2.3C-F). Importantly, an increase in cytotoxicity was also seen when wild-type NK cells were activated by SFR KO $CD4^+$ T cells and macrophages, in comparison to wild-type $CD4^+$ T cells and macrophages. Similar results were obtained when SFR KO NK cells were used as effectors against SFR KO $CD4^+$ T cells and macrophages. Of note, however, the ability of SFR KO NK cells to mediate cytotoxicity towards SFR KO $CD4^+$ T cells was slightly lower than that of wild-type NK cells. However, this difference was not statistically significant.

Expression of class I MHC on targets suppresses NK cell activation, by triggering inhibitory Ly49 or killer-cell immunoglobulin-like receptors (KIRs) on NK cells. Thus, we tested the ability of SFR KO NK cells to kill $CD4^+$ T cells deficient in class I MHC (β2 microglobulin (β2M) KO). Like wild-type NK cells, SFR KO NK cells showed increased killing of b2M KO $CD4^+$ T cells, compared to wild-type $CD4^+$ T cells (Figure 2.3G,H). The stimulatory effects of SFR deficiency in NK cells and class I MHC deficiency in targets on cytotoxicity were additive.

We also examined the impact of SFR deficiency on production of cytokines, in particular interferon (IFN)-γ. Wild-type NK cells exhibited enhanced production of IFN-γ when stimulated with SFR KO $CD4^+$ T cells, compared to wild-type $CD4^+$ T cells (Figure 2.4A). A similar effect was seen on exposure of CD107a, a marker of degranulation. Moreover, compared to wild-type NK cells, SFR KO NK cells displayed increased IFN-γ production in response to RMA (Figure 2.4B). No difference was seen when cells were activated with phorbol myristate acetate (PMA) and ionomycin, which trigger cytokine production by bypassing activating receptors.

We also tested the impact of SFR deficiency on NK cell responses triggered by activating receptors other than SFRs. Stimulation of NK cells with plate-bound antibodies against CD16 or NKRp1c (NK1.1) showed that SFR KO NK cells had a small (1.3- to 1.5-fold) increase in secretion of IFN-γ, compared to wild-type NK cells (Figure 2.4C). A similar effect was seen with PMA and ionomycin.

Thus, deletion of the *Slam* locus resulted in enhanced NK cell responses towards hematopoietic target cells, with little or no effect on non-hematopoietic target cells. This effect was seen whether the *Slam* locus was deleted in NK cells or in target cells. It was also seen whether target cells expressed or not class I MHC. Deletion of the *Slam* locus also restored the compromised responses of SAP KO NK cells towards hematopoietic target cells. The latter finding was in keeping with the idea that NK cell responses are compromised in SAP KO NK cells because SFRs become super-inhibitory in the absence of SAP.

The inhibitory effect of the *Slam* locus is mediated by 2B4 with no appreciable impact from other SFRs or *Gm10521*

To ascertain the contributions of individual SFRs to the enhanced responses of SFR KO NK cells,

the impact of removing single SFRs in NK cells was assessed. Like SFR KO NK cells, 2B4 KO NK cells displayed augmented killing of $CD4^+$ T cells, in comparison to wild-type NK cells (Figure 2.5A). This effect was seen whether wild-type or SFR KO $CD4^+$ T cells were used as targets. However, as shown in Figure 2.3C,D for SFR KO NK cells, this effect was slightly less towards SFR KO $CD4^+$ T cells, compared to wild-type $CD4^+$ T cells. Little or no difference was seen with SLAMF7 KO, SLAMF6 KO, Ly-9 KO or CD84 KO NK cells, in comparison to wild-type NK cells (Figure 2.5A-D).

Similar experiments were conducted using wild-type NK cells and, as targets, $CD4^+$ T cells from mice lacking individual SFRs or CD48. In keeping with the results described in the previous paragraph, wild-type NK cells displayed enhanced responses towards CD48 KO $CD4^+$ T cells, but not $CD4^+$ T cells lacking Ly-9 or SLAMF6, compared to wild-type $CD4^+$ T cells (Figure 2.5E). The increased responses towards CD48 KO $CD4^+$ T cells were seen with wild-type NK cells from C57BL/6, 129S1/Sv or DBA/1J mice (Figure 2.5F).

We also assessed whether SFRs other than 2B4 influenced the inhibitory function of 2B4. To this end, the ability of 2B4 KO NK cells to mediate cytotoxicity of $CD4^+$ T cells lacking other SFRs was examined. The ability of 2B4 KO NK cells to kill Ly-9, SLAMF6, SLAMF7 or CD84 KO $CD4^+$ T cells was neither increased nor decreased, compared to wild-type $CD4^+$ T cells (Figure 2.5G-I).

Lastly, we examined the possible contribution of *Gm10521* to the enhanced responses of SFR KO NK cells. For this purpose, we engineered *Gm10521*-disrupted KO mice, using CRISPR-Cas-mediated gene editing, as detailed in "Mice" section of Experimental Procedures. *Gm10521*-disrupted NK cells had no alteration of NK cell-mediated cytotoxicity towards RMA, RMA-S, YAC-1 or $CD4^+$ T cells, compared to wild-type NK cells (Figure 2.5J,K).

Therefore, the inhibitory impact of the *Slam* locus on NK cell activation by hematopoietic target cells was solely mediated by 2B4. There was no appreciable contribution, either activating or inhibitory, from the other SFRs expressed in NK cells or *Gm10521*.

Mice lacking the *Slam* locus have augmented NK cell-mediated responses during LCMV infection

A previous study showed that 2B4 KO mice displayed decreased numbers of virus-specific activated $CD8^+$ T cells during infection by lymphocytic choriomeningitis virus (LCMV) [148]. These mice also had less extensive weight loss, which is believed to reflect less severe immunopathology, which can occur during LCMV infection. Importantly, these various effects were all dependent on NK cells, and likely reflected an increased ability of cytokine-primed 2B4 KO NK cells to kill activated $CD8^+$ T cells, as well as activated $CD4^+$ T cells that provide help to $CD8^+$ T cells [147].

Thus, to address the role of the SLAM family in NK cell-mediated immune regulation *in vivo*, we examined the effect of SFR deficiency during infection by LCMV (clone 13). Mice were analyzed at day 8.5 post-infection, when control of virus-specific T cell responses by NK cells is near or at its peak. At day 8.5, SFR KO mice exhibited markedly reduced proportions and numbers of virus-specific IFN-γ-producing activated ($CD44^{hi}$) $CD8^+$ T cells in spleen, compared to wild-type mice (Figure 2.6A-C). This was shown with a variety of virus-derived antigenic peptides used in peptide re-stimulation assays (Figure 2.6B,C). Similar results were obtained when virus-specific activated $CD8^+$ T cells were detected by staining splenocytes with viral peptide-loaded class I MHC tetramers (Figure 2.6D,E).

The reduction of virus-specific T cell responses in SFR KO mice, compared to wild-type

mice, was markedly attenuated when mice were injected with anti-NKRp1c (NK1.1), which depletes NK cells (Figure 2.6F). This feature was analogous to that observed in 2B4 KO mice (Figure 2.6G), as reported [148]. SFR KO mice also displayed statistically significant less weight loss, a feature of immunopathology, in comparison to wild-type mice (Figure 2.6H). In keeping with the results previously obtained with 2B4 KO mice [148], there was no difference in viral titers between wild-type and SFR KO mice at this time point (data not shown). Effects on viral control might become apparent at later time points, as suggested by the prior study of 2B4 KO mice [148].

Thus, SFR KO mice had reduced LCMV-specific $CD8^+$ T cell responses *in vivo*. This effect was dependent on NK cells and resulted in less severe immunopathology. It was similar to the effect seen in 2B4 KO mice. These findings implied that, like the 2B4-encoding gene, the *Slam* locus had an inhibitory effect on NK cell-mediated control of activated T cell responses during LCMV infection *in vivo*.

The impact of the SLAM family requires NK cell priming by cytokines

Multiple cytokines can influence NK cell functions, including IL-15, IL-12, IL-2 and type I IFN [52, 238, 239]. The experiments depicted in Figure 2.3 were done using NK cells from non-primed mice that were expanded *in vitro* either with IL-2 or with IL-15. To examine if SFRs had an appreciable impact on NK cell functions in the absence of deliberate cytokine stimulation, similar experiments were conducted using freshly isolated NK cells that were not stimulated with any cytokine during the cytotoxicity assay. Unlike IL-2-activated SFR KO NK cells, freshly isolated SFR KO NK cells displayed no increase in cytotoxicity towards WT $CD4^+$ T cells, compared to wild-type NK cells (Figure 2.7A). We also tested the ability of non-primed mice to

eliminate *in vivo* RMA, the tumor target cell line showing the largest increase in killing by cytokine-stimulated SFR KO NK cells *in vitro* (Figure 2.3A), using a peritoneal clearance assay. There was no difference in the ability of wild-type or SFR KO mice to eliminate RMA cells in this assay (Figure 2.7B). These observations suggested that, at the steady state and in the absence of added cytokines, there was no detectable increase in NK cell responses towards hematopoietic target cells in the absence of SFRs. Thus, it appeared that the impact of the SLAM family on hematopoietic target cell killing required cytokine priming.

Inhibition of NK cell responses by SLAM family is differentially influenced by IL-12 and type I interferon

We also performed similar studies using NK cells isolated from mice treated with polyI:C, a TLR3 receptor agonist classically used to enhance NK cell functions *in vivo* [240]. Surprisingly, freshly isolated NK cells from polyI:C-primed wild-type mice did not display an increase in cytotoxicity towards SFR KO $CD4^+$ T cells, in comparison to wild-type $CD4^+$ T cells (Figure 2.7C). However, they had increased cytotoxicity towards b2M KO $CD4^+$ T cells. These findings implied that polyI:C enhanced NK cell responses towards targets lacking class I MHC, but not towards targets lacking SFRs.

As polyI:C is a potent inducer of type I IFN [240], these findings raised the possibility that the impact of SFRs on NK cell activation was regulated by type I IFN. To address directly this possibility, wild-type NK cells were purified from non-primed mice, expanded for 3 days in IL-15, and treated overnight with IL-15 alone, IL-15 plus IFN-β, a type I IFN, or IL-15 plus IL-12, another cytokine known to regulate NK cell functions. IL-15 is critical to enable survival of NK cells *ex vivo*. After extensive washing to remove the cytokines, NK cells were then

incubated with wild-type or SFR KO $CD4^+$ T cells, and cytotoxicity was measured.

Wild-type NK cells treated with IL-15 demonstrated augmented killing towards SFR KO $CD4^+$ T cells, compared to wild-type $CD4^+$ T cells (Figure 2.7D,E), in keeping with the results described in Figure 2.3. This differential response was further enhanced when IL-12 was added (Figure 2.7D,E). Strikingly, however, it was markedly diminished when IFN-β was added. A similar effect was seen with IFN-α, another type I IFN (Figure 2.7F). Addition of both IL-12 and IFN-β had the same impact as addition of IFN-β alone (Figure 2.7G). IFN-β had no effect on the responses of SFR KO NK cells towards wild-type $CD4^+$ T cells, suggesting that the impact of the cytokine was mediated by an effect on SFRs in NK cells (Figure 2.7H).

We examined the possibility that type I IFN suppressed the differential responses of NK cells towards SFR KO $CD4^+$ T cells compared to wild-type $CD4^+$ T cells, by rendering SFRs more inhibitory in NK cells when targets lack SFRs. This could result from increased *cis* interactions between SFRs on NK cells. To this end, we compared the ability of SFR KO NK cells and wild-type NK cells to kill SFR KO $CD4^+$ T cells. SFR KO NK cells and wild-type NK cells treated with IFN-β displayed equivalent cytotoxicity towards SFR KO $CD4^+$ T cells (Figure 2.7I). This finding suggested that type I IFN was not rendering SFRs more inhibitory in NK cells activated by targets lacking SFRs.

To ensure that the effect of type I IFN was dependent on IFN receptor signaling, similar experiments were conducted using NK cells from IFN-α/β receptor 1 (IFNAR1) KO mice. IFNAR1 is a critical component of the type I IFN receptor, IFNAR. Compared to wild-type NK cells, IFNAR1 KO NK cells did not display the reduction of differential responses towards SFR KO $CD4^+$ T cells compared to wild-type $CD4^+$ T cells, in the presence of IFN-β (Figure 2.7J). Loss of IFNAR1 had no impact on expression of SFRs on NK cells (data not shown).

Thus, stimulation with IL-12 further augmented the differential responses of WT NK cells towards targets lacking SFRs compared to targets expressing SFRs. The opposite effect was seen with type I IFN, in a manner dependent on expression of IFNAR1.

Cytokines regulate expression of SAP adaptors

Several possible mechanisms, either direct or indirect, could explain the impact of the cytokines on the differential responses of NK cells towards targets lacking SFRs compared to targets expressing SFRs (see Discussion). To test the possibility that cytokines were directly influencing the function of SFRs, the effect of cytokines on expression of various molecules regulating SFR functions was first examined. Addition of IL-12 or IFN-β to IL-15-stimulated NK cells had little or no impact on the expression levels of SFRs, when compared to cells treated with IL-15 alone (Figure 2.8A). However, IL-12 caused a reproducible decrease in the expression levels of SAP (by ~25%) and EAT-2-ERT (by ~60-70%) (Figure 2.8B). As EAT-2 and ERT are very similar, the available antibodies do not distinguish between the two proteins [191]. By opposition, IFN-β caused an increase (~4-fold) in expression of EAT-2-ERT, although it did not affect SAP.

The changes in expression of SAP, EAT-2 and ERT were also shown at the RNA level, using real-time PCR (Figure 2.8C). As PCR can distinguish the transcripts encoding EAT-2 and ERT, it also demonstrated that expression of both EAT-2- and ERT-encoding RNAs was affected by IL-12 and IFN-β. Of note, though, IFN-β had a more pronounced effect on expression of RNAs encoding ERT (~8-fold) compared to EAT-2 (~2-fold). This may relate to the fact that ERT, but not EAT-2, is nearly absent in freshly isolated mouse NK cells [191].

IL-12 and IFN-β had little or no impact on other effectors of SFR signaling, including the kinase Fyn, the exchange factor Vav-1, phospholipase C-g2, and the inhibitory effectors SHIP-1

and SHP-2 (Figure 2.8B). The only reproducible exception was SHP-1, which was decreased (by ~15-50%, depending on the experiment) by IL-12 and enhanced (~1.5-fold) by IFN-β. There was little or no effect on expression of NKG2D, DNAM-1, LFA-1 (CD11a), CD18 and CD11b (Figure 2.8A).

To confirm that the increase in EAT-2-ERT expression in response to type I IFN was due to IFNAR signaling, similar experiments were conducted using IFNAR1 KO NK cells. Unlike wild-type NK cells, IFNAR1 KO NK cells displayed little or no increase in EAT-2-ERT expression in response to IFN-β, compared to cells treated with IL-15 alone (Figure 2.8D). However, the decrease in EAT-2-ERT expression in response to IL-12 was still observed. The increase in SHP-1 expression in response to IFN-β was also abrogated.

As SAP adaptors prevent the inhibitory function of SFRs [37, 161, 191, 235], these results suggested that cytokines were acting at least in part by directly influencing the function of SFRs, through changes in the expression levels of SAP adaptors. To address further this idea, we focussed on IFN-β, which caused the most pronounced effect on SFR-mediated inhibition. Thus, the effect of IFN-β on differential NK cell responses towards SFR KO $CD4^+$ T cells compared to wild-type $CD4^+$ T cells was tested using NK cells from EAT-2 KO, ERT KO or EAT-2-ERT dKO mice. Unlike wild-type NK cells, the EAT-2 KO, ERT KO and EAT-2-ERT dKO NK cells displayed appreciable differences in NK cell responses towards SFR KO $CD4^+$ T cells compared to wild-type $CD4^+$ T cells, in the presence of IFN-β (Figure 2.8E).

Therefore, treatment of NK cells with IFN-β or IL-12 resulted in changes in the expression levels of SAP adaptors. These effects might explain at least in part the impact of IFN-β on the differential outcome of NK cell activation by targets expressing or lacking SFRs.

Integrin LFA-1 is required for enhancement of NK cell responses by loss of SLAM family

Based on analyses of SAP adaptor-deficient NK cells, we previously proposed that SFRs played a key role in the ability of NK cells to recognize and kill hematopoietic target cells [161]. However, the results described in Figure 2.3-2.8 clearly showed that it was not the case. Rather, SFRs inhibited this function. To identify the activating receptors mediating NK cell activation by hematopoietic target cells in the absence of SFRs, the impact of blocking antibodies towards various activating receptors was tested. Addition of blocking antibodies against NKG2D, DNAM-1 or NKp46 had little or no impact on the enhanced killing of wild-type NK cells towards SFR KO $CD4^+$ T cells, compared to wild-type $CD4^+$ T cells (Figure 2.9A). Similar effects were seen when the antibodies against NKG2D and DNAM-1 were combined. However, antibodies against the integrin LFA-1 (anti-CD11a) nearly abrogated the enhanced responses towards SFR KO $CD4^+$ T cells (Figure 2.9A,B). No effect was seen when antibodies against other integrin components, CD11b and CD11c, were used.

To validate these observations, the function of NK cells from mice lacking activating receptors was examined. Unlike wild-type NK cells, the CD11a KO NK cells, but not DNAM-1 KO NK cells, CD11b KO NK cells or NKG2D KO NK cells, failed to demonstrate augmented cytotoxicity towards SFR KO $CD4^+$ T cells, compared to wild-type $CD4^+$ T cells (Figure 2.9C-E). A similar defect was seen with CD18 KO NK cells, which lack expression of b2 integrin (CD18), another component of LFA-1 (Figure 2.9C). CD11a KO NK cells displayed no alteration of expression of SFRs or other activating receptors (data not shown).

We also examined the contribution of LFA-1 to the enhanced ability of SFR KO NK cells to kill hematopoietic cells. To this end, SFR KO mice were bred with CD11a KO mice, to generate SFR-CD11a dKO mice. Compared to SFR KO NK cells, SFR-CD11a dKO NK cells had a lower

capacity to kill wild-type $CD4^+$ T cells (Figure 2.9F). This was seen whether IL-15-stimulated or IL-2-stimulated NK cells were used. Similar results were obtained when RMA or RMA-S cells were used as targets (Figure 2.9F).

Together, these data implied that LFA-1, but not DNAM-1, NKG2D, NKp46 or other integrins, played a key role in the enhanced ability of NK cells lacking SLAM receptors to kill hematopoietic target cells.

Evidence that 2B4 inhibits induction of the "activated" form of LFA-1

These results raised the possibility that SFRs suppressed NK cell activation at least partly by inhibiting the function of LFA-1, either directly or indirectly. LFA-1 has a dual role in NK cell activation [241-243]. On the one hand, it functions as an adhesion molecule that enhances conjugate formation between NK cells and target cells. On the other hand, it triggers activating signals that promote NK cell effector responses. The activating function of LFA-1 is dependent on a modification of its conformation, which becomes "activated" and can be assessed by conformation-sensitive anti-LFA-1 antibodies.

It is nearly impossible to monitor activation of LFA-1 in mice because of lack of suitable reagents, but there are available antibodies that recognize the activated conformation of human LFA-1. Thus, to test the impact of SFRs on LFA-1 function, we developed a LFA-1-dependent human NK cell activation system, using the human NK cell line YT-S, which expresses 2B4 and SAP, and the human B cell target 721.221, which expresses CD48. The ability of YT-S cells to kill 721.221 was blocked by anti-LFA-1 (CD11a) or anti-CD18 antibodies, implying that it is LFA-1-dependent (Figure 2.10A).

To favor 2B4-mediated inhibition in YT-S cells, SAP-deficient (SAP knock-down (KD))

YT-S cells were generated, using SAP-specific small interfering (si) RNAs (Figure 2.10B). Moreover, to address the impact of 2B4-mediated inhibition in these cells, 721.221 derivatives lacking CD48 were created, using CRISPR-Cas genome editing (Figure 2.10C). Two independent CD48 KO 721.221 derivatives were generated, using two unrelated guide RNA targeting sequences. Control siRNA-transduced YT-S showed little or no difference in cytotoxicity towards parental and CD48 KO 721.221 (Figure 2.10D). This finding suggested that, in control YT-S cells exposed to 721.221, 2B4 was neither inhibitory nor activating. However, SAP KD YT-S displayed reduced cytotoxicity towards parental 721.221, compared to CD48 KO 721.221, in keeping with the inhibitory effect of 2B4 (Figure 2.10D). Hence, this system was suitable to test the impact of 2B4-mediated inhibition on LFA-1 function.

We first wanted to ascertain if 2B4-mediated inhibition resulted in diminished conjugate formation, a function dependent on LFA-1-mediated adhesion. As reported for SAP KO mouse NK cells [37], SAP KD YT-S cells displayed reduced conjugate formation with 721.221, compared to control YT-S cells (Figure 2.10E). However, this effect was of similar magnitude towards parental and CD48 KO 721.221. The latter finding implied that 2B4 was not responsible for the reduced conjugate formation of SAP KD YT-S cells. Presumably, one or more other SFRs expressed in YT-S cells were responsible. To address whether 2B4-mediated inhibition interfered with activation of LFA-1, LFA-1 activation was monitored using an antibody (m24) recognizing the activated conformation of LFA-1. SAP KD YT-S had reduced LFA-1 activation in response to parental 721.221, compared to CD48 KO 721.221 (Figure 2.10F).

To understand the mechanism by which 2B4-mediated inhibition suppressed LFA-1 activation, similar experiments were conducted using SAP KD YT-S cells in which expression of the lipid phosphatase SHIP-1, which was reported to mediate the inhibitory function of 2B4 [37],

was suppressed by siRNAs (Figure 2.10G). Down-regulation of expression of SHIP-1 corrected the lower cytotoxicity of SAP KD YT-S cells towards parental 721.221, compared to CD48 KO 721.221 (Figure 2.10H,I). Importantly, it also ameliorated the defect in LFA-1 activation (Figure 2.10J). Similar effects were seen with two other SHIP-1-specific siRNAs (data not shown).

These data implied that 2B4-mediated inhibition did not result in reduced conjugate formation with targets. However, it caused a diminution of the activation of LFA-1. The latter inhibitory effect was mediated by SHIP-1.

DISCUSSION

Herein, we created a mouse in the C57BL/6 background bearing a deletion of the entire *Slam* locus, which includes the genes coding for all 6 SFRs and CD48, the ligand of 2B4 [151, 196, 229]. NK cells isolated from SFR KO mice displayed markedly enhanced activation responses towards hematopoietic target cells, including tumor cells such as RMA and RMA-S, and normal hematopoietic cells such as activated $CD4^+$ T cells and macrophages, *in vitro*. The increase in activation responses was seen towards either class I MHC-negative or class I MHC-positive targets. Both cytotoxicity and IFN-γ production were augmented. Likewise, SFR KO mice infected with LCMV had decreased numbers of LCMV-specific activated $CD8^+$ T cells and less severe immunopathology, compared to wild-type mice. These effects were attenuated by depletion of NK cells, suggesting that they were due to enhanced killing of activated T cells by NK cells.

Although deletion of the *Slam* locus had a detectable, albeit small, impact on NK cell maturation and repertoire, it is unlikely that these changes were responsible for the enhanced responses of SFR KO NK cells. Indeed, an analogous increase in NK cell activation was seen when wild-type NK cells were incubated with SFR KO $CD4^+$ T cells or macrophages. However, it should also be noted that a small increase in cytotoxicity towards the non-hematopoietic target cell B16 melanoma was seen. B16 lacks ligands for SFRs [161]. Similarly, a small increase in IFN-γ secretion was seen when SFR KO NK cells were stimulated through CD16 or NKRp1c, or with PMA and ionomycin. These various effects may be due to altered NK cell maturation, repertoire or education. Additional studies will be needed to address this possibility.

Analyses of mice lacking individual SFRs showed that only 2B4 KO NK cells displayed augmented responses towards hematopoietic target cells *in vitro*. Conversely, only CD48 KO

$CD4^+$ T cells exhibited enhanced killing by wild-type NK cells. To ascertain the possibility that other SFRs attenuated or enhanced the impact of loss of 2B4, we examined activation of 2B4 KO NK cells by $CD4^+$ T cells lacking the other SFRs. Loss of other SFRs on targets had no influence on the enhanced responses of 2B4 KO NK cells. Thus, no SFR other than 2B4 had any appreciable effect, either inhibitory or activating, on NK cell activation. This notion was also in agreement with the observation that the reduction of LCMV-induced $CD8^+$ T cell responses in SFR KO mice was analogous to that noted in 2B4 KO mice [148].

Although SFRs other than 2B4 had no appreciable impact on NK cell activation, it is conceivable that some have alternative functions in NK cells. Perhaps, they regulate NK cell proliferation, survival, memory or migration. These possibilities disserve future consideration. It should be mentioned that we previously showed that another SFR, SLAMF6, plays a critical role in NK cell education, thereby promoting NK cell responses towards non-hematopoietic cells [38]. Whereas this effect was seen in 129S1/Sv and DBA/1J mice (and in human NK cells), it was not observed in C57BL/6 mice. Hence, it is not surprising that it was not seen in the current study.

Previous studies showed that loss of SAP adaptors strongly diminished the ability of NK cells to kill hematopoietic target cells, both in mice and in humans [37, 161, 191, 235]. Although the basis for this effect was not formally established, it was proposed to result from an increase in the inhibitory function of SFRs in the absence of SAP adaptors, which prevent inhibition by SFRs both by interfering with coupling of SFRs to inhibitory effectors and by mediating activating signals [37, 161, 191, 235]. The availability of a SFR KO mouse enabled us to test this idea. We found that breeding of SFR KO mice with SAP KO mice strongly improved the compromised NK cell responses of SAP KO NK cells, to the same level as that observed in SFR KO NK cells. This observation implied that the reduced responses of SAP adaptor-deficient NK

cells towards hematopoietic target cells are most probably due to an increase in the inhibitory function of SFRs in the absence of SAP adaptors.

Whereas studies of NK cells lacking SAP adaptors had also implied that SFRs plays key roles in the ability of NK cells to eliminate hematopoietic target cells [191, 228, 229, 235], the findings with SFR KO NK cells reported herein showed that this is not the case. Interestingly, experiments with blocking antibodies revealed that LFA-1 was critical for the enhanced responses of NK cells towards SFR KO $CD4^+$ T cells. The pivotal role of LFA-1 was confirmed by analyses of NK cells from CD11a KO and CD18 KO mice. As hematopoietic cells express high levels of ICAM-1 and ICAM-2, the ligands of LFA-1, it is plausible that LFA-1 plays a central function in NK cell-mediated responses against hematopoietic target cells.

Using the human NK cell line YT-S and the B cell target 721.221 expressing or not CD48, we obtained evidence that 2B4-mediated inhibition was accompanied by decreased induction of the activated form of LFA-1, as suggested by reactivity with the anti-LFA-1 MAb m24. This was mediated at least in part by the lipid phosphatase SHIP-1. However, 2B4-mediated inhibition had no impact on conjugate formation between NK cells and target cells, implying that the decreased activation of LFA-1 did not compromise conjugate formation. Nonetheless, it should be pointed out that activation of LFA-1 is also implicated in LFA-1-dependent signaling leading to effector functions. Hence, it is plausible that 2B4 was compromising effector functions by inhibiting the signaling potential of LFA-1. Such an effect could be due to a direct impact of 2B4 on LFA-1. Alternatively, it could reflect the influence of 2B4 on one or more other activating receptors that regulate LFA-1, although DNAM-1, NKG2D and NKp46 did not appear to be responsible. Future studies will be needed to resolve these various possibilities.

