From the Department of Medicine
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THE ONTOGENY AND REGULATION OF HUMAN NATURAL KILLER CELLS

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To my family

There is grandeur in this view of life,
with its several powers, having been originally breathed into a few forms or into one;
and that, whilst this planet has gone cycling on according to the fixed law of gravity,
from so simple a beginning endless forms most beautiful and most wonderful
have been, and are being, evolved.

Charles Darwin
On The Origin of Species, 1st edition
1859

ABSTRACT

Natural Killer (NK) cells are members of the innate lymphoid cell (ILC) family and take part in the detection and eradication of virus-infected and transformed cells. In this thesis, together with my colleagues I have investigated how NK cells and other ILCs develop and function during human fetal development, how NK cells are functionally regulated (educated) via the activating receptor KIR2DS1, and how NK cells in our body are affected during the early phase of an acute viral infection.

Little is known about the ontogeny of NK cells and other ILCs in fetal development. The characterization of ILCs has been hampered by their overlapping surface phenotypes. In contrast, ILC transcription factor expression is more specific, and by combining multicolor flow cytometry analysis of transcription factors and surface markers expressed by different fetal ILC subsets, we were able to study and model their development and differentiation. All ILC subsets were detected as early as gestational week six, and their distribution varied depending on both tissue and gestational age. Moreover, putative precursors of NK cells were identified as cells that sequentially lost CD34 and acquired CD122, Eomes, CD94/NKG2A, T-bet, and CD16. In addition putative $CD34^+$ progenitors of $ROR\gamma t^+$ ILCs were identified.

In the second trimester of fetal development, analysis of mature fetal NK cell subsets revealed that stage 4 and stage 5 NK cells differed in frequency in fetal organs, and the highest NK cell frequency was found in the fetal liver and lung. The vast majority of fetal NK cells were $NKG2A^+$, and fetal lung NK cells also frequently expressed killer-cell immunoglobulin-like receptors (KIR). Interestingly, while NKG2A educated fetal NK cells similar to adult NK cells, KIR expression on fetal NK cells was linked to hyporesponsiveness, thus contrasting education of NK cells after birth. Nevertheless, fetal NK cells were highly responsive to cytokines, as well as to antibody-coated target cells, suggesting they may take part in fetal immune responses against *in utero* infections, while remaining tolerant to maternal cells crossing the placenta.

While it is established that NK cells in adults are educated via inhibitory KIRs, it is not known how activating KIRs such as KIR2DS1 affects NK cells. By combining antibodies against four inhibitory KIRs, NKG2A, and KIR2DS1, we were able to interrogate the regulation of NK cells by KIR2DS1. We found that KIR2DS1 single-positive NK cells exist *in vivo*, and that the presence of the ligand for KIR2DS1, HLA-C2, resulted in hyporesponsiveness of such NK cells, thus ensuring self-tolerance. Our findings represent the first identification of NK cell education via activating KIR.

The human NK cell response to viral infections is not well understood. To this end we employed the live attenuated yellow fever vaccine 17D as an *in vivo* model of an acute viral infection. Our results show that the vaccine primed NK cells, and that less differentiated $CD57^-$ NK cells dominated the response, which peaked at day 6-10 post vaccination. Moreover, KIR expression on NK cells did not affect their response to the vaccine, indicating that NK cells expressing self-KIR and non-self KIR contributed equally to the NK cell response to the vaccination.

LIST OF PUBLICATIONS

This thesis is based on the following publications and manuscripts, which are referred to in the text by their Roman numerals:

I. High-resolution mapping of transcription factor expression reveals distinct developmental pathways of human fetal innate lymphoid cells.
Martin A. Ivarsson, Nicole Marquardt, Liyen Loh, Jeff E. Mold, Magnus Westgren, Erik Sundström, Elisabet Åkesson, Åke Seiger, Jenny Mjösberg, Niklas K. Björkström, Douglas F. Nixon, and Jakob Michaëlsson
Manuscript

II. Differentiation and functional regulation of human fetal NK cells.
Martin A. Ivarsson, Liyen Loh, Nicole Marquardt, Eliisa Kekäläinen, Lena Berglin, Niklas K. Björkström, Magnus Westgren, Douglas F. Nixon, and Jakob Michaëlsson
Journal of Clinical Investigation 2013;123(9):3889–3901

III. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors.
Cyril Fauriat, **Martin A. Ivarsson**, Hans-Gustaf Ljunggren, Karl-Johan Malmberg, and Jakob Michaëlsson
Blood 2010 115: 1166-1174

IV. The human NK cell response to yellow fever virus is primarily governed by NK cell differentiation independently of NK cell education.
Nicole Marquardt*, **Martin A. Ivarsson***, Kim Blom, Veronica D. Gonzalez, Monika Braun, Karolin Falconer, Rasmus Gustafsson, Anna Fogdell-Hahn, Johan K. Sandberg, and Jakob Michaëlsson
Manuscript

* Authors contributed equally

Paper II: © 2013 Journal of Clinical Investigation
Paper III: © 2010 The American Society of Hematology

LIST OF ADDITIONAL RELEVANT PUBLICATIONS

SI. Invariant natural killer T cells developing in the human fetus accumulate and mature in the small intestine
Liyen Loh, **Martin A. Ivarsson**, Jakob Michaëlsson, Johan K. Sandberg, and Douglas F Nixon
Mucosal Immunology 2014 *(Accepted for publication Feb 4th 2014)*

SII. Expression patterns of NKG2A, KIR, and CD57 define a process of $CD56^{dim}$ NK-cell differentiation uncoupled from NK-cell education.
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
Blood 2010 116: 3853-3864

SIII. Temporal dynamics of the primary human T cell response to yellow fever virus 17D as it matures from an effector- to a memory-type response.
Kim Blom, Monika Braun, **Martin A. Ivarsson**, Veronica D. Gonzalez, Karolin Falconer, Markus Moll, Hans-Gustaf Ljunggren, Jakob Michaëlsson, and Johan K. Sandberg
Journal of Immunology 2013; 190:2150-2158

SIV. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs.
Vivien Béziat, Lisa L. Liu, Jenny-Ann Malmberg, **Martin A. Ivarsson**, Ebba Sohlberg, Andreas T. Björklund, Christelle Retière, Eva Sverremark-Ekström, James Traherne, Per Ljungman, Marie Schaffer, David A. Price, John Trowsdale, Jakob Michaëlsson, Hans-Gustaf Ljunggren, and Karl-Johan Malmberg
Blood 2013 121: 2678-2688

SV. Activating killer immunoglobulin-like receptors in health and disease.
Martin A. Ivarsson, Jakob Michaëlsson, and Cyril Fauriat
Invited review. Submitted to Frontiers in Immunology

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
CMV	Cytomegalovirus
DC	Dendritic cell
Eomes	Eomesodermin
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
KIR	Killer cell immunoglobulin-like receptor
LTi	Lymphoid tissue inducer
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NK	Natural killer cell
ROR	Retinoic acid receptor-related orphan receptor
SPADE	Spanning-tree progression of density-normalized events
T-bet	Also known as “T-box transcription factor T-bet”
TGF	Tumor growth factor
TNF	Tumor necrosis factor
ULBP	UL-16 binding protein

1 INTRODUCTION

Since the dawn of life on our planet, evolution by natural selection has favored species that can stay ahead in the arms race for survival and reproduction (1). Smaller organisms have evolved ways to hide and survive inside larger plants and animals, such as humans. In turn, we and other organisms have evolved defense systems to protect us from invaders, and inside our bodies we find a sophisticated *immune system* that protects us not only against foreign invaders, but that also surveys our own cells and ensures damaged cells are removed (2). The immune system involves a multitude of cell types and interactions, and in my work I have studied the natural killer (NK) cell, and other members of the innate lymphoid cells (ILCs). NK cells are fast responding innate lymphocyte capable of detecting and killing virus-infected cells as well as tumor cells. In recent years it has also been appreciated that NK cells form a bridge between the innate and adaptive arms of the immune system (3).

1.1 FLOW CYTOMETRY

The phenotype and function of all cells in our body are defined by the receptors expressed on the cell surfaces, and what proteins and molecules are present and active inside the cells. To learn about cell function and behavior, scientists thus need to obtain information about these features, and in particular when studying NK cells, learning about the array of receptors determining their phenotype and regulating their function, is paramount.

Flow cytometry is a technique central to the work in this thesis, which allows immunologists to study immune cells in great detail. It was developed in the late 1960's and was inspired by instruments used at Los Alamos National Laboratories, to count and separate particles in nuclear fallout (4, 5). The principle of this technique is largely unchanged and briefly works by combining the coupling of a fluorochrome to an antibody specific for an antigen expressed by the cell of interest, e.g. the cell surface protein CD56 on NK cells. Single-cell suspensions of cells are mixed with such antibodies, which saturate and "stain" the cells. These are then analyzed with a flow cytometer, where cells are led through laser beams one at the time (**Figure 1**). The fluorochromes are excited when the cell passes a laser beam, and the amount of light that is subsequently emitted by the fluorochrome will be proportional to how much antibody is present, i.e. how much of the particular antigen is present on the surface (or inside) the cell. To extract this information, the emitted photons are converted into electrical signals using photomultiplier tubes, and the amount of antigen can then be visualized and analyzed with a computer (**Figure 1**). Thousands of cells can be analyzed per second, and the information from many antibodies coupled to fluorochromes with different excitation properties can be combined to provide high-dimensional information about each cell. By collecting information from many cells isolated from e.g. the peripheral blood, or from an organ, statistically representative can be obtained.

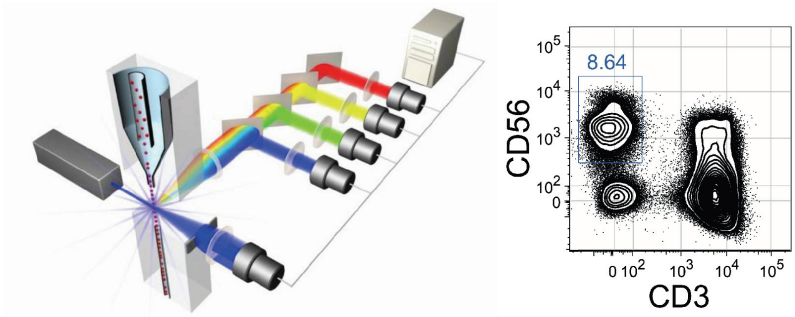

Figure 1. Schematic overview of a flow cytometer (left). Example of a two dimensional FACS-plot analysis of NK cells (inside electronic gate) separated from T cells using anti-CD56 (NK cells) and anti-CD3 (T-cells). Fluorescence intensity on a logarithmic scale is shown (right). (left: adapted from Tom Rawley's "Flow Cytometry – A survey and the Basics (http://dx.doi.org/10.13070/mm.en.2.125))

In my work I have made use of cocktails of antibodies against several antigens, providing up to sixteen-dimensional data on each cell's expression of receptors, cytosolic proteins, and transcription factors. By perturbing the cells of interest *in vitro*, for example by stimulation with cytokines, or co-culture with target cells, and then analyzing the cells by flow cytometry, it is also possible to learn about the contribution of various molecules and receptors to the phenotype and regulation of NK cells. The high-dimensional data is however hard to interpret and visualize, and we therefore made use of spanning-tree progression of density-normalized events (SPADE) (6). SPADE clusters data in an unsupervised manner, and allows us to generate models of e.g. how cell types are related based how similar they are.

1.2 NK CELL BIOLOGY

The discovery of natural killer (NK) cells was a result of researchers investigating a perplexing background killing of tumor cells that they observed in their assays with lymphocytes exposed to tumor cells (7). Normally, sensitized lymphocytes were needed to evoke killing, which had been attributed to T cells. However, "natural killing", without sensitization was also seen, and this lead to the independent identification of NK cells in 1975, by Kiessling and colleagues at the Karolinska Institutet (8), and Herberman and colleagues at the National Institute of Health in Washington (9). Subsequent investigations of NK cells were largely aimed at finding a specific activating NK cell receptor, similar to those found on T and B cell, but no such receptor was found. Moreover, it was appreciated that whereas cytotoxic T cells required MHC class I to kill its target cells, NK cells killed target cells lacking MHC class I, thus suggesting that an alternative mechanism governed NK cell function (7). Finally, studies using F1 hybrids, where the offspring rejected bone marrow and tumor grafts from either parent, also suggested MHC class I was involved in NK cell activation (10).

When seeking to consolidate the data from experiments with NK cells, during his thesis work, Klas Kärre identified a common denominator of cells resistant to NK cell killing: resistant cells expressed high levels of a full set of MHC class I (11). Kärre therefore postulated that these molecules represented "self-signatures", and that NK cells expressed inhibitory receptors interacting with the MHC class I molecules on these cells. NK cells would therefore be activated by the absence of a full set of MHC class I, which was presented as the "missing-self" hypothesis (7, 12).

Classical killing of a target cell by an NK cell has since been shown to rely on the formation of an immunological synapse, which forms via interactions of receptors on NK cells, and ligands on the putative target cell. NK cells *degranulate* and release preformed granules containing perforin and granzymes (13). Most data suggests that perforin forms pores in the target cell membrane, and that granzymes then enter the target cell where they induce apoptosis (14). Degranulation brings with it the molecule CD107a to the surface of the NK cell, and CD107a expression can thus be used as a surrogate marker of NK cell degranulation in flow cytometry assays (15, 16). Because of their potency as killers, NK cell function is tightly regulated by an array of activating and inhibitory receptors, as well as via cytokines that activate or inhibit NK cell activity (17).

1.2.1 NK cell receptors

Killer-cell immunoglobulin-like receptors
In the decade that followed the postulation of "missing-self" activation of NK cells, the sought-after inhibitory MHC class I receptors that would convey this signal were identified in mice in the form of Ly49 receptors (18), and in humans as Killer-cell Immunoglobulin-like Receptors (KIR) (19, 20). These receptor types are structurally different and therefore represent convergent evolution, indicating that they play important physiological roles.

KIR genes are found in the Leukocyte Receptor Complex (LRC) on chromosome 19 and KIRs with an "L" in their name contain a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIMs), thus making such receptors inhibitory. KIR with shorter cytoplasmic tails ("S") instead have charged amino acids in their membrane region, allowing them to interact with DAP-12, a signaling molecule conveying activating signals via immunoreceptor tyrosine-based activation motifs (ITAMs) (21). The *KIR* gene family is highly polymorphic and polygenic, and *KIR* gene combinations carried by different individuals can be separated into more than 40 haplotypes, based on their *KIR* gene content (22). Two main haplotypes have been identified; haplotype A have only inhibitory KIR, with the exception of *KIR2DS4*, whereas haplotype B carry both inhibitory and activating *KIR* genes. Because we inherit half our genes from each parent, it is possible to carry haplotype A/A, A/B, or B/B. Haplotypes can also be divided based on presence of centromeric and telomeric gene-content motifs (23).

