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TRIGGERING AND MECHANISMS OF NATURAL KILLER CELL MEDIATED CYTOTOXICITY

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In all things of nature there is something of the marvellous.
Aristotle

ABSTRACT

Natural killer (NK) cells are innate immune cells that contribute to defense against infected and transformed cells by target cell killing and cytokine release. In addition, data suggest that NK cells contribute to immune homeostasis and reproduction. In this thesis, we assessed the contribution of individual receptors and intracellular effector molecules to the function of freshly isolated, resting human NK cells.

A reductionist approach, using *Drosophila* cells transfected with ligands for human NK cell receptors, revealed that combinations of synergistic signals from distinct receptors were required to induce efficient NK cell cytotoxicity. Engagement of CD16 by IgG was sufficient to induce degranulation, whereas engagement of LFA-1 by ICAM-1 was sufficient to induce not only adhesion, but also granule polarization. Efficient antibody-dependent cellular cytotoxicity required the combination of granule polarization induced by LFA-1 and degranulation induced by CD16. Receptors NKp46, NKG2D, 2B4, DNAM-1, and CD2 have previously been implicated in natural cytotoxicity. Unexpectedly, engagement of these receptors by specific antibodies failed to induce resting NK cell cytotoxicity. For natural cytotoxicity, co-engagement of specific pairwise combinations of activating receptors synergistically induced degranulation and cytokine production. Thus, the term “co-activation receptor” has been proposed to describe natural cytotoxicity receptors that function as synergistic pairs. KIR2DL4 is an evolutionary conserved member of the KIR family of receptors. Unlike other NK cell receptors, KIR2DL4 was shown to reside in intracellular vesicles. Thus, soluble, but not solid-phase agonists of KIR2DL4, including natural ligand HLA-G, induced cytokine secretion by NK cells. Without eliciting cytotoxicity, this distinctive activation has putative implications for pregnancy.

Further, NK cells were assessed from patients diagnosed with familial hemophagocytic lymphohistiocytosis (FHL), an early onset, fatal immunodeficiency syndrome associated to mutations in genes implicated in cellular cytotoxicity. Analysis demonstrated a requirement for Munc13-4 and syntaxin 11 in resting NK cell degranulation. Remarkably, IL-2-stimulation partially restored degranulation and cytotoxicity by syntaxin 11-deficient NK cells. This could explain the later onset and less severe disease progression observed in FHL caused by nonsense mutations in *STX11*, relative to mutations in *PRF1* or *UNC13D*. In accord, an *UNC13D* mutation allowing residual degranulation and cytotoxicity was also associated with later disease onset. Our data suggest that the observed defect in NK cell degranulation may contribute to the pathophysiology of FHL, that evaluation of NK cell degranulation in suspected FHL patients may facilitate diagnosis, and that these new insights may offer novel therapeutic possibilities.

Our findings provide detailed insight into the molecular triggering and regulation of human NK cell function. Appreciation of the contribution of individual genetic elements to immune function promises increased understanding of disease. Of clinical relevance, new techniques facilitate improved diagnosis, whereas fundamental understanding may assist in development of better treatment.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

- I. **Yenan T. Bryceson**, Michael E. March, Domingo F. Barber, Hans-Gustaf Ljunggren, Eric O. Long. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells.
Journal of Experimental Medicine. 2005 Oct 3; 202 (7):1001-1012.
- II. **Yenan T. Bryceson**, Michael E. March, Hans-Gustaf Ljunggren, Eric O. Long. Synergy among receptors on resting NK cells for activation of natural cytotoxicity and cytokine secretion.
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- III. Sumati Rajagopalan, **Yenan T. Bryceson**, Shanmuga P. Kuppusamy, Daniel E. Geraghty, Anton van der Meer, Irma Joosten, Eric O. Long. Activation of NK cells by an endocytosed receptor for HLA-G.
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- IV. **Yenan T. Bryceson**, Eva Rudd, Chengyun Zheng, Josefine Edner, Daoxin Ma, Stephanie M. Wood, Anne Grete Bechensteen, Jaap J. Boelens, Tiraje Celkan, Roula A. Farah, Kjell Hulténby, Jacek Winiarski, Paul A. Roche, Magnus Nordenskjöld, Jan-Inge Henter, Eric O. Long, Hans-Gustaf Ljunggren. Defective lymphocyte degranulation in syntaxin-11-deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients.
Blood. 2007 Sep 15; 110 (6): 1906-1915. Epub 2007 May 24.
- V. Eva Rudd, **Yenan T. Bryceson**, Chengyun Zheng, Josefine Edner, Stephanie M. Wood, Kim Göransdotter Ramme, Sofie Gavhed, Aytemiz Gurgey, Marit Hellebostad, Anne Grete Bechensteen, Hans-Gustaf Ljunggren, Bengt Fadeel, Magnus Nordenskjöld, Jan-Inge Henter. Spectrum, and clinical and functional implications of UNC13D mutations in familial hemophagocytic lymphohistiocytosis.
Journal of Medical Genetics. 2008 Mar; 45 (3): 134-141. Epub 2007 Nov 9.

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FOREWORD

This thesis is divided into five main sections. Section one comprises a common introduction to the present work. It contains a basic introduction of defence reactions in general, immunology in particular, and the general aims of this thesis. This section is written so that readers outside the field can understand it. The following two sections provide an introduction the current view regarding the regulation of NK cell activity and the physiological and clinical significance of NK cells. Section two provides a detailed review of the molecular specificity of and events leading to human NK cell activation. Section three appraises the functions of NK cells as revealed by *in vitro* experiments, genetics, and clinical studies. The fourth section presents and discsses the findings contained within the work in this thesis. Finally, section five briefly derives some general conclusions and speculates on future prospects and perspectives in relation to the results in the presented work.

This thesis concerns the activation of human NK cells freshly isolated from peripheral blood. Papers I and II deal with NK cell recognition and elimination of target cells. The first papers identify the contributions of spetic receptors to NK cell activation. Paper III focus on activation of NK cells by an unusual receptor with putative implications for human reproduction. Papers IV–V assess NK cell cytotoxic function in patients suffering from a rare immunodeficiency syndrome characterized by excessive inflammation. The findings provide mechanistic insight into NK cell cytotoxicity, demonstrate applicability of laboratory research findings to the clinical diagnosis of immunodeficiency disorders, and offer clues to the biological significance of NK cell function.

Yenan T. Bryceson

Stockholm, July 22, 2008

ABBREVIATIONS

ADCC	antibody–dependent cellular cytotoxicity
ALPS	autoimmune lymphoproliferative syndrome
β2m	beta2-microglobulin
CHS	Chediak-Higashi syndrome
CIP	Cdc42 interacting protein
CMV	cytomegalo virus
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
FHL	familial hemophagocytic lymphohistiocytosis
GS	Griscelli syndrome
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HLH	hemophagocytic lymphohistiocytosis
HPS	Hermansky-Pudlak syndrome
HVEM	herpes virus entry mediator
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
ITSM	immunoreceptor tyrosine-based switch motif
KSHV	Kaposi’s sarcoma herpes virus
KIR	killer cell immunoglobulin-like receptor
LAD	leukocyte adhesion deficiency
LFA	leukocyte functional antigen
LIR	leukocyte immunoglobulin-like receptor
MAPK	mitogen–activated protein kinase
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MTOC	microtubule organizing center
NCR	natural cytotoxicity receptor

NK	natural killer
NLR	nucleotide-binding domain, leucine-rich repeat containing
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
RNA	ribonucleic acid
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SHP	Src homology 2 domain-containing phosphatase
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VCAM	vascular cell adhesion molecule
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
WIP	Wiskott-Aldrich syndrome protein interacting protein

1 INTRODUCTION

1.1 CONCEPTS OF LIFE

What life is remains an open question. However, the discovery of DNA as a common thread containing the blueprint for living organisms (1), and more recently the deciphering of whole genomes for many different species, including humans (2, 3), have provided an unprecedented framework for understanding the inner workings of life itself. Sustenance of life requires an orderly division of tasks. Evolution has given rise to increasingly complex assemblies of cells adept to a wide range of environments. Multicellular organisms strive to preserve order and integrity through intricate but precisely defined interactions. Maintaining such equilibrium, a process commonly termed homeostasis, is vital to life and requires sophisticated regulation. To counter threats to their existence and ensure biological fitness, organisms have developed a variety of genetically programmed defence reactions.

A prerequisite for a defence reaction is the recognition of an event as a threat to the wellbeing of the organism. Higher cognitive perceptions aside, an organism must recognize and discriminate between what is normal “self”, i.e. everything constituting an integral part of a given individual, and whatever “non-self”, whether foreign or altered “self”. Such recognition could, in theory, be positive or negative. In positive recognition, the organism actively recognizes “non-self”, whereas negative recognition implies reactions triggered by the failure to recognize “self”. Such discrimination is exemplified by biological systems in place to avoid self-mating in unicellular eukaryotes, whereas more complex organisms invest considerable resources in similar systems used to defend against pathogens (4). Pathogens are infectious agents that cause disease to their host. To a large extent, the experimental part of the present study deals with strategies for recognition and elimination of infected or aberrant cells that might otherwise pose a threat to the wellbeing of humans.

1.2 BASIC ASPECTS OF IMMUNOLOGY

The word “immunology” is derived from *immunis*, Latin for “exempt”. In this context, “exempt” usually is referred to being free of a particular disease. Individuals resistant to a disease were said to be immune to them. Thus, the status of a specific resistance to a disease is referred to as immunity.

Immunology covers the study of all aspects of the immune system in living organisms. The immune system is an organism’s physiological defence against infection. Infectious diseases are a leading cause of morbidity and mortality worldwide and are a major challenge for the biomedical sciences. Striving to preserve homeostasis, the immune system can also protect against cancer, another primary cause of death, by control of malignantly transformed cells. Thus, immunology aspires to improve human health.

In its broadest sense, the study of immunological defence reactions encompasses all cells in an organism. For example, cytosolic recognition

systems like the NLR (nucleotide-binding domain, leucine-rich repeat containing) family of proteins are widely expressed and sense diverse cellular insults such as microbial infections, reactive oxygen species, and crystal deposits (5). Likewise, cells ubiquitously express multiple endosomal and cytosolic receptors that sense viral nucleic acids (6). Such evolutionary conserved defence systems do not only act as cell intrinsic sentinels for pathogens. In complex organisms, these sentinels may also alert extrinsic systems consisting of specialized immune cells. Immune cells have long been the focal point of immunology. In classical terms, the study of vertebrate immunology has been divided into the study of defence reactions mediated by soluble products in the body fluids, referred to as humoral (from *humour*, Latin for “liquid”) and those mediated directly by cells, referred to as cellular. This thesis deals with cell-mediated immune reactions.

To determine appropriate action, immune cells rely on, together with soluble cues, surface receptors that engage target cell ligands and dictate the functional responses of the immune cells. To a large part, this function is dependent on specific recognition of foreign, “non-self” molecules, termed antigens. In addition, tissue damage or loss of “self” may also alert the immune system. The thousands of genes dedicated to immune function underscore the significance of the immune system to life. Based on the mechanisms by which different immune cells use to identify antigens, the immune system can logically be divided into two cooperative arms: the adaptive and the innate immune systems.

B and T lymphocytes constitute the adaptive immune system. These cells each express a unique antigen-specific receptor generated by somatic recombination of a limited number of genetic elements. In vertebrates, RAG proteins facilitate the generation of vast receptor diversity. The evolutionary appearance of the *RAG* genes in the vertebrate lineage coincided with the vertebrate species radiation approximately 500 million year ago (7). Upon encounter with a cognate antigen, cells expressing a receptor with appropriate specificity to the antigen are clonally expanded, a process involving cellular proliferation that in effect takes 1-2 weeks. Providing immunological memory, the adaptive immune system can mount a more rapid and effective response on subsequent encounters with the same antigen.

The innate immune system is often viewed as a primordial first line of defence against infection. In contrast to adaptive immune cells that undergo somatic recombination, innate immune cells rely on germline-encoded receptors that recognize conserved molecular patterns discriminating microorganisms from our own cells. Many pathogens are cleared rapidly without the aid of adaptive immune functions. In situations where the innate immune system is unable to eliminate a pathogen on its own, it acts to limit the infection until antigen specific clones of B and T cells have been sufficiently expanded to ensure elimination. Although adaptive B and T cells have been a principal focus of immunologist for their ability to confer protection to numerous pathogens, the fundamental role of innate immune cells in conferring protection and eliciting immune responses is increasingly being appreciated (8).

The adaptive and innate immune systems collaborate in a concerted fashion to destroy pathogens, reciprocally enhancing each other's actions (9). Through release of cytokines and presentation of peptides, cells of the innate immune system initiate and direct adaptive responses. Conversely, B cells secrete antibodies that activate complement and identify targets for phagocytosis or lysis by innate immune cells.

Together, immune cells possess potent weaponry to eliminate pathogens and maintain homeostasis. At the same time, mechanisms must be in place to contain such effector functions, avoiding excessive damage to host cells and tissues. Disproportionate immune reactions may culminate in pathological conditions such as autoimmunity. Therefore, a well-functioning immune system exerts self-constraint, at once counter-balancing signals for activation with others that contain or terminate responses, thereby avoiding tissue damage and limiting energy expenditure. Through evolution of genetic elements, life itself seeks a balance between efficient pathogen elimination and self-tolerance. Understanding the mechanisms underlying proper immune function, and what happens when they go awry, will hopefully provide us with knowledge and ability to manipulate the immune system for the benefit of human health.

1.3 GENERAL AIMS OF THIS THESIS

This thesis will focus on cell-mediated cytotoxicity by natural killer (NK) cells. Originally, NK cells were described as large granular lymphocytes with the ability to kill tumor cells without prior sensitization through parallel efforts by Rolf Kiessling at the Karolinska Institute, Stockholm, Sweden, and Ronald Herberman at the National Institutes of Health, Bethesda, MD, USA, respectively (10, 11).

In the 1980s, work by Klas Kärre and Hans-Gustaf Ljunggren provided evidence for NK cell recognition of target cells based on the absence of certain self-markers, rather than the presence of foreign antigen (12, 13). Later, NK cell receptors that confer protection to normal cells were identified in mice and humans by the laboratories of Wayne Yokoyama and Eric Long, respectively (14, 15). More recently, a number of receptors involved in activation of NK cells have been characterized by several different laboratories (16, 17). Still, the individual contribution of disparate activating receptors to NK cell effector function is not clear.

One general aim of this thesis was to define the minimal requirements for activation of NK cell effector functions, thereby providing insight into the strategies employed by NK cells for target cell recognition. To facilitate translation of basic findings into clinical use, the studies focused on the activation of freshly isolated NK cells from human subjects. Furthermore, techniques established through basic research were applied to the diagnosis of severe immunodeficiency syndromes, with the prospect of improving treatment of patients and facilitating further understanding of the immune system.

For details about the protocols of the experiments, the reader is referred to the material and methods section of the individual papers (I–V).

2 NK CELL ACTIVATION

2.1 DEFINITION, ONTOGENY, AND DISTRIBUTION

NK cells represent a third lymphocyte lineage and an arm of the innate immune system. NK cells were originally defined based on their ability to kill tumor cells without prior sensitization (10, 11). Nowadays, NK cells are usually defined phenotypically as CD3⁻ CD56⁺ cells in humans and CD3⁻ NKR-P1⁺ cells in mice (18). Alternatively, a definition of NK cells as NKp46⁺ cells has been proposed (19). Because NKp46 is a marker almost exclusively expressed on NK cells (20), and is conserved among mammalian species (21-24), such a definition could facilitate improved cross-species comparisons of NK cell function. Still, it should be noted that NK cells remain a heterogeneous population of cells differing in regards to expression of chemokine, adhesion, activation, and inhibitory receptors, as recently reviewed (25, 26).