Although NK cells stimulated *in vitro* with cytokines such as IL-15, IL-12 and IL-2

displayed enhanced activation responses in the absence of engagement of SFRs by hematopoietic cell targets, no obvious increase was seen when freshly isolated NK cells were analyzed. Similarly, SFR KO mice did not display any increase in elimination of RMA cells in a peritoneal clearance assay. In contrast, SFR KO mice had a better NK cell-dependent suppression of virus-specific T cell responses during LCMV infection. Coupled with the finding that SFR KO mice did not have any reduction of the steady state numbers of T cells and B cells, these observations implied that immune stimulation by cytokines or viral infection was needed to observe increased activation responses in the absence of SFRs.

Interestingly, however, NK cells isolated from wild-type mice injected with polyI:C, another immune stimulant, failed to display enhanced responses towards SFR KO $CD4^+$ T cells, compared to wild-type $CD4^+$ T cells. This was not due to lack of adequate priming by polyI:C, as these NK cells had increased responses towards b2M KO $CD4^+$ T cells. Treatment of NK cells *in vitro* with type I IFN, either IFN-α or IFN-β, dramatically attenuated the increased responses of wild-type NK cells towards targets lacking SFRs compared to targets expressing SFRs. This effect was dependent on IFNAR1.

The impact of type I IFN could be due to several effects, which directly or indirectly influenced the function of SFRs. Our results suggested that the influence of type I IFN was mediated at least in part by a decrease in the inhibitory function of SFRs, when engaged by SFRs on targets. This would be due to increased expression of EAT-2 and ERT. However, it is also possible that type I IFN altered the impact of SFRs when they were engaged in *cis* on NK cells, in particular if SFRs were lacking on targets. This could lead to changes in NK cell education, or to some other form of "pre-conditioning" of NK cell functions. This possibility might seem less likely though, given that SFR KO NK cells and wild-type NK cells treated with type I IFN

displayed similar cytotoxicity towards SFR KO $CD4^+$ T cells. Lastly, it is conceivable that cytokines influenced the function of the activating receptors repressed by SFRs, or that of inhibitory receptors cooperating with SFRs to suppress NK cell activation. Additional studies will be needed to address these various possibilities.

In summary, we found that the *Slam* locus in mice has an overall inhibitory role in NK cell activation by hematopoietic target cells. This inhibitory effect is mediated by 2B4, with no appreciable impact from any other SFR expressed in NK cells. It is influenced by cytokines such as IL-12, IL-15 and type I IFN, perhaps due to a direct effect of cytokines on SFR functions, although additional mechanisms may be involved. In addition to enhancing our understanding of the role and mechanism of action of the SLAM family in NK cell activation, these findings imply that the impact of SFRs in NK cells is likely to be dynamically regulated *in vivo*, depending on the cytokine milieu. This may be especially important in the context of immune stimulation by viruses and other pathogens.

So far, an activating role of SFRs in NK cell activation has been demonstrated primarily in experiments in which SFR ligands were ectopically expressed on non-hematopoietic target cells, or in which SFRs on NK cells were triggered with anti-SFR antibodies [231]. This is true both for mouse NK cells and for human NK cells. Whereas the data herein firmly establish the overall inhibitory role of SFRs in NK cell activation against hematopoietic target cells in mice, it is possible that SFRs are activating in mouse NK cells under physiological conditions. For instance, this may occur when levels of SFR ligands on hematopoietic target cells or the amounts of SAP adaptors in NK cells are increased. As a corollary to our findings, it is also plausible that, under physiological circumstances of stimulation, 2B4 has an inhibitory role in human NK cells.

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AUTHOR CONTRIBUTION

H.G. planned experiments, performed experiments, interpreted data and wrote the manuscript. Y.L., S.Z. and M.-C.Z. generated and characterized reagents, performed experiments, and interpreted data. J.C., R.L., N.W. and D.D. characterized reagents and interpreted data. S.A.C. and S.E.M. planned experiments, performed experiments and interpreted data. S.N.W. planned experiments, interpreted data and provided funding. A.V. planned experiments, generated reagents, interpreted data, wrote the manuscript and obtained funding.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interest.

EXPERIMENTAL PROCEDURES

Mice. To generate a mouse lacking the entire *Slam* locus (D*Slam*), the following constructs and strategy were used. The first construct contained a 5' arm bearing part of intron 1 and a 3' arm bearing exons 4-6 of the 2B4-encoding gene, *Slamf4*. *Slamf4* is the most 5' gene in the *Slam* locus. These arms flanked a single *loxP* site and the *neo* gene. This construct was transfected in C57BL/6-derived Bruce 4 ES cells and transfected cells were selected with G418. Cells with successful homologous recombination of *Slamf4* were then used for blastocyst injection to generate a 2B4-deficient (*Slamf4*$^{-/-}$) mouse. They were also transfected with a second construct that contained a 5' arm bearing exons 4-6 and a 3' arm bearing exon 8 of *Slamf6*. *Slamf6* is the most 3' gene in the *Slam* locus. These arms flanked a single *loxP* site and the *hygro* gene. Transfected cells were selected with hygromycin. Cells were homologous recombination in both *Slamf4* and *Slamf6* were then transiently transfected with the pIC-Cre (a gift from Dr. Hua Gu, Institut de recherches cliniques de Montréal, Montreal, QC, Canada). This resulted in deletion of the entire *Slam* locus, except for exon 1 of *Slamf4* and exons 8 and 9 of *Slamf6*. One *loxP* site and the *hygro* gene remained. Clones were screened by PCR to detect deletion of the *Slam* locus. Deletion of the *Slam* locus was confirmed by genomic sequencing. Cells were then injected into blastocysts to generate chimeric mice. After germ line transmission, mice were backcrossed for 6-10 generations with C57BL/6J mice (The Jackson Laboratory; Bar Harbor, ME). Deletion of the *Slam* locus was confirmed by genomic sequencing of mouse DNA. *Gm10521*-disrupted mice were generated by CRISPR-Cas-mediated genome editing, using the plasmid pSpCas9(BB) (formerly pX330, Addgene, Cambridge, MA), and the guide RNA sequence TATACCAGGGCACCTGTACCTGG. DNA was injected in fertilized oocytes of C57BL/6J mice. Mice were then screened by PCR and sequencing of the *Gm10521* locus. Mice bearing a

frameshift deletion of 5 nucleotides at the 5' end of the potential coding sequence of *Gm10521* locus were bred to homozygozity. Mice lacking Ly-9 (*Slamf3*$^{-/-}$), SLAMF6 (*Slamf6*$^{-/-}$), CD48 (*Slamf2*$^{-/-}$) or EAT-2-ERT (*Sh2b1b1*$^{-/-}$*Sh2d1b2*$^{-/-}$) in the 129S1/Sv background were described previously [38, 192]. Mice lacking b2 microglobulin (*β2m*$^{-/-}$), CD84 (*Slamf5*$^{-/-}$) or SLAMF6 (*Slamf6*$^{-/-}$) in the C57BL/6 background were obtained from The Jackson Laboratory. The 2B4-deficient mouse (*Slamf4*$^{-/-}$) used for LCMV infection experiments was reported [244]. Mice lacking SLAMF7 (*Slamf7*$^{-/-}$) in the C57BL/6 background will be reported elsewhere (Chen et. al., submitted). Mice lacking LFA-1 (CD11a; *Itgal*$^{-/-}$), CD18 (*Itgb2*$^{-/-}$), CD11b (*Itgam*$^{-/-}$) or IFN α/β receptor 1 (IFNAR1; *Ifnar1*$^{-/-}$) were obtained from The Jackson Laboratory. Mice lacking SAP (*Sh2d1a*$^{-/-}$), DNAM-1 (*Cd226*$^{-/-}$) or NKG2D (*Klrk1*$^{-/-}$) were kindly provided by Dr. Luo Yin (International Agency for Research on Cancer, Lyon, France), Dr. Marco Colonna (Washington University, St. Louis, MO) and Dr. David Raulet (University of California, Berkeley, CA), respectively [37, 245, 246]. All mice were maintained in the C57BL/6J background, unless specified. They were also kept in a specific-pathogen free (SPF) environment. Littermates were used as controls in all experiments. Animal experimentation was approved by the Animal Care Committee of IRCM and performed as defined by the Canadian Council of Animal Care, or by the Institutional Animal Use and Care Committees of Cincinnati Children's Hospital Medical Center.

Cells. Spleen NK cells were purified by positive or negative selection (StemCell Technologies, Vancouver, British Columbia, Canada). In some cases, mice were injected intra-peritoneally with polyI:C (250 μg; Sigma-Aldrich, St. Louis, MO) 24-36 hours before purification. After purification, NK cells were either used immediately for experimentation, or cultured for 3 days with IL-15 (Catalog #210-15, Peprotech, Rocky Hill, NJ), followed or not by addition of IL-12

(Catalog #210-12, Peprotech), IFN-α (Catalog #12100-1, PBL Assay Science, Pistacaway, NJ) or IFN-β (Catalog #12405-1, PBL Assay Science) overnight. Alternatively, they were expanded in IL-2 (1000 U/ml) for 5 days. Cells treated with cytokines were washed in culture medium at least three times prior to the functional assays, in order to eliminate carry over cytokines. RMA and RMA-S (mouse lymphoma), YAC-1 (mouse thymoma), B16F10 (mouse melanoma), CMT-93 (mouse rectal carcinoma) and YT-S (human NK cell line) have been described [37, 38, 161, 191]. Typically, NK cell purity was greater than 90%. 721.221 (EBV-transformed human B cell line) was obtained from the International Histocompatibility Working Group (Seattle, WA). Variants lacking CD48 were generated by CRISPR-Cas-mediated gene editing, using the plasmid pSpCas9(BB)-2A-GFP (Addgene), and the sequences TCACTTGGTACATATGACCGTGG or GTACATATGACCGTGGTCTCCGG as guide RNAs. Activated $CD4^+$ T cells and bone marrow-derived macrophages were generated as described elsewhere [161, 247]. In brief, activated $CD4^+$ T cells were produced by stimulation of purified splenic $CD4^+$ T cells in the presence of plate-bound hamster anti-CD3 MAb 145-2C11 and soluble hamster anti-CD28 MAb 37.51 for 2 days, followed by expansion in IL-2-containing medium (50 U/ml) for 2 days.

siRNAs. Previously validated siRNAs against human SAP and SHIP-1, as well as scrambled siRNAs, were purchased from Qiagen (Toronto, ON, Canada) [38]. They were transfected in YT-S cells using the AMAXA Human NK cell nucleofector kit (Lonza, Cologne, Germany), as described [38]. The following siRNAs were used: control siRNA: AllStars Negative Control siRNA (Cat #1027280); SAP-specific siRNAs: Hs_SH2D1A_4 (Cat #SI00036568), Hs_SH2D1A_5 (Cat #SI03039897); SHIP-1-specific siRNAs: Hs_INPP5D_1 (Cat #SI00078575), Hs_INPP5D_2 (Cat #SI00078582), Hs_INPP5D_5 (Cat #SI03029005).

Antibodies. Monoclonal antibodies recognizing SFRs (except 2B4), CD48, SAP and EAT-2-ERT were produced in our laboratory, as described previously [161, 192]. Monoclonal antibodies against CD3 (145-2C11), NK1.1 (PK136), CD11b (M1/70), CD27 (LG 7F9), IFN-γ (XMG1.2), CD107a (1D4B), Ly49A/D (11A8), Ly49G2 (LGL-1), Ly49C/I/F/H (14B11), CD122 (TMb1), DNAM-1 (10E5; 480.2; for blocking antibody), NKG2D (CX5), CD11a (M17/4), CD11c (N418), 2B4 (244F4), CD49b (DX5), KLRG1 (2F1), NKp46 (29A1.4), Rae-1 (CX1) and matched isotype controls were from eBioscience (San Diego, CA). Antibodies against human NKp46 (9E2) and human CD18 (TS1/18) were from BioLegend (San Diego, CA). Antibodies against activated human LFA-1 (m24) were obtained from BioLegend. Antibodies against human CD11a (TS1/22) were from Thermo Fisher Scientific (Rockford, IL). Rabbit polyclonal antibodies against Fyn, Vav-1, PLC-γ2, SHIP-1, SHP-1 and SHP-2 were reported elsewhere [37, 38, 161, 191]. Antibodies against b-actin (C4) were from Santa Cruz Biotechnology (Santa Cruz, CA).

***In vitro* NK cell assays.** ^{51}Cr release assays were performed as outlined previously, using either mouse NK cells or YT-S cells [37, 38, 161, 191]. Antibody blocking experiments were also described [38]. For measurement of IFN-γ production and CD107a exposure, splenocytes (1×10^6) were stimulated with IL-2 for 1.5 days and incubated for 4 h with activated $CD4^+$ T cells (1×10^6) or RMA cells (1×10^6). Assays were then performed as previously described [37, 38]. Splenocytes stimulated with phorbol myristate acetate (50 ng/ml) plus ionomycin (1 mM) served as controls. Antibody-induced secretion of IFN-γ was monitored as detailed elsewhere [192]. Conjugate formation assays were performed as described elsewhere [37]. To measure LFA-1 activation, YT-S cells were stimulated with 721.221 cells for 5 minutes at 37^{o}C. Cells were then stained with anti-LFA-1 MAb m24, followed by anti-NKp46 to identify YT-S cells.

Immunoblots and real-time PCR assays to detect expression of RNA encoding SAP adaptors were performed as described [37, 38, 161, 191].

LCMV infection. Clone 13 strain of LCMV was propagated in BHK21 cells [248], and titrated by plaque assay on Vero cells from American Type Culture Collection (ATCC, Manassas, VA). SFR KO and their wild-type littermate controls, or 2B4 KO mice [244], were inoculated with 2×10^6 p.f.u. of LCMV clone 13 via intravenous tail vein injection. They were then analyzed day 8.5 after infection, as described [147, 148]. Mice were weighed the day prior to infection and the day of harvest. For experiments involving *in vivo* NK cell depletion, depletion was achieved via one intra-peritoneal (i.p.) injection of 25 mg of a-NKRp1c (NK1.1) MAb PK136 per mouse, while control mice received one i.p. injection of 25 μg mouse IgG2a-isotype control antibody (C1.18A) produced by Bio-X-Cell (West Lebanon, NH) [147]. Synthetic peptides representing LCMV-encoded epitopes were purchased from 21st Century Biochemicals (Marlborough, MA). The peptides used for *in vitro* stimulation and analysis of cytokine release were as follows: D^b-presented LCMV GP_{33-41} (KAVYNFATC), LCMV $GP_{276-286}$ (SGVENPGGYCL) and LCMV $NP_{396-404}$ (FQPQNGQFI), and the K^b-presented LCMV $NP_{205-212}$ (YTVKYPNI). LCMV GP_{33-41} (KAVYNFATM)- and $NP_{396-404}$ (FQPQNGQFI)-loaded H-$2D^b$ class I MHC tetramers were produced at the NIH Tetramer Facility. Class I tetramer staining was performed at 4°C for 60 minutes.

Statistical analyses. Paired or unpaired Student's *t*-tests (two-tailed) were performed using Prism 6 (GraphPad Software).

FIGURE LEGENDS

Figure 2.1. Deletion of the *Slam* locus in mice and its impact on NK cell development. (A) Schematic diagram of the strategy used to delete the *Slam* locus. In brief, the most 5′ gene of the locus, *Slamf4*, was disrupted using a targeting construct containing the neomycin (*neo*) resistance gene, flanked by one *loxP* site. Then, the most 3′ gene of the locus, *Slamf6*, was disrupted using a targeting construct that contains the hygromycin (*hygro*) resistance gene, also flanked by a single *loxP* site. After Cre-mediated recombination, the entire *Slam* locus was deleted. The position of the potential gene of unknown function, *Gm10521*, is also depicted. (B) Expression of SLAM receptors as well as CD48 was analyzed on splenic NK cells ($CD3^{-}NK1.1^{+}$) and splenic T cells ($CD3^{+}NK1.1^{-}$) from wild-type and *Slam* locus–deleted (SFR KO) mice. Isotype control (ctrl) is shown as filled histograms. Results are representative of at least eight experiments. (C) Expression of SAP and EAT-2–ERT was determined by immunoblotting (IB) of total cell lysates of IL-2–expanded NK cells from wild-type or SFR KO mice. β-actin was used as the loading control. Note that, as the sequences of EAT-2 and ERT proteins are very similar, the available antibodies do not distinguish them. The positions of prestained molecular mass markers are shown on the left. Results are representative of three experiments. (D and E) Percentages and absolute numbers of NK cells ($CD3^{-}NK1.1^{+}$) were analyzed in the spleen (D) and bone marrow (E) from wild-type and SFR KO mice. For bone marrow, cells were pooled from the two femurs and two tibias of each mouse. Percentages and absolute numbers of T cells ($CD3^{+}NK1.1^{-}$) and B cells ($B220^{+}CD3^{-}$) are also depicted (D). Symbols represent individual mice. Results are pooled from five mice. (F and G) Expression of CD27 and CD11b was analyzed on wild-type and SFR KO NK cells ($CD3^{-}NK1.1^{+}$) from the spleen (F, left) and bone marrow (G, left). The maturation stages of NK cells were divided into $CD27^{-}CD11b^{-}$, $CD27^{+}CD11b^{-}$, $CD27^{+}CD11b^{+}$, and

$CD27^{-}CD11b^{+}$, and the percentages of each subset are shown in each quadrant. Data from multiple independent experiments are represented on the right. Symbols represent individual mice. Results are pooled from five (F) or four (G) mice. (D–G) *, $P < 0.05$; **, $P < 0.01$; unpaired Student's *t* test.

Figure 2.2. Impact of *Slam* locus deletion on NK cell repertoire. (A–D) NK cell repertoire was analyzed by flow cytometry of NK cells ($CD3^{-}NK1.1^{+}$) from the spleen (A and B) or bone marrow (C and D) of wild-type and SFR KO mice. Isotype control (ctrl) is shown as filled histograms. Statistical analyses of mean fluorescence intensity (MFI) or percentage of positive populations from A and C are shown for multiple mice in B and D. Each symbol represents an independent mouse. *, $P < 0.05$; **, $P < 0.01$; paired Student's *t* test. Data are representative of $n = 5$ (A and B) or $n = 4$ (C and D).

Figure 2.3. The SLAM family suppresses NK cell cytotoxicity toward hematopoietic target cells *in vitro*. (A) Natural cytotoxicity of IL-2–expanded NK cells purified from the spleen of wild-type, SFR KO, SAP KO, and SFR-SAP dKO mice was tested by ^{51}Cr release assay using hematopoietic (RMA, RMA-S, and YAC-1) and nonhematopoietic (B16 and CMT-93) target cells at the indicated E/T ratio. Standard deviations of duplicates are shown by error bars. (B) Statistical analyses of results at the 25:1 E/T ratio for multiple independent experiments are shown. Each symbol represents an independent mouse. (A and B) Results are representative of at least five experiments (A) and are pooled from five experiments (B). (C and D) Natural cytotoxicity of IL-2–expanded NK cells from wild-type and SFR KO mice toward activated $CD4^{+}$ T cells from wild-type or SFR KO mice was analyzed, as detailed for A and B. Each

symbol represents an independent mouse. Results are representative of at least six experiments (C) and are pooled from six experiments (D). (E and F) Natural cytotoxicity of IL-2–expanded NK cells from wild-type and SFR KO mice toward bone marrow–derived macrophages (Mϕ) from wild-type or SFR KO mice was tested, as detailed for A and B. Each symbol represents an independent mouse. Results are representative of six experiments (E) and are pooled from six experiments (F). (G and H) Natural cytotoxicity of IL-2–expanded NK cells from wild-type and SFR KO mice toward activated $CD4^+$ T cells from wild-type or β2M KO mice was tested, as detailed for A and B. Each symbol represents an independent mouse. Results are representative of three experiments (G) and are pooled from three experiments (H). (C, E, and G) Mean values with standard deviations are depicted. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; unpaired Student's *t* test.

Figure 2.4. The SLAM family suppresses NK cell–mediated cytokine production in response to hematopoietic target cells *in vitro*. (A) Splenocytes from wild-type mice were cultured with IL-2 for 1.5 d and then incubated for 4 h with medium alone, activated wild-type $CD4^+$ T cells, or activated SFR KO $CD4^+$ T cells. IFN-γ production was detected by intracellular staining (top), whereas degranulation was determined by surface staining of CD107a (bottom). NK cells were gated on $CD3^-NK1.1^+$. IFN-γ–producing or $CD107a^+$ cells are boxed, and percentages are represented above each box. (Left) Representative experiments are shown. (Right) The increase in the percentage of IFN-γ–producing (ΔIFN-γ) or $CD107a^+$ (ΔCD107a) cells between NK cells stimulated with medium alone or the indicated target for multiple independent mice is represented. Each symbol represents an independent mouse. Results are representative of five experiments. (B) Splenocytes from either wild-type or SFR KO mice were

cultured in IL-2–containing medium for 1.5 d and then incubated with medium alone, RMA cells, or PMA + ionomycin (Iono.) for 4 h. IFN-γ production was detected by intracellular staining, as detailed for A. (Left) A representative experiment is shown. (Right) The increase in the percentage of IFN-γ–producing cells between NK cells stimulated with medium alone or the indicated stimulus for multiple independent mice is represented. Each symbol represents an independent mouse. Results are representative of five experiments. (C) Wild-type or SFR KO NK cells were expanded in IL-2 and stimulated for 24 h with the indicated concentrations (in μg/ml) of plate-bound antibodies against CD16, NKRp1c (NK1.1), or class I MHC (H-2D^b). Cells were also stimulated with PMA and ionomycin. Secretion of IFN-γ was monitored by ELISA. Assays were done in duplicate and mean values of duplicates were used for statistical analyses. Data from three independent mice are included. Mean values for the three mice with standard deviations are depicted. Results are representative of two experiments with a total of five mice. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; unpaired Student's *t* test.

Figure 2.5. The inhibitory effect of the *Slam* locus is mediated by 2B4 with no appreciable impact from other SLAM receptors or *Gm10521*. (A–D) Natural cytotoxicity of IL-2–expanded NK cells from wild-type, SFR KO, 2B4 KO, SLAMF7 KO (C57BL/6 background; A), CD84 KO (C57BL/6 background; B), SLAMF6 KO (C57BL/6 background; C), or Ly-9 KO (129S1/Sv background; D) mice was analyzed toward activated CD4$^+$ T cells from either wild-type or SFR KO mice as targets, as detailed for Fig. 2.3 A. Results are representative of three (A), two (B and D), or four (C) experiments. (E) Natural cytotoxicity of IL-2–expanded NK cells from wild-type mice (C57BL/6) was tested against activated CD4$^+$ T cells from wild-type, CD48 KO, Ly-9 KO, or SLAMF6 KO mice (129S1/Sv background), as detailed for

Fig. 2.3 A. Results are representative of four experiments. (F) Natural cytotoxicity of IL-2–expanded NK cells from wild-type mice in C57BL/6, 129S1/Sv, or DBA/1J background was determined using activated $CD4^+$ T cells from wild-type or CD48 KO mice as targets, as detailed for Fig. 2.3 A. Results are representative of three experiments. (G–I) Natural cytotoxicity of IL-15–expanded NK cells from either wild-type or 2B4 KO mice was analyzed against activated $CD4^+$ T cells from wild-type, CD84 KO, SLAMF6 KO, Ly-9 KO, CD48 KO, or SLAMF7 KO mice, as detailed for Fig. 2.3 A. Results are representative of four (G), three (H), or two (I) experiments. (J and K) Natural cytotoxicity of IL-2–expanded NK cells from either wild-type or Gm10521 KO mice was tested at the indicated E/T ratio toward RMA, RMA-S, YAC-1, wild-type $CD4^+$ T cells, and SFR KO $CD4^+$ T cells. Results are representative of two experiments. Mean values of duplicates with standard deviations are depicted.

Figure 2.6. *Slam* locus–deleted mice exhibit NK cell–dependent reduction of virus-specific T cell responses during LCMV infection. (A–C) Wild-type or SFR KO mice were infected with 2×10^6 PFU LCMV clone 13. After 8.5 d, splenic T cells were restimulated *in vitro* with the indicated LCMV peptides, and IFN-γ and CD44 expression by $CD8^+$ T cells was monitored by flow cytometry. (A) A representative experiment using LCMV peptide $GP_{33–41}$ is shown. Percentage of IFN-γ–producing $CD44^{hi}$ $CD8^+$ T cells compared with total $CD8^+$ T cells is shown in the top right quadrant. (B and C) The proportions (B) and total numbers (C) of LCMV $GP_{33–41}$–, $NP_{396–404}$–, $GP_{276-286}$–, or $NP_{205–212}$–responding $CD44^+$ IFN-γ^+ $CD8^+$ T cells from four to five mice in each group are depicted. Numbers represent mean ± SEM. Results are representative of three independent experiments. (D and E) Data are as in A–C, except that virus-specific activated $CD8^+$ T cells were detected by staining splenocytes with virus peptide-loaded class I MHC

tetramers. (D) A representative experiment using $NP_{396–404}/D^b$ tetramers is shown. Percentage of tetramer-binding $CD44^+$ $CD8^+$ T cells is indicated in the top right quadrant. (E) The total numbers of $GP_{33–41}/D^b$ (left) or $NP_{396–404}/D^b$ (right) tetramer-binding $CD44^+$ $CD8^+$ T cells are shown. Four to five mice were used per group. Results are representative of three independent experiments. (F) Data are as in A–C, except that SFR KO mice were injected with 25 μg of NK cell–depleting α-NKRp1c (NK1.1; ΔNK) antibody or IgG_{2a} isotype control antibody 1 d before infection. Total numbers of $GP_{33–41}$–specific $CD44^+$ IFN-γ^+ $CD8^+$ T cells are shown. Seven to eight mice were used per group. Results are representative of two independent experiments. (G) Data are as in F, except that SFR KO and 2B4 KO mice were analyzed. Proportions of $GP_{33–41}$–specific $CD44^+$ IFN-γ^+ $CD8^+$ T cells are shown. Seven to eight mice were used per group. The gray bar represents the range for wild-type mice. Results are representative of two independent experiments. (H) Data are the same as A–C, except that the proportion of body weight loss between day −1 and day 8.5 of infection is depicted. Four to five mice per group were used. Results are representative of three independent experiments. Mean values from four to five mice (D, E, and H) or seven to eight mice (F and G) with standard deviations are depicted. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired Student's *t* test.