Thirteen *KIR* genes have been found expressed as proteins, and ligands for seven of them have so far been identified (**Table 1**). Adding to their diversity is a high variability in copy numbers of individual *KIR* genes (24), which translates into higher

frequencies of NK cells expressing KIRs with more gene-copies present (24-26). The expression of KIR2DL1 and KIR2DS1, which both bind to HLA-C2 (27), is of particular interest for **Paper III**. However, also the binding of KIR3DL1 to HLA-Bw4 (28) and KIR2DL2/3 to HLA-C1 (and some HLA-C2) (29) are relevant for **Papers II-IV**, and the role of KIRs in *education* of NK cells will be discussed in detail below. Another inhibitory receptor encoded on the same chromosome as *KIR* also expressed by NK cells is LILRB1 (also known as ILT2). It binds to HLA-A, B, C and G molecules as well as virus-encoded proteins (30, 31).

	Known HLA class I ligands					
KIR	**A3**	**A11**	**Bw4**	**C1**	**C2**	**G**
3DL1						
3DS1						
3DL2						
3DL3						
2DL1						
2DL2/3					Some alleles	
2DS1						
2DS2						
2DS3						
2SD4				Some alleles	Some alleles	
2DS5						
2DL4						
2DL5						

Table 1. Expressed KIR genes and known KIR-ligands (23)

KIR gene expression is regulated so that KIRs are expressed in a variegated manner. The molecular mechanism for this involves epigenetic regulation via methylation of promoter regions of *KIR* genes, and a molecular switch that regulates transcription of *KIR* via bidirectional promoters (32). This multifaceted system endows humans with complex and unique KIR repertoires, comprising NK cells expressing 0, 1, 2 or more KIRs. Expression of KIRs has been determined to largely follow the product rule ($\%KIR\text{-}A^{+}KIR\text{-}B^{+} = \%KIR\text{-}A^{+} \times \%KIR\text{-}B^{+}$) (33). At steady state, the presence of KIR-ligands does not affect the frequency of NK cells expressing a particular KIR. This has been described both on cord blood NK cells (34), and in adult peripheral blood NK cells (35). However, deviations from the product rule have been found, and KIR acquisition probability increases with the number of KIRs already expressed by a cell (35). Moreover we have shown that the NK cell KIR repertoire is affected by cytomegalovirus (CMV) infection (36), which is discussed more below.

As a consequence of the great variability in the KIR/HLA system, KIR/HLA combinations have been associated with the risk of acquiring and progressing in diseases of autoimmunity, cancer, viral infections, as well as pregnancy related disorders (37, 38). Most studies rely only on genetic data, but with new tools to study NK cell phenotypes, both with respect to expression of KIRs, and other receptors and

intracellular proteins, more mechanistic insights into the role of NK cells in different disease settings can likely be gained.

CD94 and NKG2A/C

Chromosome 12 carries another gene cluster encoding NK cell receptors, which is called the natural killer complex (NKC) (39). Two heterodimeric *NKC*-encoded receptor pairs are CD94:NKG2A and CD94:NKG2C. They both recognize the non-polymorphic HLA-E (40), but whereas NKG2A confers inhibitory signals (41), NKG2C is activating (42). HLA-E surface expression displays leader-peptides from the classical HLA class I molecules. As a consequence, HLA-E expression on a cell can reflect how *much* HLA class I is produced overall, whereas inhibitory KIRs monitor expression of individual HLA alleles. NK cell regulation via CD94/NKG2A/C and KIRs therefore theoretically make up a sophisticated system to sense changes in both quantity and quality of HLA expression on cells, which often occurs in disease, as viruses or tumor cells seek to avoid detection by the adaptive immune system (43).

Additional NK cell receptors

NK cells can be said to be "unleashed" when confronted with a target cell lacking inhibitory ligands, because at steady state the inhibitory signals from KIR and NKG2A are dominant. This was part of Kärre's original postulate, stating that also activating NK cell receptors must be expressed on NK cells, whose signaling becomes dominant in the "missing-self" situation (7). Indeed, many activating receptors have been identified, including the aforementioned NKG2C and activating KIR. We recently reviewed the current literature on activating KIR (*Ivarsson et al. invited review*), and their role in education of NK cells is detailed in **Paper III** and below. Moreover, NK cells express many activating NK cell receptors that interact with non-HLA ligands (44).

The first activating NK cell receptor to be identified was CD16 (45), which is expressed by the majority of peripheral blood NK cells. It binds with low affinity to the Fc-domain of IgG, and allows NK cells to perform antibody-dependent cellular cytotoxicity (ADCC) (17). CD16 signals through homo- or heterodimers of FcRγ and TCRζ, which contain ITAM motifs. NK cell activity mediated via CD16 adds another dimension to NK cell specificity, since it allows binding to target cells coated with antibodies produced by B cells, thus making NK cells an effector-component of the adaptive immune system. Most activating NK cell receptors are expressed by virtually all NK cells, including 2B4, DNAM-1, NKG2D, NKp30, NKp46, and NKp80 (46), generating an array of receptors with different ligands. Many of these ligands (e.g. the 2B4 ligand CD48) are expressed by healthy cells in the body, facilitating adhesion of NK cells to putative target cells. Other ligands are induced on stressed cells such as tumor cells and on virus-infected cells. These include MICA/B and ULBP proteins that bind to NKG2D (47). NK cells also express CD95L/FAS-ligand, and IFN-γ produced by NK cells can induce CD95/FAS expression on target cells, which in turn make these cells susceptible to FAS-mediated killing by NK cells (48).

NKp30 and NKp46, together with the activation-induced NKp44, make up the Natural Cytotoxicity Receptors (NCRs) (39). Ligands for NKp30 (B7-H6) (49) and NKp44 (MLL5) (50) have both been identified on tumor cells. NKp46 has also been suggested

to mediate recognition of tumor cells, but a ligand has not yet been identified. Both NKp46 and NKp44, have also been associated with binding to hemagglutinin of several viruses (51). Moreover, the relatives of NK cells, the non-NK ILCs, which are discussed below, can also express the NCRs (52). Together with the inhibitory receptors sensing MHC class I expression, the array of activating receptors enables NK cells to detect a wide range of target cells (46).

From the perspective of an immunologist, the most important receptor on human NK cells is CD56. Ironically, its importance is not coupled to a function, because this is not known, but CD56 has since long allowed immunologists to identify human NK cells (53). Moreover, expression intensity of CD56 separates peripheral blood NK cells into two populations, $CD56^{bright}$ and $CD56^{dim}$, and it was early on suggested that the latter differentiate from the former (54). $CD56^{bright}$ NK cells uniformly express NKG2A, lack CD16, and are the dominant NK cell subset in lymphoid organs (55). $CD56^{dim}$ NK cells are $CD16^{+}$ but diverse in their expression of NKG2A/C and KIRs (17). A $CD56^{superbright}$ NK cell subset has also been identified in the uterus (56), and a growing body of data suggests they exhibit unique functions (57, 58). Whether these tissue-resident NK cells represent unique NK cell types, or whether peripheral blood NK cells and tissue-resident NK cells are developmental stages of one type of NK cells, is not known but is currently a hot topic of research. Importantly, other members of the recently identified innate lymphoid cells (ILCs) can also express CD56, and it is thus not as specific to NK cells as initially believed (59).

NK cell regulation via cytokines

In addition to being regulated via cell-cell interactions, NK cell activity is regulated via cytokines. The cardinal interaction partners include macrophages and dendritic cells (DCs), which can produce IFN-α, IL-12, IL-15 and IL-18. These cytokines have priming effects on NK cells, and lead to a lower activation threshold, stronger response against target cells, as well as the production of IFN-γ and TNF-α (60, 61). In addition, chemokines including MIP-1α/β, RANTES, and GM-CSF are also produced by NK cells (62). Cytokines dampening NK cell responsiveness include TGF-β (63), and this provides a way for the immune system to also inhibit NK cell activity using soluble factors.

Importantly, cytokine stimulation alone, without cell-cell interactions, can activate NK cells and lead to the production of IFN-γ and other factors by NK cells. $CD56^{bright}$ NK cells have traditionally been considered more sensitive to cytokine stimulation and geared towards cytokine production compared to $CD56^{dim}$ NK cells, which instead are described as more cytotoxic (60). Importantly however, $CD56^{dim}$ NK cells, but not to the same extent $CD56^{bright}$ NK cells, produce cytokines when stimulated with target cells (61). The IFN-γ produced by NK cells can contribute to the inflammatory response, and studies have revealed that in the murine lymph node (LN), IFN-γ is important for polarization of T cells to Type 1 helper T cells (64), thus showing that NK cells can influence the adaptive immune response. A similar role in tuning of T cell function by NK cells has been found in murine other virus infection models (65, 66).

1.2.2 NK cell education

Central to regulation of NK cell function, and to the work in **Paper II-IV**, is the concept of NK cell education (67). To maintain self-tolerance while also being able to detect aberrant cells, NK cells must know when to become activated. This intuitively requires the expression of at least one self-MHC class I receptor, thus inhibiting the NK cell in a normal self-situation, and indeed this notion was supported by initial studies investigating NK cell regulation (33). Surprisingly however, mice and humans lacking MHC class I molecules (constantly "missing" inhibitor self-signals to NK cells) were found to have normal number of NK cells. Such NK cells were however hyporesponsive, indicating that absence of MHC class I is not sufficient to trigger NK cells, but rather that NK cells are *educated* to detect "missing-self" (68).

Continued investigation of NK cell regulation via MHC class I revealed that murine NK cells indeed required active engagement by inhibitory Ly49 with its ligand for the NK cell to become functionally active, or "licensed" (69). Shortly after, using multicolor flow cytometry and the CD107a degranulation assay, Anfossi and colleagues found evidence of the same phenomenon in human NK cells (70). By studying NK cell subsets defined based on co-expression of inhibitory KIRs, and NKG2A, the authors compared the functional response from $KIR^{+}NKG2A^{+}$ and KIR^{-} $NKG2A^{-}$ NK cell subsets, following stimulation with the commonly used HLA class I negative target cell line K562. NK cells expressing KIRs and NKG2A responded on average twice as good as cells lacking KIRs and NKG2A. This feature was cell-intrinsic since the same difference in response was seen when the cells were stimulated with antibodies via CD16. The authors went on to show that NK cells expressing only KIR with cognate ligand present (e.g. KIR2DL1/HLA-C2) were functionally educated, and responded more than KIR^{-} NK cells, or $KIR2DL1^{+}$ NK cells in a donor lacking HLA-C2. The implication of this was that human NK cells expressing inhibitory KIR together with the cognate ligand results in an educated NK cell, *licensed* to kill cells lacking the cognate ligand. These results were extended to include KIR3DL1 and HLA-Bw4 (71), and were also corroborated by Yawata and colleagues who also showed that NKG2A alone licenses human NK cells (72). An alternative type of NK cell education has been suggested from other studies, where NK cells are postulated to be *disarmed* by chronic stimulation via activating receptors, in the absence of self-receptors (73).

A mechanism for how inhibitory receptors mediate the education of NK cells has not been identified. However, studies indicate that signaling through ITIMs can generate both a weaker signal that efficiently blocks initiation of activating signals, and a stronger signal that is needed to generate a licensed state (74). Adoptive transfer experiments with mice have also found that the licensing of an NK cell is reversible (75, 76). This can be explained by a model where NK cells are constantly quantitatively and qualitatively *tuned* by their surrounding MHC class I, leading to a dynamic range of education levels, tuned by MHC class I like a *rheostat* (77). Such a model of education is compatible with both licensing and disarming, where the rheostat can be seen as being tuned up or down, respectively. According to the rheostat model, NK cells are not activated by a gradual or slow loss/change of MHC class I (such as in an adoptive transfer experiment in mice, or hematopoietic stem cell transplantation in

humans), whereas a relatively sudden loss or down-regulation (i.e. a virus- or tumor transformation) will be detected and will unleash appropriately tuned NK cells (46).

Interestingly, NKG2A$^-$KIR$^-$ NK cells cultured with IL-2 without accessory cells acquired both NKG2A and inhibitory KIR, which in turn licensed NK cells (78). Whether such NK cell differentiation and education takes place i*n vivo* remains to be determined, but may take place in lymph nodes during immune responses. Importantly, non-licensed NK cells are functional when stimulated with cytokines, or via CD16. In fact, it is possible that such NK cells can be the main responders to a virus infection where MHC class I expression is retained on infected cells, since they would not be inhibited, but could readily be stimulated by cytokines induced by the immune response, and sense stress ligands up-regulated by infected cells (73). Indeed, non-licensed NK cells have been found to control mouse CMV infection (however, no analysis of NKG2A on these cells was performed) (79). These authors also speculate that non-licensed NK cells used in cancer therapy might be beneficial, since they will not be inhibited by HLA, but will sense stress ligands on tumor cells.

Intuitively, expression of activating KIR2DS1 on NK cells in the absence of other inhibitory receptors, and in an individual where the cognate ligand HLA-C2 is expressed, could lead to an autoreactive situation. Prior to our study in **Paper III**, it was not known whether such NK cells were selectively low in frequency or absent, or whether they do exist *in vivo* but are differentially educated to ensure tolerance.

1.2.3 Models of NK cell development

NK cells are lymphocytes and as such they derive from CD34$^+$ hematopoietic stem cells (80). *In vitro* differentiation studies using CD34$^+$ cells isolated from adult bone marrow (81), liver (82), secondary lymphoid organs (83), peripheral blood (83), cord blood (84), fetal liver (85), and fetal gut (86), have been found to contain CD34$^+$ cells with the potential to become NK cells, but that also could develop into other lymphocytes including B and T cells.

To date, no CD34$^+$ progenitor cell that only differentiates into NK cells has been described. However, careful analysis of developmental stages of human NK cells led Freud and colleagues to propose a model of NK cell development (87), according to which CD34$^+$ cells co-expressing CD45RA and integrin β7 preferentially develop into NK cells, although this cell population still possessed T/B/DC differentiation potential. Such CD34$^+$ cells are first CD117 (c-kit) negative stage 1 NK cells, and subsequently acquired CD117 to become stage 2 NK cells (**Figure 2**). Loss of CD34 and expression of CD161, CD117, and CD127 marked stage 3 NK cells according this model. NK cells rely on IL-15 for their development and NK cell numbers are dramatically reduced in patients carrying mutations relevant for its binding and signaling (88, 89). Studies using humanized mice have further shown that trans-presented IL-15 (in complex with its receptor IL-15Rα) has a superior effect on NK cell development and differentiation (90). CD122 (IL-15Rβ) was suggested to be expressed already at stage 2 NK cells, and marked stage 3 NK cells, although it was not readily detectable by flow cytometry at these stages (87). Stage 3 NK cells were also described as committed, immature NK cells, since they readily acquired CD94 in culture with IL-15, and could

not develop into T or B cells. Acquisition of CD94/NKG2A marked stage 4 NK cells, the first mature NK cell stage, which corresponds to CD56^{bright} NK cells in blood and tonsils (87).

