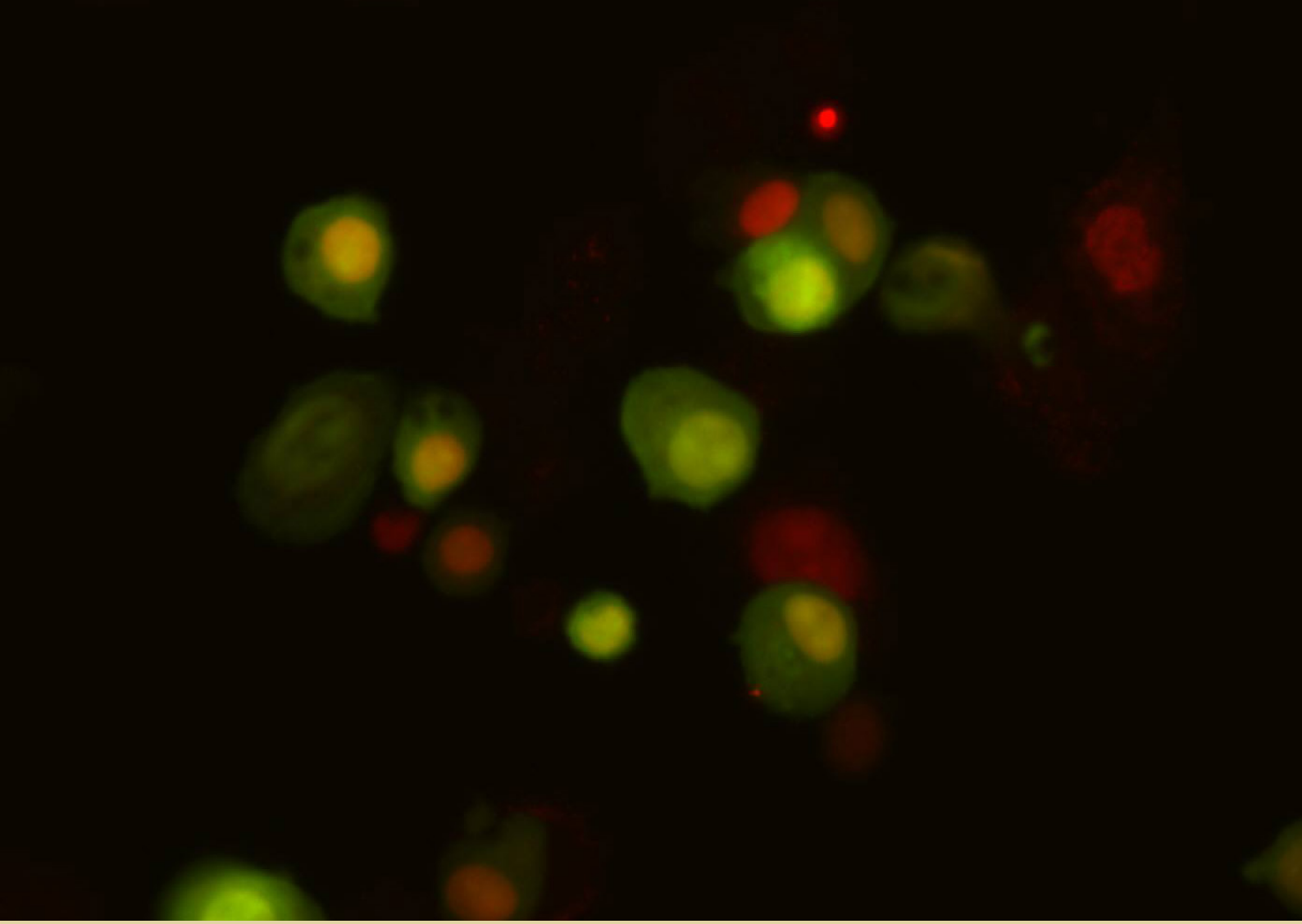

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Tese de Doutoramento em Biociências, especialização em Biologia Celular e Molecular,
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pelo Professor Doutor João Ramalho-Santos (Departamento de Ciências da Vida, FCTUC)
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Agosto de 2015

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FCTUC FACULDADE DE CIÊNCIAS
E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

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AO MEU AVÔ JOAQUIM

(1934-2010)

TO MY GRANDFATHER

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ABSTRACT

Human pregnancy is a challenge for the maternal immune system, which must maintain tolerance to a semi-foreign entity (the fetus) while keeping immunity against viral, bacterial and parasite infections. While the mechanisms involved in placental immune tolerance have been addressed for the last thirty years, very little attention has been given to the maternal immune system role in the elimination of infections.

In preparation for implantation, the uterine endometrium undergoes modifications known as decidualization. The placental cells (trophoblast) invade the decidua to facilitate nutrient and gas exchanges. In particular, the extravillous trophoblast (EVT) of fetal origin detaches from placental villi, migrates into the decidual tissue and contacts directly with maternal immune cells such as decidual natural killer cells (dNK).

dNK are the most abundant leukocyte type in decidua and have a different phenotype from peripheral NK cells (pNK). dNK have lower cytotoxicity in response to MHC-Class I – negative targets and secrete more cytokines and growth factors than pNK. dNK express high levels of killer cell immunoglobulin-like receptors (KIR) that recognize MHC Class I in maternal tissue and EVT. EVT express HLA-E and HLA-G, in addition to polymorphic HLA-C. Recently, women who lack the gene for the activating receptor KIR2DS1 and carry a fetus expressing HLA-C2 (the ligand for KIR2DS1) were found to be at increased risk of recurrent miscarriage, fetal growth restriction and preeclampsia. The molecular and cellular mechanisms involved are unknown. The current hypothesis suggests that absence of activating KIR2DS1 and expression of KIR2DL1 (inhibitory receptor for HLA-C2) results in stronger inhibition of dNK upon interaction with fetal HLA-C2. This inhibition dampens dNK secretion of growth factors involved in placentation and vessel remodeling, leading to pregnancy complications. Particular combinations of KIR haplotypes and HLA allotypes are also associated with improved clearance of viral infection. Therefore we hypothesized that the protective effect of KIR2DS1 lies in a more efficient clearance of infections during early pregnancy. This may prevent virus-induced placental pathology and subsequent fetal and maternal complications. Both hypotheses are tested in the present work. In this thesis, human dNK phenotype and function in healthy pregnancy and viral/bacterial infections are characterized, focusing on the role of KIR2DS1.

Chapter 1 of this thesis provides a general introduction to contextualize the data presented in the following chapters. Chapter 2 presents a characterization of KIR2DS1$^+$ (S1$^+$) dNK in healthy pregnancy. dNK are shown to express receptors for MHC Class I in higher frequencies than pNK, specifically KIR2DL1 and KIR2DS1, KIR2DL2/3 (inhibitory HLA-C1 receptors) and NKG2A/NKG2C (respectively, inhibitory and activating HLA-E receptors). dNK express similar levels of the cytolytic molecules granzyme A and B as pNK, but lower

levels of the pore forming protein perforin. Strikingly, dNK express much higher levels of the antimicrobial peptide granulysin than pNK. In addition, the expression of granzyme B, perforin and granulysin are increased in $S1^+$ dNK. These observations point to a higher cytolytic potential of $S1^+$ dNK. In fact, cytotoxicity of dNK from *KIR2DS1*$^+$ women was not as efficiently inhibited by HLA-$C2^+$ target cells as dNK from *KIR2DS1*$^-$ women and pNK from all donors. $S1^+$ dNK also displayed the highest levels of degranulation of all 4 subsets ($L1^+$, $S1^+$, $L1^+S1^+$, $L1^-S1^-$) in response to HLA-$C2^+$ target cells. Although dNK degranulated and secreted cytokines in response to MHC Class I-negative or HLA-$C2^+$ cell lines, co-culture with EVT did not elicit secretion of IFN-γ, VEGF or GM-CSF.

The failure of KIR2DS1$^+$ dNK to secrete cytokines in response to HLA-$C2^+$ EVT led to the hypothesis that the higher cytotoxic potential of KIR2DS1$^+$ dNK contributes to an increased clearance of placental viral infections, a possibility explored in chapter 3. Human cytomegalovirus (HCMV) is the most common congenital viral infection and can lead to miscarriages, fetal growth restriction and permanent hearing damage. In chapter 3, dNK were shown to degranulate and secrete pro-inflammatory cytokines in response to HCMV-infected decidual stromal cells (DSC). However, dNK did not respond to HCMV-infected choriocarcinoma cell line (JEG3) and sample-matched primary EVT. This demonstrates the high resistance to death by trophoblast and highlights the difficulties the maternal immune system faces to clear infections in the tolerogenic compartment of the placenta. Furthermore, KIR2DS1$^+$ dNK were demonstrated to deliver an increased cytolytic response to HCMV-infected DSC expressing HLA-C2. This observation may explain the protective effect of KIR2DS1 in human pregnancy by limiting viral infection in the placenta. KIR2DS1$^+$ dNK may control the spread of infection and reduce the risk for virus-induced pregnancy complications.

Finally, in chapter 4, the role of dNK in the elimination of bacterial infections was investigated. The high expression of antimicrobial peptide granulysin by dNK was the basis for this study. dNK constitutively secreted high levels of granulysin. In addition, dNK cells and dNK supernatants were able to kill both extracellular and intracellular bacteria in JEG3 without killing the host cell. This effect was independent of degranulation. This striking result reveals a novel function for dNK and strengthens their role as immune effector cells.

RESUMO

A gravidez humana é um desafio para o sistema imunitário materno, o qual deve garantir tolerância a um feto semi-incompatível enquanto mantém imunidade contra infeções. Os mecanismos envolvidos na tolerância têm sido bastante explorados, mas o estudo da imunidade a infeções congénitas tem sido negligenciado.

A implantação do embrião exige que o endométrio uterino sofra modificações profundas (decidualização). As células fetais da placenta (trofoblastos) invadem a decidua para permitir trocas eficientes de gases e nutrientes. Em especial, os trofoblastos extravilosos (EVT) migram para o tecido decidual, contatando directamente com linfócitos maternos como as células Natural Killer deciduais (dNK). As dNK são os leucócitos mais abundantes na decidua, mas são menos citotóxicas em resposta a células alvo que não expressam MHC Classe I que as NK do sangue (periféricas, pNK), e secretam mais citocinas e fatores de crescimento. As dNK expressam níveis elevados de recetores KIR (killer cell immunoglobulin-like receptors) que reconhecem proteínas MHC Classe I nos tecidos maternos e EVT. Os EVT expressam HLA-E e HLA-G e também HLA-C, que é polimórfico. Recentemente, foi encontrada uma associação prejudicial entre genes KIR maternos e alelos HLA-C do feto. As grávidas que não possuem o gene para o recetor ativador KIR2DS1 e cujo feto expressa HLA-C2 têm um risco elevado de aborto espontâneo, restrição de crescimento fetal e pré-eclâmpsia. Os mecanismos moleculares e celulares envolvidos não são conhecidos. A hipótese proposta atualmente sugere que a ausência de KIR2DS1 ativador e expressão de KIR2DL1 (recetor inibidor de HLA-C2) leva a uma inibição mais pronunciada das dNK aquando da interação com HLA-C2 fetal. Esta inibição reduz a secreção pelas dNK de fatores de crescimento envolvidos na placentação e remodelação de artérias uterinas, resultando em complicações na gravidez. São também conhecidas combinações de haplótipos de KIR com alelos de HLA que estão associadas a um melhor combate a infeções virais. Estes dados levaram à nossa hipótese de que o efeito protetor de KIR2DS1 está relacionado com uma resposta imunitária mais eficiente a infeções congénitas. Este fenómeno pode prevenir danos induzidos por vírus na placenta e consequentes complicações na gravidez. Ambas as hipóteses foram testadas no trabalho aqui apresentado. Nesta tese, é apresentada uma caraterização do fenótipo e função das dNK humanas em gravidezes saudáveis e afetadas por infeções virais ou bacterianas, com foco no papel de KIR2DS1.

O capítulo 1 desta tese fornece uma introdução geral para contextualizar os dados apresentados nos capítulos seguintes. O capítulo 2 apresenta uma caraterização das dNK que expressam KIR2DS1 ($S1^+$) em gravidezes saudáveis. A frequência de expressão de recetores para MHC Classe I nas dNK é mais elevada que nas pNK, em especial KIR2DL1

e KIR2DS1, KIR2DL2/3 (recetores inibidores de HLA-C1) e NKG2A/NKG2C (recetor inibidor e ativador, respetivamente, de HLA-E). As dNK contêm níveis semelhantes das proteínas citolíticas granzima A e B aos das pNK, mas níveis mais reduzidos de perforina, que forma poros nas membranas das células alvo. Em especial, as dNK contêm muito mais proteína anti-microbiana granulisina que as pNK. Ainda, a expressão de granzima B, perforina e granulisina é mais elevada em dNK $S1^+$. Estas observações revelam um potencial citolítico elevado das dNK $S1^+$. De facto, a citotoxicidade das dNK isoladas de grávidas que possuem o gene *KIR2DS1* não foi inibida por células alvo HLA-$C2^+$ com a mesma eficiência que dNK isoladas de grávidas sem *KIR2DS1* ou pNK de todos os indivíduos da amostra. As dNK $S1^+$ também demonstraram possuir a mais elevada citotoxicidade dos 4 subgrupos ($L1^+$, $S1^+$, $L1^+S1^+$ e $L1^-S1^-$) em resposta a células-alvo HLA-$C2^+$. As dNK desgranularam (libertaram moléculas citolíticas) e secretaram citocinas em resposta a células alvo MHC^- ou HLA-$C2^+$, mas não secretaram IFN-γ, VEGF ou GM-CSF após cultura com EVT.

A falta de resposta das dNK à interação com EVT levou à hipótese de que o potencial citotóxico das dNK $S1^+$ contribui para um combate mais eficiente às infeções virais na placenta. O vírus humano citomegalovírus (CMV) provoca a infeção congénita mais comum e pode levar a aborto espontâneo, restrição do crescimento fetal e danos auditivos permanentes. No capítulo 3, mostrou-se que as dNK desgranulam e secretam citocinas pró-inflamatórias em resposta a células do estroma da decidua (DSC) infetadas com CMV. No entanto, as dNK não responderam a células de coriocarcinoma (JEG3) ou EVT primários infetados com CMV. Estas observações demonstram a resistência à morte dos trofoblastos e sublinham a dificuldade que o sistema imunitário materno enfrenta para eliminar infeções na placenta. Em especial, mostrou-se que as dNK $S1^+$ são mais citolíticas em resposta a DSC HLA-$C2^+$ infetadas com CMV. Esta observação pode explicar o efeito protetor de KIR2DS1 na gravidez humana ao limitar a infeção viral na placenta. As dNK $S1^+$ podem controlar a disseminação da infeção e reduzir o risco de complicações da gravidez induzidas por vírus.

Finalmente, no capítulo 4, investigou-se o papel das dNK na eliminação de infeções bacterianas. A expressão elevada do péptido anti-microbiano granulisina em dNK constituiu a base para este estudo. As dNK secretam granulisina constitutivamente, e as células dNK e sobrenadantes das suas culturas conseguiram eliminar bactérias extracelulares e intracelulares em JEG3 sem matar as células hospedeiras. Este efeito foi independente de desgranulação. Este importante resultado revela uma nova função das dNK e reforça o seu papel como células imunitárias efetoras.

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AML	Acute myelogenous leukemia
APC	Antigen Presenting Cell
BCR	B cell receptor
CD14	Monocyte/macrophage marker
CD3	T cell marker
CD45	Lymphocyte lineage marker
CD56	NK cell marker
CD95L	Fas Ligand
CFU	Colony Forming Unit
CTL	Cytotoxic T lymphocytes
DAP10	DNAX activation protein of 10kDa
DAP12	DNAX activation protein of 12kDa
DC	Dendritic cells
dMϕs	Decidual Macrophages
dNK	Decidual NK cells
DSC	Decidual Stromal Cells
EBI3	Epstein-Barr virus-induced gene 3 (interleukin-27 subunit β)
ER	Endoplasmic reticulum
ETC	Electron Transport Chain
EVT	Extravillous trophoblast
GBS	Group B Streptococcus
GM-CSF	Granulocyte-monocyte colony stimulation factor
GNLY	Granulysin
GvHD	Graft vs Host Disease
Gzm	Granzyme
HA	Hemagglutinin
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
HLA	Human Leukocyte Antigen
HSCT	Hematopoietic Stem Cell Transplantation
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IS	Immunological Synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer cell Immunoglobulin-like Receptor
LILR	Leukocyte Immunoglobulin-like Receptor
LRC	Leukocyte Receptor Region
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex

MIC	MHC Class I polypeptide-related sequence
MOI	Multiplicity of infection
MTOC	Microtubule Organization Center
NCR	Natural Cytotoxicity Receptors
NK	Natural Killer
PFN	Perforin
pNK	Peripheral NK cells
PRR	Pattern Recognition Receptors
SAPLIP	Saposin-like proteins
SHP	Src homology region 2 domain-containing phosphatase
SYK	Spleen Tyrosine Kinase
TAP	Transporter associated with Antigen Processing
TCR	T-cell receptor
TGF-β	Transforming growth factor β
Th	T helper cell
TNF	Tumor Necrosis Factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cells
ULBP	UL16 binding sequence
VEGF	Vascular Endothelium Growth Factor
VT	Villous trophoblast
ZAP70	Zeta-chain-associated protein kinase 70

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CHAPTER 1

GENERAL INTRODUCTION

The human immune system is a complex set of cells and proteins that resulted from millions of years of co-evolution of humans and their pathogens. To allow survival of the individual and, more broadly, of human populations, each human being has a system ready to defend it from bacteria, viruses, fungi and parasites (1).

The functioning of the immune system is complex and two main arms can be identified: innate and adaptive immune system. The innate immune system constitutes the first line of defense and delivers a quick response to any invading pathogen by recognizing common patterns absent in the human body. The adaptive immune system, although slower, is more efficient, because it can recognize specific markers in each pathogen and save that information. This way the adaptive immune system can instantly eliminate subsequent infections of the same pathogen and clear the infection before any significant damage occurs. This property of the immune system to "remember" is named "immune memory" and is the basis for the many vaccines available today (1).

How the immune system attacks only foreign ("non-self") and dangerous pathogens, while remaining controlled and not damaging ("tolerating") our own (self) tissues will be discussed in detail below. This introduction will cover three main areas: self vs non-self recognition in the systemic immune system, the characteristics and role of Natural Killer cells and the complexity of the local immune system regulation during pregnancy. In the first part, the mechanisms of self and foreign tissue and pathogen recognition by immune leukocytes will be discussed. In the second part, the function of Natural Killer cells, their subsets, receptors and ligands will receive special attention. In the third part, the regulation and function of the immune system during pregnancy will be described. This last part will emphasize the double-edged challenge faced by the local immune system in the maternal-fetal interface: maintaining immune tolerance to semi-foreign fetal tissues while remaining active against potential viral, bacterial and parasite infections. The protective role of the NK cell receptor KIR2DS1 (**K**iller cell **I**mmunoglobulin-like **R**eceptor, **2** extracellular **D**omains, **S**hort cytoplasmic tail **1**) in healthy and complicated pregnancy will be addressed in detail.

1. Self vs. Non-Self recognition

The innate immune system recognizes common proteins and chemicals released by injured cells. These molecules include chemicals that induce the secretion of cytokines, activate innate immune leukocytes to secrete other chemicals or attack the pathogens, or chemokines that recruit additional leukocytes to the site of infection. These leukocytes are, for example, phagocytes (e.g. macrophages or dendritic cells) that express proteins at the surface known as "pattern recognition receptors" (PRRs). PRRs recognize common proteins

from pathogens, leading the phagocytes to engulf the pathogens and lyse them (phagocytosis) (2).

The adaptive immune system relies on Major Histocompatibility Complex (MHC) molecules that present self and non-self peptides to T and B cells (3). The MHC locus contains the most variable (polymorphic) functional genes described in vertebrates. In humans, these proteins are known as human leukocyte antigens (HLA) and two classes can be recognized: Class I and Class II. While Class I HLA are expressed by all nucleated cells, Class II are constitutively expressed only by bone marrow-derived antigen[1] presenting cells (APC), such as macrophages, dendritic cells, B cells and thymic epithelial cells (1).

