From Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet, Stockholm, Sweden

REGULATION OF NK CELL ACTIVITY

- studies of DAP12-associated receptors in immune synapse formation and in responses to cytomegalovirus infection

Hanna Sjölin

Karolinska Institutet

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To Per and Brita Sjölin,
my grandparents, for friendship, love and support

ABSTRACT

Natural killer (NK) cell effector functions are important for innate resistance against tumor cells and viral infections. NK cells display a broad range of inhibitory and activating receptors on the cell surface, which ensure specificity. Several activating NK cell receptors are co-expressed with and function through the immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecule DAP12. The general aim of this thesis was to address the role of a specific signaling pathway for activating NK cell receptors, the DAP12 pathway, in complex situations such as cellular or host-pathogen interactions.

The redistribution of inhibiting and activating receptors and their ligands to the NK cell immune synapse has recently been investigated. To determine the role of DAP12 signaling in activating NK cell immune synapse formation, we established a system based on *in vitro* co-incubation with mouse NK cells and ligand-expressing target cells, using NK cells from mice bearing DAP12 molecules with non-functional ITAMs. We showed that the recruitment of the DAP12-associated activating NK cell receptor Ly49D to the NK cell immune synapse upon ligand-interaction was independent of DAP12 signaling. Signaling was however crucial for ligand induced down-modulation of Ly49D, similar to TCR-downmodulation.

To address specific activating pathways in the regulation of NK cells in the host-pathogen interaction, we studied the role of DAP12 in the early response to murine cytomegalovirus (MCMV). In DAP12 mutant mice bearing a non-functional ITAM, we found a considerable increase in viral titers in the spleen (30-40 fold) and in the liver (2-5 fold). The difference compared to wild type mice could be attributed to NK cells. Moreover, the percentage of hepatic NK cells producing IFN-γ was strongly reduced in the absence of a functional DAP12. This was the first study showing a crucial role for a particular activating signaling pathway in the NK cell-mediated resistance to an infection *in vivo*. Our results were in line with three concurrent reports demonstrating that innate resistance to MCMV requires the presence of NK cells expressing the activating receptor Ly49H, known to associate with DAP12. The DAP12 signaling pathway was critical also for the specific expansion of Ly49H+ NK cells upon MCMV infection, most likely by enhancement of cytokine-driven NK cell proliferation, indicating an adaptive component in the NK cell response.

Upon MCMV infection, NK cell stimulating cytokines such as IFN-α/β, and to some extent IL-12, are produced by plasmacytoid dendritic cells (pDCs). Murine pDCs deficient for the signaling molecule DAP12 produced increased amounts of IFN-α/β and IL-12 in response to cytomegalovirus (MCMV) infection or to CpG challenge *in vivo*. In the case of CpG challenge, this was regulated by endogenous DAP12 signaling. However, during MCMV infection, endogenous DAP12 signaling in pDCs limited IL-12 production but did not significantly modulate IFN-α/β induction. It is possible that DAP12 signaling influences some but not all of the pathways for induction of IFN-α/β. The DAP12 mediated regulation of pDC functions may be important to allow viral defense but limit immunopathology and avoid autoimmunity. NK cells have multiple functions and our results show that these can be dissected and explored also in complex situations. DAP12 mediated signaling regulates NK cell receptor dwnmodulation, effector functions as well as proliferation.

LIST OF PUBLICATIONS AND MANUSCRIPT

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I. Hanna Sjölin, Esther Nolte-'t Hoen, Hanna Odelfors, Sungjin Kim, Katja Andersson, Wayne Yokoyama, Daniel Davis and Klas Kärre. Role of KARAP/DAP12 signaling in Ly49D specific clustering and receptor modulation in the NK immune synapse upon ligand interaction. *Manuscript.*

II. Hanna Sjölin, Elena Tomasello, Mehrdad Mousavi-Jazi, Armando Bartolazzi, Klas Kärre, Eric Vivier and Cristina Cerboni. Pivotal role of KARAP/DAP12 adaptor molecule in the natural killer cell-mediated resistance to murine cytomegalovirus infection. *Journal of Experimental Medicine, 2002, Apr 1;195(7):825-34.*

III. Anthony R. French, Hanna Sjölin, Sungjin Kim, Rima Koko, Liping Yang, Deborah A. Young, Cristina Cerboni, Elena Tomasello, Averil Ma, Eric Vivier, Klas Kärre, and Wayne M. Yokoyama. DAP12 signaling directly augments pro-proliferative cytokine stimulation of natural killer cells during viral infections. *Journal of Immunology, 2006, Oct 15;177(8):4981-90.*

IV. Hanna Sjölin*, Scott H. Robbins*, Gilles Bessou, Åsa Hidmark, Elena Tomasello, Maria Johansson, Håkan Hall, Férose Charifi, Gunilla B. Karlsson Hedestam, Christine A. Biron, Klas Kärre, Petter Höglund, Eric Vivier, and Marc Dalod. DAP12 Signaling Regulates Plasmacytoid Dendritic Cell Homeostasis and Down-Modulates Their Function during Viral Infection. *Journal of Immunology, 2006, Sep 1;177(5):2908-16.*

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*these authors equally contributed to the work

CONTENTS

LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
β2m	β2- microglobulin
cDC	conventional dendritic cell
CMV	cytomegalovirus
cSMAC	central supramolecular activation cluster
CTLs	cytotoxic T cells
EBV	Epstein-Barr virus
γ_c	common cytokine receptor γ chain
GM-CSF	granulocyte monocyte colony stimulating factor
Grb2	growth factor receptor bound protein 2
HCMV	human cytomegalovirus
HEV	high endothelial venule
HSV	herpes simplex virus
IFN	interferon
IPC	interferon producing cell
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KARAP	killer cell activating receptor-associated protein
KIR	killer cell immunoglobuline-like receptor
LCMV	lymphochoriomeningitis virus
LPS	lipopolysaccharide
MCMV	mouse cytomegalovirus
mda5	melanoma differentiation-associated gene 5
MDL	myeloid DAP12-associated lectin
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
MIP1-α	macrophage stimulatory protein 1-α
MTOC	microtubule organizing center
NK	natural killer
NKC	natural killer gene complex
PAMP	pathogen-associated molecular pattern
pDC	plasmacytoid dendritic cell
PI3K	phosphoinositide 3-kinase
PILR-β	paired immunoglobulin-like type 2 receptor-β
PLC-γ2	phospholipase C-γ2
pSMAC	peripheral supramolecular activation cluster
RIG-1	retinoic acid inducible gene 1
SHIP	SH2-containing inositol polyphosphate 5-phosphatase
SHP	SH2-containing protein tyrosine phosphatase
SIRP-β	signal regulatory protein-β
SLE	systemic lupus erythematosus
STAT	signal-transducing activator of transcription

TLR	Toll-like receptor
TNF	tumor necrosis factor
TREM	triggering receptor expressed by myeloid cells
TYROBP	tyrosine kinase binding protein

1 AIMS OF THIS THESIS

Natural killer (NK) cells have been the focus of an exciting and rapidly expanding research field over the last three decades. NK cell activity is regulated by multiple activating and inhibitory receptors, determining the outcome their interactions with target cells. At the start of the studies presented in this thesis, reductionistic research had revealed several inhibitory and activating NK cell receptors, and some of their ligands had been defined. It was further known that activating NK cell receptors require association with adaptor proteins, such as DAP12, for signal transduction. Less was known about the role of signaling via activating receptors in more complex situations, at the level of cellular interactions or host-pathogen interactions. The general aim of this thesis was to address such issues.

In addition to the combination of receptors expressed on a given NK cell and the cognate ligands on the target cell, spatial organization of these molecules at the contact area between the cells, i.e. the NK cell immune synapse, may regulate NK cell specificity. In order to investigate the requirements for an activating NK cell immune synapse formation, we set up a model system using murine NK cells, in which DAP12 signaling was abrogated, and target cells expressing a ligand for the activating receptor Ly49D. The specific aims of the first study presented in this thesis (**Paper I**) were to determine the role of DAP12 signaling in a) the attachment to the target cells, b) specific recruitment of Ly49D to the immune synapse and c) ligand-induced downmodulation of the Ly49D receptor on the NK cell surface.

When I began my work on this thesis, the role of NK cells in the early defense against certain viral infections had been established but the mode of recognition of infected cells and the possible involvement of selective NK cell subsets were not known. Different activating NK cell receptors had been shown to associate with different adaptor proteins. Thus, by identification of specific adaptor proteins and signaling pathways required for an NK cell mediated response to viral infection *in vivo*, it might be possible to deduce which receptors that are critical for NK cell activation under these circumstances. The specific aim of the second study (**Paper II**) was to investigate the role of DAP12 signaling in murine NK cells, and thus possibly the role of its associated receptors, Ly49D and Ly49H, in the NK cell mediated defense against murine cytomegalovirus (MCMV) infection *in vivo*. We found that DAP12 signaling was pivotal for MCMV resistance provided by NK cells, and this was in line with concurrent reports on a crucial role for Ly49H.

It was further reported by others that the Ly49H+ NK cell subset was specifically expanding during MCMV infection. This was an unusual finding, suggesting that an adaptive component might be involved, i.e. that signaling through a specific NK cell receptor could regulate not only effector function but also expansion of the NK cell subset most suitable to control the infection. This would be in contrast with the general view of the innate immune system as ready to act but less adjustable. The aim of the third study (**Paper III**) was to determine whether, as for the adaptive immune system, activating receptor signaling, in this case through Ly49H/DAP12, could induce specific

NK cell proliferation in response to a viral infection, MCMV. If so, it would further be interesting to understand by which mechanism this occurred, particularly in relation to different cytokines, known to drive non-specific NK cell proliferation during viral infection.

NK cell activity can also be regulated by cytokines, and several other innate immune cells express DAP12 and DAP12 associated receptors. We therefore decided, as the aim of the fourth study (**Paper IV**), to examine the role of DAP12 signaling in regulation of other innate immune cells during MCMV infection, such as dendritic cells, known to produce NK cell activating cytokines in response to the virus.

The results of these studies will presented and discussed. As the original studies, **Paper I-IV**, are included in this thesis, the discussion is written with the ambition to focus on comparison with related studies, interpretations, possible models and suggestions for follow up experiments, rather than an extensive account of the results. Before presenting the studies, I will introduce and summarize additional background information, upon which the aims were based, and necessary for discussion of the results. This introductory part thus mainly presents the status of the field as I perceived it at the time of the start of each study.

2 INTRODUCTION

2.1 Natural killer cells

2.1.1 Natural killer cells – an introduction

Natural killer (NK) cells were first described in 1975 as cells able to kill certain tumor cells *in vitro*, without prior priming. This was in contrast to cytotoxic T cells (CTLs) which require priming and clonal expansion before mounting an effective response [1-4]. Experiments further demonstrated that mouse NK cells can mediate killing of tumor cells and rejection of allogeneic bone marrow grafts *in vivo* [5-9]. NK cells, most of which are large granular lymphocytes, are now considered a part of the innate immune system and also contribute to the early defense against several intracellular pathogens [10-13]. In contrast to T and B cell lymphocytes, NK cells do not undergo receptor gene rearrangement during development. After maturation in the bone marrow, they migrate to the blood and peripheral lymphoid organs, constituting 5-15 % of the blood lymhocyte compartment, around 40% of the liver lymphocytes and 3-5 % of the spleen lymphocytes in the mouse [14]. NK cells are also found at the maternal-fetus interface in the placenta [15]. They express cell surface markers CD56 in humans, and NKR-P1 (NK1.1) and/or DX5 in mice, while lacking CD3, TCR and Ig surface expression [14, 16].

NK cells possess potent effector mechanisms for elimination of transformed, infected or allogeneic target cells. In a cell-contact dependent manner, NK cells can induce target cell lysis and apoptosis through directed release of cytotoxic granules containing perforin and granzymes, and/or ligands engaging death receptors on the target cell, such as Fas or TRAIL-R (reviewed in [17-19]). When stimulated, NK cells can further secrete cytokines, such as gamma interferon (IFN-γ), tumor necrosis factor-α (TNF-α) and granulocyte/macrophage colony stimulatory factor (GM-CSF) and chemokines, such as macrophage stimulatory protein (MIP)-1α and 1β. These can have direct anti-microbial effects, activate other cells or induce cell differentiation. IFN-γ restrains viral replication and induces upregulation of major histocompatibility complex (MHC) class I molecule expression, facilitating CD8+ T cell recognition of infected cells [12]. IFN-γ also activates myeloid cells and directs the adaptive immune system. [20].

2.2 Regulation of NK-cell activity

2.2.1 Regulation of NK cell development, homeostasis and activity by cytokines

NK cell development and activity are influenced by several cytokines and chemokines, such as the interleukins IL-15, IL-2, IL-12 and IL-18, as well as type I interferons (IFNs) produced by other cell types (reviewed by [12]). IL-15 drives proliferation of NK cells, and is essential for NK cell development in the bone marrow as well as for peripheral homeostasis [21, 22]. Mice deficient for IL-15, IL-15Rα or the common cytokine γ chain (γ_c), a subunit for receptors for IL-2,-4,-7,-9,-15,-21, all lack mature NK cells [23-26]. IFN-α/β regulate NK cell cytotoxicity and proliferation, while IL-12

and IL-18 are potent inducers of IFN-γ production by NK cells [12, 27]. Tumor necrosis factor (TNF), IL-15 and IL-1α and β also synergize with IL-12 for induction of IFNγ production [12]. Further, NK cells migrate in response to chemokines such as MIP1α, RANTES and MCP-1 [11, 28-30]. In contrast, some cytokines like IL-10 and transforming growth factor-β (TGF-β) downmodulate NK cell responses [31-37].

2.2.2 Major histocompatibility complex class I molecules

NK cell activity is also regulated through direct interactions between receptors on the NK cell and their cognate ligands on the target cell. Both T lymphocytes and NK cells express receptors that interact with molecules encoded within the major histocompatibility complex (MHC). This gene complex, located on chromosome 6 in human and 17 in mouse, includes multiple genes for each of the different types of MHC molecules: classical MHC class I (Ia), MHC class II as well as non classical MHC molecules (class Ib). These genes show allelic polymorphism, creating a highly diverse MHC repertoire among individuals. The class Ia molecules are termed HLA-A, HLA-B and HLA-C in humans, and H-2K, H-2D and H-2L in mice [38, 39].