Studies on secondary lymphoid organs such as lymph nodes suggested that CD34^{+} NK cell progenitors seed these organs, and where subsequent NK cell differentiation takes place (91). Importantly, cells that had reached a particular stage could not be differentiated backwards to a lower stage and when published in 2006, this model was satisfying and represented a big leap in our understanding of human NK cell development. However, recent years have seen the discovery of other innate lymphoid cells, alongside NK cells, and the original model of NK cell development, in particular the definition of stage 3 NK cells, has been found to be in need of updating (92).

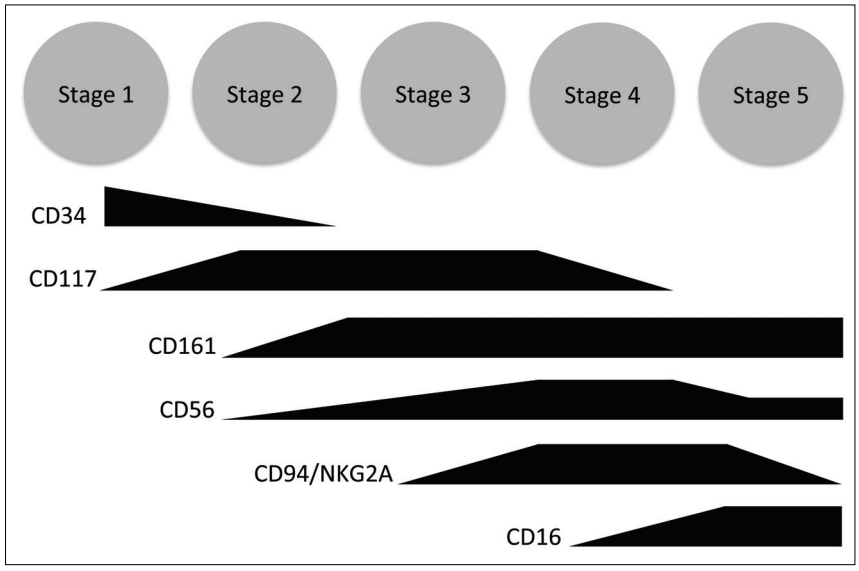

Figure 2. Model of NK cell development and differentiation based on studies by Freud et al. (87).

1.2.4 Innate lymphoid cells

Other innate lymphoid cells (ILCs) that share a developmental origin with NK cells have been identified in recent years. In contrast to NK cells, these cells are not dependent on IL-15 for their development, but instead rely on IL-7, and express the IL-7α receptor (CD127). The roles these ILCs play in health and disease is starting to be unraveled but it is my intention here to primarily discuss them in the context of their developmental relationship to NK cells. A nomenclature with three groups of ILCs has been proposed (93). These are defined based on the functional characteristics and transcription factor expression of the different ILCs (**Figure 3**). Group 1 ILCs perform type 1 immune functions, and contain T-bet^{+} IFN-γ producing ILC1, and Eomes^{+}/T-bet^{+} NK cells (that uniquely among ILCs also can be cytotoxic). Group 2 ILC contain ILC2s that produce type 2 cytokines, and group 3 ILCs contain RORγt^{+} Lymphoid Tissue inducer (LTi) cells and ILC3s (59).

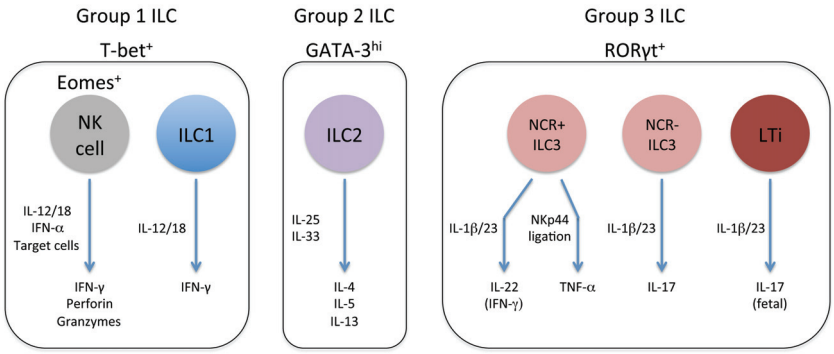

Figure 3. Innate lymphoid cell (ILC) family overview. ILC functional characteristics that have been described for human ILCs are illustrated.

Lymphoid Tissue-inducer cells

The first non-NK ILC to be discovered was the LTi cell. In the late 1990ies, Mebius et al. identified a $Lin^{-}CD4^{+}CD127^{+}$ lymphocyte in developing lymph nodes during murine embryogenesis; the LTi cell (94). These cells was among the first hematopoietic cells to develop during murine fetal development, and evidence suggest it interacts with mesenchymal cells to initiate lymphoid structure formation including lymph nodes and Peyer's patches (95). Upon culture with IL-2, a fraction of the murine LTi cells were found to give rise to cells with NK cell features (94). Subsequent studies revealed that LTi cells rely on the transcription factor RORγt (96) for their development. Moreover, both the generation of lymphoid organs (i.e. the presence of LTi cells), and the development of peripheral NK cells were found to rely on the transcription factor ID2 (97). These data thus supported a model where LTi cells potentially represented an intermediate stage of developing NK cells, and both these cell types derived from an ID2-dependent precursor.

Although quickly hypothesized to exist, the human LTi cell counterpart took more than a decade to identify (98). Similar to mouse LTi cells, these were found in the fetal mesentery where lymph nodes develop, and LTi cells expressed CD127, contained *RORγt* mRNA, and also expressed two markers associated with NK cells, namely CD7 and CD161. The cells produced IL-17 and IL-22, and just as in mice, human LTi cells could give rise to NK-like cells, thus suggesting that NK cells develop through a *RORγt* mRNA-expressing LTi-stage, prior to becoming mature NK cells (98). Importantly however, the NK cell-like cells derived from human LTi cells expressed only some features of NK cells, including NCRs and CD56. But in contrast to peripheral blood NK cells, they expressed no perforin, CD94 or NKG2D, and still retained CD127 and *RORγt* mRNA. Notably, cells with LTi cell-features were also found in adult murine lymph nodes. Using an infection model that generated immune responses that destroyed the lymph node architecture, $ROR\gamma t^{+}$ LTi cells were found to significantly shorten the time for restructuring the lymph node architecture post infection (99).

ILC3

During the same time-period as the human LTi cell was defined, lymphocytes defined as RORγt$^+$NKp46$^+$NK1.1$^-$CD127$^+$ were described in adult murine gut. These phenotypically LTi-like cells produced IL-22, but not IFN-γ, and did not contain perforin (100). Another group also described that similar cells could produce IL-17, as would be predicted by their expression of the Th17 associated transcription factor RORγt (101). A human IL-22 producing NK-like cell, referred to as NK-22, was also found in the gut and secondary lymphoid organs (102). In line with their capacity to produce IL-22, they contained *RORγt* mRNA. Not long after, with the advent of an anti-RORγt antibody for flow cytometry, using tonsil cells, it was revealed that "true" stage 3 NK cells (CD34$^-$CD94$^-$CD117$^+$CD161$^+$) do not express RORγt or CD127, which however are expressed by LTi cells, and LTi-like NCR$^+$ cells (now called ILC3s) (103). The same lineage separation based on transcription factor RORγt was also shown in mice using transgenic reporter mice (104), and have since been corroborated by *in vitro* differentiation studies of NK cells and RORγt$^+$ ILC (105). This latter study also found that similar to subsets of functional T cells, NK cells contained mRNA for transcription factors Eomes and T-bet. Together the studies of LTi cells and ILC3s have revealed that NK cells, and RORγt$^+$ ILC share a common progenitor cell and rely on ID2 for their development (59). Moreover, in contrast to LTi cells and ILC3s, NK cell development does not rely on RORγt, but instead involve transcription factors Eomes and T-bet. These two latter transcription factors will be discussed more in detail below.

More recent studies of ILC3s have revealed these cells can be both beneficial and detrimental to intestinal mucosal health (59). NCR$^+$ ILC3s produce IL-22 in response to IL-23 and IL-1β stimulation, and both studies in humans and mice indicate that ILC3-derived IL-22 promotes intestinal integrity in the gut, and tissue integrity in the lungs. However, NCR$^-$ ILC3s can produce proinflammatory IL-17, leading to immunopathology (106). The regulation, origin and physiological roles of these cells are currently areas of intense research. Not long ago it was shown that similar to NK cells, ILC3s are regulated via surface receptors in addition to cytokines. Triggering of NKp44 on tonsil ILC3s resulted in production of TNF-α, whereas cytokine stimulation with IL-23 and IL-1β resulted in IL-22 production (107).

ILC2

At the time of identification of stage 3 NK cells as RORγt$^-$, another CD127$^+$ innate lymphoid cell was identified in parallel by three groups in mice, which was called nuocyte (108), natural helper cell (109), and innate type 2 helper cell (110). This IL-5 and IL-13 producing population of cells is present in murine gut and lungs, where studies have indicated important roles for helminth parasite expulsion, and tissue repair, respectively (111). A cell type with similar function has also been identified in both fetal and adult humans (112). Just like stage 3 NK cells and RORγt$^+$ ILC, these cells express CD161, but can also uniquely express the prostaglandin receptor CRTH-2. However, they lack CD56 and other NK cell markers, and do not rely on RORγt for their development (113, 114). Instead, ILC2s express high levels of transcription factor GATA-3 and RORα, which is needed for their development and function both in humans and in mice (113, 114).

ILC1
The most recent ILC subtype to be identified is the ILC1, which completed the mirror image that ILCs represent in relation to functional T helper cell subsets (59). ILC1s are RORγt$^-$T-bet^{dim} and were found in high frequencies in inflamed human gut, and produced IFN-γ in response to IL-12 stimulation (115). Moreover, the same group showed that ILC1s could be generated from RORγt$^+$ fetal gut ILCs cultured with IL-12 and IL-18, suggesting plasticity between ILC3s and ILC1s. Notably, Fuchs et al. also reported the presence of a mucosal-associated, intra-epithelial ILC1, which expressed CD103$^+$NKp44$^+$ (116). Similar to the previous ILC1s, these cells expressed intermediate levels of T-bet, and produced IFN-γ when stimulated with IL-12 and IL-15. Interestingly however, these cells were CD127$^-$CD56$^+$, and contained *Eomes* mRNA at levels similar to NK cells, and their distinction from tonsil-resident NK cells is therefore somewhat unclear. Nevertheless, similar to the other ILC types, ILC1s represent a highly interesting topic of research.

Compared to other the B cell and T cell fields of immunology research (and except for NK cells) the ILC field is in its infancy, and the phenotype of ILCs in health and disease is just starting to be investigated. However, it is evident from the amounting murine and human studies that these cells are important for many aspects of normal and disease-related physiology. With knowledge of what transcription factors different ILC subtypes rely on for their development and function, and in light of the co-expression of many surface-markers on ILCs; we reasoned it might be advantageous to analyze ILCs based on both transcription factor and surface-marker expression. To this end, we developed and applied flow cytometry-based analysis of transcription factors and surface antigens of ILCs, which we believe will allow more confident identifications of human ILC subpopulations in general, and identification of NK cell developmental stages in particular (**Paper I**).

1.2.5 NK cell differentiation

NK cells that have acquired CD94/NKG2A, and have reached stage 4 in the development model, represent CD56^{bright} cells in the blood (117). According to the model, this is the first stage of NK cells containing cytotoxic molecules including granzymes and perforin, and capability to produce IFN-γ. With antibodies specific against transcription factors, the expression of Eomes and T-bet have also been identified in CD94$^+$ NK cells (107). These proteins have been implicated in the regulation of the expression of the aforementioned cytotoxic molecules, as well as IFN-γ, both in T cells (118) and NK cells (119). Studies of mouse NK cell development indicated that T-bet$^+$ NK cells develop first, and only later does an Eomes$^+$ NK cell population arise (120). The expression of Eomes and T-bet proteins in relation to human NK cell development has however not been investigated, and is one of the topics of **Paper I**.

In pioneering work using flow cytometry to study human NK cells, Nagler and colleagues identified three NK cell subsets; CD16$^-$, CD16^{dim}, and CD16^{bright} NK cells in peripheral blood (54). Functional analysis of the subsets indicated that the increase in CD16 expression correlated with NK cells becoming more cytotoxic, and also with loss of proliferative capacity in response to cytokines. Moreover, the CD16$^-$ cells were

CD56^{bright}, whereas the CD16^{+} were mainly CD56^{dim}, and the authors suggested that together these features might indicate that NK cells differentiate from CD56^{bright} to CD56^{dim} (54). A CD56^{bright}CD16^{+} NK cell population with intermediate functional characteristics has also been described by Béziat and colleagues (121). In the context of the NK cell development model introduced previously, CD56^{dim}CD16^{+} NK cells are referred to as stage 5 NK cells (122) (**Figure 4**). CD56^{bright} NK cells uniformly express NKG2A, whereas CD56^{dim} NK cells contain both NKG2A^{-} and NKG2A^{+} cells (40, 72), which together suggested that NKG2A could be lost during NK cell differentiation. This notion was indeed corroborated by analysis of NK cells in the recipients of hematopoietic stem cell transplantations, where NK cells were first NKG2A^{+}, and only later started loosing NKG2A expression, and gaining KIR expression on subsets of NK cells (123, 124).

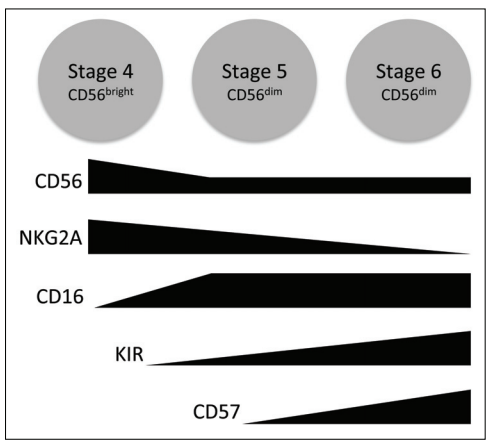

Figure 4. Model of mature NK cell differentiation.