MHC Class I molecules are surface proteins which extracellular part is composed of 3 alpha domains and a β_2-microglobulin chain. The α1 and α2 domains form a groove where a peptide can bind. The α3 domain contains a binding site for the co-receptor CD8 expressed in $CD8^+$ T cells, discussed below. During and following regular protein synthesis, a proportion of the resulting proteins are rapidly degraded into peptides by the proteasome. The resulting peptides are translocated into the lumen of the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). Within the ER, the peptides are loaded onto newly synthesized MHC I heavy chain/β_2-microglobulin heterodimers (3). MHC Class I constantly display short intracellular endogenous peptides (8-9 aminoacids long) as a sign of the status of the cell to be surveyed by T cells. When a cell is infected by an intracellular pathogen (e.g. virus), or starts producing new and damaged proteins due to tumor formation, the complex formed by pathogen-derived peptides and MHC Class I are recognized by $CD8^+$ T cells (cytolytic T lymphocytes) and elicit an immune response (4).

In humans, six MHC Class I genes can be identified: 3 classical highly polymorphic genes (HLA-A, B and C) and 3 non-classical (HLA-E, G and F). All nucleated cells express HLA-A, B and C, which main function is antigen presentation, and also HLA-E, which although non-polymorphic, has a role in Natural Killer cell inhibition. HLA-G is

Class	Gene	Alleles	Proteins
HLA Class I	A	3,192	2,245
	B	3,977	2,938
	C	2,740	1,941
	E	17	6
	F	22	4
	G	50	16
HLA Class II	DRB	1,868	1,364
	DQA1	54	32
	DQB1	807	539
	DPA1	40	20
	DPB1	550	447

Table I – MHC Class I genes, proteins and number of alleles. Source: HLA Nomenclature (http://hla.alleles.org/)

[1] Antigen – any molecule that elicits an immune response (1).

tissue restricted and is believed to be involved in immune modulation (e.g. induction of regulatory T cells). HLA-F function is still unknown (5–7) (Table I).

MHC Class II molecules, unlike MHC Class I, are tissue restricted. Class II are only expressed by professional antigen presenting cells (APC) like dendritic cells, macrophages and B cells (antibody producing cells). MHC Class II proteins have 2 heavy chains, one with two α domains and one with two β domains. The groove formed by the α1 and β1 domains can present a longer peptide than MHC Class I, which is usually derived from extracellular pathogens (e.g. phagocytosed bacteria) which proteins were subsequently degraded. MHC Class II contains a binding side for the co-receptor CD4 in $CD4^+$ T cells (T helper cells). The recognition of the MHC-peptide complex by $CD4^+$ T cells will activate them and lead to the immune response. In humans, 3 main MHC Class II proteins can be identified: HLA-DR, HLA-DQ and HLA-DP, which are also highly polymorphic (8) (Table I).

T cells are the main immune lymphocytes involved in the recognition of MHC-peptide complexes. T cells originate from hematopoietic precursors in the bone marrow and later migrate to the thymus to finish their maturation. T lymphocytes come in two flavors, $CD4^+$ and $CD8^+$, and can be further divided into several subsets with different functions. All T cells express a T cell receptor in the surface, the TCR, which is exclusive and different for each T cell and recognizes a different MHC-peptide complex. The different TCRs are formed by somatic rearrangement of the TCR genes, which leads to a different sequence and, consequently, a different specificity of each T cell. The diversity of TCR in a given individual is enormous, and since their formation is random, some TCRs recognize self-peptides that can lead to auto-immune attack (9). To avoid this, T cells need to go through a mechanism of positive and negative selection in the thymus during development. T cells that have too low affinity to self MHC-peptide complex (negligent) or too high affinity to self MHC-peptide complex (auto-reactive) are eliminated in the thymus (positive and negative selection, respectively). This way, only T cells specific for self-MHC presenting foreign peptides and can be activated by them are allowed to leave the thymus (10).

$CD4^+$ T cells are usually identified as T helper cells (Th). When in the periphery, Th cells interact with APCs expressing their specific MHC-Class II – peptide complex, leading to their activation, proliferation and differentiation into different types of T helper cells (depending on the cytokine environment) (11). $CD4^+$ T cells can differentiate into Th1 cells, which produce more inflammatory cytokines like interferon-γ (IFN- γ) and tumor necrosis factor α (TNF-α), helping $CD8^+$ T cell, NK cell and macrophage responses. $CD4^+$ T cells can also differentiate into Th2, which produce interleukins 4 and 5 (IL-4 and IL-5) that provide help to antibody-producing B cells. Upon clearing of the infection, a subset of $CD4^+$ T cells differentiates into

memory cells that will be able to respond faster to a second infection by the same pathogen (12).

T regulatory cells are also $CD4^+$, but have a very distinct phenotype (expressing high levels of CD25 and the transcription factor FoxP3). These cells are involved in immune tolerance and dampen the immune response, avoiding auto-immune reactions (13).

$CD8^+$ T cells are the major immune cells involved in tumor and viral clearance. $CD8^+$ T lymphocytes directly recognize MHC-Class I-peptide complexes, which leads to their activation, proliferation and differentiation into cytotoxic T lymphocytes (CTL) (11). CTLs directly kill infected or tumor cells by several ways, the most important being the release of cytotoxic molecules to the point of contact between the T cell and the target cell (immune synapse - IS). Among the cytotoxic molecules are perforin, a pore forming protein that creates an opening in the target cell membrane, allowing the entrance of granzymes, which lead to apoptosis of the target cell by several pathways (caspase-dependent or independent) (14, 15). Upon activation and clearance of infection, a subset of CD8 T cells differentiates into memory T cells that can elicit a rapid response upon a subsequent infection with the same pathogen (16).

The other adaptive immune lymphocyte which can play a role in the immune response are **B cells**. B cells have a B cell receptor (BCR), also highly specific to foreign epitopes[2] (since each B cell undergoes somatic rearrangements in their BCR locus) (17). However, these cells do not need to bind a MHC-peptide complex for activation. Instead, their BCR recognizes free antigen, but the B cells need interaction with primed T cells of similar antigen specificity to start their differentiation and proliferation (18, 19). Upon activation, B cells produce antibodies (immunoglobulins, Ig), highly specific molecules. The antibodies produced by a given B cell have the same specificity of the BCR, and their diversity is as high as the TCR diversity (the result of somatic rearrangements in the immunoglobulin genes) (17). Antibodies can be secreted and help in the elimination of pathogens by several ways – opsonization (coating of a pathogen, flagging it for phagocytosis), aggregation of pathogens or antibody-mediated cell death (by binding receptors in Natural Killer cells – discussed in the section below - or macrophages) (20).

Natural Killer cells, which will be discussed in more detail on section 2, are lymphocytes commonly categorized as innate immune cells, but recent studies have identified NK cell functions that place NK in the border between immune and adaptive immunity. NK cells share some characteristics with cytotoxic T lymphocytes, in particular the ability to form immune synapses and deliver cytolytic granules for target cell killing (21). Unlike T cells, NK do not recognize specific epitopes in target cells; instead, NK cells express activating

[2] **Epitope** – specific region of an antigen recognized by TCR or antibodies (1).

receptors that identify stress-induced proteins upregulated by infected or cancerous cells, and quickly eliminate them without prior stimulation (22). However, NK cells have recently been shown to possess the ability to recognize specific viral proteins expressed in infected cells, leading to their proliferation and stronger response upon second encounter with the same antigen (23). These studies have been the subject of controversy and require further development. In addition, NK cells express a wide range of inhibitory receptors that bind groups of alleles of MHC Class I, as a way of protecting healthy self tissues from NK attack (24).

1.1 HLA recognition and the allogeneic response

The MHC locus is the most variable (polymorphic) region in the human genome. These MHC polymorphisms allow for a great variety of pathogenic peptides to be presented, resulting in an increased ability to clear a large diversity of pathogens. However, these polymorphisms also underlie the extreme difficulty in finding perfectly matched organs or unrelated bone marrow donors that will not induce a strong anti-graft response (25). In the next paragraphs the mechanisms of transplant allorecognition will be discussed to illustrate how the maternal immune system could potentially respond to the allogeneic fetal tissues during pregnancy.

1.1.1 Transplant allorecognition

Transplantation is the artificial transfer of cells, tissues or organs from one individual to another. When the graft is syngeneic (genetically identical to the host) or autologous (a transplant from one individual into itself) there is perfect histocompatibility and no significant immune response is elicited. When there is incompatibility, in general an immune response is directed against the foreign antigens. Allorecognition is the term used to describe the recognition of transplanted allogeneic tissues by the host, while alloresponse denotes the effector mechanisms recruited in the reaction to the foreign tissue and the outcome of those effects (25). Allorecognition can proceed *via* two mechanisms: *direct allorecognition,* where T cells recognize epitopes on the intact donor MHC molecules displayed in the surface of transplanted cells; or *indirect allorecognition*, in which donor MHC molecules are processed and presented as peptides by self-MHC molecules (in a similar fashion to antigen processing) (26).

1.1.1.1 T cell responses to transplants

Alloreactive T cells can identify foreign tissue by both **direct allorecognition** and **indirect allorecognition**. While direct allorecognition can be carried out by both $CD8^+$ and $CD4^+$ T cells (by recognizing donor MHC Class I and MHC Class II molecules, respectively), indirect allorecognition is exclusive of $CD4^+$ T cells, which identify the recipient APC displaying MHC Class II - donor peptide complexes.

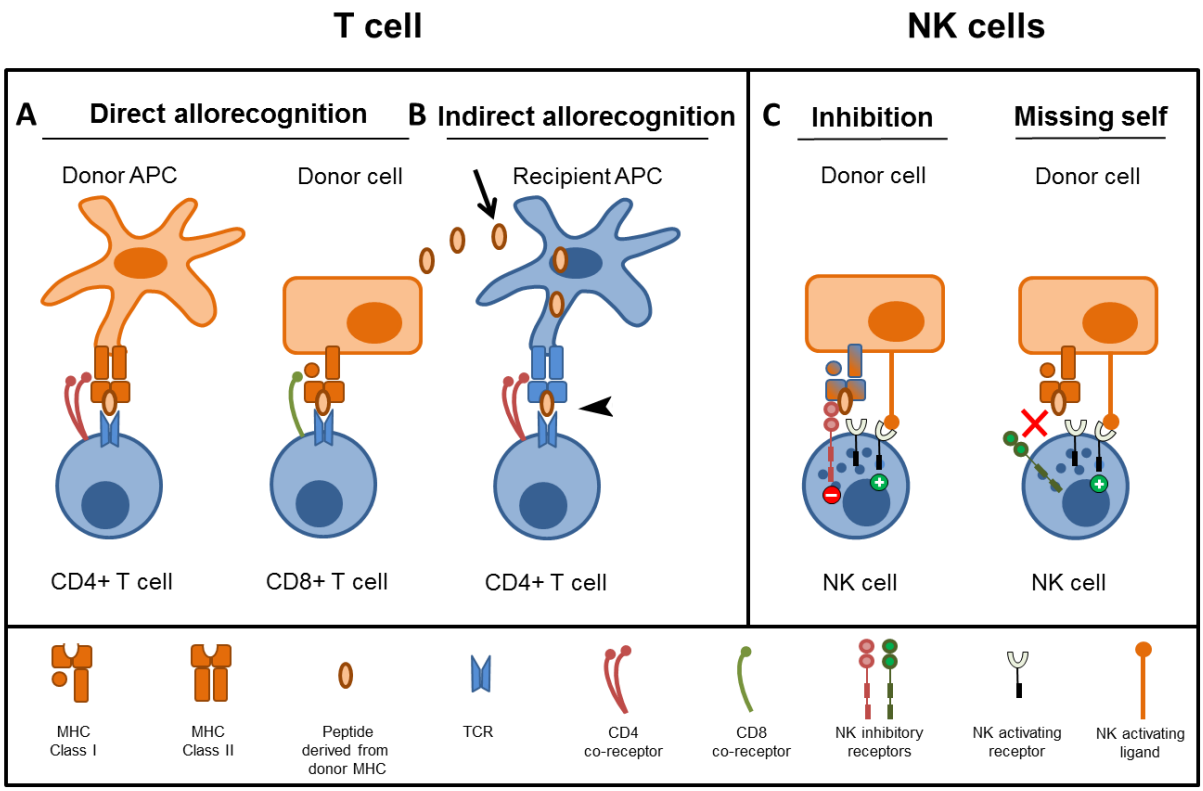

Figure 1. Transplant allorecognition mechanisms by T and NK cells. A) Direct allorecognition of MHC Class II in APC by $CD4^+$ T cells (left) and of donor MHC Class I by $CD8^+$ T cells (right). **B)** Indirect allorecognition of recipient MHC/donor peptide complex in receptor APC by $CD4^+$ T cells. Donor MHC fragments (antigens) are taken up by APCs (black arrow) and presented via MHC Class II (arrowhead) to $CD4^+$ T cells. **C)** NK recognition of donor tissue. On the left, the donor shares MHC alleles with the recipient, inhibiting NK responses. On the right, recipient NK does not recognize donor MHC (missing self), leading to activation.

The vigorous nature of the **direct alloresponse** and its immediacy (acute rejection) in comparison with the indirect pathway is the result of direct recognition of intact MHC by T cells without the need for processing and presentation of self-MHC (25) (Figure 1A).

The indirect pathway differs from the direct pathway by the requirement for antigen processing. Alloantigens shed from a graft are processed as exogenous antigens and presented by APCs in association with self-MHC Class II. These responses are considerable slower compared to the direct pathway (Figure 1B). The indirect response is likely to be responsible for long term responses to engrafted tissues once donor APCs are exhausted.

The presence of donor APCs in transplanted tissues at the time of transplantation can lead to a vigorous anti-donor alloresponse in the early period post-engraftment. The death and removal of these APCs over time reduces this response. The indirect alloresponse, on the contrary, requiring antigen capture and processing, is less rapid than the direct pathway but continues for the life of the graft, as graft derived antigens are continuously acquired and processed (chronic rejection). Acute rejection of transplanted tissues is more commonly observed in the early post engraftment period, while tolerance to grafts develops at a later time point. This correlates with demonstrations that regulatory T cells able to mediate transplant tolerance have indirect rather than direct pathway alloreactivity (26).

1.1.1.2 B cell responses to transplants

B cells can contribute to transplant rejection via two major mechanisms – as antigen-presenting cells (APC), contributing to indirect allorecognition, and as antibody producers. B cells can be activated and secrete antibodies specific to donor MHC molecules. The presence of donor-specific HLA antibodies in the circulation of transplant recipients has a negative impact on transplantation outcome (18). These alloantibodies mainly induce rejection by complement-dependent mechanisms. The complement system is a complex set of proteins that, when triggered by antibody-antigen, lead to a cascade of reactions resulting in cell lysis by membrane disruption, or chemo-attraction of macrophages and other phagocytes able to clear the foreign cells (27).

1.1.1.3 Natural Killer cell responses to transplants

NK cells express inhibitory receptors that bind groups of alleles of self-MHC Class I and are responsible for protection of self tissue from NK attack. In a MHC-mismatched transplant, MHC class I alleles expressed in the donor tissue are different from the recipient's and unable to inhibit NK (missing-self). NK cell-mediated rejection of transplants is included in the direct alloresponse, leading to acute rejection (Figure 1C) (28).

2. Natural Killer (NK) cells – between innate and adaptive immunity

Human Natural Killer cells (NK) are a subpopulation of lymphocytes, like B and T cells, that originate from hematopoietic progenitors in the bone marrow. Unlike T cells, all their development takes place in the bone marrow, from where they migrate into various tissues, including secondary lymphoid organs and the blood (circulating or peripheral NK cells – pNK) (22).

NK were initially defined by their ability to kill target cells without prior immunization. While T cells require a first encounter with a pathogen to prime their activity, differentiation and proliferation, leading to formation of memory, NK can deliver a rapid response upon first encounter (21). One of the hallmarks of NK is their lack of need to recognize specific epitopes or antigens to be activated. NK express non-rearranged somatic activating receptors that identify common ligands on target cells – stress ligands. However, NK need another layer of regulation to keep them from attacking healthy tissue. In fact, NK express inhibitory receptors that recognize self MHC Class I molecules – different receptors recognize different groups of alleles (28). Healthy cells always express MHC Class I molecules, flagging them to be spared by NK. Infected or tumor cells upregulate stress-induced surface proteins and, as a mechanism of immune evasion, also downregulate MHC molecules to escape attack by T cells. The combination of stress signals and low MHC expression works as a danger signal for NK, which take over the immune response (29).

2.1 NK cell phenotype

Two main populations of NK can be found in peripheral blood, defined by the expression of two surface markers: CD56 and CD16. About 90% of NK in the blood are $CD56^{low}CD16^{+}$, the main cytotoxic subpopulation, containing cytolytic granules as $CD8^{+}$ T cells. The remaining 10% are $CD56^{high}CD16^{-}$ and are not cytotoxic. $CD16^{-}$ NK do not contain cytolytic molecules and are defined by the secretion of cytokines (e.g. IFN-γ). $CD16^{-}$ NK are also the main population of NK found in secondary lymphoid organs. $CD56^{high}CD16^{-}$ and $CD56^{low}CD16^{+}$ subsets are also sequential stages of maturation and $CD56^{high}$ can progress to $CD56^{low}$ cells upon activation, as occurs in reactive lymph nodes or inflamed tissues (30).

NK can be further subdivided in an impressive array of subsets, based on their expression of activating and inhibitory receptors (31). The receptors belong to different families of proteins and recognize different ligands and will be addressed in more detail below. The most interesting characteristic of NK cell receptors is their random expression in the population of NK of a given individual. Each NK can express any number of receptors from zero to 30+ in different combinations. The pattern of receptors of each NK is defined during maturation and is maintained throughout the life of the cell and during division, generating clones. The balance between activating and inhibitory receptors, combined with the expression of ligands in the target cell, is crucial for the outcome of the NK cell response (32).

2.2 NK cell immune synapse and degranulation

Activation of NK triggers a complex and highly regulated response leading to cytolytic granule release, resulting in the death of the target cell. Conversely, interaction of inhibitory receptors with their ligands negatively regulates NK activity. Activation and inhibition take place at the specialized contact sites between NK and target cells, known as the immunological synapse (IS) (33). Upon encountering a target cell (i.e. a cell with downregulated MHC Class I molecules and expressing stress signals at the surface), NK form an activating synapse. This requires several steps: I. Contact and adhesion, led by integrins such as LFA-1. II. Receptor ligation and segregation linked to initial signaling. III. Actin cytoskeleton rearrangements (tight conjugation). IV. Further receptor clustering and sustained signaling (signal amplification). V. Microtubule cytoskeleton rearrangements (MTOC polarization). VI. Granule polarization and degranulation. VII. IS disassembly (34).

Similarly to T cells, NK contain cytolytic granules with molecules such as the serine proteases granzymes and the pore-forming protein perforin. Upon synapse formation, these granules exocytose their contents, releasing perforin which will polymerize in the target cell surface, opening a pore which allows granzymes to enter. Granzymes are then responsible for apoptosis by triggering caspase-dependent and independent pathways (Figure 2A) (14, 15).

2.3 Other mechanisms of NK cell target killing

Synapse formation is the main killing mechanism employed by NK, but other pathways can be followed. For example, NK express death receptor ligands such as TRAIL or Fas-L, which engage death receptors on the surface of target cells and initiate target cells apoptosis(35). Furthermore, NK cells can also secrete cytokines such as IFN-γ and TNF-α which can increase inflammation and activate T cells and dendritic cells to ensure clearance of target cells (Figure 2B and C) (36).