Classical MHC class Ia molecules consist of a MHC encoded heavy chain noncovalently associated with a subdomain, β2-microglobulin (β2m). The heavy chain contains of three extracellular domains, a transmembrane domain and a cytoplasmic tail. Two of the domains fold into a β sheet with two α helixes on top. Between these helixes a peptide is presented that together with the β2m provides stability of the MHC class I molecule. CD8+ T cells recognize antigenic peptides, derived mainly from proteins degraded in the cytosol [40] and presented by MHC class I molecules [41]. MHC class I molecules are expressed on all nucleated cells [38, 39], and the multiple genes and large polymorphism allow expression of various combinations of MHC class I molecules. This ensures presentation of a vast number of different peptides, including peptides derived from intracellular pathogens. Non-classical MHC molecules, such as HLA-G, HLA-E and MICA in humans and Qa-1b in mice, show less polymorphism. HLA-E and Qa-1b present peptides derived from the leader sequences of the classical MHC class I molecules [42].

2.2.3 Inhibitory NK-cell receptors

Over 20 years ago, Kärre proposed a model for regulation of NK cells, called the missing-self hypothesis [43-46]. According to this hypothesis, a target cell becomes susceptible to NK cell mediated killing if it fails to express sufficient autologous MHC class I molecules, i.e. the same MHC class I molecules as the NK cell. Downmodulation of the MHC class I molecules, observed on virally infected cells and tumor cells, would thus render these cells susceptible to NK cell mediated killing. A mechanistic model for how NK cells could sense the insufficient MHC class I expression predicted that regulation of NK cell activity towards a target cell is controlled by a balance between signals through activating and inhibitory receptors. The latter would recognize MHC class I molecules, and targets with reduced or no expression of these would be unable to engage putative inhibitory NK cell receptors. To test the missing self hypothesis, MHC class I deficient tumor cell lines were selected and injected in C57BL/6 mice (a commonly used inbred laboratory strain). The MHC class I deficient tumor cells were specifically rejected under conditions where wild type

cells were not [45, 47] and this rejection was shown to be NK cell dependent [46]. The model was further confirmed by several studies, including experiments where bone marrow from genetically modified mice lacking β2m, and thus MHC class I surface expression, was rejected *in vivo*, though on otherwise the same genetic background as the recipient [48, 49]. A few years later, the Ly49A molecule was shown to function as an inhibitory receptor on murine NK cells [50, 51]. An analogue receptor on human NK cells was also described [52-57] and it was concluded that self MHC class I grants target cell protection from NK cells by ligation of inhibitory receptors on NK cells.

The Ly49A molecule belongs to a C-type lectin-like receptor family of type II glycoproteins. A pseudogene is present in humans, but so far description of functional Ly49 receptors has been limited to rodents and horses. The inhibitory Ly49 receptors are expressed as disulfid-linked homodimers on NK cells [58], but also on small T cell subsets, like memory T cells and some effector CD8+ T cells [59-61]. One receptor in the family, Ly49Q, is expressed on a subset of mouse plasmacytoid dendritic cells [62-64]. The Ly49s are encoded by genes in the NK gene complex on mouse chromosome 6. The NK gene complex contains several polymorphic receptor genes. At present, Ly49A, Ly49C, Ly49G2 and Ly49I have been characterized as inhibitory receptors on NK cells in the C57BL/6 mouse strain, and specific MHC class I ligands identified [59]. The receptor expression pattern is variegated, with a certain probability for each receptor to be expressed on a given NK cell. The probability is separate for each receptor, so that an NK cell can express none to more than three inhibitory receptors. Signaling upon engagement of inhibitory Ly49 receptors is initiated by immunoreceptor tyrosine based inhibitory motifs (ITIM), V/IxYxxL/V, in the cytoplasmic domains of the receptors. Phosphorylation of the ITIMs by Src family kinases results in recruitment of tyrosine specific SH2-containing protein tyrosine phosphatases 1 and 2 (SHP-1, SHP-2) or the phospholipid specific SH2-containing inositol polyphosphate 5-phosphatase (SHIP), which can abrogate activating receptor signaling pathways [59, 65].

Another type of NK cell receptors, killer immunoglobulin-like receptors (KIRs), dominate in humans. KIRs have an analogue function to the Ly49s in the mouse but belong to the Ig superfamily of proteins. Like inhibitory Ly49s, they bind MHC class I molecules and carry ITIMs in their cytoplasmic tails, initiating inhibitory signals in NK cells upon ligation. Similar to the Ly49s, the KIR genes are polymorphic and show a variegated expression pattern within the NK cell population. Both human and mouse NK cells also express the inhibitory receptor CD94/NKG2A, a C-type lectin heterodimer complex, which binds the HLA-E in humans and Qa-1 in the mouse. Some inhibitory receptors, such as NKR-P1D and KLRG1 in the mouse, recognize other ligands than MHC molecules [65, 66].

2.2.4 Activating NK-cell receptors

The missing self hypothesis prediction of MHC class I mediated inhibition as one of the mechanisms for control of NK cell activity still holds true. Kärre and colleagues also predicted a parameter of multiple choice, according to which not one single type of interaction alone accounts for the target cell-NK cell encounter outcome [47, 67, 68]. Rather, the outcome is regulated by integrated signals from multiple receptors,

dependent on the ligands presented on the target cell. Several activating receptors are now defined, and it has been suggested that the principle model for NK cell recognition now should be extended to include that NK cell mediated killing can occur also if the target cell expresses appropriate activating ligands and activation supersedes any inhibitory signals [65].

It is noteworthy that some activating receptors, which are included in both the Ly49 family and the KIR family, actually bind MHC class I molecules, something which was not predicted by the missing self hypothesis. The activating receptors lack ITIMs and carry a comparably short cytoplasmic tail. Of the activating Ly49 receptors in the mouse, Ly49D and Ly49H are expressed in the mouse strain C57BL/6, while others are expressed in mouse strains such as Balb/c, 129 or NOD [65]. There is evidence that many of the activating receptors have originated by gene duplication or gene conversion of inhibitory receptors, and simultaneously lost the ITIM containing cytoplasmic tail. If an activating and an inhibitory receptor bind the same type of MHC, the activating receptors usually display lower affinity to the specific MHC class I ligand than the inhibitory receptor. It is possible that the low affinity interaction with self MHC by activating receptors is important to avoid autoimmunity, though this has yet to be investigated [65]. The activating receptor Ly49D has been shown to bind to the same ligand as the inhibitory receptor Ly49A, H-2D^{d}, but with lower affinity [65, 69]. The physiological relevance of this and other activating Ly49s and KIR recognizing MHC class I molecules is however not clear.

Human and mouse NK cells express a type of activating receptors distinct from the activating KIRs and Ly49s, respectively. These are termed natural cytoxicity receptors (NCRs) and consist of NKp46, NKp30 and NKp44 on human NK cells [65, 70]. A murine homologue to NKp46, MAR-1, has been characterized. Both human and mouse NK cells can express additional activating receptors such as the activating heterodimer receptor complex CD94/NKG2C, NKG2D and CD16 [65]. The NKG2D ligands are DNA-damage induced molecules, e.g. Rae-1 molecules in the mice and MICA in humans [71, 72]. CD16 is an Fc receptor that triggers NK cell mediated antibody dependent cell cytotoxicity (ADCC) of IgG-coated target cells. Additional activating receptors are CD2, 2B4, DNAM-1, KIR2DL4 and integrins like LFA-1 (CD11a/CD18), CD11b/CD18 and CD11c/CD18 [65].

2.3 The adaptor protein DAP12

2.3.1 ITAM mediated signaling

Surface expression of most known NK cell activating receptors, as well as their signaling, depends on interactions with adaptor proteins. These interactions occur through non-covalent binding between charged amino acid residues on the receptor and adaptor protein in the transmembrane region. Not all receptors associate with the same adaptor proteins, and in both mice and humans several adaptor proteins are expressed. Adaptor proteins that associate with activating NK cell receptors are CD3ζ, FcεRIγ, DAP12 and DAP10. CD3ζ, FcεRIγ and DAP12 have short extracellular domains, negatively charged amino acid-residues in the transmembrane region, and

intracytoplasmic immunoreceptor tyrosine activating motifs (ITAMs) [65, 73]. The ITAM sequence, YxxL-x6-8-YxxL, contains two tyrosine residues that both are required for signaling [65, 73, 74]. Upon crosslinking of associated receptors, the ITAMs are phosphorylated by Src family kinases, and serve as docking site for SH2-domain containing protein tyrosine kinases ZAP70 or Syk. This starts a phosphorylation cascade involving PLC-γ and MAP kinases leading to transcription factor translocation to the nucleus, Ca^{2+} influx and can consequently result in activation of the NK cell and elicitation of cytotoxicity and cytokine production (Figure 1)[65, 75, 76]. DAP10 carries a different kind of activation motif, the YxxM sequence, which upon phosphorylation can recruit phosphatidyl inositol 3 kinase (PI3K) and growth factor recptor-bound protein 2 (Grb2) [65, 75, 77].

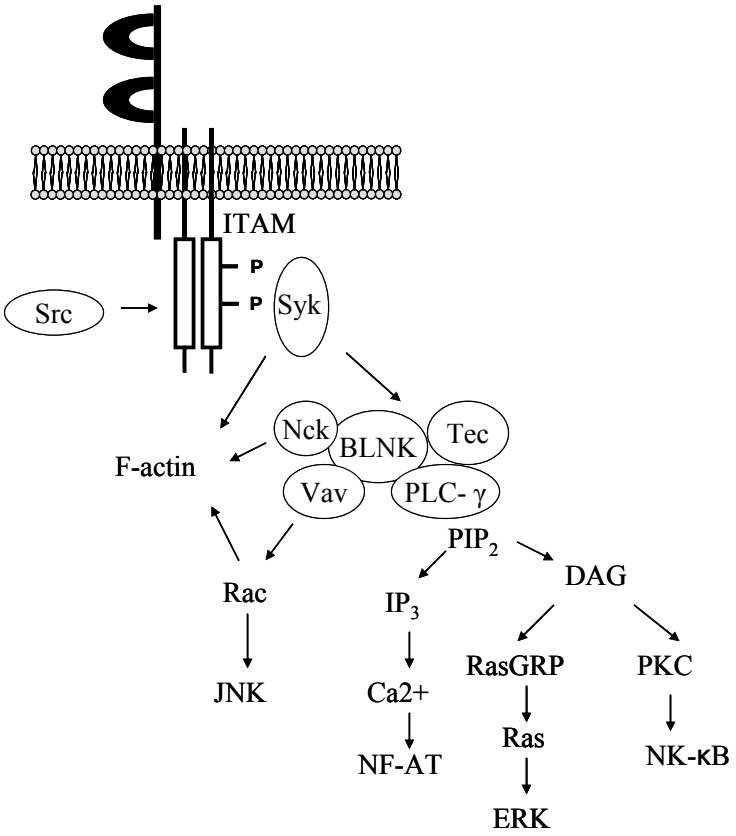

Figure 1. **Schematic outline of ITAM signaling cascade.** Receptor engagement results in ITAM phosphorylation by Src, allowing recruitment and activation of Syk family proetin tyrosine kinases. This allows subsequent phosphorylation of the BLNK or SLP-76 family of adaptor proteins. From here multiple signaling cascades are induced, including activation of PI3K, PLC-γ induction of Ca^{2+} flux and NF-AT translocation to the nucleus, ERK activation, and PKC activation resulting in activation of the transcription factor NF-κB (Modified from[78])

2.3.2 DAP12 associated receptors

The adaptor protein DAP12 associates with a variety of receptors on NK cells and myeloid cells. It is a 12kDa protein expressed as a disulfid-linked homodimer, with one ITAM per subunit [73]. The integrity of the ITAMs is required for DAP12 dependent signal transduction [74, 79]. DAP12, also called TYROBP (tyrosine kinase binding

protein) or KARAP (killer cell activating receptor-associated protein), is highly conserved in vertebrates [73]. It has a negatively charged aspartic acid in the transmembrane region [74, 80], facilitating binding to activating NK cell receptors, such as the murine receptors Ly49D, Ly49H [81] and Ly49P [82]. While otherwise associated with DAP10 as a long isoform, NKG2D can bind DAP12 when expressed as a short isoform in murine IL-2 activated NK cells. [83, 84]. Additional DAP12 associated receptors on NK cells include paired immunoglobulin-like type 2 receptor β (PILR-β) and CD200R4 in mouse, CD94/NKG2C [73, 85] and E, in both human and mouse, and NKp44 and activating KIRs in humans [73].

Although originally characterized in NK cells [74, 80], DAP12 is expressed also in other cell types, both in other lymphocytes and in cells of myeloid origin. In monocytes, macrophages and granulocytes DAP12 associates with receptors belonging to the family of triggering receptor expressed by myeloid cells (TREMs) [73, 86]. Co-ligation of TREM-1 upon LPS stimulation enhances cytokine production and inflammation, and TREM-1 triggering is involved in septic shock [87]. This indicates that DAP12 signaling can augment inflammatory reactions dependent on TLR-signaling. TREM-2, signal regulatory protein SIRP-β, PILR-β, CD200R and myeolid DAP12-associated lectin (MDL-1) are other examples of DAP12 associated receptors on myeloid cells. Whereas TREM-2 ligation can induce macrophage production of nitric oxid (NO) [88], triggering of TREM-2 on human DCs induce partial DC maturation and survival, along with CCR7 expression, presumably allowing migration to lymph nodes [89]. PILR-β ligation can induce DC production of TNF-α and NO [73, 86].

2.3.3 DAP12 deficiency

Studies of DAP12 deficient mice have revealed further aspects of DAP12 and its associated receptors in the immune system *in vivo* [90-92]. Bakker *et al* produced mice with a disruption of the gene segment coding for the transmembrane region and the first part of the ITAM sequence of DAP12, resulting in absence of the DAP12 protein [90]. Since DAP12 is required for stable surface expression of its associated receptors [73, 93], these mice show no or marginal surface expression of these receptors [90]. Tomasello *et al* produced DAP12 loss-of-function mice in which surface expression of DAP12, and its associated receptors, is maintained but one of the two tyrosine residues of the ITAM and the wild type C-terminus amino acids are lacking (Figure 2). The DAP12 molecules in these mice are thus unable to transduce activating signals [91].

