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HUMAN NATURAL KILLER CELL ACTIVATION AND DIFFERENTIATION IN HEALTH AND VIRAL INFECTION

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ABSTRACT

Natural killer (NK) cells are lymphocytes that belong to the innate immune system. They are important for the early defense against viral infections and provide tumor immune surveillance against both solid tumors and leukemias as well as in settings of hematopoietic stem cell transplantation. They also play an important role in human pregnancy via spiral artery modulation, and deliver signals that shape adaptive immune responses. However, despite these insights, several unresolved issues remain with regards to mechanisms by which NK cells respond in these and other conditions. In this thesis, NK cell activation and differentiation in healthy uninfected individuals as well as in defined acute and chronic viral infections are characterized.

Via a detailed analysis of NK cell repertoires in the healthy humans and in two specific settings of immune system development, we have provided evidence that human $CD56^{dim}$ NK cells undergo a previously undescribed differentiation process. This differentiation process can, at steady state, be defined by analysis of expression patterns of NKG2A, KIRs, and CD57 on $CD56^{dim}$ NK cells. Distinct differentiation stages identified are associated with phenotypic and functional changes. This knowledge formed a platform for studies of human NK cells in settings of defined viral infections. In acute human hantavirus infection, the NK cell response encompassed a rapid and vigorous proliferation of activated NK cells followed by long-term persistence of a differentiated NKG2C+CD57+ $CD56^{dim}$ NK cell subset. Surprisingly, this proliferation and persistence occurred only in patients on a CMV seropositive background and was mediated by IL-15 and HLA-E, providing necessary proliferative and anti-apoptotic signals to the expanding cells. When monitoring the NK cell response to immune modulatory IFN-α treatment in the context of acute and chronic hepatitis C virus (HCV) infection, we observed that $CD56^{bright}$ NK cells acquired the capacity to express the apoptosis-inducing molecule TRAIL. These differentiated $CD56^{bright}$ 'killer' cells efficiently inhibited HCV replication in Huh7.5 cells via TRAIL. NK cells could also utilize the activation receptor DNAM-1 to recognize Huh7.5 cells and suppress HCV replication. Chronic HCV-infection was found to cause disturbances in innate cellular immunity. One example of this was the differentiation of NK cells towards a functionally skewed $CD56^{neg}$ NK cell subset. Furthermore, effector CD8 T cells acquired NK cell-like properties, such as expression of CD16 and the capacity to respond independent of the TCR during chronic HCV infection.

Altogether, the described model of human $CD56^{dim}$ NK cell differentiation may serve as a framework for studies of NK cell responses in many disease conditions, here illustrated in studies of viral infections in humans.

LIST OF PUBLICATIONS

This thesis is based on six publications as well as two supplementary publications. The individual papers are referred to by roman numerals.

I. **Niklas K. Björkström**, Peggy Riese, Frank Heuts, Sandra Andersson, Cyril Fauriat, Martin A. Ivarsson, Andreas T. Björklund, Malin Flodström-Tullberg, Jakob Michaëlsson, Martin E. Rottenberg, Carlos A. Guzmán, Hans-Gustaf Ljunggren, and Karl-Johan Malmberg. Expression Patterns of NKG2A, KIR, and CD57 Define a Process of $CD56^{dim}$ NK-cell Differentiation Uncoupled from NK-cell Education.
Blood. 2010 vol 116 (19) pp. 3853-64.

II. **Niklas K. Björkström**, Therese Lindgren, Malin Stoltz, Cyril Fauriat, Monika Braun, Magnus Evander, Jakob Michaëlsson, Karl-Johan Malmberg, Jonas Klingström, Clas Ahlm, and Hans-Gustaf Ljunggren. Rapid Expansion and Long-term Persistence of Elevated NK Cell Numbers in Humans Infected with Hantavirus.
Journal of Experimental Medicine. 2011 vol. 208 (1) pp. 13-21.

III. Kerstin A. Stegmann, **Niklas K. Björkström**, Heike Veber, Sandra Ciesek, Peggy Riese, Johannes Wiegand, Johannes Hadem, Pothakamuri V. Suneetha, Jerzy Jaroszewicz, Chun Wang, Verena Schlaphoff, Paraskevi Fytili, Markus Cornberg, Michael P. Manns, Robert Geffers, Thomas Pietschmann, Carlos A. Guzmán, Hans-Gustaf Ljunggren, and Heiner Wedemeyer. Interferon-α-Induced TRAIL on Natural Killer Cells Is Associated With Control of Hepatitis C Virus Infection.
Gastroenterology. 2010 vol. 138 (5) pp. 1885-97.

IV. Kerstin A. Stegmann*, **Niklas K. Björkström***, Sandra Cisek, Jerzy Jaroszewicz, Phillipp Maliniski, Lynn B. Dustin, Charles M. Rice, Michael P. Manns, Thomas Pietschmann, Markus Cornberg, Hans-Gustaf Ljunggren, and Heiner Wedemeyer. IFNα-stimulated NK Cells from Patients with Acute HCV Infection Recognize HCV-infected and Uninfected Hepatoma Cells via DNAM-1.
*Manuscript submitted. *Equal contribution*

V. **Niklas K. Björkström**, Veronica D. Gonzalez, Karl-Johan Malmberg, Karolin Falconer, Annette Alaeus, Greg Nowak, Carl Jorns, Bo-Göran Ericzon, Ola Weiland, Johan K. Sandberg, and Hans-Gustaf Ljunggren. Elevated Numbers of $FcγRIIIA^{+}$ ($CD16^{+}$) Effector CD8 T Cells with NK Cell-Like Function in Chronic Hepatitis C Virus Infection.
Journal of Immunology. 2008 vol. 181 (6) pp. 4219-28.

VI. Veronica D. Gonzalez, Karolin Falconer*, **Niklas K. Björkström***, Kim G. Blom, Ola Weiland, Hans-Gustaf Ljunggren, Annette Alaeus, and Johan K. Sandberg. Expansion of Functionally Skewed CD56-Negative NK Cells in Chronic Hepatitis C Virus Infection: Correlation with Outcome of Pegylated IFN-α and Ribavirin Treatment.
*Journal of Immunology. 2009 vol. 183 (10) pp. 6612-8. *Equal contribution*

LIST OF SUPPLEMENTARY PUBLICATIONS

SI. **Niklas K. Björkström**, Cyril Fauriat, Yenan T. Bryceson, Johan K. Sandberg, Hans-Gustaf Ljunggren, and Karl-Johan Malmberg. Analysis of the KIR Repertoire in Human NK Cells by Flow Cytometry.
Natural Killer Cell Protocols. Methods in Molecular Biology. 2010 vol. 612 pp. 353-64.

SII. Yenan T. Bryceson, Cyril Fauriat, Joao M. Nunes, Stephanie M. Wood, **Niklas K. Björkström**, Eric O. Long, and Hans-Gustaf Ljunggren. Functional Analysis of Human NK Cells by Flow Cytometry.
Natural Killer Cell Protocols. Methods in Molecular Biology. 2010 vol. 612 pp. 335-52.

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LIST OF ABBREVIATION

ADCC	antibody-dependent cellular cytotoxicity
CMV	cytomegalovirus
DC	dendritic cell
FACS	fluorescence activated cell sorter
HSV	herpes simplex virus
EBV	Epstein-Barr virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	hepatitis C virus cell culture system
HDV	hepatitis D virus
HFRS	hemorrhagic fever with renal syndrome
HPC	hematopoietic precursor cell
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
HTNV	Hantaan virus
HUVEC	human umbilical vein endothelial cell
IFN	interferon
IL	interleukin
ISG	interferon-stimulated gene
KIR	killer cell immunoglobulin-like receptor
LFA-1	lymphocyte function-associated antigen-1
NK	natural killer
PBMC	peripheral blood mononuclear cells
SLT	secondary lymphoid tissue
TGF-β	transforming growth factor beta
TRAIL	tumor necrosis factor–related apoptosis-inducing ligand

NK CELL BIOLOGY

Natural killer (NK) cells belong to the innate immune system and represents the third arm of the lymphocyte linage next to T and B cells. They entered the immunological scene in the mid-1970ties when Kiessling and colleagues described a cell that could kill certain tumor cells without the need for previous sensitization (1, 2). Since then, many observations have moved our understanding of NK cells forward, but despite intense research many unresolved issues remain. Further, as a consequence of these cells for long being relatively poorly understood, notions about NK cells have been mistaken. For instance, the name 'killer' implies these cells to be primarily focused on cytotoxicity. Rather, NK cell functions are instead heterogeneous. In the sections below, an introduction to NK cell biology covering aspects such as NK cell development and differentiation, regulation of NK cell activation, as well as a general overview of human NK cell functions is provided.

NK cell development and differentiation

NK cells, belonging to the hematopoietic system, are derived from CD34+ hematopoietic precursor cells (HPCs). During fetal development, NK cells are amongst the first functional immune cells to develop. Cytotoxic NK cells have been found as early as at gestational week 9 in the human (3). Furthermore, with extra-medullary hematopoiesis occurring, both fetal liver and thymic CD34+ HPCs are capable of developing into NK cells (4, 5). However, in the adult, it is generally accepted that NK cell development occurs within the bone marrow. Evidence for this comes from early studies of selective bone marrow ablation in mice (6). Furthermore, bone marrow-derived human CD34+ HPCs can, *in vitro*, develop into cytolytic NK cells (7). In this setting, at least the very early stages of NK cell development are dependent on presence of bone marrow stroma (8). NK cells require interleukin (IL)-2 and/or IL-15 for their development (7-9), and flt3 ligand potentiates the effect of IL-2/15 (10). However, complete, *in situ*, bone marrow NK cell development pathways have not yet been described in the human. This raises the question as to whether NK cell precursors, formed in the bone marrow, also can traffic to distinct peripheral anatomical locations for their final stages of development. Indeed, it was recently shown that immune precursor cells with NK cell-potential are selectively enriched in human secondary lymphoid tissues (SLT), primarily in lymph nodes and tonsil (11). These CD34+CD45RA+ NK cell precursors represented more than 95% of all HPCs in SLT but were infrequent in bone marrow and blood. Based on these initial findings, a follow-up report provided evidence for a complete and continuous NK cell development pathway occurring in SLT through four discrete stages yielding mature $CD56^{bright}$ NK cells (12, 13). Stage 1 NK cells (pro-NK cells) had a CD34+CD117-CD94- phenotype, lacked expression and transcription of CD122 (IL-2/15 receptor β chain) and were, thus, unresponsive to IL-15. However, these pro-NK cells did upon stimulation with flt3-ligand, IL-3, and IL-7, possibly in a stroma cell dependent

manner, acquire a stage 2 NK cell (pre-NK cells) phenotype, CD122, and the capacity to further develop into NK cells in an IL-15-dependent manner (12). CD34+CD117+CD94- pre-NK cells are not fully committed to the NK cell linage but can also give rise to other immune cells such as T cells and myeloid dendritic cells (DCs). Furthermore, they lack characteristic NK cell functions, such as cytotoxicity and the capacity to produce interferon (IFN)-γ. However, upon IL-15 stimulation, pre-NK cells develop into stage 3 NK cells (immature NK cells, iNK cells) via the phenotypic loss of CD34 and step-wise acquisition of CD56 together with a functional commitment to the NK-cell linage via the loss of DC and T cell potential. Finally, iNK cells progress into stage 4 NK cells ($CD56^{bright}$ NK cells), which represent the first subset of mature human NK cells. This progression is marked by the acquisition of the inhibitory receptor CD94/NKG2A, NK cell-associated activating receptors such as NKp46 and NKG2D, as well as by the capacity to produce IFN-γ and release perforin and granzyme-containing granules (12).