Cells that have undergone divisions have shorter telomere lengths, and three groups in parallel showed that CD56^{dim} cells have shorter telomere lengths than CD56^{bright} cells (125-127). It was also demonstrated that CD56^{bright} cells could acquire both CD16 and KIR when cultured with cytokines *in vitro* (125). Interestingly, the efferent lymph (from paracortical/follicular hyperplasia patients) was found to contain intermediate levels of KIR^{+} and CD16^{+} NK cells, as compared to matched lymph node NK cells and peripheral blood NK cells, thus suggesting that CD56^{bright} NK cells can differentiate in, and CD56^{dim} NK cells emanate from the lymph node, and circulate to the peripheral blood (125). Moreover, the acquisition of CD16 and KIR has also been corroborated using humanized mice, where also trans-presentation of IL-15 was found important for the differentiation of CD56^{bright} to CD56^{dim} NK cells (90). As introduced above, NK cells can express 0, 1, 2 or more KIRs. By analyzing the co-expression of NKG2A and four inhibitory KIRs, the number of KIRs expressed by an NK cell correlated with a lower probability of co-expressing NKG2A, indicating that as NK cell differentiate they loose NKG2A and gain more KIRs (128) (**Figure 4**). One interpretation of this, in relation to licensing of NK cells, is that NK cells start as NKG2A^{+} and acquire one or more KIR, and subsequently down regulate NKG2A (which entails a great loss of inhibitory control) only when tolerance via self-HLA class I is ensured by the KIR(s) expressed (129).

The next step in the investigation of $CD56^{dim}$ NK cell differentiation was inspired by studies on T cells and NK cells in the context of HIV infection, where CD57 had been identified as a marker of a differentiated subset, less capable of proliferating than other subsets (130). Analysis of $CD57^{+}$ stage 6 NK cells in healthy individuals led three groups, including our own (131), to conclude that expression of CD57 indeed marks a terminally differentiated NK cell, more likely to express KIR, and less likely to express NKG2A (132, 133) (**Figure 4**). In parallel, other groups showed that also CD16 (121), CD62L (134), and CD94 (135), could be used to identify functionally distinct differentiation stages of NK cells (136). We also showed that acquisition of CD57 is a process uncoupled from NK cell education (131).

Fetal NK cells

In mice very few mature NK cells are present before birth (137), and this has been attributed to effects afforded by TGF-β in fetal and newborn mice (138). In contrast, functional human NK cells have been found in first trimester fetal organs (139, 140). Moreover, later in gestation, these cells exhibit features of differentiated NK cells, including functional CD16 (140) and expression of KIR (141). A study of fetal NK cells in relation to the data obtained on adult NK cell differentiation had however not been performed. Moreover, it was not known how fetal NK cells expressing NKG2A, KIR and CD16 are regulated. To this end, in **Paper II** we applied the stage 4 to stage 6 model of NK cell differentiation on cells isolated from second trimester fetal organs, and characterized the differentiation and functional regulation of fetal NK cells.

1.2.6 NK cells in virus infections

Following their initial identification as potent killers of tumor cells, type I interferons were also found to activate NK cells, which implicated NK cells in innate immune responses against pathogens (142). One clear example of the importance of human NK cells came from a case report of a patient lacking NK cells, who suffered from severe herpes virus infections (143). Studies in both mice and humans have subsequently shown that NK cells provide important initial control of viral replication and spread, and an important regulatory role in the shaping of the adaptive immune response. Early in a virus infection, dendritic cells (DCs), macrophages, and/or virus-infected cells produce factors that affect NK cells (144). DC subsets produce IFN-α and IL-12, and trans-present IL-15 to NK cells (144-146), and IL-18, released from DCs and macrophages (147), also stimulates NK cells. Depending on the combinations of cytokines present, this may lead to NK cell proliferation, activation, and/or production of IFN-γ and TNF-α (60, 61). These factors can in turn activate DC and promote Th1 T cell polarization. In the periphery, NK cells can also kill immature DC, whereas mature DCs are protected from killing via high expression of inhibitory ligands (148). As mentioned in the previous section, activation via the DC/macrophage derived cytokines also lowers the threshold of activation for NK cells interacting with tumor targets (61).

Arms race between viruses and their hosts

Viruses have evolved mechanisms to avoid detection by the immune system, which allows virions to be produced before the infected cell is detected and eliminated by the immune system (2). This has led to an evolutionary arms race between virus-mediated mechanisms, and immune mechanisms. Virus-infected cells seek the attention of the

immune system by presenting viral peptides on HLA class I and II molecules. $CD8^+$ T cells can detect HLA class I presenting pathogen-derived peptides, and kill the infected cell, which has promoted viral adaptations that tamper with HLA class I presentation, thusly avoiding detection by T cells. However, this loss/change of HLA class I molecules instead risks making the virus-infected cells visible to NK cells, as our immune system possibly has "counter-evolved" to still cope with the virus. To avoid this, the viruses have in turn evolved proteins such as the CMV-encoded UL18 (149), which act as decoy ligands for inhibitory NK cell receptors. Infected cells also upregulate ligands for activating NK cell receptors, again making themselves visible to NK cells. Such ligands include the aforementioned NKG2D ligands ULBPs and MICA/B. However, also here viruses such as CMV have adapted and express proteins that block such stress ligands from reaching the cell surface (149). Yet other activating NK cell receptors implicated in detection of infected cells include NKp44 in HIV (150), and NKp46 that binds to hemagglutinin on cells infected with influenza virus (151). Other ligands for activating NK cell receptors are likely to be found as new techniques and methods are developed (142).

In summary, humans and other mammals are constantly at war with viruses, which have evolved ways to avoid detection by the cells of the immune system, and the immune systems of animals have responded by evolving protective mechanisms. Many such arms races have been going on for millions of years, and it has left imprints both in the viral genomes, and in the immune cells of mammals. For instance, CMV dedicates a large part of its genome to genes encoding proteins involved in immune evasion (152), and in certain mouse strains, the Ly49H receptor seems to have evolved solely to detect the m157 CMV protein present on from CMV-infected cells (153, 154), and in humans, we have found that CMV infection imprints on the NK cell KIR repertoire (36).

Studies of human NK cells in virus infections

Human NK cell immune responses to virus infections are hard to study and often by necessity rely on samples collected after symptom debut. As a consequence, the NK cell immune response has likely already taken place, and potentially subsided. However, colleagues at our center obtained samples from patients recently diagnosed with acute hanta virus infection, and a fast expansion of NK cells in response to the virus was found. These expanded cells were $NKG2C^+CD57^+$self-KIR^+ NK cells that persisted for months in the patients (155). Interestingly, they were seen only in CMV^+ individuals, suggesting potential reactivation of CMV. Indeed, $NKG2C^+$ NK cells had previously been shown to expand in the context of CMV-infected fibroblasts (156).

Along the same lines, in analysis of cells from recipients of bone marrow transplants, individuals who reactivated CMV also showed an expansion of $NKG2C^+CD57^+$ NK cells, similar to that seen in the hanta virus infection (157). Finally, we recently used longitudinal samples collected from children prior to and after their CMV seroconversion (36). These data showed that CMV infection skewed the NK cell repertoire towards more $NKG2C^+CD57^+$self-KIR^+ NK cells (36). Together these studies clearly indicate that just as CMV (and other viruses) has adapted to our immune system, CMV infection affects the human NK cell repertoire. Whether these expanded NK cells are beneficial or not remains to be determined, and will likely depend on what

setting they arise in. Clearing of CMV reactivation in patients lacking B and T cells has however been positively correlated with expansions of NK cells, suggesting that the expanded NK cells are beneficial for virus control (158).

As previously touched upon, another role played by NK cells is in the shaping of the adaptive immune response. In a murine lymphocytic choriomeningitis virus (LCMV) infection model, virus-induced cytokines activated NK cells, which interacted with CD4 and CD8 T cells to regulate the outcome of the infection. This effect was found to be positive or negative, depending on the dose of virus used by the authors (65). In another recent study from the same group, NK cells were depleted at different time-points during the course of the same virus infection, and this also had different outcomes (159). Together these studies suggested that NK cells could act as "master-regulators" of the T cell response to this particular virus-infection. TGF-β has also been found important for NK/T cell cross-talk, and blocking TGF-β signaling in NK cells (but not in DCs) resulted in increased IFN-γ production, which subsequently led to Th1 T cell polarization, and more efficient clearing of Leishmania infection (160).

In summary, studies indicate that interaction between NK cells and T cell subsets are possibly important factors to consider both for vaccine development, and in treatment of acute and chronic viral infections. The interaction between NK cells with T cells in human settings has not been studied in detail, nor is it known whether other virus types induce similar imprints on the NK cell repertoire as those discussed above. We used yellow fever virus vaccination, a live attenuated virus, as a model of an acute viral infection, and characterized both the NK cell responses (**Paper IV**), and the T cell response (**Paper IV**) and reference (161).

1.3 METHODOLOGICAL DEVELOPMENT

All four papers in this thesis rely heavily on multicolor flow cytometry. We utilized this technique together with antibodies specific for surface receptors, cytokines and transcription factors of interest in NK cell biology. To achieve the staining combinations presented in the papers the following challenges were faced:

In all four stories, many antibodies coupled to different fluorochromes were combined, leading to problems with spectral overlap from fluorochromes with similar emission when cells were analyzed. However, by careful optimization of filter-combinations on the flow cytometer, as well as by identifying the right antibody/fluorochrome combinations to avoid overlap of signals from bright antigens, we were able to simultaneously analyze up to sixteen fluorescence-parameters (**Paper I**).

In **Paper II**, we wanted to measure the killing capacity of fetal NK cells, as a confirmation that the degranulation we could measure with CD107a upregulation, corresponded to actual killing of target cells. Because of limited possibilities to use the (51)Cr-release-assay, which is the gold standard for measuring cytotoxic killing (162), we developed a flow cytometry-based assay. Target cells were labeled with a fluorescent dye, making them easy to gate on when analyzing the data. After plating a given number of target cells (5000-10 000 per well), different number of fetal or adult effector cells were added each wells, creating different effector:target (E:T) ratios. After four hours of co-incubations, all wells were stained with a Live/Dead discriminator, and the frequency of dead target cells could be analyzed and plotted relative to the E:T ratio, thus illustrating the NK cell killing efficiency.

Due to the high degree of homology between KIR2DL1 and KIR2DS1, no specific antibody exists for KIR2DS1. To circumvent this, we used a combinatorial staining strategy previously proven successful (163, 164). We first stained cells with the specific anti-KIR2DL1 antibody, thus blocking this epitope, and then titrated and tested different conditions, to find one that allowed the promiscuous anti-KIR2DL1/S1 (clone EB6) to bind to KIR2DS1. Combining this with antibodies against inhibitory KIRs and NKG2A allowed dissection of the phenotype and function on freshly isolated KIR2DS1^{+} NK cells (**Paper III**).

For detailed information on methods used please refer to the methods sections in the individual manuscripts and papers.

2 AIMS OF THIS THESIS

Paper I. NK cells and other ILCs have previously been found to develop early in human fetal development. It was however not known how early their development starts, and whether this process varies between fetal organs, and with gestational age. Little was also known about fetal NK cell development, in particular in relation to other ILCs. The main aim of this paper was to investigate the development of ILCs in human fetal organs by analyzing the unique transcription factors expressed in different ILC types, to investigate ILCs in developing human fetal organs.

Paper II. Mature fetal NK cell differentiation and regulation had not previously been investigated. Using models of NK cell differentiation, the aim of this project was to determine in what organs and to what degree human fetal NK cells are differentiated. We also investigated whether fetal NK cells are functional, and if they are differentially regulated as compared to adult NK cells.

Paper III. NK cell education via inhibitory KIRs has been characterized, but whether *activating* KIRs have an impact on NK cell education had not been investigated at the onset of this project. The aim of this study was therefore to investigate whether the activating KIR2DS1, together with its ligands HLA-C2, had an impact on NK cell education.

Paper IV. A role for NK cells in virus infections has since long been established. However, studies of the human NK cell immune responses during acute viral infections are scarce, much because symptom debut and hospitalization often occur after the initial immune response, where NK cells primarily take part. The aim of this study was therefore to investigate the earliest phases of NK cell responses in a human model of an acute viral infection. To this end we analyzed NK cell responses in individuals vaccinated with the live attenuated yellow fever virus 17D.

3 RESULTS AND DISCUSSION

3.1 DEVELOPMENT OF FETAL NK CELLS AND OTHER ILCS

The human immune system starts to develop in the womb. However, not all its components develop simultaneously, and whereas innate cells including NK cells (139-141), and other ILCs (106), have been found already in the first trimester of gestation, T cells only develop later (165). When and where innate fetal immune cells start to develop, and how ILC function is regulated remains largely unknown.

Identification of human fetal ILCs
The role *in utero* of LTi cells in mice has been established, and these cells are important in formation of lymphoid structures including lymph nodes and Peyer´s patches (95). Less well understood is what functions fetal NK cells and other ILCs have. To be able to address questions pertaining to fetal ILCs, we established techniques to distinguish these phenotypically similar cells from each other. Both surface markers and intracellular proteins can be used for this, and the unique transcription factor expression patterns revealed by studies in transgenic mice, prompted us to combine antibodies targeting surface markers and transcription factors associated with ILC phenotype and function, and to analyze fetal cells using multicolor flow cytometry. Our first finding was that cells with group 1-3 ILC phenotypes could be identified in fetal gut, lung, bone marrow and liver as early as gestational week six (**Paper I**). Importantly, we did not perform any functional experiments on non-NK ILCs, and we thus rely only on their phenotype when we describe them.

NK cells were identified as Eomes^{+}T-bet$^{+/-}$ cells that co-expressed CD56, NKG2A or CD16 (**Figure 5**). RORγt^{+} cells lacked Eomes and T-bet, and instead expressed the highest levels of CD127 and CD117, and thus matched the phenotype of group 3 ILC (166). In line with previous studies (52), first trimester fetal RORγt^{+} cells were mainly NCR^{-} (data not shown), and thus resembled LTi cells (52). Interestingly, we also identified such cells in the connective tissue isolated from the retroperitoneal cavity, which is where the spleen and peritoneal lymph nodes later develop (167).

ILC1s have been described as CD127^{+}T-bet^{low}CD56^{-}CD161^{+} cells, present in tonsils and inflamed gut (115), and as CD127^{-}T-bet^{low}CD56^{+}NKp44^{+}CD103^{+} in tonsils (116). We found cells with similar phenotypes in fetal gut isolated at gestational week 6-10. These cells expressed CD161 and low levels of T-bet without Eomes, and could be divided into CD127^{+}CD56^{-}, and CD127^{-}CD56^{+}, thus overlapping with the ILC1 phenotypes previously described in adult tissues. However, *Eomes* mRNA was previously reported to be similar in NK cells and adult CD127^{-}CD56^{+} ILC1s, (116), making these cells less similar to our putative ILC1s, but suspiciously similar to conventional NK cells. Whether these phenotypes represent two or more distinct ILC subtypes remains to be determined, and our transcription factor staining approach can likely aid in this definition. Finally, ILC2s could be identified as GATA-3^{hi}Eomes^{-}T-bet^{-}RORγt^{-} cells. These cells were also highest in CD161 expression, and thus matched the previously described fetal ILC2s (112).