Both types of mice with modified DAP12 genes showed accumulation of DCs, in the skin and intestinal mucosa, along with impaired T cell priming. The numbers of NK cells, as well as the expression pattern and function of inhibitory Ly49 receptors, were however comparable to wild type mice. As expected, the activating Ly49s were dysfunctional, with substantial reduction of Ly49D- and Ly49H-dependent killing of target cells. In contrast, NK cell mediated killing of the tumor cell line YAC-1, which is mainly dependent on NKG2D interactions, was unaffected. Importantly, NK cells from the loss-of-function mice were also able to kill RMA-S (an NK cell susceptible cell line lacking MHC class I expression) to the same extent as wild type NK cells, indicating

that recognition of missing self MHC class I molecules is functional in these mice [90, 91].

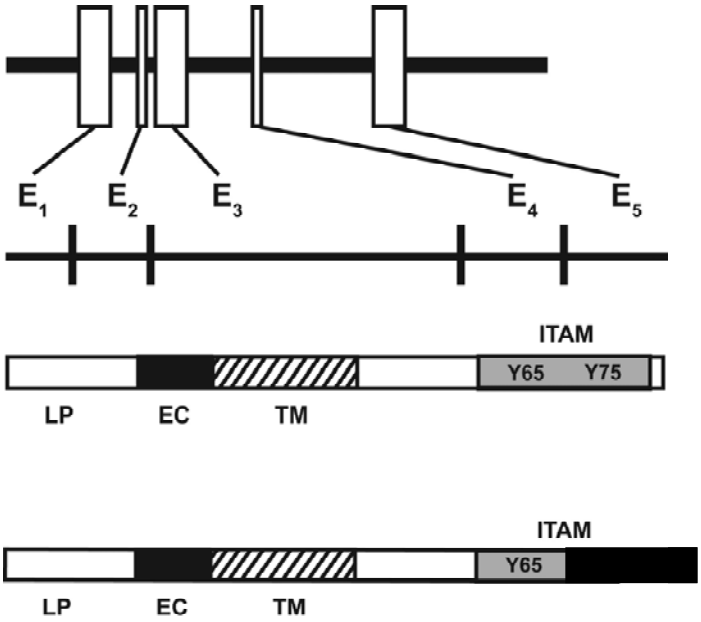

Figure 2. Modification of the DAP12 protein in DAP12-loss-of-function mice. Top: The exon/intron organization of the mouse DAP12 gene (E= exon). Corresponding DAP12 protein right below (LP= leader peptide; EC= extracellular domain; T=transmembrane domain). DAP12 ITAM with tyrosine residues Y65 and Y75 (shaded area). Bottom: DAP12 as expressed in DAP12 loss-of-function (DAP12-/-) mice, loss of Y75 and wild type C-terminus amino acids (Modified from [91]).

Osteoclasts and microglia cells also express DAP12 [86]. In humans, deficiency of DAP12 or the DAP12-associated receptor TREM-2 can give rise to the Nasu-Hakula syndrome, resulting in bone cysts and dementia [94-97]. Similar conditions developed in mice lacking DAP12 [73, 92]. Recently, a paradoxical inhibitory role of DAP12-dependent signaling was shown. Engagement of TREM-2 on macrophages dampened lipopolysaccharide (LPS)-induced cytokine production, and DAP12 deficient mice were more susceptible to a certain model of septic shock [98]. These unexpected data opened up a new field within ITAM-signaling, and will be discussed below, in relation to the studies of this thesis [76, 78]. Thus, the adaptor protein DAP12 has multiple and diverse functions in NK cells as well as in other cell types, both in the immune system and beyond [73].

2.4 NK cell immune synapses

2.4.1 Activating NK cell immune synapses

Although several activating and inhibitory NK cell receptors and their ligands are defined today, it is still unclear how the different signaling pathways are integrated. Better understanding of the spatial organization of ligands, receptors and signaling molecules at the contact area between the NK cell and its target, i.e. the NK cell immune synapse, may provide clues as to how specificity of NK cell activation is

achieved. Techniques such as electron or confocal microscopy have therefore been applied to study the molecular arrangements and the polarisation of the cytolytic machinery. The interactions and formation of the immune synapses can be analysed in different model systems, e.g. in lipid bilayer models of cell membranes, by staining for molecules on fixed cell conjugates or by live cell imaging using cells transfected with fluorescently tagged receptors and ligands [99, 100].

The term immune synapse was first used to describe the contact area between T cells and antigen presenting cells (APCs), and the subsequent polarised secretion of cytokines. T cell immune synapses have been extensively studied. However, transient interactions with specific spatio-temporal accumulation of receptors and signaling molecules seem to be a general hallmark of immune effector cell function and reciprocal communication between cells. Some general themes observed for T cell synapses may thus be applicable to the activation of NK cells. Organization of the T cell immune synapses ranges from mere local enrichment of receptors at the site of contact with target cells or APCs to microdomains formed in a so called mature T cell synapse. The latter is characterized by TCR enrichment in the central supramolecular activation cluster (cSMAC), surrounded by a ring of adhesion molecules, such as the β2 integrin LFA-1, at the peripheral supramolecular activation cluster (pSMAC). Large molecules such as CD43 and CD45 are excluded from the synapse. Multifocal synapses, with several discrete clusters of the TCR in the cSMAC within the pSMAC have also been observed [101, 102].

The processes involved in TCR recruitment to the synapse are not completely clear. Polarized recycling of TCRs, together with passive lateral diffusion, and size exclusion based on extracellular parts of receptor and ligand complexes may contribute to formation of the T cell synapse [101, 102]. Further, if receptors are linked to the cytoskeleton, they may be recruited to the synapse upon actin polymerisation and cytoskeleton movements towards the synapse [103]. TCR signaling is initiated in the peripheral parts of the synapse before TCR accumulation in the cSMAC. Quality and quantity of antigen are important for TCR recruitment to the cSMAC, and downstream signaling molecules of the TCR such as Vav1 and Rac1 are important for modulation of the actin cytoskeleton and acetylation of microtubuli. This indicates that TCR signaling is involved in synapse formation [101, 102, 104]. Engagement of adhesion molecules or co-receptors such as CD28 can also influence molecular organization at the synapse [101-103]. Accumulation of lipid rafts, i.e. lipid- and cholesterol-rich plasmamembrane micro-domains, occurs at the T cell synapse and activating NK cell synapses and is involved in cytotoxicity and phosphorylation of activating receptors [100, 105, 106]. Although the concept of lipid rafts can be questioned due to the relatively intrusive methods available to study them, several reports address this type of membrane subdomains. Signaling molecules such as the Src family kinase Lck can associate with lipid rafts, which are considered as potential signaling platforms, bringing receptors and downstream signaling molecules together at the synapse. The accumulation of lipid rafts is also dependent on actin polymerisation, suggesting that it may be induced by receptor signaling [100].

Similar to CTLs, NK cells can form activating synapses allowing directed secretion of cytolytic granules [102, 104, 107]. For NK cells, two scenarios have been studied

(Figure 3): activating immune synapses, when activation prevail and the target is killed, and inhibitory immune synapses, when NK activity is inhibited and the target cell spared [107]. For activating NK cell synapses, actin polymerisation is required for recruitment of lipid rafts, certain receptors and adhesion molecules to the contact area [108]. In addition to actin polymerisation, microtubuli movements and relocalisation of the microtubuli organizing center (MTOC) towards the immune synapse are important for further synapse formation and polarised secretion of cytolytic granules. Signaling molecules, like Lck, are recruited to the activating NK cell immune synapse [109, 110], and activating signaling is necessary for cytoskeleton movements [111] and synapse assembly of lipid rafts [105]. The reports on signaling molecules involved in the recruitment of activating receptors to the NK cell immune synapse are however scarce and not much is known about the underlying mechanisms.

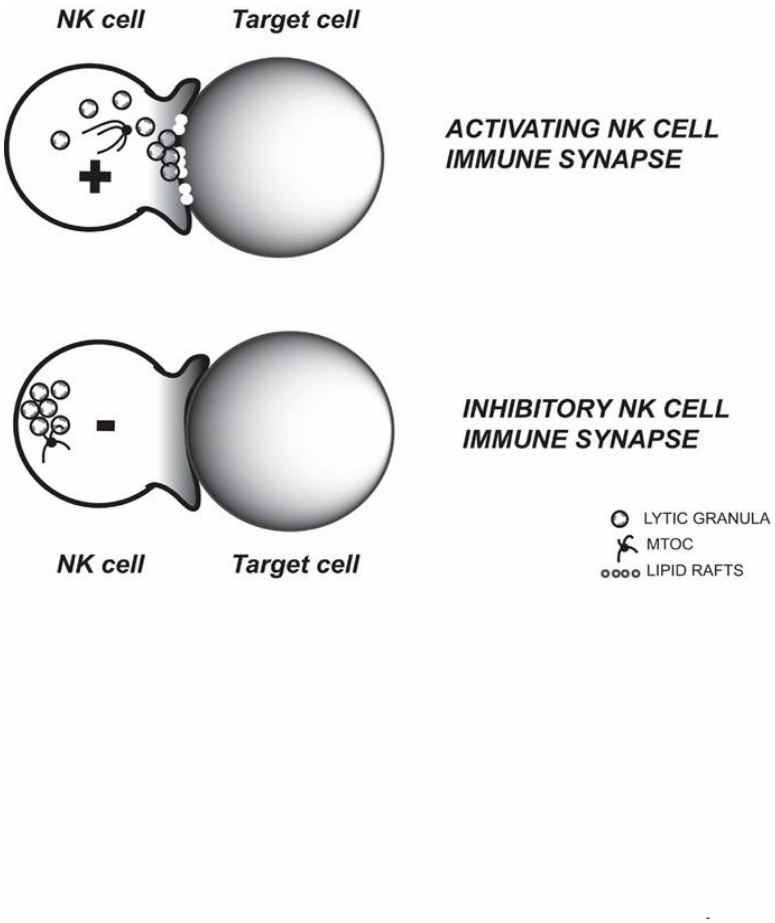

Figure 3. Schematic picture of the NK cell immune synapse. Top: Activating NK cell immune synapse, with polarisation of MTOC and lytic granula, as well as lipid raft accumulation. Bottom: Inhibitory NK cell immune synapse where polarisation of MTOC and lytic granula does not occur and lipid raft accumulation is blocked.

2.4.2 Inhibitory NK cell immune synapses

The rate by which inhibitory receptors are recruited to the inhibitory immune synapse is partly dependent on ITIM signaling and actin polymerisation, but other factors may also contribute [111-114]. Lck is initially recruited also to the inhibitory synapse, but is quickly removed, while SHP-1 phosphatases co-localize with the inhibitory receptors [110, 111, 115, 116]. Inhibitory signaling, as shown for CD94/NKG2A and human KIR2DL1, abrogates assembly of lipid rafts at the synapse [110, 111, 116], and polarisation of the MTOC and cytolytic granula does not occur at the inhibitory synapse[107]. This indicates that inhibitory signaling is able to interfere upstreams of these events. Although phosphatase activity from SHP-1 and SHP-2 may influence phosphorylation status of ZAP70, Syk, PLC-γ, LAT and SLP76, Vav1 is so far the only defined direct substrate for SHP-1 [117]. Coligan *et al* recently reported that the inhibitory receptor CD94/NKG2A, through dephosphorylation of Vav1 and ezrin-radixin-moesin (ERM) proteins (linking transmembrane proteins to the cytoskeleton), disrupts actin polymerisation at the inhibitory immune synapse and thus impairs the settings for activating signals already at the level of lipid raft recruitment [118]. Importantly, inhibitory signaling at one synapse does not inhibit actin polymerisation at interaction sites with other target cells on the same NK cell, supporting former data on the capacity of an NK cell to simultaneously form activating and inhibitory synapses with different target cells [119].

As for the T cell synapse, several factors may be involved in the recruitment of both inhibitory and activating receptors and the formation of the NK cell synapse. These factors include receptor-ligand affinity, cytoskeleton reorganization, size exclusion (dependent of the length of extracellular part of the receptors and ligand) and accumulation of lipid rafts [100, 120, 121]. The studies done so far on NK cell immune synapses have been based on various systems with human and murine NK cells, and it is difficult to draw conclusions about general mechanisms. Separate signaling pathways are probably regulated in different ways, and the formation of the activating synapse may depend on the combination of receptors involved [122, 123]. Is signaling through an NK cell activating receptor, as reported for inhibitory receptors and the TCR, necessary for this receptor to be recruited to the synapse? If not, are activating receptors recruited also in the presence of inhibitory signaling, as long as there are specific activating ligands on the target cell? The requirements for receptor clustering may also differ depending on the type of activating receptor. Knowledge about the requirements for NK cell receptor recruitment, the synapse formation and the putative functions of these processes may help us to better understand how NK cell activity is regulated. Ultimately, imaging of cells migrating and interacting within tissue samples or *in vivo* may further elucidate activation mechanisms of NK cells involved in tumor clearance or resistance against infection [124].

2.5 Innate responses to cytomegalovirus

2.5.1 NK cells in innate responses to viral infections

The innate immune system counteracts viral infection through multiple mechanisms, where soluble factors, such as anti-microbial peptides, cytokines and the complement

system, act in concert with innate immune cells, such as phagocytes and NK cells. [125]. The role of NK cells in the innate defense against viral infections was first implicated through the augmented NK cytotoxicity and NK cell blastogenesis in virus infected and interferon treated mice [27, 126-129]. Studies of viral infections such as herpes simplex virus (HSV)-1 [130], and mouse hepatitis virus (MHV) [131, 132], and of NK cell mediated cytotoxicity towards infected target cells *in vitro* also provided evidence of an NK cell mediated defense against viral infections [133-135]. Depletion of NK cells in mice caused susceptibility to murine cytomegalovirus (MCMV) and MHV, but not to lymphocytic choriomeningitis virus (LCMV), indicating selective importance of NK cell activity in the defense against certain viral infections [136-138]. NK cell transfer experiments [139] and studies of NK cell deficient mice later strengthened the insights on the role of NK cells in viral resistance [132, 140, 141]. Interestingly, studies also showed that interferons could induce target cell protection from NK cell mediated killing [135, 142-144].