Several key aspects of NK cell development occur in the bone marrow. These include commitment of hematopoietic precursors to the NK cell lineage, education of immature NK cells towards self markers, acquisition of receptors involved in target cell recognition, and establishment of functional competence, as has been reviewed (26-28). Recent evidence suggest that NK cells may also develop in the thymus and secondary lymphoid organs (29-31). Nonetheless, athymic humans and mice have functionally competent NK cells (32-34). Thus, the ontogenic relationship between different NK cell subsets and relevance of thymic NK cell development is not clear (26).

In regards to distribution, NK cells are widespread throughout lymphoid and non-lymphoid tissues, as has been recently reviewed (35). In mice, the frequency of NK cells in relation to all lymphocyte subsets is highest in non-lymphoid organs such as the liver and lung (35). Human NK cells are also abundant in liver (36). Effector memory CD8⁺ cytotoxic T lymphocytes (CTL) display a similar pattern of distribution in peripheral tissues (37). Of note, at birth, human cord blood NK cells are functionally mature, whereas T cells are predominately naïve. Effector CTL develop gradually as a result of infections, accumulating in peripheral tissue in an age-dependent manner (38). In humans, peripheral blood NK cells are readily accessible for *ex vivo* analysis and constitute approximately 5-20% of adult circulating peripheral blood lymphocytes (39). Human NK cell turnover in blood is around 2 weeks (40), consistent with data in the mouse (20, 41). Noteworthy, NK cells are the predominant lymphocyte population in the placenta during pregnancy (42), where they constitute a phenotypical and functional unique NK cell subset (43, 44). A fundamental enigma of pregnancy it that the fetal cells constitute an allograft. Yet, in normal pregnancies, they are in effect not perceived as foreign and are not rejected by the maternal immune system.

2.2 MOLECULAR SPECIFICITY OF TARGET CELL RECOGNITION

NK cells contribute to host defence by their ability to rapidly secrete cytokines and chemokines, as well as to directly kill infected or malignant host cells. Distinct from T and B lymphocytes, NK cell function is controlled by a limited repertoire of germline-encoded receptors that do not undergo somatic recombination (16, 17). Together with CTL, NK cells share a common mechanism for target cell killing that relies on directed exocytosis of secretory lysosomes that contain lytic proteins such as perforin, granzymes, Fas ligand, and TRAIL (45-47). In addition, NK cells are a major source of chemokines, such as macrophage inflammatory protein (MIP)-1 α (CCL3) and MIP-1 β (CCL4), and cytokines, such as tumor necrosis factor (TNF)- α and interferon (IFN)- γ . MIP-1 α and MIP-1 β recruit other immune cells to sites of inflammation (48). Impeding their function can impair the generation of adaptive CTL responses (49). TNF- α initiates pro-inflammatory cytokine cascades (50), while IFN- γ promotes Th1 differentiation (51), enhances major histocompatibility class (MHC) I expression (52), and has potent anti-mycobacterial, anti-viral, and growth inhibitory effects (53, 54). In addition to target cell recognition, NK cells produce cytokines and chemokines in response to soluble mediators, such as IL-12 and IL-18 (55).

According to the prevailing view, NK cells distinguish normal, healthy cells from sensitive target cells by a balance between signals from numerous activating and inhibitory receptors (56-58). The net income of key positive and negative signaling events is thought to determine the capacity of NK cells to kill target cells. However, the precise molecular checkpoints where signals from inhibitory receptors abrogate activating receptor pathways are not well defined (59). Most receptors either belong to families of genes encoding highly polymorphic extracellular domains, or bind to polymorphic ligands. The apparent plasticity in NK cell receptor recognition is further underlined by the fact that genetic context varies among individuals in a population and functional orthologs of some genes are not conserved among different mammals. Thus, the genetics of NK cell receptors and the actual recognition of target cells are complex processes. The following sections will examine the specificity and proximal signaling of human NK cell receptors.

2.2.1 Inhibitory receptors

In combat with rapidly evolving pathogens, NK cells must achieve specific recognition of infected or transformed cells, yet maintain tolerance for self. The 'missing-self' hypothesis (60) advocates a central role for NK cell inhibitory receptors and target cell major histocompatibility (MHC) class I expression in determining NK cell specificity. MHC class I molecules present endogenous peptides to T cells for adaptive immunity to intracellular pathogens, a strategy for recognition of "non-self". Thus, by eliminating cells with decreased MHC class I expression, NK cells form a functional complement to T cell-mediated immunity. Inhibitory receptors expressed on human resting NK cells and their ligands are listed in *Table 1*.

Receptor	Cellular ligand	Function	
KIR2DL1 (CD158a)	HLA-C group 2	MHC	Assess loss of MHC class I alleles
KIR2DL2/3 (CD158b1, b2)	HLA-C group 1		Assess loss of MHC class I alleles
KIR3DL1 (CD158e1)	HLA-B alleles		Assess loss of MHC class I alleles
KIR3DL2 (CD158k)	HLA-A alleles		Assess loss of MHC class I alleles
LIR-1/ILT2 (CD85j)	Multiple HLA class I		Assess loss of MHC class I expression
ITIM NKG2A (CD94/CD159a)	HLA-E	Non-MHC	Gauge MHC class I expression
KLRG1	E/N/P-cadherin		Assess loss of tissue integrity
NKR-P1 (CD161)	LLT1		?
LAIR-1 (CD305)	collagen		Control activation in extracellular matrix
Siglec-7 (CD328)	sialic acid		?
Siglec-9 (CD329)	sialic acid		?
IRp60 (CD300a)	?		?

Table 1. *Human NK cell inhibitory receptors. Inhibitory receptors expressed by freshly isolated resting NK cells, their ligands, and their function are listed.*

Surprisingly, inhibitory receptors for classical MHC class I molecules in humans (killer cell immunoglobulin-like receptors, KIR, CD158) and rodents (lectin-like Ly49), separated by 70 million years of evolution, are structurally distinct (14, 15). However, the genetic loci that encode receptors for MHC class I in the two species represent a striking example of convergent evolution (61). First, both KIR and Ly49 loci contain rapidly evolving genes that have arisen through extensive gene duplications (62). Second, the loci are highly polymorphic among different individuals at the level of gene content (63). Some alleles even encode an activating counterpart to an inhibitory receptor. Third, different receptors for MHC class I are expressed on distinct peripheral blood NK cell subsets. Other inhibitory NK cell receptors for human leukocyte antigens (HLA, or human MHC class I), such as NKG2A (CD159a) and leukocyte immunoglobulin-like receptor (LIR)-1 (ILT2, CD85j), also display variegated expression patterns. Recent analysis has revealed that all possible subsets of inhibitory receptor combinations are expressed within the NK cell population of any given individual (64). Fourth, in spite of diversity in the extracellular ligand binding domains, NK cell inhibitory receptors appear to use a common mechanism for inhibition. Upon engagement of classical MHC class I molecules (HLA-A, -B, -C), KIR can mediate inhibition of NK cell responses through the recruitment of the Src homology 2 domain-containing phosphatases (SHP)-1 and SHP-2 to phosphorylated, cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) (65-67). Similarly, CD94/NKG2A, LIR-1, and mouse Ly49 receptors also contain cytoplasmic ITIMs that are capable of recruiting SHP-1 and SHP-2 (68). The ligand of the CD94/NKG2A lectin heterodimer is the non-classical MHC class I molecule HLA-E, which in turn serves as a gauge of classical MHC class I expression through its unique requirement for stabilization by leader peptides from HLA molecules (69-71). LIR-1 is an Ig-superfamily receptor that binds several alleles

of classical MHC class I, in addition to the non-classical MHC class I molecules HLA-G and HLA-F (72-74). Functional and crystallographic studies suggest that KIR may exhibit a degree of peptide selectivity (75-79). Interestingly, as a result of cellular stress, peptides derived from heat-shock proteins may replace MHC class I leader peptides in HLA-E, abrogating NKG2A binding to HLA-E and inhibition of NK cells (80). The high resolution crystal structure of CD94/NKG2A, in combination with results from mutagenesis studies, has led to a model for the CD94/NKG2A–HLA-E complex. According to the model, the CD94 chain has a more dominant role in the interaction with HLA-E, as compared to NKG2A (81). Thus, in spite of a degree of peptide selectivity, these receptors are generally considered to bestow NK cells with means of evaluating target cell expression of multiple “self” molecules.

Inhibitory receptors for MHC class I are thought to mediate NK cell self-tolerance (82, 83). However, in spite of defective MHC class I expression, NK cells are self-tolerant in $\beta 2m$ -deficient mice (84, 85) or TAP-deficient humans and mice (86, 87). Remarkably, defective MHC class I expression leads to attenuated NK cell responses (84-87). Furthermore, a subpopulation of NK cells that lack known inhibitory receptors for self-MHC class I exists in humans and mice, but display reduced responsiveness relative to NK cells expressing inhibitory receptors (88, 89). Likewise, expression of inhibitory receptors specific for self-MHC confers greater responsiveness to NK cells (89, 90), a property termed “licensing”, which requires functional ITIMs (90). Thus, NK cell reactivity is somehow “calibrated” by the MHC class I environment. The potency with which NK cells reject cells with aberrant MHC class I expression appears to correlate with the number and strength of inhibitory receptor – MHC class I interactions (90, 91).

Furthermore, non-MHC class I ligands for other ITIM-containing inhibitory receptors have been identified. The inhibitory lectin-like receptor KLRG1, expressed on a subset of NK cells (92, 93), binds members of the ubiquitously expressed cadherin family of cell-junction proteins in both humans and mice (94, 95). Loss of E-cadherin expression during metastasis and invasiveness of epithelial tumors has been suggested to facilitate NK cell surveillance of epithelial tumors (95). Indeed, mutations in E-cadherin that abrogate KLRG1 binding have been detected in diffuse type gastric carcinomas (96). Another inhibitory lectin-like receptor, NKR-P1 (CD161), binds the related lectin-like molecule LLT1 in humans or other LLT1-homologues in mice (97-100). LLT1 is expressed on activated plasmacytoid and monocyte-derived dendritic cells, in addition to B cells stimulated through Toll-like receptor (TLR) 9, surface Ig, or CD40 (101). LAIR-1 (CD305) is an inhibitory receptor that binds collagen and is widely expressed on immune cells (102). Notably, LAIR-1 has a unique ability to inhibit independently of tyrosine phosphatases SHP-1 and SHP-2. Even though its phosphorylated ITIM binds to the SH2 domain of SHP-1 and SHP-2, as is typical for ITIM-containing receptors, LAIR-1 can also deliver inhibitory signals by binding the SH2 domain of tyrosine kinase Csk (103), which negatively regulates Src-family kinases by phosphorylation of a C-terminal tyrosine (104). Subsets of human NK cells also express inhibitory, sialic acid-binding Siglec-7 (CD238) and Siglec-9 (CD239) receptors (105-108).

Furthermore, the inhibitory receptor IRp60 (CD300a) is expressed by all resting NK cells (109), but ligands have not been identified. Whether inhibitory receptors for non-MHC class I ligands contribute to NK cell calibration has yet to be investigated.

2.2.2 Activating receptors

The discovery of ITIM-containing inhibitory receptors suggested that their interaction with MHC class I governed the specificity of NK cells for target cells. However, it has become clear that activation receptors contribute substantially to NK cell specificity. NK cells kill preferentially hematopoietic cells, whereas many tumors derived from other tissues are resistant to NK cells (110). This property has been exploited to improve the outcome of bone marrow transplantation. NK cells in T cell-depleted allogeneic hematopoietic grafts can mediate beneficial graft-versus-leukemia effects, without necessarily causing graft-versus-host disease (111, 112). These and other data imply that NK cell reactivity can be limited even in the absence of MHC class I on target cells. Although inhibitory receptors for non-MHC class I ligands may also control NK cells, the available evidence suggests that NK cells are not pre-wired to kill any encountered cell but depend on the expression of sufficient ligands for positive recognition.

A large number of structurally distinct activating NK cell receptors have been characterized (113, 114). In contrast to inhibitory receptors, most activating receptors are expressed by all NK cells. Furthermore, activating receptors induce diverse signaling cascades, whereas inhibitory receptors appear to use a common mechanism for inhibition. Some of the activating receptors expressed on human resting NK cells are listed, together with their ligands, in *Table 2*.

Activating receptors associated with immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor proteins propagate strong activation signals through the recruitment of the tyrosine kinases Syk and ZAP-70 (113, 114). Such receptors can be further subdivided into two groups; the first includes rapidly evolving receptors expressed on subsets of NK cells, such as KIR2DS, KIR3DS, and NKG2C (CD159c). The extracellular domains of these receptors are closely related to MHC class I-specific inhibitory receptor counterparts. These receptors associate with the ITAM-containing adaptor chain DAP12 (115). Some activating KIRs bind classical MHC class I (116), whereas NKG2C binds HLA-E (69, 117). Generally, binding of activating receptors to MHC class I exhibit lower affinity than that of their related inhibitory receptor counterparts. Interestingly, one report suggests that KIR2DS1 recognizes particular MHC class I-peptide complexes expressed on Epstein-Barr virus (EBV) infected cells (116). Conservation of homologous activating and inhibitory receptor pairs through evolution may be important for maintaining immune system equilibrium (68), or may result from the selective pressure imposed by pathogens (118, 119).

	Receptor	Cellular ligand	Function
ITAM	CD16 (Fc γ RIIIA)	IgG	Elimination of antibody-coated cells (ADCC)
	NKp30 (CD337)	BAT-3	Surveillance of genotoxic stress/transformation
	NKp46 (CD335)	?	Surveillance of mitotic cells
	KIR2DS1–2	HLA-C (low affinity)	?
	KIR2DS3–6	?	?
	KIR3DS1 (CD158e2)	?	?
	NKG2C (CD94/159c)	HLA-E	?
	NKG2D (CD314)	ULBPsMICA, MICB	Surveillance of tumor cells and genotoxic stress
	NKp80	AICL	NK cell-myeloid crosstalk
	DNAM-1 (CD226)	PVR (CD155), CD112	Surveillance of tissue integrity
Non-ITAM	2B4 (CD244)	CD48	Interaction with hematopoietic cells
	CRACC (CD319)	CRACC (CD319)	Interaction with hematopoietic cells
	NTB-A	NTB-A	Interaction with hematopoietic cells
	CD2	LFA-3 (CD58)	Interaction with hematopoietic cells and endothelial cells
	CD7	SECTM1, Galectin	?
	CD59	C8, C9	Complement regulatory protein
	BY55 (CD160)	HLA-C	?
	KIR2DL4 (CD158d)	HLA-G (soluble)	Trophoblast-induced vascular remodeling?
	CD44	Hyaluronan	Interaction with extracellular matrix
	LFA-1 (α L β 2, CD11a/18)	ICAM-1–5	Recruitment and activation during inflammation, polarization
Integrin	MAC-1 (α M β 2, CD11b/18)	ICAM-1, iC3b, Fibrinogen	Adhesion
	CD11c/18	ICAM-1, iC3b	Adhesion
	VLA-4 (α 4 β 1, CD49d/29)	VCAM-1, Fibronectin	Recruitment during inflammation, adhesion to matrix
	VLA-5 (α 5 β 1, CD49e/29)	Fibronectin	Adhesion to extracellular matrix

Table 2. *Human NK cell activating receptors. Activating receptors expressed by freshly isolated resting NK cells, their ligands, and their function are listed.*

The second group of ITAM-associated receptors, includes CD16, NKp30 (CD337), and NKp46 (CD335), that are expressed on most resting NK cells. CD16 signals through the Fc ϵ RI γ -chain and the CD3 ζ -chain and induces antibody-dependent cellular cytotoxicity (ADCC) (120–122). CD16 binds the lower hinge region of IgG (123). Natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 were identified for their role in natural cytotoxicity towards tumor cells (21, 124, 125) *in vitro*. NKp44 is expressed only on IL-2–cultured NK cells (124). NKp30 and NKp46 are not structurally related, but contain a transmembrane arginine residue, which forms salt-bridges with transmembrane aspartate residues in CD3 ζ -chain homodimers (126). The nature of the ligands for NCRs is still unclear. Although NKp46 has been reported to bind viral hemagglutinins via sialic acid modifications on infected cells (127, 128), cellular ligands have not been defined. NKp46 contributes to the enhanced killing of mitotic cells by NK cells, suggesting a role for NKp46 in controlling expansion of rapidly dividing cells (129). NKp30 mediates killing of immature dendritic cells by NK cells (130). Surprisingly, an intracellular protein implicated in the induction of apoptosis after DNA damage or endoplasmic reticulum stress, called BAT3, was recently described as a ligand for NKp30 (131). How BAT3 becomes exposed at the cell surface is not known. Furthermore, immunostaining of several tumor cells with soluble forms of NKp30 and NKp44 resulted in intracellular straining, suggesting that translocation from the inside to the surface of cells may be a common theme among ligands for NCRs (132). In support of this notion, the human cytomegalovirus tegument protein pp65,

which is not expressed at the cell surface of infected cells, has also been identified as a ligand for NKp30 (133). However, binding of pp65 results in the inhibition of NK cell cytotoxicity induced by NKp30, which may represent one of the many evasion tactics developed by human cytomegalovirus to counter detection by NK cells.