2.4 Activating and Inhibitory receptors in NK cells – a balance

NK express several receptors responsible for modulating NK function when facing a target cell. The most important receptors can be subdivided in 5 families: Natural Cytotoxicity Receptors (**NCR**), Fc receptors (**FcR**), C-type lectins, Leukocyte Immunoglobulin-like receptors (**LILR**) and Killer cell Immunoglobulin-like receptors (**KIR**). While some families include only activating (NCR and FcR) or inhibitory receptors (LILR), others include both

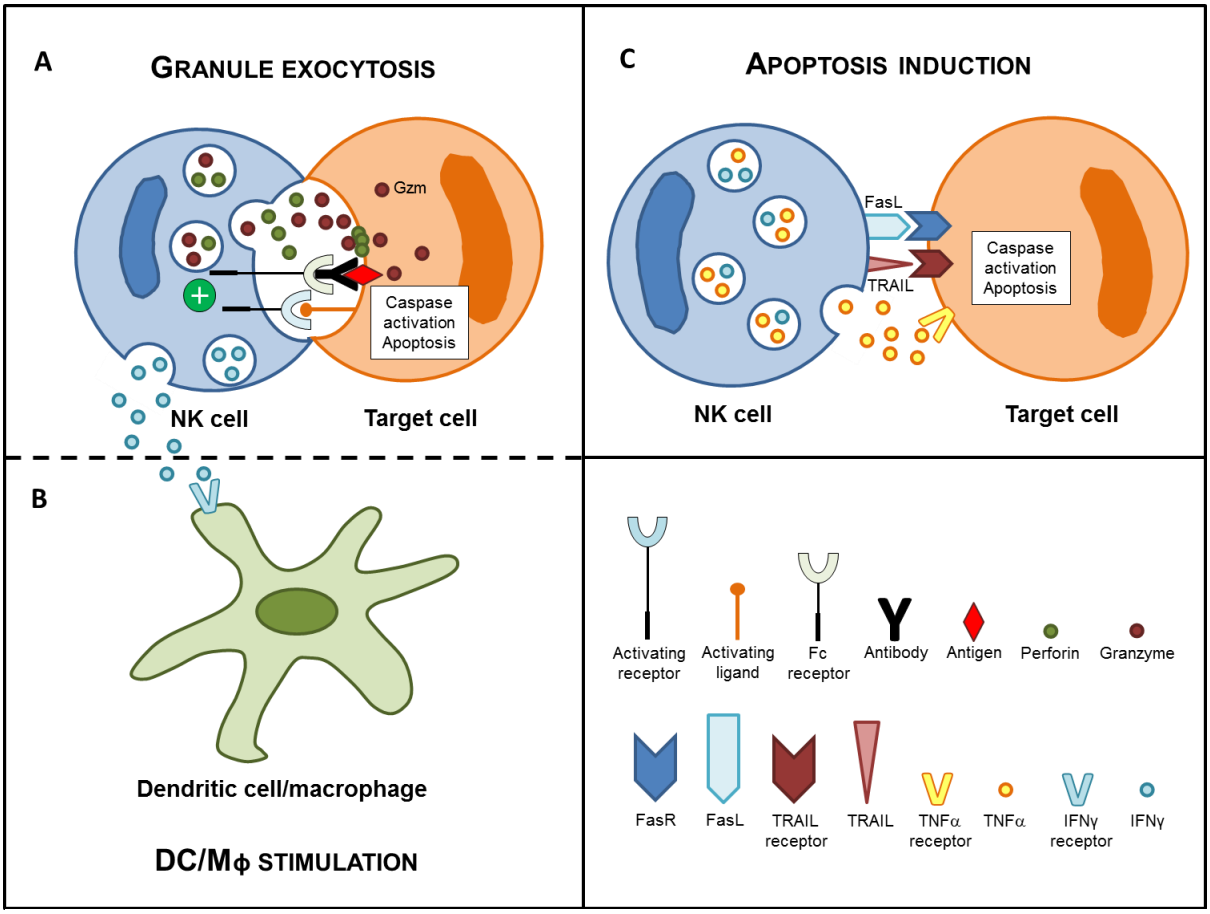

Figure 2. NK cell target killing mechanisms. A) Granule exocytosis. The recognition of activating ligands (and lack of MHC Class I molecules) or antibodies bound to target cell antigens (antibody dependent cell cytotoxicity) leads to immune synapse formation and degranulation of NK. The same stimuli lead to cytokine secretion (e.g. interferon-gamma, IFN-γ) that increase inflammation and activate T cells, dendritic cells (DC) and macrophages (Mϕ) **(B). (C)** Apoptosis induction. The engagement of death-inducing receptors (FasR, TRAIL receptor) by NK ligands (FasL, TRAIL) and tumor necrosis factor (TNF)-α production by NK lead to apoptosis of target cells.

types (KIR and C-type lectins) (Figure 3 and Table II). NK cells express several other receptors (e. g. adhesion molecules, integrins, etc.), that contribute to NK activation but, for clarity, such proteins will not be addressed here.

2.4.1 Natural Cytotoxicity Receptors (NCR)

The major NK receptors able to induce NK-mediated killing are the natural cytotoxicity receptors (NCRs) belonging to the Immunoglobulin-like superfamily — **NKp46** (**NCR1**), **NKp44** (**NCR2**) and **NKp30** (**NCR3**). The activating receptors are organized into multi-chain complexes where the ligand-binding and signal-transducing sub-units are separate. The ligands for these receptors are mostly unknown but believed to be viral proteins or stress ligands expressed at the surface of infected cells. The signal transducing units are diverse, and include CD3ζ, FcεRγ and DAP12 (DNAX activation protein of 12kDa). DAP12 contains an intracellular immunoreceptor tyrosine-based activation motif (ITAM) that recruits protein kinases involved in activating signaling such as Spleen tyrosine kinase (SYK) and Zeta-chain-associated protein kinase 70 (ZAP70). These receptors also use different downstream pathways leading to the NK activation (28).

2.4.2 C-type lectins

C-type lectins are a family of transmembrane proteins characterized by a type II membrane orientation (extracellular C terminus) and the presence of a C-type lectin domain. This family includes both activating and inhibitory receptors of NK.

The best-characterized activating C-type lectin is **NKG2D**. NKG2D recognizes “induced-self” ligands – molecules not expressed or expressed at low levels on normal cells, but upregulated on unhealthy cells due to the activation of pathways associated with cancer, infection or stress. NKG2D ligands include MHC class I polypeptide-related sequence A and B (MICA/MICB) and UL16-binding proteins (ULBP) 1-6 in humans. NKG2D associates with the signal transducing proteins DAP10 or DAP12 (Figure 3). NKG2D recognition of target cells is one of the most important pathways of killing of tumor cells (37).

In addition, two other C-type lectins have been well studied: **NKG2A** and **NKG2C**. Both of these proteins form a heterodimeric receptor in association with another type II transmembrane molecule: CD94 (Table II). Both CD94/NKG2C and CD94/NKG2A are specific for the non-classical MHC class I (class Ib) molecules HLA-E (38). While NKG2C is an activating receptor associated with DAP12 (Figure 3), NKG2A is an inhibitory receptor that transduces negative signaling through immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences present in its cytoplasmic tails (Figure 3). Upon tyrosine phosphorylation, ITIM interacts with Src homology region 2 domain-containing phosphatase (SHP)-1/2 that disrupt the activating signaling pathways (39).

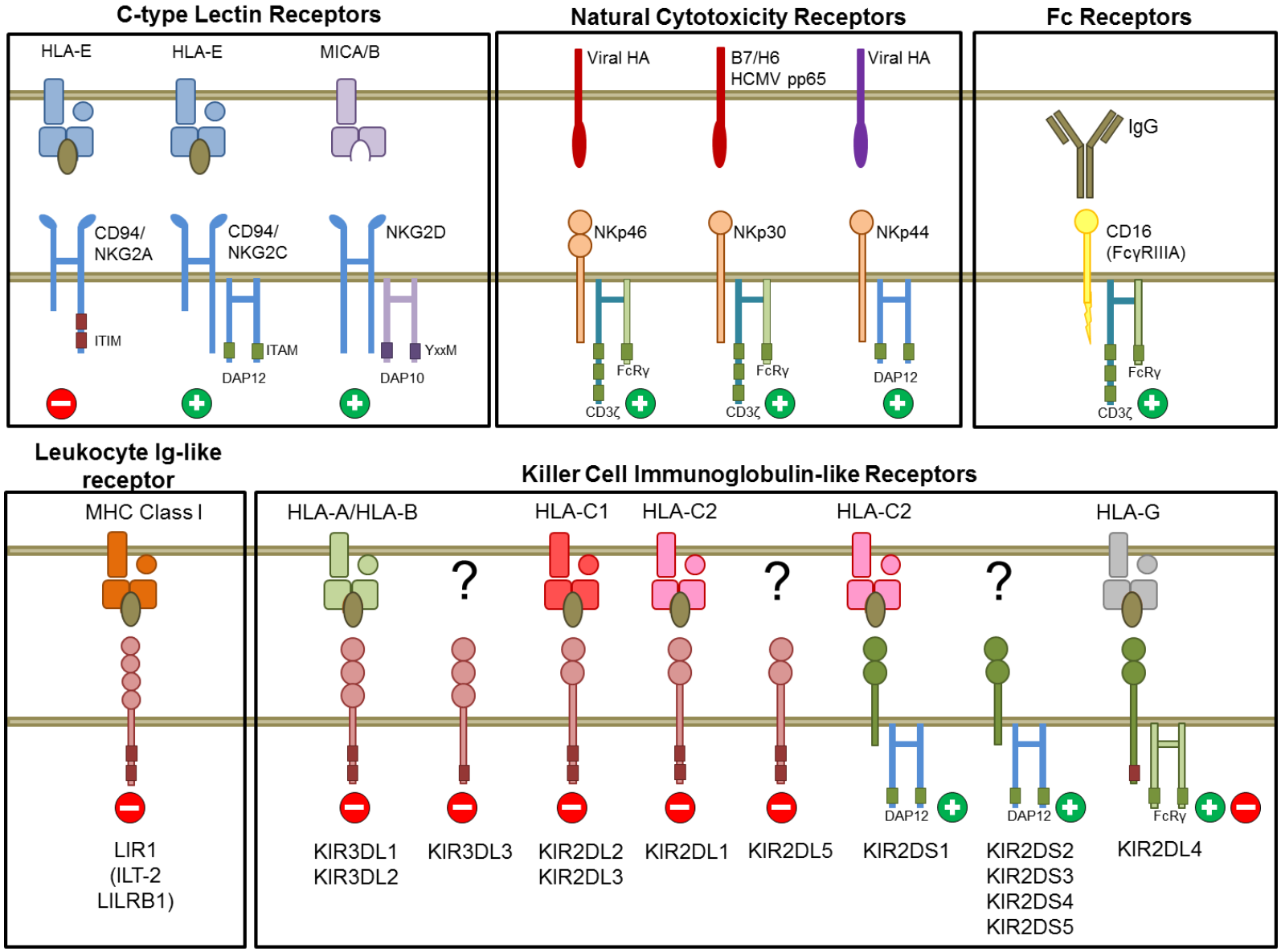

Figure 3. NK cell receptors and ligands.

2.4.3. Fc Receptors

Fc receptors are commonly expressed in macrophages and bind the Fc common part of antibodies bound to antigens on the surfaces of other cells. NK express the Fc receptor **FcγRIII** (**CD16**), which triggers antibody-dependent cell cytotoxicity (ADCC). FcγRIII signals through FcεRγ and CD3ζ transducers (Figure 3), leading to degranulation and cytokine production (32).

2.4.4 Leukocyte Immunoglobulin-like Receptors (LILR)

The Leukocyte immunoglobulin-like receptor genes (**LILRB1** and **LILRB2**) are mapped in the chromosome 19 Leukocyte Receptor Complex (LRC) region. LILRB1/LIR1 and LILRB2/LIR2 are inhibitory receptors that display four extracellular Ig-like domains and a cytoplasmic portion characterized by ITIM motifs that mediate association with the SHP-1 phosphatase (Figure 3) (40). These receptors are expressed on monocytes and dendritic

Family	Receptor	Ligand	Function
Natural Cytotoxicity Receptors (NCR)	NKp46 (NCR1)	Viral HA	Activating
	NKp44 (NCR2)	HCMV pp65, B7/H6	Activating
	NKp30 (NCR3)	Viral HA	Activating
C-type Lectins	NKG2D	MICA/B, ULPBs	Activating
	CD94/NKG2C	HLA-E	Activating
	CD94/NKG2A	HLA-E	Inhibitory
Fc Receptors	FcγRIII (CD16)	Antibody Fc	Activating
Leukocyte-Ig like receptors (LILR)	LILRB1	MHC Class I	Inhibitory
	LILRB2	MHC Class I	Inhibitory
Killer Cell Immunoglobulin-like Receptors (KIR)	KIR2DL1	HLA-C2 (Lys 80)	Inhibitory
	KIR2DL2/3	HLA-C1 (Asn 80)	Inhibitory
	KIR2DL4	HLA-G	Activating
	KIR2DL5	Unknown	Inhibitory
	KIR2DS1	HLA-C2 (Lys 80)	Activating
	KIR2DS2	Unknown	Activating?
	KIR2DS3	Unknown	Activating?
	KIR2DS4	HLA-Cw4	Activating
	KIR2DS5	Unknown	Activating?
	KIR3DL1	HLA-Bw4	Inhibitory
	KIR3DL2	HLA-A3/A11	Inhibitory
	KIR3DS1	Unknown	Activating

Table II – NK cell receptors and ligands.

cells and LILRB1 is also expressed on B-cells, some T-cells and subsets of NK. They bind to the non-classical class I molecules HLA-G, HLA-F and a similar broad spectrum of HLA-A, -B and -C alleles (41).

2.4.5 Killer cell Immunoglobulin-like Receptors (KIR)

Killer cell Ig-like Receptors (**KIRs**) are the major MHC Class I NK receptors and have specificity for discrete groups of HLA Class I alleles. Their genes map in the leukocyte receptor complex (LRC) located on human chromosome 19q13.4 (42). Although most of the functional KIRs are inhibitory, a few have activating function. The ~150 kb KIRs cluster consists of 14 genes and 2 pseudogenes. However, due to rearrangements that lead to deletions and duplications during evolution, each individual possesses a different number of genes in this locus. Based on their gene content, two haplotypes have been defined: haplotype A characterized by identical genes number and content (with mostly inhibitory receptors and only one activating receptor gene), and haplotype B, highly variable in terms of gene number (Figure 4). The KIRs cluster may have evolved almost 50 million years ago from an ancestral gene (KIR3DL0 also known as KIR3DX1) (43).

The inhibitory KIR receptors have an extracellular portion with two (**KIR2D**) or three (**KIR3D**) Ig-like C2-type domains, a transmembrane region characterized by non-polar amino acid residues, and a long (**L**) cytoplasmic tail containing two ITIM sequences (Figure 3). Upon recognition of sufficient levels of MHC class I molecules, KIRs generate src kinase-dependent tyrosine phosphorylation of ITIMs. Phosphorylated ITIMs serve as docking motifs for SHP1 or SHP2 through the formation of a ternary complex with α-arrestin-2. This complex finally blocks the signaling cascades induced by activating receptors (44). The inhibition of NK-mediated cytotoxicity requires co-aggregation with triggering receptors and it is focused in the immune-synapse area and not over the whole cell membrane. This way, an NK cell, although inhibited in the limited area of contact with a particular target cell, may still survey for transformed/infected tissues and maintain their killing potential (45).

Among the 14 KIR genes described so far in humans, five encode receptors associated with activating rather than inhibitory potential (Table II) (43). These molecules have a shorter (**S**) cytoplasmic tail not containing ITIM sequences, and a transmembrane portion displaying a polar lysine residue. This residue forms a salt bridge with the opposite charged amino acid present in the transmembrane region of the ITAM–containing adaptor DAP12 (Figure 3) (46).

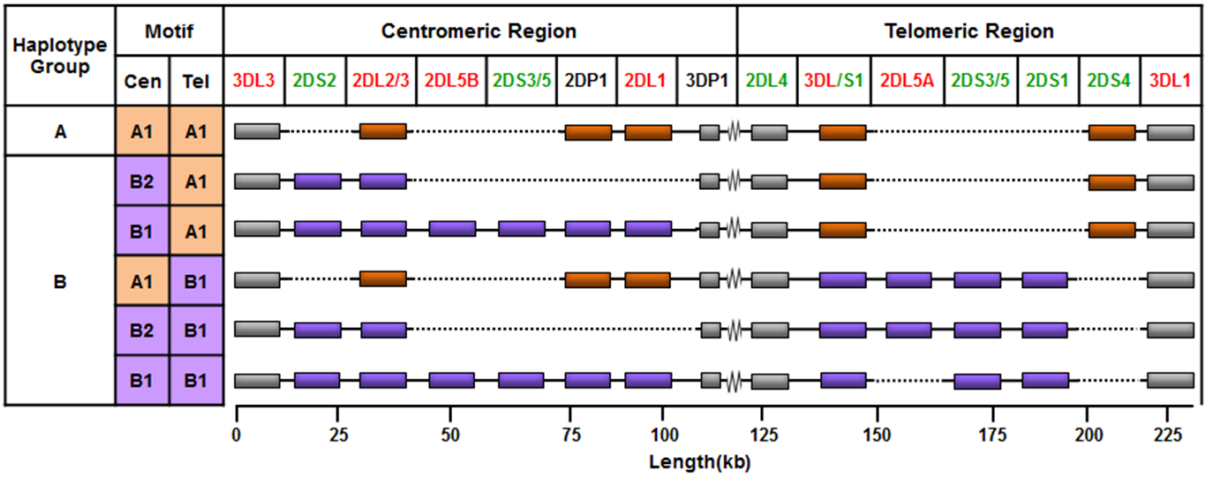

Figure 4. Human KIR haplotypes. The human KIR locus consists of centromeric and telomeric regions that recombine to produce KIR-A and KIR-B haplotypes with a distinctive gene content. Structures of common haplotypes are shown. Gray boxes indicate conserved framework genes; orange boxes indicate genes characteristic of KIR-A haplotypes; purple boxes indicate KIR-B haplotype–specific genes. The centromeric (Cen) and telomeric (Tel) gene-content motifs are listed at left. Adapted from reference (212).

Activating KIRs are unevenly distributed between KIR haplotypes. KIR-B haplotypes are the ones with higher, although variable, numbers of genes encoding activating KIR, while haplotype KIR-A is characterized by a single gene coding for triggering Ig-like receptor (KIR2DS4) (47).

Different from the other long-tailed KIR receptors, KIR2DL4 triggers NK activation rather than inhibition. This is possible because, although it displays a single non-functional ITIM-like sequence, it may associate with FcγRI chain through the formation of a transmembrane-embedded salt bridge (Figure 3). KIR2DL4 is able to bind soluble HLA-G molecules inducing the release of proinflammatory/proangiogenic mediators, thus promoting vascularization in maternal decidua, site of HLA-G expression, and facilitating implantation during pregnancy (48, 49).

2.4.5.1 KIR specificity

Different KIR family members, particularly the ones with inhibitory function, have been characterized for their HLA class I allele specificity. The distal domains of KIR receptors have been found to be responsible for defining the HLA class I allele specificity. Most KIR2D molecules are specific for the recently evolved HLA-C alleles (Table II). KIR/HLA-C alleles recognition is based on dimorphic residues present in the HLA-C α1-helix at position 80 (Table II and Figure 3). Thus, KIR2DL1 and KIR2DS1 bind HLA-C2 alleles (Cw2, Cw4, Cw5, Cw6, Cw15 and Cw16) characterized by the presence of a Lys80, while both KIR2DL2 and KIR2DL3 recognize HLA-C1 alleles represented by Cw1, Cw3, Cw7, Cw8, Cw12, Cw13 and Cw14 characterized by an Asn80 (Table II) (32). The three-domain receptor KIR3DL1 specifically binds HLA-B alleles sharing the Bw4 epitope (50). Other HLA-

A alleles (HLA-A3 and -A11) bind KIR3DL2 and apparently display some peptide specificity (Table II) (51).

2.5 NK cell education

Until recently, it was thought all NK expressed at least one inhibitory receptor for self-MHC class I molecules; this model provided a satisfactory explanation for self-tolerance and for the involvement of NK in missing-self recognition (52). However, studies of humans and mice that lacked MHC class I molecules had shown they were hyporesponsive, even though these individuals had normal numbers of NK. These NK were not able to release their cytotoxic granules to kill MHC Class I – negative target cells (53). Hyporesponsive NK were thought to be unique to MHC class I-deficient individuals, but work from several laboratories has now shown they also exist in normal humans and mice, and are characterized by a lack of inhibitory receptors (53). The active engagement of inhibitory receptors on NK by self-MHC class I molecules during maturation is the key event that determines whether an NK will be functionally capable of mediating missing-self recognition (education), or whether it will be of low cytotoxicity following stimulation (54). There are several models to explain how this education/licensing occur. However, the **rheostat model** is the most widely accepted.

The rheostat model proposes NK have increased or decreased responsiveness depending on the strength of the inhibitory signal received during maturation. For example, NK with no or low expression of inhibitory KIR for self HLA molecules will establish weak interactions with their ligands during maturation, leading to lower cytotoxicity upon later encounter of target cells in the periphery. This suggests NK education operates in a quantitative manner (cytotoxicity is increased or decreased) and states that NK cytotoxicity can either be ‘tuned up’ (arming) or ‘tuned down’ (disarming) rather than in a binary manner (e.g. cytotoxicity is completely shut down or turned on). The tuning of NK thus depends on the inhibitory signal received by individual NK cells (55).