Increased susceptibility to viral infections due to NK cell deficiency or malfunction has been reported also in humans. Lack of efficient NK cell responses can originate from several genetic defects, of which some have been identified and others are unknown. The conditions include complete and selective lack of NK cells, severe combined immune deficiency (where T and B lymphocyte differentiation are impaired as well) and defective NK cell function [145]. The first evidence for an important role of NK cells in resistance to human herpesviruses was provided in a case report in 1989. The patient, a young girl who was monitored for several years, developed severe primary herpes virus infections including varicella zoster, human cytomegalovirus (HCMV) and HSV. She was diagnosed with selective and complete NK cell deficiency [145, 146], (for each infection she eventually developed virus specific adaptive immunity). The second case so far of selective NK deficiency was reported recently, describing fatal varicella infection as result of the impaired NK cell response [147]. Reports of four additional patients diagnosed with impaired NK cell activity, and suffering from widespread HSV disease, have also published, but in these cases other immmunodeficiences may also have contributed to the lack of viral resistance [145]. In the studies mentioned above, there is no information on the genes involved or other causes behind the NK cell deficiency. However, patients with certain CD16 receptor alleles suffered from recurrent HSV, varicella or Epstein-Barr virus (EBV) infections, displaying a genetic disorder as the cause of NK cell deficiency and suggesting an important role for ADCC as NK cell effector mechanism is viral defense. Moreover, selective NK cell deficiency linked to a specific region on human chromosome 8 has recently been reported. This deficiency resulted in increased susceptibility to virus related disease such as Epstein-Barr virus lymphoproliferative disorder, further emphasizing the role of NK cells in anti-viral immunity [145, 148].

2.5.2 Cytomegalovirus

The most thoroughly studied infection in NK cell mediated defense against pathogens is cytomegalovirus (CMV). CMV belongs to the β-herpesvirus family and is an ancient, species-specific group of viruses with a long history of co-evolution between microbe and hosts. Like other β-herpesviruses, CMV has a slow cycle of replication and is enveloped. Human CMV (HCMV) has a large double stranded DNA genome of 230

kb, leaving room for several genes not directly necessary for replication and latency, e.g. genes encoding immunoevasion proteins. The virus spreads via body fluids, such as saliva, breast milk, urine and blood, with initial infection commonly occuring during childhood. Infection results in lifelong latency, with latent virus likely residing in cells of myeloid lineage. Between 30 and 70% of the population in developed countries carries human CMV, while over 90% show seroprevalance in developing countries. HCMV can be reactivated occasionally, but is relatively harmless in healthy individuals. Reactivation may be induced for example during pregnancy and breast feeding. If the immune system is deficient or suppressed, e.g. in association with cancer, transplantation or AIDS, HCMV can however give rise to severe symptoms and even life-threatening multiple organ failure. By crossing the placenta and infecting the fetus, HCMV may also cause damages such as mental retardation, loss of hearing and failure of liver and spleen of the fetus [149-152].

Murine CMV (MCMV) shows significant analogy to HCMV, both in terms of molecular and pathological aspects. In acute MCMV infection, the virus disseminates to the liver and spleen, and can similarly to HCMV induce conditions such as hepatitis and pneumonia if not controlled [153, 154]. Although many of the MCMV proteins are quite different from the ones encoded by HCMV, many of the immune evasion mechanisms they confer are comparable, such as downmodulation of MHC class I molecules and expression of inhibitory ligands for NK cell receptors. These analogue strategies, together with the similar pathology of HCMV and MCMV, make acute primary infection of MCMV in mice a useful model for further studies of the immune responses against CMV [152].

2.5.3 NK cell responses to MCMV

Upon primary infection, mostly studied after intra-peritoneal injections, MCMV titers in spleen peak around day 2-5 and is resolved around day 6 (depending on the infection dose). The NK cell response peaks 3-5 days after virus injection, and includes cytotoxicity and production of cytokines and chemokines [153, 155-157]. NK cells migrate, infiltrate and accumulate in several organs such as the lungs, the spleen and the liver [158, 159]. The importance of the NK cell effector mechanisms for viral clearance vary in an organ-dependent manner. Although both effector mechanisms contribute in each of the organs [160], in the spleen the NK cell mediated defense is mainly perforin dependent, whereas it is mainly IFN-γ dependent in the liver [161]. In salivary glands, another site for MCMV replication, infection is controlled by NK cells in a perforin and granzyme dependent manner [162]. Natural cytoxicity results in contact dependent killing of MCMV infected target cells, while IFN-γ interferes with viral replication and assembly of virus particles [163]. Although IFN-γ is produced systemically, proximal NK cell production of IFN-γ is required for viral load control in the liver [11, 12].

Some mouse strains, like C57BL/6 mice, are resistant to MCMV and can clear the infection quite efficiently. Resistance is dependent on a functional NK cell defense, though final control of MCMV infection requires an efficient T cell response [164-167]. Other mouse strains, like Balb/c or 129, are MCMV susceptible [168]. The discovery of the Cmv1r locus mediating MCMV resistance in the spleen [168, 169],

and its location within the NK complex, close to the Ly49s, argued for the role of specific receptor recognition. MHC class I molecules surface expression can be reduced on virally infected cells [169-174], possibly as part of viral immune evasion from T cells. This would allow virus infected cells to escape recognition, even though MHC class I expression is augmented in most cells by IFN-α/β. According to the missing self hypothesis, this would however render an infected cell more susceptible to NK cell mediated cytotoxicity, since it entails reduction of the capacity for NK cell inhibition. This concept was tested in mice genetically deleted for β2-microglobulin, thus not able to further downmodulate MHC expression on infected cells or upregulate them in normal cells [139, 175]. However, these mice showed similar early control of viral titers as resistant wild type mice, arguing against the idea [175, 176]. NK cell mediated resistance could thus be activated through other still unidentified direct recognition strategies, or NK cells might not be able to discriminate infected cells from uninfected cells through direct interaction. In the latter case, local cytokines may act as main regulators of NK cell selective reactivity in areas of infection. At the start of the work presented in this thesis, the gene and protein of the Cmv1r locus were unknown. During the last five years this field has been the subject of intense studies, which will be presented and discussed later in relation to two of the original papers included in this thesis.

2.5.4 IFNα/β production upon viral infection

A high production of type I interferons IFNα/β is commonly detected early during viral infections, and this is vital for host defense against the infection [177-179]. IFN-β is encoded by a single gene, while distinct genes encode the 13 different IFN-α proteins [180]. Both IFN-α and IFN-β are recognized by a heterodimeric receptor composed of IFNAR1 and IFNAR2 subunits, expressed on most cells. Receptor binding triggers phosphorylation of Jak and Tyk tyrosine kinases, and subsequent induction and phosphorylation of several signal transducers and activators of transcription (STAT), such as STAT1 and STAT2 forming homo- or heterodimers. STAT1/STAT2 translocate into the nucleus, and ultimately induces transcription of the IFN inducible genes [177, 180].

During viral infection, IFNα/β have multiple and vital functions. Most cells are capable of producing type I IFNs in response to intracellular virus infection. Type I IFNs upregulate an anti-viral response in the infected cells but also in neighbouring uninfected cells in a paracrine fashion, leading to a block of protein translation and degradation of cellular and viral RNAs [177]. A broad range of viruses, including both RNA and DNA viruses, are sensitive to IFN-α/β mediated anti-viral effects [12, 180]. In addition to the direct anti-viral effects, IFN-α/β cytokines modulate both innate and adaptive immune cells and their functions. For instance, they upregulate MHC class I expression [144, 181], induce DC maturation [182] and mediate changes in immune cell distribution, such as cellular arrest in the lymph nodes [183-186] and proliferation of IL-15 responsive specific memory CD8+ T cells [177].

2.5.5 Cytokines produced in response to MCMV

Upon acute MCMV infection, IFN-α/β strongly enhance NK cell cytotoxicity [187, 188], and promote NK cell proliferation via IL-15 induction [188]. IFN-α/β produced in

the liver trigger macrophage secretion of MIP-1α [185]. This in turn promotes NK infiltration of the liver and formation of inflammatory foci, mainly consisting of NK cells and to a lesser extent of macrophages [11, 156, 185]. The serum levels of IFNα/β peak after 36 hours upon MCMV infection, though production at lower levels is seen also after 48 hours [189-192]. Other cytokines, such as IL-12, also affect NK cell activity during MCMV infection. IL-12 is pivotal for NK cell production of IFN-γ [189, 193], in synergy with cytokines such as IL-18 (in serum and spleen) [194]. Biologically active IL-12p70, which consists of two subunits, IL-12p35 and IL-12p40, signals through the IL-12R complex via STAT4 to induce NK cell production of IFN-γ [188]. Interestingly, it has been shown that activating Ly49 receptor signaling can overcome otherwise dominating inhibitory signals through co-operation with IL-12 and IL-18 [195]. Whether this or similar processes occur during MCMV infection and other viral infections, is however not clear, and will be discussed later in relation to the studies presented in this thesis. IL-12 is a potent inducer of TH1 immune responses and if uncontrolled secretion occurs, such as in the absence of IL-10 production, IL-12 production can result in immunopathology and/or impaired TH2 responses [196, 197].

2.5.6 Plasmacytoid dendritic cells

The major producers of IFN-α/β early during MCMV infection, as well as in many other viral infections, are plasmacytoid dendrititic cells (pDCs) [198-200]. Originally, these cells were characterized independently in several studies, at separate time points. In these different studies they were given various names, such as plasmacytoid T cells or natural interferon producing cells (IPCs). Not until later, when these cells were further characterized, it became apparent that they were same type of cell population (as reviewed by [201-203]. Plasmacytoid dendritic cells possess a unique capacity to rapidly produce very high amounts of type I IFNs, up to 1000 times higher levels than other cell subsets. Upon maturation, they express increased levels of MHC class II and co-stimulatory molecules, though not as high as myeloid or conventional dendritic cells (cDCs), and provide a link between innate and adaptive immune responses [202-206]. Murine pDCs, characterized more recently, are distinguished by low CD11c expression, positive expression for markers such as PDCA, or B220 and Ly6G/C, and an absence of the myeloid marker CD11b (expressed on cDCs) [207] [208, 209]. Recently, the DAP12 associated receptor, SiglecH, has been shown to specifically be expressed on mouse pDCs [210-212]. In humans and mice, pDCs mature in the bone marrow in a FLT3-L dependent manner [213-215], and reside in blood and lymphoid tissues. Less than one percent of the leukocyte population are pDCs, though the precise frequency varies in differerent mouse strains [209, 216]. pDCs travel from the blood through the high endothelial venules (HEV) to peripheral lymphoid tissues instead of via the lymphatic system, thus showing a different migration pattern than cDCs [217]. pDCs are also recruited to sites of inflammation [202, 203].

2.5.7 Regulation of pDCs responses

To sense infections and determine which type of immune response should be elicited, innate immune cells express receptors to pathogen associated molecular patterns (PAMPs), such as the Toll-like receptors (TLRs) [218]. Without necessarily being infected themselves, pDCs can detect nucleic acids of viruses through ligation of TLR7-TLR9 located in endosomes. TLR7 and TLR8 bind single stranded RNA [219]

[220] [221], while TLR9 binds unmethylated DNA sequences with immunostimulatory CG motifs, commonly found in microbes [222-226]. Together, these receptors enable pDCs to sense RNA viruses and DNA viruses, respectively [177]. TLR7/8 and 9 signal in an MyD88-dependent manner, activating the transcription factor IRF-7 [227]. This induces expression of all the type I IFN family genes [228, 229]. IRF-7 is constitutively expressed in both human and mouse pDCs [230]. Induction of IFN α/β production early upon MCMV infection is dependent both on TLR9-dependent, MyD88-dependent and independent signaling pathways [191, 231, 232]. IL-12 induction is dependent on MyD88 and mainly on TLR9, in both pDCs and cDCs in the mouse [191, 192]

In most cells, viral replication can also be detected by cytosolic receptors, such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (mda5) [233, 234]. This induces activation of transcription factors IRF-3, NFκB and AP-1, and their subsequent induction of type I IFN production. A positive autocrine and paracrine feedback loop via IFN-α4 (in mice) and IFN-β further induces *de novo* production of transcription factors such as IRF7, which helps to drive transcription of IFN-β and the multiple IFNα variants, thus amplifying the response [235-237]. These pathways may contribute to the production of type I IFN also in pDCs, though these cells are capable of mounting a strong IFN-α/β response also in the absence of the feedback loop [238-240]. Recent reports further show that TLR and RIG-I independent pathways may contribute to recognition of cytoplasmic DNA [241, 242].

The exact mechanism behind the capacity of pDCs to promptly produce so high levels of type I IFNs is not fully clarified. Nevertheless, this capacity may be crucial for a rapid onset of an IFN-α/β response before infection is established and other cells can be activated. However, the production of IFN-α/β and IL-12, as well as their effects on the immune response must be controlled to avoid autoimmunity [180, 201, 243, 244], and/or to induce the appropriate direction of the adaptive immune response and to prevent immunopathology [196, 197]. Regulation of the different cytokines includes inhibition of IL-12 production by cDCs at high levels of IFN-α/β [192, 245, 246], in a STAT1-dependent manner [246]. Upon TLR-induced IL-12 production in DCs, phosphoinositide 3-kinases (PI3Ks) are also induced and exert an intrinsic limiting function on the IL-12 production [247]. However, little is known about the negative regulation of pDC activity.