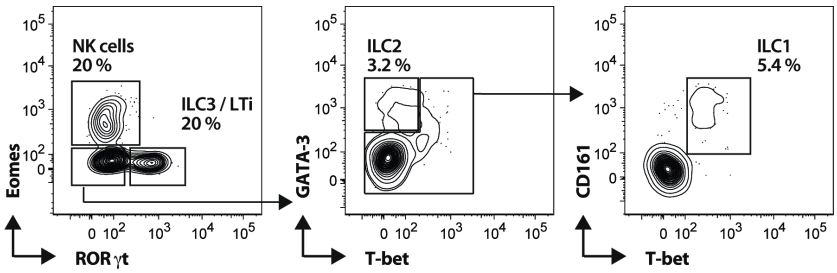

Figure 5. Representative staining of Lin$^-$CD34$^-$CD45$^+$ fetal gut cells isolated at gestational week 8.

Using these phenotypes we could measure the frequency of each ILC type in different tissues in relation to gestational age (**Paper I**), and found that the frequency varied both between ILCs, tissues, and with gestation. NK cells and group 3 ILCs were the dominating populations among total ILCs in all tissues from gestational week 6-22. Notably, in the organs associated with pronounced lymphoid organ development, i.e. the gut and retroperitoneal space, we found more LTi cells than NK cells. Here it is noteworthy that the gastrointestinal tract increases in length in a linear fashion early in gestation (168), and the number of Peyer's patches increases from on average 60 before gestational week 30, to over 240 at puberty (167), thus likely requiring LTi cells to help in the formation of these structures. In contrast, NK cells were most frequent in the liver and the lung. ILC2s could not be defined using GATA-3 expression in the second trimester samples, since the combinatorial staining for transcription factors was not available when those samples were analyzed. However, ILC2s cells make up a large part of the Lin$^-$CD34$^-$CD94$^-$RORγt$^-$CD161$^+$ cells, and as such we could estimate the frequency of ILC2s. However, the frequency of ILC2s was low in all organs, and did not significantly change during the first and second trimester (**Figure 5** and **Paper I**).

Fetal gut contained CD34$^+$CD45RA$^+$CD7$^+$CD117$^{+/-}$ cells, resembling stages 1 and 2 of the model for NK cell development suggested by Freud and colleagues (87). Notably, the CD34$^+$CD117$^+$ cells found here contained a subset of cells co-expressing RORγt, suggesting they are committed to develop into group 3 ILCs, rather than NK cells. Moreover, many of these cells expressed CD45RA and CD7, thus still closely resembling stage 2 NK cells, and supporting the notion that ILCs have a common progenitor. The expression of CD45RA has not been investigated on group 3 ILC before, and the expression of CD7 has been debated, as *in vitro* differentiated RORγt$^+$ cells lacked expression of CD7 (105), whereas *ex vivo* analyzed fetal LTi cells expressed CD7 (98). Our analyses of fetal RORγt$^+$ group 3 ILCs suggest they might start as CD34$^+$CD45RA$^+$CD7$^+$CD117$^+$CD127^{dim}, and gradually loose CD45RA and CD7 as they differentiate they. In line with this hypothesis, the CD34$^-$RORγt^{hi}CD117$^+$ CD161$^+$CD127$^+$ group 3 ILCs in fetal organs had the lowest expression of CD7 and CD45RA (data not shown).

Expression of Eomes and T-bet in NK cell development
The presence of NK cells so early in fetal development is intriguing, and to learn more about these cells we characterized fetal NK cell development, function and regulation in relation to gestational age, organ and differentiation stage (**Paper I** and **II**).
As introduced above, human NK cells have been found to develop through stages defined by expression of combinations of cell surface markers (87). However the expression of the transcription factors Eomes and T-bet had not been placed in this model. In contrast, these factors have been studied in murine NK cells, and studies indicate that T-bet precedes Eomes in mouse NK cell ontogeny (120), since T-bet^{+} NK cells in the liver were present before Eomes^{+} NK cells in the spleen could be detected. Interestingly, although we did not analyze Eomes and T-bet expression in fetal NK cells from the spleen, NK cells from all fetal organs contained T-bet$^{+/dim}$ NK cells, only among Eomes^{+} cells, whereas Eomes^{+}T-bet^{-} NK cells also were detected (**Paper I**). Moreover, T-bet was expressed by the vast majority of CD16^{+} NK cells, which represents a later stage of human NK cell development (117, 125). This suggested that human fetal NK cells express Eomes prior to T-bet as they develop. NK cells in adult peripheral blood expressed both Eomes and T-bet, but whereas CD56^{bright} NK cells expressed more Eomes, CD56^{dim}CD16^{+} NK cells tended to have less Eomes relative to their T-bet expression (unpublished observations).

Eomes and T-bet act as regulators of expression of genes associated with NK cell function (119). In support of this, further analysis of NK cells using antibodies against Eomes, together with functionally related molecules including granzymes and perforin, revealed that Eomes^{+}CD94^{-}CD16^{-} NK cells expressed low levels of granzymes and perforin, as well as NKG2D. This pointed to an initiation of expression of functional molecules prior to acquisition of CD94/NKG2A, but after expression of Eomes. Further functional studies of the fetal NK cells showed that only Eomes/T-bet^{+} NK cells responded to cytokine and target cell stimulation (**Paper I**).

These data thus suggest that Eomes and T-bet expression is initiated at different time-point in mouse and human NK cell ontogeny. An alternative interpretation of the mouse data instead suggests that two different NK cell lineages may exist; one T-bet^{+}Eomes^{-} liver NK cell, and one later developing T-bet^{+}Eomes^{+} splenic NK cell (169). Notably, mice transgenically modified to lack Eomes or T-bet still had functional NK cells, which suggests both NK cells from early mouse ontogeny (Eomes^{-} T-bet^{+}) and later (Eomes^{+}T-bet^{+}) are functional (120). However, in another mouse study, tumor-infiltrating exhausted NK cells were detected, which exhibited an Eomes^{low}T-bet^{low} phenotype (170). The exhaustion could be partly rescued by forced over expression of Eomes, but not T-bet. Together these functional studies indicate that murine NK cell function is influenced by both Eomes and T-bet, but that their functions are not entirely overlapping.

It is noteworthy that in spite of the overlap in functions of Eomes and T-bet, ILC1s seem to rely solely on T-bet, and not Eomes (**Paper I**). Since ILC1s and NK cells share the capacity to produce IFN-γ, but NK cells alone are cytotoxic, it is tempting to speculate that the expression of Eomes is what endows NK cells with this effector function.

Stage 1-3 fetal NK cell development in relation to other ILCs
Of the human ILCs, NK cell development has been studied in greatest detail. The aforementioned model of NK cell development suggests that $CD34^{+}CD45RA^{+}CD117^{-}$ stage 1 and $CD34^{+}CD45RA^{+}CD117^{+}$ stage 2 NK cell precursors sequentially acquire features of mature NK cell as they progress through $CD34^{-}CD117^{+}CD161^{+}CD94^{-}$ stage 3, to $CD94^{+}$ stage 4 (117). In light of our findings that Eomes and T-bet are expressed seemingly sequentially, we wanted to investigate the pattern of expression of other markers associated with NK cell development. Our multicolor flow cytometry approach provided means to further decipher fetal NK cell development.

No Eomes or T-bet positive cells were found among $CD34^{+}$ cells, suggesting these transcription factors are expressed only later in development of NK cells. However, among putative fetal stage 3 NK cells ($CD34^{-}CD94^{-}CD127^{-}ROR\gamma t^{-}CD7^{+}CD161^{+}$), a $CD117^{-}CD122^{-}Eomes^{-}T\text{-}bet^{-}$ population could be found. Based on the presence of cells with intermediate expression, they seemed to sequentially acquire CD122, Eomes, and CD94, as they differentiated to stage 4. Analysis of such transition states using two-dimensional FACS-plots is however not optimal, because the analysis risks becoming tainted with the subjective views of the operator. To avoid this, we analyzed the aforementioned sequential expression of markers with a more objective tool, and used SPADE (6) to perform unsupervised cluster analysis of the FACS data. This algorithm used twelve-dimensional fluorescence data for each cell to cluster similar events in nodes, which are connected to other nodes based on similarity. The size of the nodes is proportional to the number of cells with that particular phenotype.

This resulted in a model with three main arms containing $CD34^{+}$ hematopoietic cells, $Eomes^{+}$ NK cells, and $ROR\gamma t^{+}$ group 3 ILCs, respectively (**Figure 6** and **Paper I**). In the beginning of the $Eomes^{+}$ arm, nodes from $CD161^{+}CD122^{+}Eomes^{-}$ cells, followed by $CD161^{+}CD122^{+}Eomes^{+}$ cells, were clustered. This suggested a sequential expression pattern as NK cell precursors approach mature NK cell status, and express Eomes and CD94/NKG2A. CD56 expression also clustered together with CD122 in the SPADE model, but its expression could also be seen elsewhere in the model, in line with its previously demonstrated expression on group 3 ILCs. In contrast, $NKG2A^{+}$ and $CD16^{+}$ cells clustered only within the $Eomes^{+}$ cells, and our data indicating CD16 expression on $T\text{-}bet^{+}$ NK cells was also recapitulated by the SPADE analysis. Together, our data supports a model where surface markers and transcription factors are expressed in a sequential fashion as NK cells differentiate **(Figure 7**). Although low in frequency in relation to NK cells and group 3 ILCs, SPADE also captured ILC2s, and as expected identified them as $CD161^{hi}GATA\text{-}3^{hi}Eomes^{-}T\text{-}bet^{-}ROR\gamma t^{-}$ (**Paper I**).

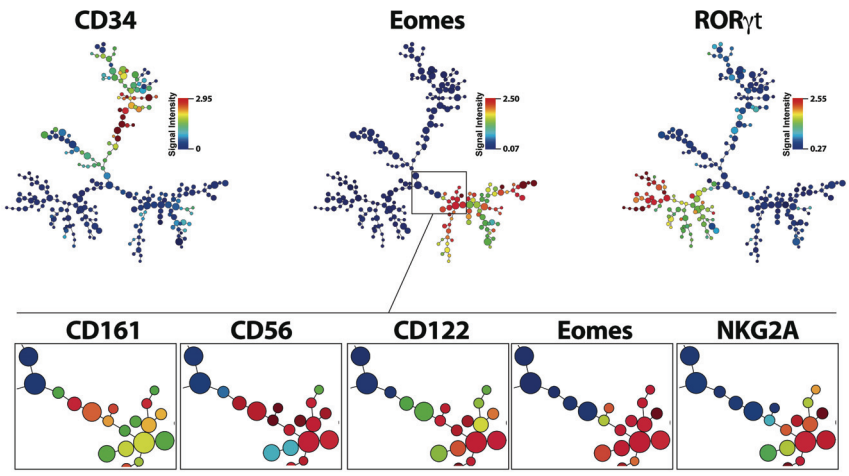

Figure 6. SPADE analysis of fetal gut Lin^{-}CD45^{+} cells, illustrating clustering of events into three main branches based on expression intensities of CD34, Eomes and RORγt. Zoom-boxes illustrate the sequential acquisition of NK cell-associated markers. Color indicates relative expression ranging from blue to red.

Importantly, although SPADE was a useful tool to interpret our high-dimensional cytometry data, our model of NK cell differentiation does not prove the existence of any of the suggested intermediate stages. This and other models do however serve as useful guides when devising FACS-sorting strategies for future experiments, where putative ILC precursors can be sorted and analyzed for differentiation capabilities. A first step in such an undertaking is to identify surrogate surface markers for the transcription factors, which will allow sorting of living cells.

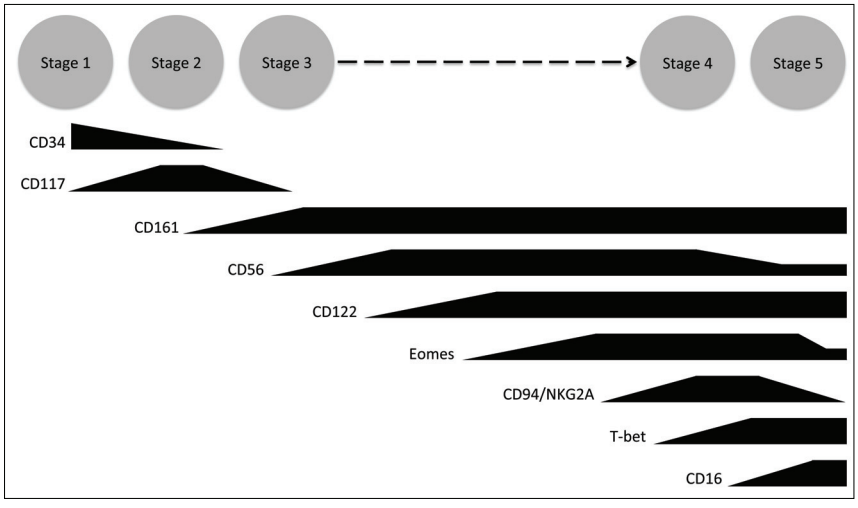

Figure 7. Extended model of human NK cell development and differentiation based on our studies of human fetal NK cells.

3.2 FETAL NK CELL DIFFERENTIATION AND FUNCTIONAL REGULATION

Following the identification of mature NK cells in the fetus, we asked whether they are functional, and whether similar to T cells (171), fetal NK cells exhibit unique features as compared to their adult counterparts. To this end we analyzed the phenotype and function of mature NK cells in second trimester fetal tissues, as described in **Paper II**. We studied the later stages in fetal NK cell development, namely the CD94/NKG2A and CD16 expressing stage 4 to stage 6. NK cells have previously been described in fetal liver and spleen isolated at this time in fetal development, but no study had applied the current model of mature NK cell differentiation to fetal cells.

Identification of second trimester fetal NK cells
We used conventional surface markers, and the Boolean algorithm "CD56 or NKG2A or CD16", to identify NK cells among $CD7^{+}CD3^{-}CD14^{-}CD19^{-}CD34^{-}$ cells. As we have seen in the previous paragraphs, CD56 can be expressed on non-NK ILCs, and to control for this we verified that $CD56^{+}NKG2A^{-}CD16^{-}$ non-NK cell ILCs did not make a large contribution to our analysis. In fact, this population reached maximum 5%, and only in mesenteric lymph nodes (mLNs), and the vast majority of these cells co-expressed RORγt.