Figure 2.7. Cytokines influence the functions of SLAM receptors. (A) Natural cytotoxicity of either freshly isolated or IL-2–expanded NK cells (lymphokine-activated killer (LAK)) from wild-type or SFR KO mice was tested toward activated $CD4^+$ T cells from wild-type mice, as detailed for Fig. 2.3 A. Results are representative of at least three experiments. (B) The ability of nonprimed wild-type or SFR KO mice to eliminate RMA cells (10^6 cells injected) expressing GFP was evaluated in a peritoneal clearance assay. Residual RMA cells were counted 18 h after

injection. Each symbol represents a different mouse. Unpaired Student's *t* test was used. Results are representative of three experiments. (C) Wild-type mice were primed or not primed for 36 h with polyI:C. Cytotoxicity of freshly isolated NK cells toward activated $CD4^+$ T cells from wild-type, SFR KO, or β2M KO mice was evaluated, as detailed for Fig. 2.3 A. IL-2–activated NK cells were analyzed in parallel as control. Results are representative of at least three experiments. (D and E) IL-15–expanded NK cells from wild-type mice were treated or not treated overnight with either IL-12 or IFN-β. (D) Natural cytotoxicity toward activated $CD4^+$ T cells from wild-type or SFR KO mice was then examined, as detailed for Fig. 2.3 A. (E) Statistical analyses of results at the 10:1 E/T ratio from multiple independent experiments are shown. Results are representative of nine experiments (D) and are pooled from nine experiments (E). (B and E) **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; unpaired Student's *t* test. (F) Data are as in D, except that IL-15–expanded NK cells from wild-type mice were treated or not treated with IFN-α. Results are representative of three experiments. (G) Data are as in D, except that IL-15–expanded NK cells from wild-type mice were treated with IL-12 alone or together with IFN-β. Results are representative of three experiments. (H, left) NK cells from SFR KO mice were expanded in IL-15 and stimulated overnight with or without IL-12 or IFN-β. Natural cytotoxicity toward wild-type $CD4^+$ T cells was determined at the indicated E/T ratio. (Right) Statistical analyses of results at the 10:1 E/T ratio are shown. Unpaired Student's *t* test was used. Results are representative of five experiments. (I) Data are as in D, except that NK cells from wild-type or SFR KO mice were expanded in IL-15 and stimulated overnight with IFN-β. (Left) Natural cytotoxicity toward $CD4^+$ T cells from SFR KO mice was determined at the indicated E/T ratio. (Right) Statistical analyses of results at the 10:1 E/T ratio from several independent experiments are shown. Unpaired Student's *t* test was used. Results are representative of four

experiments. (J) Data are as in D, except that IL-15–expanded NK cells from IFNAR KO mice were treated or not treated with IL-12 or IFN-β. Results are representative of three experiments. (A, C, F, G, and J) Mean values of duplicates with standard deviations are depicted. (B, D, H, and I) Mean values with standard deviations are depicted.

Figure 2.8. Evidence that cytokines influence SFR-mediated inhibition by regulating expression of SAP adaptors. (A) Expression of SLAM receptors, CD48, NKG2D, DNAM-1, and integrin subunits on IL-15–expanded wild-type NK cells treated with or without IL-12 or IFN-β was examined. NK cells were gated on TCR-β^-NK1.1$^+$ cells. Isotype control (ctrl) is shown in filled histograms. Results are representative of four experiments. (B) Expression of various positive or negative effectors of SLAM receptor signaling was analyzed by immunoblotting (IB) of total cell lysates with the indicated antibodies. IL-15–expanded NK cells treated or not treated with IL-12 or IFN-β were used. Numbers below each lane indicate levels of protein expression relative to those of cells cultured with IL-15 alone. Quantification was normalized to β-actin. Results are representative of seven experiments. (C) Data are the same as B, except that levels of RNA encoding SAP (*Sh2d1a*), EAT-2 (*Sh2d1b1*), and ERT (*Sh2d1b2*) were analyzed by quantitative real-time PCR. Levels were compared with those of cells expanded in IL-15 alone. Levels of *Gapdh* RNA were used for normalization. Results are representative of three experiments. (D) Data are the same as B, except that wild-type or IFNAR KO NK cells were analyzed. Results are representative of three experiments. (E) Data are the same as Fig. 2.7 D, except that NK cells from wild-type, EAT-2 KO, ERT KO, or EAT-2–ERT dKO mice expanded in IL-15 and IFN-β were used. Results are representative of three experiments. (C and E) Mean values of duplicates with standard deviations are depicted.

Figure 2.9. Integrin LFA-1, but not other activating receptors, is required for enhancement of NK cell responses upon loss of SLAM receptors. (A) Data are as in Fig. 2.7 D, except that natural cytotoxicity of IL-15–expanded wild-type NK cells toward activated $CD4^+$ T cells from wild-type or SFR KO mice was tested at the 10:1 E/T ratio in the presence or the absence of various blocking antibodies during incubation (final concentration of 10 μg/ml). *, $P < 0.05$; ****, $P < 0.0001$; unpaired Student's *t* test. Results are pooled from six experiments. (B) Data are as in A, except that natural cytotoxicity of IL-15–expanded wild-type NK cells toward activated $CD4^+$ T cells from wild-type or SFR KO mice was tested at the indicated E/T ratio in the presence of blocking anti-CD11a antibody or control IgG antibody. Results are representative of six experiments. (C–E) Data are as in Fig. 2.7 D, except that natural cytotoxicity of IL-15–expanded NK cells from wild-type, CD11a KO, CD18 KO, DNAM-1 KO, CD11b KO, and NKG2D KO mice was determined. Results are representative of three experiments. (F) Data are as in Fig. 2.7 D, except that natural cytotoxicity of IL-15–expanded or IL-2–expanded NK cells from wild-type, SFR KO, CD11a KO, or SFR-CD11a dKO mice toward activated $CD4^+$ T cells from wild-type mice, RMA, or RMA-S was determined. Results are representative of three experiments. Mean values of duplicates with standard deviations are depicted.

Figure 2.10. 2B4-mediated inhibition results in decreased activation of LFA-1. (A, left) The ability of the human NK cell line YT-S to mediate cytotoxicity of the B cell line 721.221 was assessed at the indicated E/T ratio in the presence of blocking antibodies against CD11a (LFA-1) or CD18 or control IgG. Standard deviations of duplicates are shown by error bars. (Right) Statistical analyses of results at the 10:1 E/T ratio for three independent experiments are shown.

****, $P < 0.0001$. (B) Expression of SAP in YT-S cells transfected with scrambled siRNA (control (siCT)) or siRNAs against SAP (siSAP) was analyzed by immunoblot (IB) analysis. Results are representative of four experiments. (C) Expression of SLAMF6, SLAMF7, and CD48 was analyzed on parental (WT) or CD48 KO 721.221 cells. Two different targeting sequences were used to generate two different CD48 KO derivatives of 721.221 through CRISPR-Cas–mediated genome editing. Isotype control is shown in red. Results are representative of three experiments. (D) Natural cytotoxicity of YT-S cells transfected with control siRNAs (left) or siRNAs against SAP (right) was analyzed toward wild-type or CD48 KO 721.221 cells at the indicated E/T ratio. For CD48 KO 721.221 cells, a pool of CD48 KO 721.221 no. 1 and no. 2 cells was used. Mean values of duplicates with standard deviations are depicted. Results are representative of four experiments. (E) YT-S cells transfected with control or SAP-specific siRNAs were labeled with Brilliant violet 421–conjugated anti–human NKp46 and then incubated for the indicated times at 37°C with parental (WT) or CD48-deficient 721.221 cells labeled with PE-conjugated anti–human CD19. Conjugate formation was assessed by flow cytometry. A representative experiment is depicted on the left, whereas a statistical analysis of duplicates with mean values and standard deviations is shown on the right. Data are representative of three experiments. (F) YT-S cells transfected with control siRNAs or SAP-specific siRNAs were incubated with medium alone, wild-type 721.221, or CD48 KO 721.221 for 5 min at 37°C. After stimulation, cells were stained with anti-CD11a conformational antibody (m24) for 30 min at room temperature, followed by anti-NKp46 staining for 15 min at room temperature. YT-S cells were gated on $NKp46^+$ cells and analyzed for m24 staining. Results are representative of three experiments. (G) Expression of SAP and SHIP-1 in YT-S cells transfected with control siRNA, siRNAs against SAP, or SHIP-1 was analyzed by

immunoblot analysis. β-actin was used as the control. Results are representative of three experiments. (H) Natural cytotoxicity of YT-S cells transfected with the siRNAs detailed in G was determined at the indicated E/T ratio toward wild-type or CD48 KO 721.221 cells. (I) Statistical analyses of the results at the 10:1 E/T ratio from multiple independent experiments are shown. (J, left) YT-S cells transfected with the siRNAs detailed in G were incubated with medium alone, WT 721.221, or CD48 KO 721.221 and stained with the anti-CD11a conformational antibody (m24), as outlined for F. (Right) Statistical analyses of the results from multiple independent experiments are shown. (H–J) *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired Student's *t* test. Mean values with standard deviations are depicted. Results are representative of four experiments.

Figures

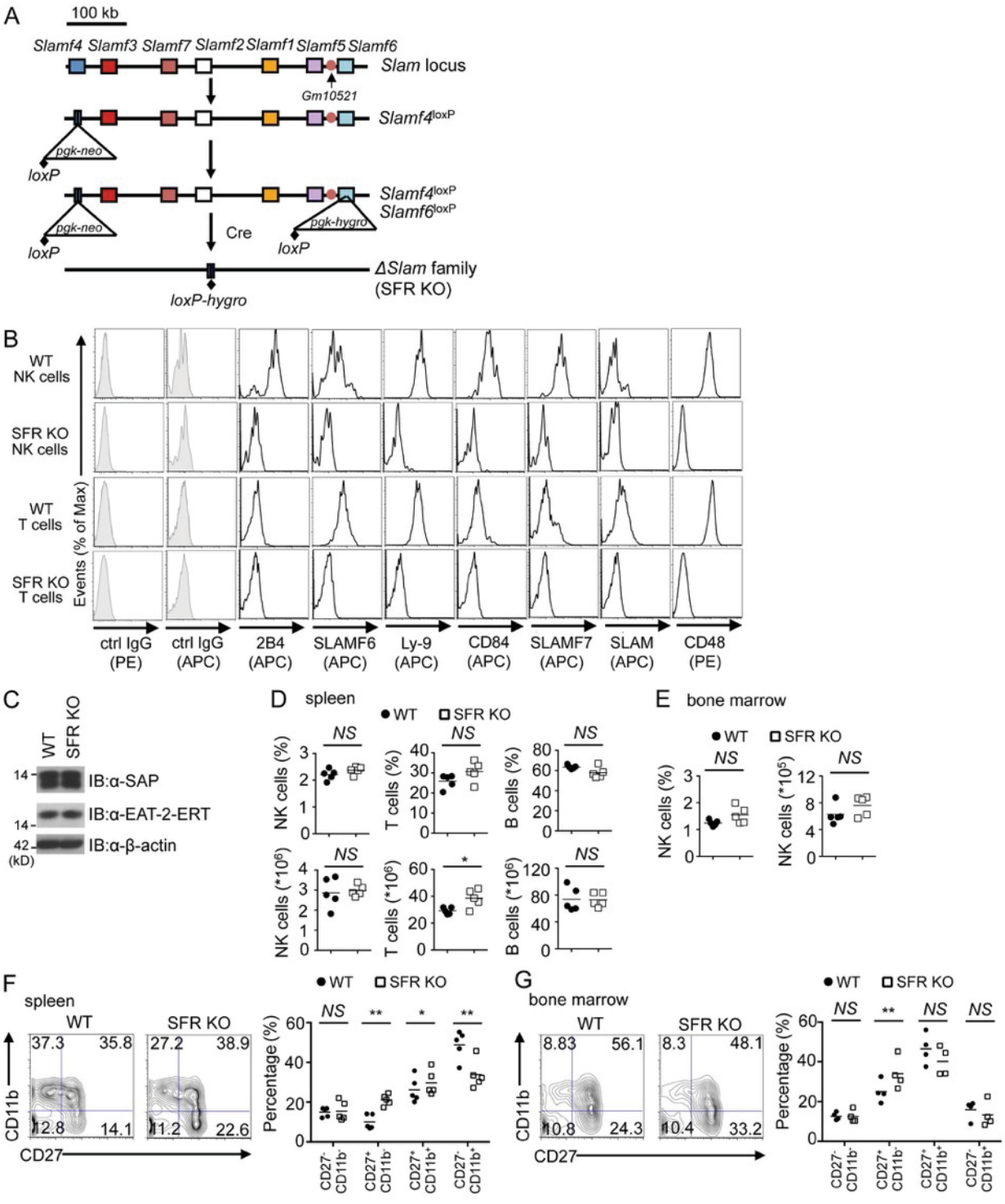

Figure 2.1

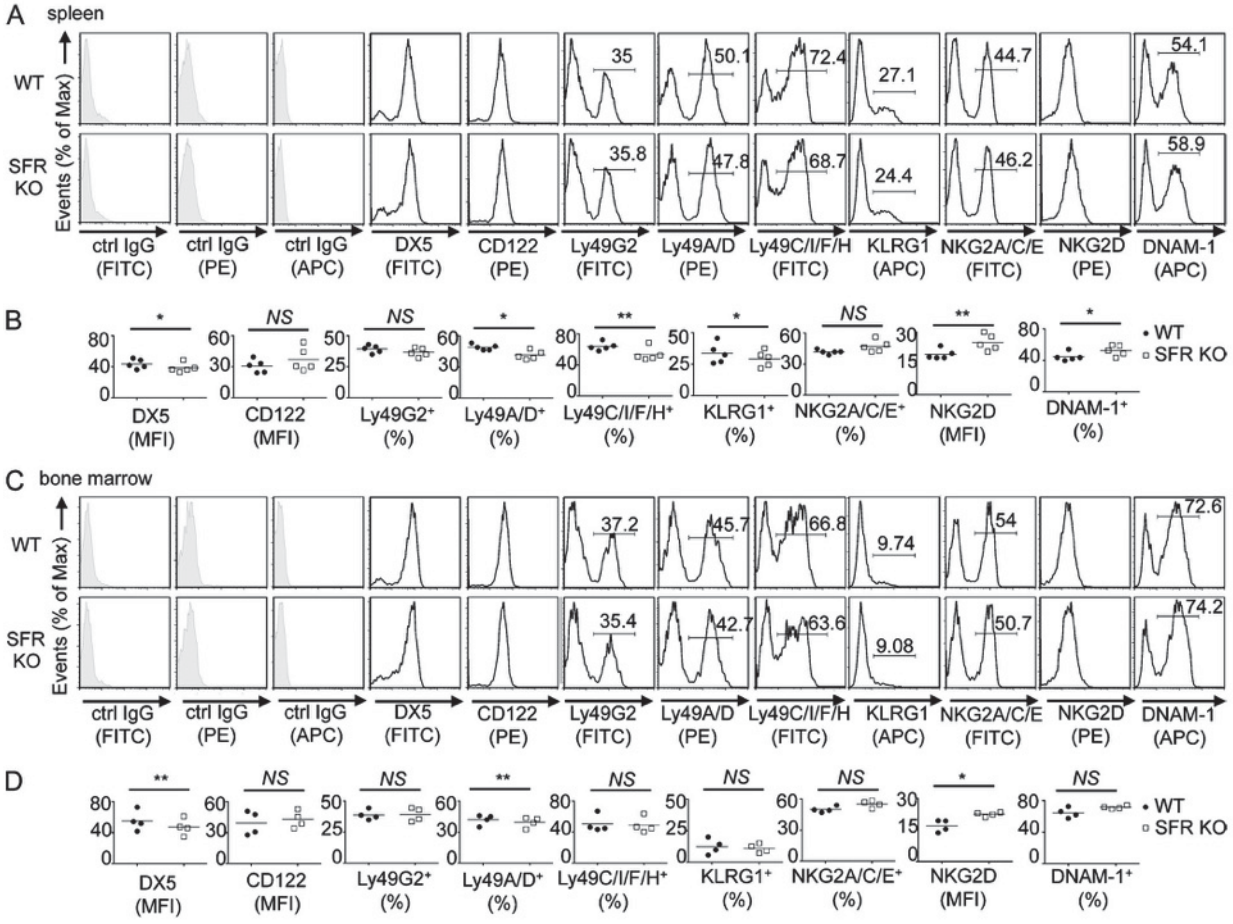
A
spleen
WT
SFR KO
Events (% of Max)
35
50.1
72.4
27.1
44.7
54.1
35.8
47.8
68.7
24.4
46.2
58.9
ctrl IgG (FITC)
ctrl IgG (PE)
ctrl IgG (APC)
DX5 (FITC)
CD122 (PE)
Ly49G2 (FITC)
Ly49A/D (PE)
Ly49C/I/F/H (FITC)
KLRG1 (APC)
NKG2A/C/E (FITC)
NKG2D (PE)
DNAM-1 (APC)
B
DX5 (MFI)
CD122 (MFI)
Ly49G2+ (%)
Ly49A/D+ (%)
Ly49C/I/F/H+ (%)
KLRG1+ (%)
NKG2A/C/E+ (%)
NKG2D (MFI)
DNAM-1+ (%)
WT
SFR KO
C
bone marrow
37.2
45.7
66.8
9.74
54
72.6
35.4
42.7
63.6
9.08
50.7
74.2
D

Figure 2.2

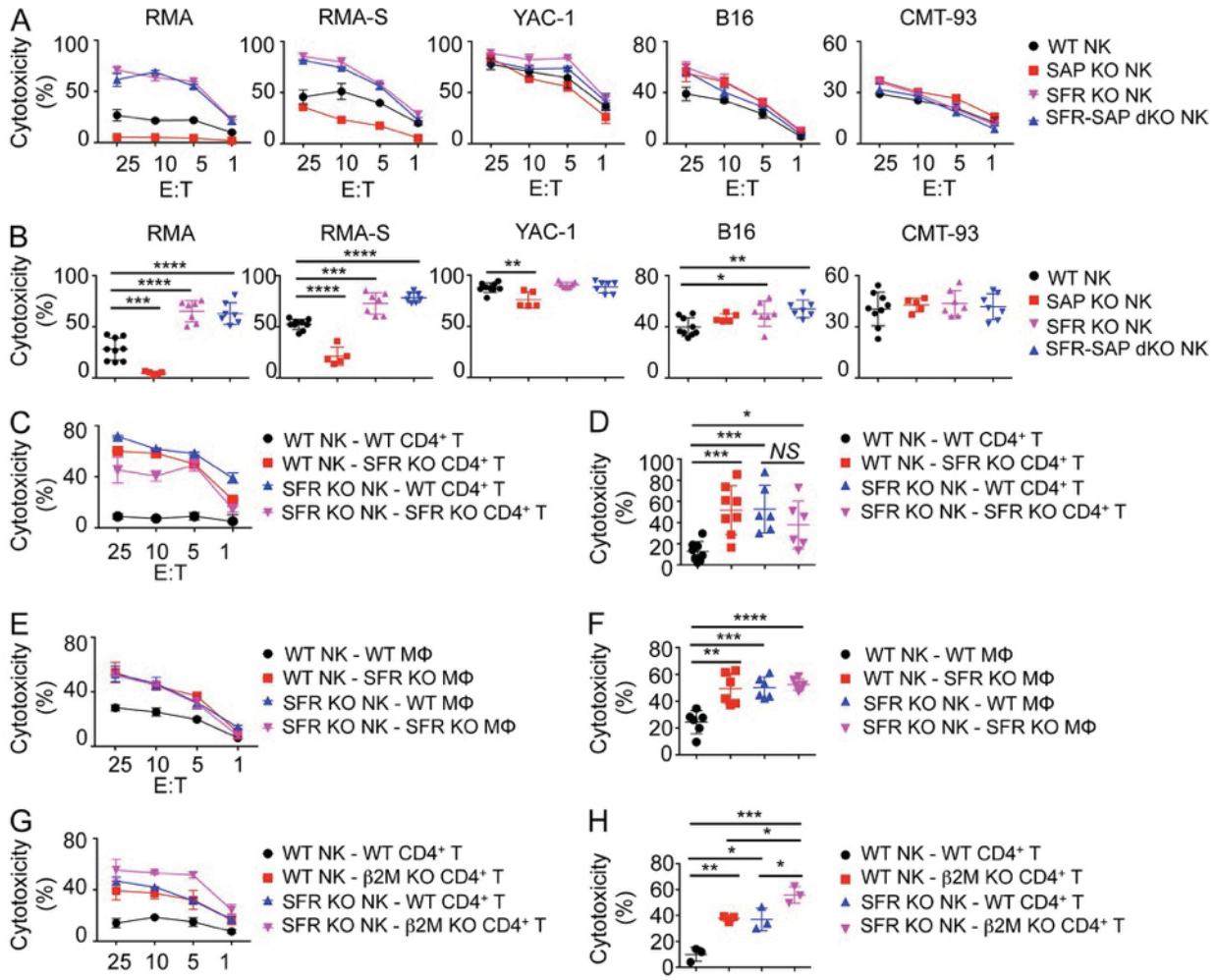
A
RMA
RMA-S
YAC-1
B16
CMT-93
Cytotoxicity (%)
E:T
WT NK
SAP KO NK
SFR KO NK
SFR-SAP dKO NK
B
C
WT NK - WT CD4+ T
WT NK - SFR KO CD4+ T
SFR KO NK - WT CD4+ T
SFR KO NK - SFR KO CD4+ T
D
NS
E
WT NK - WT MΦ
WT NK - SFR KO MΦ
SFR KO NK - WT MΦ
SFR KO NK - SFR KO MΦ
F
G
WT NK - β2M KO CD4+ T
SFR KO NK - β2M KO CD4+ T
H

Figure 2.3

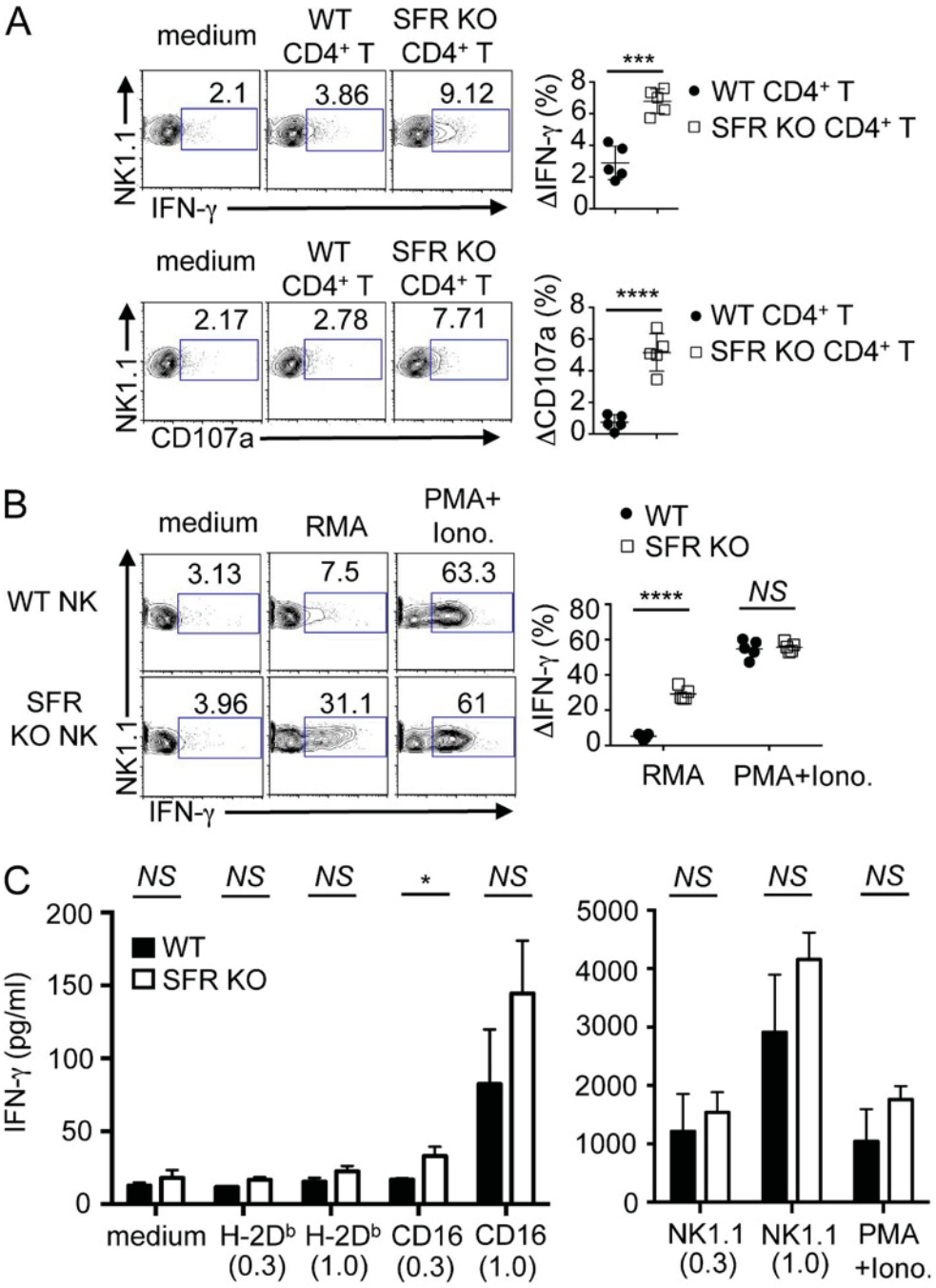
A
medium
WT CD4+ T
SFR KO CD4+ T
2.1
3.86
9.12
NK1.1
IFN-γ
***
ΔIFN-γ (%)
WT CD4+ T
SFR KO CD4+ T
2.17
2.78
7.71
CD107a
****
ΔCD107a (%)
B
RMA
PMA+ Iono.
WT NK
SFR KO NK
3.13
7.5
63.3
3.96
31.1
61
NS
ΔIFN-γ (%)
RMA
PMA+Iono.
C
IFN-γ (pg/ml)
WT
SFR KO
*
medium
H-2Db (0.3)
H-2Db (1.0)
CD16 (0.3)
CD16 (1.0)
NK1.1 (0.3)
NK1.1 (1.0)
PMA +Iono.