The licensing or education of NK by their receptors adds a level of sensitivity to self MHC ligand expression. Productive licensing through inhibitory signaling provides a two-fold benefit to NK function. It serves to simultaneously enhance effector responses (e.g. IFN-γ secretion and cytotoxicity) and broaden the NK cell’s target specificity to include aberrant cells that would not be detected by stimulatory receptors alone (56).

2.6 NK cell response to viruses, tumors and transplants

NK play a key role in the immune fight against viral infections and tumor cells and can both have a beneficial or harmful role during transplantation. Due to the high polymorphism of MHC molecules and the diversity of KIR haplotypes in the population, certain combinations of MHC-KIR can lead to different outcomes (Figure 5).

2.6.1 Viral responses

Viruses are intracellular pathogens that take advantage of the host cell machinery to replicate. Cells infected with viruses will enter a state of stress leading to the expression of ligands for activating NK receptors (e. g. MICA/B, recognized by NKG2D). Certain viruses, such as influenza and human cytomegalovirus (HCMV) express proteins that are displayed at the surface of infected cells and act as ligands for NK receptors [e.g. influenza hemagglutinin (HA) – NKp46 and HCMV pp65-NKp30]. To escape T cell immune responses, most viruses interfere with the MHC Class I processing and surfacing, leading to a lower expression of MHC by infected cells. This phenomena leads to "missing-self" recognition by NK, tipping the balance towards activation instead of inhibition (Figure 5A) (57).

Interestingly, human studies provide evidence that individuals of matched KIR/HLA class I may exhibit enhanced viral control. These correlations have been observed in immune responses to HIV-1 (3DL1:HLA-Bw4), hepatitis C virus (HCV) (2DL3:HLA-C1), influenza (3DL1:HLA-Bw4;2DL2/DL3:HLA-C1), and HCMV (58). The combined presence of KIR3DS1 and HLA-Bw4 alleles was associated with significantly slower progression to AIDS, lower viral load and slower decline of $CD4^+$ T cells (59–61). In addition, KIR3DS1 and KIR2DS1

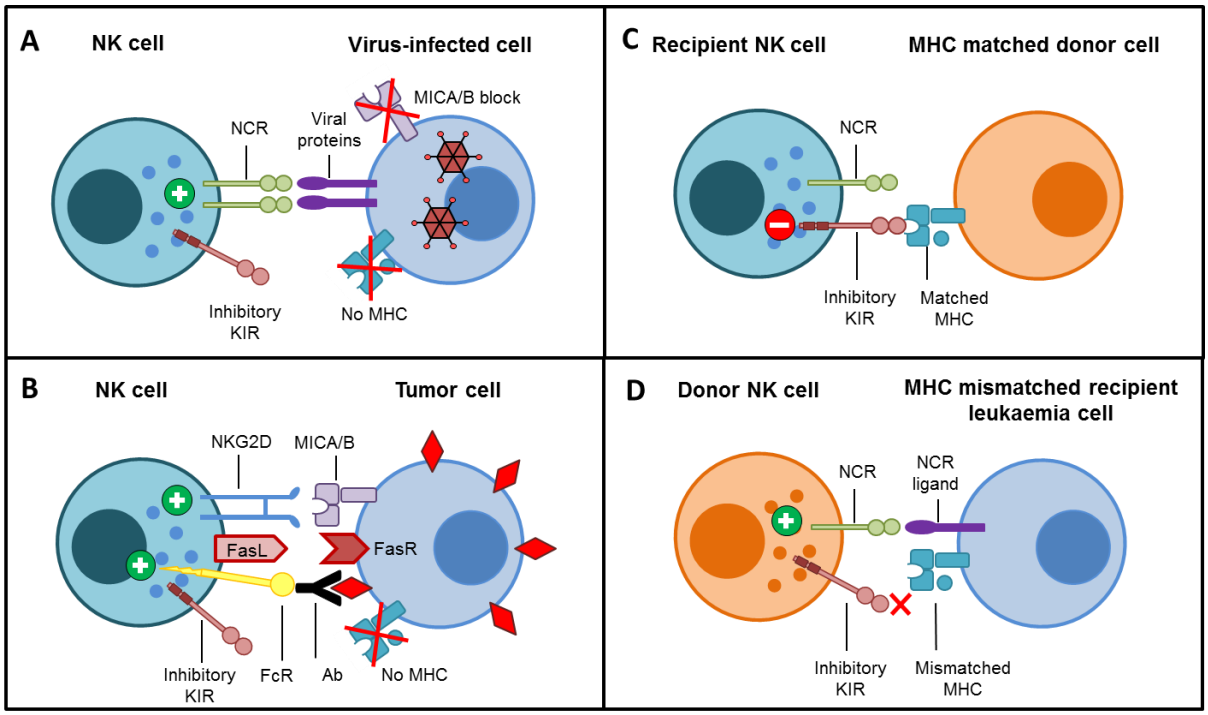

Figure 5. NK cell response to viruses, tumors and transplants. **A)** Viral infected cells express viral and stress proteins that serve as activating ligands for natural cytotoxicity receptors (NCR) in NK and downregulate MHC Class I, leading to NK activation and degranulation. **B)** Tumor cells upregulate stress ligands (e.g. MICA/B), death receptors (e.g. FasR) and misfolded proteins that can be recognized by antibodies. In addition, tumor cells downregulate MHC Class I. NK cells are activated through NKG2D (MICA/B receptor) and FcR (antibody receptor, triggering ADCC), leading to degranulation, and can engage death receptors in tumor cells through TRAIL and FasL expression. **C)** MHC-matched transplant donor cells are protected from NK attack due to inhibitory KIR engagement. Mismatched receptors (missing self) do not inhibit NK and are rejected. **D)** Missing self recognition by donor NK, in addition to engagement of activating ligands expressed by recipient leukaemia cells, lead to donor NK activation and elimination of leukaemia cells.

were shown to be protective against respiratory papillomatosis caused by human papillomavirus (62). Finally, activating KIRs characteristic of B haplotypes have been shown to play a role in NK mediated clearance of HCMV infection following hematopoietic stem cell (HSC) or solid organ transplantation (63–66) (Table III).

2.6.2 Tumor responses

Tumor (or cancer) cells are modified cells that proliferate uncontrollably and accumulate uncorrected mutations. This phenomenon can lead to the production of new and misfolded proteins and generates new epitopes which can be presented via MHC to T cells, or recognized by antibodies produced by B cells. NK can then eliminate the tumor cells by antibody-dependent cell cytotoxicity (ADCC). In addition and similarly to virus-infected cells, the high stress level of tumor cells leads to the upregulation of proteins that serve as activating ligands to NK receptors (80). Tumor cells also downregulate of MHC Class I molecules, disrupting T cell responses and leading to NK activation through missing self recognition. Tumor cells may also upregulate FasR and other death inducing receptors for which NK express ligands like FasL, leading to caspase-dependent apoptosis of the tumor cell (Figure 5B) (81).

Receptor	Haplotype	Ligand	Function	Clinical Effects
KIR2DL1	A^+B	HLA-C2	Inhibitory	Recurrent HCMV infection, severe influenza (67, 68)
KIR2DL2/3	A^+B	HLA-C1	Inhibitory	Severe influenza (69) Resolution of HCV infection (70)
KIR2DS1	B	HLA-C2	Activating	Protection from pregnancy complications (71) Decreased incidence of HCMV infection after hematopoietic stem cell and solid organ transplantation (63) Susceptibility to psoriasis (72, 73)
KIR2DS2	B	Unknown	Activating	Scleroderma and rheumatoid vasculitis (74, 75)
KIR2DS3	B	Unknown	Activating	Decreased incidence of HCMV infection after hematopoietic stem cell and solid organ transplantation (63)
KIR2DS5	B	Unknown	Activating	Protection from pregnancy complications in African population (76, 77)
KIR3DS1	B	HLA-Bw4?	Activating	Slower progression to AIDS (59, 78)
NKG2C	A^+B	HLA-E	Activating	Increased clearance of HCMV-infected cells through ADCC (79)

Table III – NK cell receptors, ligands and clinical effects.

2.6.3 NK cells in haploidentical haemopoietic stem cell transplantation (HSCT)

In some cases of hematopoietic stem cell transplantation (HSCT), there are mismatches between the KIR-HLA combinations of donor and recipient. This situation is a classical "missing-self" setting, which leads the recipient NK to attack the donor cells due to lack of inhibition (Figure 5C). However, HSCT is a special transplant because it contains immune cells from the donor, including NK cells. A fraction of NK from the donor may be able to kill target cells from the recipient and lead to Graft-vs-Host Disease (GvHD) (82). Remarkably, the contrary has been observed: the presence of alloreactive donor-derived NK have been correlated with highly improved survival after HSCT for acute myelogenous leukaemia (AML) and alloreactive NK promote engraftment and decrease leukaemic relapses (83). Leukaemic cells express high levels of ligands for NCR that promote lysis by NK, and combined with missing self, leads to their elimination by donor NK cells (Figure 5D). In particular, Moretta and others showed a mismatch between homozygous HLA-C1$^+$ and HLA-C2$^+$ donor/receptor pairs lead to the elimination of leukemia blasts (e.g. donor KIR2DL1$^+$ NK kill leukemia blasts from a HLA-C2$^-$ receptor) (84). (Figure 5D).

Recent experimental evidence has revealed some activating KIR are also involved in the graft-vs-leukemia effect. In a recent study, Moretta and others provided direct evidence that donor KIR2DS1$^+$ NK play a crucial role in the lysis of receptor C2/C2 leukaemia blasts (85). Cytolysis was inhibited by monoclonal antibody (mAb)-mediated blocking of KIR2DS1. These data strongly support the clinical relevance of activating KIRs in the elimination of leukaemic cells and the benefit of HLA-C mismatched HSCT.

3. Pregnancy - an immunological paradox

Human reproduction occurs by viviparity. This carries obvious advantages for the fetus, which enjoys protection and efficient exchange of gas and nutrients with the maternal blood (86). Human placentation is classified as haemochorial, the most invasive type of placentation. In preparation for implantation, the human uterine mucosa is transformed into **decidua**, a highly specialized tissue able to support placental invasion. Following implantation, the blastocyst develops into an inner cell mass (the fetus precursor) and an external layer of cells (trophoblast) which gives rise to the placenta and fetal membranes (Figure 6). Over gestation, the trophoblast evolves into a fully formed placenta, constituted by several layers of cells. **Villous trophoblasts** (VT) form tree-like structures that extend the surface of the placenta in contact with maternal blood. VT include **cytotrophoblast** (proliferating cells composing the inner cell layer of the villi) and **syncytiotrophoblast** (composed by cells that fuse together to form a single layer over the villi). In addition, the placenta contains **extravillous trophoblast (EVT**). EVT is the most invasive type, detaching from villi and infiltrating the uterine tissue and the maternal vessels. EVT replaces the

endothelium of uterine spiral arteries, while the smooth muscle cell layer is degraded. These processes open the maternal vessels and reduce resistance to allow for a low pressure blood flow into the placenta and intervillous space. The maternal blood surrounds the syncytiotrophoblast and facilitates the exchange of nutrients and gases with the fetus (86) (Figure 7). Shallow placentation or inadequate maternal vessel transformation can lead to serious conditions later in pregnancy that include intrauterine growth restriction, stillbirth and preeclampsia, a hypertensive disorder of pregnancy (87–89).

The evolution of hemochorial placentation allowed for a better support of fetal development. However, such an intimate contact between mother and fetus exposes the fetal trophoblast (a semi-allogeneic entity) to the maternal immune system. In previous sections, the immune responses that can be raised against mismatched transplants were discussed; however, in human pregnancy, the mother tolerates a semi-allogeneic organ for 9 months. This phenomenon raises a question that has fascinated immunologists for 60 years, starting with Sir Peter Medawar. In his monolog “Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates,”(90) he proposed three explanations for maternal immunological tolerance: physical separation of maternal and fetal tissues, the antigenic immaturity of the fetal tissues, and immunological inertness of the mother. Although none of those explanations have held up, they had a profound influence on research concerned with maternal-fetal tolerance and mechanisms of fetal immune evasion (91).

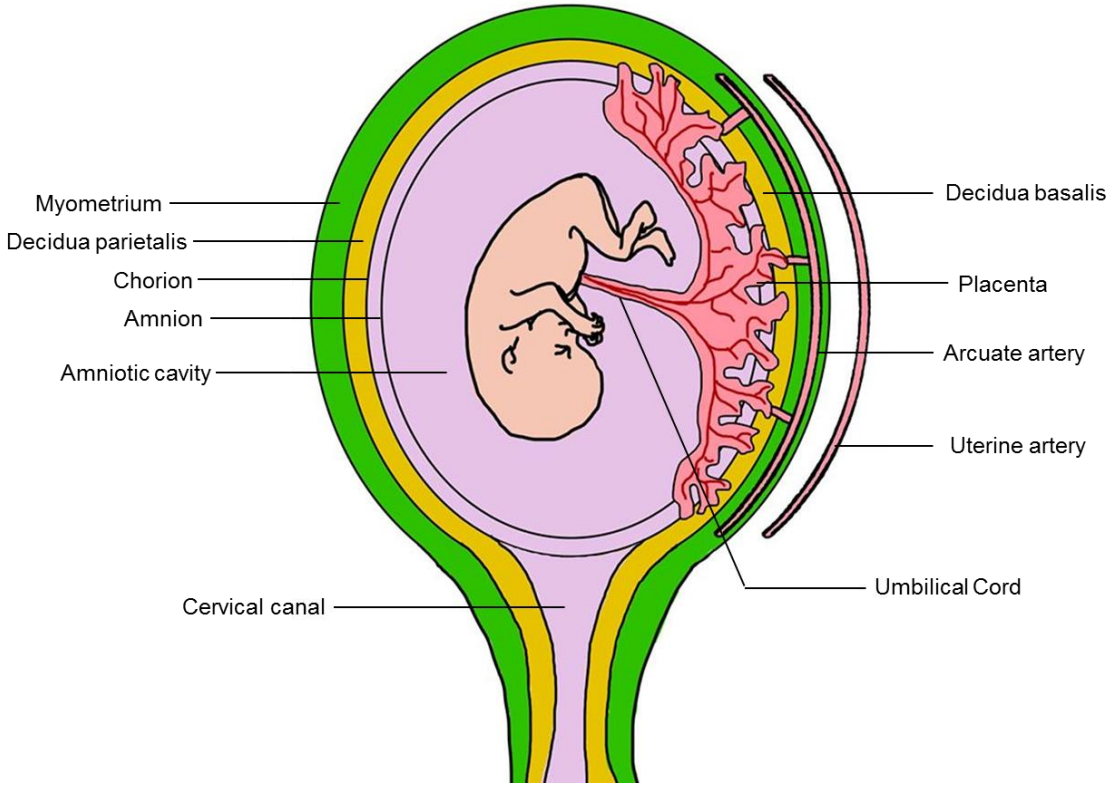

Figure 6. Diagram of the gravid uterus showing the placenta, extraembryonic membranes and decidualized uterus wall. Image courtesy of Ana Haydeé Linares, adapted from reference (94).

3.1 MHC class I and II expression in human placenta

The fetal-placental cells (trophoblasts) come in close contact with the maternal immune system in two interfaces. The syncytiotrophoblast is surrounded by maternal blood, while the extravillous trophoblast invades the uterine decidua and encounters the tissue resident immune cells (Figure 7). To understand the mechanisms involved, both the MHC status of trophoblast cells and the maternal leukocytes present in the lining of the uterus are important considerations.

The **syncytiotrophoblast layer**, which is in direct contact with the maternal immune cells, does not express any MHC molecules or NK stress ligands. This way, the syncytiotrophoblast can be considered immunologically inert, not being able to elicit $CD8^+$ T cell or NK cell-mediated immune responses (92).

Extravillous trophoblasts (EVT), which are in contact with the immune cells in the decidua, express an unusual combination of HLA-C, HLA-E and HLA-G molecules. While all cells in the body express HLA-C and HLA-E, HLA-G is restricted to trophoblast and believed to modulate NK and T cell functions towards tissue tolerance(93). The HLA-A and HLA-B

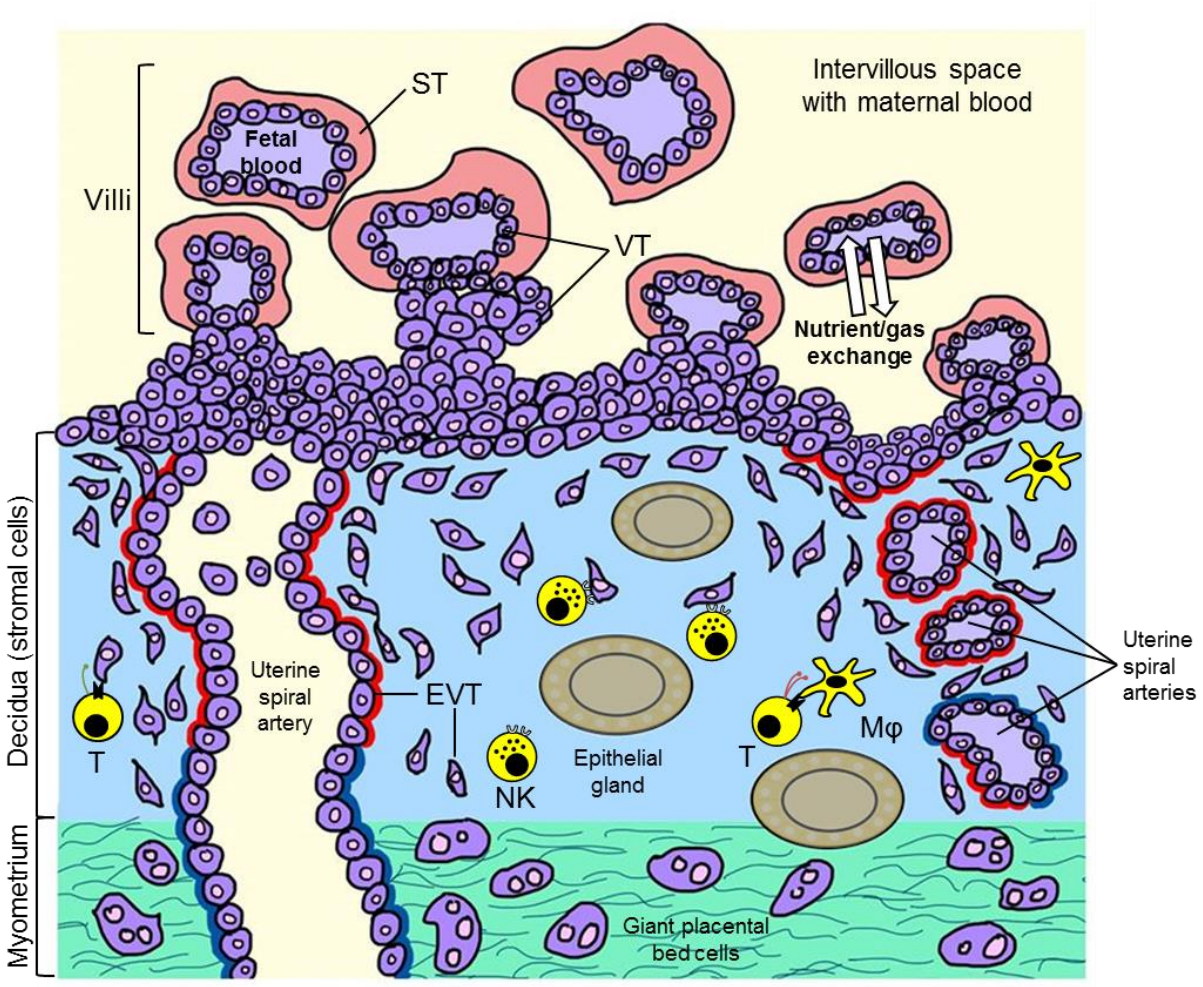

Figure 7. Anatomy of the interface between placental trophoblast and maternal decidua. The different types of trophoblast cells and maternal immune cells (yellow) present in the first trimester are shown. The regions of uterine spiral arteries where epithelium was substituted by EVT are shaded in red. Image courtesy of Ana Haydeé Linares, adapted from reference (94).

molecules, which initiate allograft rejection, are not expressed, which leaves HLA-C as the only polymorphic molecule in the trophoblast surface that could represent a mismatch between mother and fetus. All 3 MHC Class I molecules interact with receptors — such as KIRs, CD94/NKG2 and LILR — expressed by NK and subsets of T cells (94). Interestingly, microarray and functional gene set enrichment analysis revealed a striking immune-activating potential for EVT that is absent in villous trophoblast (95).