In humans, mature NK cells are primarily divided into $CD56^{bright}$ and $CD56^{dim}$ NK cells, defined by different levels of surface expression of CD56 (14-16). These two subsets are present at varying proportions in different compartments of the human body. In blood, bone marrow, and spleen, $CD56^{dim}$ NK cells dominate representing around 90% of all NK cells (14-16). On the other hand, tonsils and SLT are enriched for $CD56^{bright}$ NK cells whereas the liver contains equal proportions of dim and bright NK cells (17-19). The $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets differ in phenotype and function. In brief, $CD56^{bright}$ NK cells are negative, or express only low levels, of the low affinity Fc-receptor CD16, have a low expression of inhibitory killer cell immunoglobulin-like receptors (KIRs) and of cytotoxic molecules such as perforin and different granzymes (16). On the other hand, $CD56^{bright}$ NK cells are uniformly positive for inhibitory CD94/NKG2A, as well as CD62L and express the high affinity IL-2 receptor α chain (CD25). $CD56^{dim}$ NK cells display a variegated expression of KIRs, NKG2A, and CD62L, have high levels of CD16 on their surface, and contain intracellular granules with large amounts of perforin and granzymes (16). With respect to function, it has for long been considered that $CD56^{bright}$ NK cells are prominent cytokine producers with little capacity to perform cytotoxicity, whereas $CD56^{dim}$ NK cells are efficient killers but poor at producing cytokines (17, 20). However, recent work, including work presented in this thesis, instead suggests that both $CD56^{bright}$ and $CD56^{dim}$ NK cells are efficient in performing cytotoxicity and in producing cytokines (**papers I and III**) (21).

Lanier and colleagues originally postulated some 25 years ago that the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets represent two sequential stages of NK cell differentiation with $CD56^{bright}$ cells being the less mature cells (14). Despite the inherent difficulties to formally prove this linage relationship in the human by demonstrating that $CD56^{bright}$ NK cells can give rise to $CD56^{dim}$ NK cells and not *vice versa*, several lines of evidence support this original hypothesis. First, $CD56^{bright}$ NK cells appear earlier during immune reconstitution compared to $CD56^{dim}$ NK cells where they represent up to 70-80% of all peripheral blood NK cells after hematopoietic stem cell transplantation (HSCT) (15, 22). Second, $CD56^{bright}$ NK cells have a more

immature phenotype compared to $CD56^{dim}$ NK cells with, for instance, expression of CD117 (c-kit) (23), which is typically expressed by immature HPCs, display longer telomeres (24) and morphologically contain both granular and agranular cells, whereas all $CD56^{dim}$ NK cells are granular (14). Third, purified $CD56^{bright}$ NK cells was recently shown to differentiate into $CD56^{dim}$ NK cells in humanized mice engrafted with human hematopoietic stem cells (HSCs) (25).

Regulation of NK cell activation

Upon activation, NK cells can mediate potent perforin/granzyme and IFN-γ-dependent effector functions. These processes are tightly regulated on a multitude of levels. Belonging to the innate immune system, NK cells express a repertoire of germline-encoded activation and inhibitory receptors that do not undergo somatic recombination (26, 27). In accordance to the 'dynamic equilibrium concept', the net signaling input from these arrays of activation and inhibitory receptors will determine whether or not NK cells are activated to kill target cells or secrete IFN-γ (27, 28). These repertoires of receptors have evolved to ensure for the proper detection of pathogen-affected and/or transformed cells, but yet to prevent deleterious effects if NK cells would start to target normal self cells in an uncontrolled fashion. Besides the signaling input via activation and inhibitory receptors, the NK cell threshold of activation can be influenced in positive and negative manners by previous cytokine-priming as well as by previous encounters with regulatory immune cells such as DCs (29, 30). Furthermore, specificity for activation will be determined in accordance with the 'missing-self' hypothesis and the level of NK cell education will have an impact on NK cell responsiveness (31-33).

Recent work using a *Drosophila* insect cell system and resting unmanipulated NK cells has revealed new knowledge on the discrete steps taking place during NK cell activation (27). In this system, NK cells, after making contact with a susceptible target cell, adhere and polarize its granules in a lymphocyte function-associated antigen-1 (LFA-1)-dependent manner (34). On the other hand, single activation receptor signaling did not result in granule polarization (34). Instead, engagement of individual activation receptors did, at this stage during NK cell activation, yield inside-out signaling promoting an LFA-1 conformational change to an open high-affinity conformation and LFA-1 clustering (35). Finally, directed degranulation occurs. In resting, unmanipulated, NK cells the engagement of at least two different activation receptors is necessary for this (36). The mechanism behind this dependence on synergistic coactivation was recently identified (37). It was shown that signaling through two different activation receptors yielded strong enough Vav1 activation to overcome c-Cbl-mediated inhibition (37).

However, resting unmanipulated NK cells behave differently compared to cytokine-activated NK cells. NK cells readily respond with a higher level of activation to cytokines such as IFN-α, IL-2, IL-12, IL-15, and IL-18 (38). This encompasses, for instance, the loss of need for synergistic coactivation upon target cell encounter. Instead, engagement of single activation receptors now suffices for the induction of

directed degranulation (27, 36). On the other hand, the threshold for NK cell activation can also be negatively tuned by cytokines such as transforming growth factor-β (TGF-β) (39). Thus, it will likely be of importance to consider the level of NK cell activation when studying NK cells in different medical conditions and clinical contexts. For instance, an NK cell responding during an acute viral infection is likely to require less signaling through activation receptors and have a lower threshold of inhibition to overcome for activation to occur compared to an NK cell that performs its tumor-surveillance duties in an otherwise healthy unaffected host.

NK cells express a multitude of different activation receptors on their surface and most of these are homogeneously expressed on the surface of all NK cells (26). The proximal signaling from these receptors occurs through highly divergent signaling domains and through a multitude of different adaptors. Schematically, NK cells signal activation through three major proximal pathways, through the NKG2D-DAP10-PI3K/Grb2 complex, through ITAM-bearing receptors, such as CD16, NKp46, and NKG2C, that pair up with the adaptors DAP12, CD3ζ, or FcεRγ, or through SLAM-family member receptors such as 2B4 and CRACC that recruit SAP and Fyn through a cytoplasmic ITSM (27, 40, 41). Taken together, activation receptors recognize a wide array of molecular structures. For instance, NKG2D recognizes the ligands MICA/B and ULBP1/2/3/4/5/6 on target cells (42). These ligands are typically expressed at low levels during steady state but become upregulated in settings of cellular stress, such as viral infection or tumor transformation (42). Another example is CD16 (FcγRIIIA), which is a low to medium affinity Fc-receptor that binds most subclasses of IgG antibodies with the resulting antibody-dependent cellular cytotoxicity (ADCC) (43, 44). However, for many activation receptors, the cellular ligands are still unknown, e.g., the activating KIRs KIR2DS2 and KIR3DS1. Thus, despite having extracellular domains that closely resemble those of the MHC-class I binding inhibitory counterparts, it is still unknown what these receptors recognize.

Compared to the activation receptors, inhibitory receptors have conserved features with respect to their signaling and specificity. All major inhibitory receptors signal through ITIMs, recognize classical or non-classical MHC class I molecules, and are heterogeneously expressed on resting NK cells (45, 46). Kärre and colleagues predicted the existence of these inhibitory receptors in a seminal study 25 years ago where NK cells were shown to be able to kill MHC class I negative tumor cells, yet sparing their MHC class I expressing counterparts, and with this formed the platform for the 'missing-self' hypothesis (47). It predicts that NK cells can engage in the surveillance of virus-infected cells or transformed tumor cells that have specifically downregulated expression of MHC class I in an attempt to avoid recognition by CD8 T cells (31). From this point, it took another 9 years until the first inhibitory NK cell receptors, KIRs, were identified in the human (48). However, NK cells are not only able to sense loss of MHC class I through the absence of inhibitory signals, intriguingly, they are also dependent on inhibitory signaling through MHC class I during their development and differentiation to become fully functional. Evidence for such MHC-dependent functional maturation first came from studies of MHC class I deficient mice where NK cells were hyporesponsive (49-51). Furthermore, it was

shown, both in human and mice, that NK cells lacking expression of inhibitory receptors altogether exhibit a hyporesponsive phenotype as well as NK cells expressing inhibitory receptors with no self-ligand present in the host (32, 33, 52). Thus, for an NK cell to become functionally competent, it needs to express at least one inhibitory receptor that recognizes a self-ligand present in the host, a process referred to as NK cell 'education'. However, the exact mechanism behind this, when it occurs, as well as the functional implications of NK cell 'education' on a grand scale for the host are still unknown.

Functions of human NK cells

Research performed over the last decade in the human have substantiated previous work in experimental models and provided solid evidence for a clear role of NK cell in several human disease and normal physiological conditions. NK cell function during viral infection will in more detail be reviewed in sections below. Despite being originally identified for the capacity to spontaneously kill tumor cells (1), it has been difficult to provide solid *in vivo* evidence for the biological function of NK cells in tumor elimination and antitumor immune surveillance. However, a multitude of studies have described the *ex vivo* capacity of freshly isolated human NK cells to recognize primary tumor cells (53). Furthermore, the genetic removal of key NK cell activation receptors, such as NKG2D and DNAM-1, have in experimental systems of spontaneous tumor development further substantiated the importance of these receptors, and possibly NK cells, in antitumor immune surveillance (54, 55). In line with this, a large epidemiological survey with >3000 healthy individuals over an 11-year follow-up period showed a clear connection between NK cell cytolytic activity in peripheral blood and the appearance of cancer (56). In this study, individuals with low NK cell activity had an increased risk of developing cancer (56). NK cells are also believed to exhibit a graft versus tumor effect in settings of HSCT. KIR-HLA ligand mismatching appears to be beneficial when patients with acute myeloid leukemia are treated with an allogeneic HSCT (57). Finally, human pregnancy has been considered a physiological condition associated with increased risk for tumor development as well as tumor dissemination. Interestingly, it was recently shown in an experimental model that decreased NK cell function, as a consequence of general immune suppression during pregnancy, was associated with tumor appearance and dissemination (58).

Another, somewhat unexpected, role for NK cells have lately been revealed in studies of human pregnancy. A specialized subset of decidual NK cells constitute up to 50-90% of all lymphocytes in the uterine decidua early during pregnancy (59). These decidual NK cells seem to regulate trophoblast invasiveness through chemokine production as well as uterine spiral artery remodeling via angiogenetic factors, of which both are vital events occurring during the first trimester of pregnancy (60). Furthermore, human pregnancy-associated disorders, such as preeclampsia, fetal growth restriction, and recurrent miscarriages, are linked to certain maternal and fetal KIR-HLA combinations (61, 62). However, the exact mechanism behind why these

clinical conditions occur and whether decidual NK cells play a role in them remain a matter of debate.

NK CELL IMMUNITY AGAINST VIRUSES

Perhaps the most convincing evidence for a role of NK cells in the defense against viral infections comes from case reports of humans with selective NK cell immunodeficiencies (63-66). These patients often suffer from respiratory viral infections and have problems controlling infection by herpesviruses, such as cytomegalovirus (CMV), varicella zoster virus, Epstein-Barr virus, and herpes simplex viruses (63, 64). Another line of evidence supporting an important role for NK cells in viral infections is the multitude of studies that have identified viral immune evasion mechanisms that specifically target NK cells (67, 68). Furthermore, experimental mouse models show that NK cells contribute to the control of acute viral infections, and in humans, genetic epidemiological findings advocate for a role of NK cells in the long-term outcome of chronic viral infections (45, 69-71). However, it is important to bear in mind that NK cell do not control all viral infections. For instance, the NK cell contribution to defense against LCMV and the γ-herpesvirus MHV-68 in the mouse, as well as against SIV in rhesus monkeys, are reported to be minimal (72-74). The sections below will cover how NK cell responses develop during acute and chronic viral infections. A more detailed outlook into two specific human infections, hepatitis C virus (HCV) infection and hantavirus infection, of relevance for the subsequent discussion on work included in this thesis, will also be provided.

NK cells in acute viral infections

A role for NK cells in the defense against acute viral infections was early implicated by numerous reports in the 1980ties. In these studies, mice that had received the depleting antibody anti-asialo GM1 exhibited increased sensitivity to infection with murine CMV (72, 75, 76), mouse hepatitis (72), vaccinia (72), herpes simplex (77), influenza (78), and Coxsackie virus infection (79). Despite that anti-asialo GM1 efficiently depletes NK cells from many mouse strains, it also removes smaller subpopulations of CD4 and CD8 T cells as well as basophils and other myeloid subpopulations (80). Thus, interpretations of these early studies should be made with some caution (81, 82). Nevertheless, more recent studies focused on characterizing the development of the NK cell response, in particular against murine CMV. NK cells were shown to both specifically and unspecifically proliferate and expand early after murine CMV infection (83). This response terminated by a rapid contraction of the NK cell population, much similar to the behavior of effector T cells upon clearance of virus (84-86). In humans, early *ex vivo* and *in vitro* studies revealed that virus-induced IFN-α enhanced NK cell cytotoxicity and ADCC and indicated a role for NK cells in the defense towards viruses (87, 88). Also, in the 1980ties, abnormal low NK cell function was linked to severe

herpesvirus infections such as Epstein-Barr virus (EBV) (89, 90), CMV (91), and herpes simplex virus (HSV)-1 (92). Having said this, however, the most convincing data for a role of human NK cells in the defense against viral infections has, as reviewed above, come from studies of patients with primary NK cell immunodeficiencies (63-66).