Figure 2.4

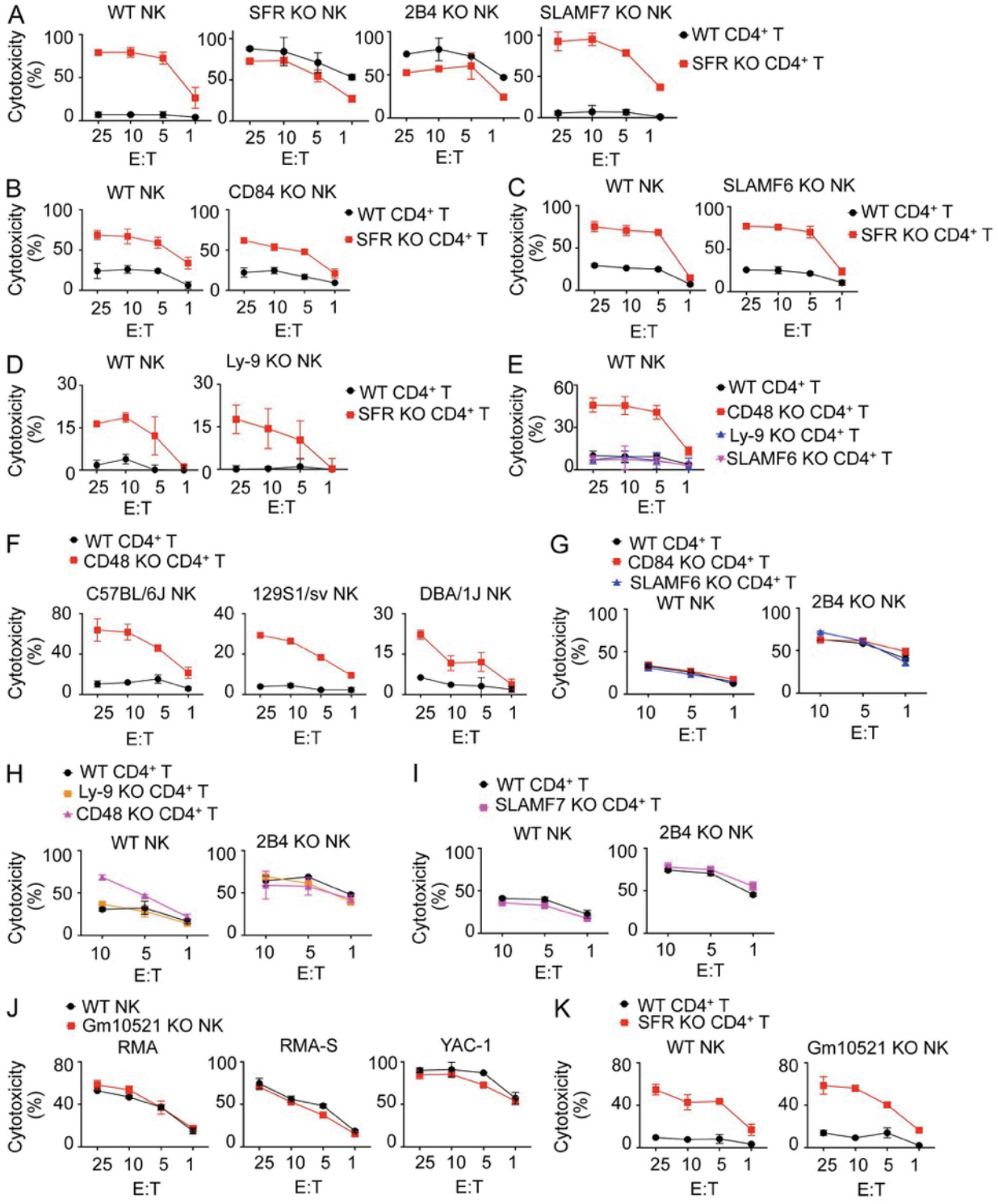
A
WT NK
SFR KO NK
2B4 KO NK
SLAMF7 KO NK
WT CD4+ T
SFR KO CD4+ T
B
WT NK
CD84 KO NK
C
WT NK
SLAMF6 KO NK
D
WT NK
Ly-9 KO NK
E
WT NK
CD48 KO CD4+ T
Ly-9 KO CD4+ T
SLAMF6 KO CD4+ T
F
C57BL/6J NK
129S1/sv NK
DBA/1J NK
G
WT NK
2B4 KO NK
H
Ly-9 KO CD4+ T
I
SLAMF7 KO CD4+ T
J
WT NK
Gm10521 KO NK
RMA
RMA-S
YAC-1
K
Gm10521 KO NK
Cytotoxicity (%)
E:T

Figure 2.5

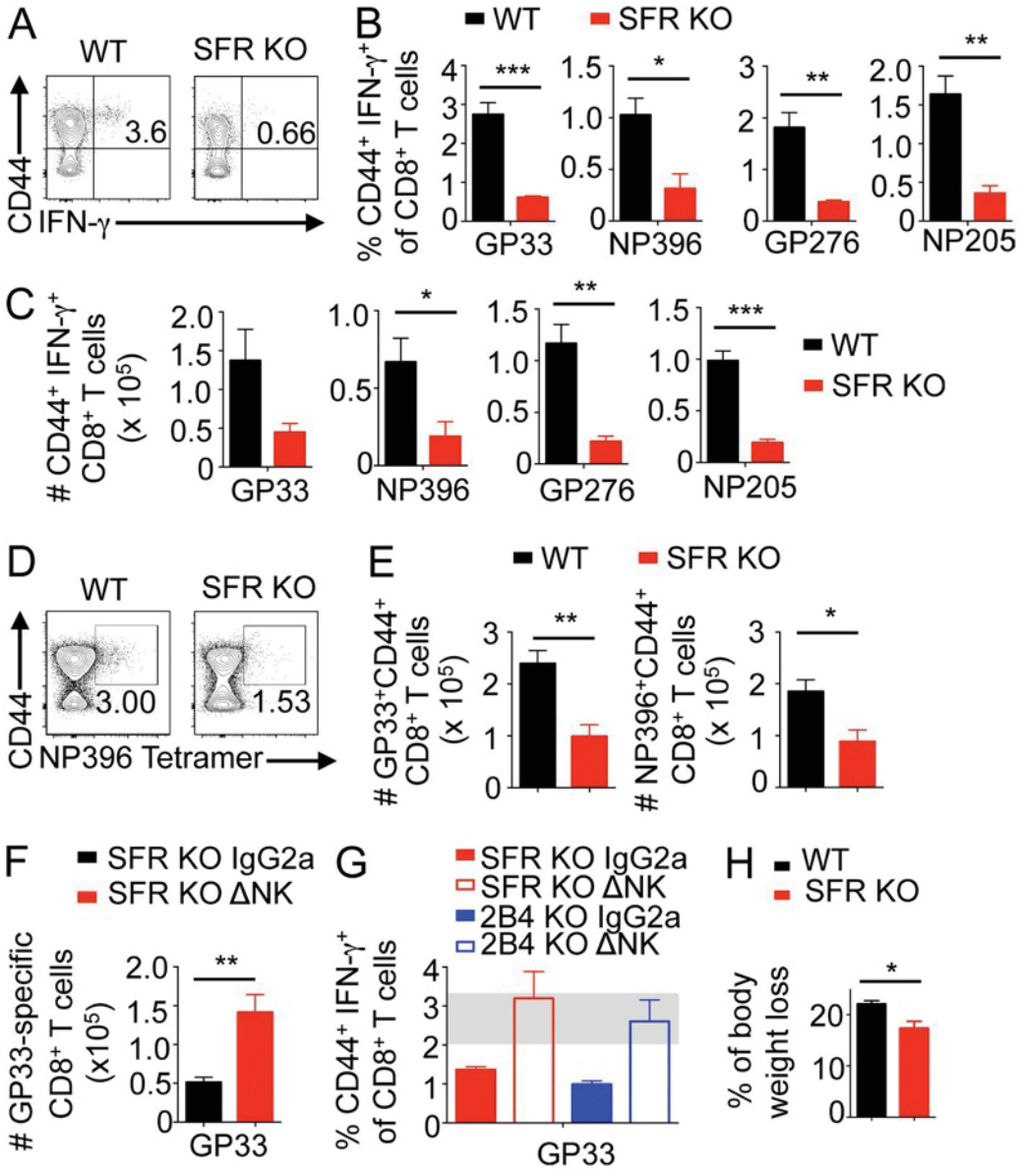
A
WT
SFR KO
3.6
0.66
CD44
IFN-γ
B
WT
SFR KO
% CD44+ IFN-γ+ of CD8+ T cells
***
*
**
**
GP33
NP396
GP276
NP205
C
CD44+ IFN-γ+ CD8+ T cells (x 10^5)
*
**
***
GP33
NP396
GP276
NP205
WT
SFR KO
D
WT
SFR KO
3.00
1.53
CD44
NP396 Tetramer
E
WT
SFR KO
GP33+CD44+ CD8+ T cells (x 10^5)
**
NP396+CD44+ CD8+ T cells (x 10^5)
*
F
SFR KO IgG2a
SFR KO ΔNK
GP33-specific CD8+ T cells (x10^5)
**
GP33
G
SFR KO IgG2a
SFR KO ΔNK
2B4 KO IgG2a
2B4 KO ΔNK
% CD44+ IFN-γ+ of CD8+ T cells
GP33
H
WT
SFR KO
% of body weight loss
*

Figure 2.6

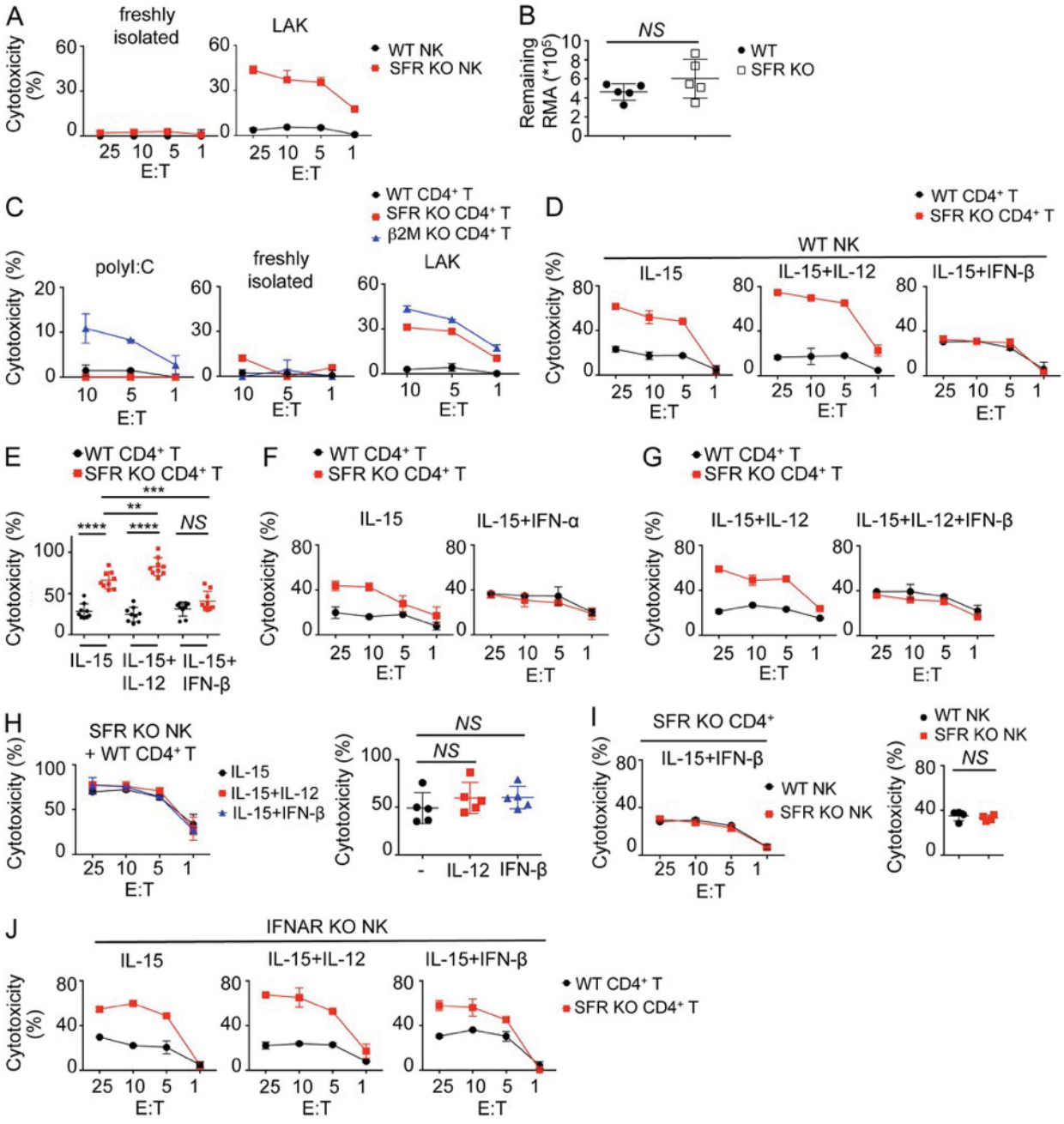
A
freshly isolated
LAK
Cytotoxicity (%)
E:T
WT NK
SFR KO NK
B
NS
Remaining RMA (*10^5)
WT
SFR KO
C
WT CD4+ T
SFR KO CD4+ T
β2M KO CD4+ T
polyI:C
freshly isolated
LAK
D
WT NK
IL-15
IL-15+IL-12
IL-15+IFN-β
E
****
**
***
NS
IL-15
IL-15+ IL-12
IL-15+ IFN-β
F
IL-15
IL-15+IFN-α
G
IL-15+IL-12
IL-15+IL-12+IFN-β
H
SFR KO NK + WT CD4+ T
IL-15
IL-15+IL-12
IL-15+IFN-β
- IL-12 IFN-β
I
SFR KO CD4+
IL-15+IFN-β
J
IFNAR KO NK
IL-15
IL-15+IL-12
IL-15+IFN-β

Figure 2.7

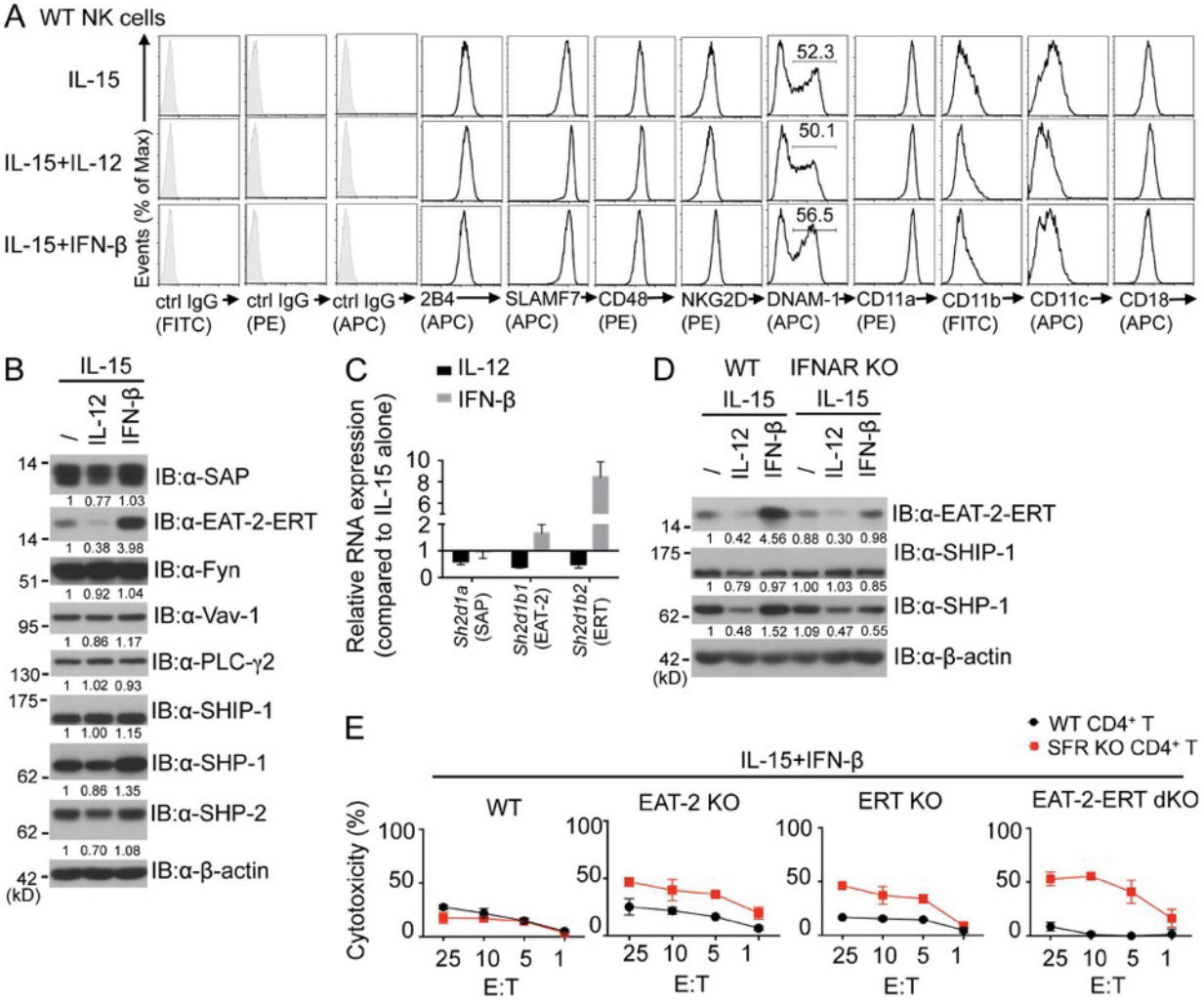
A WT NK cells
IL-15
IL-15+IL-12
IL-15+IFN-β
Events (% of Max)
52.3
50.1
56.5
ctrl IgG (FITC)
ctrl IgG (PE)
ctrl IgG (APC)
2B4 (APC)
SLAMF7 (APC)
CD48 (PE)
NKG2D (PE)
DNAM-1 (APC)
CD11a (PE)
CD11b (FITC)
CD11c (APC)
CD18 (APC)
B
IL-15
/
IL-12
IFN-β
IB:α-SAP
IB:α-EAT-2-ERT
IB:α-Fyn
IB:α-Vav-1
IB:α-PLC-γ2
IB:α-SHIP-1
IB:α-SHP-1
IB:α-SHP-2
IB:α-β-actin
(kD)
C
IL-12
IFN-β
Relative RNA expression (compared to IL-15 alone)
Sh2d1a (SAP)
Sh2d1b1 (EAT-2)
Sh2d1b2 (ERT)
D
WT
IFNAR KO
IL-15
IL-15
IB:α-EAT-2-ERT
IB:α-SHIP-1
IB:α-SHP-1
IB:α-β-actin
(kD)
E
WT CD4+ T
SFR KO CD4+ T
IL-15+IFN-β
WT
EAT-2 KO
ERT KO
EAT-2-ERT dKO
Cytotoxicity (%)
E:T

Figure 2.8

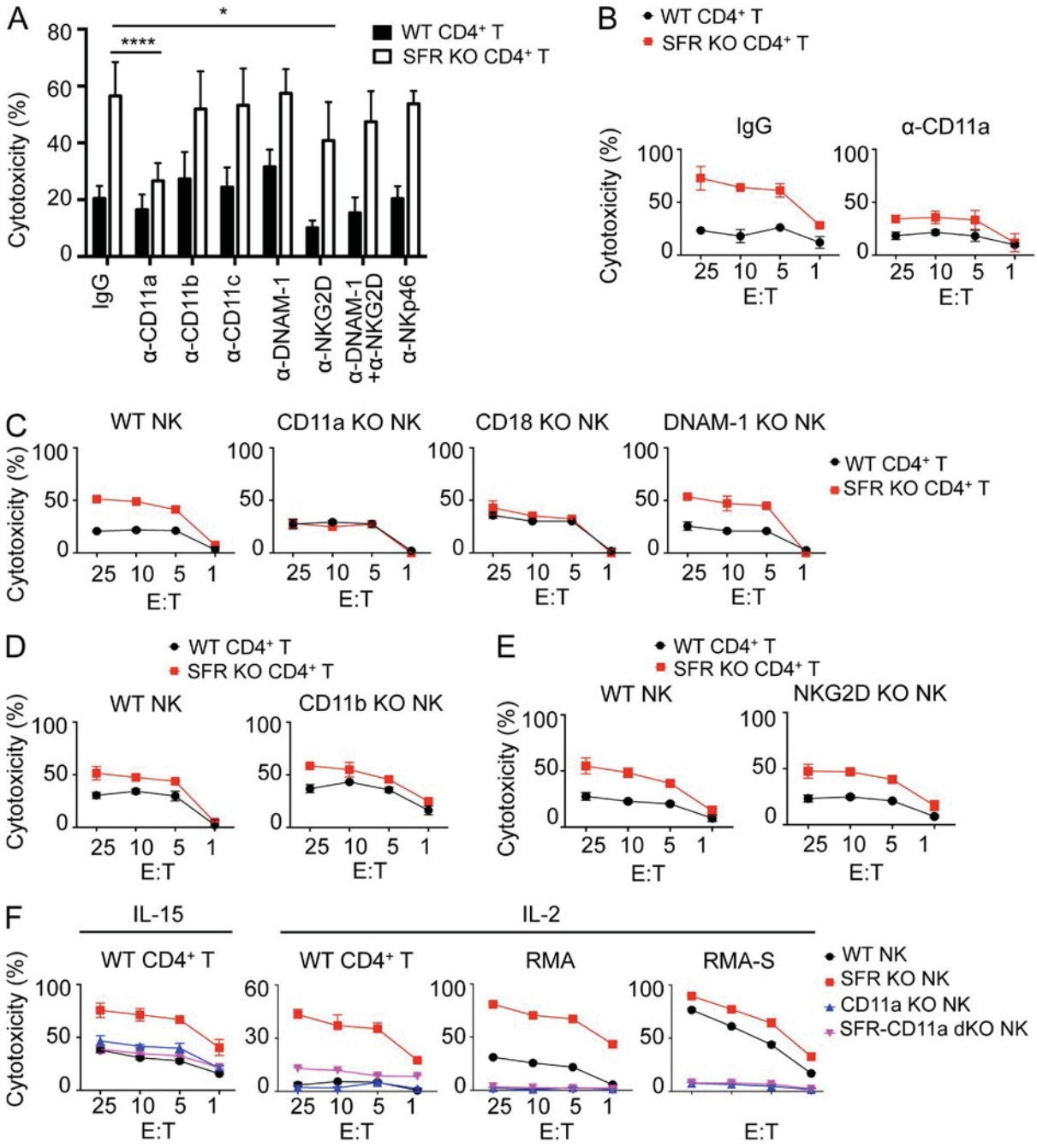
A
Cytotoxicity (%)
WT CD4+ T
SFR KO CD4+ T
IgG
α-CD11a
α-CD11b
α-CD11c
α-DNAM-1
α-NKG2D
α-DNAM-1 +α-NKG2D
α-NKp46
B
WT CD4+ T
SFR KO CD4+ T
IgG
α-CD11a
E:T
C
WT NK
CD11a KO NK
CD18 KO NK
DNAM-1 KO NK
D
WT NK
CD11b KO NK
E
WT NK
NKG2D KO NK
F
IL-15
IL-2
WT CD4+ T
RMA
RMA-S
WT NK
SFR KO NK
CD11a KO NK
SFR-CD11a dKO NK

Figure 2.9

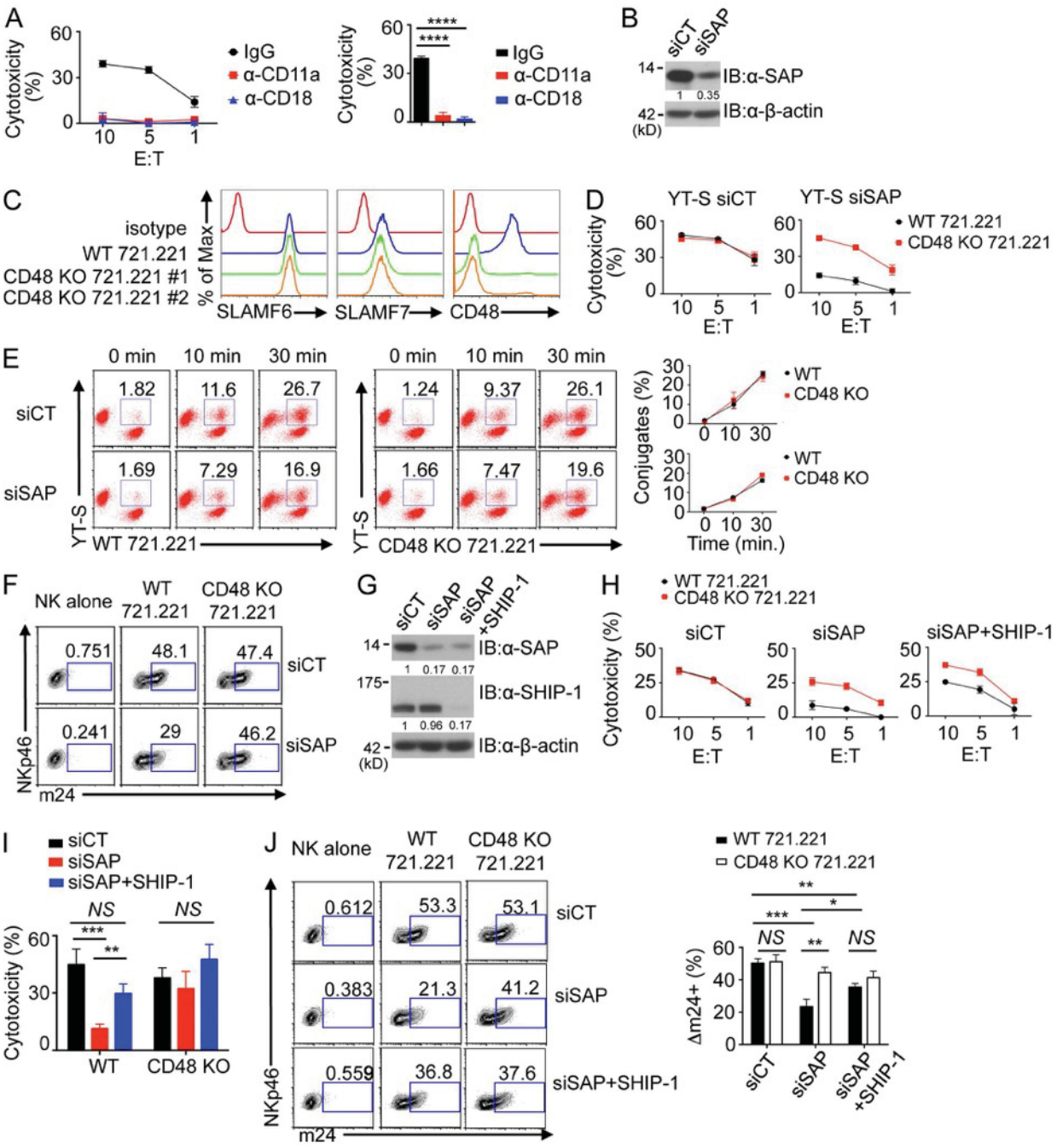
A
Cytotoxicity (%)
60
30
0
10 5 1
E:T
IgG
α-CD11a
α-CD18
****
****
B
siCT
siSAP
14-
IB:α-SAP
1 0.35
42-
(kD)
IB:α-β-actin
C
isotype
WT 721.221
CD48 KO 721.221 #1
CD48 KO 721.221 #2
% of Max
SLAMF6
SLAMF7
CD48
D
YT-S siCT
YT-S siSAP
WT 721.221
CD48 KO 721.221
E
0 min
10 min
30 min
siCT
siSAP
1.82
11.6
26.7
1.69
7.29
16.9
1.24
9.37
26.1
1.66
7.47
19.6
YT-S
WT 721.221
CD48 KO 721.221
Conjugates (%)
WT
CD48 KO
Time (min.)
F
NK alone
WT 721.221
CD48 KO 721.221
0.751
48.1
47.4
0.241
29
46.2
NKp46
m24
G
siCT
siSAP
siSAP +SHIP-1
IB:α-SAP
1 0.17 0.17
175-
IB:α-SHIP-1
1 0.96 0.17
IB:α-β-actin
H
WT 721.221
CD48 KO 721.221
siCT
siSAP
siSAP+SHIP-1
Cytotoxicity (%)
I
siCT
siSAP
siSAP+SHIP-1
NS
***
**
Cytotoxicity (%)
WT
CD48 KO
J
NK alone
WT 721.221
CD48 KO 721.221
0.612
53.3
53.1
0.383
21.3
41.2
0.559
36.8
37.6
NKp46
m24
WT 721.221
CD48 KO 721.221
Δm24+ (%)
siCT
siSAP
siSAP +SHIP-1

Figure 2.10

Chapter 3: Immune cell inhibition by SLAMF7 is mediated by a mechanism requiring Src kinases, CD45, and SHIP-1 that is defective in multiple myeloma cells

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Immune cell inhibition by SLAMF7 is mediated by mechanism requiring Src kinases, CD45 and SHIP-1 defective in multiple myeloma cells

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ABSTRACT

Signaling lymphocytic activation molecule (SLAM) F7 is a receptor present on immune cells including natural killer (NK) cells. It is also expressed on multiple myeloma (MM) cells. This led to development of an anti-SLAMF7 antibody, elotuzumab, showing efficacy against MM. SLAMF7 mediates activating or inhibitory effects in NK cells, depending whether cells express or not the adaptor EAT-2. Since MM cells lack EAT-2, we elucidated the inhibitory effectors of SLAMF7 in EAT-2-negative NK cells, and tested whether these effectors were triggered in MM cells. SLAMF7-mediated inhibition in NK cells lacking EAT-2 was mediated by SH2 domain-containing inositol phosphatase (SHIP)-1, which was recruited via tyrosine 261 of SLAMF7. Coupling of SLAMF7 to SHIP-1 required Src kinases, which phosphorylated SLAMF7. Although MM cells lack EAT-2, elotuzumab did not induce inhibitory signals in these cells. This was at least partly due to lack of CD45, a phosphatase required for Src kinase activation. A defect in SLAMF7 function was also observed in CD45-deficient NK cells. Hence, SLAMF7-triggered inhibition is mediated by a mechanism involving Src kinases, CD45 and SHIP-1 that is defective in MM cells. The latter defect might explain why elotuzumab eliminates MM cells by an indirect mechanism, involving activation of NK cells.