A second category of activating receptors do not contain ITAMs or associate with ITAM-carrying adaptors. They include NKG2D (CD314), the CD2 family members CD2, 2B4 (CD244), CRACC (CD319), and NTB-A, DNAM-1 (CD226), and NKp80. Human NKG2D associates with the adaptor protein DAP10 (134-137), which carries a phosphatidylinositol-3 kinase (PI3K) binding motif. The phosphorylated form of this tyrosine motif can bind the p85 subunit of PI3K and Grb2 (138). Ligands for NKG2D, such as MICA, MICB, and ULBP, are expressed on some tumor cells, and on infected or stressed cells (139-141). NKG2D ligands can be induced by genotoxic stress and stalled DNA replication, conditions that activate DNA damage checkpoint pathways (142). Detection of tumor cells by NKG2D can be counteracted by soluble NKG2D ligands, which are shed from the cell surface after cleavage by the plasma membrane associated protease Erp5 (143). While NKG2D provides an important defence against tumors (144, 145), it can also contribute to autoimmunity (146, 147).

CD2 signaling in NK cells is largely unknown. CD2 binds to CD58 (148). 2B4 (CD244) can recruit SAP and Fyn through cytoplasmic immunoreceptor tyrosine-based switch motifs (ITSMs) (149, 150). The ligand of 2B4 is CD48, which is expressed on hematopoietic cells (151). CRACC and NTB-A also contain ITSMs, and are involved in homotypic interactions between hematopoietic cells (152-154). The crystal structures of CRACC homophilic interactions and 2B4 in complex with CD48 were recently solved (155, 156). At 11 and 11.5 nm, the membrane spacing required for homophilic CRACC interactions and 2B4-CD48 interactions, respectively, is similar to the space required for KIR-MHC class I interactions (77, 155, 156). Thus, activating receptors such as 2B4 and CRACC could potentially intermix with inhibitory KIR at the NK cell immune synapse, facilitating dynamic assessment of activation thresholds. DNAM-1 is associated with leukocyte functional antigen (LFA)-1 in NK cells (157), is phosphorylated by a protein kinase C (PKC) (158), and binds to the polio virus receptor (PVR, CD155) and Nectin-2 (CD112) (159). On NK cells, DNAM-1 may facilitate surveillance of damaged endothelium and transformed cells (160, 161). NKp80 is another NK cell activation receptor with unknown signaling properties (162). The cellular ligand of NKp80 was recently identified as activation-induced C-type lectin (AICL) (163). The NKp80 and AICL genes are closely linked on in the NK cell gene complex on chromosome 12. Expression of AICL is confined to granulocytes and macrophages, and is up-regulated by inflammatory stimuli (163). Thus, NKp80-AICL interactions may be important for NK cell-myeloid cell crosstalk during immune reactions. Signaling by NKp80 has so far not been characterized. Similar to ITAM-associated receptors, receptors within this category are capable of inducing target cell lysis by IL-2-cultured NK cells in redirected lysis assays (139, 153, 154, 158, 162, 164-167). A possible caveat

of several of these studies is that IL-2–cultured NK cells might not fully resemble physiological NK cells.

Additional activating receptors expressed by all resting NK cells include CD7, CD44, CD59, KIR2DL4 (CD158d), and BY55 (CD160). CD7 encodes a cytoplasmic PI3K binding motif, binds SECTM1 or Galectin-1 (168, 169), can enhance NK cell cytokine secretion and β_1 -integrin–dependent adhesion to fibronectin, but does not induce cytotoxicity (170). CD44 binds hyaluronan, a constituent of the extracellular matrix. Engagement of CD44 on does not induce cytotoxicity, but can co-stimulate CD16–dependent cytotoxicity by resting NK cells (171, 172). Engagement of CD44 on IL-2 or IL-12–activated NK cells can induce cytotoxicity (172). CD59 lacks a cytoplasmic tail but associates with NKp30 and NKp46 (173). Engagement of CD59 induces CD3 ζ -chain phosphorylation (173). Generally, CD59 binds complement C8 and C9, whereby formation of a membrane attack complex is prevented. Engagement of CD59 co-stimulates human NK cells (173). KIR2DL4 is an evolutionary conserved framework member of the KIR gene family (174). Atypical among KIRs, KIR2DL4 is expressed by all KIR haplotypes and in all NK cells (175). KIR2DL4 contains both a cytoplasmic ITIM and encodes a transmembrane arginine residue, through which it can associate with the Fc ϵ R γ -chain (176, 177). KIR2DL4 binds the non-classical MHC class I molecule HLA-G (178, 179). HLA-G exhibits limited polymorphism and has a unique expression pattern restricted mainly to trophoblast cells that invade the maternal decidua during early pregnancy (180). HLA-G expression may be inducible in other cell types in response to inflammation, infection, or transformation (181). Engagement of KIR2DL4 does not induce cytotoxicity but cytokine production by freshly isolated NK cells (182). Signaling by BY55 (CD160) is not well characterized. BY55 binds HLA-C and induces cytokine production by NK cells (183). Of note, recent data suggest that BY55 on T cells can bind HVEM and inhibit T cell activation (184).

Integrins represent a different category of NK cell activating receptors, which are heterodimers of α and β subunits, such as the α_L and β_2 subunits of LFA-1 (CD11a/CD18). LFA-1 binds intercellular adhesion molecules (ICAM)-1 through -5 (185). LFA-1 facilitates natural cytotoxicity and ADCC, as anti-LFA-1 blocking antibodies impair these processes (186–189). NK cells also express lower levels of β_2 -integrins Mac-1 (CD11b/CD18) and CD11c/CD18.

The β_1 -integrins expressed on NK cells, namely $\alpha_4\beta_1$ (very late antigen (VLA)-4, CD49d/CD29) and $\alpha_5\beta_1$ (CD49e/CD29), contribute activation signals upon binding to their ligands, vascular cell adhesion molecule (VCAM)-1 and fibronectin (190). Fibronectin coated on plates is sufficient to induce activation of mitogen–activated protein kinases (MAPK) in NK cells, specifically Erk and p38 (191). Interestingly, β_1 -integrin engagement induces IL-8 production by NK cells, through a signaling pathway that involves Vav1/Rac1 and p38 MAPK activation (191).

Engagement of $\alpha_4\beta_1$ integrin activates Pyk2 and tyrosine phosphorylation of paxillin (192), and co-stimulates NK cell cytotoxicity (193). The complexity of intersecting signaling pathways in NK cells is illustrated by the inhibition of

CD16–induced phospholipase D activation and degranulation, upstream of Ca^{2+} release, by co-ligation of $\alpha_4\beta_1$ integrin (194). The reason for this β_1 integrin–mediated negative regulation is unknown. In addition, LFA-1–dependent migration of T cells is transactivated by $\alpha_4\beta_1$ through binding of paxillin to the α_4 cytoplasmic tail and activation of Pyk2 (195). These data suggest that β_1 -integrins may also regulate LFA-1–dependent signals in NK cells. Trans-regulation is mutual, as LFA-1 engagement up-regulates ligand binding by β_1 -integrin (196).

Inhibitory NK cell receptors, which display variegated expression patterns on resting NK cell populations, may on the one hand potentiate NK cell effector function through calibration (82, 83), and on the other restrict activation towards targets expressing ligands for inhibitory receptors. Further, it is likely that cells in many tissues normally are not susceptible to NK cell mediated surveillance, because they do not express sufficient levels of ligands to induce NK cell activation. Which of the many receptor–ligand interactions are sufficient or required for NK cell activation, and how receptors integrate to mediate NK cell activation requires further knowledge of the activation process. The multiplicity of NK cell activation pathways may in part have been selected to counteract attempts by pathogens to circumvent NK cell-mediated immune surveillance. The next section will review the molecular events involved in activation of NK cells.

2.3 DISCRETE EVENTS IN NK CELL ACTIVATION

Recruitment of NK cells to sites of inflammation is a prerequisite for participation of NK cells in immune responses. NK cells express a number of chemokine receptors that can facilitate extravasation and recruitment to sites of inflammation in response to chemokines released by tissue resident cells (197, 198). This section will focus on events that comprise NK cell recognition of target cells and activation of effector function.

The induction of NK cell effector functions, including cytotoxicity, requires contact of NK cells with target cells. This ensures precise targeting of the cytolytic response to individual aberrant cells, thereby limiting potential damage to bystander cells. Several discrete events occurring on interaction between cytotoxic effector cells and target cells have been described (199-201). A central concept is the immunological synapse, relating to an organized arrangement of receptor-ligand interactions at the interface between the effector and the target cell (202) (*Figure 1*). In NK cells, accumulation of F-actin, intracellular signaling molecules, adhesion receptors, activating receptors, and inhibitory receptors has been observed upon target cell recognition (203-205). The immunological synapse itself has been proposed to serve as a platform for integrating signaling and directing secretion (202, 206). Clearly, further understanding of the significance of immunological synapses necessitates studies addressing the formation of immunological synapses and their function in live cells. The following sections will consider tangible events leading to NK cell effector functions and discuss their molecular basis.

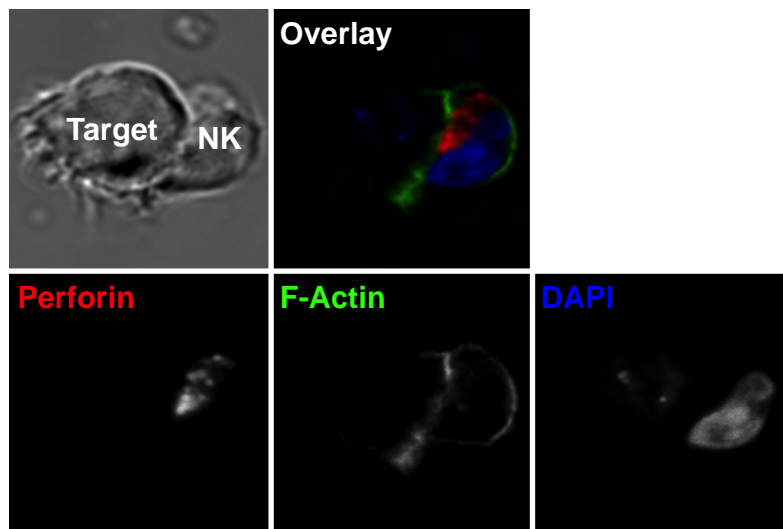


Figure 1. *The NK cell immune synapse.* Images depict a resting NK cell in conjugate with a target cell susceptible to NK cell lysis, as indicated. Cells were fixed, permeabilized, and stained with perforin (red) for visualization of secretory lysosomes, phalloidin (green) for visualization of polymerized actin, and nuclear stain DAPI (blue).

2.3.1 Contact

It is not clear which receptors provide initial signals upon NK cell contact with target cells. Studies of T and B cells have suggested a prominent role for antigen-specific receptor signaling in the initiation of adhesion. Which of the many NK cell activation receptors signal upstream of LFA-1-mediated adhesion? Using a reductionistic model target cell system, where ligands for human NK cell receptors are expressed in *Drosophila* cells, Barber *et al.* (207) have demonstrated that expression of human ICAM-1 is sufficient to induce signaling-dependent adhesion by resting NK cells. Moreover, recombinant, plate-coated ICAM-1 also induces adhesion of resting NK cells (207). Together, this suggests that LFA-1 can provide autonomous signals for adhesion in resting NK cells. Importantly, the potential of numerous other NK cell receptors to initiate contact and provide signals for adhesion remains to be assessed.

2.3.2 Adhesion

Adhesion is thought to be a prerequisite for NK cell effector functions, providing stable contact with the target cell and leading to the formation of an immune synapse. Interaction of integrins with ligands on target cells must be regulated dynamically, as release from adherence is required for lymphocyte movement. Importantly, these initial stages in NK cell recognition likely occur prior to the molecular patterning observed in immune synapses.

LFA-1 has previously been attributed a central role in NK cell adhesion (208). In the *Drosophila* cell system, adhesion was evaluated by formation of conjugates between NK cells and target cells expressing specific ligands. Engagement of LFA-1 by ICAM-1 is sufficient to induce adhesion by human resting NK cells (207). LFA-1–dependent adhesion can be augmented by stimulation with exogenous IL-2 and IL-15 (207). Resting NK cell adhesion is also augmented by the co-expression of ligands for CD2 and 2B4 (207). Engagement of CD2, or 2B4 alone does not induce adhesion (207). The use of pharmacological inhibitors of the actin cytoskeleton, Src-family kinases, or PI3K indicated a signaling–dependent role for CD2 and 2B4 in enhancing LFA-1–dependent adhesion (207). In LFA-1–deficient mice, IL-2–activated NK cells have a profound deficiency in target cell adhesion (209). Interestingly, the immunoglobulin superfamily molecule CD44 facilitates LFA-1–dependent adhesion, as LFA-1–dependent adhesion is diminished in CD44–deficient mice (209). Analysis of cells from patients affected by leukocyte adhesion deficiency (LAD) type 1, caused by mutations in the gene encoding the β_2 -subunit, revealed that IL-2-cultured NK cells from LAD1 patients can kill target cells (210). However, lysis of murine target cells by human IL-2 cultured NK cells from these patients was impaired (210). Moreover, previous studies of similar patients have noted a defective lysis of human NK cell-sensitive target cells by peripheral blood lymphocytes from affected individuals (211).

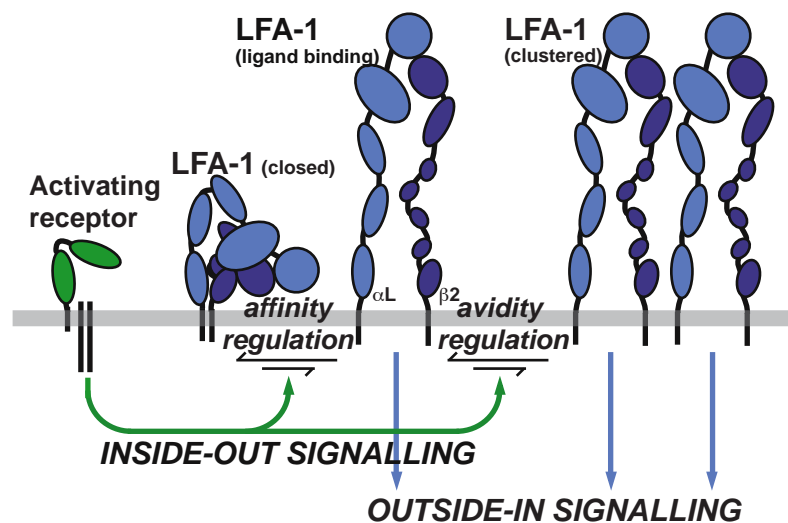


Figure 2. *Regulation of LFA-1–mediated adhesion.* Inside-out signals from NK cell activating receptors may promote conformational changes leading to a high-affinity, ligand-binding conformation of LFA-1. They may also promote LFA-1 avidity through signals for clustering of LFA-1. Upon ligand binding, LFA-1–mediated outside-in signals are conveyed into the cell.