NK cells in chronic and latent viral infections

More amenable to study are human NK cells in settings of chronic or latent viral infection. For instance, having certain KIR genes in compound with HLA, or certain KIR and HLA polymorphisms, significantly influence the progression to AIDS in HIV-infected individuals (93, 94). Although attempts have been made to find functional evidence for these associations (95), the exact mechanisms behind the protective KIR and HLA effects remain elusive. Furthermore, it is tempting to speculate that recent reports, on how polymorphisms that affect HLA-C surface expression levels and consequently progression from HIV to AIDS (96, 97), might find its underlying explanation in varying levels of NK cell functionality. Thus, according to the rheostat model of tuning of NK cell education (98), the strength of the inhibitory input (high or low HLA-C surface expression) might, in a quantitative fashion, tune the level of functionality in individual NK cells (99). In relation to these association studies, it has been shown that HIV-1 *Nef* specifically downregulated surface expression of HLA-A and -B, while not exhibiting this effect on HLA-C and –E (100). In theory, this evasion strategy renders the virus less attractive to cytotoxic T lymphocytes (CTLs) whereas it still efficiently inhibits NK cells through KIR2DL1/2/3 and NKG2A.

Given the relative accumulation of NK cells in the human liver compared to peripheral blood (101), it is easy to make the case of studying the role of NK cells during hepatotropic viral infections. The role of NK cells during acute and chronic HCV infection has rendered much attention lately (in detail covered in the upcoming section). On the other hand, relatively less is known with respect to NK cell function in acute and chronic hepatitis B virus (HBV) infection. A transgenic mouse model of human HBV has been used to study immune pathology during HBV infection (102). In this model, it is clear that NK cells play a role in causing liver pathology, however, whether this occurs indirectly and is dependent on CD1d-restricted NKT cells or occurs directly, through the activating receptor NKG2D, is unclear (103, 104). Nevertheless, the human liver contains very few CD1d-restricted invariant NKT cells (Dr. E. Heeregrave, personal communication), and NK cells have been implicated to mediate liver damage via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in eAg negative patients with chronic hepatitis B (105). Another, somewhat neglected, hepatotropic infection is hepatitis D virus (HDV) infection (106, 107). HDV is a small defective RNA virus that can only infect individuals already infected with HBV. Compared to chronic infection with HBV or HCV, chronic HBV and HDV co-infection much rapidly lead to liver cirrhosis, and these patients have a higher risk of developing hepatocellular carcinoma (HCC) (106, 107). Despite this, and despite an

estimated more than 15 million co-infected individuals worldwide, the literature is devoid of reports assessing NK cell function in this disease setting.

Besides the conclusive evidence obtained under conditions of human NK cell deficiencies supporting a role for NK cells in the early defense against many herpesviruses, also when latency of these infections establishes, there is likely a battle between the host and the virus that goes on for many years (68). These viruses have devised numerous strategies to avoid the recognition by NK cells. Reciprocally, one can speculate that this has influenced the evolution of receptors on NK cells and their ligands (68). Perhaps the most striking evidence for such co-evolution comes from the murine CMV model where NK cells expressing the activating Ly49H receptor specifically recognizes, responds and proliferates towards, and can develop innate 'memory' against, infected cells expressing the murine CMV protein m157 (83, 108, 109). A similar imprinting is also evident in the human. Expression of the activation receptor NKG2C is tightly linked to CMV serostatus. NK cells from CMV seronegative individuals have low, or absent, expression of NKG2C on their surface, whereas expression is considerable higher in CMV seropositive individuals (110). However, the function of NKG2C on NK cells during primary CMV infection, or CMV reactivation, remains elusive. One strategy employed by herpesviruses is to modulate expression of NK-cell receptor ligands. For instance, CMV, EBV, and Kaposi's Sarcoma-associated herpesvirus, all transcribe separate microRNA's that target and downregulate MICB, a ligand for the activation receptor NKG2D (111, 112). Given this seemingly perfect balance that have been achieved between NK cells (and other host immune cells) and the herpesviruses, it is not surprising that consequences of viral reactivation in settings of immune suppression often have severe implications and that the elderly population, likely to have a weaker and more narrower immune system, suffer more from these pathogens.

Outlook: hepatitis C virus infection

Hepatitis C virus, originally discovered in 1989, has been estimated to chronically infect between 130-170 million individuals worldwide (113, 114). The virus, with a narrow tropism for human hepatocytes, is a small single stranded positive sense RNA virus of the *Flaviviridae* family. It has a high rate of replication with up to 10^{12} virions produced in the infected host every day. Interestingly, due to lack of proofreading by the RNA polymerase of HCV, the mutation rate of the virus is considered to be exceptionally high (115, 116). In the natural history of infection, roughly 75% of infected individuals will develop chronic disease and out of these, 25% will progress to an end-stage liver disease characterized by liver cirrhosis and a significant risk for HCC (113, 114). Combinatorial treatment with IFN-α and ribavirin is effective in subgroups of patients but associated with side effects (117). From an immunological point-of-view, a robust adaptive cellular response has been correlated to clearance of acute infection (118, 119), whereas chronic disease is associated with impaired CD4 and CD8 T cell function and an exhausted immune profile of these cells (115, 116).

Several lines of evidence support a role for NK cells in the control and clearance of HCV infection. First, NK cells are accumulated at the site of infection, the human liver, representing up to 30-45% of all intrahepatic lymphocytes (18, 101). Second, genetic epidemiological data on liver diseases in general, and HCV in particular, advocate for a role of NK cells and NK cell receptors in conditions of liver disease (120-125). Khakoo and colleagues made an important first observation when they showed that individuals having the *KIR2DL3* gene together with two HLA group C1 alleles are more prone to clear acute HCV infection (124). This beneficial effect has subsequently been shown to protect in additional cohorts as well as pinpoint patients with chronic disease that are more likely to clear infection upon treatment (125). Third, a recent adoptive immunotherapy trial, in which donor-derived intrahepatic NK cells, after short-term *in vitro* propagation and stimulation, were administered intravenously to HCV-positive individual undergoing orthotropic living donor liver transplantation, came down with promising results showing that these allogeneic NK cells has the potential to reduce serum viral load (126). However, the exact mode of action behind this NK-cell anti-HCV effect still remains largely unknown.

Numerous studies have assessed the phenotypic properties and function of peripheral blood and intrahepatic NK cells during acute and chronic HCV infection, and to a lesser extent, during IFN-α and ribavirin treatment of acute and chronic HCV. In general, NK cells are activated and display enhanced function during acute HCV (127, 128), however a recent report could not detect any functional differences comparing NK cells from patients with acute HCV with uninfected controls (129). During chronic HCV, on the other hand, NK cell function is generally attenuated and both immature and dysfunctional subsets of NK cells appear at greater frequencies than in healthy individuals (70). Nevertheless, some of these aspects, including the appearance of dysfunctional NK cell subsets, NK cell-interactions with HCV-infected target cells, and how NK cells respond during IFN-α + ribavirin treatment will be covered in more detail in the discussion section below.

Outlook: hantavirus infection

The prototypic hantavirus, Hantaan virus, was identified 1978 (130). However, reports of hantaviral-like diseases date as far back as 960 A.D., “War nephritis” was a major problem during the first World War, and many thousands of UN soldiers fell ill in a diseases named Korean hemorrhagic fever during the Korea war. More than 20 serologically and genetically distinct hantavirus serotypes have been identified of this viral *genus* that belongs to the *Bunyaviridae* family of negative single-stranded RNA viruses (131). Hantaviruses are maintained in rodents, believed to be persistently infected, and transmitted to humans through inhalation of contaminated excreta. Spread within the human population has only been documented for Andes hantavirus (132). Hantaviruses causes two distinct diseases in the human, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome. The latter is much more infrequent, with only around 1,000 cases reported this far, however with a case fatality rate of 50% (133). On the other hand, it is estimated that 150,000 cases of HFRS

involving hospitalization occurs every year (133). For HFRS, the case fatality rates are lower and much dependent on the serotype of the virus, ranging from 0.5-1% for Puumala hantavirus up to 10-15% for Hantaan hantavirus (131). From a clinical point of view, HFRS is characterized by five distinguishable phases occurring after a rapid onset of disease (131). The first phase involves flu-like symptoms; this is followed by a hypotensive phase with proteinuria and decreased levels of platelets. The third phase is the oliguric phase with reduced urine production as a sign of pronounced renal involvement and an elevation of blood creatinine levels. This is followed by a polyuric stage and finally, a convalescent phase of recovery occurs, typically during the second week of disease. However, full recovery can take many more weeks.

In the human, hantaviruses have been shown to infect endothelial cells and the ensuing viremia lasts up to two weeks after symptom debut (134, 135). Interestingly, hantaviruses does not, *per se*, cause cytopathic effects in the infected cells (135). Instead, current-thinking advocates for clinical symptoms, as a consequence of capillary leakage, to be immune mediated (135, 136). Furthermore, clearance of this acute viral infection from the host is believed to occur by a robust adaptive cellular immune response that also provides life-long memory (135). Indeed, the literature is void of studies reporting on re-infection to occur in the human (135). During the acute phase of infection, up to 50% of peripheral blood CD8 T cells are responding (N.K. Björkström, accepted for publication July 16 2011, Journal of Virology), and hantavirus-specific memory CD8 T cells can be detected in individuals years after primary infection occurred (137, 138). However, both the exact mechanism behind clearance of virus, and what is causing immune pathology, still needs to be established.

RESULTS AND DISCUSSION

This thesis is made up of six papers and two supplementary articles. In the following sections, the content of the thesis will be discussed in more detail. The first section will involve a general discussion on methodology developed in relation to this thesis and outlooks on future prospects with respect to detailed analysis of human lymphocytes. The second and third sections will focus on how NK cell differentiation occurs in the healthy human and how this is affected by an acute viral infection. Furthermore, these sections will also include a discussion on how an individual's infectious disease history might play a role in shaping the NK cell repertoire. The fourth section describes results and discusses NK cell activation in response to viral infections and the treatment thereof. The aim of the fifth section is to dissect NK cell – target cell interactions in relation to two *in vitro* model systems that have been used. Finally, the sixth section will focus on how immune exhaustion affect NK cells and T cells during chronic HCV infection. For extensive materials and methods, results, and discussion, please see the respective articles.

GENERAL AIMS OF THIS THESIS

The overall aim of this thesis was to gain more knowledge on how human NK cells function in health and disease. In particular, the work was focused on studying basic aspects of NK cell differentiation and activation at steady state. Furthermore, specific aims were to investigate the role of NK cells during acute, chronic, and latent viral infections in humans.

METHODOLOGICAL DEVELOPMENT

The ways in which immune cell function can be analyzed are immense. Over the years, many specific assays and techniques have been developed, some only to rapidly disappear again, while others persist over decades. One clever example of this is the ^{51}Cr-assay, originally developed by Wigzell and colleagues in the mid 1960s, as an indirect way to measure target cell killing (139), still used in many laboratories now close to 50 years later. New approaches, such as those presented by O'Garra and colleagues with rapid transcriptional profiling of the immune system (140), or Nolan and colleagues with a system-wide approach to study immune compartments (141), represent the other extreme in methodological development where we now, instead of getting a single value of cytotoxicity or an E:T ratio curve, can start to visualize complete signaling cascades in hundreds of distinct subsets of immune cells simultaneously.

Flow cytometry have been utilized as an important tool through out this thesis (**papers I-IV, SI, SII**) and efforts have been spent developing this technology in order to permit a detailed phenotypic and functional characterization of human NK cells (**papers SI and SII**) (142). Flow cytometry, and fluorescence activated cell sorting (FACS), initially developed in the 1940-50s by Gucker and Coulter and in the 1970s commercialized through the work of the Herzenberg's and Becton Dickinson, have over the past two decades become a widely used technique for studies of immune cells (143, 144). In particular, flow cytometry is useful for assessing phenotype and function of immune cells. Using this technology, a plethora of receptors and other surface and intracellular molecules has been defined that regulate the function of immune cells. Furthermore, technological advances, such as multi-color flow cytometry, have greatly facilitated our understanding of the immune cell as such and also helped to accurately isolate immune cells with defined properties.