3.2 Decidual leukocytes and potential immune responses

During pregnancy, the decidua accumulates several types of immune cells. The relative amounts and the phenotype of these cells are very different from the ones found in blood. Decidual leukocytes are composed of 60-80% NK cells, followed by 15-20% macrophages and 5-10% T cells in first trimester of pregnancy (Figure 7). Low percentages of dendritic cells, γδ T cells and NKT cells can also be found, but B cells are virtually absent. Below is a short description of the most important immune cells found at the fetal-maternal interface (Figure 7).

3.2.1 T helper cells, cytotoxic T cells and regulatory T cells

T cells are the main immune cell population found in blood (70%), which is in stark contrast to decidua, where they represent only 5-10% in first trimester, but this percentage increases to 60-80% at term. Their low frequency is considered a mechanism of immune evasion, since T cells could recognize mismatched HLA-C alleles in EVT and elicit rejection. Both $CD4^+$ and $CD8^+$ T cells in decidua display an activated phenotype; however, the percentage of regulatory T cells found in decidua is much higher than in blood (96). Studies in abortion-prone mice where Tregs were adoptively transferred to the site of pregnancy, avoiding fetal resorption, showed the vital importance of these cells in pregnancy outcome (97). In addition, Tilburgs *et al* demonstrated an HLA-C mismatch between mother and fetus leads to an increase in the number and activity of activated T cells in the maternal-fetal interface. As a compensatory mechanism, the function of Tregs in the mismatched pregnancies is also enhanced. (98). Moreover, EVT have the ability to induce Treg differentiation in decidua (95).

Some of the mechanisms employed by EVT to avoid T cell attack involved expression of Fas ligand (which can induce apoptosis in Fas receptor-expressing cells like activated $CD4^+$ and $CD8^+$ T cells) and expression of B7 family members (co-stimulatory molecules) that have an inhibitory effect on T cell activation (B7-H1, B7-H2, B7-H3, B7-DC) (91). In addition, trophoblast cells and APC produce the tryptophan catabolizing enzyme **IDO**, which inhibits T cell proliferation by tryptophan depletion or production of toxic metabolites (99).

3.2.2 Macrophages and dendritic cells

Macrophages (Mϕ) comprise the second most abundant leukocyte in human decidua (10-15%). The large abundance of $CD14^+$ decidual macrophages (dMϕs) and the near absence of $CD14^-$ dendritic cells (DC) suggest they are the most important professional APCs in the decidua (100). Therefore, dMϕs, which express MHC class II, are likely to play a crucial role in antigen presentation to decidual T cells and may contribute in the establishment of fetal–maternal immune tolerance. In addition to their antigen presenting function, dMϕs have been shown to express receptors important for phagocytosis of degraded extracellular matrix products (101) as well as in apoptotic cell clearance and tissue remodeling (100, 102). Furthermore, $CD14^+$ dMϕs produce immunomodulatory proteins such as TGF-β, but also proinflammatory IL-6 and IL-8 when stimulated by cell lines expressing the fetal-trophoblast HLA-G homodimer (48). Recently, our lab identified two distinct populations of dMϕs in decidua: $CD11c^{high}$ and $CD11c^{low}$. $CD11c^{high}$ dMϕs express genes associated with lipid metabolism and inflammation, whereas $CD11c^{low}$ dMϕs express genes associated with extracellular matrix formation, muscle regulation, and tissue growth. The $CD11c^{high}$ dMϕs also differ from $CD11c^{low}$ dMϕs in their ability to process protein Ag and are likely to be the major APCs in the decidua(103).

3.2.3 Natural killer cells

The infiltration of decidual NK (dNK) cells is part of the cyclical changes of the endometrium and is clearly influenced by sex hormones, particularly progesterone. While in blood NK cells represent about 10% of lymphocytes, in decidua they are particularly abundant, constituting up to 70% of leukocytes. dNK start accumulating even before implantation, which may implicate a role in placentation. During early pregnancy, dNK accumulate as a dense infiltrate around the trophoblast and they progressively disappear from mid-gestation onwards and at term represent only 10-20%. Therefore, their presence is coincident with the period of trophoblast invasion (placentation is complete by about 20 weeks gestation) (94).

Our lab and others have shown dNK form a distinct NK cell population that has many differences in gene expression profile, cytokine secretion profile and cytolytic capacity in comparison with peripheral blood NK cells (pNK) (Table IV). While 90% of pNK are $CD56^{low}CD16^+$, all dNK are $CD56^{high}CD16^-$. dNK express KIRs and NKG2 receptors, and contain high levels of the cytolytic molecules perforin, granzyme B, and granulysin (104, 105). These characteristics, however, do not lead to high cytotoxicity, as they are not able to kill MHC Class I negative targets as efficiently as pNK (e.g. cell lines K652 or 721.221). The low cytotoxicity of dNK is due to an intrinsic deficiency in polarizing cytolytic granules to the immune synapse (106).

dNK have been shown to secrete high levels of Interleukin-6 (IL-6), IL-8 and vascular endothelial growth factor (VEGF) in response to HLA-G transfected 721.221 cells (221.HLA-G) and antibody stimulation (48, 107). The potential to secrete cytokines led to the hypothesis that dNK facilitate implantation and placental growth by producing chemokines for EVT invasion and growth factors involved in vascular remodeling of the spiral arteries. However, our lab recently showed that contact with EVT alone does not induce secretion of VEGF, IFN-γ or the granulocyte-monocyte colony-stimulating factor GM-CSF by dNK, which indicates other stimuli are involved in the activation of dNK cytokine secretion (95). In mice, uterine NK cells have in fact been shown to be crucial for proper vascularization of the maternal-fetal interface(108).

dNK express many of the receptors that can interact with the MHC Class I expressed in the EVT. HLA-G was thought to modulate NK responses, but more recent findings have added controversy to that claim (109, 110). While HLA-E could potentially inhibit NK responses against the trophoblast (111), mismatched paternal HLA-C alleles could lead to missing self recognition by dNK (112).

Characteristics	pNK CD56 ^{low}	pNK CD56 ^{hi}	dNK CD56 ^{hi}
Percentage of CD45+ in tissue	10-20%	1-2%	60-80%
CD16	+++	+/-	-
NKG2A/NKG2C/NKG2E	+/-	+	++
KIRs	+	-	++
Granzyme A	++	-	+++
Granzyme B	++	-	++
Perforin	+++	-	++
Granulysin	+	-	+++
Galectin 1	+	+	++
PP14	-	-	+
Tetraspanins (CD151, CD9)	-	-	+
Integrins (ITGAD, CD11d, ITGB5)	+	+	++
Activity	**High**	**Low**	**Low**

Table IV – Differences between pNK and dNK.

3.3 HLA-KIR interactions in maternal and fetal health

Similarly to pNK, dNK express both activating and inhibitory receptors, especially MHC-Class I receptors. Interestingly, they are found at higher frequencies and higher expression levels in decidua (113). While KIR and MHC can influence viral clearance or HSCT outcome, pregnancy outcome and complications can also be traced down to KIR-HLA interactions between maternal dNK and trophoblast.

In 2004, Hiby *et al* reported that mothers homozygous for KIR-A haplotypes (genotype AA) were at greater risk for preeclampsia than mothers having one (AB) or two (BB) KIR-B haplotypes (114). The risk was further increased for the subset of KIR-AA mothers carrying babies bearing the HLA-C2 epitope. KIR-A haplotypes are characterized for the presence of the inhibitory receptor (KIR2DL1) and the absence of the activating receptor (KIR2DS1) for HLA-C2 (Figure 4). More recently, by comparing the pregnancies of mothers whose KIR-B haplotype genes are only centromerically or telomerically located (Figure 4), Hiby *et al* focused the protection effect within the telomeric KIR-B haplotype genes: KIR3DS1, KIR2DL5, KIR2DS3/5, and KIR2DS1 (71). Although genetic analysis cannot distinguish between them, because of strong linkage disequilibrium, functional studies point to *KIR2DS1* as the protective gene. KIR2DS1 is a well-defined HLA-C2 receptor and protection increased when the fetus expressed HLA-C2. In addition, the protective effect was extended from only pre-eclampsia to recurrent miscarriage and fetal growth restriction (Table III) (71). Interestingly, the detrimental association of KIR-AA with HLA-C2 may have been a strong evolutionary force for the distribution of KIR and MHC haplotypes in human populations. Frequency of AA haplotype and HLA-C2 epitope is inversely correlated. For example, the Japanese have high KIR AA and low C2 frequencies (115). In the US Caucasian population, KIR AA has a frequency of 30%, while HLA-C2 epitope frequency is about 50%. The *KIR2DS1* gene frequency in the Eastern US population is of 33%(116).

The cellular mechanisms involved in this genetic association are mostly unknown, but the current hypothesis is that dominance of the inhibitory HLA-C2 receptor (KIR2DL1) in women with KIR-AA genotype reduces dNK stimulation of EVT and the depth of EVT invasion. This hypothesis relies on the paternal HLA-C2 as responsible for the inhibition of dNK and was tested in a mouse model of these associations. However, the mouse studies demonstrated the parental origin of the inhibitory MHC is irrelevant (117). On the other hand, women with telomeric KIR-B haplotypes are protected, because dNK are activated through KIR2DS1/HLA-C2 interaction, counterbalancing inhibition and leading to the secretion of cytokines facilitating trophoblast invasion and placental growth (118).

A recent study addressed the cytokine secretion capacity of KIR2DS1^{+} dNK and identified GM-CSF as a growth factor secreted by KIR2DS1^{+}, but not KIR2DS1^{-} dNK. GM-CSF was shown to promote the migration of trophoblast cell lines (117, 118). This increase in cytokine

production was observed when dNK were stimulated with KIR2DS1 antibodies and classical MHC negative target cells, but our recent study demonstrated EVT do not elicit cytokine responses by dNK even when KIR2DS1 and HLA-C2 are present (95). Therefore, these genetic associations demand further investigation into the molecular mechanisms underlying the protective effect of activating KIRs, and in particular KIR2DS1, from pregnancy complications (described in chapters 2 and 3).

3.4 Viral, bacterial and parasite infections in pregnancy

The tolerant environment of the placenta allows the development of the fetus, but also makes it an ideal place for pathogens to evade an immune attack. Viral [e. g. HCMV, HIV, herpes simplex virus (HSV) and influenza virus], bacterial [e. g. *Listeria monocytogenes*, Group B Streptococcus, *Treponema* (syphilis)] and parasitic infections (*Toxoplasma*, malaria) can cause severe maternal and fetal disease and morbidity when they occur during pregnancy (119–121). Infections have been reported to cause recurrent spontaneous abortions at a rate of about 4%. However, difficulties in demonstrating the pathogenic role of a wide variety of viral pathogens may result in under-diagnosis (122).

3.4.1 Viral infections

HCMV is the most common congenital viral infection and occurs in 0.5–2% of all live births. HCMV causes placental thickening and insufficiency, leading to fetal growth restriction (123–126). HCMV-infected individuals often have detectable virus specific T cell immunity, but a process of immune tolerance combined with viral escape mechanisms overpower the efficient eradication of the virus (127). Therefore, primary infection with HCMV leads to life-long persistence of the virus in the infected host and 50 to 80% of all people in the United States are infected with HCMV. HCMV infection is latent in the majority of the hosts, but episode of reactivation are known to occur, particularly in immunocompromised patients such as recipients of hematopoietic stem cell transplantation (82). HCMV significantly inhibits the expression of HLA-A and HLA-B molecules on the cell surface of infected cells (128), diminishing viral peptide presentation and subsequent immune recognition by $CD8^+$ effector T cells. The low numbers of $CD8^+$ T cells in decidual tissue and the HCMV-induced down regulation of HLA-A and HLA-B molecules limit the effectiveness of $CD8^+$ T cells to respond to HCMV at the maternal-fetal interface. This leaves dNK cells as the predominant immune cell able to control viral infections in decidua and placental tissue.

Interestingly, similar to HIV infection, HCMV specifically retains HLA-C molecules on the cell surface (129). This allows for interaction with NK cells through KIR that bind to HLA-C2 (KIR2DL1, KIR2DS1) or HLA-C1 (KIR2DL2, KIR2DL3) group molecules. Infection of *HLA-C2*$^+$ placental cells may be more efficiently cleared by dNK expressing activating KIR2DS1 (present in women with KIR-B haplotypes) preventing pregnancy complications caused by

viral infection in later gestation. dNK from women with KIR-AA haplotypes (lacking the *KIR2DS1* gene) would be primarily inhibited by HLA-C2, preventing viral clearance. This hypothesis lays the basis for the work presented in Chapter 3.

3.4.2 Bacterial infections

Bacterial infections are also common in pregnancy, and can lead to fetal loss, premature labor, congenital anomalies and fetal distress. GBS colonize the vagina and can cause an ascending infection in pregnant women, spontaneous abortion and neonatal meningitis and sepsis (130). *Listeria monocytogenes* infection among pregnant women is 17-fold higher than among the general population (12 per 100,000 vs. 0.7/ 100,000 respectively) (131). *L. monocytogenes* is a Gram-positive rod that causes severe fetal sequelae with spontaneous abortion in 10–20%, intrauterine fetal death in 11%, and preterm birth in 50% (132). The mechanism of fetal death may result from impaired suppression of maternal T cells to fetal antigens by maternal $Foxp3^+$ T regulatory cells (133). Infection by *L. monocytogenes* during pregnancy can result in miscarriage, stillbirth, preterm delivery, and maternal and neonatal sepsis (134). Intracellular bacterial infections are primarily eliminated by killer cells (CTL and NK), in a mechanism involving synapse formation with the infected target cells and the release of perforin and granzymes, which lead to infected cell death (135). In addition, CTL and NK cells release granulysin, a microbicidal protein that leads to bacterial cell death in combination with granzymes (136, 137). Interestingly, dNK contain high levels of granulysin (138), which indicates a possible role in placental bacterial infection and will be the focus of Chapter 4 of this thesis.

3.4.4 Parasite infections

Parasites such as *Toxoplasma gondii* and the malaria-causing *Plasmodium falciparium* can also affect pregnancy and cause fetal disorders. *Toxoplasma gondii* is a highly prevalent infection throughout the world with human transmission generally occurring after ingestion of raw meat or exposure to cat feces; fetal damage is characterized by brain and eye injury. *T. cruzi*, also known as Chagas disease, is endemic in Mexico and Central and South America. Congenital *T. cruzi* infection is associated with low birth weight, hepatosplenomegaly, anasarca, and stillbirth (132). *Plasmodium falciparum* infection is especially prevalent in regions of Africa, and leads to chronic anaemia and placental malaria infection, reducing the birth weight and increasing the risk of neonatal death(139, 140).

The severity of these infections, both for mother and fetus, requires immediate action to improve maternal and fetal health. To understand how and where to act, the achievement of a better understanding of the balance between fetal tolerance and immune response to infections is crucial.

AIMS

The aim of this thesis is to provide a detailed characterization of the decidual NK cell responses to healthy and viral and bacterial-infected placental cells. A particular focus is placed on the interaction between placental (fetal) HLA-C and maternal KIR receptors expressed by dNK. Chapter 2 presents a study on the phenotype and function of dNK that express the activating receptor KIR2DS1. Specifically, the influence of KIR2DS1 expression in the cytolytic granule content, degranulation in response to HLA-$C2^+$ targets and cytokine secretion in response to primary trophoblast were investigated. In Chapter 3, the KIR2DS1/HLA-C2 interactions were further studied in a model for placental viral-infection. dNK and pNK response to HCMV-infected decidual stromal cells and fetal trophoblast, in particular $KIR2DS1^+$ dNK, were studied. Finally, Chapter 4 introduces a preliminary study on dNK responses to bacterial-infected placental cells and the role of dNK-derived granulysin in bacterial clearance. The data presented in this thesis contributes for a better understanding of the dNK role in human pregnancy and aim to help establish dNK as true immune effector cells, in addition to their function in the production of growth factors for facilitation of trophoblast invasion.

CHAPTER 2

CHARACTERIZATION OF THE PHENOTYPE, CYTOTOXICITY AND CYTOKINE SECRETION CAPACITY OF KIR2DS1^{+} DECIDUAL NK CELLS

INTRODUCTION

One of the most interesting observations in the last decade in reproductive immunology research is the association between the presence of the killer cell immunoglobulin-like receptor 2DS1 (KIR2DS1) in the mother's genome and a lower risk of developing pregnancy complications. In contrast, women who lack KIR2DS1 are at increased risk (71, 114). The protective effect of maternal KIR2DS1 expression is most obvious when the fetus expresses the ligand for KIR2DS1, the HLA-C2 group allotype of HLA-C (71). This association was also found to increase birthweight (76). Recently, presence of KIR2DS5 was also identified as a protection factor in pregnancies of African women, while the effect of KIR2DS1 seems to be characteristic of European populations (77). The presence or absence of the KIR2DS1 gene is due to the various rearrangements suffered by the KIR locus during human evolution (141). These rearrangements resulted in two main KIR haplotypes, KIR-A and KIR-B. The KIR-A haplotype lacks most activating KIR, including KIR2DS1, and is associated with increased risk for pregnancy complications. In contrast, the KIR-B haplotypes include one or more activating KIR genes and reduce the risk of developing complications. Protection from pregnancy complications include severe maternal and fetal syndromes that occur in high frequencies in the population such as pre-term birth (5-9%), fetal growth restriction (3-10%) and preeclampsia (3-5%).

KIR2DS1 is an activating NK receptor expressed with increased frequency on decidual NK cells (dNK) at the maternal-fetal interface compared to peripheral blood NK cells (pNK) (113). The current hypothesis states that the protective effect of KIR2DS1 lies in the activation of dNK through binding to its ligand, HLA-C2 group molecules, expressed by fetal extravillous trophoblasts (EVT). The activation through HLA-C2/KIR2DS1 provides dNK with the ability to secrete beneficial cytokines and growth factors, especially granulocyte-monocyte colony stimulation factor (GM-CSF), to facilitate trophoblast invasion and placental growth (71, 118). This increase in cytokine production was observed when dNK were stimulated with KIR2DS1 antibodies and classical MHC negative target cells, but one recent study demonstrated EVT do not elicit GM-CSF or even IFN-γ or VEGF secretion by dNK (95).

dNK are by far the most abundant decidual leukocyte subset present at the maternal-fetal interface in early pregnancy (60-80%). Our lab and others have shown dNK form a distinct NK population which has many differences in gene expression profile, cytokine secretion profile and cytolytic capacity in comparison with pNK (48, 105–107, 118). We recently demonstrated that many dNK form immune synapses with one EVT in which perforin is not localized to the synaptic region, typical for a non-lytic synapse. During the immune synapse of dNK and EVT, dNK acquired HLA-G from the EVT through trogocytosis (Tilburgs *et al*,

provisionally accepted). Interestingly, KIR2DS1^{+} dNK incorporated significantly increased levels of HLA-G into their membrane, suggesting a strong interaction between KIR2DS1^{+} dNK and EVT. Prolonged intracellular signaling and possibly distinct functional properties may result.

The protective association between maternal *KIR2DS1* and fetal *HLA-C2* suggests the activation of dNK through KIR2DS1 elicits dNK functions involved in the establishment and maintenance of a successful pregnancy. However, activation may lead to higher cytotoxicity which may be detrimental for placental cells if not controlled. To analyze the function of KIR2DS1^{+} dNK in healthy pregnancy, the cytolytic capacity and cytokine secretion of dNK from *KIR2DS1*$^{+}$ versus *KIR2DS1*$^{-}$ women was studied. In particular, the expression of NK receptors, cytolytic granule content, signaling molecules, degranulation in response to HLA-C2^{+} cell lines, as well as cytokine production in response to primary EVT, were analyzed. Our results reveal a higher cytolytic potential of KIR2DS1^{+} dNK which does not translate into EVT-induced cytokine secretion.