We started by observing that fetal NK cells were present in multiple fetal organs, including the liver, lung, spleen, bone marrow, thymus, skin and mLN (**Paper II** and unpublished observation). The frequency of NK cells within each organ varied greatly, and the liver and lung contained the highest frequencies of NK cells. Next we analyzed the expression of NKG2A, CD16 and KIR, to obtain information on the differentiation status of fetal NK cells. More than 90% of fetal NK cells expressed NKG2A, which is similar to cord blood (172). Many fetal NK cells also expressed $CD16^{+}$ (30-80%), indicating differentiation to stage 5, however this frequency was not as high as in cord blood (>80-90%) (173). Moreover, the distribution of NK cells in the three different subsets defined by NKG2A and/or CD16 expression differed between organs, with more differentiated cells found in peripheral organs such as the lungs, and more immature NK cells residing in organs associated with fetal hematopoiesis, including mLN and liver (**Figure 4** and **8**). Together with our data in **Paper I**, this suggests possible *in situ* differentiation of NK cells, possibly attributed to local cytokine-milieu, promoting NK cell development in organs such as the liver and lungs, whereas group 3 ILCs are promoted in the gut.

Differences between organs was also observed with regards to KIR expression, and bulk KIR expression was found to range from 10% in fetal liver and mLN, up to 35% in the fetal lung. These distributions of KIR^{+} NK cells fit with data from adults, where more KIR^{+} NK cells are found in peripheral non-lymphoid compartments organs (e.g. the lungs) compared to lymph nodes, tonsils, bone marrow, and also cord blood (unpublished observation).

Fetal NK cell differentiation
NK cell differentiation is coupled to acquisition of CD16, KIR and CD57, but loss of NKG2A (136). Analysis of the co-expression of NKG2A, CD16 and KIR on fetal cells revealed that $NKG2A^{+}CD16^{-}$, $NKG2A^{+}CD16^{+}$, and $NKG2A^{-}CD16^{+}$ cells contained increasing frequencies of KIR^{+} NK cells, respectively, thus indicating that the model developed for adult NK cell differentiation is also valid for fetal NK cells

(**Figure 8**). Moreover, the number of KIRs co-expressed on each NK cell also increased in the manner previously observed by us on adult NK cells (131). Surprisingly, fetal lung NK cells, which expressed the most KIRs, had more NK cells expressing multiple KIRs, than adult peripheral blood NK cells analyzed in the same manner (analyzing only $CD57^-$ cells).

CD57 is expressed primarily by highly differentiated NK cells, and has been found to be very low on cord blood NK cells (131, 173). In line with this, only few $CD57^+$ stage 6 fetal NK cells were found in fetal tissues. However, second trimester fetal lung did contain a small population of $CD57^+$ NK cells, which expressed CD16 (**Paper I**) and KIRs (unpublished observation), indicating that CD57 marks highly differentiated NK cells also in the fetus. Together this suggests that the fetal NK cell pool is largely naïve in terms of NKG2A and CD57 expression, which resembles immature NK cells in adult peripheral blood. However, the expression of KIR more resembles differentiated NK cells, and although relatively few $KIR^+NKG2A^+CD57^-$ NK cells are found in adult peripheral blood, NK cells with this phenotype has been found in e.g. the uterus, indicating that different KIR^+ NK cell phenotypes exist at different stages of our life, and in different tissues.

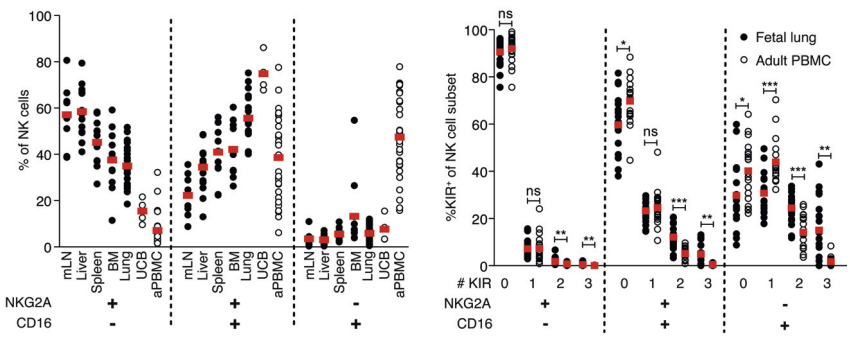

Figure 8. Distribution of NKG2A and/or CD16 expressing NK cells (left), and number of KIRs co-expressed on subsets of NK cells (right). Second trimester fetal organs. mLN: mesenteric lymh node, BM: Mone marrow, UCB: Umbilical cord blood, aPBMC: adult PBMC.

Possible consequences of KIR expression on fetal NK cells

It is noteworthy that human fetal NK cells display a differentiated phenotype, including expression of cytotoxic molecules, and receptors known to endow NK cells with functionality, including the education via NKG2A and KIR. In mice, mature NK cells are few in the fetus (137), and $Ly49^+$ NK cells are virtually absent (138) (whereas NKG2A expression on fetal mouse NK cells has not been reported). This has been interpreted as evidence of adaptations to ensure fetal-maternal tolerance (138). Indeed, maternal cells can cross over the placenta, together with nutrients, antibodies and pathogens (174). Such maternal cells create interesting demands on the involved immune cells, and the reverse is seen with fetal cells crossing over to the mother (175). Maternal cells in the fetus could be semi-allogeneic, and express HLA that fetal NK cells have not seen. If fetal NK cells were regulated similarly to adult NK cells, including education via inhibitory receptors with polygenic ligands (i.e. KIRs), it is plausible that fetal NK cells may interact with maternal cells, and not be inhibited. Such NK cell activity could be harmless, and take place so rarely that it has not affected the reproductive success of our species. Alternatively, such activation

could have unfavorable consequences and affect the outcome of the pregnancy negatively, which might have given rise to adaptations that minimize such effects. One way to learn which of these scenarios is closest to the truth was to investigate whether fetal NK cells are differentially regulated as compared to adult NK cells.

Functional regulation of fetal NK cells

Previous studies had shown that fetal NK cells are functional but exhibit lower levels of activity than adult NK cells (140). We could corroborate these data regarding NK cells both from first (**Paper I)** and second trimester (**Paper II**). It had however not been investigated how fetal NK cells are regulated via inhibitory receptors. NK cells in cord blood express more NKG2A than NK cells in adult blood (172), and Schönberg et al. found that cord blood NK cells were educated via both NKG2A and inhibitory KIRs (176). To investigate if fetal NK cells exhibit this conventional type of NK cell education, we stimulated fetal NK cells with K562 cells. In line with previous studies, fetal NK cells degranulated in response to such stimulation, but to a lower degree than adult NK cells on a bulk level (**Paper II)**.

Next we investigated whether fetal NK cells are educated. We first analyzed KIR^- NK cells expressing or lacking NKG2A. This showed that NKG2A does educate fetal NK cells, since $NKG2A^+$ NK cells degranulated more than $NKG2A^-$ NK cells. Because the frequency of $NKG2A^-$ NK cells was so low (<5-10%), we analyzed education via inhibitory KIR within the $NKG2A^+$ subset, thus measuring the potential added effect afforded by KIR-education, on top of the NKG2A-mediated education. To this end, DNA was isolated from fetal cells and the presence of KIR-ligands was determined by PCR. NK cells expressing self-KIR, non-self KIR, or no KIR (using antibodies for KIR2DL1, KIR2DL3 and KIR3DL1) were then compared in terms of response to K562. Following this analysis, we obtained the perhaps most fascinating result in this thesis: fetal NK cells expressing KIR were hyporesponsive in relation to KIR^- NK cells, irrespective of presence of KIR-ligands **(Figure 9)**. In fact, similar to the tuning process of inhibitory receptors in adult humans and in mice (77), expression of one or more KIRs on fetal NK cells was associated with decreased responsiveness, in a seemingly dose-dependent manner. Colleagues of ours have described a similar effect of KIR expression on CD8 T cells expressing KIR (177), potentially pointing to common pathways of KIR-mediated hyporesponsiveness in fetal NK cells and adult T cells. In contrast, adult NK cells increased in functional responsiveness when more KIRs were expressed (**Figure 9**).

We next investigated if only natural cytotoxicity was affected by this differential regulation, or whether also antibody-dependent cytotoxicity (ADCC) and cytokine stimulation of fetal NK cells was affected. To this end we analyzed fetal NK cells after co-culture cells with 721.221 cells (a $CD20^+$ B cell line) together with Rituximab (an anti-CD20 antibody). Fetal NK cells degranulated and killed target cells, but also in this setting KIR^+ NK cells responded less than KIR^-. Finally, also cytokine-primed fetal NK cells expressing KIRs remained lower in response to target cell stimulation, corroborating the idea that KIR expression on fetal NK cells is associated with a hyporesponsive phenotype, and that this is an intrinsic feature. However, cytokine stimulation induced a strong IFN-γ production in all fetal NK cell subsets, which together with their capacity to mediate ADCC suggests that fetal NK cells might contribute to immune responses *in utero*.

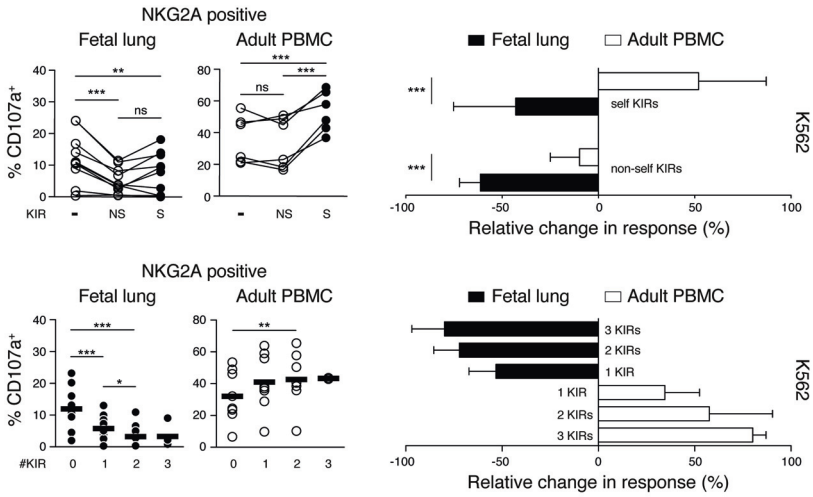

Figure 9. Functional responses of fetal lung NK cell subsets, as compared to adult peripheral blood NK cells, following co-culture with K562 target cells. Self-KIR (S), non-self KIR (NS), KIR negative NK cells (-). Right panels summarizes the relative changes in response relative to KIR negative NK cells.

In relation to the suggested scenarios introduced above, one interpretation of our results is that this alternative education of fetal NK cells represents evolutionary adaptations to pressures from the presence of maternal cells crossing the placenta, leading to a demand for a tolerance mechanism. The same pressure would not have acted on education via NKG2A, because its ligand, HLA-E, is not polymorphic and will be the same on both maternal and fetal cells. The education via NKG2A could instead be beneficial, since it would allow fetal NK cells to sense infected or transformed cells that have lost HLA-E, irrespective of origin, while remaining tolerant to maternal cells. Indeed, perhaps evolutionary pressure has driven fetal NK cells to almost uniformly express NKG2A. The propensity to express many KIRs on the same fetal NK cell might also be the result of selective pressures to ensure tolerance; expression of many KIRs is likely to ensure tolerance via one or more KIR/KIR-ligand pairs, if a fetal NK cells interacts with a maternal cells with semi-allogeneic HLA class I. However, if fetal KIR expression is only associated with problems, it is reasonable to ask why they are expressed at all *in utero*, and why their expression is not initiated until closer to birth, like their counterparts in mice.

Effects of TGF-β on fetal NK cell function

Fetal lymph nodes have been found to contain elevated levels of TGF-β transcripts (178), and this cytokine is known to be highly expressed in the placenta, likely leading to relatively high levels also in the fetus (179). Moreover, murine NK cell ontogeny is influenced by TGF-β, and NK cells lacking TGF-β signaling had more mature NK cells at birth than controls, indicating a role in mouse NK cell maturation (138). In adult humans, NK cell function is inhibited by TGF-β (180, 181). Based on this data we hypothesized that increased susceptibility to TGF-β could explain the hyporesponsiveness exhibited by KIR^+ fetal NK cells. We were however unable to test this using fetal organ cells, due to logistic reasons, and instead we used cord blood isolated routinely from *in utero* blood transfusions of fetuses in gestational week 18-31. These blood transfusions are performed due to anti-RhD immune responses from the mother to fetal erythrocytes, and the isolated cells do therefore not

represent healthy cord blood. Nevertheless, KIR^+ fetal blood NK cells were confirmed to be hyporesponsive in relation to KIR^- NK cells, whereas $NKG2A^+$ NK cells were educated, indicating that the fetal blood NK cells exhibited the same features as fetal organ NK cells.

We co-cultured fetal and adult peripheral blood NK cells with either TGF-β or a TGF-β receptor signaling inhibitor for 48 hours, and subsequently added 721.221 cells coated with Rituximab, thus exposing the NK cells to an ADCC situation. Fetal blood NK cells were significantly inhibited by TGF-β stimulation, whereas the inhibitor had the opposite effect, indicating TGF-β signaling is active in the cells. However, both KIR^- and KIR^+ NK cells showed the same response to TGF-β or the signaling inhibitor, indicating the KIR-associated hyporesponsiveness is not a TGF-β mediated feature. Nevertheless, it was notable that fetal NK cells were affected by TGF-β *in vivo*, thus potentially functioning as an additional tolerance inducing mechanism, together with the NKG2A expression on fetal NK cells, and their differential regulation via KIR.

Interpretation of fetal ILC data

As discussed above and in **Paper I**, it is possible that the frequency of LTi cells is correlated to the formation of lymphoid structures including Peyer's patches in the gut, as well as in the peritoneal cavity where lymph nodes and the spleen is formed. While this notion is intuitively pleasing, in contrast, the reason for the early presence of NK cells and ILC2s, and possibly ILC1s, at this stage in fetal development is still not known. Whereas mice lack mature NK cells *in utero* until late in gestation (137), humans develop them very early in pregnancy, and they are also differentiated and functional, thus pointing to different demands on the human ILC components.

Of the many differences between the mice and humans, one that might be relevant is the fact that mice have many pups, thus lessening the demand for each pup to survive, from an evolutionary point of view. Our species instead invests all our energy into (usually) only one offspring. Moreover, whereas mouse gestation is only three weeks, the longer time in the womb needed for human fetal development increases the risk of *in utero* infections. It is thus possible, as was suggested above, that human fetal innate cells develop early to aid in defense against *in utero* infections, and to maximize the likelihood of survival of our offspring.

As an example, $CD16^+$ fetal NK cells could potentially use maternal antibodies that cross the placenta, to find and eliminate virus-infected cells. Moreover, other innate cell subsets, including macrophages, DCs and eosinophils have been also been identified in the developing fetus (182), and as we have illustrated, fetal NK cells can be a source of IFN-γ in response to stimulation via IL-12 and IL-18 produced by these other innate cells. The IFN-γ can possibly activate fetal macrophages to kill engulfed pathogens, thus functioning just as in adult immune responses to pathogens. Moreover, NK cell immune surveillance via NKG2A-mediated licensing might be used to detect aberrant proliferating cells that likely arise during the many cell divisions that per definition take place during organogenesis and fetal development. ILC2s are also present early in human fetal development and have been demonstrated to produce IL-5 and IL-13 *ex vivo* (112), and might thus also take part in immune responses *in utero*.