The developmental work in this thesis has been necessary much because of the complex receptor repertoires that regulate human NK cells and the multitude of effector functions they perform (26, 27). One example of this is the KIR family of receptors. Briefly, 15 *KIR* genes exist and the expressed receptor pairs KIR2DL1/KIR2DS1, KIR2DL2/KIR2DL3/KIR2DS2, and KIR3DL1/KIR3DLS1 respectively have almost identical extracellular domains. Thus, many commercially available monoclonal antibodies cross-react between inhibitory (KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1) and activating (KIR2DS1, KIR2DS2, KIR3DS1) forms of these receptors (**papers II and SI**) (145). Knowing this, different approaches can be utilized to study the expression of KIRs and the function of KIR-expressing NK cell subsets. One approach is to study NK cell clones (145), since individual clones uniformly will contain only certain *KIR* genes, and thus in most cases the corresponding receptors (146, 147). Other approaches, instead analyzing primary human NK cells or polyclonal cultures, involves the assessment of total-KIR expression without getting information on how individual KIRs are expressed as well as the focused analyses of NK cells expressing only a single KIR where in the latter approach, all other KIR-expressing NK cells are

removed from the analysis through a combinatorial staining of multiple KIRs (33). However, for global analysis of this polygenic and polymorphic family of activating and inhibitory receptors, the use of multicolor flow cytometry is necessary together with genetic information disclosing the presence or absence of particular genes (**paper SI**). In particular, a high-resolution analysis is warranted for studies of NK cell licensing and differentiation since the number of KIRs expressed by NK cells on a single cell level as well as the nature of the expressed KIRs (activating or inhibitory, licensed or non-licensed) will have an impact on result interpretation (**paper I**) (33, 148-152). Furthermore, to in detail dissect the growing body of epidemiological studies implicating a role for epistatic KIR – HLA interactions in anti-viral immunity (93, 94, 124), reproductive success (61, 62), and graft-versus-leukemia effects in allogeneic HSCT (57, 153), more complete information on KIR expression repertoire as well as the function of NK cell subsets with distinct KIR profiles are likely necessary.

Work in the current thesis strived towards the development of optimized multicolor immunofluorescence panels (154) for high-resolution analysis of KIR expression (**paper SI**) (142). Indeed, these techniques were subsequently employed for studies of NK cell differentiation in individuals homozygous for the group A *KIR* haplotype (**paper I**) as well as to start dissecting up populations of NK cells expressing inhibitory and activating KIR2DL1/S1 and KIR2DL2/L3/S2 receptors in hantavirus infected patients with group B *KIR* haplotypes. The group A *KIR* haplotype contain fewer *KIR* genes compared to group B *KIR* haplotypes, and only one activating KIR (KIR2DS4). This makes repertoire analyses of group A *KIR* haplotype homozygous individuals more straightforward (**paper SI**). This is also the breaking point were current instrumentation is starting to become unpractical. With increasing numbers of parameters included, the level of spectral overlap will significantly increase, and consequently the need for compensation that might create confounding variability (**paper SI**) (142, 155, 156). Whereas a simultaneous measurement of 6-10 parameters readily can be achieved, once the analysis reaches the 11- to 16-parameter range, it becomes increasingly more difficult (155, 156). For instance, if the aim would be to simultaneously assess all KIRs in a single experiment, eleven unique antibodies, and thus 11 parameters, would be required (15 *KIR* genes in total, *KIR2DP1* and *KIR3DP1* are pseudogenes (157), KIR2DS3 is expressed but retained in the cytoplasm (158), and KIR2DL4 is expressed on all NK cells (159). If this is feasible or not, and furthermore, whether the extension of such an approach, also including the inhibitory and activation receptors NKG2A, LILRB1, and NKG2C, now needing 14 parameters for the MHC class I binding NK cell receptors only, is possible, remains to be shown in the future. Nevertheless, recent work deciphering expression and co-expression patters of KIR2DL1- and KIR2DS1-expressing NK cells allowed for detailed studies of KIR2DS1 single-positive primary NK cells in the context of NK cell education yet taking other KIRs expressed into account (149).

On top of phenotyping NK cells with respect to expression of KIRs, which has the potential to provide information on the KIR repertoire, for instance to be used when assessing different models of KIR acquisition (147, 150, 160), as well as in settings of KIR repertoire perturbations, a second layer of information can be obtained when

combining this with the functional analysis of distinct NK cell subsets. With these combined approaches, the principle behind NK cell education was revealed (32, 33, 148, 152). Obviously, many functional parameters exist to study. For example, early work in developing the CD107a degranulation assay, first for cytotoxic CD8 T cells (161), and later on validated for NK cells (162), have been a valuable supplement to non-flow cytometry-based killing assays. Furthermore, both $CD56^{bright}$ and $CD56^{dim}$ NK cell cytokine and chemokine production can in comprehensive manners be quantified with flow cytometry (**paper SII**) (21, 142). Another interesting functional parameter is detection of inside-out signaling yielding a LFA-1 conformational change from the less active closed conformation to the more effective open form (35). A recent methodological paper describes this assay in detail including the epitope-sensitive anti-CD18 antibody that allows for quantification of this early parameter of NK cell activation (163). Functional parameters can also be assessed in combination (142), and, although, correlates between NK cell multifunctionality and disease outcome (e.g. increased capacity to control infection, better anti-tumor immune surveillance capabilities) still are missing, similar approaches assessing T cell function have proven successful. Multifunctional CD4 T cells are important determinants of protection in an experimental model of Leishmania major infection (164) and the quality of the CD8 T cell response seem to predict the rate of HIV disease progression (165). With respect to NK cells, interestingly, the level of murine NK cell education, modulated with varying levels of inhibitory input, is not only reflected on the quantitative level with higher degranulation and IFN-γ production, when assessing quality of the response, these subsets of NK cells also produce more IFN-γ on a per cell basis and secrete higher numbers of granules (98, 99). In the current thesis, we assessed up to four functional parameters simultaneously on NK cells (**paper SII**), this included MIP-1β, IFN-γ, TNF-α, and CD107a. Using this, the activation threshold of $CD56^{dim}$ NK cell cytokine production upon target cell stimulation was dissected, revealing the lowest level of activation needed for MIP-1α and MIP-1β production whereas strong stimulation was needed to yield NK cell IFN-γ production (21). Furthermore, specific triggering of NKG2C with its natural ligand HLA-E on NKG2C+ $CD56^{dim}$ NK cells that had expanded during acute hantavirus infection resulted in a strong multifunctional response (**paper II**). This would suggest that distinct NK cell populations might exhibit specific functional response profiles. Recent unpublished work corroborates this view (Dr. V. Beziat, personal communication). Again NKG2C+ NK cells from patients with chronic hepatotropic viral infection, upon stimulation with the natural ligand for NKG2C or assayed for ADCC, exhibited a unique multifunctional response profile simultaneously producing higher levels of cytokines and degranulating in a stronger fashion compared to other subsets of NK cells (Dr. V. Beziat, personal communication). Nevertheless, these observations have to be put into a bigger context. Thus, even if it is tempting to speculate that, for instance the level of NK cell functionally early after infection will have an impact on rate of resolution and/or likelihood of development of chronic infection, these are important questions that deserve future consideration in appropriate settings.

Because of the obvious limitations in performing human experiments *in vivo*, over the last decades much effort has been put into developing small animal xenotransplantation models. Although early reports were promising, adoptively transferring human fetal HSCs or human peripheral blood cells to SCID mice, the level of reconstitution as well as the functionality of the developing human immune system was low (166, 167). A breakthrough came, however, when Manz and colleagues used conditioned neonatal BALB/c $Rag2^{-/-}\gamma cR^{-/-}$ mice and performed intrahepatic infusions with CD34+ HSCs (168). Since then, much work has been performed optimizing these humanized mice models, where for instance mouse to human cytokine cross-reactivity have been taken into account (169). With respect to NK cells, the addition of human transpresented IL-15 seem to be a prerequisite for *in vivo* development and differentiation (25). With IL-15 supplemented, functional, *in vivo* developed, NK cells can be obtained (170). Nevertheless, results obtained from these models should be interpreted with caution because of interspecies differences. In the current thesis, we established and used the BALB/c $Rag2^{-/-}\gamma cR^{-/-}$ mice model for a specific question (**paper I**). Here, the aim was to determine whether developed NK cells, and developed KIR-positive NK cells, started to express the differentiation marker CD57 (**paper I**). On the other hand, before these models can be used in more complex disease settings, such as viral infection (171), more work is probably needed elucidating the differences between the human immune system developed in these mice and the authentic one. Nevertheless, the future prospects in establishing more complex systems, such as a combined xenotransplantation model for human immune system and human liver holds promise (172).

In summary, technological development over the last decades has greatly increased our detailed understanding of individual components of the human immune system. The next step that we need to take, however, likely involves moving beyond the assessment of a single cell type, or a single function. Instead, new techniques that will allow for combined assessment of many different immune cells and the interactions between these cell types, in either more complex *in vitro* systems or *in vivo* models such as mice with a human immune system, is bound to deliver new important knowledge.

HUMAN NK CELL DIFFERENTIATION AT STEADY STATE

The mature human NK cell compartment is made up of $CD56^{bright}$ and $CD56^{dim}$ NK cells (14, 16, 17). Evidence from different models of immune system development as well as from steady-state conditions in healthy humans strongly suggests that $CD56^{bright}$ NK cells are predecessors that, upon a yet to be defined stimulation/trigger, differentiates into $CD56^{dim}$ NK cells. In more detail, such linear differentiation can be observed in patients following hematopoietic stem cell transplantation (HSCT) (15, 22) as well as during immune reconstitution in humanized mice engrafted with human HSCs (25). Work in this thesis has substantiated these findings. We show that $CD56^{bright}$ NK cells are more common early after reconstitution in HSCT as well as in

engrafted humanized mice (**paper I**). Besides this, however, the common notion has been that differentiated CD56^{dim} NK cells retain fixed functional and phenotypic properties during their lifespan. This is in contrast to the complex differentiation that both T and B cells undergo upon activation after antigen encounter (173, 174). For instance, differentiating CD8 T cells undertake multiple phenotypic and functional changes where more differentiated cells have less requirements for co-stimulation as well as a different need for survival factors as compared to naïve CD8 T cells (173, 174).

With these differences in complexity between different lymphocytes as a starting-point, in **paper I**, we asked the question whether the transition from CD56^{bright} to CD56^{dim} NK cells represent a final step of mature human NK cell differentiation or whether CD56^{dim} NK cells continue to differentiate beyond previously defined stages. As a point of departure, we evaluated the proliferate responses of CD56^{dim} NK cells, this since a major functional difference between CD56^{dim} and CD56^{bright} NK cells is the reduced proliferative capacity of CD56^{dim} NK cells (**paper I**) (24, 175). Taking different CD56^{dim} NK cell subsets into consideration, we found that expression of CD57, NKG2A, and KIRs independently of each other correlated with CD56^{dim} NK cell proliferative responses. By FACS-sorting NKG2A+KIR-CD57-, NKG2A-KIR-CD57-, NKG2A-singleKIR+CD57-, and NKG2A-singleKIR+CD57+ CD56^{dim} NK cell subsets, we could show that expression of NKG2A was associated with a higher capacity to proliferate, whereas NK cell subsets expressing KIRs and/or CD57 were less prone to divide upon cytokine stimulation (**paper I**). Amongst these three molecules, expression of CD57 had the most profound effect on proliferative capacity. Furthermore, and intriguingly, when assessing the distribution between subsets of CD56^{dim} NK cells expressing CD57, NKG2A, and KIRs, we found that CD57 and NKG2A in many respects are expressed on separate subsets of NK cells. In agreement with previous studies (148), expression of NKG2A decreases with the sequential expression of more KIRs. Thus, an NK cell expressing zero or a single KIR has a higher NKG2A expression as compared to an NK cell expressing three or four KIRs. The opposite pattern of expression emerged for CD57, with a strong positive correlation between CD57 expression and multiple KIR expression.