INTRODUCTION

Signaling lymphocytic activation molecule (SLAM) family receptors are hematopoietic cell-specific receptors playing critical roles in normal immune regulation [219, 249-251]. They have also been firmly implicated in many human diseases, including immune deficiencies, autoimmunity and hematological malignancies. SLAM family receptors can mediate either activating or inhibitory effects in immune cells, depending in part on whether they are co-expressed with members of the SLAM-associated protein (SAP) family of Src homology 2 (SH2) domain-only adaptors. Typically, SLAM family receptors are activating in the presence of SAP family adaptors, but are inhibitory in the absence of SAP family adaptors. Whereas much is known of the molecular mechanisms by which SLAM family receptors mediate activating effects, little is known about how they mediate inhibitory effects.

SLAMF7 (also named CS1 (CD2 subset 1), CRACC (CD2-like receptor-activating cytotoxic cell) and CD319) is a member of the SLAM family [219, 249-251]. The other members of the family are SLAM, 2B4, NTB-A/Ly108, Ly-9 and CD84. Like most SLAM receptors, SLAMF7 is a self-ligand, i.e. it recognizes as ligand another SLAMF7 molecule on another cell. The only exception is 2B4, which recognizes CD48. SLAMF7 is found on natural killer (NK) cells, activated T cells, most B cells including antibody-producing plasma cells and myeloid cells [250, 252]. It is also abundantly present in most cases of multiple myeloma (MM), a nearly universally fatal malignancy of plasma cells, either freshly isolated cells or cell lines [219, 251].

In NK cells, SLAMF7 is usually a positive regulator of NK cell activation [252, 253]. This activity requires expression of the SAP family adaptor, Ewing's sarcoma-associated transcript (EAT)-2. SLAMF7 binds EAT-2 via phosphorylated tyrosine 281 (Y281) in its

cytoplasmic segment, thereby triggering activating signals involving phospholipase C (PLC)-γ [254]. In the absence of EAT-2, SLAMF7 mediates inhibitory effects; these effects were documented in NK cells from EAT-2-deficient mice and normal activated T cells, which lack EAT-2 [252]. However, the molecular basis of this inhibition is undetermined. Depending on the SLAM family receptor studied, it was suggested that inhibition might be mediated by SH2 domain-containing protein tyrosine phosphatases (SHP)-1, SHP-2 or SH2 domain-containing inositol phosphatase (SHIP)-1. However, firm genetic evidence in support of this idea has not been reported. Moreover, how any of the SLAM family receptors couples to its inhibitory effectors has not been addressed.

The nearly universal expression of SLAMF7 in MM led to development of a humanized anti-SLAMF7 monoclonal antibody (MAb), elotuzumab [219, 251]. Pre-clinical studies using transplanted human MM cells in mice showed that elotuzumab caused MM elimination *in vivo* [255]. The efficacy of elotuzumab in combination with lenalidomide was subsequently demonstrated in phase 1 and 2 trials of patients with refractory and relapsed MM [256-259]. Phase 3 studies are ongoing. Surprisingly, elotuzumab had little or no direct inhibitory effects on MM cells *in vitro*, implying that its efficacy occurred through an indirect mechanism. Accordingly, maximal activity of elotuzumab in mice was NK cell-dependent [255]. Furthermore, elotuzumab directly activated NK cells, through antibody-dependent cellular cytotoxicity (ADCC) via CD16 (FcγRIII) and triggering of SLAMF7 on NK cells [219, 251, 255, 260].

As MM cells lack EAT-2 [249, 260], we wanted to ascertain the molecular mechanism by which SLAMF7 mediates inhibition in NK cells and evaluate if this mechanism is functional in MM cells. Our studies showed that the inhibitory function of SLAMF7 in EAT-2-negative NK

cells was mediated by a molecular mechanism implicating lipid phosphatase SHIP-1, Src kinases and protein tyrosine phosphatase CD45. Interestingly, coupling of SLAMF7 to SHIP-1 was severely compromised in MM cells. This defect correlated with lack of CD45, which is required for activation of Src family kinases in hematopoietic cells and was needed for initiation of SLAMF7 inhibitory signaling.

MATERIALS AND METHODS

Mice. C57BL/6J, 129S1/Sv and CD45-deficient (B6.129-Ptprc^{tm1Holm}/J) mice were from The Jackson Laboratory (Bar Harbor, ME). EAT-2-deficient mice (in 129/Sv) were described [261]. In all experiments, normal littermates were used as control. The IRCM Animal Care Committee approved all experimentation in accordance to the Canadian Council for Animal Care.

Cells. YT-S, Cos-1, HeLa, B16 and DT40 were described [37, 252, 262]. B16 expressing mouse (m) SLAMF7 or mCD48 were described [252]. IM-9, U266 and NCI-H929 were from Jerry Pelletier, McGill University (Montreal, Quebec, Canada). MM1S was from American Type Culture Collection (Manassas, VA). OPM2 and KMS-12-PE were from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Isolation of NK cells from mice primed with polyI:C and generation of interleukin-2 (IL-2)-expanded mouse NK cells was described [252, 263]. YT-S expressing wild-type (WT) or mutant mSLAMF7, human (h) SLAMF7 or hEAT-2, were reported [252], [254]. HeLa or DT40 expressing green fluorescent protein (GFP) alone or in combination with SLAMF7 were produced by retroviral infection, as detailed [252]. SHIP-1-deficient DT40 was reconstituted with mSHIP-1, as outlined [37]. For expression of EGFR-CD45, OPM2 and MM1S were electroporated with pAW-EGFR-CD45 [264] (provided by Arthur Weiss, UCSF, CA), and selected in G418-containing medium. Cells expressing EGFR-CD45 were sorted using anti-EGFR antibody. Cos-1 transfections were performed as detailed [265].

Antibodies. Antibodies recognizing m2B4 (eBio244F4), mCD48 (HM48-1), mNKG2D (CX5), mDNAM-1 (10E5), mCD45 (30F11), h2B4 (C1.7), hNTB-A (NT-7), hNKp46 (9E2), hCD45 (2D1 and HI30), hCD84 (CD84.1.21), hLy-9 (HLy9.1.25) or hCD148 were from eBioscience (San Diego, CA) or BioLegend (San Diego, CA). Anti-hEGFR antibody was from BD

Biosciences (Mississauga, Ontario, Canada). Mouse anti-chicken IgM (M-1) antibody was from Southern Biotech (Birmingham, AL). Polyclonal rabbit anti-rat IgG, rabbit anti-mIgG and rabbit anti-hIgG antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Antibodies recognizing mSLAMF7 (4G2), mCD84 (1D3), mLy-9 (Ly9.2), mLy108 (3E11), mSLAM (12F12), phosphotyrosine (4G10), hEAT-2 (10F7), mEAT-2 (8F12) and SAP (12C4) were produced as reported [252, 254, 263]. Hybridomas producing anti-hSLAMF7 (162) and anti-hDNAM-1 (11A8) were provided by Marco Colonna, Washington University (St. Louis, MO) [253, 266]. Elotuzumab (HuLuc63) was provided by Bristol-Myers Squibb (Lawrenceville, NJ) [251]. Polyclonal rabbit antibodies recognizing SHIP-1, Shc, Vav-1, Fyn, Lyn, Src, Lck, Syk, ZAP-70 and c-Cbl were produced in our laboratory. Flow cytometry was performed using a BD BioSciences FACSCalibur flow cytometer and CellQuest software (BD BioSciences). Immunoprecipitations and immunoblots were performed as detailed [267].

Antibody-mediated stimulation. For immunoblot analyses, $5x10^6$ cells were incubated with 6 μg anti-SLAMF7 antibody for 5 minutes at room temperature (RT). After washing, cells were stimulated with 10 μg of the relevant secondary antibody for the indicated periods of time at 37°C. After lysis, SLAMF7 and SHIP-1 were immunoprecipitated. Unstimulated cells were processed similarly, except that the primary antibody was added after cell lysis. For inhibition of Src kinases, cells were incubated with PP2 (dissolved in dimethysulfoxide (DMSO); and purchased from Calbiochem, Darmstadt, Germany) for 1 hour at 37°C, and then stimulated as outlined above. To study Ca^{++} fluxes, cells were loaded with Indo-1 (Invitrogen, Burlington, Ontario, Canada) for 20 minutes at 37°C. After washing, $2x10^6$ cells were incubated with 2 μg of anti-BCR antibody, with or without 2 μg anti-SLAMF7 antibody, for 5 minutes at RT, and then loaded on BD LSR cell analyzer. After adding the relevant secondary antibody, changes in

intracellular Ca^{++} were monitored over time at 37°C using the Fluo-4 (FL4) and Fluo-5 (FL5) ratio. Control cells were stimulated with only the secondary antibody.

NK cell cytotoxicity. NK cell-mediated cytotoxicity was assayed as detailed [263]. Duplicates were used in each experiment.

RNA interference. Scrambled siRNA (1027280) and SHIP-1-specific siRNAs (SI00078575, SI03029005) were from Qiagen (Valencia, CA). Transfection of YT-S cells was performed using Amaxa Cell Line Nucleofector Kit R (VCA-1001) from Lonza (Basel, Switzerland), according to the manufacturer's protocol and the program O-017. Transfected cells were grown for 36-48 hours and processed for cytotoxicity or immunoblot assays.

RT-PCR. RNA was purified with TRIzol (Invitrogen) according to the manufacturer's protocol, and reverse transcribed into cDNA using random primers and Superscript II (Invitrogen). PCR reactions were performed using single-stranded cDNAs, gene-specific primers and Taq polymerase (Invitrogen). The primers to distinguish the human SLAMF7 isoforms were: CS1 F727 (5-TCTCTTTGTACTGGGGCTATTTC-3) and CS1 R955 (5-TTTTCCATCTTTTTCGGTATTT-3), as described [268]. The primers to detect human GAPDH were: 5-AGGTCGGAGTCAACGGATTTG-3, 5-GTGATGGCATGGACTGTGGT-3.

Statistical analysis and quantitation. Unpaired Student's *t* tests (two-tailed) were performed using the Prism software program. Bands in autoradiograms were quantified with the Image J software program.

RESULTS

SLAMF7-mediated inhibition in NK cells is accompanied by tyrosine phosphorylation of SHIP-1

It was proposed that inhibition by SLAM family receptors might be mediated by various effectors, including SHP-1, SHP-2, SHIP-1 and Csk [269, 270]. To identify the effectors of SLAMF7-mediated inhibition, we used the human NK cell line YT-S, ectopically expressing or not WT mSLAMF7 (Figure 3.1A). As YT-S lacks EAT-2, SLAMF7 is inhibitory in these cells [252]. Since SLAM family signaling is initiated by protein tyrosine phosphorylation [37, 252, 263, 269, 271, 272], we focused on tyrosine phosphorylation signals (Figure 3.1B). Engagement of SLAMF7 by anti-SLAMF7 antibodies resulted in tyrosine phosphorylation of two polypeptides of 55 and 145 kilodaltons in total cell lysates. These effects were not seen in YT-S cells lacking SLAMF7.

Based on their apparent masses, p55 and p145 were consistent with SLAMF7 and SHIP-1, respectively. To test this idea, immunoprecipitation experiments were conducted (Figure 3.1C). Although SHIP-1 was not detectably tyrosine phosphorylated in unstimulated YT-S cells, it became tyrosine phosphorylated following engagement of SLAMF7. Likewise, although some tyrosine phosphorylation of SLAMF7 was detected in unstimulated YT-S cells, this phosphorylation was augmented in stimulated cells. Vav-1, a positive regulator of NK cell activation [37], did not undergo tyrosine phosphorylation upon SLAMF7 triggering. SLAMF7 migrated as a broad band or multiple bands in protein gels, presumably reflecting differential glycosylation.

Similar experiments were performed using NK cells from WT or EAT-2-deficient mice (Figure 3.1D). While stimulation of SLAMF7 on WT NK cells increased SHIP-1 tyrosine

phosphorylation, this increase was more prominent in EAT-2-deficient cells. By opposition, tyrosine phosphorylation of SLAMF7 was equivalent, in keeping with the report that EAT-2 is not required for SLAMF7 tyrosine phosphorylation [252].

Thus, an increase in SLAMF7-mediated inhibition was consistently paralleled by an enhancement of SLAMF7-triggered tyrosine phosphorylation of SHIP-1.

SHIP-1 is critical for SLAMF7-mediated inhibition

To examine if SHIP-1 was critical for inhibition, the impact of down-regulating SHIP-1 expression in YT-S cells was tested (Figure 3.2A-C). YT-S cells expressing mSLAMF7 were independently transfected with two small interfering (si) RNAs against SHIP-1, or irrelevant siRNAs. SHIP-1-specific siRNAs resulted in a ~80% reduction of SHIP-1 levels (Figure 3.2A). Cells were then tested for their ability to kill HeLa targets, expressing or not the ligand of SLAMF7, i.e. SLAMF7 itself. SHIP-1-specific siRNAs resulted in an increased ability to kill HeLa (Figure 3.2B). In keeping with the involvement of SHIP-1 in multiple inhibitory pathways [37, 273, 274], this effect was observed whether HeLa expressed or not SLAMF7. Nonetheless, calculation of the extent of inhibition conferred by expression of SLAMF7 on targets showed that SLAMF7-mediated inhibition was attenuated by loss of SHIP-1 (Figure 3.2C). This finding represented the first direct genetic evidence for a specific effector of SLAM family receptor-mediated inhibition in an immune cell function.

We also tested the role of SHIP-1 using the DT40 system. DT40 is a chicken B cell line having variants lacking SHIP-1 or other inhibitory effectors such as SHP-1, SHP-2 or Csk [275]. It lacks EAT-2 [37]. Therefore, parental or mutant DT40 cells expressing mSLAMF7 were generated. These cells expressed equivalent amounts of SLAMF7 and B cell antigen receptor

(BCR) (data not shown).

As engagement of SLAMF7 resulted in inhibition of BCR-triggered Ca^{++} fluxes in DT40 (data not shown), we focussed our studies on Ca^{++} fluxes. Contrary to parental DT40, cells lacking SHIP-1 displayed a near abolition of the ability of SLAMF7 to inhibit BCR-evoked Ca^{++} fluxes (Figure 3.2D). Cells devoid of Csk, or SHP-1 and SHP-2, had no defect. A quantitation of the extent of inhibition is depicted in Figure 3.2E. To confirm that this defect was due to lack of SHIP-1, SHIP-1-deficient cells were reconstituted with SHIP-1 (Figure 3.2F; data not shown). Re-expression of SHIP-1 restored SLAMF7-mediated inhibition (Figure 3.2G and H).

SLAMF7 contains two conserved intra-cytoplasmic tyrosines, Y261 and Y281. Y261 is critical for SLAMF7-mediated inhibition when NK cells lack EAT-2, whereas Y281 is needed for SLAMF7-mediated activation when NK cells express EAT-2 [252]. Therefore, the roles of Y261 and Y281 in tyrosine phosphorylation of SHIP-1 were also assessed (Figure 3.2I). Engagement of SLAMF7 resulted in robust tyrosine phosphorylation of SHIP-1 in cells expressing WT or tyrosine 281-to-phenylalanine 281 (Y281F), but not Y261F, SLAMF7. Mutation of either Y261 or Y281 resulted in reduced SLAMF7 tyrosine phosphorylation, implying that both sites contributed to SLAMF7 phosphorylation. Hence, Y261, but not Y281, was needed for tyrosine phosphorylation of SHIP-1 in response to SLAMF7 engagement.

Therefore, SHIP-1, but not SHP-1, SHP-2 or Csk, was responsible for SLAMF7-mediated inhibition.

Src kinases are critical for SLAMF7-induced inhibitory signals

The kinase(s) responsible for initiation of SLAMF7-induced tyrosine phosphorylation was(were) identified. Using transient transfection in Cos-1 cells, we found that the Src family kinases Fyn,

Lyn and Src triggered an increase in SLAMF7 tyrosine phosphorylation (Figure 3.3A). No effect was seen with Lck, Syk and ZAP-70. We also examined the impact of a pharmacological inhibitor of Src kinases, PP2, in YT-S (Figure 3.3B). PP2 completely eliminated the ability of SLAMF7 to evoke tyrosine phosphorylation of SHIP-1 in these cells. A full effect was seen at 5 mM. PP2 also caused a reduction of SLAMF7 tyrosine phosphorylation. Although only a minimal effect was seen at 5 mM, a near complete elimination was seen at 50 mM.

We also tested the roles of various PTKs in the DT40 system, by expressing hSLAMF7 in DT40 variants lacking the kinases Lyn (the only Src kinase in DT40), Btk or Csk. All cells expressed equivalent amounts of hSLAMF7 (data not shown). Stimulation with anti-SLAMF7 MAb revealed that tyrosine phosphorylation of SLAMF7 and SHIP-1 was eliminated in cells lacking Lyn, but not in cells lacking Btk or Csk.

Thus, Src kinases, in particular Fyn and Lyn, were responsible for SLAMF7-triggered protein tyrosine phosphorylation.

Elotuzumab fails to trigger tyrosine phosphorylation of SHIP-1 in multiple myeloma cells

MM cells express high levels of SLAMF7, but lack EAT-2 [219, 249, 251, 255, 260] (Figure 3.4A,B). Hence, we tested if SLAMF7-mediated inhibitory signals identified in EAT-2-negative NK cells also occurred in MM cells. Several human MM cell lines were stimulated with the therapeutic anti-SLAMF7 MAb elotuzumab and SHIP-1 tyrosine phosphorylation was examined (Figure 3.4C and D). IM-9, an Epstein-Barr virus-transformed B cell line also expressing SLAMF7 but lacking EAT-2 was used as control.

Elotuzumab triggered prominent tyrosine phosphorylation of SHIP-1 in IM-9 cells (Figure 3.4C). However, it failed to evoke a similar response in MM cells, although SHIP-1 was

constitutively tyrosine phosphorylated in some cell lines (in particular U266). Similar findings were made with anti-SLAMF7 MAb 162 (Figure 3.4D). In contrast to the defect in SLAMF7-evoked SHIP-1 tyrosine phosphorylation, there was a more variable compromise in tyrosine phosphorylation of SLAMF7 (Figure 3.4D). The latter was severely reduced in KMS-12-PE, NCI-H929, OPM2 and MM1S, but not in U266.

Hence, unlike NK cells and the B cell line IM-9, MM cells had a defect in SLAMF7-evoked tyrosine phosphorylation of SHIP-1 and, to a lesser extent, SLAMF7.

Multiple myeloma cells express the long isoform of SLAMF7 and Src kinases, but lack the phosphatase CD45

We tested whether any of the intermediates linking SLAMF7 to SHIP-1 in NK cells was defective in MM cells (Figure 3.5). Human SLAMF7 exists as two isoforms produced by alternative splicing: the long isoform (SLAMF7-L), which contains all tyrosines including Y261, and the short isoform (SLAMF7-S), which bears an alternative intra-cytoplasmic domain lacking Y261 [268, 276, 277]. In DT40, SLAMF7-L, but not SLAMF7-S, was inhibitory (data not shown). Therefore, we ascertained whether MM cells expressed the long isoform of SLAMF7. RT-PCR analyses showed that, like YT-S and IM-9, all MM cells predominantly expressed SLAMF7-L (Figure 3.5A). We also verified expression of Fyn and Lyn, which were most effective at triggering SLAMF7 tyrosine phosphorylation in Cos-1 cells (Figure 3.3A). Immunoblot analyses showed that all MM cells contained Fyn and Lyn (Figure 3.5B). Of note, however, levels of Fyn tended to be lower in MM cells, in comparison to IM-9 cells.

These data suggested that the activity, rather than the expression, of Src kinases might be defective in MM cells. In B cells, activation of Src kinases requires two transmembrane protein

tyrosine phosphatases (PTPs), CD45 and CD148 [278-280]. These PTPs dephosphorylate the inhibitory tyrosine of Src kinases. Whereas IM-9 expressed both CD45 and, to a lesser extent, CD148, MM cells expressed CD148, but not CD45 (Figure 3.5C). The only exception was U266, which contained both CD45-positive and CD45-negative populations. It should also be noted that, whereas MM cells expressed CD148, levels were lower than in IM-9 cells.

Therefore, the defect in SLAMF7-triggered tyrosine phosphorylation of SHIP-1 and, to a lesser extent, SLAMF7 in MM cells correlated at least in part with lack of CD45.

CD45 is required for SLAMF7-triggered tyrosine phosphorylation of SHIP-1

To assess if lack of CD45 was responsible for defective SLAMF7-evoked protein tyrosine phosphorylation in MM cells, two approaches were taken (Figure 3.6). Firstly, limiting dilution was used to generate CD45-positive and CD45-negative variants of U266, which contains a mixture of the two populations (Figure 3.6A). Stimulation of a CD45-negative variant of U266 with anti-SLAMF7 resulted in minimal tyrosine phosphorylation of SHIP-1 and SLAMF7 (Figure 3.6B). In contrast, triggering of a CD45-positive variant resulted in prominent tyrosine phosphorylation of SHIP-1 and SLAMF7. Intermediate responses were observed in a variant containing CD45-positive and CD45-negative cells. This variant also expressed lower amounts of SLAMF7.

Secondly, expression of CD45 was reconstituted in the CD45-negative cell lines OPM2 and MM1S (Figure 3.6C-H). As full-length CD45 is notoriously difficult to express in transfected cells, we used a chimeric CD45 in which the extracellular and transmembrane domains of the epidermal growth factor receptor (EGFR) are fused to the CD45 cytoplasmic region, which contains the phosphatase domain [264]. After transfection with a cDNA encoding EGFR-CD45,

~50% of OPM2 and ~80% of MM1S expressed the chimeric receptor (Figure 3.6C and F). Expression of EGFR-CD45 in OPM2 and MM1S resulted in a marked increase in SHIP-1 tyrosine phosphorylation in response to SLAMF7 stimulation by MAb 162 or elotuzumab (Figure 3.6D,E,G and H). A smaller effect was seen on tyrosine phosphorylation of SLAMF7.

Thus, lack of CD45 was responsible at least in part for the defect in SHIP-1 tyrosine phosphorylation upon SLAMF7 engagement in MM cells. Absence of CD45 also reduced the ability to evoke tyrosine phosphorylation of SLAMF7, although this effect was less pronounced.

CD45 is required for the function of SLAMF7 in NK cells

To obtain evidence that CD45 was needed for SLAMF7 function, we tested the impact of CD45 on the ability of SLAMF7 to control NK cell activation, using NK cells from CD45-deficient mice. Since these mice express EAT-2, the activating function of SLAMF7 was analyzed (Figure 3.7). It was not possible to analyze the inhibitory function of SLAMF7 using NK cells lacking both CD45 and EAT-2, since the mice lacking CD45 or EAT-2 suitable for these studies were generated in a different genetic background (C57BL/6 and 129/Sv, respectively). NK cell functions are drastically different between the two strains.

As reported elsewhere [281-283], lack of CD45 had little impact on NK cell development (data not shown). Moreover, it had no effect on expression of SLAMF7, the activating NK cell receptors NKG2D and DNAM-1, or the effectors of SLAMF7 signaling PLC-γ2 and SHIP-1 (Figure 3.7A,B). Rather, expression of EAT-2 was enhanced in CD45-deficient NK cells. Paradoxically, expression of the SLAM family receptors 2B4 and CD84 was reduced. The basis for the latter alterations remains to be clarified.

NK cells were incubated with B16 melanoma cells, expressing or not SLAMF7, and

cytotoxicity was measured (Figure 3.7C). B16 cells expressing CD48, the ligand of 2B4, were analyzed as control. Unlike SLAMF7, 2B4 mediates its activating function through EAT-2 and its relative SAP [263]. NK cells lacking CD45 displayed reduced killing of B16 lacking SLAMF7 or CD48. Since cytotoxicity towards B16 is largely mediated by DNAM-1 [284-286], this finding suggested that the function of DNAM-1 might be compromised. More importantly, enhancement of cytotoxicity caused by expression of SLAMF7 on B16 was reduced in CD45-deficient NK cells. This was not the case for expression of CD48 on B16 that still augmented cytotoxicity by CD45-deficient NK cells. A quantitation of these findings is shown in Figure 3.7D.

Hence, CD45 was required for the activating function of SLAMF7, but not of 2B4, in NK cells.

DISCUSSION

Although the ability of SLAM family receptors, including SLAMF7, to mediate inhibitory effects in immune cells is well established, the molecular mechanism triggering these effects is largely undetermined. To identify the effectors of SLAMF7-mediated inhibition, we first used the human EAT-2-negative NK cell line YT-S. Stimulation of SLAMF7 on YT-S with anti-SLAMF7 resulted in tyrosine phosphorylation of two proteins, SLAMF7 and SHIP-1. Tyrosine phosphorylation of SHIP-1 was also observed upon triggering of SLAMF7 on mouse NK cells, and this effect was enhanced in EAT-2-deficient mice. Thus, SLAMF7 was coupled to SHIP-1 in NK cells and this effect was enhanced in cells lacking EAT-2.

Several findings firmly supported the idea that SHIP-1 was the critical effector of SLAMF7-mediated inhibition. First, down-regulation of SHIP-1 expression using siRNAs attenuated SLAMF7-mediated inhibition in YT-S. Second, the ability of SLAMF7 to inhibit BCR-triggered Ca^{++} fluxes in DT40 was eliminated in cells lacking SHIP-1, but not SHP-1 and SHP-2 or Csk. This defect was corrected by re-introduction of SHIP-1. And third, the tyrosine required for SLAMF7-mediated inhibition in YT-S, Y261, was needed for coupling to SHIP-1.

Although these data implied that SHIP-1 was the inhibitory effector of SLAMF7 in EAT-2-negative NK cells, this may not be true for all SLAM receptors or all cell types. In studies using the heterologous system DT40, it was suggested that the inhibitory effectors of 2B4 were SHIP-1 and, to a lesser extent, SHP-1 and SHP-2 [37]. Conversely, experiments with a pharmacological inhibitor suggested that the inhibitory function of SLAMF6 (Ly108) in $CD8^{+}$ T cells and NK-T cells is SHP-1 [234, 236]. Hence, the inhibitory effectors of SLAM receptors may vary from one family member to the other or from one cell type to the other.