Adhesion and signaling by LFA-1 is a carefully orchestrated process. Activating receptors may provide inside-out signals, which increase LFA-1 affinity through conformational changes (Figure 2) (212, 213). Alternatively, signals from activating receptors may also induce LFA-1 clustering, whereby LFA-1 avidity

is enhanced (*Figure 2*) (212, 213). In the resting state, the α_L and β_2 cytoplasmic domains are in close proximity and the extracellular domain is closed, but either inside-out signaling or ligand binding induces an extended conformation of the extracellular domain coupled to a spatial separation of the cytoplasmic domains (214). In the ligand binding conformation, LFA-1 itself can transduce outside-in signals (*Figure 2*). Apart from the LFA-1 outside-in signals themselves promoting LFA-1 adhesion (thereby conferring inside-out signals) (207), evidence of inside-out signaling by other NK cell receptors has not been directly assessed in terms of LFA-1 affinity or avidity. Data remain circumstantial, demonstrating a combined contribution by LFA-1 and other receptors such as CD2 and 2B4 in augmenting LFA-1–dependent adhesion.

Many signaling molecules and pathways have been implicated in the modulation of LFA-1 affinity (215). In other cell types, LFA-1 affinity is intimately coupled to regulation of the actin cytoskeleton. For example, LFA-1 affinity can be promoted by calpain, a Ca^{2+} –dependent protease (216). Calpain–mediated cleavage of talin, a cytoskeletal component, produces a talin fragment that binds the cytoplasmic tail of integrin β chains, thereby inducing separation of the cytoplasmic tails and augmenting LFA-1 affinity (214, 217). Evidence suggests competition for β chain binding between talin and another actin-binding protein, filamin. Binding of filamin inhibits integrin affinity. Talin and filamin binding sites on the β chain overlap, and talin binding might be promoted by phosphorylation of threonine residues in the filamin binding site that would displace filamin (218, 219). PKC δ and β I/II are the major kinases in lymphocyte extracts able to phosphorylate β -chain residues involved in filamin binding (220).

In T cells, a distinct role has been described for the GTPase Rap1 in regulation of LFA-1 avidity. In resting T cells, a fraction of LFA-1 is phosphorylated on α_L Ser1140. Phosphorylation of this residue is required for induction of LFA-1 clustering by Rap1 (221). Activation of Rap1 by chemokine stimulation or T cell receptor engagement can induce RAPL binding to the α_L cytoplasmic tail, which in turn leads to redistribution of LFA-1 to the immunological synapse (222).

Thus, although genetic evidence suggests a major contribution of LFA-1 to NK cell adhesion, the potential contribution of other receptors to NK cell adhesion and signaling pathways that regulate NK cell adhesion remain to be assessed.

2.3.3 Polarization

For cytotoxic cells in general, polarization of the secretory lysosomes (also called cytotoxic granules) precedes target cell cytotoxicity (223). Early studies of the interaction between NK cells and sensitive target cells revealed that adhesion was accompanied by NK cell polarization of the actin cytoskeleton, the Golgi apparatus, and microtubules towards the target cell interface (224–226). Live cell imaging experiments during natural cytotoxicity by lymphokine–activated killer cells demonstrated that NK cells establish cytoskeletal polarity in a stepwise fashion, suggesting a series of checkpoints, as opposed to cytolytic T cells where antigen induces rapid and robust cellular polarity (199).

In T cells, engagement of the antigen receptor is required to induce polarization (227). The receptor–ligand interactions required or sufficient for polarization in NK cells were until recently undefined. In IL-2–cultured NK cells, target cell expression of ICAM-1, leading to LFA-1 engagement on NK cells, is sufficient to induce granule polarization (228).

The signals that regulate granule polarization in NK cells are not well defined, but expression of dominant–negative Rac1 and RhoA does inhibit polarization of perforin in IL-2–cultured NK cells, whereas over-expression of Vav1 enhances polarization (229). Further, pharmacological inhibitors of PI3K inhibited polarization of perforin and suppressed cytotoxicity in an IL-2–dependent NK cell line (230). Moreover, expression of dominant negative PYK-2 or inhibitors of Erk interfere with microtubule organizing center (MTOC) polarization and NK cell cytotoxicity (231, 232). As highlighted, LFA-1 is sufficient for both adhesion and polarization in NK cells. In both IL-2–activated NK cells and T cells, engagement of LFA-1 by ICAM-1 induces activation of a Vav–Rac–PAK1 pathway (233–235). Furthermore, chemoattractants can induce PI3K activity associated with LFA-1 in a manner dependent on the association of the Src-family kinase Fyn with the LFA-1 cytoplasmic tail (236). In T cells, Fyn has been demonstrated to be upstream of Vav1–mediated signals for T cell polarization (237). Supporting these findings, Fyn-deficient mice have defective tubulin cytoskeleton rearrangements in T cells and granule polarization in mast cells (238, 239). NK cell function has been studied in Fyn–deficient mice. Notably, Fyn is required for efficient, NK cell-mediated lysis of target cells which lack both self-MHC class I molecules and ligands for NKG2D (240). In contrast, NK cell inhibition by the MHC class I-specific receptor Ly49A was independent of Fyn, suggesting that Fyn is specifically required for NK cell activation (240).

Studies of T cells have shown that upon actin-dependent formation of an immunological synapse, perforin-containing granules move towards the minus-end of microtubules (241). The centrosome becomes juxtaposed to the target cell by an actin-dependent process and secretory lysosome delivery is independent of plus-ended motility. Recently, a link between the actin and microtubule cytoskeleton formed by the Cdc42 interacting protein 4 (CIP4) has been shown in NK cells (242). Knockdown of endogenous CIP4 impairs granule polarization to the immune synapse, but does not impair actin reorganization (242). Upon mixing with susceptible target cells, the formation of a WIP, WASp, actin-, and myosin IIA complex was observed in the NK cell line YTS (243). RNA interference-based knockdown of WIP demonstrated a pivotal role in granule polarization and NK cell cytotoxicity (243, 244). Future studies using genetic approaches will hopefully elucidate the signaling pathways responsible for granule polarization in NK cells.

2.3.4 Inhibitory signals

The potent inhibition of NK cells by ITIM-containing receptors is mediated by a block at an early step of the signaling pathway for activation. Phosphorylated ITIMs represent optimal sequences of direct binding of SHP-1 and SHP-2 Src homology 2 (SH2) domains (245). Moreover, the crystal structure of SHP-2 has

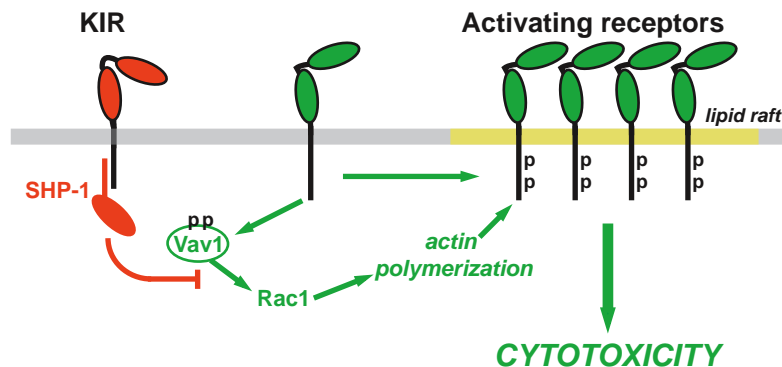


Figure 3. *Model for inhibitory receptor signaling by KIR in NK cells. Early, actin-independent, dephosphorylation of Vav1 prevents actin-dependent processes, such as recruitment of activating receptors to lipid rafts, and receptor tyrosine phosphorylation.*

shown that recruitment of tyrosine phosphatases through binding of their SH2 to phosphorylated peptides releases them from an inhibitory intramolecular interaction (246). The engagement of inhibitory receptors prevents actin cytoskeleton dynamics (243, 247), thereby preventing actin-dependent processes, such as coalescence of lipid rafts (248), recruitment and phosphorylation of co-activation receptors 2B4 and NKG2D to lipid rafts (249, 250), and dephosphorylation of ezrin-radixin-moesin proteins, which connect actin filaments to membrane structures (247). A direct substrate of SHP-1 during inhibition is Vav1, which is an essential regulator of actin dynamics (251). Interestingly, Vav1 and its close relatives Vav2 and Vav3 have been implicated in different signaling pathways downstream of several NK cell activation receptors, such as CD16, NKG2D (252), 2B4 (149), and β_2 -integrin (234). Therefore, it is possible that dephosphorylation of Vav during inhibition by KIR is a way to stop different signaling pathways at a common point. Trapping of Vav1 was insensitive to cytochalasin D, suggesting that dephosphorylation of substrates occurs independently of actin polymerization (251). As phosphorylation of activation receptor 2B4 is dependent on actin polymerization, the inhibition mediated by KIR may precede full engagement of receptor 2B4. The revised view of the inhibitory pathway is one where KIR operates independently of activation signals, thereby preventing activation at a very early step, including signals delivered by LFA-1 (Figure 3). Adding complexity to inhibitory receptor signaling, a recent report has demonstrated the involvement of β -arrestin 2, a intracellular scaffolding molecule, in inhibitory receptor signaling (253). The mechanisms whereby β -arrestin 2 facilitates ITIM-mediated inhibition remains to be elucidated.

Much remains to be learned about the precise way in which ITIM-based inhibitory signals intersect the many signals received by NK cells, and how inhibitory receptors control the various steps in NK cell activation, such as inside-out signals to LFA-1, signals for granule polarization, and signals for degranulation.

2.3.5 Degranulation and cytokine production

Unless antagonized by inhibitory receptor signaling, the interaction of an NK cell with a target cell may culminate in a productive response. Degranulation (exocytosis of secretory lysosomes) can be measured by the release of hexosaminidase or granzyme B into supernatants. Another assay, which can quantitate degranulation at the single cell level, is based on the appearance of CD107a (LAMP-1) at the cell surface upon degranulation. CD107a is a lysosomal membrane protein that colocalizes with perforin in secretory lysosomes and redistributes to the cell surface when granules fuse with the plasma membrane (254, 255).

Although F-actin reorganization is required for MTOC and lytic granule polarization, degranulation is thought to require actin disassembly and clearance of conduits in the cortical actin cytoskeleton (204, 205, 256). The transit of secretory lysosomes or even the MTOC together with secretory lysosomes through such conduits might require cytoskeletal motor functions. Myosin IIA is proposed to facilitate this process, as biochemical inhibition of myosin IIA or down-regulation by small interfering RNA specifically impairs degranulation, but not polarization (257).

NK cell degranulation requires calcium and is induced by PKC and G protein-dependent pathways (258). Patch clamp experiments have shown that cytosolic Ca^{2+} is sufficient to induce degranulation in an NK cell line (259). PLC- γ 2-deficient mice have defective NK cell natural cytotoxicity and ADCC, and display increased viral loads upon infection with cytomegalovirus (260-262). Although NK cells from PLC- γ 2-deficient mice polarize granules towards sensitive target cells, no intracellular calcium mobilization is observed after engagement of multiple activating receptors, and degranulation induced by sensitive target cells is abolished (262). While mouse NK cells predominantly express PLC- γ 2, human NK cells express both PLC- γ 1 and PLC- γ 2 (261, 263). Experiments in our laboratory also suggest that inhibition of PLC- γ with the pharmacological compound U73122 abrogates resting NK cell degranulation induced by both ITAM-dependent and -independent pathways (200). Inhibition of Src-family kinases by PP2 also blocks degranulation. Further, pharmacological inhibition of PLC- γ by U73122 also inhibits Ca^{2+} mobilization and cytotoxicity induced by mAb-mediated cross-linking of CD16 alone, or NKG2D and 2B4 together, in resting NK cells. Likewise, pharmacological inhibitors of PI3K abrogate NK cell degranulation, cytotoxicity, and cytokine production, but do not necessarily impair mobilization of intracellular calcium (200). The study of PI3K function in NK cells is complicated by the fact that knocking out two out of the four p110 subunits results in embryonic lethality. Analysis of viable p110 γ and p110 δ knockout mice has revealed a requirement

for p110 δ in NK cell cytokine production, while NK cells from p110 γ and p110 δ double knockout mice demonstrate impaired cytotoxicity as well (264, 265). Thus, signals from PLC- γ and PI3K are important for induction of NK cell degranulation, leading to killing of target cells, as well as the induction of cytokine release by NK cells.

The point at which signals for degranulation and cytokine production diverge is emerging (59). The final steps in secretory lysosome release will be dealt with in the results and discussion section, as they are a major theme of the work in this thesis.

In regard to cytokine production, an important role of the Carma1/Bcl10/Malt1 complex in activation of NF- κ B and induction of multiple cytokines by ITAM-coupled receptors has been demonstrated in mouse knockout models (266, 267). Absence of Bcl10 or Malt1 impaired also activation of p38 and JNK (267). In contrast cytokine production induced by IL-12 and IL-18 was normal in Bcl10-deficient mice (267). CD45-deficient mice exhibit decreased cytokine production but normal cytotoxicity (268-270). Receptor tyrosine phosphatase CD45 deficiency augments tyrosine phosphorylation upon stimulation of ITAM-associated receptors, but impairs phosphorylation and activation of Erk, and JNK, abrogating cytokine transcription (269). IFN- γ production is exacerbated in adaptor protein MIST-deficient CD4⁺ T cells, suggesting that MIST negatively regulates IFN- γ production (271). Expression of the Src-family kinase Fgr paralleled the suppressive effect of MIST in NK cells, and an Fgr-MIST interaction is required for the suppression of NK cell receptor-induced IFN- γ expression (271). Several soluble factors, such as IL-12 and IL-18, induce cytokine production by NK cells. IFN- γ release by NK cells in response to interleukins is augmented by the protein SET (272). SET mediates this effect by suppressing PP2A phosphatase activity, a negative regulator of NK cell cytokine production (272). Thus, proteins specifically implicated in the induction of NK cell cytokine responses are being unravelled. Understanding the divergence of pathways for NK cell effector functions such as cytotoxicity and cytokine secretion can have ramifications for the role of NK cells in disease, as particular balances in NK cell responses to challenges might be required in order to maintain immune homeostasis.

3 FUNCTIONS OF NK CELLS

Several lines of research have provided insight into the physiological role of NK cells in immune function. NK cells were first recognized for their ability to kill certain autologous tumors in experimental model systems. Later, the ability of NK cells to provide a first line of defence against viruses was recognized. In addition, NK cells are also implicated in immunity to certain bacterial and parasitic infections. Furthermore, recent data reveal an important role for NK cells in the regulation of other immune cell subsets. Relating NK cell function to clinical conditions provide invaluable insights to the role of NK cells in human disease. However, the paucity of NK cell-selective deficiencies underlines a division in thinking of immunobiology in cellular or genetic terms. After all, genes are the elementary units selected upon through evolution. In terms of disease, dissecting the contribution of individual genes, rather than particular cell types, is of high clinical relevance. However, in terms of NK cell immunotherapy, an increased understanding of cellular functions is still required. The next sections will review facets of NK cell function as revealed by different experimental and clinical approaches, followed by a more comprehensive review of immunodeficiencies where NK cell dysfunction has been characterized.