With these functional and phenotypic observations, and taking the phenotype and function of CD56^{bright} NK cells into account (high proliferative capacity; uniformly NKG2A+CD57- with low KIR expression), we hypothesized that sequential loss of NKG2A and acquisition of KIRs and CD57 might represent a process of human CD56^{dim} NK cell differentiation. To show that this process occurs over time, we took use of two dynamic models of human NK cell development. When monitoring NK cell reconstitution in patients after HSCT and in mice that had received HSCs, we could observe that NKG2A decreased, CD57 increased over time, and proliferating cells were primarily CD57 negative (**paper I**) (Figure 1). Furthermore, it was clear from the humanized mouse model, and from *in vitro* experiments that cytokine stimulation (IL-2 and/or IL-15) of CD57- NK cells could promote the appearance of CD57+ NK cells. However, this step seemed irreversible since we could not detect loss of CD57 expression in any of the studied systems.

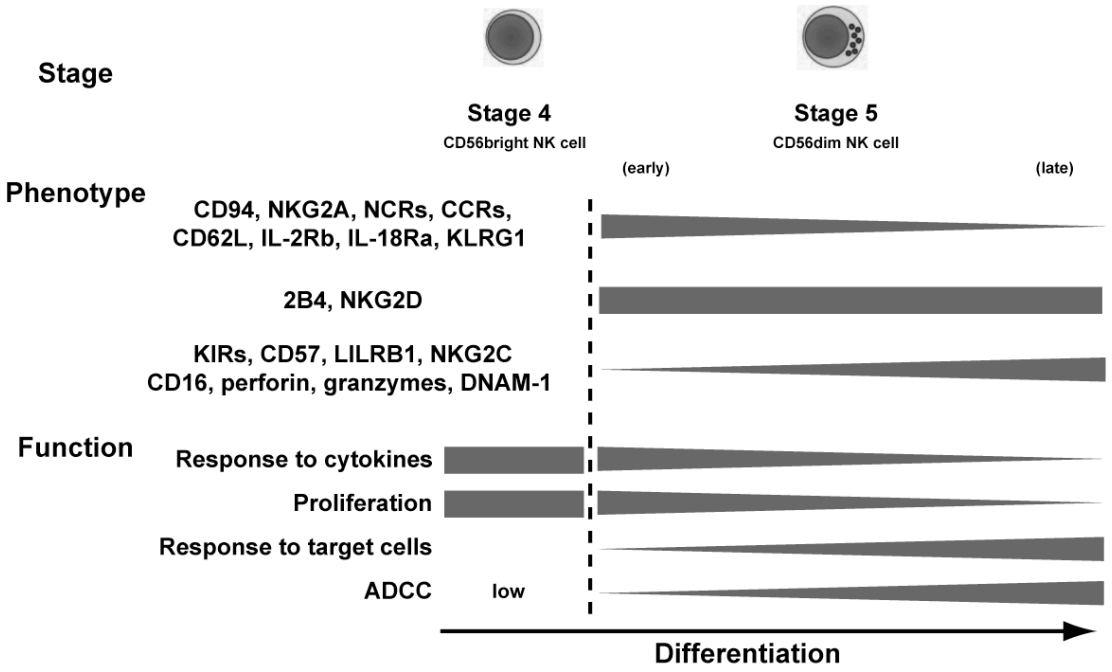

Figure 1. Proposed model of human NK cell differentiation (**paper I**).

What is the mechanism behind the proliferative incapacity of CD57+ NK cells, and what is the role for CD57? Both T cells and NK cells can express CD57. Previous literature has linked CD57 expression to terminal CD8 T cell differentiation (176, 177). Similar to the results obtained here on CD57+ NK cells, these CD57+ CD8 T cells are replicative senescent. Apoptosis sensitivity is another variable that have been investigated on CD8 T cells. Contrasting results exist, CD57+ CD8 T cells have been shown to be more sensitive to activation-induced cell death but on the other hand more resistant to spontaneous or death receptor-induced apoptosis (177, 178). It still remains unclear if CD57 affects NK cell apoptosis rates. Furthermore, CD57+ NK cells are absent in cord blood but increase with age in the adult population (179). Since mice do not possess CD57, and since the ligand(s) of CD57 is / (are) still unknown, it has been difficult to define a role for CD57 itself. A recent report investigated the transcription signature of CD57+ and CD57- NK cells (180). Strikingly, only 33 genes were differently expressed between the two subsets and most of these were implicated in receptor signaling. Indeed, we could show that CD57+ NK cells express lower levels of the common IL-2Rβ receptor subunit as well as of the IL-18Rα subunit (**paper I**). These phenotypic changes might in part explain the reduced proliferative capacity of CD57+ NK cells. However, whether or not CD57 by itself is responsible for the differences in proliferative capacity is unclear.

Except for CD57 as a molecule expressed on terminally differentiated NK cells (**paper I**) (180, 181), several other markers have recently been suggested to identify subsets of NK cells with distinct functions, including CD94, CD62L, CD6, and CD27 (182-185). Briefly, CD94, CD62L, and CD27 are highly expressed on $CD56^{bright}$ NK cells but low on terminally differentiated $CD56^{dim}$ NK cells whereas CD6 show the opposite pattern of expression. Furthermore, in murine NK cells, a four-stage

differentiation program has been suggested for mature NK cells based on surface expression of CD11b and CD27. $CD27^{high}CD11b^{low}$ murine NK cells share some characteristics with $CD56^{bright}$ NK cells, whereas the $CD27^{high/low}CD11b^{high}$ subsets are more similar to $CD56^{dim}$ NK cells (186, 187). Results from these studies are well in line with our presented results. However, many of the findings in these reports would suggest NK cells to differentiate along a fixed linear scheme. Our data rather support NK cells undergoing a continuous differentiation with many possible pathways. We find that all intermediates of this process, characterized by a gradual loss of NKG2A paralleled with a sequential acquisition of KIRs and CD57, are present at steady state in the healthy human and make up the total $CD56^{dim}$ NK cell repertoire (**paper I**). Nevertheless, superimposing CD94, CD62L, CD6, and CD27 on the model we propose with NKG2A, KIRs, and CD57, might reveal a more precise view of different stages of NK cell differentiation.

Since we could show that NK cell differentiation was associated with significant functional changes (discussed in detail below), we also assessed the relationship between NK cell differentiation and NK cell education. During development (and/or differentiation) NK cells are believed to go through an education process where interactions between KIR and/or NKG2A with self MHC class I tunes NK cell responsiveness (32, 33). Thus, NK cells negative for KIRs and NKG2A as well as NK cells expressing KIRs without a self MHC class I ligand present in the host will turn out to be hyporesponsive (33, 148, 152). Results obtain here suggest that NK cell differentiation and NK cell education are two parallel but non-related events (**paper I**). For instance, an NK cell can acquire a self-KIR without changing the likelihood of that NK cell being more or less differentiated. Furthermore, education had no impact on NK cell proliferative capacity as well as capacity to respond after cytokine stimulation. On the other hand, and in line with the concept of education, educated NK cells more efficiently responded with degranulation against susceptible target cells. In fact, having differentiation and education as uncoupled events might be beneficial. Thus, if educated NK cells would be more differentiated, that would also suggest these cells to have a lower proliferative capacity and a lower capacity to respond to cytokine stimulation. Whereas NK cell education seems to exist as a mechanism that allows for tolerant cells to be functional, it would be counterproductive to restrain these cells from being able to proliferate. On the other hand, recent work in the murine CMV-model showed that the response from 'unlicensed' NK cells was critical for protection (188). In line with this, a striking finding of our work was that seemingly hyporesponsive, KIR and NKG2A negative, NK cells still were more efficient in responding to cytokines with proliferation and as efficient in responding with IFN-γ production, as were educated KIR+ NK cells (**paper I**). Nevertheless, more work is needed on elucidating the mechanisms behind NK cell education and NK cell differentiation. How NK cell differentiation is affected during viral challenges as well as what consequences alterations in NK cell differentiation might have for the host are discussed in more detail in upcoming sections.

ACUTE AND CHRONIC VIRAL INFECTIONS INFLUENCE HUMAN NK CELL DIFFERENTIATION

Although, as reviewed above, case reports on selective NK cell deficiencies strongly suggest for a role of NK cells in the early defense against viral infections (63-66), the development of this response has rarely been studied in the human. Instead, we know from murine models that the NK cell response encompasses a rapid and robust proliferation of activated NK cells that peaks within days after infection (83). This is, after clearance of infection, followed by an active contraction of the NK cells, much in line with the dynamics of an effector T cell response (84). Nevertheless, after an outbreak of Puumala hantavirus infection in the human population, we were given the opportunity to study the development of the NK cell response in these patients (189). We believe this to be an appropriate human model of acute viral infection for several reasons. First, symptom debut is brisk making it conceivable for early identification of infected patients (131). Second, many patients need to be hospitalized for 1-2 weeks after symptom debut, allowing for longitudinal sampling of these patients. Third, the virus has a tropism for endothelial cells (135). Thus, when investigated peripheral blood NK cells, we will be studying NK cells at the site of infection. We followed 16 patients from the presentation at the emergency unit with acute symptoms until up to 15 months following disease onset and long after viral clearance (**paper II**). When assessing dynamics in the NK cell compartment during the acute phase of infection, we could document a rapid expansion in $CD56^{dim}$ NK cell numbers occurring during the first week after symptom debut. This expansion was probably a result of ongoing proliferation since a large number of NK cells expressed the proliferation marker Ki67 (**paper II**). Time wise, this rapid expansion is in line with what has been reported from experimental models (83, 84). However, the expanded NK cells in the hantavirus-infected individuals persisted at elevated levels for up to, at least, 2 months after symptom debut. This observation is in contrast to the rapid contraction seen after murine CMV infection in mice. One of the mechanisms behind specific NK cell and T cell contraction is increased sensitivity to apoptosis. This can be visualized by the loss of Bcl-2 expression (84, 190). Indeed, expanding CD8 T cells in the hantavirus infected individuals showed evidence of Bcl-2 downregulation (**paper II**). On the other hand, expanding NK cells retained, and interestingly, seemed to upregulate Bcl-2. This might be part of the explanation behind the observed NK cell persistence. A facilitator for this could be the increased levels of IL-15 detected in the patients during the acute phase of their disease (**paper II**). IL-15 is important for NK cell homeostasis and has been shown, *in vitro,* to upregulate Bcl-2 (29, 191, 192). However, it is likely so that Bcl-2 upregulation only is one out of many changes occurring with respect to apoptosis sensitivity. Other factors, such as Bim, PUMA, Bcl-xL, FlipL, FlipS, and Bax need to be assessed in the future.

Activation of NK cells can be assessed in many ways. For instance, NK cells from patients with acute hantavirus infection expressed high levels of CD69 (data not shown). Another sign of activation is upregulation of activation NK cell receptors. Here, increased expression of NKG2D, 2B4, and, to a lesser extent, NKp30 was

detected in the hantavirus infected individuals during the acute phase of infection as compared to expression levels on NK cells from uninfected individuals (Figure 2). A similar pattern of activation receptor upregulation could be seen in patients with acute HCV infection (Figure 3). Analogous to this, it was recently reported by Amadei and colleagues that NK cell activation during acute HCV infection encompasses the upregulation of NKG2D (128). Except for upregulation of activation receptors, NK cells from patients with acute hantavirus infection also express higher levels of perforin and granzymes as compared to uninfected individuals (data not shown). It is interesting that NK cells from patients with acute hantavirus and HCV infection share a common activation phenotype with NKG2D and 2B4 upregulation but with only diminutive changes in NCR expression. This could suggest that similar inflammatory conditions exist in the host during these two acute infections.