MATERIALS AND METHODS

Isolation of NK and trophoblast

Discarded human placental and decidual material (gestational age 6-12 weeks) were obtained from women undergoing elective pregnancy termination at a local reproductive health clinic. Peripheral blood leukocytes were isolated from discarded Leukopacks® from healthy volunteer blood donors at the Massachusetts General Hospital in Boston, MA. All of the human tissue used for this research was de-identified, discarded clinical material. The Committee on the Use of Human Subjects (the Harvard IRB) determined this use of placental and decidual material is Not Human Subjects Research. The procedure to isolate EVT, dNK and pNK has recently been described (95). Briefly: Decidual and villous tissues were macroscopically identified and separated. Decidual tissue was washed with PBS, minced and thereafter digested with 0.1% collagenase type IV and 0.01% DNase I (Sigma-Aldrich) gently shaking in a water bath for 60-75 min at 37°C. Released lymphocytes were washed with RPMI 10% FBS (8 min, 1800 rpm) and filtered through 100µm, 70µm and 40µm sieves (BD, Labware; NJ). Lymphocytes were dissolved in 20 ml 0.830 g/ml Percoll (GE Healthcare) and layered on a Percoll gradient (10 ml 1.085g/ml; 15 ml 1.054g/ml) for density gradient centrifugation (30min/2000rpm). Lymphocytes were isolated from the 1.085 – 1.054g/ml interface, washed twice and directly stained for flow cytometric analysis or FACS sort. Peripheral blood NK were isolated using a RosetteSep™ human NK enrichment cocktail (StemCell Technologies) followed by Ficoll (GE Healthcare) density gradient centrifugation (20 min, 2000 rpm).

Villous (VT) and extravillous (EVT) trophoblast were obtained as described previously (142). Briefly, villous tissue was macroscopically identified and gently scraped from the basal membrane. Thereafter the tissue was digested for 8 min at 37°C with a trypsin (0.2%) EDTA (0.02%) solution. Trypsin was quenched with F12 medium containing 10% Newborn Calf Serum (NCS) and 1% Pen/Strep (all from Gibco) and filtered over a gauze mesh. Filtrate was washed once (7 min, 1800 rpm) and layered on Ficoll (GE Healthcare) for density gradient centrifugation (20 min at 2000 rpm). Cells were collected, washed once (7 min, 1800 rpm) and incubated 20 min at 37°C in a 30 mm tissue culture dish for removal of macrophages. Cells were collected and thereafter directly stained for flow cytometric analysis or FACS sort. Decidual stromal cell lines were obtained as described previously (143). HLA typing was performed by the American Red Cross tissue typing facility, Dedham, Massachusetts.

Cell culture

pNK and dNK were cultured in X-VIVO 10™ media (Lonza) supplemented with gentamycin, 5% human AB serum (Corning) and 20 ng/ml recombinant IL-15 (Biolegend). 50.000 CD45-sorted VT and EVT were plated in 48-well cell culture plates (Costar) pre-coated with fibronectin (100µl of 20ng/ml for 45 min, BD), in DMEM/F12 medium (Gibco) supplemented with 10% NCS, 1x Pen/Strep and 1x Glutamine, insulin, transferrin, selenium (Gibco), 5 ng/ml EGF (Peprotech) and 400 units human gonadotropic hormone (Sigma) as described previously (95) . 721.221 (221) and K562 immortalized cell lines were cultured in RPMI 10% FCS, while 721.221-HLA-Cw3 (221.Cw3), 721.221-HLA-Cw7 (221.Cw7), 721.221-HLA-Cw4 (221.Cw4), 721.221-HLA-Cw6 (221.Cw6) (144), K562-Cw1 and K562-Cw6 were cultured in RPMI 10% FCS supplemented with 1.6 g/L G418.

Flow Cytometry

Antibodies used for flow cytometric analysis and FACS sort are listed in Supplemental Table S1. For surface staining, cells were stained for 30 minutes on ice in the dark in PBS 1%FCS. For KIR2DS1 staining, NK were stained with KIR2DL1 first (Clone 143211, 1.5µg/ml, 20 min) and thereafter with KIR2DL1/S1 (Clone EB6, 0.5µl, 15 min) as described previously (145). Cells were analyzed immediately or fixed in 1% paraformaldehyde (PFA) for 10 minutes for next day analysis. For intracellular staining, cells were fixed and permeabilized using the BD CytoFix/CytoPerm kit (BD). Analysis was performed on a FACSCalibur (BD) or LSR II (BD) and FACS sort was done using a BD Aria III, MoFlo XDP or MoFlo Astrios (Beckman Coulter).

Phosphorylation assessment

Freshly isolated pNK and dNK cells were incubated at 1 million/ml in X-VIVO 10 media (Lonza) for 30 min at 37ºC with or without 100 µM pervanadate solution. Cells were subsequently fixed for 10 minutes in equal volume of BD Cytofix buffer (BD) at 37ºC, washed, and stained for surface markers for 30 min. The cells were then washed and permeabilized with prechilled (-20ºC) BD Phosflow Perm Buffer III for 30 min on ice, followed by intracellular staining with BD Phosflow antibodies for 20 minutes.

Degranulation assays

For degranulation assays, dNK and pNK were cultured overnight in X-VIVO 10™ supplemented with 20ng/ml IL-15 (Biolegend). The dNK and pNK (effectors) were harvested, counted and co-cultured with 721.221 (221), 221-HLA-C1 (221.C1) and 221.HLA-C2 (221.C2) in X-VIVO 10 ™ in 96 well plates in a 1:3 (effector:target) ratio for 2 hours. For NKG2A blocking experiments, dNK were incubated with anti-NKG2A antibody or isotype

control for 30 min at 37ºC prior to co-culture with targets. 250ng/ml CD107a PerCP-Cy5.5 antibody was added to all co-cultures. NK were collected and fixed for 10min in 1% PFA and subsequently stained for all relevant surface markers for FACS analysis.

Cytokine secretion analysis

dNK were co-cultured for with 221, 221.C2 and EVT in a 1:1 mix of trophoblast media and X-vivo media (supplemented with 20 ng/ml IL-15) for 18h. Supernatants were snap frozen at -80 °C and all cytokines were analyzed using a multiplex cytokine assay (Magpix, Bio-Rad) according to manufacturer's protocols.

Statistical analyses

All data was analyzed using GraphPad Prism (version) software. To determine differences between 2 unpaired groups, a non-parametric Mann-Whitney test was performed, whereas between 2 paired groups a non-parametric Wilcoxon Signed Rank test was performed. To determine differences among more than 2 unpaired groups, a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-test was performed. To determine differences among more than 2 paired groups, a non-parametric Friedman's test with Dunn's multiple comparison post-test was performed. For correlation analyses, Spearman correlation analysis was used. All data are depicted with median and interquartile range. P-values <0.05 were considered to reflect significant differences.*-$p<0.05$;**-$p<0.01$; *** $p<0.005$

TABLE I

Epitope	Clone	Fluorochrome	Manufacturer
CD45	HI30	PerCP	Biolegend
	HI30	Alexa Fluor 700™	Biolegend
	HI30	Pacific Orange™	Life Technologies
	HI30	APC	Biolegend
CD56	HCD56	Alexa Fluor 488™	Biolegend
	HCD56	Alexa Fluor 700™	Biolegend
	HCD56	PE	BIolegend
	HCD56	PE-Cy7	Biolegend
	MEM-188	PE-Texas Red™	Life Technologies
CD14	M5E2	FITC	BD Biosciences
	TüK4	PE-Texas Red™	Life Technologies
	M5E2	PE-Cy7	Biolegend
	HCD14	Pacific Blue™	Biolegend
CD3	SK7	Pacific Blue™	Biolegend
	UCHT1	PE-Texas Red™	Life Technologies
NKG2A	Z199	PE	Beckman Coulter
NKG2C	134591	PE	R&D Systems
KIR3DL1	DX9	PE	BD Biosciences
KIR2DL1	143211	APC	R&D Systems
KIR2DL1/S1	EB6	PE	Beckman Coulter
	EB6	PE-Cy7	Beckman Coulter
KIR2DL2/3	CH-L	FITC	BD Biosciences
	CH-L	PE	BD Biosciences
	DX27	PerCP	Miltenyi Biotec
Granzyme A	CB9	Alexa Fluor 488™	Biolegend
Granzyme B	GB11	Alexa Fluor 488™	Biolegend
	GB11	PE-Texas Red™	Life Technologies
Perforin	dG9	PE	Biolegend
	dG9	Pacific Blue™	Biolegend
Granulysin	DH2	PE	Biolegend
IgG1	MOPC-21	Alexa Fluor 488™	Biolegend
IgG1	MOPC-21	PE	Biolegend
IgG2b	MPC-11	Pacific Blue™	Biolegend
SYK	4D10	FITC	BD Biosciences
ZAP-70	1E7.2	PE	BD Biosciences
ZAP-70(PY319)/SYK(PY352)	17A/P-ZAP70	Alexa Fluor 488™	BD Biosciences
CD107a	H4A3	PerCP-Cy5.5	Biolegend
IgG1	MOPC-21	PerCP-Cy5.5	Biolegend
HLA-G	MEM-G/9	PE	Abcam
EGFR	ICR10	FITC	AbD Serotec

RESULTS

Frequency of KIR2DS1⁺ NK cells is significantly increased in decidua

The extracellular domains of KIR2DL1 and KIR2DS1 are almost identical, making the development of specific antibodies a challenging task. No specific antibodies for KIR2DS1 are available, but the combination an antibody clone specific for KIR2DL1 (143211) with a clone that binds both KIR2DL1 and KIR2DS1 (EB6) allows the identification of KIR2DL1 and KIR2DS1 gene carriers (145). This strategy also discriminates four NK populations in *KIR2DL1*⁺ and *KIR2DS1*⁺ gene carriers: KIR2DL1 single positive (L1⁺ S1⁻), KIR2DS1 single positive (L1⁻S1⁺), KIR2DL1⁺ and KIR2DS1⁺ double positive (L1⁺S1⁺) and double negative (L1⁻S1⁻). In *KIR2DL1*⁺ but *KIR2DS1*⁻ gene carriers, two NK populations can be identified: KIR2DL1 single positive (L1⁺) and KIR2DL1 negative (L1⁻) (Figure 1A). Among the *KIR2DS1*

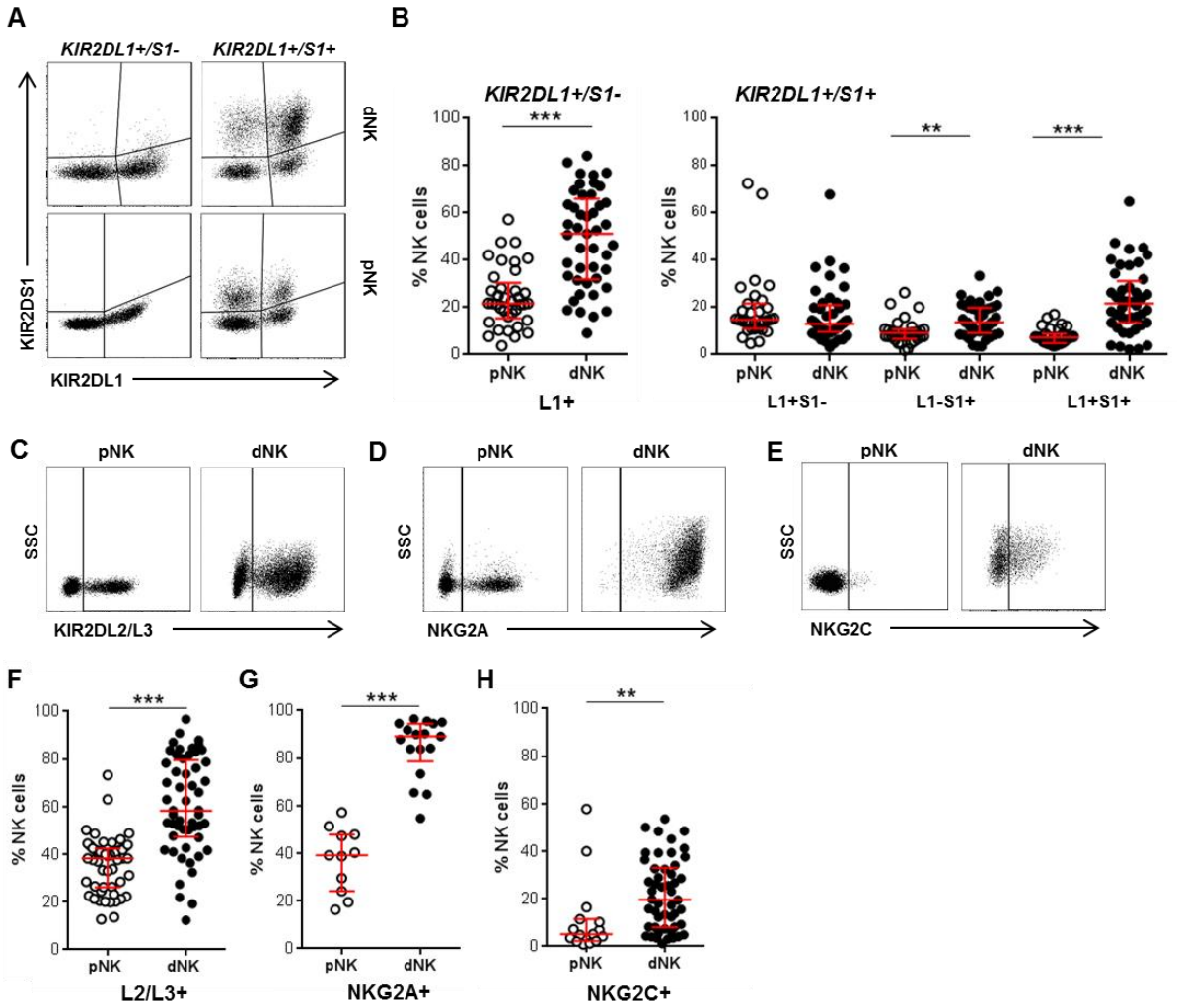

Figure 1. Frequency of MHC Class I receptor-positive NK cells is significantly increased in decidua
A) Representative FACS plots of KIR2DL1 and KIR2DS1 expression in dNK from *KIR2DS1*⁻ (top left) and *KIR2DS1*⁺ (top right) women, and in pNK from *KIR2DS1*⁻ (bottom left) and *KIR2DS1*⁺ (bottom right) donors. Cells were gated in Live cells →CD45⁺CD56⁺**. B**) Graphs depict the percentages of dNK and pNK subsets defined by KIR2DL1 (L1) and KIR2DS1 (S1) expression. Representative FACS plots of KIR2DL2/L3 **(C)**, NKG2A **(D)** and NKG2C **(E)** expression in pNK and dNK. Graphs depict the percentages of dNK and pNK expressing KIR2DL2/L3 (L2/L3) **(F)**, NKG2A **(G)** and NKG2C **(H).**

gene carriers, the frequency of both $L1^-S1^+$ and $L1^+S1^+$ NK was significantly higher in decidua than blood (Figure 1B). In *KIR2DS1*$^-$ individuals, $L1^+$ NK were more frequent in decidua than in blood (Figure 1B). In addition, KIR2DL2/L3$^+$, NKG2A$^+$ and NKG2C$^+$ NK frequencies were also increased in decidua (Figure 1 C-H). These results confirm previous reports demonstrating a skewing of dNK receptor expression towards recognition of MHC Class I, especially HLA-C, and particularly, the potential of dNK to develop an activating response to HLA-C2 through KIR2DS1 (118, 146).

KIR expression influences granule content in dNK

To determine whether the presence of KIR2DS1 influenced the cytolytic potential of NK cells, freshly isolated dNK and pNK were stained for KIR2DS1 and KIR2DL1 and examined for the intracellular expression of the cytolytic molecules granzyme A, granzyme B, perforin and the 9 kDa active form of granulysin (147). pNK express significantly higher levels (as demonstrated by mean fluorescence intensity, MFI) of perforin compared to dNK, whereas a significantly higher percentage of dNK expressed granulysin compared to pNK. Granulysin was also expressed at a significantly higher level in dNK. No difference was found between expression of granzyme A and B by dNK and pNK (Figure 2 A-C).

pNK and dNK of *KIR2DL1*$^+$*/S1*$^+$ individuals were divided in the four NK subtypes and a significantly higher percentage of $S1^+$ dNK ($L1^-S1^+$ and $L1^+S1^+$) expressed granzyme B, perforin and granulysin compared to the $L1^-S1^-$ dNK subset (Figure 2 D and F). In addition, $L1^-S1^+$ and $L1^+S1^+$ dNK also expressed significant higher levels (MFI) of granzyme B, perforin and granulysin compared to the $L1^-S1^-$ dNK subset (Figure 2D and H). The increase of cytolytic molecules in $S1^+$ cells was specific for dNK and was not observed in $S1^+$ pNK (Figure 2 D, E and G).

dNK have a higher potential for activation of signaling pathways downstream of KIR2DS1

The binding of KIR2DS1 to its ligand HLA-C2 triggers DAP12 phosphorylation and subsequent recruitment and phosphorylation of adaptor proteins SYK and ZAP-70 (148). To determine the potential for activation of these signaling pathways, dNK and pNK from *KIR2DS1*$^+$ and *KIR2DS1*$^-$ donors were stimulated with pervanadate and the levels of phosphorylated SYK and ZAP70 (p-SYK/p-ZAP70) were determined by Phosflow assay as described in Methods. A significantly higher percentage of dNK had p-SYK/p-ZAP70 expression than pNK after pervanadate stimulation (Figure 3A-B). The expression levels (MFI) of p-SYK/p-ZAP70 were also significantly increased in dNK compared to pNK (Figure 3B). No difference was found between total expression of SYK or ZAP70 between pNK and dNK (data not shown). This data may suggest a previous activation of dNK *in vivo* or a

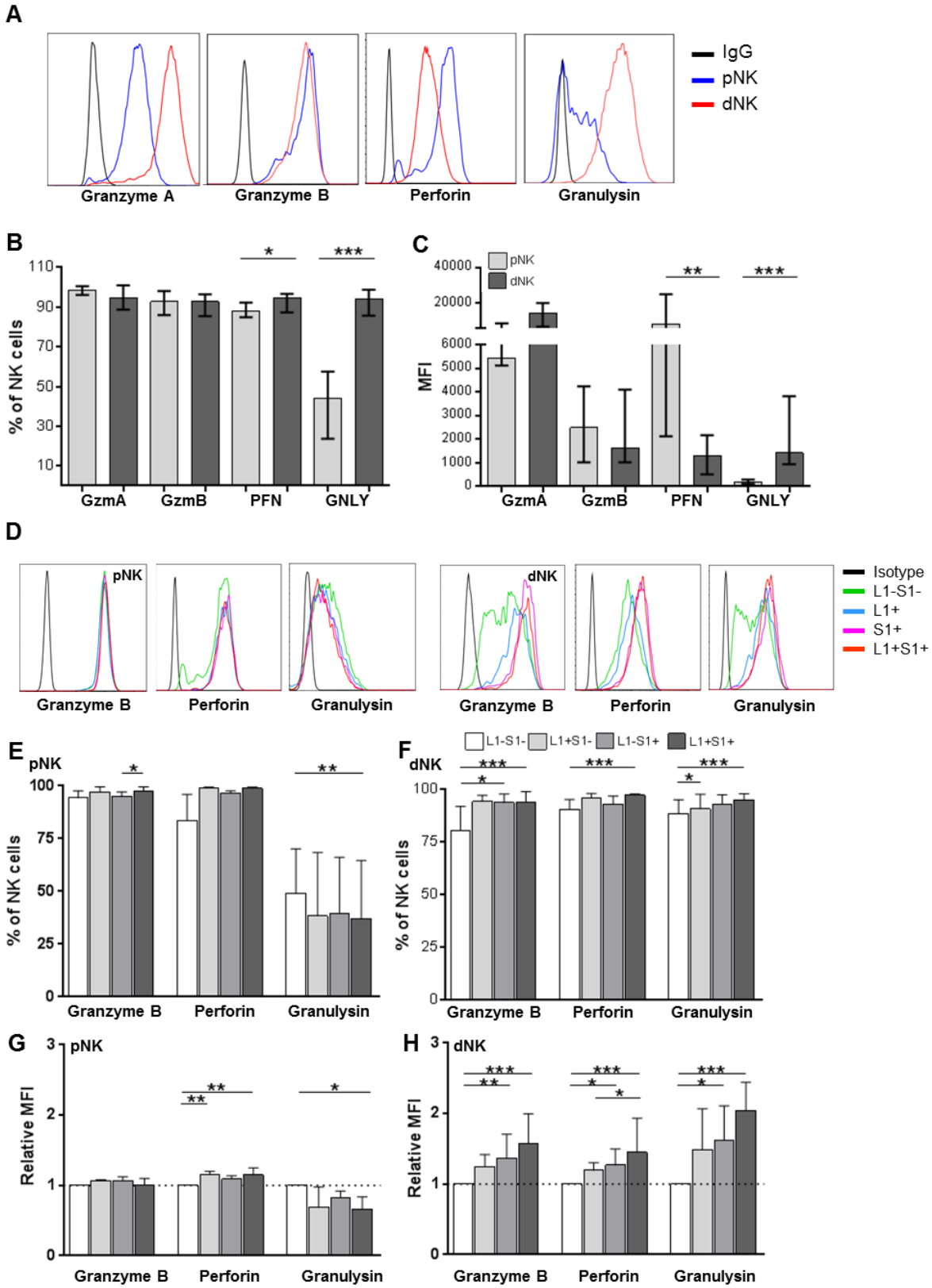

Figure 2. KIR2DS1 expression increases granule expression in dNK
A) Representative FACS plots of granule expression in pNK and dNK. **B)** Percentages of pNK and dNK expressing cytotoxic granules. **C)** Mean Fluorescence Intensity (MFI) for each cytolytic molecule in pNK and dNK. **D)** Representative FACS plots of granzyme B, perforin and granulysin expression in each of the four L1/S1 subsets in dNK and pNK. **E)** Percentage of positive pNK and **F)** dNK and **G)** the expression level (MFI) for each of the three cytolytic molecules in the four L1/S1 pNK and **H)** dNK subsets. The expression level is depicted as the relative MFI compared to the double negative subset (dNK n=10, pNK n=7).

higher capacity for dNK to signal through these proteins. Furthermore, phosphorylation levels of p-SYK/p-ZAP70 were analyzed within *KIR2DS1*$^+$ and *KIR2DS1*$^-$ individuals. No differences in p-SYK/p-ZAP70 were found in dNK from *KIR2DS1*$^+$ and *KIR2DS1*$^-$ donors, whereas pNK from *KIR2DS1*$^+$ donors have a small but not significant increase in p-SYK/p-ZAP70 when compared with pNK from *KIR2DS1*$^-$ donors (Figure 3C).