3.1 NK CELLS AND CANCER

3.1.1 Experimental evidence

Since the discovery of NK cells, numerous *in vitro* studies have demonstrated NK cell killing of different tumor cell lines. Several studies in rodents have documented a role for NK cells in the eradication of grafted tumor cell lines, as revealed by the antibody-mediated depletion of NK cells or use of mice with deficiencies in the development of NK cells (273, 274). More specifically, in mice tumor cells can be rendered susceptible to NK cell mediated lysis by the lack of MHC class I expression or induced expression of ligands for NKG2D or CD27 ligands (12, 275-277). Intriguingly, elimination of tumor cells by NK cells can induce subsequent tumor-specific T cell responses to the parental tumor (275, 277).

What is the biological relevance of NK cells in tumor development? In 1909, Paul Ehrlich postulated a role for the immune system in protecting the host against cancer. This concept was modified in the 1950s by Macfarlane Burnet and Lewis Thomas, as recently reviewed (278). However, studies of tumor development in nude mice, which lack T cells and B cells, surprisingly revealed that spontaneous tumors and carcinogen-induced tumors developed at a similar frequency to that of wild-type mice (279). Thus, these results lead to abandonment of the tumor immunosurveillance hypothesis. However, recently, a growing body of evidence is providing support for the immune system in having an important role in tumor surveillance, as recently reviewed (280-282). Key findings include the demonstration of increased spontaneous tumor incidence in mice lacking an intact IFN- γ receptor (283, 284), and increased

incidence of spontaneous B cell lymphomas in perforin-deficient mice (285, 286). These studies have led to a further refinement in the theory now termed “cancer immunoediting” (287). According to this theory, cancer development encompasses three phases: elimination, equilibrium, and escape. During the elimination phase, nascent tumor cells are destroyed by elements of the innate and adaptive immune systems, including NK cells. Providing the first genetic evidence for immunosurveillance of primary tumors by an NK cell activating receptor, David Raulet and colleagues (145) elegantly demonstrated that NKG2D-deficiency in mice results in increased incidence of epithelial and lymphoid malignancies when crossed to tumor prone backgrounds. The elimination phase is complete when all tumor cells are cleared, or incomplete when only a portion of tumor cells are eliminated. In the case of partial tumor elimination, the theory of immunoediting is that a temporary state of equilibrium can then develop between the immune system and the developing tumor, with the tumor being dormant or continuing to evolve. The pressure exerted by the immune system on the tumor during this phase is sufficient to control tumor progression. Eventually, if the immune response still fails to completely eliminate the tumor, the process results in the selection of tumor variants that through various mechanisms are able to resist, avoid, or suppress the antitumor immune response. This process is defined as the escape phase (288).

In humans, experiments using freshly explanted tumors and peripheral blood NK cells are elucidating specific receptor ligand interactions involved in NK cell recognition of tumor cells. NK cells from KIR–HLA incompatible donors can kill solid tumors, including melanoma cells, renal cell carcinoma, and ovarian carcinoma cells *in vitro* (289-291). In addition to NKG2D, NCRs and DNAM-1 have been implicated in positive recognition of tumor cells (291-293). Importantly, delineating the activation of NK cells may allow prediction of tumor cell sensitivity to NK cell killing based on phenotypic analysis of ligand expression, and thus predict the potential efficacy of NK cell-mediated immunotherapy.

3.1.2 Clinical insights and perspectives

In humans, evidence for NK cell targeting of human tumors have also come from the clinical studies in the settings of hematopoietic stem cell transplantation (HSCT) and adoptive transfer of NK cells to cancer patients. NK cells can kill allogeneic cells in hematopoietic transplantation (294) and have clinical potential by conveying graft-versus-leukemia activity (111, 295, 296). Interestingly, a Japanese 11-year follow-up epidemiological study revealed an association between increased cancer risk and low NK cell activity, as assessed by lysis of susceptible cell lines by peripheral blood lymphocytes (297). Thus, given the evidence for NK cell anti-tumor activity, there is considerable interest in harnessing NK cells for cancer immunotherapy (298, 299). Several prospects for the use of NK cells in the clinic have been put forth. The next paragraph will examine such possibilities in more detail.

Cytokines such as type I IFN, IL-2, IL-12, and IL-18 enhance NK cell activity (300, 301). Controlled exogenous administration of cytokines could provide a

valuable tool for up-regulating NK cell effector functions *in vivo*. Moreover, therapeutic blockade of inhibitory receptor–ligand interactions could potentially allow reactivity from wider NK cell subsets and facilitate the use of NK cells in an autologous setting.

In addition to inhibitory receptor blockade, natural cytotoxicity against tumors can potentially be aided by preferential engagement of activating NK cell receptors. One strategy is to design bispecific proteins that can simultaneously engage tumor markers and NK cell activating receptors. In a recent article a fusion protein of ULBP2 and an anti-CD138 antibody fragment was described that can mediate antitumor activity in a xenograft model of multiple myeloma (302). Another approach would be to adoptively transfer gene-modified NK cells with targeted chimeric receptors for tumor antigens fused to potent activating NK cell receptor components, such as a CD4- ζ chain chimera that binds to HIV-infected cells (303).

We have argued that the engagement of CD16 would be the most applicable and affordable approach to harness NK cells for immunotherapy (200). Indeed, several human IgG mAb–based treatments are increasingly applied in immunotherapy against haematological and non-haematological malignancies (304, 305). Examples include rituximab (anti-CD20) for B cell lymphomas, alemtuzumab (anti-CD52/CAMPATH) for B cell chronic lymphoid leukemia, trastuzumab (anti-HER2) for breast cancer, and adecatumumab (anti-EpCAM) for prostate and breast cancer. The extent to which these antibody–based tumor therapies are NK cell–mediated is not clear, but all are capable of triggering NK cell–mediated cytotoxicity *in vitro* (306, 307). The most convincing evidence for CD16 and possible NK cell involvement comes from studies with rituximab. In patients undergoing treatment for large-cell non-Hodgkin's lymphoma, rituximab (anti-CD20) administration induces NK cell degranulation *in vivo* (307). A positive correlation between better clinical responses to rituximab and a CD16 polymorphism with higher affinity for IgG have been reported (308, 309). Moreover, in a study of a combination therapy of rituximab and recombinant IL-2, NK cell expansions correlated with a favourable clinical response (310). In primary breast cancer, a recent report advocates an *in vivo* role for NK cells in the mechanisms of trastuzumab action, since the treatment significantly increased the numbers of tumor-associated NK cells (311). A consequence of NK cell activation by ADCC is production of T cell recruiting chemokines. They are increased in sera from patients with clinical benefit from rituximab treatment and have been shown to induce T cell migration (312). Thus, NK cell–mediated ADCC could promote beneficial adaptive immune responses.

Appreciation of NK cell biology could also encourage new, combinatorial therapeutic approaches. Specifically, enhancing NK cell effector function through up-regulation of inducible ligands, for receptors such as LFA-1 or NKG2D might be feasible. Proinflammatory cytokines can rapidly up-regulate ICAM-1 on several tissues (313), while NKG2D ligands can be induced by radiation or chemotherapeutic drugs (314). In combination with engagement of

specific triggering receptors on NK cells, desired cytolytic activity could be accomplished.

3.2 NK CELLS AND INTRACELLULAR PATHOGENS

3.2.1 Viral evasion strategies

In animal models, NK cells can control infections by many viruses, such as herpes simplex virus (HSV), cytomegalovirus (CMV), influenza virus, or the ecromelica poxvirus (315). Several viruses have evolved strategies to avoid NK cell recognition of virally infected cells (316, 317). The rapid evolution of MHC class I receptors provides insight into an interesting strategy in the conflict opposing microorganisms and the vertebrate immune system. Viral subversion of T cell-mediated immune surveillance through MHC class I down-regulation can render infected cells susceptible to NK cell lysis. Therefore, NK cell inhibitory receptors are targeted by counter-acting viral immune evasion strategies. Certain viruses can express MHC class-like decoy molecules that can engage certain NK cell inhibitory receptors or enhance expression of endogenous MHC class I molecules.

Genetic associations between combinations of KIR and HLA genotypes and susceptibility to viruses imply that KIR–HLA interactions are crucial to anti-viral immunity (318). Human CMV expresses US2 and US11 proteins that preferentially down-regulate HLA-A, without interfering with HLA-E expression (319). Moreover, human CMV encodes UL18 which can bind LIR-1 with high affinity (73, 320), and UL40 which can enhance expression of endogenous HLA-E (a ligand for CD94/NKG2A) (321, 322). However, due to the variegated expression pattern of inhibitory receptors, effective NK cell inhibition requires co-ordinated targeting of several inhibitory modalities. Individuals display considerable variation in the numbers of inhibitory receptor–MHC class I interactions. Most individuals possess a minimum of three interactions (KIR2DL–HLA-C, CD94/NKG2A–HLA-E, LIR-1–HLA class I) that would have to be circumvented by pathogens for evasion of NK cell activation. Thus, the variation in NK cell inhibitory receptor interactions provides robustness to the organisms both at individual and species level. Conceivably, viral evasion of inhibitory receptors could be a driving force for the rapid genetic evolution of these receptor systems (62).

To avoid viral escape from NK cell-mediated immunosurveillance, redundancy in recognition systems is employed by NK cells. Again, studies of human CMV proteins have demonstrated interference with expression of ligands for activating NK cell receptors NKG2D and DNAM-1. Human CMV proteins UL16, UL141, and UL142 proteins interfere with expression of ULBPs, CD155, and MICA, respectively, suggesting that NKG2D and DNAM-1 contribute to NK cell-mediated immune responses to CMV (140, 323, 324). A recent paper suggests that human CMV also encodes a micro RNA, miR-UL112, responsible for preventing translation of MICB mRNA (325). Notably, both NKG2D and DNAM-1 can bind several divergent ligands, complicating viral efforts to block recognition by ligand down-regulation. Using a different

mechanism, human CMV also encodes UL83/pp65, a protein that can directly bind the activating receptor NKp30 and block NKp30–dependent immune activation (133).

Another member of the herpes virus family, Kaposi's sarcoma herpes virus (KHSV) also interferes with the recognition by activating NK cell receptors. As a central mediator of NK cell adhesion and granule polarization, LFA-1 is an attractive target for viral escape of NK cell recognition. Indeed, KHSV encodes a protein that selectively down-regulates ICAM-1 expression on virally infected cells (326). In addition, KSHV encodes a protein with ubiquitin E3 ligase activity that down-regulates MICA and AICL expression, ligands for activating NK cell receptors NKG2D and NKp80, respectively (327).

Infection of dendritic cells with influenza virus induces IFN- γ production by NK cells *in vitro* (328). NK cell cytokine production is dependent on secretion of type I interferon and IL-12 by dendritic cells (328). The NK cell activation receptor NKp46 can mediate NK cell recognition of influenza virus hemagglutinin on infected cells (127). In mice, NKp46-deficiency confers higher mortality relative to wild-type mice upon infection with influenza virus (329). Thus, genetic studies provide clues to multiple evolutionary struggles between viruses and host immunity mediated by particular NK cell receptors.

Epidemiological studies have demonstrated an intriguing relationship between polymorphic HLA and KIR genes in the resolution of hepatitis C virus (HCV) infection (330). Moreover, as for HCV, KIR and HLA polymorphisms have been reported to significantly influence the progression to disease in human immunodeficiency virus (HIV)-infected individuals. HIV-infected individuals possessing the HLA-B alleles with the Bw4 epitope in addition to certain KIR3DL1 or KIR3DS1 alleles demonstrate a significantly delayed onset of disease (331). The mechanisms whereby certain HLA and KIR combinations protect against HIV disease progression are not clear. Of note, the HIV Nef protein selectively down-regulates HLA-A and HLA-B, sparing HLA-C (332), potentially providing a mechanism for HIV-infected cells to avoid CTL recognition, but engage inhibitory receptors on NK cells.

It is possible that ADCC may contribute to resistance to viruses, in addition to the protection provided by natural cytotoxicity. However, individuals with mutations leading to CD16–deficiency or polymorphisms with reduced binding of IgG are not reported to have increased viral infections, but increased incidence of autoimmunity (333, 334). CD16 mutations have been reported that do not abrogate NK cell–mediated ADCC, but impair natural cytotoxicity and viral immunity, suggesting a potential ADCC–independent role for CD16 (335). Nonetheless, other studies suggest that NK cell–mediated ADCC might contribute to HSV, human immunodeficiency virus (HIV)-1, and influenza virus protection (336–339). Physiologically, the availability of IgG against target cell epitopes is probably limiting in early adaptive immune responses.

3.2.2 NK cell recognition of intracellular parasites and bacteria

There is evidence for NK cells contribution to host protection from intracellular parasites and bacteria (340, 341).

NK cells have been implicated in the response to *Plasmodium falciparum*, the parasite that causes malaria. Experimental infection of non-immune volunteers with *P. falciparum* has revealed elevated levels of soluble granzyme A 1-2 days prior to clinical symptoms and microscopically detectable parasitaemia (342). Elevated granzyme A in serum coincided with increases in IFN- γ , IL-12 p40 and IL-8, while granzyme B and IL-10 levels increased 24-48 h later (342). The elevation of soluble granzyme A and IFN- γ in non-immune volunteers suggests that NK cells are activated upon release of parasites by infected liver cells and subsequently during blood stage infection. Additional evidence for NK cells involvement in innate immune human host resistance in the early phase of a malaria infection comes from *in vitro* experiments. NK cells IFN- γ production in response to infected erythrocytes cells varies among donors and is dependent on myeloid cell derived IL-12 and IL-18 production (343, 344). Specific NK cell receptors involved in the recognition of infected erythrocytes are not well characterized, but KIR receptors might play a role because associations were found between the KIR genotype and donor susceptibility to malaria (345). Interestingly, polymorphisms in NKp30 have been associated to malaria (346), and some evidence exist for direct recognition of *P. falciparum* proteins by NK cell receptors NKp30 and NKp46 (347).

Mouse models have suggested a role for NK cells in protection against *Trypanosoma cruzi* infection, that causes sleeping-sickness in humans. Antibody-mediated depletion of NK cells does not affect survival with low doses of *Trypanosoma cruzi* (348), however reduced survival was observed in NK cell-depleted mice when a higher dose of parasite was administered (349).

Although NK cells have been implicated in response to other intracellular parasites and bacteria, their role in control of infection is not clear and warrants further study (341).

3.3 IMMUNE REGULATION BY NK CELLS

Increasingly, roles for NK cells in interactions with other immune cells are being appreciated (350). NK cells may instruct and shape adaptive immune responses through cytokine release (300, 351) or by direct interaction with dendritic cells or T cells (352, 353).

NK cells interact with dendritic cells in peripheral tissues and secondary lymphoid organs. In both humans and mice, NK cells can kill immature dendritic cells, thereby influencing dendritic cell homeostasis and potentially limiting immune responses (354, 355). Furthermore, NK cells can promote dendritic cell maturation through release of cytokines such as IFN- γ and TNF- α . Reciprocally, NK cell activity during immune responses triggered by various pathogens are regulated by dendritic and myeloid cell-derived cytokines (356, 357).

NK cell secretion of IFN- γ promotes priming of T helper cell type 1-mediated immunity (358, 359). In addition, NK cells can also kill activated T cells (360,

361). Thus, by suppressing immune reactions, NK cells may have an important role in preventing autoimmunity (362).