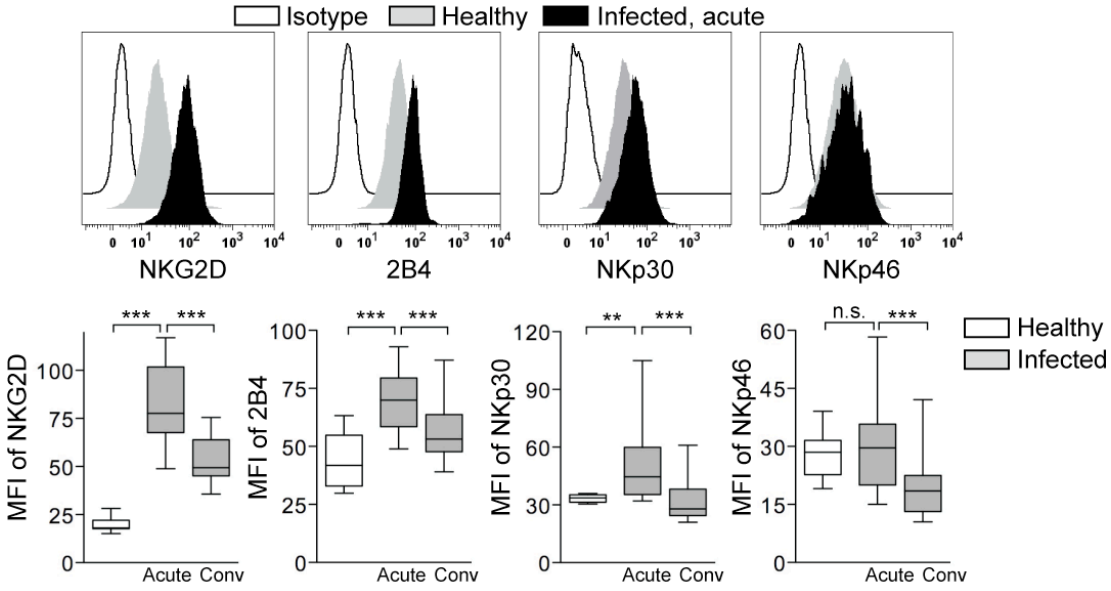

Figure 2. Expression of activation NK cell receptors during acute hantavirus infection (Björkström and Braun et al., manuscript in preparation). Acute represent analysis performed on samples acquired 3-5 days after symptom debut and convalescent represent samples acquired on average 60 days after symptom debut.

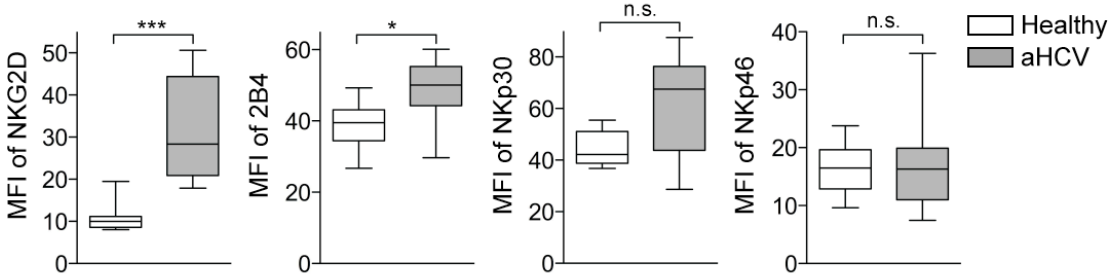

Figure 3. Expression of activation NK cell receptors during acute HCV infection (Stegmann and Björkström et al., manuscript in preparation). Patients with acute HCV were recruited according to the criteria of the HEP-NET Acute-HCV-II Study (193).

Most humans carry a number of chronic viruses, such as viruses that belong to the herpesvirus family, polyoma, and, to a lesser extent, papilloma viruses (174). We often refer to these viruses as latent viruses and they do not cause significant disease in the healthy human. It is only in the compromised host that polyoma, papilloma and herpes viruses will yield severe problems. Indeed, the seroprevalence of some of these viruses is so high within the population that it would even be hard to identify 'normal' uninfected individuals to study the impact these viruses have on the immune system. What this tells us, is that what we in most situations are studying, and referring to as negative controls / uninfected controls, instead are individuals that constantly are carrying any given number of these chronic viruses. Also, since many of them only cause disease when the immune system is weak, it is likely that the immune system constantly engage these pathogens on the micro level. Is it so that some of the phenotypic changes we find in the immune system of adults and elderly as compared to infants, is a consequence of a constant pressure from these latent viruses? One example might be the absence of CD57 expression on both T cells and NK cells in the developing fetus or in the cord blood, compared to the varying, but often high, expression found in healthy adults (**paper I,** M.A. Ivarsson, personal communication). In line with this, the T cell compartment in most infants is entirely made up of naïve T cells, whereas these rapidly decline in frequency and number with time, and aging (194, 195).

When studying the development of the NK cell response in patients with hantavirus infection, we made the striking observation that a subset of NKG2C+ $CD56^{dim}$ NK cells specifically expanded and persisted (**paper II**). Previous work has associated expression of NKG2C with CMV seropositivity at steady state in healthy individuals (110). CMV seronegative individuals have close to undetectable levels of NKG2C, whereas CMV seropositive individuals have NKG2C expressed at varying frequencies (110, 196). Furthermore, *in vitro* culturing of NK cells from CMV+ individuals together with CMV-infected fibroblasts led to the specific expansion of NKG2C+ NK cells in a CD94 and IL-15-dependent manner (197). In line with this, our work showed that Hantaan hantavirus (HTNV) infected human umbilical vein endothelial cells (HUVECs) strongly upregulated HLA-E, hantavirus infected patients had increased levels of plasma IL-15, and NKG2C+ NK cells specifically proliferated upon combined stimulation with IL-15 and HLA-E (**paper II**). Interestingly, this expansion of NKG2C+ NK cells was only observed in CMV seropositive hantavirus-infected patients. This would suggest that an individual's previous infectious disease history, in this case CMV, might have an impact on how NK cell responses develop during subsequent acute viral infections. However, the exact mechanism behind this, and whether CMV have the capacity to specifically prime NKG2C+ NK cells for efficient expansion, still remain unknown.

Increased levels of NKG2C+ NK cells have not only been reported in relation to CMV. Studies investigating patients with HIV (198), HBV (199), and HCV (200) infection have all reported on the increased frequency of NKG2C+ NK cells. Although it is plausible that these changes are dependent on CMV serostatus as a confounding factor (196), a detailed investigation of the NKG2C+ NK cell subset in these infections

might provide clues as to the mechanism underlying NKG2C+ NK cell expansion. Indeed, recent work shows that HBV or HCV patients with high levels of NKG2C all are CMV seropositive (Dr. Vivien Beziat, personal communication). More interestingly, perhaps, is the phenotype of the expanded NKG2C+ subset in these patients. The NKG2C+ NK cell subset in the HBV and HCV-infected patients had a clonal expression pattern of inhibitory KIRs specific for self-HLA class I molecules (Dr. Vivien Beziat, personal communication). This is in line with the profile of the NKG2C+ NK cells from patients with acute hantavirus infection (**paper II**). Here, a single inhibitory 'licensing' KIR dominated in the NKG2C+ NK cells, whereas the NKG2C- NK cells in the same patients displayed a variegated distribution of KIR expression (**paper II**). Whether this unique KIR-profile merely has the commission to ensure tolerance or if NKG2C+ NK cell expansion is dependent on a licensing-signal remains unknown. However, it is tempting to speculate that the presence of an inhibitory KIR might provide this subset with a survival benefit allowing for the specific expansion upon acute viral infections.

Lopez-Botet and colleagues reported on the association between CMV serostatus and NKG2C-expression, whereas EBV and HSV-1 had no impact on NKG2C expression (110). In the current thesis, we had the opportunity to evaluate NK cell differentiation in relation to HSV-2 infection (Figure 4). We studied two age- and sex-matched cohorts of HSV-2 seropositive patients: asymptomatic patients identified during a screening-study that after thorough interviewing had no recollection of experiencing HSV-2-like symptoms, and patients classified as symptomatic having six or more annual relapses with symptoms of HSV-2 infection. Interestingly, disseminating HSV-2 infections have previously been linked to transitory NK cell deficiencies (201). Additionally, NK cells have, in an experimental model, been suggested to protect against HSV-2 in an IL-15-dependent manner (202). However, in humans, symptomatic HSV-2 infection appears to have no effect on peripheral blood NK cell differentiation (Figure 4). In more detail, asymptomatic individuals and symptomatic patients had similar levels of KIR negative, NKG2A+, and CD57+ $CD56^{dim}$ NK cells in peripheral blood. Furthermore, symptomatic HSV-2 infection did not yield increasing numbers of NKG2C+ NK cells. However, the patients did express NKG2C, suggesting them to be CMV seropositive. Although the hypothesis would be for NKG2C+ NK cells to expand as a consequence of the recurrent HSV-2 infections combined with an underlying CMV seropositivity, it might be so that this is only a local effect. Thus, if NK cells had been investigated in the mucosa of these patients, an increase in NKG2C might have been observed. Support for this comes from studies of patients with celiac disease (203). Intraepithelial CD8 T cells in the gut lumen of patients with active disease express high levels of NKG2C whereas the corresponding T cells in healthy controls or patients on a gluten-free diet expressed low levels of NKG2C (203). Interestingly, this increase in NKG2C-expression was only evident at the site of inflammation, whereas when peripheral blood CD8 T cells were investigated, patients with active disease as well as health individuals had low to undetectable levels of NKG2C (203).

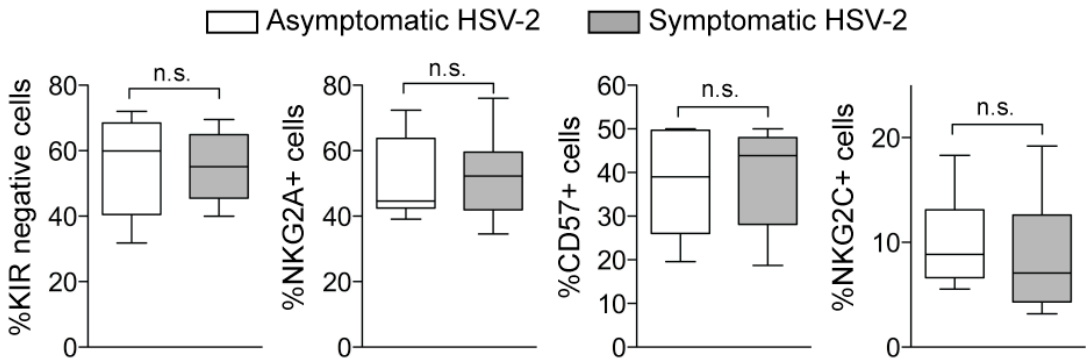

Figure 4. Assessing NK cell differentiation during HSV-2 infection (Björkström et al., manuscript in preparation).

CD56^{BRIGHT} 'KILLERS' EMERGE IN RESPONSE TO IFN-α TREATMENT OF HCV

As reviewed above, mature NK cells are typically divided into CD56^{bright} and CD56^{dim} subsets (14, 16, 17). Historically, this division was based on both phenotypic and functional differences between these two subsets. Also, tissue localization differs. CD56^{bright} NK cells are abundant in SLT and tonsils (>90% of all NK cells), in the liver it is an equal distribution, whereas CD56^{dim} NK cells dominate in blood, spleen, and bone marrow (17, 20) (Figure 5). From a functional point-of-view, CD56^{bright} NK cells have been considered prominent cytokine producers but inferior in target cell killing. Whereas the opposite profile has been shown for CD56^{dim} NK cells. However, recent work has challenged this rigid classification into cytokine-producers and killers. It rather seems that the function of these cells are dependent on the type of stimulation they receive. Upon engagement of activation receptors, CD56^{dim} NK cells efficiently produce large quantities of cytokines and chemokines whereas CD56^{bright} NK cells show a low level of responsiveness (21). However, when stimulated with cytokines, such as IL-12, -15, and -18, CD56^{bright} NK cells rapidly responded to a higher extent compared to CD56^{dim} NK cells (21). NK cell killing can be the result either from a directed exocytosis of cytotoxic molecules or from the induction, expression, and sometimes release, of apoptosis-induced molecules such as TRAIL and FasL (27, 204). CD56^{dim} NK cells have preformed vesicles containing perforin and granzymes whereas CD56^{bright} NK cells, at most, express low levels of these molecules (27). On the other hand, the capacity of CD56^{bright} and CD56^{dim} NK cells to induce killing through death receptors have not been thoroughly investigated.