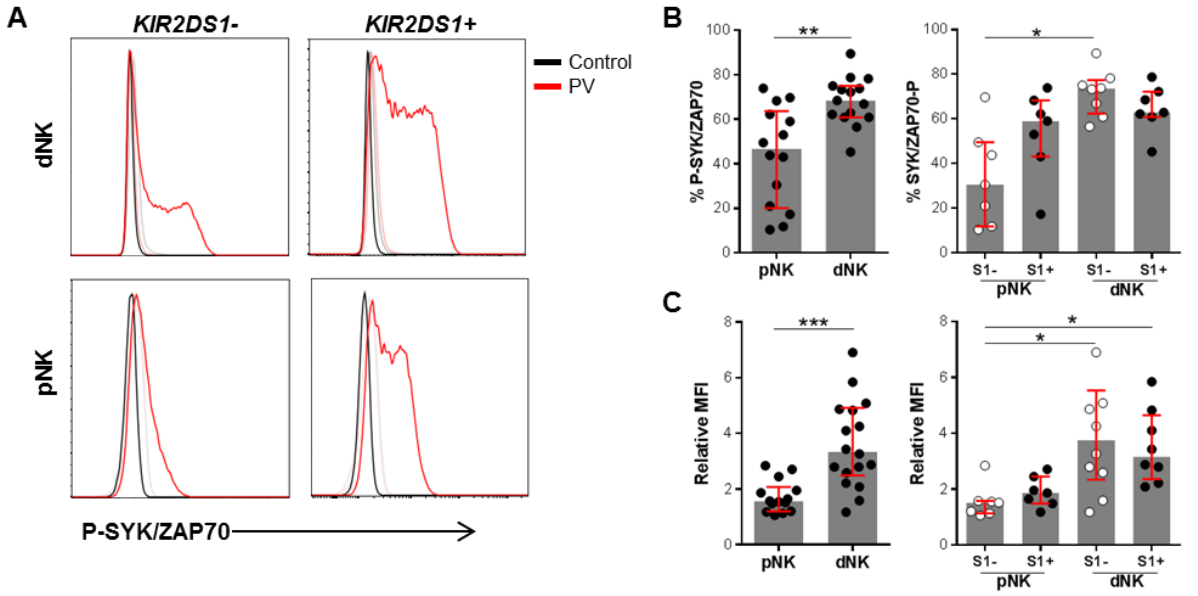

Figure 3. dNK have higher phosphorylation potential of SYK and ZAP-70 than pNK
A) Representative FACS plots of p-SYK/p-ZAP-70 expression in pNK and dNK after pervanadate stimulation. NK from *KIR2DS1*$^-$ and *KIR2DS1*$^+$ individuals are depicted. **B)** Percentage of pNK and dNK with p-SYKp-ZAP-70 expression from all donors (left) and discriminated in *KIR2DS1*$^-$ and *KIR2DS1*$^+$ individuals (right). **C)** Expression level (MFI) of p-SYK/p-ZAP70 in pNK and dNK from all donors (left) and discriminated in *KIR2DS1*$^-$ and *KIR2DS1*$^+$ individuals (right). Values are depicted as the ratio between MFI of pervanadate-stimulated cells over unstimulated cells.

dNK from KIR2DS1$^+$ *individuals are not efficiently inhibited by HLA-C2*

Next, cytotoxicity of dNK and pNK from *KIR2DS1*$^-$ and *KIR2DS1*$^+$ individuals in response to MHC class I negative targets 721.221 (221) and 221 that express either HLA-C1 (221.C1) or HLA-C2 (221.C2) molecules were compared. As reported previously, degranulation levels of pNK in response to 221 were significantly higher than dNK (Figure 4B) (118). HLA-C1 binds the inhibitory KIR2DL2/3 receptors and its expression on 221.C1 significantly inhibited cytotoxicity of NK cells from all donors compared to 221.

In contrast, HLA-C2 can inhibit NK cytotoxicity by engaging with KIR2DL1 or activate NK cells through KIR2DS1. Expression of HLA-C2 on 221.C2 significantly inhibited cytotoxicity of all pNK and dNK obtained from *KIR2DS1*$^-$ donors and pNK from *KIR2DS1*$^+$ donors (Figure 4C, E, F). However, dNK from *KIR2DS1*$^+$ women were not as effectively inhibited by 221.C2 (Figure 4D). In addition, the percentage of S1$^+$ single positive dNK, but not pNK, positively correlated with the degranulation level of total dNK in response to 221.C2 (R^2 0.418 ; p= 0.047 – Figure 4G). These results suggest the activation of dNK through KIR2DS1/HLA-C2 interaction outweighs the inhibition of L1$^+$ by HLA-C2 in dNK but not pNK.

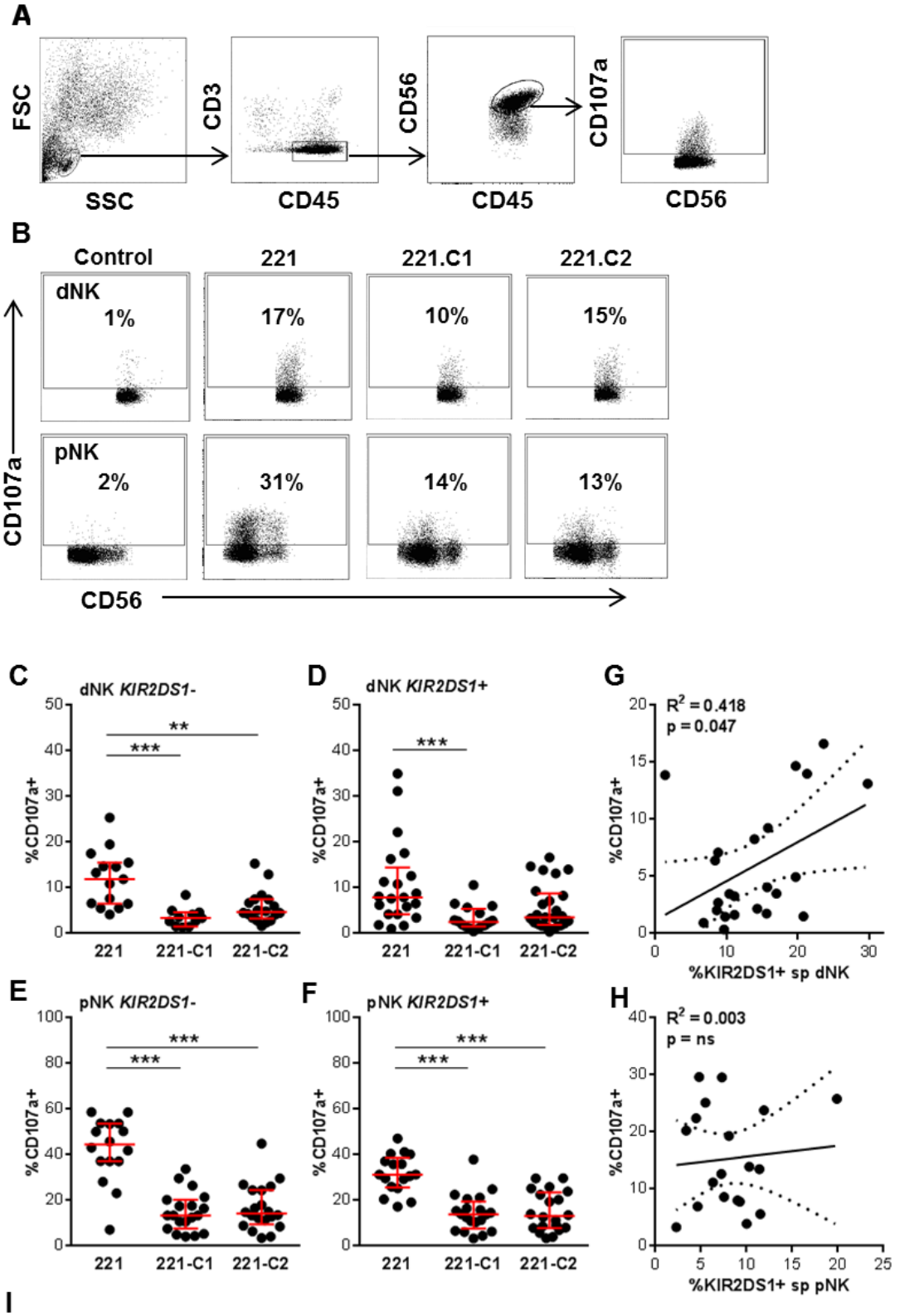

Figure 4. dNK cells from *KIR2DS1*⁺ women are not efficiently inhibited by HLA-C2⁺ targets. A) Gating strategy for analysis of CD107a⁺ dNK following co-culture with target cells. **B)** Representative FACS plots of degranulation of dNK (top) and pNK (bottom) cells in response to 221, 221.C1 and 221.C2. Percentage of CD107a⁺ cells in **C)** dNK from *KIR2DS1*⁻, **D)** dNK from *KIR2DS1*⁺, **E)** pNK from *KIR2DS1*⁻ and **F)** pNK from KIR2DS1⁺ donors in response to 221, 221.C1 and 221.C2. Correlation between the percentage of S1⁺ single positive cells and percentage of CD107a⁺ cells in **G)** dNK and H) pNK in response to 221.C2**. I)** Ratios of the degranulation in response of dNK to 221.C2 versus 221, from *KIR2DS1*⁻ or *KIR2DS1*⁺ mothers pregnant with a HLA-C2⁻ or HLA-C2⁺ fetus.

Hiby *et al*'s studies demonstrated the importance of the presence of the *KIR2DS1* gene in the maternal genome for a successful pregnancy, and this effect was enhanced if the fetus expressed HLA-C2 (71). To determine the effect of the fetal genotype in the dNK response to 221.C2 versus 221, fetal and maternal tissues were genotyped for HLA-C. No difference was found between the degranulation response of dNK from *KIR2DS1*$^-$ or *KIR2DS1*$^+$ women with HLA-C2$^-$ or HLA-C2$^+$ fetuses (Figure 4I). These results indicate previous contact with semi-allogeneic HLA-C has no influence in the cytotoxicity of *KIR2DS1*$^-$ or *KIR2DS1*$^+$ dNK. Moreover, the four L1/S1 dNK subsets were analyzed separately for degranulation in response to 221, 221.C1 and 221.C2. The degranulation of S1$^+$ single positive dNK in response to 221.C2 was significantly higher than all other dNK subsets (Figure 5A, D). The response of S1$^+$ single positive dNK to 221.C1 and 221 was comparable to the double negative subset and lower than the L1$^+$ subsets, as expected due to NK licensing (53) (Figure 5B, C). These data demonstrate HLA-C2-induced activation of S1$^+$ dNK resulting in downstream signaling and degranulation.

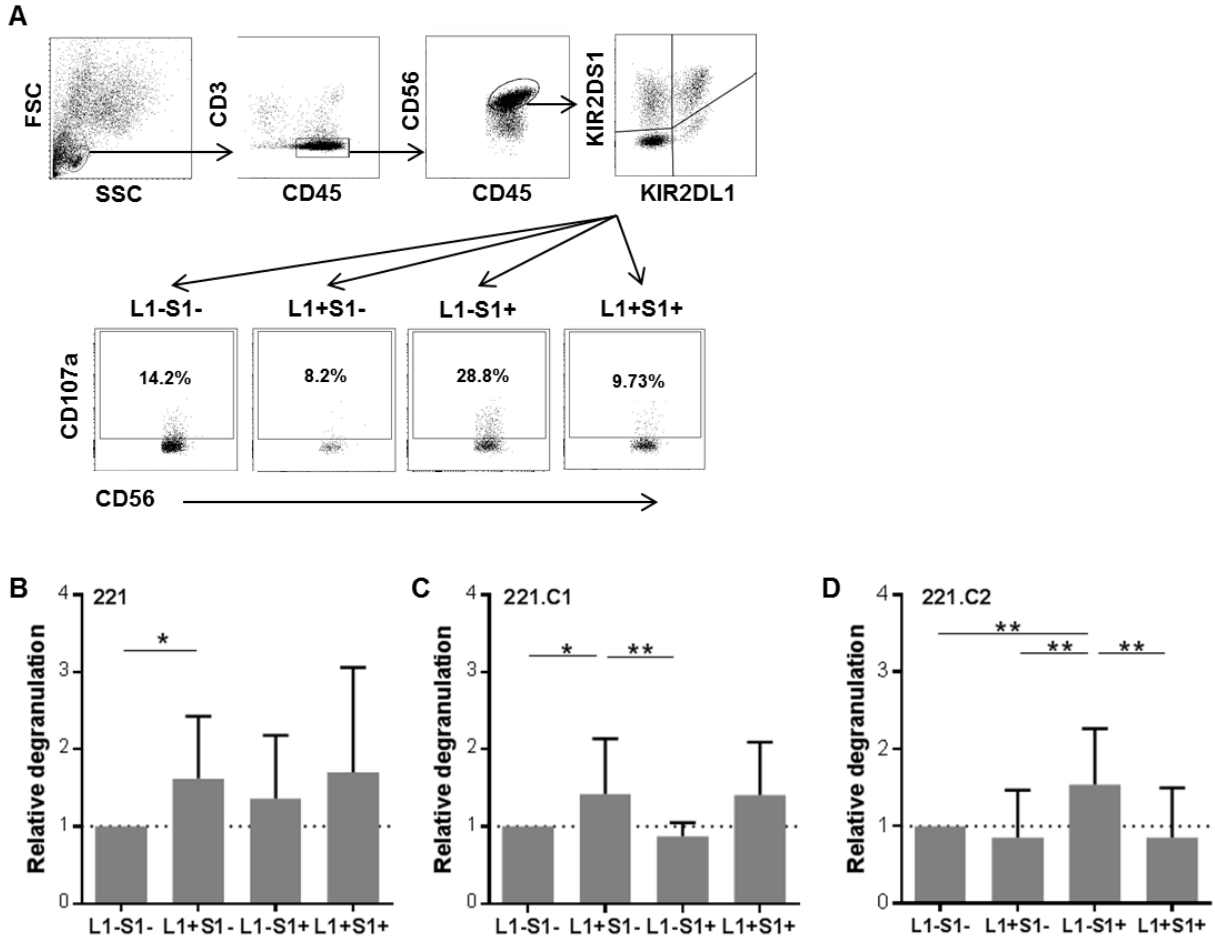

Figure 5. KIR2DS1$^+$ single positive dNK have higher levels of degranulation in response to HLA-C2$^+$ targets **A)** Gating strategy and representative FACS plots of CD107a dependent degranulation of four dNK subsets (L1$^-$S1$^-$, L1$^+$S1$^-$ L1$^-$S1$^+$ and L1$^+$S1$^+$) in response to 221.C2 targets. Graphs depict degranulation levels of L1$^+$, S1$^+$ and L1$^+$S1$^+$ subsets relative to L1$^-$S1$^-$ subset in response to **B)** 221 (n=21) **C)** 221-C1 (n=16) and **D)** 221.C2 (n=24).

HLA-E expression in 221.C2 has no influence in dNK and pNK degranulation

The 721.221 cell line has genomic deletions in the HLA-A, B and C locus, but not in HLA-E (149). HLA-E presents peptides derived from the leader sequence of other MHC Class I molecules. 221 do not express MHC class I and this prevents the surfacing of HLA-E. However, the transfection of 221 with HLA-C1 and HLA-C2 provide such leader peptides and increases the expression of HLA-E on the cell surface (150). dNK and pNK express inhibitory receptors for HLA-E (NKG2A); while clear NKG2A$^-$ and NKG2A$^+$ populations can be identified among pNK, virtually all dNK express NKG2A (Figure 1D, G). To examine if HLA-E had an effect in the degranulation results observed in response to 221.C1 and 221.C2, NKG2A$^-$ and NKG2A$^+$ pNK responses were compared. No difference in the percentage of CD107a$^+$ NK cells in response to 221.C1 and 221.C2 was found between NKG2A$^-$ and NKG2A$^+$ pNK (Figure 6A). A clear NKG2A$^-$ population cannot be identified in dNK and thus blocking of NKG2A was necessary. No difference in the percentage of CD107a$^+$ cells in response to 221.C2 was found between dNK previously incubated with anti-NKG2A blocking antibody or isotype control (Figure 6B).These results suggest the effect observed is due to HLA-C expression only.

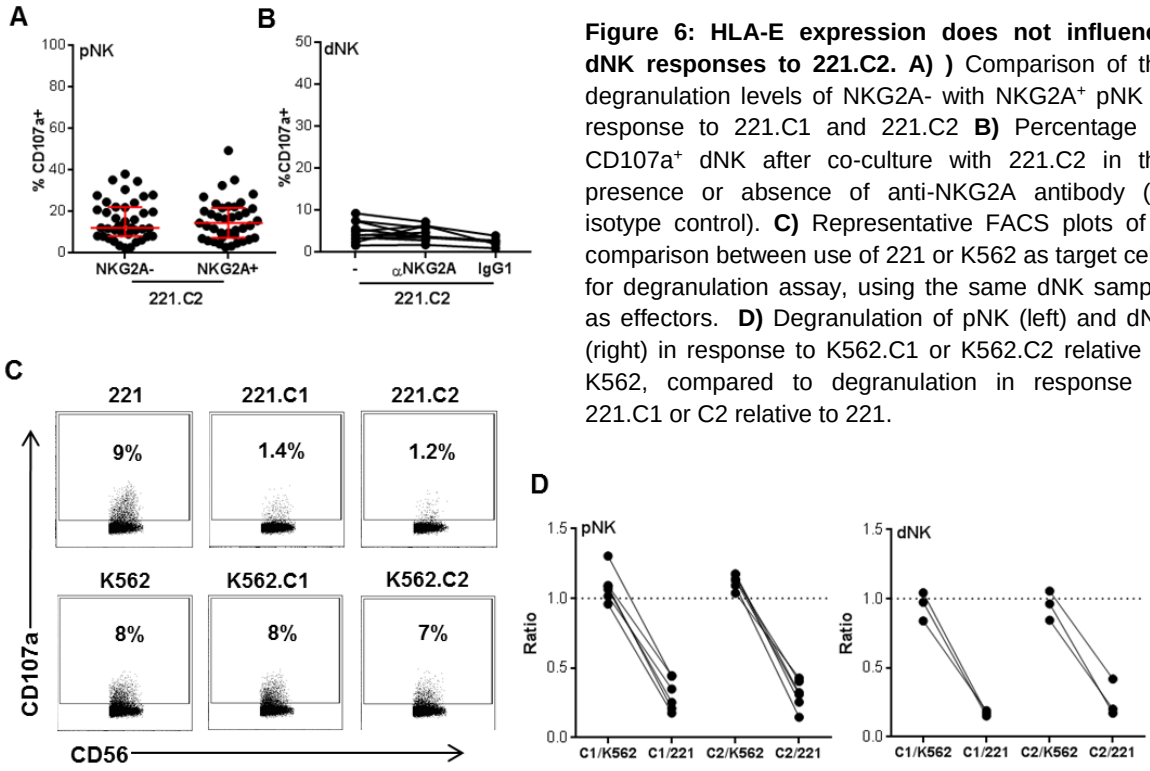

Figure 6: HLA-E expression does not influence dNK responses to 221.C2. A)) Comparison of the degranulation levels of NKG2A- with NKG2A$^+$ pNK in response to 221.C1 and 221.C2 **B)** Percentage of CD107a$^+$ dNK after co-culture with 221.C2 in the presence or absence of anti-NKG2A antibody (or isotype control). **C)** Representative FACS plots of a comparison between use of 221 or K562 as target cells for degranulation assay, using the same dNK sample as effectors. **D)** Degranulation of pNK (left) and dNK (right) in response to K562.C1 or K562.C2 relative to K562, compared to degranulation in response to 221.C1 or C2 relative to 221.