3.4 NK CELLS AND IMMUNODEFICIENCY SYNDROMES

Studies of NK cell function in primary immunodeficiencies has been an approach to gaining insight into the regulation of NK cells and their involvement in disease predisposition. Primary immunodeficiencies provide invaluable insight into *in vivo* immune responses to microorganisms, dependent on the variable vulnerability to pathogens and opportunistic infections. Although most primary immunodeficiencies described follow a Mendelian inheritance, mutations of a given gene can lead to an array of phenotypes based on factors such as the type of mutation, genetic background, environmental factors, and infection history, thereby adding considerable complexity to the diagnosis, management, and understanding of disease (363). Although exceedingly informative, only a few patients with selective NK cell-deficiencies have been described in the literature (364). Two patients with NK cell deficiency have been reported to die from herpes virus infections (365, 366). Interestingly, a primary immunodeficiency with reduced frequency of circulating NK cells and susceptibility to viral infections was recently described (367). The genetic basis for these immunodeficiencies is currently not known. The paucity of patients described with selective NK cell-deficiencies highlights the difficulty with which the contribution a particular cell type to immunity can be dissected through studies of immunodeficiencies. One obvious reason being that the expression of most genes is not restricted to any given cell type.

Other immunodeficiency syndromes caused by mutations in defined genes, that, in addition to other immunological abnormalities, are associated with absent or functionally deficient NK cells are providing clues to the role of NK cells in the immune system (335, 364). One group of such immunodeficiencies includes patients with severe combined immunodeficiency syndrome (SCID). A phenotype caused by mutations in the genes *IL2RG* or *JAK3*, encoding the common cytokine receptor γ -chain and the Janus kinase 3, respectively, present with defective NK cell development and reduced numbers of NK cells in circulation (368, 369). SCID represents a rare syndrome that may impair development of all lymphocyte lineages, and diagnosis typically occurs in infants suffering life-threatening disease from opportunistic infections.

Another group of immunodeficiencies concerns mutations in surface receptors and ligands involved in regulating NK cell function. In bare lymphocyte syndrome, where MHC class I expression is severely reduced due to mutations in *TAP1* or *TAP2* (370), NK cell-mediated natural cytotoxicity is impaired (87, 371). Interestingly, ADCC is not impaired (87). Therefore, impaired natural cytotoxicity in bare lymphocyte syndrome likely reflects defective licensing of NK cells, as has been observed in MHC class I-deficient mice (84-86). Remarkably, bare lymphocyte syndrome patients are usually characterized by chronic bacterial infections of the upper and lower airways, and in half of the cases, also skin ulcers with features of a chronic granulomatous inflammation.

The onset is generally late and even asymptomatic cases have been described (372). Despite the defect in MHC class I-mediated presentation of viral antigens to CTLs, the patients do not suffer from severe viral infections. Presumably, other efficient antiviral defence mechanisms such as antibodies, NK cells, and CTL responses to TAP-independent antigens compensate for the lack of endogenous peptide presentation, highlighting immune system redundancy and resilience. Of note, a single patient with mutations in *CD8A*, which encodes CD8 α that is critical for high avidity CTL recognition of MHC class I, has been described in the literature (373). The CD8 α -deficient patient presented with a chronic lung inflammation reminiscent of infections observed in bare lymphocyte syndrome patients. Thus, the clinical manifestations of bare lymphocyte syndrome might predominately relate to CTL, rather than NK cell, deficiencies. Individuals with leukocyte adhesion disorder (LAD) type 1, a syndrome due to β_2 -integrin subunit deficiency, suffer severe recurrent bacterial infections, susceptibility to herpes simplex virus (HSV) infections, and impaired immunity (374-376). Severely affected patients often die of infection in childhood or early adulthood unless a bone marrow transplantation is successfully accomplished. Lastly, a case of a patient with recurrent HSV-infections has been attributed to mutations in *CD16* (377). Astonishingly, natural cytotoxicity, but not ADCC, was impaired in this patient (377).

A major group of immunodeficiencies with impaired or defective NK cell function typically present with similar clinical manifestations. A familial case involving two children suffering from fever, hepatosplenomegaly, and café-au-lait pigmentation of the skin was first reported by Farquhar and Claireaux in 1952 (378). In current literature the condition is generally termed hemophagocytic lymphohistiocytosis (HLH), a rare heterogenous sepsis-like disorder with both familial and acquired forms (379-381). The symptoms include prolonged fever and hepatosplenomegaly. Clinical guidelines for diagnosis of the disease have been established (382). Frequently, neurological symptoms such as seizures and ataxia are evident (383, 384). Clinical markers comprise elevated ferritin, triglycerides, and soluble IL-2 receptor α -chain (sCD25), in addition to low fibrinogen (385). Notably, a characteristic laboratory finding is defective NK cell-mediated cytotoxicity (386, 387). Hemophagocytosis is not necessarily demonstrable at onset, but is more frequently observed in advanced disease (388). Failure to demonstrate hemophagocytosis does not negate the diagnosis of HLH (382). Familial hemophagocytic lymphohistiocytosis (FHL) has an autosomal recessive inheritance, typically affects infants, has an incidence of 1/50000 births, and is usually fatal unless treated by chemo-immunotherapy and subsequent hematopoietic stem cell transplantation, which is the only curative treatment of FHL (389). Besides defective cytotoxicity, patients typically display polyclonal CTL expansion, a lymphohistiocytic infiltration of visceral organs associated with macrophage activation, and systemically elevated concentrations of pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-6, and IL-18 (387, 390, 391). In regards to etiology, frequent triggers are infectious agents, in particular viruses of the herpes family (392, 393). The autosomal recessive inheritance FHL has facilitated elucidation of a genetic basis for the disorders. In humans,

a linkage analysis initially associated FHL with the genomic region 9q21.1-22 (FHL1) (394), but a gene accountable for the disease has so far not been identified at this locus. Concurrently, linkage with 10q21-22 was reported and loss-of-function mutations in the perforin (*PRF1*) gene were attributed as causative for a large proportion of cases of FHL (FHL2) (395). Perforin is indispensable for delivery of granzymes to target cells, which in turn cleave cytosolic proteins and induce DNA fragmentation (396, 397). Upon infection with certain herpes viruses, perforin-deficient mice display similar characteristics as FHL patients (398-400). Animal models, together with data from patients, point to an important role for perforin-dependent mechanisms in maintaining immune homeostasis by controlling activated immune cells. Two additional human loci on chromosome 17q25 and 6q24 have subsequently been associated with FHL (401, 402). Mutations impeding the function of the Munc13-4 (*UNC13D*) gene are causative of FHL3 (401), while loss of function-mutations in the syntaxin 11 (*STX11*) gene are causative of FHL4 (402). Recently, a mouse random mutagenesis screen for genes implicated in resistance to murine lymphocytic choriomeningitis virus identified a splice-site mutation in mouse *Unc13d* as causative of features similar to human HLH upon infection (403). Mutations in *PRF1* or *UNC13D* account for approximately 15-50% and 15-25% of FHL patients, respectively, depending on geographic region (404). Mutations in *STX11* were initially identified in patients of Turkish origin, where they account for approximately 20% of FHL patients (404, 405). Other autosomal recessive syndromes associated with clinical manifestations similar to FHL, but additionally presenting hypopigmentation, include Griscelli syndrome type 2 (GS2), Chediak-Higashi syndrome (CHS), and Hermansky-Pudlak syndrome type 2 (HPS2) which result from mutations in genes encoding Rab27a (*RAB27A* on 15q15-21), Lyst (*LYST* on 1q42.1-42.2), and adaptor protein 3 β subunit (AP-3, *ADTB3A* on 5q14.1), respectively (406-408). While HLH is a common feature of patients diagnosed with GS2 and CHS (406, 409), only a single HPS2 patient has been reported to develop HLH (410). The discovery that pigmentation defects were associated with defective NK cell cytotoxicity in humans and mice was first reported in 1980 by Tony Fauci and colleagues (411). NK cell cytotoxicity is also defective in GS2 and HPS2 (412, 413). However, a recent article suggests that only natural cytotoxicity is affected in GS2, whereas ADCC is intact (414). Furthermore, HLH is also prevalent in patients suffering from X-linked lymphoproliferative disease (XLP), an Epstein-Barr virus (EBV)-associated immunodeficiency. Patients with XLP type 1 (XLP1), caused by mutations in the gene encoding SAP (*SH2D1A*) (415, 416), may also manifest with HLH (417). Another recently identified locus associated with X-linked lymphoproliferative type 2 (XLP2) encodes the gene for X-linked inhibitor of apoptosis (XIAP, *XIAP*) (418). Patients with mutations in *XIAP* typically manifest with HLH (418). SAP is an intracellular adaptor molecule that signals down-stream of several receptors involved in interactions between lymphocytes (419, 420). Two studies of NK cells from XLP1 patients have revealed that 2B4 requires SAP in order to transmit signals for activation (421, 422). In the absence of SAP, 2B4 inhibits NK cell function. Moreover, immature NK cells contain low levels of SAP and engagement of 2B4 can therefore mediate tolerance of immature NK cells that

have not acquired expression of inhibitory receptors for MHC class I (423). These data partially corroborate divergent findings from 2B4-deficient and SAP-deficient mice, in which 2B4 has been attributed both positive and negative signaling functions (424, 425). Adding further complexity to the possible mechanisms of XLP1, a structural homologue of SAP that competes for binding to ITSMs has been described. EAT-2 is expressed in human NK cells and can bind ITSMs of 2B4 and other CD2-related receptors (426). In an NK cell line, Tassi *et al.* (426) observed that EAT-2 preferentially binds 2B4 in non-activated cells, whereas SAP binds with higher affinity after cell activation. Tentatively, 2B4-mediated activation could therefore be dynamically regulated by SAP expression and competition for ITSM binding with EAT-2. How SAP and EAT-2 competition in receptor signalling relates to disease remains to be established. The molecular mechanisms underlying disease caused by mutations in *XIAP* require further investigation.

Papillon-Lefevre syndrome is characterized by early onset of periodontitis, which is in some cases thought to be viral in origin (364). Recent studies of patients with Papillon-Lefevre syndrome caused by mutations in the gene encoding cathepsin C, *CTSC*, suggest a role for this peptidase in mediating activation of granzyme B (427). Granzyme B, an effector of target cell apoptosis and major constituent of secretory lysosomes, requires proteolytic cleavage for activation. Interestingly, the stimulation of NK cells with IL-2 restored the ability to process granzyme B (427), consistent with a previous study that did not find any cytotoxic defect in cultured NK cells from Papillon-Lefevre patients (428).

Defective NK cell function has also been noted in other immunodeficiencies caused by mutations in intracellular signaling proteins, such as Wiskott-Aldrich Syndrome (WAS) caused by mutations in WAS protein (WASp), *WASP*, autoimmune lymphoproliferative syndrome (ALPS) caused by mutations in caspase 8, *CASP8*, and NEMO-deficiency caused by mutations in the inhibitor of NF- κ B complex, *IKBKG*, as recently reviewed (364).

Formerly, primary immunodeficiencies were considered to be limited to a few rare, familial, monogenic, and recessive traits impairing the development and function of one or several immune cell types and resulting in opportunistic and fatal infections in infancy (429). These days, with more advanced techniques to assess genetics and immune function, the spectrum of individuals considered to suffer from immunodeficiencies is expanding. In the broadest sense, Jean-Laurent Casanova and Laurent Abel argue that most individuals suffer at least one of a multitude of primary immunodeficiencies (430). Thus, with the advance of genetic and diagnostic techniques, the spectrum of immunodeficiency syndromes is expanding. Exemplifying the broadening notion of immunodeficiency disorders, extensive studies of KIR polymorphisms are revealing multiple disease associations (431).

4 RESULTS AND DISCUSSION

4.1 ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

Due to the multiplicity of receptor–ligand interactions between NK cells and target cells, it has been difficult to assign specific functions to individual receptors and assess their relative contribution to NK cell effector functions. To overcome this complexity a reductionist target cell system was employed. *Drosophila* cells expressing individual or combinations of human NK cell ligands were generated and used as target cells for human NK cells (207, 228) (Figure 4). Unlike mammalian cells, insect cells are not expected to express a multitude of ligands for adhesion and activation receptors of human NK cells. Therefore, they are better suited for investigations on the individual contribution of, and crosstalk among NK cell receptors. A notable advantage of such a reconstituted target cell system is that activation of normal, unmanipulated NK cells can be studied with physiological ligands. The goal of this approach was to characterize the contribution of individual receptors NK cell activation and define a minimal requirement for ADCC by resting NK cells.

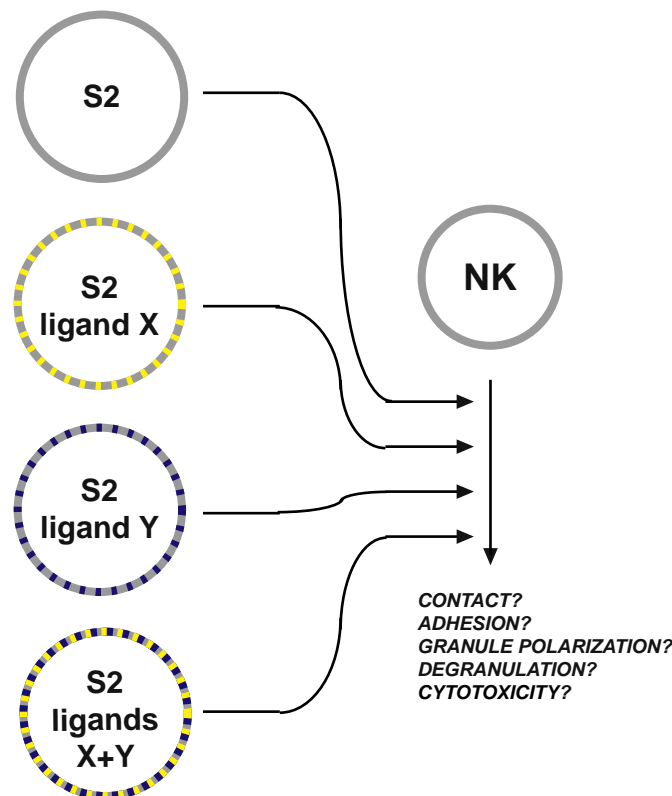


Figure 4. *Reductionist Drosophila target cell system for assessment of individual contribution of receptors to NK cell activation.* *Drosophila* S2 cells are transfected with individual or combinations of constructs encoding human NK cell receptor ligands. In cell mixing experiments with primary, resting NK cells, NK cell activation events or target cell lysis can be evaluated.

The focus of **Paper I** is the contribution of three NK cell receptors to cytotoxicity by human freshly isolated, resting NK cells. Receptors CD16, LFA-1, and 2B4 are generally considered as activation, adhesion, and co-stimulation receptors, respectively. Different assays were used to measure cytolytic granule polarization and degranulation separately, rather than overall target cell lysis. Polarization of perforin-containing granules towards target cells was assessed by confocal microscopy, whereas degranulation was assessed by CD107a surface staining. CD107a is a transmembrane protein that usually resides in secretory lysosomes, but upon degranulation of secretory lysosomes, CD107a is exposed on the plasma membrane.

Antibody-coated *Drosophila* cells were sufficient to induce CD16-mediated degranulation by resting human NK cells, as determined by granzyme B release, induction of CD107a, and Fas ligand surface expression (*Figure 5*). Resting NK cell degranulation was observed within minutes of mixing with sensitive target cells, and parallels the rapid mobilization of calcium by interaction with target cells (199). CD16-mediated degranulation occurred in spite of very low target cell adhesion. Furthermore, examination of conjugates between resting NK cells and IgG-coated target cells did not reveal granule polarization. Alone, engagement of LFA-1 by ICAM-1 on *Drosophila* cells was sufficient to induce low adhesion and granule polarization (*Figure 5*). Efficient ADCC by resting NK cells required the combined presence of IgG and expression of human ICAM-1 on *Drosophila* cells (*Figure 5*). The results define two separable signals for adhesion/polarization (LFA-1) and degranulation (CD16) that are required for target cell killing. Notably, ICAM-1 expression on target cells did not increase CD16-induced degranulation. Results are in agreement with a study of T cells (432), which concluded that LFA-1–ICAM-1 interactions are dispensable for degranulation, but essential for effective target cell lysis through enhancement of TCR-dependent granule polarization towards target cells. In another report, perforin release induced by soluble ICAM-2 and ICAM-3 Fc fusion proteins, but not ICAM-1–Fc, was observed in a CD8⁺ subset of human NK cells (433). Because the Fc portion of the ICAM fusion proteins was derived from human IgG1, it is not possible to exclude co-engagement of and activation by CD16, as has been described with other human IgG1 fusion proteins (434).