3 RESULTS AND DISCUSSION

3.1 Role of activating receptor signaling in NK cell immune synapse formation

3.1.1 Experimental system

The combination of interacting receptors and ligands as well as cytokines control the outcome of an NK cell interaction with a potential target cell. However, regulation of NK cell activity may also be under the influence of the duration of contact, as well as the spatial organization and dynamics of the receptor interactions involved. As the requirements for formation of the activating NK cell synapse are not yet fully determined, we decided to investigate whether signaling through an activating receptor is involved in a) conjugate formation, b) recruitment of the receptor to the contact area between the NK cell and the target cell, and c) subsequent surface downmodulation of the receptor. In order to test this we set up an *in vitro* model system with murine NK cells from C57BL/6 mice (wild type) and NK cells expressing non-signaling DAP12 adaptor protein (DAP12 -/- NK cells) [91]. The NK cells were separately co-incubated with Chinese hamster ovarian tumor cell line (CHO), expressing a hamster MHC class I molecule, Hm1-C4. Hm1-C4 binds the murine activating Ly49D NK cell receptor [248]. Since DAP12 signaling is required for Ly49D mediated signaling and cytotoxicity towards CHO cells [93], we were able to investigate the role of Ly49D/DAP12 signaling in activating synapse formation, studying conjugate formation, recruitment of Ly49D to the synapse and finally, Ly49D downmodulation **(Paper I**).

3.1.2 Conjugate formation

Immune synapse formations have been studied extensively for T cells, and it is possible that NK cell conjugate formation with target cells at some levels resembles T cell interactions with target cells or other immune cells. For T cell synapse formation with APCs or target cells, several scenarios are possible, depending on the cell types (CD4+ T cells, CD8+ T cells, and types of APCs) their activation status and the surrounding environment e.g. tissue or liquid cell suspension [101]. Duration of contact and stability of conjugates are also influenced by the concentration of antigenic peptides presented to the T cell and the strength of signaling has been shown to correlate with conjugate stability to DCs [249]. NK cell formation of conjugates with potential target cells may be regulated in similar ways, in spite of differences between T and NK cells such as type and number of receptors and ligands involved in the cell interactions. In an experimental *in vitro* system, inhibitory KIR signaling via SHP-1 rapidly disrupted conjugates between the NK cell line YTS and HLA-C expressing target cells [250]. In another system, an NK cell line expressing the inhibitory receptor Ly49A formed conjugates for various lengths of time with target cells positive or negative for D^d, the MHC class I ligand for Ly49A. Target cells not expressing D^d were susceptible to NK cell mediated killing and formed conjugates with NK cells for longer times (>10 minutes) than D^d expressing (resistant) target cells, indicating that also NK cells stay longer attached if the activating signals dominate [119].

In our system, activating signals through Ly49D was directly abrogated due to the dysfunctional DAP12. We thus hypothesized that loss of DAP12 signaling would decrease the time of NK cell interaction with normally sensitive CHO cells, reflected by a lower percentage of NK cells forming conjugates at a given time point. However, we found no significant differences between the capacity of IL-2 activated DAP12-/- NK cells and wild type NK cells to form conjugates with CHO cells, as studied at various time points after initiated co-incubation, indicating that Ly49D/DAP12 signaling is not mandatory for conjugate formation with CHO cells (data not shown, **Paper I**). Graham et al reported comparable results for NKG2D/DAP10 signaling (through Vav-1) upon interaction with Rae-1 expressing cells; a system where cytotoxicity was dependent on NKG2D. IL-2 activated wild type or Vav1-/- NK cells formed similar percentage of conjugates with Rae-1+ target cells [251]. It is thus possible that other activation receptors or adhesion molecules control the conjugate formation rather than Ly49D and NKG2D in our study and the other study respectively.

In contrast to previously mentioned reports and our data, it has been reported that the adherence to YAC-1 target cells by IL-12/IL-18 activated murine NK cells negatively correlates with killing of these target cells. If killing occurred, the target cell interaction was short, up to 14 minutes, but if the target was not killed the interaction could last for up to an hour [252]. The discrepancy between these results may reflect that interaction time is not correlated to the final outcome but rather the time it takes to come to that decision. It should be noted here that the study on IL-12/IL-18 activated NK cells as well as our study is based on whole NK cell populations, rather than an NK cell line where every NK cell has the same set of receptors. The time it takes before final outcome is determined may depend on the combination of receptors and ligands involved and the activation status of the NK cells, providing either settings were the choice is easy, and the interaction short, or more complex, resulting in a long interaction. Although we did not further dissect the different NK cell subpopulations in our system, we performed the same type of conjugation assay using freshly isolated NK cells, to see if the activation status of the NK cells affected the requirements of signaling for conjugate formation with CHO cells. For these cells, lower percentages of NK cells forming conjugates with CHO cells was observed in general, but we did not detect any significant difference between wild type and DAP12-/- NK cells (**Paper I)**. However, there was a trend towards lower percentages of conjugate forming NK cells at two of the time points tested.

For neither IL-2 activated nor freshly isolated NK cells, can we exclude that both adhesion rate and dissociation rate differ between DAP12-/-NK cells and wild type NK cells, resulting in the same percentage of conjugates at any given time point. Experiments using live-cell imaging can be used to elucidate the nature of the interactions, e.g. by calculating the time each NK cell stay in contact with a target cell, if the NK cell stays still or moves and if so, how fast it moves across the target cell. In conclusion, we observed a normal frequency of conjugates for DAP12-/- NK cells as compared to wild type NK cells, indicating that Ly49D signaling through DAP12 was not necessary for conjugate formation to ligand expressing target cells. The formation of conjugates also for DAP12-/- NK cells further allowed us to analyse these

conjugates by using confocal microscopy, to study receptor recruitment to the area of contact between effector cell and target cell.

3.1.3 Receptor recruitment to the NK cell immune synapse

Several mechanism may contribute to specific receptor accumulation at the NK cell immune synapse. For T cells, actin cytoskeleton reorganization is essential for organization of signaling components and recruitment of surface proteins to the intercellular contact, and this can be influenced by TCR and co-receptor signaling [101-103]. Though specific clustering of inhibitory KIRs and their ligands at the inhibitory NK cell synapse has been observed in the presence of drugs inhibiting actin polymerisation or blocking all ATP dependent processes [111-114], Standeven *et al* showed that actin cytoskeleton movements are involved in both activating and inhibitory conjugate formations, as well as in regulation of the rate of KIR recruitment to the synapse [114]. For activating synapses, evidence that the recruitment of at least some activating receptors and adhesion molecules is dependent on actin polymerisation has been presented [108]. Thus, actin polymerisation induced by receptor signaling may be involved in the recruitment of receptors to the NK cell immune synapse.

To assess the role of DAP12/ITAM signaling in specific recruitment of the activating receptor Ly49D, we used confocal microscopy to determine the frequency of clustering, i.e. the percentage of the conjugates analysed in which Ly49D was scored as clustered at the synapse. For Ly49D+ NK cells in conjugates with CHO cells, we detected similar frequencies of conjugates showing specific Ly49D receptor recruitment, and the same extent of clustering, for both wild type and DAP12-/- NK cells (**Paper I**). Our results thus indicated that recruitment of Ly49D is independent of a functional ITAM on DAP12. This was in contrast to the study presented by Standeven *et al* in which the rate of KIR clustering at inhibitory synapses was dependent on intact ITIMs. The evidence for a role of the ITIM sequence in specific recruitment of KIR to the inhibitory synapse is however based on experiments conducted by using live-cell imaging and NK cell clones transfected with GFP-tagged KIR, facilitating detection of receptor movements and conjugate formation over time [114]. In our system, we cannot exclude that clustering of Ly49D is dependent on DAP12 signaling at earlier time points during synapse formation, as we may have studied mainly late conjugates. A detailed time course study of receptor clustering may elucidate if DAP12 signaling affects Ly49D clustering at early stages. Nevertheless, considering also the role of receptor signaling in T cell synapse formation, our data are somewhat unexpected and suggest that the role of signaling for specific recruitment of activating NK cell receptors may differ from TCR and KIR recruitment.

The study of NK cell immune synapse formation is a relatively young research field. Due to the biology of NK cells, there may be different pre-existing conditions in the different experimental systems studied, making it difficult to draw firm general conclusions at this point. Specific clustering of activating receptors may occur partially through lateral diffusion due to ligand binding, as reported for TCR [101]. Even if DAP12 signaling is not involved, the clustering of Ly49D may also in part be dependent on actin polymerisation induced by other activating receptors or adhesion molecules. Signaling by the integrin LFA-1 can induce Vav1 phosphorylation, actin

polymerisation and lipid raft assembly at the immune synapse [253], and this event is thus a possible candidate for induction of actin polymersation-dependent receptor recruitment. Clustering of the inhibitory receptor KIR2DL1 was independent of LFA-1/ICAM-1 interactions [114, 254], but the requirements for activating receptor clustering may differ from the requirements for inhibitory receptor assembly at the synapse.

One can speculate on a couple of scenarios for assembly of receptors at the synapse: 1) If the inhibitory interactions are strong, inhibitory signaling may prevent full recruitment of activating receptors. If activating receptors need to associate with lipid rafts for synapse assembly, signaling by inhibitory receptors may hinder recruitment of activating receptors by inhibiting receptor association with lipid rafts or by preventing lipid raft accumulation at the synapse. In this scenario, regardless of the mechanism, activating receptors would not fully cluster to an inhibitory synapse even if activating ligands were present on the target cell. 2) Activating receptors and inhibitory receptors may be recruited to the synapse independently of each other. This would imply that inhibitory and activating receptors both cluster, independently of the final outcome. Our model, with disruption of activating DAP12 signaling, may be comparable to the situation in which inhibitory signaling disrupts the activating signaling cascades. Assuming this, one would expect impaired clustering of Ly49D if the first scenario above was true. Since Ly49D clustering occurred also for DAP12-/- NK cells, our results rather supports the second scenario.

However, these models may be extreme, and the true scenario is perhaps an intermediate of the two. NK cell activation status, type of receptors, combination of receptors and ligands as well as ligand affinity and expression levels may influence the requirements for receptor recruitment and synapse formation. To test these different parameters, a system with defined activating and inhibitory receptors in which their respective ligands could be titrated, thus controlling the impact of activating and inhibitory signaling, would be preferable. Considering the variegated receptor expression within the NK cell population, setting up such a system may however prove to be very difficult.

3.1.4 Receptor downmodulation

Immune synapse formation can, in addition to assembly of receptors and signaling molecules, lead to downmodulation of receptors from the cell surface. Specific interaction with MHC/peptides complexes induces downmodulation of TCR, accomplished by a change in the TCR recycling process. Studies have shown that TCR downmodulation is the result of increased rate of degradation, by lysosomes and proteasome involvement [255-258], and that TCR signaling via ZAP70 and the CD3 chains are involved in these processes [259-261]. This suggests that ITAM signaling may have a role in inducing the downmodulation of the TCR. For NK cells, Ogasawara *et al* have reported that NKG2D downmodulation, after interaction with target cells expressing the NKG2D ligand Rae-1, in part depends on signaling through DAP10 activation motifs and PI3K activity [262]. These results indicates that the role of signaling in activating NK cell receptor downmodulation is not restricted to ITAM-

mediated signaling, but that receptor downmodulation can be induced also through the signaling from other adaptor proteins carrying different activation motifs.

To study whether DAP12/ITAM signaling is required for ligand induced Ly49D downmodulation, we compared surface staining of Ly49D on freshly isolated DAP12-/- NK cells and wild type NK cells after interaction with CHO cells. Ly49D surface expression was gradually reduced over time for wild type cells, reaching 42 ± 1% of the original level after 4 hour co-incubation. This reduction was not seen for DAP12-/- NK cells, on which Ly49D expresion was only affected marginally (**Paper I**). Similar results were obtained also after prior IL-2 stimulation of the NK cells. For freshly isolated wild type NK cells, as depicted in Figure 4, the receptor downmodulation was not complete, but instead resulted in a Ly49D^{low} NK subpopulation. A more uniform reduction was observed for IL-2 activated NK cells, indicating that activation status of the NK cell may affect ligand induced receptor downmodulation (data not shown).

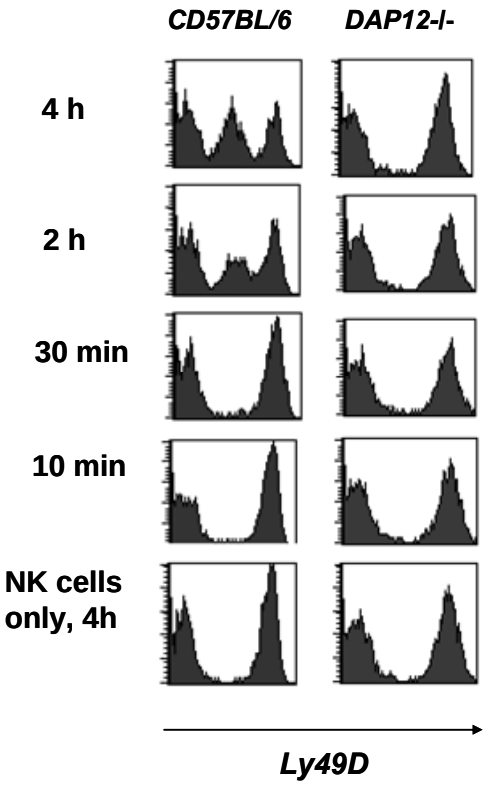

Figure 4. Surface downmodulation of the activating NK cell receptor Ly49D. Ly49D is downmodulated, after interaction between freshly isolated splenocytes with CHO cells, through a process requiring DAP12 signaling. Gate on NK1.1+CD3- lymphocytes. Histograms showing Ly49D surface staining intensity for wild type (C57BL/6) and DAP12-/- NK cells at different timepoints after co-incubation with CHO cells.

Ly49H surface expression is extensively reduced after ligand interaction [263, Bubic, 2004 #2095, 264]. We studied the role of DAP12 signaling in downmodulation of Ly49H *in vitro*, after co-incubation of NK cells and target cells expressing the ligand for Ly49H, m157. Ly49H was downmodulated for wild type NK cells, as expected, but less so on the DAP12-/- NK cells (Figure 5). In this assay, we also performed intracellular staining of IFN-γ to study NK cell activation. Interestingly, IFN-γ+ NK cells were only found in the subset with reduced Ly49H expression, suggesting that

activation of cytokine production is accompanied by receptor downmodulation. This does not exclude that downmodulation can occur in the absence of activation of cytotoxicity and cytokine production.