Starting their developmental program early will also ensure that NK cells, ILC2s and other innate cells will be in place at birth, where the transition from life in the womb to the outside world involves the risk of pathogens invading the newborn, in particular through the lungs and gastrointestinal tract. Here an important aspect is also the establishment of the commensal flora of the gut and lungs, which likely involve innate cells in many ways yet to be discovered. Importantly, none of these suggested roles of fetal innate cells are mutually exclusive, but might together have been beneficial to the successful development and survival of fetuses, as our species has evolved. As discussed previously, this seems to have been worth the risk of potential fetal-maternal immune activation, which however appears to have resulted in adaptations involving differential regulation of NK cells via KIR, and inhibition via TGF-β.

How fetal NK cells can be different than adult NK cells in terms of their regulation can be interpreted in the context of a model suggesting that "layers" of immune cells develop at different time-points in our ontogeny (171, 183, 184). The features of these layers have possibly been shaped by the unique demands put on fetal immune cells, in contrast to adult immune cells, such as the need for fetal-maternal tolerance. Members of our group have shown that fetal T cell progenitors had a much higher propensity to become regulatory T cells, as compared to phenotypically similar progenitor cells isolated from adult humans (185). It is tempting to speculate, as we do in **Paper II**, that also human NK cell (and possibly non-NK ILC) ontogeny is layered. A prediction from this hypothesis would then be that fetal NK cell progenitors, in spite of exhibiting similar phenotypes as adult progenitors (e.g. CD34, CD45RA, and CD117 expression), would give rise to NK cells with unique features, including those described by us in second trimester fetal tissues. Future studies are warranted to address this intriguing prospect.

3.3 EDUCATION OF NK CELLS VIA ACTIVATING KIR

As introduced above, NK cells are educated by the presence of their cognate KIR-ligands, and are activated when inhibitory signals are missing. The inhibitory receptors maintaining NK cell inhibition at steady state include NKG2A and inhibitory KIR, whose ligands are HLA class I molecules expressed on healthy normal cells. In addition to the inhibitory KIR, NK cells can express activating KIR, including KIR2DS1. The ligand for KIR2DS1 is HLA-C2, which is the only known ubiquitously expressed ligand for an activating KIR. Given the stochastic nature of KIR expression on NK cells, it is possible that NK cells express only KIR2DS1, without co-expression of inhibitory receptors. However, in an individual homozygote for HLA-C2/C2, such an NKG2A$^-$KIR2DS1sp (single-positive) NK cell could potentially be autoreactive.

One possibility to avoid such a situation would be to negatively select against NK cells with this phenotype, a scenario that would be implied by the absence of KIR2DS1sp NK cells in the NK cell pool of HLA-C2/C2 individuals. Alternatively, such NK cells might be differentially educated *in vivo*, and be hyporesponsive in individuals expressing the cognate ligand. Interestingly, prior to our study (**Paper III**), *in vitro* expansion experiments where KIR2DS1$^+$ NK cell clones were generated showed that KIR2DS1 is functional and activates the NK cell via HLA-C2 (164). This confirmed the potential risk posed by KIR2DS1$^+$ NK cells present in HLA-C2$^+$ donors. However,

whether KIR2DS1sp NK cells existed *in vivo*, or whether they were selected against due to the risk of autoreactivity, remained unknown. To investigate the existence and regulation of KIR2DS1sp NK cells *in vivo*, in relation to NK cells positive for other KIRs, we first had to overcome the problem posed by the close homology of KIR2DS1 and KIR2DL1. Because of their similarity, no specific antibody for KIR2DS1 exists, but other studies had shown that combinations of anti-KIR antibodies with different specificities allowed detection of KIRsp NK cells (163).

To this end, by first staining cells with an anti-KIR2DL1 antibody we found that this partially blocked KIR2DL1 binding sites. We then shortly after added an anti-KIR2DL1/S1 antibody, which then was able to bind only to KIR2DS1 binding sites (**Figure 10**). By combining this strategy with antibodies against inhibitory KIRs and NKG2A, this allowed us to show that KIR2DS1sp NK cells are present *in vivo*. Moreover, we could use this staining technique to study the impact of this receptor on NK cell regulation and function (**Paper III**).

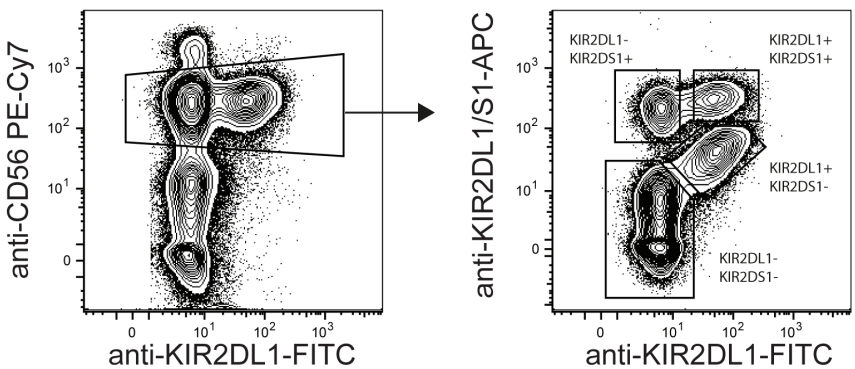

Figure 10. Combinatorial staining of NK cells with anti-KIR2DL1 and anti-KIR2DL1/S1 antibodies permits detection of KIR2DL1 and KIR2DS1 co-expressing subsets of NK cells.

Influence of HLA-C2 on KIR2DS1 expression and KIR2DS1$^+$ NK cell function
Previous work had shown that KIR repertoires are formed independently of the presence of KIR-ligands (35, 176). To investigate if this applied also to KIR2DS1, we analyzed the frequency of NK cells expressing KIR2DS1 in *KIR2DS1* positive, HLA-C1/C1, C1/C2 and C2/C2 donors. This revealed that the frequency of KIR2DS1$^+$ NK cells did not significantly differ between donors with different HLA-C status, and suggested that KIR2DS1 expression is not affected by the presence of its ligand HLA-C2.

Next we co-cultured cytokine-primed NK cells with K562 cells, and compared the responses in individuals where the ligand for KIR2DS1 was present (HLA-C2/C2) or absent (HLA-C1/C1). Notably, KIR2DS1sp NK cells in HLA-C2/C2 individuals were hyporesponsive compared to NKG2A$^-$KIR$^-$ NK cells. In contrast, no such effect was seen in HLA-C1/C1 donors. Furthermore, the hyporesponsiveness of KIR2DS1sp NK cells in HLA-C2/C2 individuals was also evident after stimulation via CD16, even without cytokine priming. Together this indicated that KIR2DS1/HLA-C2 conferred hyporesponsiveness, and that KIR2DS1sp NK cells are differentially regulated compared to conventional education via inhibitory KIRs and

NKG2A, presumably to ensure tolerance in HLA-C2/C2 individuals. Interestingly, HLA-C1/C2 heterozygote donors displayed an intermediate hyporesponsiveness, suggesting a dose-dependent effect of the KIR2DS1-related education. The concept of *disarming* of NK cells following chronic stimulation via an activating receptor offers one interpretation of these data (73). On that note, mouse studies have demonstrated that continuous engagement of the activating receptor Ly49H results in dampened responsiveness of Ly49H expressing NK cells (186, 187). In line with our data, this process was found to be dependent on interaction between receptor and ligand *in vivo* (188).

Effects of KIR2DS1 on NK cell education
Experiments with *in vitro* generated NK cell clones expressing KIR2DS1 suggested that these cells are hyporesponsive when interacting with HLA-C2$^+$ targets, if the donor is HLA-C2/C2 (164, 189). We had identified KIR2DS1sp NK cells *in vivo*, and we therefore next investigated whether these cells indeed were tolerant to target cells expressing HLA-C2. To test this, we co-cultured freshly isolated NK cells from HLA-C2/C2 donors with HLA-C2$^+$ tumor target cells, and found that KIR2DS1sp NK cells were hyporesponsive, which suggests they are tolerant to self (HLA-C2) *in vivo*. Interestingly, KIR2DS1sp NK cells from HLA-C1/C1 donors were not hyporesponsive, which may have beneficial effects in some transplant settings, which is discussed more below.

The array of HLA class I-binding inhibitory receptors including KIR and NKG2A, and activating receptors together transmit both inhibitory and activating signals upon an interaction with target cells, and the integrated signals determines the level of response following stimulation. This process has been referred to as *tuning* of the NK cell response, analogous to a rheostat (68). We next investigated whether KIR2DS1 affected the level of responses from NK cells co-expressing KIR2DS1 with inhibitory receptors. Indeed, analysis of the effect from KIR2DS1 on subsets expressing other KIRs and NKG2A indicated that KIR2DS1 tunes down the responsiveness of NK cells educated via KIR3DL1, KIR2DL3 and NKG2A, but only if HLA-C2 is present. In contrast, co-expression with KIR2DL1, which also binds to HLA-C2, did not lead to a significant reduction in responsiveness. This suggests that KIR2DL1-signaling has a dominant effect over KIR2DS1-signaling when these two receptors are co-expressed.

Up to this point our data indicated that KIR2DS1 conferred hyporesponsiveness, and we wondered whether the receptor is at all functional on NK cells from HLA-C2$^+$ individuals. To this end, we stimulated NK cells with anti-KIR2DL1/S1 antibody-coated P815 cell, thus directly stimulating KIR2DS1. Interestingly, this resulted in robust degranulation from KIR2DS1$^+$KIR2DL1$^-$ NK cells, irrespective of HLA-C status. Moreover, stimulation with IL-12/15 resulted in IFN-γ production in KIR$^-$ and KIR2DS1sp NK cells alike. Together, these functional experiments suggested that while being differentially educated to ensure tolerance, KIR2DS1 is a functional receptor *in vivo*, and KIR2DS1sp NK cells can be activated by cytokines to produce proinflammatory cytokines including IFN-γ. It also remains possible that hitherto unidentified ligands for KIR2DS1 exist *in vivo*. These could for instance be virus peptides presented by HLA-C2, which could make HLA-C2 into a strong ligand for KIR2DS1. Alternatively, tumor-induced ligands for KIR2DS1 might exist. In both cases, KIR2DS1sp NK cells in both HLA-C1 and HLA-C2 individuals might then be advantageous *in vivo*.

Implications of KIR2DS1/HLA-C2-mediated education
The use of NK cells and information about KIR is increasing in the field of bone marrow transplantation of cancer patients (190). Here, transplantation with $CD34^+$ cells from *KIR2DS1*$^+$ individuals is correlated with a lower risk of relapse in patients with leukemia, as long as the donor is not HLA-$C2^+$ (191). This data fits with our data discussed above; KIR2DS1 educates NK cells to be tolerant and hyporesponsive in the presence of its ligand HLA-C2. Furthermore, it suggests that it might be advisable to avoid donors positive for both *KIR2DS1* and *HLA-C2*, where anti-tumor effects are the goal, since these NK cells will be hyporesponsive. Future studies using the staining combination to analyze $KIR2DS1^+$ NK cells could prove to be useful when matching donors and recipients. Notably, following transplantation of $CD34^+$ cells, the KIR repertoire of the donor has been found to largely be recapitulated by the recipient (123). Choosing donors with larger $KIR2DS1^+KIR2DL1^-$ populations of NK cells in HLA-$C2^-$ donors might thus be beneficial if transplanted into an HLA-$C2^+$ recipient.

Research focusing on tissue-resident NK cells and other ILCs is coming of age (106). In particular, the aforementioned uterine NK cells have been implicated in pregnancy, in the process of implantation of the embryo (57). Here, fetal trophoblasts (expressing HLA-C, E and G, but not HLA-A and B) invade into the uterine wall (which is also called the decidua) and widen maternal spiral arteries, thus establishing and ensuring sufficient supply of nutrients to the growing placenta and fetus. Decidual NK cells are suggested to interact with the trophoblasts, and to take part in regulating the depth of trophoblasts invasion. Too little invasion can lead to low supply of nutrients to the fetus, which in turn is associated with reproductive disorders including recurrent abortions and preeclampsia. Deeper invasion by the trophoblasts is beneficial to a certain degree, but can also have negative consequences (192).

Decidual NK cells are $CD56^{superbright}CD9^+NKG2A^+$ and predominantly express KIR2DL1/S1 and KIR2DL2/3 (193), which gives them a phenotype distinct from conventional KIR^+ $CD56^{dim}$ NK cells in the peripheral blood. Using the staining strategy that combined anti-KIR2DL1 and anti-KIR2DL1/S1 antibodies, it was recently revealed that decidual KIR2DL1sp, KIR2DS1sp and $KIR2DL1^+KIR2DS1^+$ NK cell subsets are all functional, but that activation of them leads to different genes being transcribed (194). In particular, stimulation of KIR2DS1sp cells (but not to the same extent $KIR2DL1^+KIR2DS1^+$ cells) with an HLA-$C2^+$ target cell resulted in GM-CSF gene transcription, and KIR2DS1 cross-linking translated into GM-CSF secretion. This GM-CSF could in turn be used to attract trophoblasts in a transwell system, thus implying that activation of decidual NK cells can affect the chemotaxis of fetal trophoblasts. Importantly, the results suggest that such activation will depend on the KIR-ligand status of the fetus, and of the KIR repertoire of the mother, since this will determine whether KIR2DS1 and HLA-C2 will be present. Notably, studies from the same group had previously linked combinations of *KIR/KIR-ligand* genes with risk of recurrent abortion and preeclampsia, and maternal *KIR2DS1* had been found to be protective in the presence of fetal *HLA-C2* (38). Together, these genetic and functional studies indicate an important role for decidual NK cells regulated via KIR/KIR-ligand interactions.

No other ubiquitously expressed ligand for activating KIR has been identified, and the role played by KIR2DS1 in reproduction is the strongest data to date implying a role of KIRs in healthy human physiology. Given the central role pregnancy and fetal development plays for the survival and evolution of a species, it has been speculated

that KIR2DS1 evolved for this purpose. Only the great apes (except orangutans) have *KIR2DS1*, and one theory suggests that KIR2DS1 might have evolved to counteract the inhibitory role that KIR2DL1 plays in decidual NK cell interaction with trophoblasts (23). Supporting this notion, KIR2DL1 inhibited GM-CSF production when interacting with HLA-C2, whereas co-expression of KIR2DS1 overcame this inhibition, and resulted in production of GM-CSF (194). A potential consequence of this, suggested by Parham and Moffett, is that the evolution of *KIR2DS1*, and KIR2DS1$^+$ decidual NK cells in our ancestors, might have allowed deeper invasion of trophoblasts, leading more nutrients to pass over to the developing fetus. This in turn might have contributed to the evolution of the large brains found in modern humans (195).