In YT-S, Y261, but not Y281, of SLAMF7 was needed for SLAMF7-triggered tyrosine

phosphorylation of SHIP-1. As SHIP-1 contains an SH2 domain, one scenario is that, upon SLAMF7 engagement, phosphorylated Y261 recruits SHIP-1 via its SH2 domain. Intriguingly, however, a synthetic SLAMF7 peptide phosphorylated at Y261 did not interact with SHIP-1 *in vitro* (data not shown), suggesting that SLAMF7 does not bind directly to SHIP-1. Possibly, an intermediate molecule, such as another adaptor like Grb2, Grap or Shc, mediates binding of SLAMF7 to SHIP-1. Alternatively, Y261 may recruit the PTK phosphorylating SHIP-1.

To understand further how SLAMF7 coupled to its inhibitory effector, we elucidated the PTKs responsible for SLAMF7 tyrosine phosphorylation. Co-transfection experiments in Cos-1, experiments with DT40 lacking PTKs and studies with pharmacological inhibitors indicated that Src kinases, in particular Fyn and Lyn, were responsible for tyrosine phosphorylation of SLAMF7. No effect was observed with Lck, ZAP-70, Syk, Btk or Csk. Therefore, Src kinases initiated SLAMF7-mediated inhibitory signaling. Since SLAMF7 tyrosine phosphorylation also triggers the activating function of SLAMF7 [252, 253], Src kinases are probably required as well for initiation of SLAMF7-mediated activation.

Although MM cells lack EAT-2 [219], engagement of SLAMF7, including by elotuzumab, failed to trigger tyrosine phosphorylation of SHIP-1. Tyrosine phosphorylation of SLAMF7 was also partially reduced. This was in contrast to the EBV-transformed B cell line IM-9, in which prominent tyrosine phosphorylation of SHIP-1 and SLAMF7 was evoked. The defect in SHIP-1 tyrosine phosphorylation in MM cells was not due to lack of SLAMF7-L, Fyn and Lyn, or SHIP-1. Rather, it correlated with lack of CD45, a PTP required for Src kinase activation in hematopoietic cells. Whereas we documented lack of EAT-2 in all MM cell lines tested including U266, it should be noted that another group stated that U266 expressed EAT-2, although the data to that effect were not reported [219, 249, 251, 255, 260]. The basis for this

distinction is not known, but could relate to clonal variation between the U266 lines used.

Subcloning of CD45-positive and CD45-negative variants of U266, as well as transfection of EGFR-CD45 in CD45-negative OPM2 and MM1S, indicated that absence of CD45 explained at least in part the defect in SLAMF7 signaling in MM cells. In all likelihood, CD45 was needed for activation of Src kinases in these cells. However, CD45 had a more variable impact on tyrosine phosphorylation of SLAMF7. Perhaps, the pool of Src kinases responsible for SLAMF7 phosphorylation was less dependent on CD45 for activation.

Elotuzumab mediates its anti-MM effect by activating immune cells like NK cells, rather than by having direct effects on MM cells [219, 251, 255]. Our finding that elotuzumab failed to trigger inhibitory signaling in MM cells might explain at least in part why elotuzumab has no direct effect in these cells. Nonetheless, it should be mentioned that EGFR-CD45 was unable to render MM cells susceptible to a direct anti-tumor effect from elotuzumab (data not shown). Possibly, the levels of CD45 achieved by EGFR-CD45 were insufficient. Alternatively, additional defects might contribute to the resistance of MM cells to a direct effect from elotuzumab. The lower levels of CD148 and Fyn observed in these cells could contribute.

MM cells, both primary samples from patients and cell lines, frequently lack CD45 [287, 288]. This is also true for normal plasma cells. Compared to CD45-positive MM cells, CD45-negative MM cells have been linked to disease progression and poor treatment response [289]. Xenotransplantation experiments also suggested that CD45-negative MM cells contained a greater proportion of long-lived and/or myeloma-initiating cells [290]. Interestingly, these cells tended to have higher PI3'K activity [291]. Considering our finding that SLAMF7-mediated inhibition involved SHIP-1, a negative regulator of PI3'K, it is tempting to speculate that increased PI3'K activity in MM cells lacking CD45 was due at least in part to reduced

SLAMF7-dependent activation of SHIP-1.

A clear impact of CD45 on SLAMF7 function was documented in mouse NK cells. Whereas SLAMF7 was activating in WT NK cells, this activating effect was lost in CD45-deficient NK cells. This was presumably because CD45-dependent activation of Src kinases was needed for tyrosine phosphorylation of SLAMF7 or, possibly, EAT-2 and PLC-γ2. Surprisingly, this was not the case for another SLAM receptor, 2B4, which was still activating in CD45-deficient NK cells. Perhaps, this distinction relates to the fact that 2B4 is also coupled to SAP [37], which binds Fyn via the Fyn SH3 domain and, thus, may activate Fyn in a CD45-independent manner [269].

In summary, our data indicated that SLAMF7-mediated inhibition in EAT-2-negative NK cells was mediated by the lipid phosphatase SHIP-1. SLAMF7 was coupled to SHIP-1 by way of Y261, which was phosphorylated by Src family kinases. This mechanism was defective in MM cells, at least in part due to a lack of PTP CD45, which is needed for activation of Src kinases in hematopoietic cells and for initiation of SLAMF7 inhibitory signaling. In addition to clarifying the mechanism of inhibition by SLAMF7 and, possibly, other SLAM family receptors, these data help understand why elotuzumab has no direct effect on MM cells, but rather causes elimination of these cells by activating normal immune cells such as NK cells.

ACKNOWLEDGMENTS

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Figure Legends

Figure 3.1. SHIP-1 is tyrosine phosphorylated in response to engagement of SLAMF7. A-C. YT-S cells were transfected with a vector encoding the puromycin resistance marker (puro) alone or in combination with mouse (m) SLAMF7. Polyclonal populations were used for analyses. Expression of SLAMF7, 2B4, NTB-A, DNAM-1 and NKp46 (open black lines) was analyzed by flow cytometry (A). Isotype controls are shown as filled grey lines. Representative of n=3. Cells were stimulated or not for 3 minutes with anti-SLAMF7 MAb 4G2 and rabbit anti-rat IgG. After lysis, overall protein tyrosine phosphorylation was determined by immunoblotting of total cell lysates with anti-phosphotyrosine (a-p-Tyr) antibodies (B). The positions of prestained molecular mass markers are shown on the right. Tyrosine phosphorylation of SHIP-1, SLAMF7 and Vav-1 was determined by immunoprecipitating the indicated proteins with specific antibodies and subsequent immunoblotting with a-p-Tyr antibodies (C). The abundance of SHIP-1, SLAMF7 and Vav-1 in the immunoprecipitates was verified by probing parallel immunoprecipitates or reprobing the immunoblot membrane with substrate-specific antibodies. It is noteworthy that the amount of SLAMF7 recovered from cells stimulated with anti-SLAMF7 was frequently lower than that obtained from unstimulated cells. This was due to the fact that a proportion of SLAMF7 becomes detergent-insoluble upon SLAMF7 stimulation with antibodies. The position of the heavy chain of IgG, which is recognized by the immunoblotting antibodies, is shown by an arrowhead on the left. Representative of n=3. D. NK cells from wild-type (WT) or EAT-2-deficient (knock-out; KO) mice were stimulated for 3 minutes with anti-SLAMF7, and tyrosine phosphorylation of SHIP-1 and SLAMF7 was analyzed as detailed for panels A-C. A quantitation of the relative tyrosine phosphorylation of SHIP-1 is shown. Representative of n=2.

Figure 3.2. SHIP-1 is required for SLAMF7-mediated inhibition in NK cells and DT40 B cells. A-C. Expression of SHIP-1 in YT-S cells expressing mouse (m) SLAMF7 was down-regulated using two different SHIP-1-specific small interfering (si) RNAs, #1 and #2. Expression of SHIP-1 was determined by immunoblotting of total cell lysates with anti-SHIP-1 antibodies (A). Vav-1 was used as loading control. For cells transfected with scrambled siRNAs, various amounts of lysates were loaded to confirm linearity of the immunoblot assays. Cytotoxicity towards HeLa target cells, expressing green fluorescent protein (GFP) alone or in combination with mSLAMF7, was determined using a ^{51}Cr release assay (B). The effector-to-target ratios (E:T) are shown at the bottom. Average values of duplicates are shown, while error bars depict standard deviations. Representative of at least n=3. Percentage of decreased killing was calculated as ((cytotoxicity towards GFP^+ targets - cytotoxicity towards $SLAMF7^+$ targets)/ cytotoxicity towards GFP^+ targets)) x 100 at the 25:1 E:T ratio (C). Average values from 3 independent experiments with standard deviations are represented. *: $p<0.05$. D,E. Wild-type (WT) DT40 cells or variants lacking the indicated inhibitory molecules were transduced with a cDNA encoding mouse SLAMF7. Cells were loaded with the Ca^{++} indicator dye Indo-1, and then stimulated with anti-B cell receptor (a-BCR) antibodies alone or in combination with anti-SLAMF7. Changes in intracellular Ca^{++} were analyzed over time by determining the FL4/FL5 ratio using flow cytometry (D). Arrows indicate the time at which receptor cross-linking was triggered. Stimulation with only the secondary antibody was used as control. A quantitation of inhibition of Ca^{++} levels in cells stimulated with anti-BCR plus anti-SLAMF7, in comparison to anti-BCR alone, at different times is represented graphically (E). Representative of n=3. F-H. SHIP-1-deficient DT40 were transfected with a plasmid encoding

the puromycin resistance marker (puro) alone or in combination with mouse (m) SHIP-1. SHIP-1 expression was determined by immunoblotting of total cell lysates with anti-SHIP-1 antibodies (F). Ca^{++} fluxes were analyzed as detailed for panels D and E (G,H). I. YT-S cells expressing WT, tyrosine 261-to-phenylalanine 261 (Y261F) or Y281F SLAMF7 were stimulated with anti-SLAMF7. Tyrosine phosphorylation of SHIP-1 and SLAMF7 was examined as detailed for Figure 3.1C. A quantitation of the relative tyrosine phosphorylation of SHIP-1 is shown. Representative of n=3.

Figure 3.3. Src family kinases are responsible for SLAMF7-dependent tyrosine phosphorylation of SHIP-1. A. Cos-1 cells were transiently transfected with cDNAs encoding the indicated protein tyrosine kinases, without or with SLAMF7. Tyrosine phosphorylation of SLAMF7 was determined by immunoblotting of SLAMF7 immunoprecipitates with anti-phosphotyrosine antibodies. A quantitation of the relative tyrosine phosphorylation of SLAMF7 is shown. Representative of n=3. B. YT-S cells expressing mSLAMF7 were pretreated with the indicated concentrations of PP2, a Src family kinase inhibitor, or with vehicle (dimethylsulfoxide; DMSO) alone. Cells were then stimulated with anti-SLAMF7 and tyrosine phosphorylation of SHIP-1 and SLAMF7 was determined as detailed for Figure 3.1C. A quantitation of the relative tyrosine phosphorylation of SLAMF7 is shown. Representative of n=2. C. Wild-type (WT) or kinase-deficient DT40 cells expressing SLAMF7 were stimulated or not with anti-SLAMF7 antibody. Tyrosine phosphorylation of SHIP-1 and SLAMF7 was analyzed as detailed for Figure 3.1C. Representative of at least n=5.

Figure 3.4. Engagement of SLAMF7 on multiple myeloma cells fails to induce tyrosine

phosphorylation of SHIP-1 and, to a lesser extent, SLAMF7. A,B. Expression of SLAMF7, EAT-2 and SHIP-1 in several multiple myeloma (MM) cell lines, in addition to YT-S cells ectopically expressing human (h) SLAMF7 or hEAT-2 and IM-9 cells, was examined by flow cytometry (A) or immunoblotting of total cell lysates (B). Staining with anti-SLAMF7 is depicted as open black lines, whereas staining with isotype controls is shown as filled grey lines (A). Representative of at least n=3. C,D. Cells were stimulated with elotuzumab (C) or anti-SLAMF7 MAb 162 (D), followed by the relevant secondary antibody. Tyrosine phosphorylation of SHIP-1 and SLAMF7 was determined as outlined for Figure 3.1C. Note that, as elotuzumab is a humanized antibody, it was inefficient at immunoprecipitating SLAMF7 with the currently available secondary reagents. As a result, SLAMF7 could be efficiently immunoprecipitated only from cells stimulated with MAb 162. The differences in electrophoretic mobility of SLAMF7 between cell lines were presumably due to differential glycosylation. Quantitations of the relative tyrosine phosphorylation of SHIP-1 (C,D) and SLAMF7 (D) are shown. Representative of at least n=3.

Figure 3.5. Multiple myeloma cells express the long isoform of SLAMF7 and Src family kinases, but not CD45. A. Cells were examined for expression of the long (SLAMF7-L), short (SLAMF7-S) isoforms of SLAMF7 and GAPDH using RT-PCR, as detailed in Methods. DT40 transfectants expressing either SLAMF7-L or SLAMF7-S, and YT-S transfectants expressing SLAMF7-L, were used as controls. Representative of n=3. B. Expression of Src family kinases Fyn and Lyn was analyzed in cells shown in (A). It should be noted that Lyn exists as two isoforms, p53 and p56, which are generated by alternative splicing. Representative of at least n=3. C. Expression of CD45 and CD148 was examined on cells shown in (A). Staining with

anti-CD45 or anti-CD148 is depicted as open black lines, whereas staining with isotype controls is shown as filled grey lines. Representative of n=3.

Figure 3.6. CD45 is critical for SHIP-1 tyrosine phosphorylation in multiple myeloma cells. A,B. Sub-clones of U266 were tested for CD45 and SLAMF7 expression (A). Staining with anti-CD45 or anti-SLAMF7 is depicted as open black lines, whereas staining with isotype controls is shown as filled grey lines. Cells were then stimulated with anti-SLAMF7 MAb 162, and tyrosine phosphorylation of SHIP-1 and SLAMF7 was tested as detailed for Figure 3.1C (B). A quantitation of the relative tyrosine phosphorylation of SHIP-1 is shown. Representative of n=2. C-H. (C-E) OPM2 cells or MM1S cells (F-H) were stably transfected with a vector encoding the epidermal growth factor receptor (EGFR)-CD45 chimera or empty vector. Expression of surface EGFR and SLAMF7 was detected by flow cytometry (C,F). Staining with anti-EGFR or anti-SLAMF7 is depicted as open black lines, whereas staining with isotype controls is shown as filled grey lines (A). Cells were then stimulated for the indicated times with anti-SLAMF7 MAb 162 (D,G) or elotuzumab (Elo; E,H), and tyrosine phosphorylation of SHIP-1 and SLAMF7 was tested as detailed for Figure 3.1C. It is noteworthy that the amount of SLAMF7 recovered from cells stimulated with anti-SLAMF7 was lower than that obtained from unstimulated cells. This was due to the fact that a proportion of SLAMF7 becomes detergent-insoluble upon SLAMF7 stimulation with antibodies. Quantitations of the relative tyrosine phosphorylation of SHIP-1 are shown (D,E,G,H). Representative of n=4.

Figure 3.7. CD45 is required for the function of SLAMF7. A,B. NK cells from spleen of wild-type (WT) or CD45-deficient (knock-out; KO) mice were analyzed for expression of

various cell surface molecules by flow cytometry (A). Staining with specific antibodies is depicted as open black lines, whereas staining with isotype controls is shown as filled grey lines. They were also assessed for expression of several intracellular signalling molecules, by immunoblotting of total cell lysates with the indicated antibodies (B). A quantitation of the relative expression of EAT-2 is shown. Representative of n=3 (A,B). C. NK cells from the indicated mice were tested for their ability to kill B16 melanoma cells expressing the puromycin resistance marker (puro) alone or in combination with SLAMF7 or CD48, as described for Figure 3.2B. The effector-to-target ratios (E:T) are shown at the bottom. Average values of duplicates are shown, while error bars depict standard deviations. Representative of at least n=5. D. The enhancement of cytotoxicity by expression of the ligands of SLAMF7 or 2B4 (CD48) on targets was quantitated for multiple independent experiments. Enhanced killing was calculated as cytotoxicity towards SLAMF7 or $CD48^{+}$ targets - cytotoxicity towards puro targets at the 10:1 E:T ratio. Average values from 3 independent experiments with standard deviations are represented. **: $p<0.01$; ***: $p<0.001$.

Figures

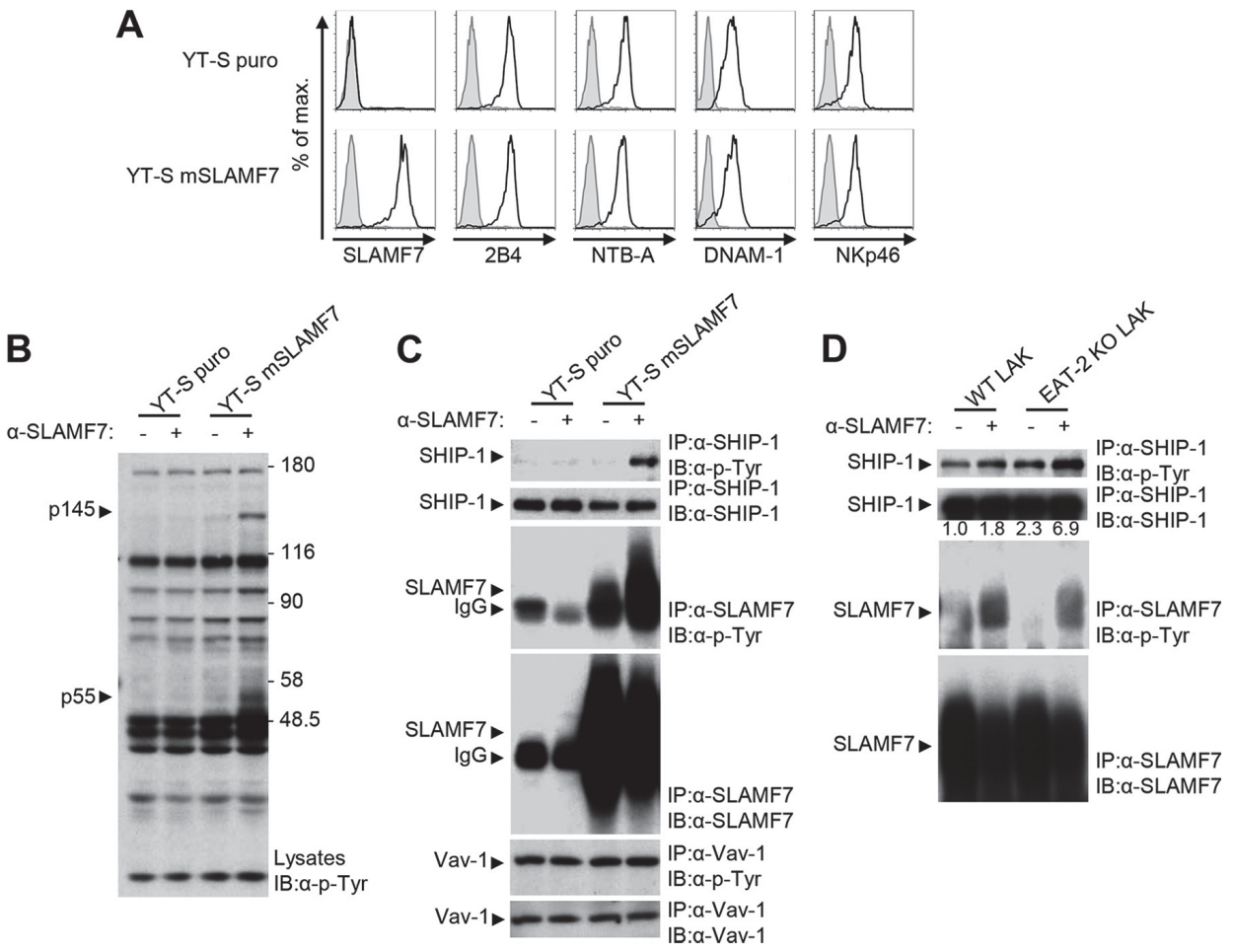

Figure 3.1

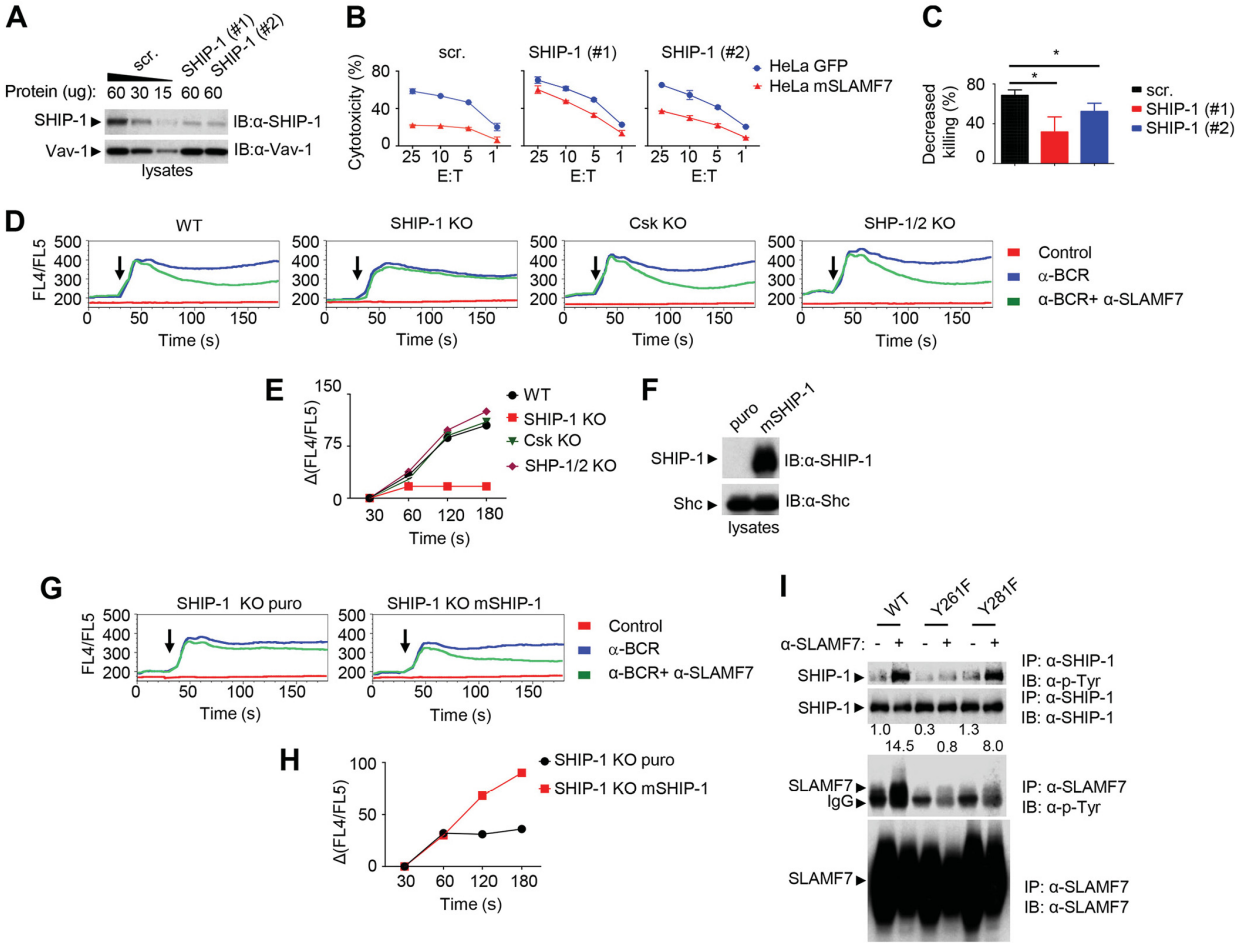
A
scr.
SHIP-1 (#1)
SHIP-1 (#2)
Protein (ug): 60 30 15 60 60
SHIP-1
IB:α-SHIP-1
Vav-1
IB:α-Vav-1
lysates
B
scr.
SHIP-1 (#1)
SHIP-1 (#2)
Cytotoxicity (%)
E:T
HeLa GFP
HeLa mSLAMF7
C
Decreased killing (%)
scr.
SHIP-1 (#1)
SHIP-1 (#2)
D
WT
SHIP-1 KO
Csk KO
SHP-1/2 KO
FL4/FL5
Time (s)
Control
α-BCR
α-BCR+ α-SLAMF7
E
Δ(FL4/FL5)
Time (s)
WT
SHIP-1 KO
Csk KO
SHP-1/2 KO
F
puro
mSHIP-1
SHIP-1
IB:α-SHIP-1
Shc
IB:α-Shc
lysates
G
SHIP-1 KO puro
SHIP-1 KO mSHIP-1
FL4/FL5
Time (s)
Control
α-BCR
α-BCR+ α-SLAMF7
H
Δ(FL4/FL5)
Time (s)
SHIP-1 KO puro
SHIP-1 KO mSHIP-1
I
WT
Y261F
Y281F
α-SLAMF7: - + - + - +
SHIP-1
IP: α-SHIP-1
IB: α-p-Tyr
SHIP-1
IP: α-SHIP-1
IB: α-SHIP-1
1.0 0.3 1.3
14.5 0.8 8.0
SLAMF7
IgG
IP: α-SLAMF7
IB: α-p-Tyr
SLAMF7
IP: α-SLAMF7
IB: α-SLAMF7

Figure 3.2

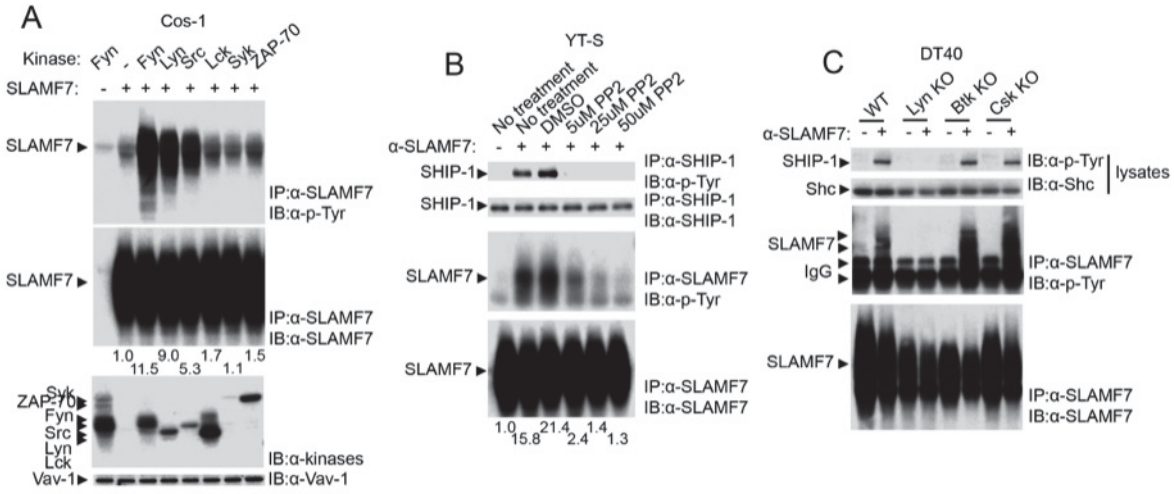
A
Cos-1
Kinase: Fyn - Fyn Lyn Src Lck Syk ZAP-70
SLAMF7: - + + + + + + +
SLAMF7
IP:α-SLAMF7
IB:α-p-Tyr
SLAMF7
IP:α-SLAMF7
IB:α-SLAMF7
1.0 9.0 1.7 1.5
11.5 5.3 1.1
Syk
ZAP-70
Fyn
Src
Lyn
Lck
IB:α-kinases
Vav-1
IB:α-Vav-1
B
YT-S
No treatment
No treatment
DMSO
5uM PP2
25uM PP2
50uM PP2
α-SLAMF7: - + + + + +
SHIP-1
IP:α-SHIP-1
IB:α-p-Tyr
SHIP-1
IP:α-SHIP-1
IB:α-SHIP-1
SLAMF7
IP:α-SLAMF7
IB:α-p-Tyr
SLAMF7
IP:α-SLAMF7
IB:α-SLAMF7
1.0 21.4 1.4
15.8 2.4 1.3
C
DT40
WT
Lyn KO
Btk KO
Csk KO
α-SLAMF7: - + - + - + - +
SHIP-1
IB:α-p-Tyr
lysates
Shc
IB:α-Shc
SLAMF7
IgG
IP:α-SLAMF7
IB:α-p-Tyr
SLAMF7
IP:α-SLAMF7
IB:α-SLAMF7