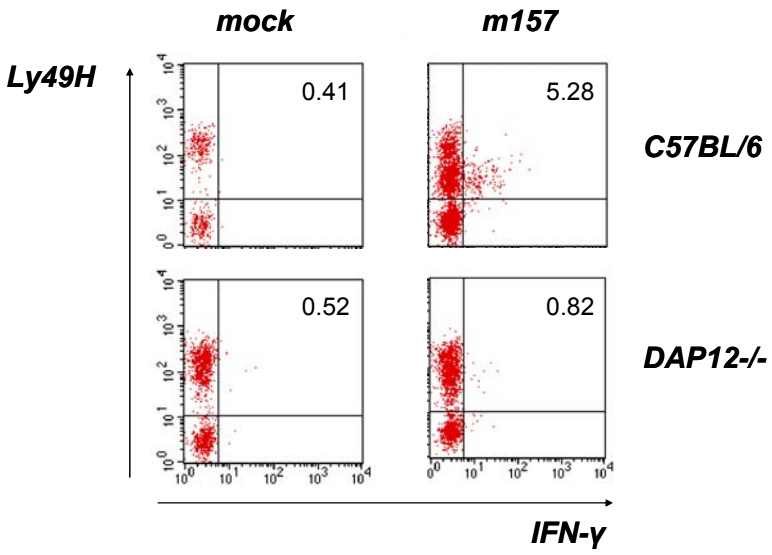

Figure 5. Surface downmodulation of Ly49H, and activation of IFN-γ production requires DAP12 signaling. Ly49H is downmodulated, after interaction between freshly isolated splenocytes with m157 expressing target cells, through a process requiring DAP12 signaling. Gate on NK1.1+CD3- lymphocytes. Histograms showing Ly49D surface staining intensity for wild type and DAP12-/- NK cells after 8 hours of co-incubation with target cells.

The consequence and biological significance of receptor downmodulation, possibly followed by internalization and degradation, is not clear. It could result in enhancement of signaling, but also in limitation of the NK cell activation; e.g. as a way to disrupt activating signaling. If there is a physiological role of receptor downmodulation, our results can be interpreted to mean that downmodulation occurs only when the final outcome of the interaction is activation, such as cytotoxicity or cytokine production; mere clustering of the receptor during target cell contact is not sufficient. Could it be that downmodulation is a negative feedback mechanism to dampen NK cell function, tuned to feedback on delivery of NK cell effector function rather than detecting of activating signals per se? Constitutively high expression levels of Rae-1 molecules in NOD mice induce downmodulation of NKG2D *in vivo* and limits the responsiveness of NOD NK cells [262]. Continuous stimulation through NKG2D negatively regulates NK cell cytotoxicity, through declined transcription of DAP10 and DAP12, correlating with reduced NKG2D surface expression. Thus, reduced receptor expression may cause less responsiveness. Intriguingly, also transcription of the adaptor protein CD3ζ is reduced. CD3ζ involved as a signaling adaptor for other receptors than DAP10 and DAP12, suggesting that the ligation of one receptor could influence the overall NK cell capacity to respond to other types of targets [265].

Reduction of NKG2D surface expression and function is reversible if the ligand is removed [262, 265]. We have seen similar results for Ly49D; receptor expression increases after removal of the CHO cells (data not shown). This suggests a temporary dampening of NK cell cytotoxicity, perhaps involved in reducing NK cell mediated immunopathology during an inflammatory response, but possibly reverted once the pathogen is cleared away. In fact, acute infection with increasing titers of MCMV, likely to induce increasing amounts of infected cells expressing the Ly49H ligand m157, has been shown to induce Ly49H downmodulation on spleen NK cells *in vivo*. This effect was only observed during the first days of infection and not after day 4-5 (French, AR; personal communication). According to our data presented above, one would expect this receptor downmodulation not to occur in absence of Ly49H ligation and signaling *in vivo*.

3.2 Role of DAP12 signaling in NK cell activation in response to MCMV

3.2.1 A specific signaling pathway for NK cell activation upon MCMV infection

As described in the introduction of this thesis, over two decades ago, NK cells were found to be a major component in the innate defense against MCMV. However, it was not clear whether, and if so how, NK cells could selectively recognize infected cells. As missing self recognition appeared less likely (discussed in section 2.5.3), interest turned towards activating NK cell receptors. Since different activating receptors depend on different adaptor proteins, one approach was to test the role of these adaptor proteins, to further narrow down the search for possible receptor(s) involved. In collaboration with Eric Viver and Elena Tomasello, we decided to investigate the role of DAP12 associated receptor signaling in the NK cell mediated defense against MCMV. We infected DAP12-loss-of-function mice [91], on C57BL/6 genetic background (normally MCMV resistant), and measured viral titers in spleen and liver three days later. Viral titers were significantly elevated in both spleen and liver in the DAP12-/- mice (Figure 6), and in additional experiments we depleted NK cells and demonstrated that the increase in viral titer was NK cell-dependent (**Paper II)**. This showed that DAP12 signaling pathways were crucial for NK cell activation upon MCMV infection.

During the course of this study, an essential role for Ly49H+ NK cells in the defense against MCMV was demonstrated. The gene within the Cmv1r locus responsible for the resistance was found to encode Ly49H [266, 267], and specific depletion of the Ly49H+ NK cells rendered otherwise resistant CD57/Bl6 mice susceptible [266, 267] [268]. Our data were well in line with and extended these results, as Ly49H is one of the receptors associating with DAP12 [93]. The role of Ly49H was further supported by introduction of the gene encoding for Ly49H in 129 mice, converting them from an MCMV susceptible to an MCMV resistant phenotype [269]. Combined, our data and the reports on Ly49H strongly suggested that Ly49H signaling through DAP12 regulates NK cell activity in response to MCMV. For the first time, a specific activating signaling pathway critical for regulation of NK cell activity upon viral infection was identified.

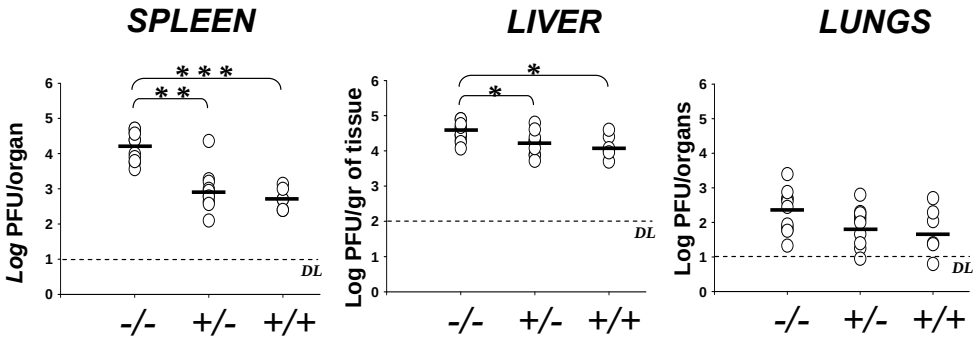

Figure 6. Increased viral titers in DAP12 (-/-) mice compared to wild type mice (+/+).
3 d after infection with 10^5 PFU of MCMV, organs were harvested, homogenized, and viral titers were measured. Each point represents the average titer determined for an individual mouse. Horizontal bars depict mean viral titers for each group. The limit of detection of the assay is shown (DL) and for mice with titers below this limit the minimum number of detectable PFU was assumed to determine the mean. This assumption overestimates the mean of the group having titers below detectable levels. This figure combines five independent experiments.

The identification of the Ly49H gene as responsible for MCMV resistance also explained why β2m-/- mice can control viral replication, as they were tested on a C57BL/6 background carrying the Ly49H gene. However, this does not exclude a role for interaction between MHC class I molecules and inhibitory receptors on NK cells as part of the regulation of NK cell activity in C57BL/6 mice. In fact, it does not even exclude the contribution of other, yet unidentified, activating pathways, though Ly49H is the vital one to tip the balance. In the PWK mouse strain, likely to be distantly related to the C57BL/6 mouse strain, it was recently shown that the Cmv4 locus, distinct from Ly49H, Ly49D and NKG2D but linked to the NK complex, confers MCMV resistance. This resistance was also to a lesser degree influenced by certain MHC genes [270]. Interestingly, additional recent reports have suggested other NK cell dependent, but Cmv1r locus independent, mechanisms for MCMV resistance in other mouse strains. One of the loci responsible for resistance, the Cmv3 locus corresponding to Ly49P, mediated resistance only when expressed in combination with the H-2k haplotype. *In vitro* data were consistent with Ly49P-mediated recognition of MCMV antigens in the context of the H-2k haplotype [271-273].

Not long after the reports on Ly49H and DAP12, the MCMV encoded protein termed m157, with an MHC class I like fold, was shown to be a ligand for Ly49H [263, 274] and crucial for NK cell mediated MCMV resistance in vivo [264]. This was surprising; why would a virus encode a protein that facilitated NK cell recognition of the virus? As discussed by Arase *et al*, m157 may originally have evolved to inhibit NK cells, as it not only binds Ly49H in C57BL/6 mice, but also the inhibitory receptor encoded by the Ly49I allele, present in MCMV susceptible 129/SvJ mice. Ly49H may have later evolved through gene duplication of Ly49I, keeping the ligand specificity but mutating

or exchanging the sequence encoding the cytoplasmic domain, thus ensuring MCMV resistance via an activating receptor [274]. Alternatively, as discussed by Tripathy *et al*, the development of m157 may have provided a possibility for MCMV to replicate without killing its host too fast, facilitating spread of the virus between mice. The last speculation is based on the natural infection route being the mucosal linings of the oropharynx, were there are relatively few NK cells. The virus may thus replicate substantially before reaching the spleen, where the NK cells through Ly49H signaling prevent uncontrolled spread of the virus and lethal pathogenesis [174].

3.2.2 Regulation of MCMV induced NK cell activity in the liver

Though Ly49H signaling confers control of viral load in the spleen, the role of Ly49H in the liver is less obvious, and the Cmv1r locus has accordingly not been proved to provide resistance in the liver [168, 169]. While it was conluded by Brown *et al* that depletion of Ly49H+ NK cells had no significant effect on viral titers in the liver [267], Daniels *et al* demonstrated that the majority of IFN-γ producing NK cells in the liver were Ly49H+ NK cells, and that viral titers were increased 5-fold (compared to wild type mice) in the liver after depletion of this subset [268]. Though we observed an NK cell dependent increase in viral titers also in the liver of the DAP12-/- mice, there was a quantitative difference in the effect of DAP12 signaling between the liver (2-5 fold increase in viral titers) and the spleen (30-40 fold increase in viral titers) (**Paper II**). The most important NK cell effector mechansim in the liver is IFN-γ production, in contrast to the spleen where the defense is mainly perforin-depedent [161]. To further analyse the role of DAP12 signaling in the NK cell mediated defense in the liver, we investigated the IFN-γ production early after infection. We observed a substantial decrease in the percentage of IFN-γ producing NK cells in the liver of DAP12-/- mice, reduced by more than half compared to wild type controls. Together with the increased liver viral load in DAP12-/- mice, this result supported a role for DAP12 signaling, and thus possibly a role for Ly49H, in the NK cell mediated defense in the liver **(Paper II).**

In a later series of experiments (unpublished), however, I was able to study the IFN-γ production in Ly49H+ and Ly49H- NK cell subsets after MCMV infection in DAP12-/- mice. While expecting the reduction of IFN-γ-producing NK cells to occur mainly in the Ly49H+ subset, I found the percentage of IFN-γ producing NK cells to be decreased for both Ly49H+ and Ly49H- NK cells. Further, the IFN-γ production was not preferentially carried out by the Ly49H+ subset. This result indicates contribution from other DAP12-associated receptors, either on NK cells or possibly other cell types, such as dendritic cells. Finally, in these experiments, I compared IL-12p35-/- mice, DAP12-/- and mice deficient for both DAP12 signaling and IL-12p35. Neither the IL-12-/- NK cells nor the double deficient mice produced IFNγ at day 2 post infection, as reported previously [189, 193], supporting cytokine mediated activation as the foremost mechanism for control of the liver NK cell response. In line with this, deletion of m157 did not cause higher viral titer in the liver than wild type virus [264]. Combined, the data show that Ly49H in C57BL/6 mice may contribute, but does not have a major role in regulation of NK cell activity in the liver upon MCMV infection.

The reason for a limited influence of Ly49H in the liver versus the spleen is not known. The tissue structure in the liver might necessitate a soluble, cytokine mediated

activation of the NK cell response rather than direct cell contact-dependent initiation. However, m157 ligation of Ly49H induces IFN-γ production *in vitro*, as shown by others [264, 274] and by the data presented above. It is plausible that Ly49H exhibit a more central role later during the infection, when the myeloid cytokine response has declined. Assessment of liver viral titers of DAP12-/- mice on day 5 or 6 post infection may indicate if this is the case. Finally, a minor role of Ly49H in the liver does not rule out the possibility of other DAP12-dependent receptors mediating activation, or a receptor mediated defense in the liver in other resistant mouse strains. In fact, phospholipase C-γ2 (PLC-γ2) has been reported to be critical for MCMV resistance in the liver but dispensible for IL-12 induced IFN-γ production, suggesting a role of an activating receptor NK cell mediated defense mechanism also in the liver [275]. On the other hand, in another study the role of PLC- γ in the liver was questioned [276].

3.2.3 The role of DAP12 signaling in MCMV- induced NK cell proliferation

The relative size of the NK cell subsets change during MCMV infection [158], and NK cell numbers in the spleen are dramatically reduced 2 days after of infection [158, 277]. This suggests MCMV induced proliferation and/or trafficking of NK cells, and the reduction in the spleen indicates either migration elsewhere or NK cell apoptosis, perhaps by activation induced cell death. Liver recruitment of NK cells and formation of inflammatory foci, mainly consisting of NK cells and to some extent macrophages, are induced by the chemokine MIP1-α produced by macrophages in the liver during early MCMV infection [11, 156, 185]. To investigate whether the formation of inflammatory foci was impaired in the absence of DAP12 signaling, we studied liver tissue sections from infected DAP12-/- mice. Inflammatory foci were formed in DAP12-/- mice livers in numbers comparable to infected wild type mice **(Paper II).** Livers from DAP12-/- mice further contained similar number of NK cells as wild type mice at day 2 after infection. This suggests that neither DAP12 signaling nor Ly49H-mediated activation of NK cells, for example in the spleen or peripheral blood, are required for NK cell recruitment to the liver.