3.4 NK CELL RESPONSES IN A MODEL OF ACUTE VIRAL INFECTION

To develop vaccines against pathogens and different cancers represent a holy grail for immunologists. One important component of this work is to identify correlates of immune-mediated protection. Only with knowledge of such correlates can we design and evaluate vaccines in an effective manner. Adaptive immunity in the form of B and T cell memory against a pathogen are considered the main factors of a vaccine response. However, it is well established that components of the innate immune system, including NK cells, play important parts in shaping the adaptive immune response (64-66). Moreover, adaptive "memory" features of NK cells have recently been shown to exist (196). Together, this makes it interesting to characterize NK cell responses to virus infections (**Paper IV**).

Although NK cells are often mentioned as important players in control of virus infections, few human studies have followed the early immune response of NK cells to a virus infection. Patients suffering from a virus infection commonly do not seek help early enough for NK cell responses to be detected, and thus patient cohorts are hard to establish. Moreover, for obvious reasons humans, unlike mice, cannot be infected with a virus in a controlled manner. However, certain existing vaccines consist of live attenuated viruses, and by collecting blood from individuals receiving a vaccine, it is possible to follow the early immune response, including the contribution by NK cells, and also to potentially identify correlates to protective immunity.

Yellow fever virus vaccine induces NK cell activation and proliferation
In **paper IV** we analyzed the NK cells of 21 individuals vaccinated against yellow fever virus 17D (YF17D). Using multi-color flow cytometry we analyzed NK cells at different stages of differentiation (NKG2A and CD57 co-expression), as well as their education status (NKG2A and KIRs). We analyzed PBMC isolated on day 0, 3, 6, 10, 15, and 90 from vaccinated individuals.

It is well established that hematopoietic cells upregulate CD69 when activated (197). Although a clear physiological role for CD69 is still being investigated, it is commonly used as a marker of activated lymphocytes, including NK cells. To this end we analyzed its expression on NK cell subsets in relation to YF17D vaccination. This revealed an increase in CD69$^+$ NK cells on day six post vaccination, and in particular in the NKG2A$^+$CD57$^-$ subset (**Paper IV)**. At steady stage, this subset is relatively immature, and has a high proliferative capacity, but relatively low KIR expression

(136), indicating YF17D vaccination leads to activation of preferentially immature NK cells.

In addition to activation, we also analyzed the proliferation of NK cells in response to vaccination, by analyzing the expression of nuclear protein Ki-67. This protein is exclusively expressed in cells undergoing replication (198). Here we found an increase in Ki67^{+} NK cells at day ten (**Figure 11**). Also this response was seen predominantly in immature CD56^{dim}CD57^{-} NK cells. In another study using a similar vaccination model, CD56^{dim}CD62L^{+} cells (largely overlapping with the NKG2A^{+}CD57^{-} NK cells (131, 134)) predominantly upregulated Ki67 at day seven post vaccination (134). Interestingly, the Ki67^{+} NK cells did not overlap with the CD69^{+} NK cells, indicating that at a given time-point post vaccination, different subsets of preferentially immature CD56^{dim} NK cells are activated (CD69^{+}), whereas others are proliferating (Ki67^{+}).

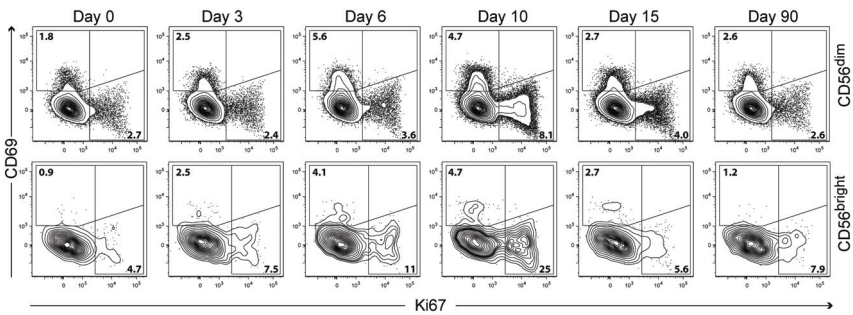

Figure 11. Expression of CD69 and Ki67 on CD56^{bright} and CD56^{dim} NK cells following vaccination against Yellow Fever.

Possible events leading to YF17D induced NK cell responses

Wild type yellow fever is known to infect DCs in lymph nodes, prior to its spread to Kupffer cells in the liver (199). Interestingly, a systems biology approach, investigating the change in expression of hundreds of genes in PBMC following vaccination with 17D, revealed increases in e.g. CD86 (a marker of DC activation) and pro-inflammatory IL-1β on day seven post-vaccination (200). Subsequent *in vitro* experiments with monocyte-derived DC co-incubated with 17D virus showed that IL-1β protein was produced within 24 hours. This suggests that shortly after YF17D vaccination, DCs are activated and IL-1β is produced.

As previously discussed, NK cells are primed by IL-12 in combination with IL-15 or IL-18. Interestingly, IL-1β belongs to the same family of cytokines as IL-18, and studies have indicated that (predominantly) CD56^{bright} NK cells are susceptible to activation via IL-1β in combination with IL-12 (201). IL-12 could not be detected by us (data not shown) or by others performing similar studies with YF17D (202) in the plasma of vaccinated individuals. Nevertheless, the elevated levels of type I/III interferons and viral load found in vaccinated individuals day six post vaccination, suggests activation of DCs. This in turn makes it possible that IL-12 was produced, but perhaps only in physiologically relevant (and detectable) concentrations locally, i.e. in the lymph node where infection takes place.

Together this suggests that YF17D virus infects and/or activates DC in the draining lymph nodes of vaccinated individuals, leading to IL-1β, type I/III IFN and IL-12 production. What effect this DC activation has on NK cells is not clear, but from our data we know that NK cells are both activated (CD69), and stimulated to proliferate (Ki67). However, different NK cell subsets expressed CD69 and Ki67, suggesting they have been affected in different manners, perhaps because they exhibit different phenotypes, or that the same NK cells are first activated (CD69), and then proliferate four days later (Ki67). Lymph node NK cells are predominantly immature $CD56^{bright}$ and have been suggested to differentiate into $CD56^{dim}$ NK cells (87).

One possible scenario is thus that $CD56^{bright}$ NK cells might be activated by DC-derived factors, and upregulate CD69, and initiate differentiation. Possibly, these NK cells then egress the lymph node, and can be detected on day six in the peripheral blood. The same cells might then also proliferate, and appear in peripheral blood as $Ki67^{+}$ immature $CD56^{dim}$ NK cells on day 10 (or on day seven as reported by Juelke et al. (134). CD69 upregulation is described as transient (197), but to the best of my knowledge, it is not known whether activation of NK cells and upregulation of CD69 is followed by proliferation. Alternatively, the $CD69^{+}$ and $Ki67^{+}$ NK cell populations represent distinct subsets of NK cells, that for unknown reasons are affected differently by the vaccination.

In vivo priming of NK cells following vaccination
Stimulation of NK cells with IFN-α, or IL-12 in combination with IL-15, IL-18, IL-1β, or IL-21 activates NK cells (60, 201). Subsequent challenge of the NK cells with target cells leads to increased degranulation and killing, as well as production of e.g. IFN-γ and TNF-α, both with and without target cells present (61).

In the vaccinated individuals we found that NK cell responses to IL-12 alone; IL-12 and IL-18; and K562 target cells, peaked on day six post vaccination, thus coinciding with activation as indicated by CD69 expression. This increase in response is indicative of an *in vivo* priming of NK cells sampled at this day, leading to a higher response when challenged *ex vivo*. Relating this data to the hypothesis of NK cell activation discussed in the previous paragraph, it is interesting to note that IL-12 alone induced a strong response *ex vivo* after vaccination. A potential explanation for this is that NK cells sampled at day six post vaccination have been primed *in vivo* by DCs, which in turn had been activated by the virus during the first day post vaccination. This might have lowered the threshold for subsequent NK cell activation. Priming of NK cells by cytokine exposure has also been described to be long lasting and has been called cytokine-induced *memory* (196). However, although we saw a clear priming effect on day six, this effect was transient, and evidence of cytokine-induced NK cell memory was not found. The priming did however translate into both increased IFN-γ production and degranulation against K562 cells (**Paper IV**).

NK cell education and KIR repertoires in vaccinated individuals
NK cell responses to CMV virus leads to an expansion of self-KIR^{+} cells, as well as an increase in differentiated NK cells as measured by frequency of $CD57^{+}$ NK cells (36, 155). Flaviviruses are known to increase the MHC class I expression in infected cells, thus potentially inhibiting NK cell activation by increasing inhibition via KIRs and

NKG2A (203). To this end, it was possible that the virus differentially affected NK cells expressing different KIR repertoires. We therefore used multicolor flow cytometry panels with antibodies targeting all educating KIRs, including activating KIR2DS1. However, we found no evidence of effects on the KIR repertoire following YF17D vaccination; the KIR receptor profile of NK cells isolated from vaccinated individuals was instead stable from day 0 to day 90, similar to the stability of KIR repertoires described previously by us in healthy individuals (36). Moreover, we could find no vaccine-induced difference in responsiveness when stimulating the cells *ex vivo*, indicating that NK cell priming *in vivo* affected educated and non-educated NK cell subsets equally.

Considerations for future studies

Several possible NK cell receptor ligands have been identified on infected cells. NKp44 is induced on activated NK cells and it has been suggested to interact with cells infected with influenza, HIV, HCV, and mycobacteria (204). It has also been found to interact with the envelope glycoprotein (E-protein) of members of the flavivirus family (205), suggesting activated NK cells may be able to kill virus-infected cells. During the course of natural yellow fever infection, liver cells are eventually infected, and in studies of paraffin-imbedded liver sections from yellow fever victims, increased numbers of NK cells were found close to the liver lesions (206). However, NK cells were here identified as $CD57^+$ cells, and a more rigorous characterization is warranted. Nevertheless, together this suggests that activated NK cells expressing NKp44, may be important players in elimination of virus-infected cells in the liver of later stages of yellow fever disease. Whether this interaction can take place in other sites of the body, such as the lymph node, where the primary immune response to flaviviruses takes place (203), remains to be investigated. Notably, also ILC3s express NKp44, and ligation led to production of TNF-α (107). Moreover, ILC3s are present in the human liver (J. Mjösberg personal communication) and it thus possible that they take part in yellow fever immune responses.

4 CONCLUDING REMARKS

The field of NK cell research has for long been trailing that of other lymphocytes including B and T cells. However in recent years, many discoveries of NK cell development, differentiation and regulation have been made, and NK cells have been grouped as a member of the innate lymphoid cells (ILCs).

Key findings in our investigations are:

- Human fetal NK cells, ILC2s and group 3 ILCs could be detected using transcription factor analysis by flow cytometry, and were found in multiple fetal organs as early as gestational week six.

- Fetal stage 3 NK cells did not express CD127, CD117 or RORγt, and were thus distinct from $CD127^{+}GATA\text{-}3^{hi}$ ILC2s, and $ROR\gamma t^{+}$ group 3 ILCs. Cluster analysis of FACS-data suggest a model where stage 3 NK cells sequentially acquire CD161, CD56, CD122, Eomes, CD94/NKG2A, T-bet and CD16, as they differentiate to mature NK cells.

- Mature fetal NK cells were hyporesponsive in natural cytotoxicity settings, compared to NK cells from adults. CD94/NKG2A educated fetal NK cells, but KIR^{+} NK cells were hyporesponsive to target cells, indicating a fundamental difference between NK cell education *in utero* as compared to after birth. Fetal NK cells were also highly sensitive to TGF-β mediated inhibition. Notably, all fetal NK cells subsets produced high amounts of IFN-γ in response to cytokine stimulation, and $CD16^{+}$ fetal NK cells killed antibody-coated target cells. Together these findings suggest a unique regulation of fetal NK cells that potentially allows them to take part in immune responses *in utero*, while still remaining tolerant to maternal cells crossing the placenta.

- Adult NK cells expressing KIR2DS1 are not negatively selected by the presence of its cognate ligand HLA-C2, but $KIR2DS1^{+}$ NK cells negative for inhibitory HLA class I receptors, were hyporesponsive in $HLA\text{-}C2^{+}$ individuals, thus ensuring tolerance. KIR2DS1 receptor is however functional in $HLA\text{-}C2^{+}$ individuals, and $KIR2DS1^{+}$ NK cells can be stimulated via cytokines, and possibly via ligands that remain to be discovered.

- Yellow fever virus vaccination results in activation and proliferation of predominantly less differentiated $CD57^{-}$ NK cells, on day 6-10 post vaccination. The immune response leads to *in vivo* priming of both educated and non-educated NK cells, resulting in a transient increased responsiveness to target cell stimulation. Moreover, no changes in KIR repertoire following vaccination were detected.

Outlook
At the time of writing, "big data" is causing a commotion in the media, with too much personal information being collected by authorities including the American NSA.

In science, we have seen the same revolution towards big data. However, in contrast to the negative sentiments this causes for society in general, for us scientists it creates great opportunities. Although daunting at first, having access to big data about e.g. a particular cell type like an ILC, with information about its phenotype, genotype, transcriptome, KIRome(!) et cetera, is a blessing. It shifts some of our focus in research from *generating* data, to *analyzing and interpreting* data. Interpreting data means answering scientific questions, and with bigger data, we can dare to ask bigger questions.

In contemporary immunology, big data is represented by for instance multiparameter cytometry, including flow cytometry and mass cytometry (CyTOF). With access to such sophisticated high-dimensional analysis tools, more and more features of ILCs can be studied on a single-cell level. Such possibilities will allow even deeper dissections of e.g. NK cell receptor functions and mechanisms, as well as the developmental relationships between immune cells. I wish to highlight two areas of research that are likely to be touched by these techniques, and that I hope to contribute to by asking and (to some degree) answering big questions.

Future investigations into the development and differentiation of immune cells during fetal development will likely reveal more dissimilarities between cells in the womb, and cells with seemingly identical phenotypes after birth. In line with what we hypothesized in **Paper II**, such findings will shed light on why fetal cells look and behave like they do. The answer to such a "why" question will be reached only by interpreting our results through the "lens of evolution". This will hopefully let us grasp the pressures from natural selection that have resulted in the unique evolutionary adaptations of the fetal immune system.
As discussed in **Paper III**, the establishment of a fetal-maternal interface in human pregnancy is a fascinating area of research. By elucidating the roles that maternal and fetal cells play in the early stages of pregnancy, and learning to monitor and possibly manipulate them, this research can be beneficial for the individual woman, her developing fetus, and for society in terms of healthcare costs.

From an academic standpoint, the unique physiological adaptations in humans are enticing, and by collecting big data about its components, i.e. the cells, we can find the big answers to how the cells interact during pregnancy, and how they have evolved.

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