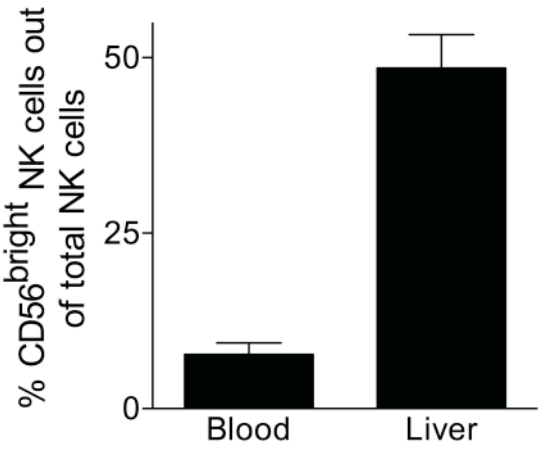

Figure 5. Levels of $CD56^{bright}$ NK cells out of total NK cells in human blood and liver (Björkström et al., manuscript in preparation).

In **paper III**, we studied the role of TRAIL on NK cells in the context of HCV infection. HCV can, in some cases, be efficiently treated with the combination of IFN-α and ribavirin. IFN-α has many immune modulatory effects. Interesting in this context are reports that IFN-α specifically can induce TRAIL on human T cells, whereas IFN-γ, IL-2, -12, -15, and -18 did not have this effect (205). Furthermore, on murine NK cells, the *TRAIL* gene promoter contains an IFN-stimulated response element that the IFN-stimulated gene (ISG) ISGF3 can bind to (206). Indeed, corroborating these results using human NK cells, after performing gene expression profiling studies, we could show that TRAIL was among the most upregulated genes on NK cells in response to IFN-α (**paper III**). We also found that TRAIL was, to a higher degree, induced on $CD56^{bright}$ NK cells both *in vitro* and *in vivo* after IFN-α stimulation as compared to $CD56^{dim}$ NK cells. Furthermore, in experiments using FACS sorted $CD56^{bright}$ and $CD56^{dim}$ NK cells, on a single cell level, $CD56^{bright}$ NK cells were as efficient as $CD56^{dim}$ NK cells in inducing killing of TRAIL-sensitive Huh7.5 cells (**paper III**). With these findings, we propose that $CD56^{bright}$ NK cells, expect for efficiently responding with cytokine production upon cytokine stimulation, also are prominent killers via TRAIL. Whether these results also extend to FasL-induced apoptosis remains to be investigated.

Given the abundance of $CD56^{bright}$ NK cells in the liver (Figure 5), what are the implications of death receptor-induced apoptosis in the context of liver disease, and in particular during HCV-infection? Strikingly, in an experiment performed 1993 by Ogasawara and colleagues, where healthy mice were given an anti-Fas antibody, they all rapidly succumbed due to massive liver apoptosis (207). These results and many subsequent studies have concluded that hepatocytes have an increased sensitivity to death receptor-induced apoptosis (208, 209). It is further believed that persistent hepatocyte apoptosis play a role in fibrogenesis, chronic liver failure, and perhaps even development of hepatocellular carcinoma (HCC) (209). Whereas normal hepatocytes are sensitive to FasL, they seem protected against TRAIL-induced apoptosis (210).

Interestingly, and in contrary to this, hepatocytes from HCV-infected patients have upregulated TRAIL-receptor expression and are highly sensitive to TRAIL-induced apoptosis (210). Also hepatocytes from patients with HBV-infection seem to have induced expression of TRAIL-receptors (105). In the current study, we employed a cell culture system for HCV (HCVcc) based on the TRAIL-receptor expressing Huh7.5 cells to evaluate if NK cells could inhibit HCV-replication via TRAIL (**paper IV**) (211). Indeed, IFN-α stimulated NK cells efficiently induced apoptosis of HCV-infected Huh7.5 cells and consequently reduced viral load in this system (**paper III**). A limitation of these studies is the usage of peripheral blood $CD56^{bright}$ NK cells as compared to intrahepatic $CD56^{bright}$ NK cells. In this context, it has previously been shown that mouse intrahepatic NK cells constitutively express TRAIL and that these cells, in a TRAIL-dependent manner, can target and eliminate liver metastasis (212, 213). However, TRAIL on NK cells have mostly been studied in an anti-tumor context (204, 212, 213). Thus, more thorough investigations on the role of NK cells expressing TRAIL during viral challenge are mandated in the future.

NK CELL – TARGET CELL INTERACTIONS DURING ACUTE AND CHRONIC VIRAL INFECTION

NK cells can exert both target-cell contact and target-cell contact-independent effector functions (26, 27). For instance, IFNγ can be produced after NK cell cytokine-stimulation alone or after NK cell encounter with a susceptible target cell. NK cells can, upon stimulation and activation, express, but also secrete, FasL and TRAIL, both of which have the capacity to induce apoptosis of target cells (214). Furthermore, upon target-cell contact, NK cells can in a directed fashion release granules containing perforin and different granules (27). In this thesis, these effector functions have been analyzed in different *in vitro* systems, both in disease-related context, but also in order to try to answer more basic questions of NK cell biology (**papers I-IV**). In many cases, the ambition has been to try to dissect the NK cell – target cell interaction to reveal the molecular basis for NK cell recognition.

In **paper I**, we assessed changes in activation receptors during NK cell differentiation (Figure 1). We could show that differentiation, determined by acquisition of CD57, was associated with a substantial reduction of surface expressed NKp30 and NKp46, whereas expression of NKG2D and CD16 only exhibited minor changes (**paper I**) (180). On the other hand, more differentiated, CD57+ $CD56^{dim}$ NK cells, express higher levels of DNAM-1 compared to less differentiated CD57- $CD56^{dim}$ NK cells (Dr. K.J. Malmberg, personal communication). These differentiation-associated phenotypic alterations did not result in any functional changes when measuring capacity to degranulate against K562 cells (**paper I**) (180, 183). However, this might be due to the complex nature of the target cell, being an 'optimized' NK cell target by expressing high levels of ligands for many distinct NK cell receptors. Instead, differentiated NK cells were more efficient in performing

cytotoxicity after CD16 engagement, and specific engagement of DNAM-1 and 2B4 in a co-activation receptor dependent manner (36) yielded higher levels of degranulation (180) (Dr. K.J. Malmberg, personal communication). The increased cytotoxicity might be due to the higher levels of perforin and granzymes expressed by differentiated NK cells (180, 183). The other side of this coin is obviously the reduced responsiveness to cytokine stimulation exhibited by differentiated NK cells (**paper I**) (180, 183, 184). Based on these functional and phenotypic differences present at steady state in the healthy human, and given that the level of NK cell differentiation varies significantly in-between individuals (**paper I**), these parameters will likely be important to take into consideration in future studies assessing NK cell function in different systems of disease. For instance, one could speculate that a stronger response to cytokines (having less differentiated NK cells), such as IL-12 and IL-18, might be advantageous during certain viral infections, whereas a greater capacity to perform cytotoxicity (having more differentiated NK cells) might be relevant for tumor immune surveillance at steady state.

During viral challenge, it is plausible to assume that the infection will affect the NK cell – target cell interaction on a number of levels. First, the inherent antiviral response will likely yield production of cytokines such as IFN-α, IL-12, and IL-18 (69). These will have the capacity to stimulate NK cells with changes both affecting transcription and surface expression of activation and inhibitory receptors, but probably also in how intracellular signaling pathways convey their messages. For instance, resting NK cells need triggering through at least two activation receptors to respond with degranulation whereas cytokine-stimulated NK cells can respond through a single receptor (36, 37, 66). Furthermore, in a chronic setting of disease, where by definition the immune system fails to clear the virus, a dysregulation of immune cell function might occur (see next section for a more thorough discussion on this). Finally, the virus will probably also affect the immunogenicity of infected cells. One can envision that the infected cell will, through different danger signals, try to alert the immune system of the ongoing infection, whereas the virus will strive towards counteracting these effects. One example relevant for NK cells is upregulation of stress-induced ligands for activation NK cell receptors, such as the ligands MICA and MICB that are recognized by the activation receptor NKG2D (42, 215).

In **papers II** and **IV**, the function of NK cells from virus-infected patients has been investigated in different *in vitro* systems of viral infection. With respect to activation of NK cells during these two acute infections, and as discussed above, both acute hantavirus and acute HCV infection results in an upregulation of activation receptors such as NKG2D and 2B4 on NK cells (Figures 2 and 3). However, whether these changes are redundant or not in these two infections are not clear. For hantavirus infection, we established an *in vitro* infection system where primary HUVECs were used and infected with the prototypic hantavirus, Hantaan hantavirus (HTNV) (**paper II**). Human primary kidney epithelial cells and human primary lung endothelial cells were used in parallel to the HUVECs and the results obtained were identical in-between the three cell types (unpublished observations). Importantly, endothelial cells are major targets for hantaviruses during infection in humans (135). When assessing

changes in expression of NKR ligands on infected endothelial cells, no induction of classical stress-induced ligands for NKG2D could be observed (**paper II**). One explanation for the absence of changes might be the weak IFN-response believed to be induced by hantaviruses during infection (216, 217). It has been shown that endothelial cells pretreated with IFNs induce ISGs (216, 217). However, once infection has been established, the virus seems to inhibit the IFN-response (217). Instead, infection resulted in a robust upregulation of both classical and non-classical MHC class I molecules (**paper II**) as well as of trans-presented surface IL-15 (Dr. M. Braun, personal communication). The consequences of this with respect to NK cell expansion, differentiation, and survival have already been discussed above. However, as hypothesized, with an infected target cell expressing higher levels of HLA class I but with no change in expression of ligands for activation receptors, hantavirus-infected endothelial cells were protected against NK cell killing (Figure 6). Furthermore, also NK cell IFN-γ and TNF-α production was efficiently inhibited against the virus-infected target cells (unpublished observations).

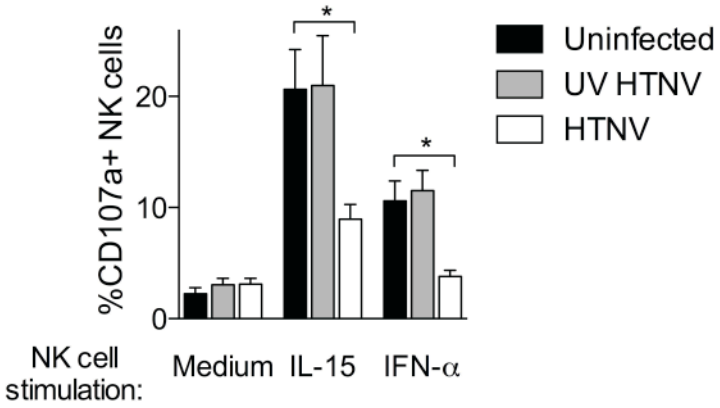

Figure 6. NK cell degranulation is inhibited by hantavirus infected endothelial cells (Braun and Björkström et al., manuscript in preparation).

It is important to consider that these changes are obtained from an *in vitro* infection-system. If possible, they need to be further validated *ex vivo* during acute infection. Except for these effect, it seems that hantaviruses employ numerous other mechanisms to avoid the immune system, for instance, both granzyme- and caspase-activity are inhibited by the virus and infected cells downregulate expression of TRAIL receptors (unpublished observations and Dr. J. Klingström, personal communication). In summary, during acute hantavirus infection, NK cells become highly activated (upregulation of activation receptors and effector molecules, high levels of CD69), yet they are not allowed to react against infected target cells. Instead, it seems that the infected target cells, through high expression of MHC class I, HLA-E, trans-presented IL-15, and ICAM-1, convey further activation, and perhaps also survival signals, to the NK cells. What is then the fate for these activated NK cells? Could this be the mechanism explaining NK cell persistence during acute hantavirus infection? Interestingly, despite the virus being non-cytopathic to infected cells, the clinical symptoms during HFRS stem from capillary leakage secondary to endothelial cell

damage (135, 218). Thus, it is tempting to speculate that activated NK cells might contribute to bystander killing of non-infected endothelial cells, thereby being harmful to the host by causing immune pathology.