Previous studies using cells from LFA-1-deficient patients or experiments with LFA-1-blocking mAbs have demonstrated a role for LFA-1 in ADCC. NK cells from LAD patients display attenuated ADCC (374, 435) and anti-LFA-1 blocking antibodies inhibit lysis of anti-CD16 expressing hybridomas (188, 189). The interpretation of these results used to be that LFA-1 is required to provide target cell adhesion, in order for activating receptors such as CD16 to trigger NK cell cytotoxicity.

In the absence of LFA-1 engagement, CD48 expression by *Drosophila* cells enhanced ADCC (*Figure 5*). At comparable IgG concentrations, co-engagement by target cell ligands of CD16 and 2B4 induced target cell killing as efficiently as co-engagement of CD16 and LFA-1. The mechanisms used by 2B4 or LFA-1-dependent co-stimulation of ADCC are different. In contrast to

LFA-1-mediated co-stimulation, IgG in combination with CD48 expression on *Drosophila* cells did not induce strong adhesion. Instead, co-engagement of CD16 by IgG and 2B4 by CD48 augmented the signals for polarization and degranulation. CD48-mediated co-stimulation lowered the IgG concentration required to induce resting NK cell degranulation.

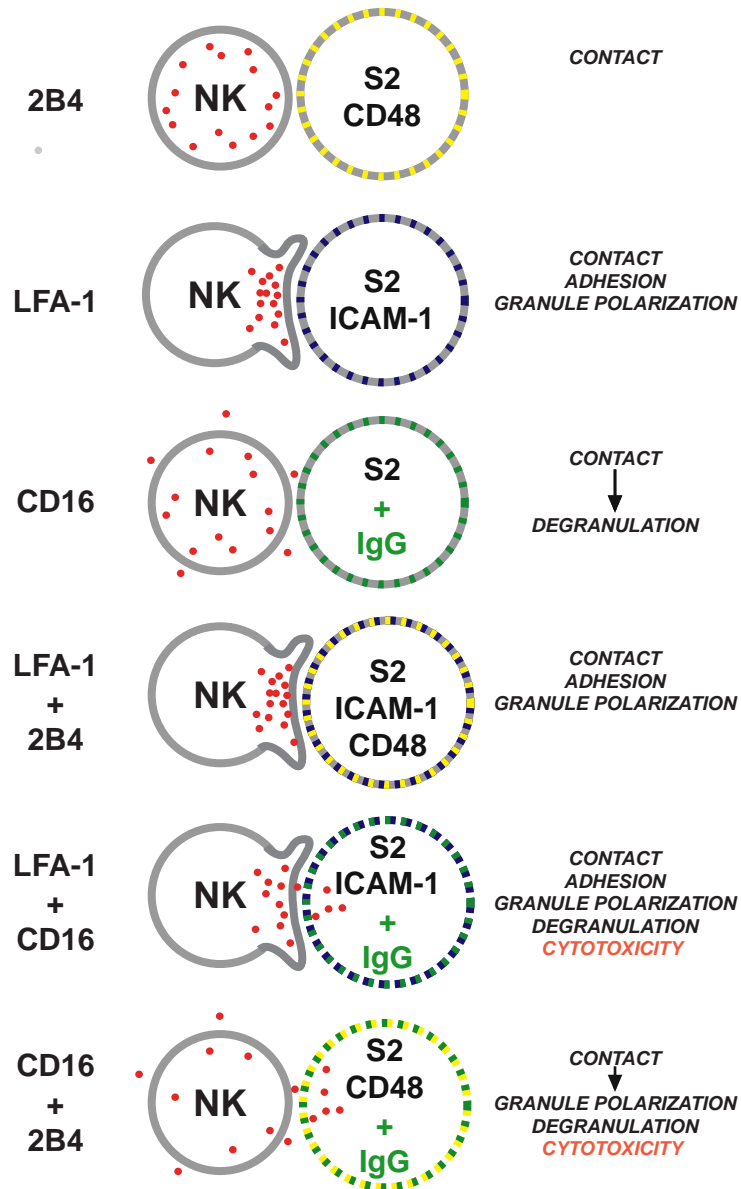


Figure 5. Summary of NK cell activation by *Drosophila* cells expressing ligands for human receptors (Paper I). Ligands expressed on S2 cells are indicated in yellow (CD48), blue (ICAM-1), and green (IgG). The outcome of interaction with NK cells is listed on the right.

So far, data obtained with the *Drosophila* target cell system suggest that no single receptor–ligand interaction is sufficient to trigger all activation steps. Conceptually, it could be envisaged that certain steps might be required for the triggering of consecutive events; for example, a sequence of adhesion followed by granule polarization and degranulation. However, *in vitro* mixing experiments with *Drosophila* cells and resting NK cells suggest that receptors may trigger discrete activation steps independently of each other. Thus, NK cell activation does not necessarily follow a sequence of events, but is guided by the engagement of receptor or receptor combinations upon encounter with target cells. Admittedly, requirements for NK cell activation could be more stringent *in vivo*, under conditions of shear flow and limited ligand availability.

Although degranulation appears to be necessary for cytotoxicity by resting NK cells, it is not synonymous with target cell lysis. Neither granule polarization nor degranulation alone is sufficient for cytotoxicity. Rather, combinations of NK cell receptors cooperate to induce efficient elimination of target cells. The data imply a central role for LFA-1, not only in target cell adhesion, but also in signaling for cytotoxicity, and suggest that LFA-1 can prime NK cells for cytotoxicity.

4.2 NATURAL CYTOTOXICITY

A large number of receptors have been implicated in natural cytotoxicity, as discussed in the preceding chapters. Two commonly used approaches to characterize NK cell activating receptors have been to identify mAbs that interfere with NK cell–mediated lysis of sensitive target cells and evaluate whether mAbs to such NK cell structures trigger redirected lysis of the FcR⁺ target cell line P815. In such experiments, IL-2–activated NK cells are commonly used as effectors. Remarkably, using *Drosophila* cells expressing ligands for different NK cell activation receptors, alone or in combination with ICAM-1, we did not observe any target cell lysis (**Paper I** and manuscript in preparation). Therefore, we hypothesized that receptors might cooperate for activation of resting NK cells. In **Paper II**, instead of generating an exhaustive repertoire of *Drosophila* cells expressing all possible combinations of ligands for activating NK cell receptors, well-characterized mAbs to activating NK cell receptors were used to study the requirements for activation of natural cytotoxicity and cytokine production.

Analysis of mAbs to NKp46, NKG2D, DNAM-1, 2B4 and CD2 with resting NK cells demonstrated that these receptors do not induce efficient lysis. Nonetheless, K562 cells triggered both degranulation and lysis by resting NK cells, suggesting no impairment of natural cytotoxicity in resting NK cells *per se*. In addition, mAbs to CD16 induced lysis of P815 cells by both resting and IL-2–activated NK cells. Of note, the FcR⁺ mouse cell line P815 used as target cells in these redirected-ADCC assays express mouse ICAM-1, which binds human LFA-1 (436), thereby corroborating the results obtained in **Paper I**.

In contrast to the degranulation induced by CD16 ligation, ligation of NKG2D or 2B4 by Abs bound to the FcR⁺ mouse cell line P815 did not induce

degranulation. When mAbs were combined, NKG2D and 2B4 synergistically induced degranulation in resting NK cells (*Figure 6*). However, as P815 cells express mouse ICAM-1, which binds human LFA-1 (436), it is possible that recognition of mouse ligands by human NK cells contributed to activation.

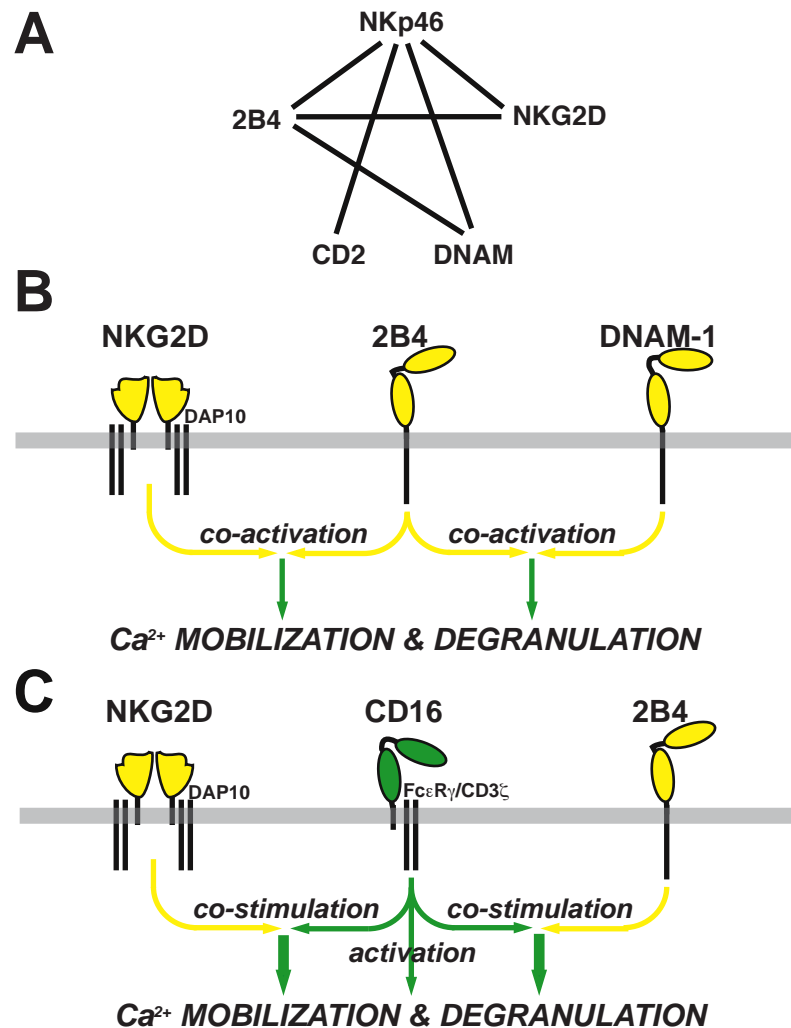


Figure 6. **Coactivation of resting NK cells (Paper II).** (A) Schematic representation of synergies among coactivation receptors for Ca²⁺ mobilization for receptors expressed on resting NK cells. (B) Coengagement on non-ITAM-associated receptors can synergistically induce Ca²⁺ mobilization and degranulation in resting NK cells. (C) Engagement of CD16 is sufficient to induce Ca²⁺ mobilization and degranulation in resting NK cells. Ca²⁺ mobilization and degranulation are enhanced by coengagement of costimulatory receptors such as NKG2D and 2B4.

Upon closer examination, we found that mAb-mediated crosslinking of NKp46, NKG2D, 2B4, DNAM-1, and CD2 only induced weak intracellular Ca²⁺ mobilization, as compared to Ca²⁺ mobilization induced by mAb-mediated crosslinking of CD16. However, co-crosslinking of specific, pairwise combinations of receptors can induce synergistic Ca²⁺ mobilization. Results revealed a hierarchy of receptors for activation of resting NK cell cytotoxicity and cytokine secretion, as depicted (*Figure 6*). The unique pattern of receptor

combinations that provide synergy is consistent with the use of different signaling modules by each receptor to induce activation. We propose the term “co-activation” receptors, as they do not by themselves induce strong activation signals, but depend on co-engagement of other co-activating receptors for activation of NK cell function. Generally, the same combinations of mAbs that synergize for Ca^{2+} mobilization also enhance resting NK cell cytotoxicity and cytokine production. Moreover, while engagement of neither receptor alone induces degranulation, co-engagement of 2B4 with NKG2D or DNAM-1 by mAbs can induce strong synergistic signals that lead to degranulation. Thus, we speculate that receptor co-activation as observed between 2B4 and NKG2D, or 2B4 and DNAM-1, may be responsible for the ITAM-independent NK cell cytotoxicity observed in mice deficient in both SYK and ZAP-70 (437). In these mice, NKG2D can contribute to target cell lysis by IL-2-activated NK cells (438).

So how might NKG2D and 2B4 signals synergize for PLC- γ recruitment, Ca^{2+} mobilization, degranulation, and cytotoxicity? NKG2D can recruit PI3K through DAP10 (135). In IL-2-activated NK cells recruitment of PI3K by DAP10 leads to activation of Vav, Rho family GTPases, and PLC- γ (439). In resting NK cells, this pathway only induces a minor, but reproducible Ca^{2+} mobilization that can be inhibited by wortmannin or Ly294002, which are pharmacological inhibitors of PI3K (200 and unpublished data). Similar to NKG2D, 2B4 activates PLC- γ in IL-2-activated NK cells (440). Unlike NKG2D and CD16 crosslinking, however, Ca^{2+} mobilization induced by 2B4 crosslinking is insensitive to PI3K inhibitors in resting NK cells (200). The synergy of 2B4 and NKG2D-DAP10 signals could result from enhanced PI3K-mediated membrane recruitment of PLC- γ through the PLC- γ pleckstrin homology (PH) domain. Surprisingly, the synergistic Ca^{2+} mobilization induced by NKG2D and 2B4 co-activation is insensitive to PI3K inhibitors in resting NK cells. Therefore, the NKG2D signal that augments Ca^{2+} mobilization in co-ordination with 2B4 signals is PI3K-independent. Although PI3K inhibition only partially inhibits CD16 and has no effect on NKG2D and 2B4 synergistic Ca^{2+} mobilization, it abolishes resting NK cell degranulation (200) and cytotoxicity (unpublished data). The data demonstrate that NKG2D and 2B4 co-activation of Ca^{2+} mobilization is PLC- γ -dependent and PI3K-independent, while resting NK cell cytotoxicity requires both PLC- γ and PI3K for degranulation. In line with these findings and providing mechanistic insights, a recent study (138) showed that NKG2D-DAP10 recruitment of both a Grb2-Vav complex and the p85 subunit of PI3K is required for NKG2D-mediated cytotoxicity in IL-2-activated NK cells. Substantiating these findings, PLC- γ is activated independently of PI3K, but associates with Vav and SLP-76 in activated human mast cells (441). Thus, PLC- γ and PI3K are emerging as two critical signaling components for NK cell degranulation, and PI3K appears to be downstream of PLC- γ activation.

The perception that ITAM-mediated signaling induces potent NK cell activation, similar to how T cell and B cell activation depends on antigen receptor signaling, is challenged by these results. Although engagement of ITAM-containing receptors by specific mAbs induces lysis of FcR^+ cells in redirected lysis assays with IL-2-activated NK cells, this is not necessarily the

case in assays with resting NK cells. Comparison of cytotoxicity by IL-2-activated and resting NK cells in redirected lysis assays revealed that mAbs to NKp30 and NKp46 do not efficiently trigger cytotoxicity by resting NK cells. This was not due to an intrinsic incapability of resting NK cell to mediate ITAM-dependent cytotoxicity, as mAbs to CD16 efficiently triggered lysis by resting NK cells. Under some circumstances, signaling by ITAM has even been shown to inhibit the function of other cell types (442, 443). Furthermore, signaling by ITAMs is not required for NK cell effector function. Cytotoxicity towards certain target cells proceeds independently of ITAM, as NK cells from mice deficient in both Syk and ZAP-70 or a combination of the ITAM-containing adaptors DAP12, CD3 ζ -chain, and Fc ϵ R γ -chain can mediate cytotoxicity (437, 444).