Figure 3.3

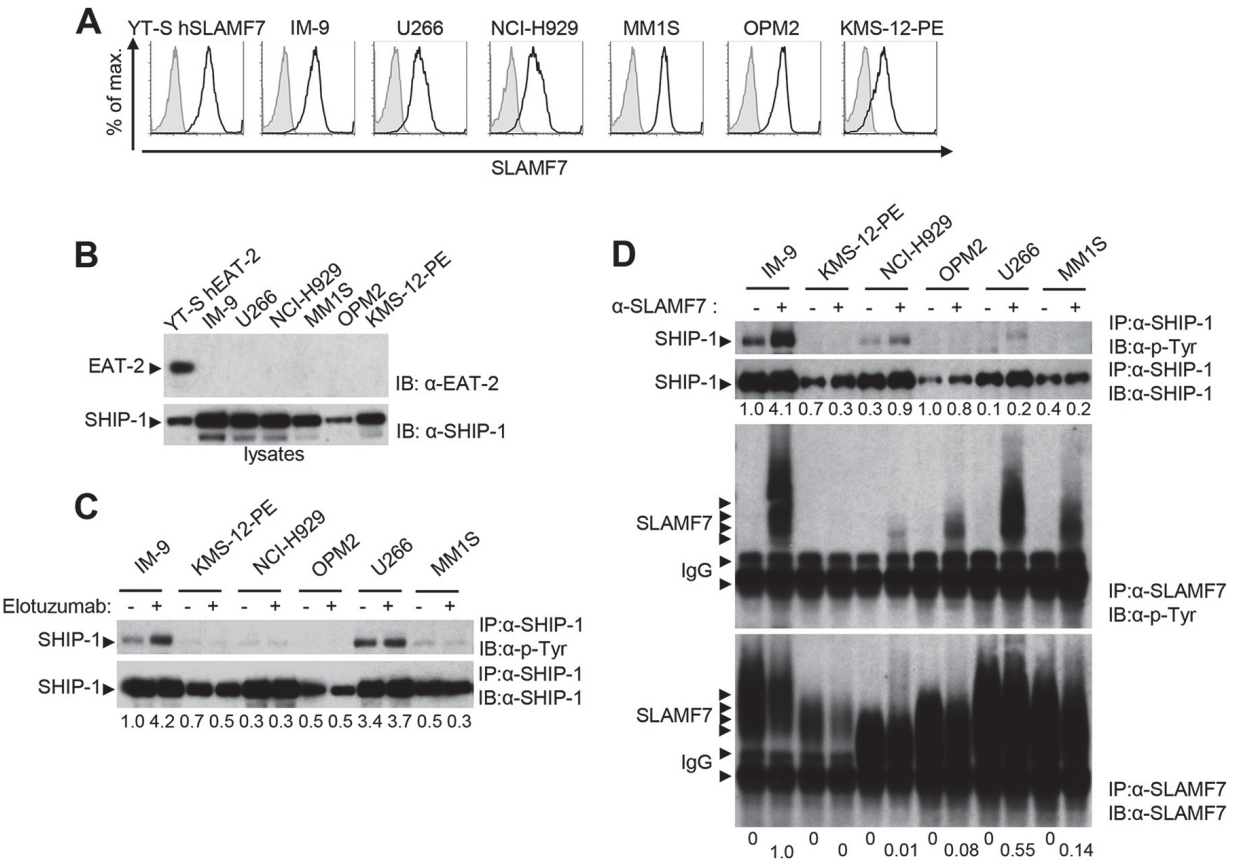
A
YT-S hSLAMF7
IM-9
U266
NCI-H929
MM1S
OPM2
KMS-12-PE
% of max.
SLAMF7
B
YT-S hEAT-2
IM-9
U266
NCI-H929
MM1S
OPM2
KMS-12-PE
EAT-2
IB: α-EAT-2
SHIP-1
IB: α-SHIP-1
lysates
C
IM-9
KMS-12-PE
NCI-H929
OPM2
U266
MM1S
Elotuzumab: - + - + - + - + - + - +
SHIP-1
IP:α-SHIP-1
IB:α-p-Tyr
SHIP-1
IP:α-SHIP-1
IB:α-SHIP-1
1.0 4.2 0.7 0.5 0.3 0.3 0.5 0.5 3.4 3.7 0.5 0.3
D
IM-9
KMS-12-PE
NCI-H929
OPM2
U266
MM1S
α-SLAMF7 : - + - + - + - + - + - +
SHIP-1
IP:α-SHIP-1
IB:α-p-Tyr
SHIP-1
IP:α-SHIP-1
IB:α-SHIP-1
1.0 4.1 0.7 0.3 0.3 0.9 1.0 0.8 0.1 0.2 0.4 0.2
SLAMF7
IgG
IP:α-SLAMF7
IB:α-p-Tyr
SLAMF7
IgG
IP:α-SLAMF7
IB:α-SLAMF7
0 1.0 0 0 0 0.01 0 0.08 0 0.55 0 0.14

Figure 3.4

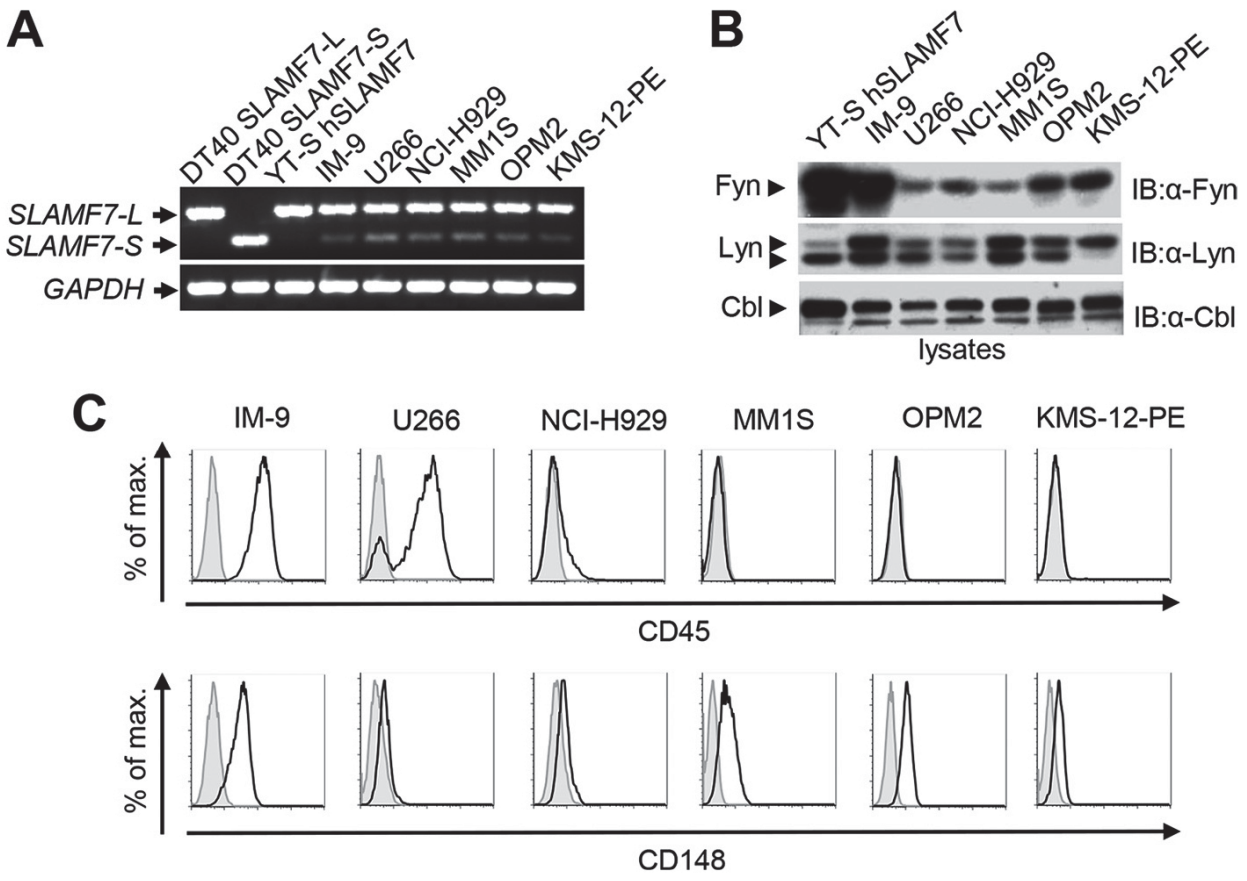
A
DT40 SLAMF7-L
DT40 SLAMF7-S
YT-S hSLAMF7
IM-9
U266
NCI-H929
MM1S
OPM2
KMS-12-PE
SLAMF7-L
SLAMF7-S
GAPDH
B
YT-S hSLAMF7
IM-9
U266
NCI-H929
MM1S
OPM2
KMS-12-PE
Fyn
IB:α-Fyn
Lyn
IB:α-Lyn
Cbl
IB:α-Cbl
lysates
C
IM-9
U266
NCI-H929
MM1S
OPM2
KMS-12-PE
% of max.
CD45
% of max.
CD148

Figure 3.5

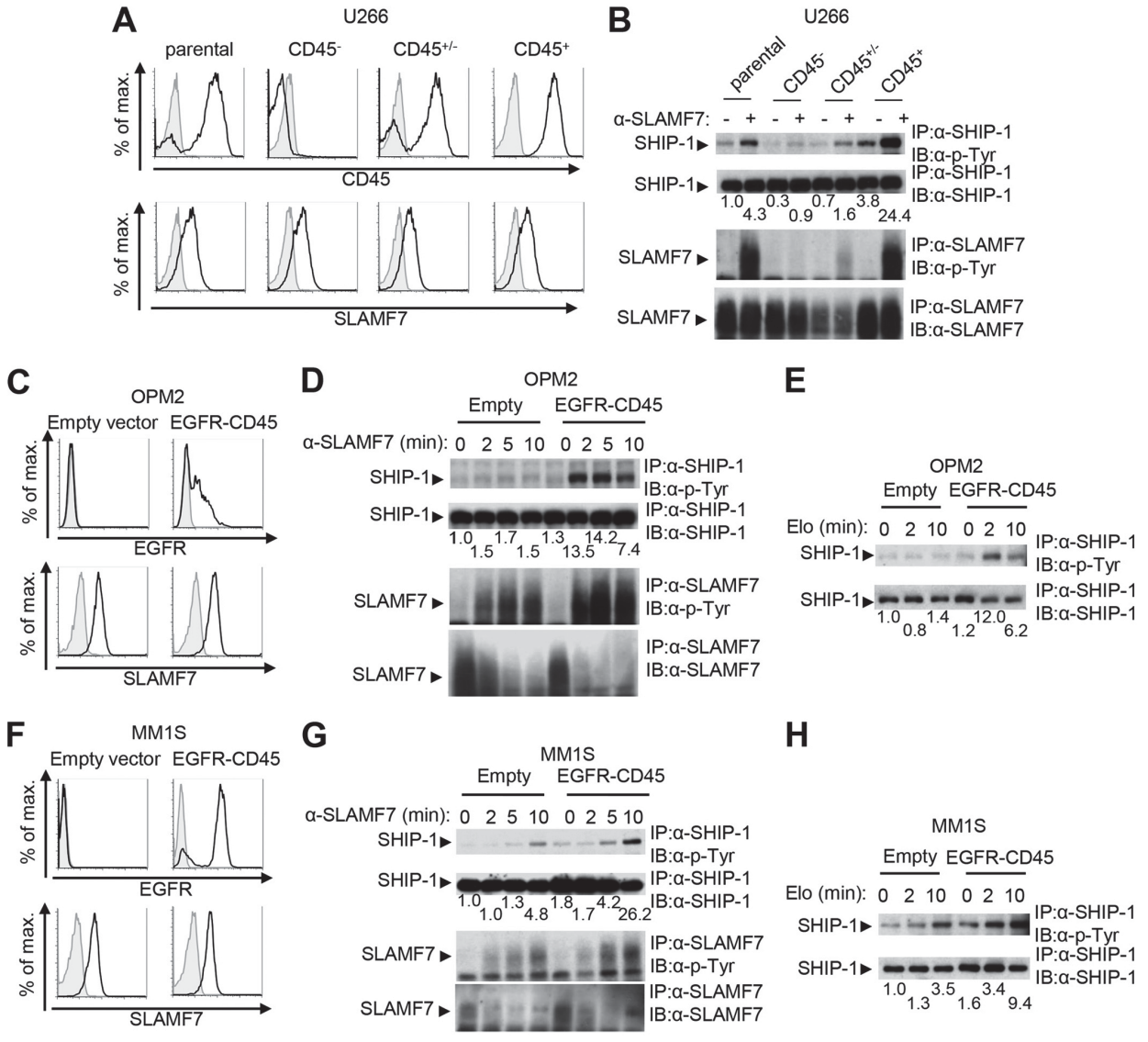
A
U266
parental
CD45-
CD45+/-
CD45+
% of max.
CD45
SLAMF7
B
U266
α-SLAMF7: - + - + - + - +
SHIP-1▶
IP:α-SHIP-1
IB:α-p-Tyr
IB:α-SHIP-1
1.0 4.3 0.3 0.9 0.7 1.6 3.8 24.4
SLAMF7▶
IP:α-SLAMF7
IB:α-p-Tyr
IB:α-SLAMF7
C
OPM2
Empty vector
EGFR-CD45
EGFR
D
OPM2
Empty
α-SLAMF7 (min): 0 2 5 10 0 2 5 10
1.0 1.5 1.7 1.5 1.3 13.5 14.2 7.4
E
OPM2
Elo (min): 0 2 10 0 2 10
1.0 0.8 1.4 1.2 12.0 6.2
F
MM1S
G
MM1S
1.0 1.0 1.3 4.8 1.8 1.7 4.2 26.2
H
MM1S
1.0 1.3 3.5 1.6 3.4 9.4

Figure 3.6

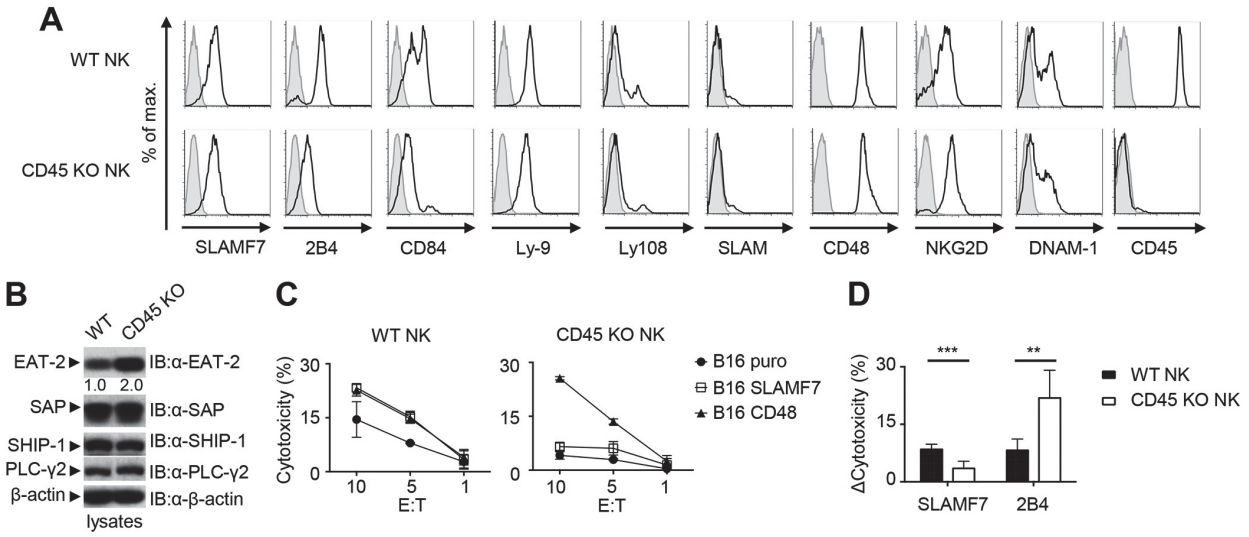
A
WT NK
CD45 KO NK
% of max.
SLAMF7
2B4
CD84
Ly-9
Ly108
SLAM
CD48
NKG2D
DNAM-1
CD45
B
WT
CD45 KO
EAT-2
IB:α-EAT-2
1.0 2.0
SAP
IB:α-SAP
SHIP-1
IB:α-SHIP-1
PLC-γ2
IB:α-PLC-γ2
β-actin
IB:α-β-actin
lysates
C
WT NK
CD45 KO NK
Cytotoxicity (%)
E:T
B16 puro
B16 SLAMF7
B16 CD48
D
ΔCytotoxicity (%)
***
**
SLAMF7
2B4
WT NK
CD45 KO NK

Figure 3.7

Chapter 4: Discussion

SLAM family receptors (SFRs) are homotypic receptors that are widely expressed on hematopoietic cells. The general role of SFRs on immune cells remain largely unknown, because single SFR-deficient mice often exhibit few defects, probably due to the redundant role of SFRs. We are unable to investigate the role of multiple SFRs by breeding single SFR-deficient mice together because the genes encoding SFRs are all located on chromosome 1. Hence, we generated a mouse lacking the entire ~400 kilobase (kb)-*Slam* locus on chromosome 1 using a conventional gene-disrupting strategy. Briefly, we disrupted the most 5' gene of the locus, *Slamf4*, using a targeting construct containing the neomycin resistance gene (*neo*) flanked by one *loxP* site. Additionally, we disrupted the most 3' gene of the locus, *Slamf6*, using a targeting construct bearing the hygromycin resistance gene (*hygro*) flanked by another *loxP* site. After Cre-mediated recombination, the entire *Slam* locus was deleted. The *Slam* locus contains genes encoding the six SFRs and CD48 (the ligand of SFR 2B4), as well as a short non-conserved open reading frame (ORF) of an unknown gene named *Gm10521*. The *Slam* locus-disrupted mice (SFR KO) were viable and fertile. Deletion of SFRs in SFR KO mice were verified by staining the six SFRs and CD48 in immune cells, such as T cells, B cells and NK cells.

NK cells are innate immune cells with potent cytotoxic capacity against tumor cells, virus-infected cells and self-transformed cells. The action of NK cells toward target cells relies on the integrated signaling mediated by activating receptors and inhibitory receptors. NK cells express five SFRs (2B4, SLAMF7, SLAMF6, CD84, Ly-9), which are able to transduce activating signals via SAP family adaptors, as well as inhibitory signals via phosphatases (e.g., SHIP-1, SHP-1). Hence, SFRs are important signaling receptors on NK cells, the function of which remains largely undetermined.

The role of SFRs on NK cells has been investigated in three different systems: (1) NK cell

cytotoxicity toward nonhematopoietic cells with or without ectopic expression of SFRs; (2) NK cell activation with or without antibody crosslinking against SFRs; (3) NK cell cytotoxicity toward hematopoietic cells with or without SFR-SFR interactions. In the first system, wild-type NK cells showed enhanced killing toward the melanoma cell line B16 ectopically expressing SLAMF7, SLAMF6, Ly-9, CD48 or CD84, compared with non-transfected B16 cells[161, 183]. In the second system, antibody crosslinking to 2B4 or SLAMF7 induced intracellular calcium influx in wild-type NK cells. In addition, 2B4 stimulation synergized CD16-induced calcium influx in wild-type NK cells[37, 161, 183]. SFRs behave as activating receptors in wild-type NK cells when exposed to strong stimulation (e.g., overexpression of ligands on nonhematopoietic target cells or strong antibody crosslinking). However, the role of SFRs exposed to ligands on hematopoietic cells (the third system) is more complicated and less well described. NK cells from SLAMF7-deficient mice displayed decreased cytotoxicity toward SLAMF7-negative hematopoietic cell lines, RMA-S (MHC I negative lymphoma) and YAC (MHC I negative thymoma), compared with wild-type NK cells[183]. This finding indicates that the SLAMF7-SLAMF7 interaction (either *cis* or *trans*) in NK cells activates them prior to encountering SLAMF7 negative hematopoietic cells. During LCMV infection, 2B4 knock-out (KO) NK cells displayed increased killing toward activated CD4 T cells, compared to wild-type NK cells, indicating that 2B4 plays an inhibitory role in this setting[148]. Another group also suggested an inhibitory role of 2B4 in NK cells by showing that a deficiency of 2B4 or CD48 on NK cells elevated NK cell fratricide (NK cell killing of neighboring NK cells)[292]. The role of SFRs (either activating or inhibitory) in wild-type NK cells that express SAP family adaptors is still debated. Undoubtedly, however, SAP-EAT-2-ERT-deficiency renders SFRs inhibitory, suppressing NK cell activation.

The generation of *Slam* locus-disrupted (SFR KO) mice enabled us to test the overall role of SFRs in NK cell development and function. Herein, we showed that SFR KO mice had no alterations in NK cell percentages and absolute numbers in the spleen and bone marrow, compared with wild-type mice. However, there was a slight change in NK cell maturation (SFR KO NK mice have more $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ immature NK cells and less $CD27^{-}CD11b^{+}$ mature NK cells in the spleen, but this difference is less apparent in the bone marrow). SFR KO NK cells also exhibit slight differences in the NK cell repertoire (less DX5, Ly49A/D, Ly49C/I/F/H and KLRG1 in the spleen, but this is less significant in bone marrow). As mature NK cells often have increased expression levels of DX5, KLRG1 and Ly49 receptors, these findings suggested that SFR might affect NK cell maturation to some extent. In fact, a recent report proposed that SFRs are critical for NK cell education against hematopoietic cells[115], even though the phenotypes in our study (to be discussed in the following paragraphs) appeared to be less dependent on the educational effect.

Our previous studies demonstrated that SAP family adaptors are essential for NK cell cytotoxicity toward hematopoietic cells[37, 161, 183]. Given that SFRs transduce activating signals through SAP family adaptors, we proposed that SFRs serve as important activating receptors for NK cell surveillance against hematopoietic cells. Hence, we expected SFR KO NK cells to exhibit the same phenotype as SAP KO NK cells. Surprisingly, however, SFR KO NK cells displayed enhanced killing toward RMA (MHC I-positive lymphoma), RMA-S (MHC I-negative variant of RMA) and YAC (MHC I-negative thymoma), in contrast to SAP NK cells, which displayed diminished killing. In addition, SFR-SAP double KO (dKO) NK cells had a phenotype similar to SFR KO NK cells, leading us to re-propose the role of SFRs in wild-type NK cells as follows. SFRs are primarily inhibitory in the presence of SAP family adaptors in NK

cells, and in the absence of SAP family adaptors, SFRs become super-inhibitory. In addition to using hematopoietic tumor cell lines, we also used activated CD4 T cells and bone marrow-derived macrophages as target cells. SFR KO NK cells exhibited elevated killing, compared with wild-type NK cells, toward activated CD4 T cells and macrophages from wild-type mice. The enhanced cytotoxicity of SFR KO NK cells could be explained by one of two reasons: (1) SFR (on NK cells) - SFR (on targets) interaction inhibits NK function; (2) SFR (on NK cells) - SFR (on NK cells) interaction educates NK cells to be hyperresponsive. The former reveals the direct inhibitory role of SFRs on NK cells, whereas the latter underlies NK cell education prior to encountering target cells. To investigate these hypotheses, we used NK cells from wild-type mice as effector cells and activated CD4 T cells (or macrophages) from wild-type or SFR KO mice as target cells. In this setting, NK cells were only from wild-type mice to avoid an effect on NK cell education. Accordingly, wild-type NK cells exhibited enhanced killing toward SFR KO activated CD4 T cells (or macrophages) compared with wild-type activated CD4 T cells (or macrophages). Hence, we concluded that SFRs are inhibitory receptors in wild-type NK cells.

To determine the role of SFRs in NK cell education, we used plate-bound antibody to stimulate the activating receptors CD16 and NK1.1 on NK cells from wild-type or SFR KO mice. Although SFR KO NK cells displayed a slight increase (1.3 to 1.5-fold) in IFN-γ production in response to stimulation compared with wild-type NK cells, this difference was not significant. Of note, SFR KO NK cells also exhibited a slight enhancement of killing toward nonhematopoietic tumor cells B16 (C57BL/6-derive melanoma) but not toward CMT-93 (polyploid carcinoma). Since nonhematopoietic cells do not express any SFR, the augmented ability of SFR KO NK cells to kill nonhematopoietic cells in this case is due to NK cell education. Therefore, SFRs

might participate in NK cell education, although it is not the main explanation for the observed hyperresponsiveness of SFR KO NK cells in our study.

Slam locus-disrupted mice lack six SFRs, CD48 and *Gm10521*. NK cells express all SFRs except SLAM. To figure out which receptor(s) is (are) important for the inhibitory function, we tested NK cells that were deficient in 2B4, SLAMF7, SLAMF6, Ly-9, CD84, *Gm10521* and the *Slam* locus (as a control). Interestingly, the inhibitory effect of the *Slam* locus was mediated solely by 2B4, with no appreciable impact from the other SLAM receptors or *Gm10521*. To confirm the dominant role of 2B4 in NK cells, we adopted a mouse model of LCMV infection. During infection of the LCMV clone 13, 2B4 inhibited NK cell cytotoxicity against virus-specific T cells. Thus, 2B4 KO mice suffered less from immune pathology compared with wild-type mice, due to the enhanced killing of virus-specific T cells by NK cells in 2B4 KO mice. SFR KO mice provided results that were comparable to 2B4 KO mice, indicating that the inhibitory effect of SFRs was mainly mediated by 2B4. Although only 2B4 inhibits NK cell cytotoxicity, other SFRs may still play a role in NK cell function, e.g., NK cell proliferation, survival, memory, migration or conjugate formation. In addition to the NK cell cytotoxicity assessments, which were most commonly applied here, further studies are required to investigate these effects.