As for HCV, it was more challenging to perform *in vitro* studies on the interaction between NK cells and HCV-infected target cells. Although it has recently been shown that HCV can infect primary hepatocytes in a microscale tissue culture system (219), we did not have access to this elaborate system. Instead, we decided to evaluate the HCVcc as target for NK cells (211, 220, 221). It was for many years cumbersome to establish an *in vitro* model for HCV. This, probably because of the narrow cellular tropism and consequently the high demands HCV has on entry factors (222, 223). The HCVcc is based on an HCV-strain cloned from a patient that presented with fulminant hepatitis (224) and the hepatoma cell line Huh7.5. Huh7.5 cells are a variant of the original Huh7 cells and are, with a defect in RIG-I signaling, and consequently in the IFN-response, more permissive to HCV infection (225, 226). In **paper IV** we investigate the expression of NK cell receptor ligands on HCV-infected and uninfected Huh7.5 cells. Surprisingly, we could not detect any major differences in expression of these ligands upon infection (**paper IV**). One explanation for this finding might be defect IFN-signaling in the Huh7.5 cells (225, 226). Probably as a direct result of the unchanged NK receptor ligand profile, NK cells from patients with acute HCV, which needed to be short-term stimulated with IFN-α in order to recognize Huh7.5 cells, did not display enhanced functions against HCV-infected Huh7.5 cells. On the other hand, nor did HCV manage to reduce NK cell functionality in this system. In the context of HCV-mediated NK-cell inhibition, soluble HCV E2 protein binding to CD81 has previously been shown to inhibit NK cell function (227, 228). A recent report using hepatitis C virions, instead of soluble protein, challenged these findings by reporting intact NK cell function upon exposure to HCV (229). Our results further substantiate this report, that HCV, by itself, does not inhibit NK cell function. Nevertheless, we could identify the activation receptor DNAM-1 as the critical receptor for NK cell recognition in this system (**paper IV**). At the moment, it is not clear if this has any relevance for the detection of HCV, or if this merely is the activation receptor needed for recognition of the tumor cell line Huh7.5. Rather, it is probably so that the HCVcc is not a suitable *in vitro* model for studying NK cell recognition of HCV. Nevertheless, in this system, NK cells readily inhibited HCV replication in a DNAM-1-dependent manner (**paper IV**). Thus, not only TRAIL, as discussed in the previous section (**paper III**), but also degranulation, IFN-γ, or TNF-α produced by NK cells have the capacity to inhibit HCV replication.

There is a substantial literature on the specific recognition by NK cells of viral infected target cells. For instance, Ly49H recognizes the MCMV protein m157 (108) and NKp46 seem to bind to haemagglutinin of Influenza virus (230). Furthermore, NK cells have the capacity to sense MHC class I downregulation as well as the induction of ligands to activation receptors on target cells. In the current thesis, two *in vitro* models have been evaluated, both of which brought along somewhat unexpected findings. Human hantavirus efficiently inhibited NK cell effector functions, but also promoted NK cell activation, and perhaps NK cell survival. On the other hand, in the HCVcc,

HCV itself had no effect on NK cell recognition. It is important to remember that both of these models are simplified versions of the *in vivo* situation. However, with the basic understand of how these reductionist systems work, and in the absence of small animal models, this work should open up for future studies of more complex models, such as microscale tissue culture systems using primary hepatocytes for HCV infection, and perhaps 3D-engineered tissue models (219, 231).

DYSREGULATION OF NK CELLS DURING CHRONIC VIRAL DISEASE

Except for chronic viruses that the immune system manages to control and suppress into latency, there are also a number of chronic viral infections that more rapidly cause disease and where the viruses exhibit a negative impact on the function of the immune system (174). Lately, much effort has been spent studying how these viruses affect the T cell compartment. From these studies, it is clear that a hallmark of chronic, disease causing, viral infections such as HIV and HCV in humans, and LCMV in the mouse, is extensive T cell exhaustion (232). This exhaustion reveals itself as a gradual loss of T cell function, probably in part through active suppression from the viruses themselves, but also because of more global effects in signaling and metabolism of the affected cells (173, 174). With respect to exhaustion of NK cells, much less is known. An initial paper published some 15 years ago reported on the loss of cytolytic $CD56^{dim}CD16+$ NK cells during chronic HIV infection on the expense of a CD56-CD16+ NK cell population (hereafter referred to as $CD56^{neg}$ NK cells) (233). Since then, these $CD56^{neg}$ NK cells have been thoroughly characterized during acute and chronic HIV (234). Observations from this field stimulated us to ask the question whether these cells also expand during chronic HCV infection. An initial finding was that patients co-infected with HIV and HCV had an increased frequency of $CD56^{neg}$ NK cells but that combinatorial IFN-α + ribavirin therapy reverted this subset of NK cells back to levels found in healthy individuals (235). Indeed, also when assessing a cohort of 32 untreated HCV-patients, we could detect an increased frequency of $CD56^{neg}$ NK cells as compared to uninfected controls (**paper VI**). Furthermore, these cells had a restricted functional profile upon target cell or cytokine stimulation as compared to $CD56^{dim}$ or $CD56^{bright}$ NK cells. We assessed for MIP-1β, IFN-γ, TNF-α, and CD107a upregulation, and the $CD56^{neg}$ NK cells only managed to produce MIP-1β upon stimulation (**paper VI**). When examining $CD56^{dim}$ NK cells from healthy individuals, the threshold for activation has been reported to be the lowest for MIP-1β compared to other functional parameters such as IFN-γ, TNF-α and CD107a upregulation (21). Thus, one explanation for the restricted functionality of $CD56^{neg}$ NK cells might be that the threshold the cells need to overcome before responding is elevated. Another plausible hypothesis would be an inherent loss of capacities to degranulate and produce cytokines. This would be more in line with the gradual loss of functions that T cells undergo when they differentiate towards exhaustion (174, 232).

The mechanism behind the appearance of $CD56^{neg}$ NK cells is not fully understood. However, by studying the phenotype of these cells, we would at least get an idea as to from where they might originate. Interestingly, during chronic HCV, $CD56^{neg}$ NK cells share many phenotypic features with the corresponding $CD56^{dim}$ NK cells in these patients (**paper VI**). A major difference is the very low expression of CD57, which would argue against the idea that $CD56^{neg}$ NK cells represents a population that have emerged from terminally differentiated CD57+$CD56^{dim}$ NK cells (**paper I**). This is further emphasized from both *in vitro* and *in vivo* studies of the dynamics of CD57 expression (**paper I**). Acquisition of CD57 seems to be an irreversible step during $CD56^{dim}$ NK cell differentiation (**paper I**). Thus, a low expression of CD57 together with a retained capacity to proliferate (236), would instead suggest the $CD56^{neg}$ NK cells to have emerged from less differentiated $CD56^{dim}$ NK cells. One of the phenotypic and functional differences between less and more differentiated $CD56^{dim}$ NK cells is their cytokine receptor expression as well as their capacity to functionally respond upon cytokine stimulation (**paper I**) (183). Indeed, less differentiated CD57-NKG2A+$CD56^{dim}$ NK cells are much more potent in responding to cytokines (**paper I**). Given this, and given the phenotype of the $CD56^{neg}$ NK cells being similar to less differentiated $CD56^{dim}$ NK cells, it is tempting to speculate that dysregulation of the NK cell compartment during chronic HCV is cytokine-driven. Nevertheless, many outstanding questions remain on the biology of $CD56^{neg}$ NK cells (234).

Chronic inflammation and immune activation, can, besides exhausting the immune system also have other effects. The chronic inflammatory milieu in patients with autoimmune disorders such as rheumatoid arthritis and celiac disease seem to drive a reprogramming of T cells with the acquisition of more innate-like features. For instance, CD4+CD28null CD4 T cells, expanded in CMV seropositive patients with rheumatoid arthritis (237), express high levels of many activation NK cell receptors (238). A similar phenotype has been reported for intraepithelial CD8 T cells in patients with active celiac disease where IL-15 has been suggested to be the driving cytokine (203, 239, 240). In these patients, CD8 T cells can respond with killing through the activation receptor NKG2D, independently of their TCR (239, 240). Except for sharing characteristics with NK cells, these 'innate' CD4 and CD8 T cells, present during chronic inflammation, also share features with innate CD1d-restricted NKT cells. Indeed, CD1d-restricted NKT cells were recently shown to express high levels of activation NK cell receptors that they could utilize in a TCR-independent manner to perform functions (241).

In line with the thinking that chronic inflammation and activation not only induces T cell exhaustion, but also reprogram T cells towards more innate-like cells, we evaluated whether any traits of such processes were present during chronic HCV infection. Interestingly, we found that up to 10% of all CD8 T cells in blood and in the liver express CD16 during chronic HCV infection (**paper V**). CD16+ CD8 T cells had a restricted TCR profile and were terminally differentiated. Interestingly, and similar to the function of other activation NK cell receptors on T cells in settings of chronic inflammation (238, 239), CD16+ CD8 T cells responded with ADCC, independently of

the TCR (**paper V**) (242, 243). Furthermore, the level of CD16+ CD8 T cells in these patients correlated with T cell activation and could be induced on CD16- CD8 T cells *in vitro* after cytokine stimulation.

An obvious question to ask is whether CD16 expression on CD8 T cells is beneficial or not in chronic HCV. It was a substantial inter-individual spread in the levels of CD16+ CD8 T cells present during chronic HCV infection indicating that some patients were more prone to acquire CD16+ CD8 T cells compared to others (**paper V**). Except for being increased in patients with chronic HCV infection, a recent report documented a high frequency of CD16+ CD8 T cells in patients with ongoing immune activation, determined as a hyperlymphocytosis (243). This could indicate that the level of CD16+ CD8 T cells in HCV patients could be an indicative of the capacity of the CD8 T cell compartment to respond to certain stimuli with immune activation. Thus, high levels of CD16+ CD8 T cells might identify HCV-infected patients that still have an intact unaffected CD8 T cell compartment capable of responding with activation. This may serve as an opposing biomarker compared to biomarkers that quantify the level of CD8 T cell exhaustion. However, this is speculative and needs to be assessed in future studies. Nevertheless, it is also tempting to envision that high levels of $CD56^{neg}$ NK cells, on the other hand, is reflective of a disturbance of the innate cellular immune system. Indeed, elevated levels of $CD56^{neg}$ NK cells in peripheral blood correlated with HCV treatment failure (**paper VI**). However, this was a retrospective analysis performed in a fairly small cohort of patients (n=42). To in detail validate the significance of these two findings as potential prognostic biomarkers of HCV-treatment response (elevated levels of $CD56^{neg}$ NK cells and CD16+ CD8 T cells), it will be important to perform larger prospective studies, were additional known risk factors such as IL-28B polymorphisms (244), KIR/HLA status (124), and viral genotype, are taken into account.

CONCLUDING REMARKS

This thesis provides data that argue for the existence of a differentiation process that mature human $CD56^{dim}$ NK cells undergo during their lifespan. Furthermore, results presented in this thesis explore how mature NK cell differentiation and activation is modulated during acute and chronic human viral infections and how this might impact NK cell-mediated recognition of virus-infected target cells. Below, I have listed the major conclusions from the present work.

- Expression patterns of NKG2A, KIR, and CD57 define a continuous process of $CD56^{dim}$ NK-cell differentiation characterized by loss of NKG2A, and sequential acquisition of KIRs and CD57 (**paper I**).
- $CD56^{dim}$ NK cell differentiation, and the associated functional imprint, is uncoupled from NK-cell education (**paper I**).
- NK cell activation in response to acute hantavirus infection encompasses rapid expansion and long-term persistence of highly differentiated NKG2C+CD57+ $CD56^{dim}$ NK cells (**paper II**).
- Hantavirus-responding NKG2C+CD57+ $CD56^{dim}$ NK cells uniformly express one single HLA-C-specific inhibitory KIR (**paper II**).
- Immune modulatory IFN-α treatment during HCV-infection promotes the appearance of activated $CD56^{bright}$ 'killer' cells (**paper III**).
- $CD56^{dim}$ and $CD56^{bright}$ NK cells can inhibit HCV-replication in Huh7.5 cells through TRAIL or via DNAM-1-medaited effector functions (**papers III and IV**).
- Chronic HCV-infection stimulates terminal effector CD8 T cells to acquire NK cell-like functional properties, including expression of CD16 (**paper V**).
- NK cell differentiation is skewed towards expansion of functionally altered $CD56^{neg}$ NK cells during chronic HCV-infection (**paper VI**).

Taken together, data in this thesis shed new light on basic processes that regulate the formation of diverse and functional NK cell repertoires in the healthy human. Furthermore, the results explore NK cell responses during viral infections and how this might affect NK-cell anti-viral functions. In summary, a framework, NK cell activation and differentiation in health and during viral infection, is provided and evaluated. The implementation of this framework in future studies on human NK cell biology in health and disease might yield significant new findings. With respect to the "bigger picture", the detailed understanding of how the immune system develops, differentiates, and counteracts pathogens is an important prerequisite for understanding pathogenesis, and for the design of new treatment and intervention protocols to achieve efficient pathogen eradication.

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