The NK cell numbers in both spleen and liver increase successively from day 2 post MCMV infection, peaking around day 6 [277]. At this time point of infection the subset distribution has shifted so that a majority of the NK cells are positive for Ly49H, as reported by Dokun *et al* shortly after the publications on the role of Ly49H. At the start of MCMV infection, a non-specific proliferation occurs within all NK cell subsets, most likely driven by pro-inflammatory cytokines [188, 278]. However, a specific proliferation of Ly49H+ NK cells later dominates, which may account for the shift in subset distribution observed at day 6 [277]. While cytokine-driven expansion of NK cells in general had been observed, specific expansion of an NK cell subset in response to acute infection had not been previously reported. To explain this phenomenon, at least three possibilities were at hand. 1) Ly49H+ NK cells might constitutively display intrinsic responsiveness to cytokines, by expressing high levels of certain cytokine receptors or signaling molecules involved in cytokine signaling. 2) Alternatively, interaction with infected cells and ligation of Ly49H might induce upregulation of critical cytokine receptors and thus specific responsiveness to pro-proliferative cytokines. 3) Yet another possibility was that ligation of Ly49H and signaling through

DAP12 directly drives not only activation of effector functions, such as cytoxicity and cytokine production, but also proliferation.

To determine if the specific proliferation of Ly49H+ NK cells actually was driven by signaling through the Ly49H receptor, we first studied accumulation of NK cells in DAP12-/- mice. Uninfected DAP12 -/- mice have normal numbers of NK cells in spleen and liver compared to wild type mice. However, after 5-6 days of MCMV infection, we found increased numbers in spleen and liver of wild type mice only, whereas the DAP12-/- mice had the same or lower numbers than uninfected controls in these organs (data not shown). This indicated a role for DAP12 signaling in the expansion of NK cells upon MCMV infection. To further eluciate whether Ly49H/DAP12 signaling plays a role in the expansion, we studied specific proliferation in the spleen in DAP12-/- mice. We saw impaired specific proliferation in the DAP12-/- mice, showing that DAP12 signaling is necessary for the specific proliferation of Ly49H+ NK cells upon MCMV infection, and thus suggesting a role for Ly49H receptor signaling itself (**Paper III**).

Concurrently, it was reported by Andrews *et al* that the Ly49H+ NK cell accumulation at day 6 after infection, was strongly reduced in C57BL/6 mice deficient for IL-12, IL-18 or both [278], thus indicating a role for these cytokines in the specific proliferation. To investigate the role of these and other cytokines, we tested proliferation of Ly49H+ and Ly49H- NK cells in a panel of mice deficient for various cytokines or cytokine receptors. Specific proliferation was observed in all of these mouse strains, and no significant differences in cytokine receptor expression on Ly49H+ NK cells compared to Ly49H- NK cells were revealed. We further investigated a panel of mice deficient for cytokine receptors, and as the specific proliferation was unaffected also in these mice **(Paper III)**, this showed that none of these cytokines were crucial to drive the specific proliferation. In fact, the specific proliferation of Ly49H+ NK cells in MyD88-/- mice, unable to trigger IL-12 production by DCs, is comparable to wild type mice, further arguing against a role of IL-12 [191]. There is of course a possibility that some of these cytokines contribute but in a redundant fashion *in vivo*, which might be possible to test by infecting mice deficient for two or more of these cytokines, or cytokine receptors.

To study the role of Ly49H/DAP12 signaling in an isolated system, we tested the capacity of m157 (expressed in a transfected cell line) to stimulate proliferation of NK cells, in combination with a panel of exogenous cytokines, added alone or in combination, *in vitro*. As shown for IL-15, at high concentrations of cytokine all NK cells proliferated, regardless of m157 stimulation or Ly49H expression. However, at lower cytokine concentrations, not able to support NK cell proliferation by themselves, there was specific expansion of Ly49H+ NK cells when m157 expressing target cells were added to the cultures. Importantly, specific proliferation of Ly49H+ NK cells *in vitro* was not detected for DAP12-/- NK cells (**Paper III**), supporting our results from the *in vivo* experiments. Our study thus showed that Ly49H/DAP12 signaling is crucial for the specific proliferation of Ly49H+ NK cells during MCMV infection. Levels of pro-inflammatory cytokines peaks early during MCMV infection, and later declines. We propose that Ly49H/DAP12 signaling critically augments otherwise low cytokine-

induced proliferation of NK cells, promoting the specific proliferation of the Ly49H+ NK cell subset.

As described in the introduction, cytokines such as IFN-α/β and IL-12 are important for viral load control also in MCMV resistant mice. Further, IL-12/18 can work in synergy to enhance signaling of another DAP12 associated receptor, Ly49D, to dominate over inhibitory signaling from receptors binding to the same ligand, H-2D^d [195, 279], both *in vivo* and *in vitro*. It is tempting to speculate that cytokines, in combination with Ly49H, may help scewing the balance towards activation and proliferation, to overcome inhibitory signals from Ly49C or Ly49I, which potentially bind MHC class I molecules on infected cells. Inhibitory signals may also be induced by inhibitory receptors binding virally encoded decoy MHC class I molecules, as reported both for HCMV and MCMV [280, 281]. Thus, cytokines and the Ly49H receptor may be necessary but separately not sufficient for optimal NK cell response to MCMV. This scenario is compatible with the MCMV resistance in the β2m-/- mice, and it would be interesting to study the effect of cytokine deficiency in these mice to see if Ly49H signaling is sufficient to drive the specific proliferation in absence of MHC class I mediated inhibition. The role of inhibitory receptor interactions in regulation of NK cell activity in response to MCMV infection could be further studied by analysing activation and proliferation of NK cells co-expressing one or more inhibitory receptors with or without Ly49H in wild type mice. A third approach is to block inhibitory interactions *in vivo* during infection, and study NK cell activity and proliferation. This would perhaps help us further investigate whether inhibitory receptors and missing self recognition at all play a role in the NK cell response during MCMV infection.

Specific expansion of an NK cell subset, regardless of the mechanism involved, is a fascinating indication of an adaptive component, i.e. expansion of the cells best suitable to eliminate the infection, in the NK cell system. In contrast to the adaptive immune response, however, there is no evidence that the specific expansion upon MCMV leaves any permanent imprint [277, 282]. The concept of specific proliferation of an NK cell subset in response to viral infection and receptor triggering may not be restricted to MCMV and Ly49H. Human CD94/NKG2C+ NK cells were recently shown to expand specifically in response to HCMV [283, 284], but it remains to be tested if signaling through CD94/NKG2C plays an important role for the expansion and if cytokines influence this expansion. Since human KIR is the dominating receptor family analogue to Ly49s it would also be interesting to investigate if one or several of the activating KIRs play a role similar to Ly49H in the NK cell mediated response to specific viral infections in humans. Interestingly, the activating KIR allele encoding for KIR3DS1, in combination with the allele for a HLA-Bw4, is associated with delayed progression of AIDS [285]. However, the mechanism is not identified and it is not even known whether this phenomenon is related to NK cells, since KIR can be expressed also on T cells.

3.3 DAP12 mediated downmodulation of pDC responses to MCMV

3.3.1 Increased levels of innate cytokines in DAP12-/- mice upon MCMV infection

Although we could conclude that the abrogated signaling in the DAP12-/- mice upon MCMV infection impaired the NK cell mediated response (**Paper II**), we could not exclude that this was due to altered cytokine production by other cells. Plasmacytoid DCs are the major IFN-α/β producers in early MCMV infection, with production peaking at day 1.5, while several DC subsets produce IL-12 [198, 200, 286]. DAP12 associated receptors are expressed also on DCs [73]. In order to test the role of DAP12 in cytokine production early during MCMV infection, we measured levels of IFN-α/β and IL-12, in the spleen as well as in serum, 1.5 days post MCMV infection. As DAP12 signaling can induce activation of myeloid cells, we expected to find decreased cytokine levels, if changed at all. Surprisingly, we found strongly increased levels of IFN-α/β in the serum and of IFN-α/β and IL-12p40 in spleen homogenates in the infected DAP12-/- mice, compared to infected wild type mice (**Paper IV**). To further analyse the production of cytokines by DCs in DAP12-/- mice, we isolated DC subsets after infection and measured intracellular cytokines. We showed that absence of DAP12 elevated the production of IFN-α/β in pDCs (no production of IFN-α/β was detected in cDCs at this time point) and IL-12 in both pDCs and cDCs. Twice as high percentages of cytokine producing cells were found within each subset in the DAP12-/- mice. In addition, we also discovered a higher peripheral proportion and absolute number of pDCs in the DAP12-/- mice compared to wild type mice (**Paper IV**). The higher cytokine levels may thus have been the consequence of a a higher number of pDCs as well as higher responsiveness within this cell population.

We demonstrated this effect also in an experimental set up not involving viral infection. TLR9 stimulation by CpG ODN *in vivo* gave rise to elevated levels of IFN-α/β, as well as higher percentage of cytokine producing pDCs in DAP12-/- mice. During the course of our study, partly similar results were reported by Blasius *et al* [211]. Crosslinking of the pDC specific DAP12 associated receptor Siglec H by antibody treatment *in vitro* and *in vivo*, inhibited CpG induced IFN-α/β production. In accordance, DAP12 knock out mice, lacking surface expression of Siglec H, exhibited higher than wild type serum levels of IFN-α/β in response to CpG. As expected, injection of Siglec H specific antibodies had no further effect on the production of IFN-α/β in these mice. The study did however not show which cell subset that was responsible for the elevated cytokine levels in serum of the DAP12 knock out mice. (**Paper IV**). As shown in another concurrent report, Siglec H is involved in endocytosis and cross-presentation. In this study Siglec H had no effect on IFN-α/β production induced by inactivated Sendai virus or influenza virus [287], indicating that other DAP12 associated receptors may regulate pDC function in response to virus. We detected DAP12 mRNA in freshly isolated pDCs, and in addition to Siglec H we readily detected mRNA for other DAP12 associated receptors such as CD200R4, NKG2D and MAIR-II, indicating that several receptors binding DAP12 can be expressed in pDCs (**Paper IV**). More than one may be involved in dampening the pDC production of IL-12 and IFN-α/β during viral infection or upon CpG stimulation.

A noteworthy finding within this study is also that higher IFNα/β serum and spleen levels, though known to enhance NK cell cytotoxicity, could not compensate for the lack of Ly49H/DAP12 signaling in NK cell mediated control of viral load in the spleen. This argued in line of the speculation raised in the last section (3.2) about critical synergy between cytokines and activating receptors in activation of NK cells upon viral infection. Recently, Andoniou *et al* showed how myeloid, conventional DCs (cDCs) rather than plasmacytoid DCs (pDCs) can activate NK cell cytotoxicity during MCMV infection by IFNα/β production at day 2 after infection [288] It would be interesting to determine if DAP12 signaling affects also IFNα/β by cDCs day 2 post infection. To activate IFN-γ production by NK cells *in vitro* DCs can deliver IL-12 in a synapse formation dependent way [289]. Further, a role for DAP12 on DCs has been reported in DC-NK contact dependent activation of NK cell activity [290]. If DAP12 signaling is required for contact and directed secretion of IFN-α/β and IL-12 and thus cytokine-mediated activation of NK cells *in vivo* upon MCMV infection, DAP12-/- DCs might be inefficient primers of NK cells in spite of their higher secretion of cytokines. This may be an alternative explanation, not necessarily contradictory, to the increased viral titer observed in the spleen and liver in the DAP12-/- mice upon MCMV infection (**Paper II**). One could further speculate that inefficient contact between DCs and NK cells, and thus impaired delivery of cytokines, could be responsible for the loss of specific proliferation of Ly49H+ NK cells in DAP12 deficient mice (**Paper III**), rather than lack of Ly49H signaling. This would not, however, explain our results on the role of Ly49H/DAP12 signaling in NK cells for specific proliferation *in vitro*, and I thus favor the interpretation that Ly49H/DAP12 mediate an augmentation of cytokine stimulation of proliferation.

3.3.2 Role of intrinsic DAP12 signaling for control of pDC cytokine production

It could be argued that other cell types expressing DAP12 might be responsible for regulating activation of pDCs. Further, upon MCMV infection, the viral titers were higher in DAP12-/- mice due to impaired NK cell mediated control of replication and this may also contribute to pDC activation **(Paper II)**. To elucidate whether the regulating role of DAP12 signaling was intrinsic to the pDCs, and in order to avoid a difference in the viral titers surrounding DAP12 proficient and DAP12 dysfunctional pDCs, we produced mixed bone marrow chimeric (BMC) mice. These were constructed so that wild type recipient mice harboured bone marrow and peripheral immune cells of both wild type and and DAP12 deficient type. Within each mouse, wild type and DAP12-/- pDCs thus encountered the same environment, including other cell types and viral load upon infection. By using wild type donors and recipients that expressed leukocyte common marker CD45.1 and DAP12-/- donors expressing CD45.2, we were able to distinguish between wild type pDCs and DAP12-/- pDCs in single cell analysis. (Figure 7).

Analysis of infection induced cytokine production in the mixed bone marrow chimeras showed higher percentages of IL-12 producing cells within the DAP12-/- pDC population compared to wild type pDCs, while IFN-α/β production was similar between the two populations. This suggests that production of IL-12 was negatively regulated due to intrinsic DAP12 signaling, i.e. within the pDCs, whereas IFN-α/β production was not. Following CpG injection however, there were higher percentages

of both IL-12 and IFN-α/β producing cells within the DAP12-/- pDC population compared to wild type pDCs. (**Paper IV**). In conclusion, DAP12 signaling in pDCs can regulate pDC functions, but may do so differently depending on the type of stimuli. Our study further shows a role for intrinsic DAP12 mediated regulation of pDC function in response to a viral infection *in vivo*.