In our study, NK cells must be primed by cytokines *in vitro* or *in vivo* to be able to kill SFR KO target cells. Hence, we examined the impact of cytokines on SFR-mediated inhibition. By binding to the cytokine receptors expressed on NK cells, various cytokines can have different roles in NK cell activation. IL-15 is the most important cytokine for NK cell development *in vivo* and for the expansion of NK cells *in vitro*. IL-2 is the most commonly used cytokine to expand human or mouse NK cells *in vitro*. NK cells cultured with IL-2 are often termed

lymphokine-activated killer (LAK) cells. Additionally, IL-12 and type I IFN (IFN-α, IFN-β) are frequently used to activate NK cells. As depicted in our study, IL-2- or IL-15-expanded NK cells exhibited increased killing toward SFR KO-activated CD4 T cells in comparison to wild-type activated CD4 T cells. The difference in killing toward wild-type and SFR KO activated CD4 T cells revealed an inhibitory function mediated by SFRs. Interestingly, IL-12 enhanced, whereas type I IFN suppressed, this difference. This finding was also correlated with the altered expression of EAT-2 and ERT in IL-12- or type I IFN-treated NK cells. In brief, IL-12 promotes SFR-mediated inhibition via downregulating SAP/EAT-2/ERT expression, whereas type I IFN diminishes SFR-mediated inhibition by upregulating EAT-2/ERT expression. Given that SAP family adaptors serve as positive effectors downstream of SFRs, cytokines can regulate SFR-mediated functions by modulating the expression level of SAP family adaptors. This finding provides deep insight into the mechanism by which NK cell function is controlled by SFRs in immune stimulations involving different cytokine milieus. For example, during LCMV infection, IL-12, IL-15 and IL-18 are strongly induced and promote the inhibitory role of SFRs. As a consequence, NK cells are less capable of killing virus-specific T cells, which in turn contributes to the control of the virus to protect the body. In another case, during murine cytomegalovirus (MCMV) infection, type I IFN is supposed to be the most abundant cytokine, suppressing SFR-mediated inhibition. Thus, NK cells are more activated and are responsible for clearing the virus by activating receptor Ly49H.

In NK cells, the most important inhibitory receptors are killer cell immunoglobulin-like receptors (KIR) in humans, inhibitory Ly49 receptors in mice and CD94/NKG2A in both species. These receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and inhibit NK cell activation by binding to MHC I. Herein, we defined SFRs as another critical group of

inhibitory receptors in NK cells. Compared with MHC I-mediated inhibition, SFR-mediated inhibition is more dynamic and flexible. SFR, via its ITSM, is able to interact with both positive effectors (SAP family adaptors) and negative effectors (SHIP-1, SHP-1, SHP-2, Csk). By modulating the expression level of SAP family adaptors or tuning the SFR aggregation intensity, the function of SFRs can be dynamically regulated. As shown in Figure 4.1, strong stimulation by antibody crosslinking or overexpression of ligands on nonhematopoietic target cells activates SFRs in wild-type NK cells[37, 191, 293]. However, moderate stimulation by engaging normal levels of SFRs on hematopoietic cells renders SFR inhibitory, even in the presence of SAP family adaptors[148, 237, 292]. In fact, in response to hematopoietic cells, SFRs are primarily inhibitory, but they are also capable of providing an activating stimulus in certain situations.

Role of SFR	Aggregation intensity	SAP family adaptors	
		+	-
antibody crosslinking	strong	activating	inhibitory
nonhematopoietic cells (ectopic expression of SFR)	strong	activating	inhibitory
hematopoietic cells (normal amount of SFR)	moderate	inhibitory	super-inhibitory

Figure 4.1. The role of SLAM family receptors is dynamically regulated. The role of SLAM family receptors (SFRs) was determined in three types of stimulations: (1) antibody crosslinking; (2) nonhematopoietic cells with ectopic expression of SFR; (3) hematopoietic cells with normal levels of SFR. The aggregation intensity of SFRs in response to different stimuli is shown. The role of SFRs in the presence (+) or absence (-) of SAP family adaptors is depicted. IL-12 deceased the expression level of SAP family adaptors (red), whereas type I IFN increased the expression level of SAP family adaptors (blue).

Up to here, we have demonstrated that SFRs are inhibitory receptors toward hematopoietic cells. Our next question concerned the identification of the activating receptor(s) that is (are) suppressed by SFRs. Using blocking antibodies against a variety of activating receptors (DNAM-1, NKG2D, NKp46) and integrin subunits (CD11a, CD11b, CD11c), we found that CD11a was essential for the enhanced killing of activated CD4 T cells mediated by SFR deficiency. CD11a is the alpha unit of lymphocyte function-associated antigen 1 (LFA-1), which promotes cell adhesion, immunological synapse formation and activating signals. LFA-1 is a heterodimer composed of CD11a (αL) and CD18 (β2). Unlike NK cells from wild-type mice, NK cells from CD11a KO and CD18 KO mice did not exhibit enhanced killing toward SFR KO activated CD4 T cells in comparison to wild-type activated CD4 T cells. Breeding SFR KO mice with CD11a KO mice also diminished the increased NK cell killing in SFR KO mice toward activated CD4 T cells, RMA and RMA-S. This finding indicates that LFA-1 is essential for NK cell activation and serves as an activating or co-activating receptor that triggers NK cell recognition toward hematopoietic target cells.

LFA-1 is known as an adhesion receptor, not only in NK cells but also in other immune cells such as T cells, B cells, macrophages and neutrophils. In NK cells, the significance of LFA-1 is often assessed in studies investigating the NK cell immunological synapse (IS). The NK IS is the dynamic interface that forms between the NK cell and the target cell, and it is a prerequisite for NK cell cytotoxicity toward target cells. During the initiation stage of NK IS, LFA-1 is located in the peripheral supramolecular activation cluster (pSMAC), which binds to ICAM-1 and ICAM-2 to promote NK cell-target cell adhesion[294]. Additionally, in the effector stage, a stable interaction between NK cell and target cell facilitates the recruitment of activating receptors to the NK IS, triggering NK cell activation and degranulation toward target cells. In the

second stage, LFA-1 is also capable of transducing "outside-in" signals, which activate NK cells[66]. Hence, SFRs, as inhibitory receptors, can inhibit the function of LFA-1 by either interfering with NK cell-target cell conjugate formation (the initiation stage) or by inhibiting LFA-1-mediated activating signals (the effector stage). To examine these two possibilities, we adopted an LFA-1-dependent model to test NK cell cytotoxicity by using the human NK cell line YT-S as the effector cell and the human EBV-transformed B cell line 721.221 as the target cell. Given that 2B4 is the only SFR involved in this inhibitory effect, we assessed NK cell-target cell conjugate formation in the presence or absence of the 2B4-CD48 interaction. Clearly, 2B4 did not inhibit conjugate formation, suggesting it does not suppress LFA-1-mediated adhesion. Moreover, we evaluated whether 2B4 inhibited LFA-1-mediated signaling by monitoring LFA-1 activation using an antibody that specifically recognizes the active conformation of LFA-1. Interestingly, 2B4 diminished the generation of the active form of LFA-1, indicating that it suppresses LFA-1-mediated signaling. Indeed, 2B4-mediated inhibition is, at least in part, due to activation of the inositol phosphatase SHIP-1. As shown in Figure 4.2, SHIP-1 hydrolyzes the 5' phosphate of phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$) into $PI(3,4)P_2$. Since $PI(3,4,5)P_3$ can ultimately promote LFA-1 activation by the recruitment of Wiskott-Aldrich Syndrome protein (WASP) to the site of LFA-1 ligation[66], 2B4 can inhibit this process by decreasing the amount of $PI(3,4,5)P_3$ via SHIP-1. This notion requires verification in future studies, but minimally, it provides a potential hypothesis to explain the mechanism by which 2B4 inhibits LFA-1 via SHIP-1.

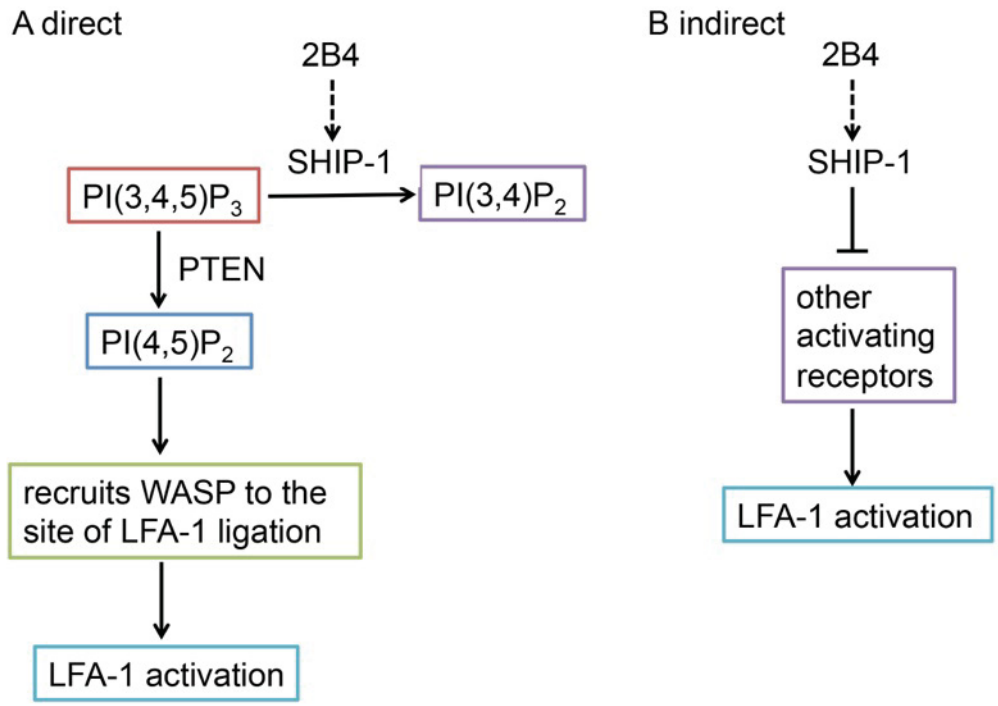

Figure 4.2. Hypothesis for the mechanism of 2B4-mediated inhibition on LFA-1. 2B4 can inhibit the function of LFA-1 directly (A) or indirectly (B). (A) Via the phosphatase and tensin homolog (PTEN), phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$) generates $PI(4,5)P_2$, which recruits Wiskott-Aldrich Syndrome protein (WASP) to the site of LFA-1 ligation. This phenomenon promotes LFA-1 activation. SH2-containing inositol phosphatase-1 (SHIP-1), which lies downstream of 2B4, hydrolyzes $PI(3,4,5)P_3$ to $PI(3,4)P_2$. The decreased levels of $PI(3,4,5)P_3$ lead to less activation of LFA-1. (B) 2B4 inhibits the function of other activating receptors through SHIP-1. The diminished activating signals result in reduced activation of LFA-1.

In summary, in Chapter 2, we examined the role of SFRs on NK cells toward hematopoietic cells, by generating a mouse lacking the entire *Slam* locus. SFR does not alter NK cell development and has little impact on the NK cell repertoire. As indicated in Figure 4.3, SFR inhibits NK cell cytotoxicity in response to hematopoietic cells, and 2B4 is the sole receptor involved in SFR-mediated inhibition. The inhibitory role of SFRs on NK cells was confirmed by

the LCMV infection model *in vivo*, in which SFR KO mice exhibited the same phenotype as 2B4 KO mice. IL-12 enhances SFR-mediated inhibition by downregulating the expression level of SAP/EAT-2/ERT, whereas type I IFN suppresses the inhibitory effect of SFR by upregulating the expression level of EAT-2/ERT. SFR modulates NK cell function, in part by inhibiting the generation of the active conformation of LFA-1, but not by reducing LFA-1-mediated adhesion.

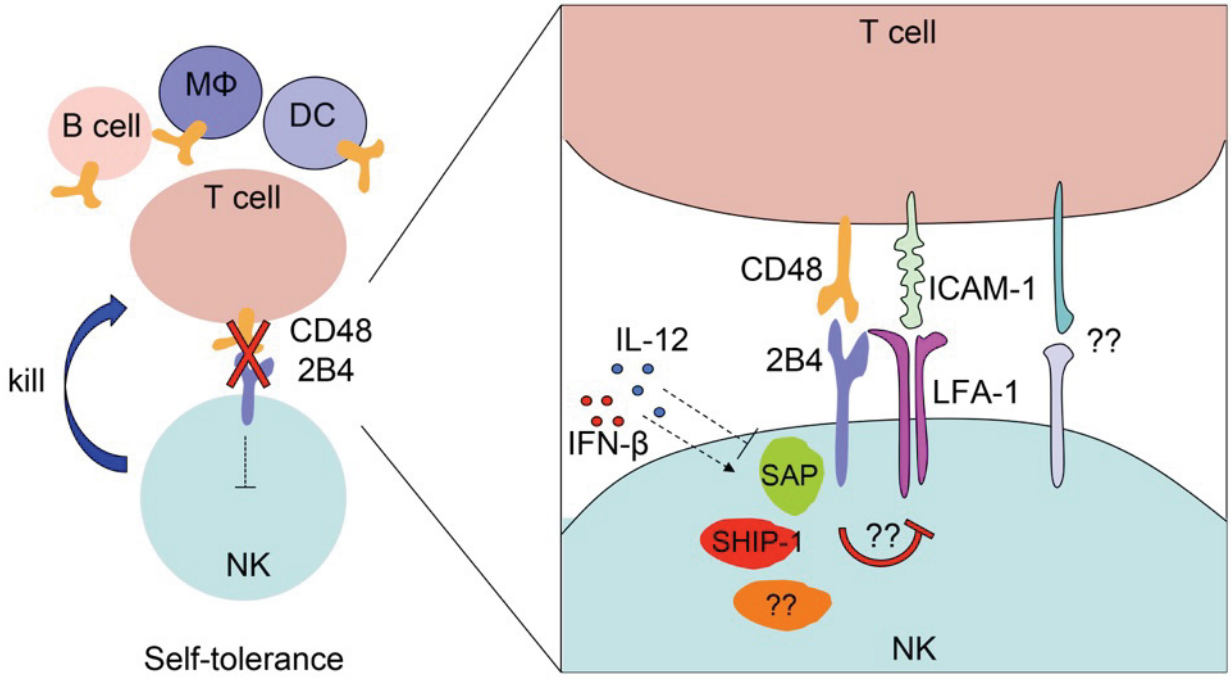

Figure 4.3. Model of NK cell regulation by receptors and cytokines. 2B4 inhibits NK cell cytotoxicity toward activated T cells, B cells, macrophages (Mϕs) and dendritic cells (DCs). Considering activated T cells as an example, 2B4 interacted with CD48 and suppressed the function of LFA-1, in part via SHIP-1. IL-12 reduced the expression level of SAP family adaptors, whereas type I IFN (e.g., IFN-β) increased the expression level. Whether SHIP-1 inhibits the function of LFA-1 directly or indirectly, and whether other undescribed, activating receptors are also involved in NK cell cytotoxicity toward activated T cells remain unknown.

Despite the striking findings discussed above, several features require further elucidation.

First, it is still not clear whether LFA-1 is the only activating receptor that is able on its own to trigger sufficient signaling for NK cell activation, or whether it serves as a co-activating receptor that requires the presence of other activating receptors. Although we excluded the possible involvement of common activating receptors, such as DNAM-1, NKG2D and NKp46, there is still a possibility that other, unidentified receptors are critical for this process. Additionally, whether 2B4 inhibits LFA-1 activation directly or indirectly remains unclear. As depicted in Figure 4.2, 2B4 may mediate the direct inhibition of LFA-1 via SHIP-1. It may also suppress the function of other activating receptors, in turn generating fewer activating signals and leading to reduced activation of LFA-1. Further studies are required to unravel this mechanism. In addition, because SFR-mediated signaling can be dynamically regulated, it is not appropriate to define it as an inhibitory receptor in all circumstances. Rather, it should be analyzed on a case-by-case basis. For example, in our study, SFR was primarily inhibitory in NK cells toward hematopoietic cells. However, it may become activating with the upregulation of SAP family adaptors or overexpression of the SFR ligand. In fact, stimulation of SFR by strong aggregation via antibody crosslinking or ectopic expression of ligand on nonhematopoietic cells causes it to function as an activating receptor. Hence, we conclude that SFR-mediated signaling is flexible and dynamic. Intriguingly, the SFR-mediated effect is distinct toward hematopoietic cells (inhibitory) and nonhematopoietic cells (activating). This phenomenon might rely on different activating receptors involved in recognition of these two cell populations. Granted that most hematopoietic cells express ICAM-1 and ICAM-2, NK cells recognize these cells and trigger cytotoxicity via LFA-1. In this case, SFR works as an inhibitory receptor that suppresses the function of LFA-1. Regarding the nonhematopoietic cells, NK cells mediate killing, typically through DNAM-1 or NKG2D. In this case, SFR may be able to serve as a co-activating receptor, synergizing the

effect of DNAM-1 and NKG2D. Finally, in humans, SFR is termed an activating receptor based on studies utilizing antibody stimulation or ectopic expression of ligand on nonhematopoietic cells. However, in some situations, it can still become inhibitory, even in the presence of SAP family adaptors.

In the study of multiple myeloma (MM) described in Chapter 3, we investigated the role of SLAMF7 in NK cells and in MM cells, both of which express high levels of SLAMF7 on their surface. SLAMF7 transduces activating signals through EAT-2, but not SAP. In the absence of EAT-2, SLAMF7 inhibits NK cell cytotoxicity[183]. In our study, we showed that antibody stimulation of SLAMF7 triggered the phosphorylation of SLAMF7, as well as SHIP-1. By using the genetically modified chicken B cell line DT40, which lacks SHIP-1, SHP-1, SHP-2 or Csk, we demonstrated that only SHIP-1 was responsible for SLAMF7-mediated inhibition. Furthermore, we revealed that Src family kinases were required for the phosphorylation of SLAMF7 and that the tyrosines (Y261, Y281) in the cytoplasmic domain of SLAMF7 were necessary for its function. Interestingly, Y261 was responsible for SLAMF7-mediated inhibition, whereas Y281 was critical for the recruitment of EAT-2 to activate NK cells[183]. We also found that Y261 was essential for the phosphorylation of SHIP-1, further confirming the role of Y261 and SHIP-1 in the inhibitory effect of SLAMF7.

In parallel, we investigated the action of SLAMF7 in MM cells. Most MM cell lines express high levels of SLAMF7, including U266, NCI-H929, MM1S, OPM2 and KMS-12-PE, and they also lack EAT-2 expression. Hence, SLAMF7 might act as an inhibitory receptor in MM cells, based on our findings in NK cells. Strikingly, however, engagement of SLAMF7 only induced mild phosphorylation of SLAMF7 but no phosphorylation of SHIP-1. However, the

EBV-transformed B cell line IM-9 (as a positive control) was able to induce strong phosphorylation of SLAMF7 and SHIP-1 upon antibody stimulation of SLAMF7. To understand the underlying mechanism, we tested whether the MM cell lines expressed different patterns of SLAMF7 isoforms. In humans, SLAMF7 exists as two isoforms, the long isoform (SLAMF7-L) and the short isoform (SLAMF7-S), due to alternative splicing. SLAMF7-L is capable of transducing signals, whereas SLAMF7-S is incapable because it lacks tyrosines (Y261, Y281) in the cytoplasmic domain. Similar to the positive control IM-9, all MM cells dominantly expressed SLAMF7-L. Moreover, we analyzed the expression level of Src family kinases, which is critical for the phosphorylation of substrates such as SLAMF7 and SHIP-1. All MM cells express the Src family kinases, Fyn and Lyn. Of note, the expression level of Fyn in MM cells is lower compared with IM-9. Hence, the lack of SLAMF7-mediated signaling in MM cells is neither due to altered expression of SLAMF7 isoforms nor to a lack of Src family kinases.

In B cells, the activity of Src family kinases largely depends on two transmembrane protein tyrosine phosphatases, CD45 and CD148[295]. As shown in Figure 4.4, CD45 and CD148 can dephosphorylate the C-terminal tyrosine (Y508) of Lyn, generating basally active Lyn from inactive Lyn. This is the prerequisite step for the full kinase activity of Lyn, which leads to the phosphorylation of its substrates. Hence, we evaluated the expression levels of CD45 and CD148 in MM cells. Surprisingly, CD45 was absent in most MM cells (NCI-H929, MM1S, OPM2, KMS-12-PE), except for U266, which partially expressed CD45. Compared with the B cell line IM-9, the expression level of CD148 in MM cells was also lower. Thus, no expression of CD45, together with low expression levels of CD148 could result in inefficient Src family kinase activity, leading to reduced phosphorylation of SLAMF7 and SHIP-1 in MM cells. To test this hypothesis, we restored CD45 activity in OPM2 and MM1S by introducing EGFR-CD45 on the

surface of the cells. As expected, upon engagement of SLAMF7, the phosphorylation of SHIP-1 was rescued in cells expressing EGFR-CD45. In addition, CD45-deficient NK cells exhibited less SLAMF7-mediated functions compared with wild-type NK cells. Hence, the engagement of SLAMF7 on MM cells was unable to induce a signal due to the absence of CD45 and low levels of CD148.

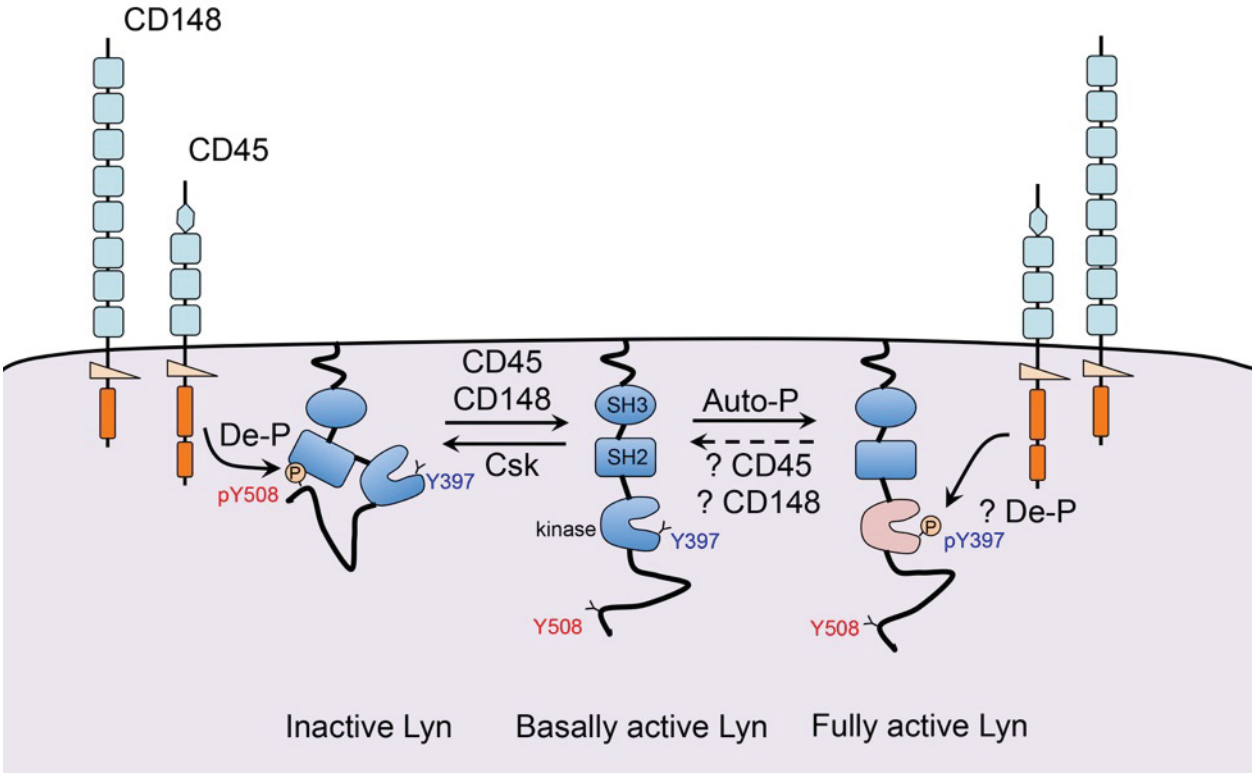

Figure 4.4. Regulation of Src family kinases by CD45 and CD148 in B cells. In B cells, CD45 and CD148 dephosphorylate (De-P) the phosphorylated C-terminal tyrosine 508 (pY508) of Lyn, which in turn leads to basal activation of Lyn from its inactive form. Autophosphorylation (Auto-P) of tyrosine 397 (Y397) in kinase domain renders Lyn fully activated. In contrast, CD45 and CD148 can also dephosphorylate the phosphorylated Y397 (pY397) to transform fully active Lyn into basally active Lyn, and the C-terminal Src kinase (Csk) phosphorylates Y508 to transform Lyn into an inactive form.

In brief, in Chapter 3, we studied the mechanism underlying SLAMF7-mediated inhibition in NK cells in the absence of EAT-2, and we determined that SHIP-1 is involved in the inhibitory effect of SLAMF7. Src family kinase and tyrosine 261 in the cytoplasmic region of SLAMF7 are two critical factors for the inhibitory effect of SLAMF7. We also tested whether SLAMF7 was able to mediate inhibitory signals in MM cells expressing SLAMF7 but not EAT-2. Strikingly, engagement of SLAMF7 in MM cells only induced mild phosphorylation of SLAMF7 but not SHIP-1. This defect was not due to altered expression of SLAMF7 isoforms but to the lack of CD45 in MM cells. The absence of CD45 contributes to inefficient Src family kinase activity, which in turn leads to poor phosphorylation of SLAMF7 and SHIP-1. This phenomenon explains why no studies have reported direct cytostatic or cytotoxic effects of elotuzumab on MM cells, and it facilitates our understanding of the activity of elotuzumab for the treatment of MM.

Nevertheless, several issues remain to be addressed. Firstly, it is not clear whether SHIP-1 binds directly to SLAMF7 or it is indirectly recruited to SLAMF7 via other molecules. Our studies showed Y261 is essential for the inhibitory role of SLAMF7. However, we were unable to demonstrate the direct recruitment of SHIP-1 to Y261. It is possible that the immunoprecipitation assay used *in vitro* was not sufficiently sensitive to detect direct binding, or another molecule, such as the SH2 domain-containing protein SHC, bridged the interaction of SLAMF7 and SHIP-1. Further studies are needed to resolve this issue. Moreover, the cytostatic or cytotoxic role of SHIP-1 in MM cells is unknown. Although we were able to restore SLAMF7 signaling by introducing EGFR-CD45 in MM cells, we failed to detect any cytotoxic effects upon engagement of SLAMF7, e.g., effects in cell proliferation, survival, apoptosis and

migration. The expression level of EGFR-CD45 was likely not comparable to the endogenous expression level of CD45 in normal cells, thus SLAMF7-mediated signaling was not sufficient to provide any visible effects. Alternately, the MM cell lines used in our study have been previously adapted to the culture system *in vitro* and are able to bypass any cytotoxic effect regulated by SLAMF7. The samples from MM patients would be of great help to evaluate the cytotoxic effect. Finally, although MM cells do not express EAT-2, it is still possible that SLAMF7 might carry some tumor-promoting role in MM cells, rather than being inhibitory. Activated B cells (plasmablasts, plasma cells, MM cells, among others) upregulate the expression level of SLAMF7 in comparison to naïve B cells. SLAMF7 might contribute to B cell activation and survival in a manner that has not yet been described. In fact, a previous study showed that SLAMF7 induces proliferation and cytokine production in human B cells[184], suggesting a positive role for SLAMF7.

SLAM family receptors, as homotypic receptors, are widely expressed on hematopoietic cells. They act dynamically and elastically in NK cell regulation. Like receptors that recognize MHC I, SLAM family receptors are primarily inhibitory in NK cells, even in the presence of SAP family receptors. However, they are able to switch their function to exert activating effects. In fact, the immune cell functions are actively regulated by SLAM family receptors via modulation of the expression level of SLAM family receptors or SAP family adaptors. Clearly, more work is needed to elucidate the role(s) of SLAM family receptors in immune cell regulation.

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