In terms of early signaling events, co-crosslinking of CD16 and 2B4 by specific mAbs synergistically augmented intracellular calcium mobilization relative to cross-linking of CD16 alone. MAb-mediated co-engagement of other NK cell receptors, such as NKG2D, DNAM-1, and CD2 also augmented CD16-induced calcium fluxes. Therefore, expression of ligands for other activating NK cell receptors might also synergistically co-stimulate CD16-triggered degranulation and reduce the concentration of IgG required to trigger resting NK cell degranulation. Of interest, ITAM-mediated signals from different receptors do not enhance each other, as co-engagement of NKp46 with CD16 did not result in enhanced responses.

It should be emphasized that the outcome of specific receptor engagement on NK cells is not clear-cut. NK cell responses are not merely a function of engaged receptors, but also represent the expression levels and distribution of intracellular signaling molecules present in any given NK cell. The availability of signaling components is influenced by cell maturation stage, and is potentially modulated by inhibitory receptor calibration and extrinsic inflammatory signals. These factors combine to fine-tune and provide distinctiveness to the reactivity of individual NK cells.

In conclusion, resting NK cells are not inherently non-responsive, but the regulation of their activation is far more stringent than that of IL-2-activated NK cells. Receptors can signal independently in resting NK cells, but cytotoxicity requires a combination of signals for adhesion, granule polarization, and degranulation, supplied by two or more interactions between different receptor-ligand pairs. It appears that no receptor alone, but co-engagement of certain combinations of co-activating receptors induces efficient cytotoxicity, signifying redundancy in NK cell recognition.

4.3 ACTIVATION OF NK CELLS BY KIR2DL4

KIR2DL4 is an enigmatic NK cell receptor with both activating and inhibitory potential, and thus represents an anomaly among NK cell receptors. KIR2DL4 contains a functional cytoplasmic ITIM, in addition to harboring a transmembrane arginine residue through which it can associate with the Fc ϵ R γ -chain (176, 177). KIR2DL4 is an evolutionary conserved framework member of the KIR gene family, is present in all KIR haplotypes, and is expressed by all

NK cells suggests it might subserve an important function (174, 175). Remarkably, engagement of KIR2DL4 does not induce cytotoxicity, but rather IFN- γ production by freshly isolated NK cells (182).

In **paper III**, we explored the cellular distribution, signaling mechanisms, and transcriptional profile induced by KIR2DL4 engagement in further detail. Unlike other KIR family members, KIR2DL4 was constitutively internalized to intracellular Rab5⁺ compartments via a dynamin-dependent process. Thus, cell-surface staining of KIR2DL4 was weak, as KIR2DL4 resides in intracellular compartments. In contrast to mAbs to other NK cell activating receptors such as CD16, soluble but not solid phase mAbs to KIR2DL4 induced IFN- γ secretion. Microarray profiling of genes induced by mAbs to KIR2DL4 revealed a proinflammatory/proangiogenic response. Soluble HLA-G was endocytosed into KIR2DL4-containing compartments in resting NK cells and induced secretion of a similar set of cytokines and chemokines. Soluble HLA-G is produced naturally by alternative splicing (445, 446), and by proteolytic cleavage of membrane-bound HLA-G (447). Interestingly, chemokine secretion induced by KIR2DL4 transfection into 293T cells occurred only with recombinant forms of KIR2DL4 residing in endosomes, and was not dependent on the transmembrane arginine residue. Thus, KIR2DL4 appears to selectively signal from endosomes, recently emerging as a specialized signaling compartment (448, 449). However, the signaling pathways induced by KIR2DL4 require further study.

Successful pregnancy implies accommodation of fetal cells that constitute an allograft. In normal pregnancies, fetal cells are in effect not perceived as foreign and are not rejected by the maternal immune system. Invading trophoblasts do not express classical class I HLA-A or HLA-B molecules and HLA class II molecules. Instead trophoblasts selectively express HLA-C, HLA-E, and HLA-G molecules (450, 451). Dysregulation of NK cells has been associated with reproductive failure, such as recurrent spontaneous abortions (452), infertility (453, 454), and pre-eclampsia (451). In conjunction with NK cells being the predominant lymphocyte population in uterine mucosa, the finding that KIR2DL4 binds HLA-G has led to speculation of a role for KIR2DL4 in pregnancy (178, 179). IFN- γ contributes to initiation of uterine vascular modification and decidual integrity during normal murine pregnancy mice (455). Thus, IFN- γ and other factors released after KIR2DL4 engagement could contribute to successful pregnancy. Hopefully genetic associations studies can clarify the putative role for KIR2DL4 and HLA-G in human pregnancy, or in infectious diseases.

4.4 MECHANISMS OF NK CELL GRANULE EXOCYTOSIS

Studies of the minimal requirement of ADCC and natural cytotoxicity by resting NK cells raised several questions regarding the mechanisms whereby resting NK cells release secretory lysosomes. Due to the inefficiency of current technology to modify primary, freshly isolated NK cells *in vitro*, we wished to study the mechanisms of NK cell cytotoxicity by use of NK cells isolated from individuals with defined mutations affecting the ability of NK cells to kill target

cells. Patients diagnosed with HLH typically present with defective NK cell cytotoxicity. Importantly, understanding the molecular pathophysiology of immune disorders such as HLH does not only contribute to a better knowledge of the immune system, but may also aid development of improved diagnostic procedures and favor new therapeutic approaches.

The identification of mutations in *PRF1*, *UNC13D*, *STX11*, *RAB27A*, *LYST*, and *ADTB3A* as causative of hemophagocytic syndromes combined with studies of the biological function of their protein products have provided a compelling link between impaired lymphocyte cytotoxicity and HLH (456, 457). The perforin gene is transcribed by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Such cytotoxic lymphocytes store perforin in secretory lysosomes, specialized granules that mediate cytotoxic function. Upon degranulation, perforin facilitates granzyme-mediated apoptosis of target cells (458, 459). The protein products of other genes associated with hemophagocytic syndromes have been shown to be involved in biogenesis, trafficking, and regulation of lytic granule release, thereby providing mechanistic insight into cytotoxic lymphocyte effector function. Lyst-deficiency interferes with lysosome biogenesis and degranulation (460), whereas AP-3-deficiency impairs secretory lysosome movement along microtubules, polarization and release at the immune synapse (461). Rab27a, a small GTPase, and Munc13-4 are ubiquitously expressed and are localized to the cytosolic face of secretory lysosomes (401, 462). Rab27a and Munc13-4 may associate with each other (462-464), and deficiency of either Rab27a or Munc13-4 impairs docking and degranulation of secretory lysosomes (401, 406, 464, 465). Syntaxin 11 is a protein of 287 amino acids containing a Qa soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) motif from amino acid 201 to 277 (466, 467). The SNARE protein syntaxin 11 localizes to punctuate intracellular structures, which correspond to late endosomes and the trans-Golgi network, as determined by co-localization with mannose-6-phosphate receptor (466-468). Unlike Rab27a and Munc13-4, syntaxin 11 was reportedly expressed in monocytes, but not lymphocytes (402). Therefore, syntaxin 11 was proposed to regulate cytotoxic lymphocyte function indirectly through cell-cell interactions with antigen-presenting cells (402).

Due to the strong link between defective NK cell cytotoxicity and HLH, we decided to examine NK cell function in syntaxin 11-deficient patients in more detail. Providing insight into the general mechanisms of lymphocyte cytotoxicity, studies focusing on NK cell functional deficits in HLH offer several advantages. As opposed to T cells, where antigen receptor specificities are clonally distributed, NK cells can be activated through a number of uniformly expressed activating receptors. Unlike T cells, where only a subset of effector CD8⁺ T cells express low levels of perforin and stimulation is required to augment lytic content, all NK cells contain abundant intracellular perforin (469). Accordingly, NK cells are particularly well suited for studies of distinct steps in cytotoxicity by freshly isolated, primary lymphocytes.

In **paper IV**, we assessed the expression and function of syntaxin 11 in freshly isolated, resting NK cells. In contrast to a previous study (402), expression of

syntaxin 11 transcript and protein was demonstrated in cytotoxic lymphocytes, including resting NK cells and a cytotoxic NK cell line. Importantly, degranulation by resting NK cells in patients with disease-causing, homozygous mutations in STX11 was defective. The findings are corroborated by a study demonstrating that knockdown of syntaxin 11 results in impaired NK cell cytotoxicity (470). Thus, these studies were the first to identify a member of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) domain containing family member in lymphocyte granule exocytosis. The formation of a SNARE domain complex consisting of four different SNARE domains contributed by distinct effector proteins facilitates fusion of vesicle membranes (471). The exact vesicular fusion step that syntaxin 11 mediates and the partners involved in forming a SNARE complex required for membrane fusion remain to be elucidated.

The steps leading to fusion of lytic granules with the plasma membrane still represent a conundrum in cytotoxic lymphocyte biology (472). This process was first assumed to entail direct fusion of lytic granules with the plasma membrane. Loss-of-function mutations in *RAB27A*, a member of the Rab GTPase family that regulate discrete steps of vesicle trafficking, cause defective CTL degranulation and cytotoxicity by impairing docking of granules at the membrane (406, 465, 473). Munc13-4, belonging to a family of proteins that mediate vesicle priming at synapses, is required for docking and fusion of perforin-containing granules with the plasma membrane in CTL and NK cells (401, 473, 474). *In vitro* binding assays have identified Munc13-4 as an effector of GTP-bound Rab27a (462, 463). Moreover, studies of a mast cell line have provided evidence for localization of transfected and tagged Rab27a and Munc13-4 to the limiting membrane of secretory lysosomes, as determined by electron and confocal microscopy (462). In platelets, however, endogenous Rab27a is associated with dense granules, while Munc13-4 is present on other membrane fractions (463). Recently, a study of cytotoxic T cells by Menanger *et al.* (475) revealed that Munc13-4 initially mediates assembly of Rab11⁺ recycling endosomes with Rab27a⁺ late endosomes independently of Rab27a, and thereafter primes granule fusion, possibly through Rab27a. The results imply that lytic granule exocytosis does not necessarily involve direct fusion of granules with the plasma membrane. Instead, the process appears to be more complex than previously appreciated. Furthermore, molecular pathways leading to lymphocyte cytotoxicity have been thought to converge for lytic granule release, with T cell receptor-mediated, Fc receptor-mediated, and natural cytotoxicity all sharing the same cytolytic machinery. Perplexingly, a dichotomy in the requirement for Rab27a in NK cell natural cytotoxicity versus antibody-dependent cellular cytotoxicity (ADCC) was recently described, suggesting that ADCC is Rab27a-independent (414). In contrast, Munc13-4 is indispensable for NK cell mediated degranulation and cytotoxicity induced by several stimuli (474). Clearly, further study of the intracellular components that instigate membrane fusion and specific role of syntaxin 11 are warranted.

Unexpectedly, IL-2 stimulation partly restored degranulation and cytotoxicity by NK cells from syntaxin 11-deficient patients, which could explain the less severe disease progression observed in FHL4 patients relative to FHL2 and

FHL3 patients (405). Since the T cell compartment is still immature in infants, we speculated in **Paper IV** that the observed defect in NK cell degranulation contributes to the pathophysiology of FHL. Although genetic and functional evidence provides a strong link between HLH and perforin–dependent cellular cytotoxicity, the expression and function of syntaxin 11 in other cell types could also contribute to the pathophysiology observed in FHL4 (476).

The results in **Paper IV** also have implications for diagnosis and therapy of FHL2, FHL3, and FHL4. Diagnostically, the CD107a assay for degranulation, combined with evaluation of cytotoxicity, offer a rapid and highly reproducible method for diagnosis of FHL and subclassification of FHL2 from FHL3 and FHL4, thereby providing guidance for genetic analysis, as also suggested by Marcenaro *et al.* (474). Therapeutically, our results suggest that reagents which would specifically activate NK cells or CD8⁺ T cells may be beneficial in the treatment of FHL4, by partially restoring cytotoxic function. Further, as we argue that NK cells are the major perforin expressing cell population in infants, an alternative therapeutic approach could be NK cell donor lymphocyte infusion to FHL patients lacking cytotoxic activity. Donor NK cells might stabilize patients prior to transplantation, by contributing to restoration of immune homeostasis. Thus, donor NK cell infusion could facilitate successful transplantation of patients.

In **Paper V**, we described *UNC13D* mutations, causative of FHL3, in a cohort of patients and related genotype to phenotypic analysis. Most notably, *UNC13D* mutations in two siblings with late, adolescent onset disease were identified. The patients in this family were homozygous for a splice site mutation. NK cell degranulation in a patient from this family was severely impaired. However, compared to patients with early onset disease and *UNC13D* nonsense mutations, low levels of degranulation were evident. Thus, we speculate that mutations conferring some, residual protein activity might predispose to the development of HLH later in life. Therefore, mutations in *PRF1*, *UNC13D*, and *STX11*, in addition to other still unidentified genes could explain cases of late onset, yet unaccountable secondary HLH. Moreover, patients with mutations that impair cytotoxic lymphocyte function might predispose to other infectious diseases or malignancies due to reduced capacity for immunosurveillance. Supporting such a notion, both homozygous and heterozygous perforin mutations have been identified in lymphoma patients (477). Prospectively, classification of patients with reduced cytotoxic function could facilitate prognosis and treatment of disease.

5 CONCLUDING REMARKS

The immune system, a pillar of life, is a mesmerizingly complex entity. However daunting, NK cell biology represents a fascinating view into the evolutionary struggles between intracellular pathogens and the immune system, with clinical implications for immunity to infection. Moreover, accumulating evidence suggests a significant role for NK cells in reproduction and tumor surveillance. The regulation of NK cell function is remarkably diversified in terms of recognition strategies and genomic diversity. By relating genetic studies of disease with carefully designed functional studies of immune cells, it is possible to decipher the role of individual genetic elements to proper immune function.

By use of different experimental approaches, the work in this thesis has focused on the individual contribution of several gene products to human NK cell function. With reductionist target cell systems, we have defined requirements of receptor engagement for ADCC and natural cytotoxicity by physiological NK cells. Further, the contribution of individual receptors to these processes was dissected. Through study of NK cells from immunodeficient patients, we have assessed the role of intracellular effector proteins to NK cell function and identified critical mediators of NK cell granule release.

Several important questions remain. In terms of NK cell biology, reductionist systems can feasibly expand our knowledge in relation the point at which inhibitory signaling intersects activating receptor signals and how diverse ligand engagements regulate NK cell functions, such as degranulation, cytokine release, and proliferation. Furthermore, modulation of NK cell function by extrinsic cytokines is of interest. Specifically, how do cytokines affect the thresholds for NK cell effector function? The expression of so-called NK cell receptors is seldom confined to NK cells. Therefore, assessment of their function on other cell types is also of interest. Arguably, NK cells represent a useful model system for studying the mechanisms of cellular cytotoxicity. The process of cytotoxic granule release remains convoluted. How does syntaxin 11 facilitate granule release, and by which means is this requirement surpassed by cellular activation? Which SNARE proteins facilitate granule release and how are these proteins mobilized by different NK cell receptors?

Meticulous studies will hopefully provide answers to such fundamental questions of NK cell biology. In addition, they should provide clinically relevant insight. The realm of immunodeficiency syndromes is expanding. Detailed understanding of NK cell activation promises to elucidate genetic causes of infectious and inflammatory disease, such as the many unaccountable cases of secondary HLH. In addition, predisposition to cancer and autoimmunity may be explained. Hypothetically, although redundancy and resilience prevails in NK cell recognition, defects in particular NK cell receptor systems should produce more subtle clinical phenotypes, compared to fatal immunodeficiencies such as FHL. Importantly, advanced knowledge of the immune system's checks and balances will encourage new and improved treatment modalities.

Surely, nature has plenty of marvel to entice our further pursuit of wisdom!

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