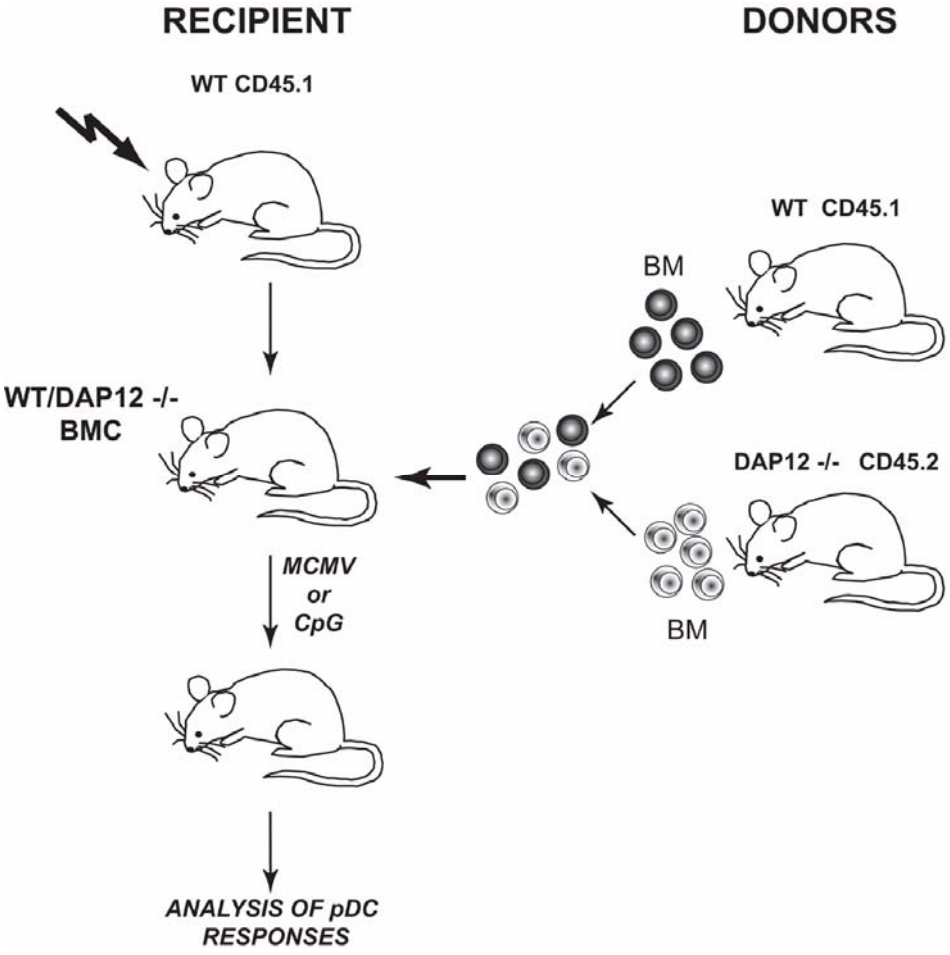

Figure 7. Generation of bone marrow chimeric mice. Bone marrow chimeric (BMC) mice were generated by irradiation of CD45.1+ C57BL/6 and reconstitution by i.v. injection of bone marrow (BM) cells; CD45.1 wild type (WT) and CD45.2 DAP12-deficient (DAP12-/-) BM cells at a ratio of 1:1.

The discrepancy in the pDC intrinsic regulation by DAP12 for IFN-α/β and IL-12 during viral infection may result from different triggering pathways for production of IFN-α/β and IL-12. Induction of IFN-α/β upon MCMV can be mediated by MyD88-dependent TLR9 signaling but also by MyD88-independent signaling, and possibly by other pathways [191, 232]. Production of IL-12, on the other hand, is strictly dependent on MyD88 signaling and mainly on TLR9 signaling, both upon CpG and MCMV stimulation [191, 232, 288]. DAP12 signaling may influence the cytokine triggering

pathways with different efficiency or may influence only the TLR9 depedent pathway. Since CpG triggers both IFNα/β and IL-12 through TLR9 [222], this may explain why DAP12 signaling in pDCs can regulate IFN-α/β in this situation. Why then did IFN-α/β levels increase in absence of DAP12 signaling in the DAP12-/- mice, if it was not due to DAP12 signaling within the pDC? The most straightforward answer is that the higher viral titers in these mice compared to the control mice, due to impaired NK cell response, provides higher concentration of for example TLR ligands and thus higher activation of pDCs.

However, a second interpretation of the experiments in the BM chimeric mice is possible. If part of the regulation of IFN-α/β production is through a positive feedback loop as described previously [237], DAP12 signaling may regulate the initial induction of IFN-α/β and DAP12-/- pDC might experience a more powerful feedback, thus further increasing their IFN-α/β production. This extra amount of IFN-α/β in the feedback loop from the DAP12-/- pDCs may also dominantly influence the surrounding wild type pDCs. This could be the reason why we did not observe a difference in percentage of IFN-α/β producing cells between wild type pDCs and DAP12-/- pDCs within the BMC mice. In this way, the settings for a powerful feedback loop would be more favorable also in the DAP12-/- mice, causing the raised cytokine levels.

A third interpretation is available, closely related with and not mutually exclusive to the first interpretation. If the DAP12 mediated regulation of IFN-α/β is dose-dependent, as shown for DAP12 mediated negative regulation of CpG induced IFN-α/β by pDCs *in vitro* [211], perhaps we would have detected a pDC intrinsic effect of DAP12 signaling on the IFN-α/β production had we infected the BM chimeric mice with a lower viral dose. We have recently performed experiments indicating that DAP12-/- pDCs produce more IFN-α/β, and IL-12, in response to HSV-1 *in vitro*, indicating that DAP12 mediated signaling can influence virally induced IFN-α/β production in a pDC intrinsic manner. The pathways for HSV-1 triggering of pDCs may differ from MCMV induced pathways [291], making it difficult to compare the HSV-1 results with the MCMV setting. A better experiment might be to expose *ex vivo* purified pDCs to a various virus doses and analyse cytokine production.

3.3.3 On the role of DAP12 mediated regulation of pDC responses

If, as our results suggested, the DAP12 mediated regulation of pDC produced IFN-α/β is different from the regulation of pDC production of IL-12 upon viral infection *in vivo*, what is the physiological gain by this difference? If both cytokine types are under the control of DAP12 signaling in the absence of infection, what would be the purpose of this regulation? The IFN-α/β system is a powerful anti-viral agent limiting viral replication and protecting most cells during viral infection [177]. It is possible that regulation of IFN-α/β is less stringent, to avoid the risk of insufficient production. This may partly be guaranteed by the existence of multiple genes encoding for different subtypes of IFN-α, possibly regulated in the separate ways [180]. IL-12 on the other hand is a potent pro-inflammatory cytokine and may cause septic shock and tissue damage if not tightly controlled [196, 197]. In absence of viral infection, however, it may be advantageous to more strictly control also IFN-α/β secretion, as both IFN-α/β

and IL-12 can be involved in the development of autoimmunity, such as reported for systemic lupus erythematosus (SLE) and insulin-depedent diabetes mellitus [180, 197]. Thus, depending on the circumstances, DAP12 signaling in pDCs may be an important pathway to limit pDC production of these cytokines.

3.3.4 ITAM-mediated inhibition

In contrast to what has previuosly been considered an activating pathway, the DAP12/ITAM signaling can thus confer negative regulation of immune cell activity. ITAM mediated inhibition of TLR signaling is however not restricted to pDCs and DAP12 (reviewed in [76, 78]). Recently, ITAM mediated inhibition has also been shown in macrophages, microglia cells, B cells, mast cells and human pDCs [76, 78. For macrophages, TREM-2 crosslinking can negatively regulate production of inflammatory cytokines, such as IL-6, TNF and IL-12p40 {Hamerman, 2005 #2240, 292, 293]. DAP12 knock out mice are further more susceptible of septic shock, if induced by LPS and D-galactosamine, and show enhanced cytokine response to Listeria monocytogenes [98]. For human pDC, engagement of BDCA-2 [294] or the DAP12-associated receptor NKp44 inhibits secretion of IFN-α [295]. Further, crosslinking of the pDC specific receptor ILT7-FcεRIγ inhibits production of IFN-α and TNF-α, induced by CpG or influenza in primary human pDCs, through ITAM signaling [296]. The inhibition of influenza through ITAM signaling, along with our data on HSV-1, demonstrates that inhibition is not restricted to MCMV, but can occur upon other viral infections, at least *in vitro*.

The observation that TNF-α production can be elevated in absence of DAP12 further gives rise to a second interpretation of the liver data in **Paper II**. We saw substantially increased numbers of necrotic areas in liver tissue sections in the DAP12-/- mice after MCMV infection, as compared to infected wild type mice. This might have been due to increased viral titers as a result of the impaired NK cell mediated defense, as was our conclusion at the time. However, in light of the data on a role of DAP12 signaling in dampening production of innate cytokines, there is a possibility that the increased necrosis was caused by elevated TNF-α levels, as previously demonstrated [297].

None of the reports mentioned above, nor our study on pDCs, describe the mechanism for this paradoxal role of ITAM signaling. In **Paper IV** we speculate on the possibility that DAP12-/- pDCs may be primed for increased TLR responsiveness by higher expression of signaling molecules involved. Constitutive ITAM signaling may reduce transcription of TLRs expression or other signaling molecules, as seen for crosslinking of the high affinity IgE receptor associated with FcεRIγ which inhibits TLR9 transcription in pDCs [298, 299]. However, evidence for ITAM dependent receptor signaling directly interfering with the other stimuli, TLR-signaling or other activating pathways, is now emerging [76, 78, 211, 292, 293, 296]. One or multiple pathways downstream of the ITAM sequence may be involved. DAP12 mediated inhibition of TLR responses in macrophages involve Syk kinase activity, and TLR induced ERK phosphorylation increases in absence of DAP12 [98]. The inhibition of human pDC IFN-αβ production by BDCA-2 crosslinking is dependent on Ca^{2+} flux, indicating that PLC-γ may be involved [294]. PI3K activity, induced both by TLR signaling itself and by DAP12 signaling, can rapidly inhibit TLR induced transcription of IL-12p35 and IL-

p40 [300]. A bifunctional role of the ITAM motif has also been suggested as a possible explanation, where phosphatase SHP-1 might be recruited to a sequence within the ITAM and dephosphosrylate other signaling molecules [78, 296, 301]. So far, however, the exact biochemical mechanism for the inhibition remains to be elucidated.

The general aspect of negative regulation induced by ITAM signaling makes it interesting but also difficult to speculate about the ligands involved in regulation of ITAM signaling receptors, cell types and settings. Low affinity ligands, inducing a constitutive low level of DAP12 signaling through Siglec H has been suggested as a mechanism for regulation of mouse pDC, in absence of infection [211]. A ligand for TREM-2, regulating macrophage activity, is potentially expressed on macrophages themselves [292]. Such ligands could exhibit immune downmodulating functions to prevent autoimmune reactions to reagents like chromatin-containing immune complexes [302]. Other ligands, with higher affinity, may induce activation through the same receptor in case of infection [303]. The ligands may be host encoded or encoded by the pathogen. However, our results from MCMV infection as well as the reports on Listeria monocytogenes show that DAP12 associated receptors may interact with ligands inducing inhibition also in the presence of a pathogen infection *in vivo*. The future characterization of the ligands involved will improve our understanding of this phenomenon.

4 CONCLUDING REMARKS

It has been thrilling to, for the past five years, conduct and follow the research on NK cells and the mechanisms regulating their activity. During these years a detailed analysis of the contribution of separate signaling pathways and effector molecules has been possible through studies in genetically modified mice and by the use of specific antibodies detecting cell surface markers and effector molecules. Techniques such as confocal microscopy and flow cytometry have been applied to elucidate the underlying processes of NK cell specificity and functions. To summarize and clarify the specific contributions of my thesis to this field, I here list the major conclusions and further interpretations:

The first study addressed whether signaling via an activating NK cell receptor affects its behaviour during immune synapse formation. Upon Ly49D+ NK cell interaction with susceptible, Ly49D-ligand expressing target cells, adaptor protein DAP12 signaling was not required for initial conjugate formation or for recruitment of the activating DAP12-associated receptor Ly49D to the synapse. This indicates that signaling through an activating NK cell receptor may not be required for synapse assembly of this receptor. In line with what has been previously described for specific engagement of the TCR and for other activating NK cell receptors, Ly49D/DAP12 signaling was however required for ligand-induced Ly49D downmodulation (**Paper I**).

Turning to a more complex *in vivo* situation, we demonstrated a pivotal role of DAP12 signaling in the NK cell mediated defense against MCMV infection. This was in line with reports, published by others, of a crucial role for Ly49H+ NK cells in innate resistance to MCMV. Combined, these studies showed for the first time that a specific activating receptor signaling pathway is essential for the regulation of NK cell activity upon viral infection *in vivo* (**Paper II**). We further revealed a critical role of Ly49H/DAP12 signaling in the triggering of specific proliferation of the Ly49H+ NK cell subset during MCMV infection. This shows that direct target cell interactions and subsequent activating receptor signaling can induce proliferation of a selective NK cell subset in response to a viral infection *in vivo*, reminiscent of the first expansion that occurs among specific cells in responses of the adaptive immune system. Our results indicate that the mechanism may depend on synergy between activating receptor signaling with low, subtreshold levels of cytokines that otherwise, at higher concentrations, are able to drive non-specific proliferation of the general NK cell population (**Paper III**).

We unexpectedly found DAP12/ITAM signaling to mediate negative regulation of pDC activity, such as production of cytokines modulating the NK cell activity, in response to CpG stimulation and MCMV infection *in vivo*. This showed that, in spite of the activating signaling pathways downstreams of the ITAM sequence, the outcome of the combination of these and other innate activating pathways such as TLR signaling may be dampening. This may reflect that an integrated balance rather than the additive effect of activating signaling may be required to avoid excessive stimulation of the immune system (**Paper IV**).

For the future, the knowledge regarding what controls NK cell activation and specificity, gained by these studies and the extensive studies published by others, need to be compiled and evaluated to form a complete picture. The fundamental research on molecular mechanisms must further be combined with clinical studies of NK cell activity and its consequences for innate immunity. Can we manipulate NK cell activation and expansion in patients in order to increase protection against viruses, or even against other infections or malignancies? Clinical studies on human NK cell regulation in virally infected patients are being performed. Important findings from basic research on NK cell regulation upon transplantation and cancer treatment are already being followed up at a clinical level. Finally, to complement the mouse model systems, studies of human genetic disorders causing impaired NK cell responses are vital to help us unravel the relevance of cytokines and NK cell receptors in regulation of NK cell activity in humans.

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