K562 is not a suitable cell line for the study of MHC influence in dNK responses

To overcome some of the disadvantages of the use of 221 (MHC Class II and HLA-E expression – Appendix I), the MHC Class I and II-negative cell line K562 (151) (and K562 transfected with HLA-C1 (K562.C1) and HLA-C2 (K562.C2)) were also used as targets for

degranulation assay. K562, K562.C1 and K562.C2 were co-cultured with dNK and pNK in parallel with 221, 221.C1 and 221.C2. The degranulation of pNK and dNK in response to K652 was similar to 221. However, while MHC Class I transfected 221 were able to inhibit degranulation, MHC Class I transfected K562 were not (Figure 6C-D). These results showed 221 are a more reliable NK target for the study of the effect of MHC Class I molecules in NK responses.

KIR2DS1 does not influence cytokine secretion of dNK in response to EVT

dNK have been shown to secrete both pro- and anti-inflammatory cytokines, as well as growth factors and chemokines involved in trophoblast invasion and vascular remodeling (48, 107, 118). However, all of these studies used cell lines, antibodies or mitogens to stimulate dNK. Recently, it was demonstrated that co-culture of dNK with primary EVT did not lead to secretion of the cytokines identified in these studies (IFN-γ, VEGF) (95). To determine if KIR2DS1 expression has an influence in the cytokine response to HLA-C2^{+} EVT, dNK were co-cultured alone or with 221, 221.C2 and matched primary EVT. dNK constitutively secreted high levels of IL-8 (neutrophil chemotactic factor), and the secretion was amplified by co-culture 221 and 221.C2, but only slightly with EVT (Figure 7F). In fact, EVT alone also constitutively secreted IL-8 (data not shown). Co-culture of dNK with 221 induced secretion of IFN-γ, TNF-α, GM-CSF, VEGF and IL-6 confirming previous studies (48, 107) (Figure 7A-E). Interestingly, expression of HLA-C2 in 221

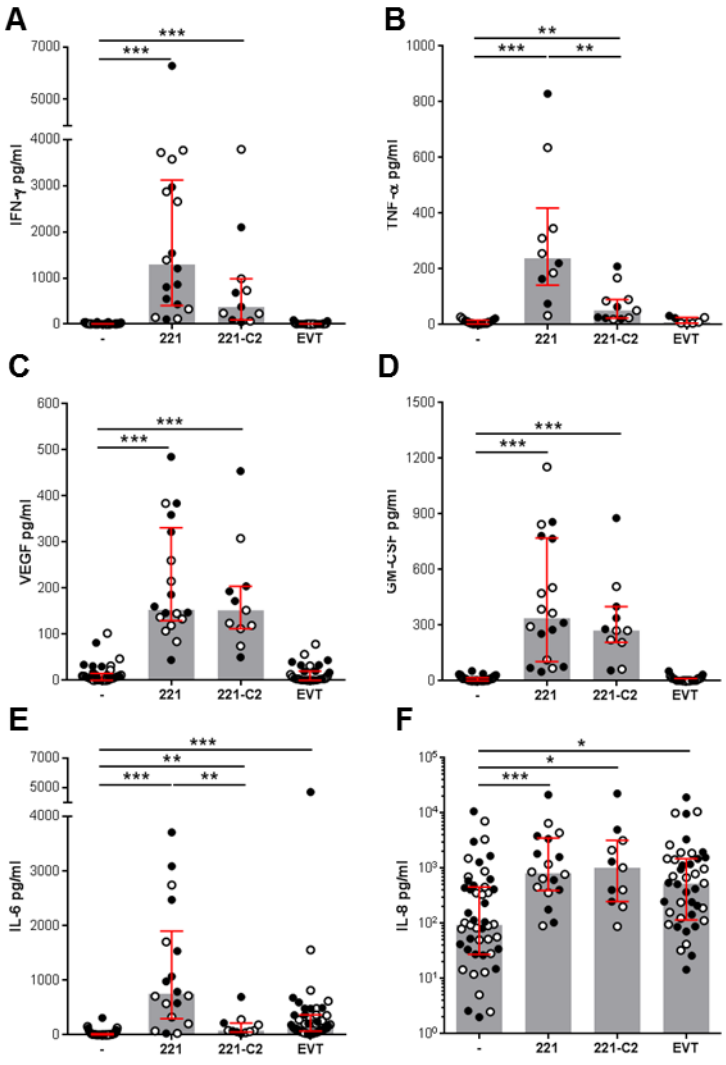

Figure 7. . KIR2DS1 does not influence cytokine secretion of dNK in response to EVT. Cell culture supernatants of dNK incubated alone or with 221, 221.C2 and EVT analyzed for **A)** IFNγ, **B)** TNFα, **C)** VEGF, **D)** GM-CSF, **E)** IL-6 and **F)** IL-8.

partially inhibited the secretion of IFN-γ, TNF-α and IL-6, but not GM-CSF and VEGF (Figure 7A-E), confirming MHC class I may play a direct role in the suppression of pro-inflammatory cytokines. Co-culture of dNK with EVT stimulated dNK secretion of IL-6 (Figure 7E), but not to the same levels of 221, and did not elicit secretion of IFN-γ, TNF-α, GM-CSF or VEGF (Figure 7A-D).

No significant differences were found in cytokine secretion of dNK from *KIR2DS1*- vs *KIR2DS1*$^{+}$ individuals when co-cultured with 221.C2 and HLA-C2^{+} EVT (Figure 7A-F), contrarily to previous published studies regarding GM-CSF secretion by KIR2DS1^{+} dNK (118).

Together, these data indicate HLA-C expression by EVT has no influence on cytokine secretion by dNK, suggesting other stimuli at the maternal-fetal interface may activate dNK to secrete cytokines and growth factors.

DISCUSSION

This chapter demonstrates dNK from *KIR2DS1*$^+$ individuals acquire higher cytotoxic function than dNK from *KIR2DS1*- individuals when exposed to HLA-C2-expressing targets, an effect not observed in pNK. Furthermore, the cytotoxic function was correlated with the frequency of KIR2DS1$^+$ dNK. KIR2DS1$^+$ dNK had an increased capacity to degranulate in response to 221 cells that expressed HLA-C2 than KIR2DS1$^-$ dNK. This effect was independent of the HLA-C genotype of the fetus with which the dNK had had contact *in vivo*. In addition, HLA-C2$^+$ EVT did not elicit GM-CSF, IFN-γ or VEGF secretion by dNK from either *KIR2DS1*- or *KIR2DS1*$^+$ individuals.

HLA-C expressed by fetal EVT has a unique role in pregnancy, as the only polymorphic molecule that can present peptides to maternal T cells and as the main molecule to which immune tolerance needs to be established (127). The importance of HLA-C was further demonstrated by the observation that the combination of the HLA-C allotype (HLA-C2, expressed by fetal trophoblasts) and the expression of its receptor KIR2DS1 in maternal NK cells reduce the risk for pregnancy complications and regulate fetal birth weight (71, 76, 114).

The previous observation that dNK express higher levels of KIR2DL1 and KIR2DS1 than pNK was confirmed. In addition, it was shown expression of KIR2DS1 (both L1$^-$S1$^+$ and L1$^+$S1$^+$ subsets) increased the expression of key cytolytic molecules (perforin, granzyme B and granulysin) stored intracellularly in dNK but not pNK. Furthermore, dNK from *KIR2DS1*$^+$ individuals were less efficiently inhibited by HLA-C2 expression on target cells. HLA-E/NKG2A interaction did not influence the HLA-C2 effect. Overall, these data indicate a skewing of dNK receptor expression towards recognition of HLA-C, and particularly, the potential of dNK to develop an activating response through KIR2DS1 and its interaction with HLA-C2.

Interestingly, the cell line K652 proved not to be a suitable target to study the effect of MHC Class I on NK cell cytotoxicity. K562 is an erythroleukemia cell line that does not express MHC Class II or MHC Class I (151, 152). Because of this lack of MHC Class I molecules, K562 are widely used as classical targets for T cells and NK cells in a variety of cytotoxicity assays (153). However, expression of MHC Class I molecules on transfected K562 lines, even at very high levels, was not enough to inhibit dNK and pNK degranulation against K562. K562 is a tumor cell line and is known to express very high levels of molecules that serve as activating ligands for NK receptors, (e. g. MICA and MICB ligands for NKG2D) (154). The expression of MHC Class I was not sufficient to tip the balance towards inhibition of NK cells.

Although dNK have a higher potential to be activated through the p-SYK/ZAP70 signaling pathway, this does not result increased cytolytic activity. On the contrary, dNK have reduced ability to degranulate and kill target cells including classical MHC-negative 221. Requirements for activation by pro-inflammatory signals may be different between pNK and dNK, possibly due to different maturation conditions. The origin of uterine NK cells is not completely clear. Several lines of evidence point to an origin in mature peripheral NK cells that migrate to the uterus, where their phenotype is modified due to the low inflammatory environment of the placenta. Some studies have developed protocols involving the addition of cytokines abundant in the maternal fetal interface (such as TGF-β) and low oxygen pressure that are able to convert pNK into dNK-like cells (155–157). However, a recent study in mice demonstrated mature NK cells do not migrate to the uterus. Instead, immature NK precursors migrate from the bone marrow to the endometrium where maturation is completed (158). Independently of the origin, NK cells have high plasticity and ability to adapt to the surrounding environment to prevent tissue damage due to excess of inflammation (53, 159). The threshold for dNK activation is thought to be higher than pNK to avoid detrimental effects to pregnancy. However, the expression of high levels of cytolytic molecules in dNK shows a cytotoxic potential that may be employed in situations such as viral or bacterial infections.

The increased activation state of $S1^+$ dNK was previously linked to increased ability to secrete cytokines and growth factors that facilitate trophoblast invasion and placental growth (6). However co-culture of dNK with primary EVT did not enhance cytokine secretion in dNK and no differences were found in cytokine responses between dNK or pNK from *KIR2DS1*$^+$ and *KIR2DS1*$^-$ individuals toward any of the target cells, including 221, 221.C2 and EVT (7). This suggests other stimuli present in the maternal-fetal interface may be involved in dNK function. Interaction of dNK with other immune cells (e.g. macrophages) or decidual stromal cells (DSC), in combination with the cytokine environment of the placenta, may lead to dNK secretion of growth factors. EVT interaction alone was not sufficient to elicit these functions, but may modulate the dNK phenotype. Future focus will be placed on the analysis of the cytokine secretion of dNK stimulated with mitogens (e.g. PMA – Ionomycin) after or during co-culture with EVT or decidual stromal cells. Furthermore, the analysis of cytokine content in the dNK cell culture supernatant only shows the secretion of the total sample of dNK. To analyze the contribution of the $KIR2DS1^+$ dNK subset, intracellular staining of cytokines following stimulation will be included.

The protective effect of maternal KIR2DS1 and fetal HLA-C2 was previously thought to be related to activation of dNK to secrete growth factors. In this chapter, $KIR2DS1^+$ dNK demonstrate a high activation and cytolytic potential that does not translate into cytokine

secretion following stimulation with EVT. In the next chapter, the role of KIR2DS1^{+} dNK in the clearance of placental HCMV infection will be discussed.

CHAPTER 3

EXPRESSION OF KIR2DS1 BY DECIDUAL NK CELLS INCREASES THEIR ABILITY TO CONTROL PLACENTAL HCMV INFECTION

INTRODUCTION

The establishment of a healthy human pregnancy requires the maternal immune system to tolerate fetal alloantigens. HLA-C is the only polymorphic MHC molecule expressed by extravillous trophoblast (EVT) that can be directly recognized by maternal NK and T cells and elicit an allogeneic response in case of a mismatch between mother and fetus. Tolerance is achieved via several mechanisms, such as reduced numbers of effector T cells in the maternal decidua, high activity of regulatory T cells, lower cytotoxicity of decidual NK and $CD8^+$ T cells and a primarily anti-inflammatory microenvironment with high levels of TGF-β and IL-10 (92, 160). However, the immune system must still be able to fight viral and bacterial infections that arise during gestation. The balance between fetal tolerance and infection clearance is one of the greatest challenges the maternal immune system faces during pregnancy.

Viral infections [e.g. human cytomegalovirus (HCMV), HIV, herpes simplex virus (HSV) and influenza virus], can cause severe maternal and fetal disease and morbidity (119–121). However, most infections are under-diagnosed due to the high variety of pathogens and the difficulty in demonstrating the pathogenic role of the viral pathogens in pregnancy complications (122). The most common congenital infection HCMV, occurring in 0.5–2% of all live births. HCMV infects the placenta and can inhibit trophoblast differentiation and invasion, which can lead to placental insufficiency and fetal growth restriction (123–126). If the infection reaches the fetus, it can cause long-term health problems and disabilities such as hearing and vision loss, mental disability and seizures (121). $CD8^+$ effector T cells are the most important cell type to respond and clear viral infections in the periphery, recognizing MHC-viral peptide complexes in infected cells (161). However, in early pregnancy decidual tissue, the frequency of $CD8^+$ T cells is very low (2-7% of total $CD45^+$ lymphocytes). In addition, decidual effector-memory T cells contain reduced levels of the cytolytic molecules perforin and granzyme B, which may limit their cytotoxicity (162). This leaves dNK cells as the main potential actors in the clearance of placental viral infections. Decidual NK cells represent 60-80% of lymphocytes in decidua. In spite of their low cytotoxicity , dNK express very high levels of cytolytic granules (granzymes, perforin, granulysin) and can become cytotoxic when activated by pro-inflammatory cytokines (e.g. IL-15, IL-2 IL-12 and IL-18) (105, 106). A recent study demonstrated dNK are capable of killing HCMV-infected placental fibroblasts (163). Nevertheless, whether or not dNK or pNK can respond and kill HCMV-infected HLA-G^+ EVT has not been addressed.

The presence of activating KIRs, especially *KIR2DS1*, in the maternal genome has been associated with protection from pregnancy complications such as miscarriage, fetal growth restriction and preeclampsia (71, 114). In addition, individuals who carry the KIR-B

haplotype (with more activating KIR) have a significantly improved outcome after viral infections such as HIV and HCMV (64, 164). The combined presence of *KIR3DS1* and its putative ligand *HLA-Bw4* was associated with significantly slower progression to AIDS, lower viral load and slower decline of $CD4^+$ T cells (59–61, 164). In addition, *KIR3DS1* and *KIR2DS1* were shown to be protective against respiratory papillomatosis caused by human papillomavirus (62). Activating KIRs have also been shown to play a role in NK mediated clearance of HCMV infection following hematopoietic stem cell (HSC) or solid organ transplantation (63–66). We therefore postulated the activation of $KIR2DS1^+$ dNK may lead to an earlier and more efficient clearing of virus-infected placental cells. This avoids virus induced pathology and prevents development of complications in later gestation and would provide an explanation to the protective effect of *KIR2DS1* in human pregnancy.

In this chapter, $KIR2DS1^+$ dNK are shown to acquire higher cytotoxicity than $KIR2DS1^-$ dNK when exposed to HCMV-infected decidual stromal cells (DSC), particularly when DSC expresses HLA-C2. Furthermore, dNK were unable to degranulate or secrete cytokines in response to HCMV-infected JEG3 and primary EVT. This emphasizes the immunological challenge to clear placental viral infections within the immune privileged compartment of the placenta. Additional activation of dNK through KIR2DS1/HLA-C2 interaction may contribute to a more efficient control of pregnancy-associated HCMV infection and prevent the spread of the infection through the placenta and fetal tissues.

MATERIALS AND METHODS

Isolation of NK, decidua and trophoblast

Discarded human placental and decidual material (gestational age 6-12 weeks) were obtained from women undergoing elective pregnancy termination at a local reproductive health clinic. Peripheral blood leukocytes were isolated from discarded Leukopacks® from healthy volunteer blood donors at the Massachusetts General Hospital in Boston, MA. All of the human tissue used for this research was de-identified, discarded clinical material. The Committee on the Use of Human Subjects (the Harvard IRB) determined this use of placental and decidual material is Not Human Subjects Research. The procedure to isolate dNK, pNK and trophoblast was described in Chapter 2. Decidual stromal cell lines were obtained as described previously (143). HLA typing was performed by the American Red Cross tissue typing facility, Dedham, Massachusetts.

Cell culture

pNK and dNK were cultured in X-VIVO 10^{TM} media (Lonza) supplemented with gentamycin, 5% human AB serum (Corning) and 2.5 ng/ml rIL-15 (Biolegend). Decidual stromal cells were cultured in OPTI-MEM media (GIBCO) supplemented with 3% FCS and 25µg/ml gentamycin and passaged once to twice a week. 50.000 CD45- sorted EVT were plated in 48 well cell culture plates or 8-chamber slides (Costar) pre-coated with fibronectin (100µl of 20ng/ml for 45 min, BD), in supplemented DMEM/F12 medium (Gibco) as described previously (95). The choriocarcinoma cell line JEG-3 was cultured in RPMI 10% FCS.

Flow Cytometry

Antibodies used for flow cytometric analysis and FACS sort are listed in Supplemental Table S1. For surface staining, cells were stained for 30 minutes on ice in the dark in PBS 1%FCS. For KIR2DS1 staining, NK were stained with KIR2DL1 first (Clone 143211, 1.5µg/ml, 20 min) and thereafter with KIR2DL1/S1 (Clone EB6, 0.5µl, 15 min) as described previously (145). Cells were analyzed immediately or fixed in 1% PFA for 10 minutes for next day analysis. Analysis was performed on a FACSCalibur (BD) or LSR II (BD) and FACS sort was done using a BD Aria III, MoFlo XDP or MoFlo Astrios (Beckman Coulter).

Degranulation assays

For degranulation assays, freshly isolated dNK and pNK were co-cultured with uninfected and HCMV-infected DSC and JEG-3. For co-culture with EVT, dNK and pNK were cultured in X-vivo media (Lonza) supplemented with 5% human serum (Corning) and 2.5ng/ml IL-15 (Biolegend) for 24h to allow optimal HCMV infection of dNK-matched primary EVT. 150.000

dNK or pNK were added to confluent DSC, JEG3 or 50.000 EVT plated in 48 well plates in X-VIVO 10^{TM} supplemented with 2.5 ng/ml IL-15 for 10h. 250ng/ml CD107a PerCP-Cy5.5 antibody was added to all co-cultures. NK were collected and fixed for 10min in 1% PFA and subsequently stained for all relevant surface markers for FACS analysis.

Cytokine secretion analysis

Supernatants from the same co-cultures used for degranulation assays were snap frozen at -80 °C and all cytokines were analyzed using a multiplex cytokine assay (Magpix, Bio-Rad) according to manufacturer's protocols.

Infection of DSC, JEG3 cells and EVT

High titer virus stocks of HCMV AD169 (IE-1-GFP) (a gift from Prof. Donald Coen at Harvard Medical School) were obtained by infecting Human Foreskin Fibroblasts (HFF) (ATCC) and collecting supernatants. Supernatants were aliquoted and snap frozen in liquid nitrogen until use (46). DSC at 80% confluence in 48 well plates were infected at a MOI 0.5 for 1 hour at 37ºC with frequent shaking, followed by normal culture for 48h before use in assays. JEG-3 and primary EVT were infected at a MOI 3-4 for 2 or 3 periods of 12 hours (including 1h of centrifugation at 2800rpm) before assays.

Fluorescence microscopy

HCMV-AD169-GFP - infected DSC, JEG3, and EVT were fixed with 4% PFA for 10 minutes followed by a 10 min permeabilization with PBS/0.01% Triton-X. Purified primary anti-pp65 antibody (Abcam) or mouse IgG2a (Biolegend) were added in a 1:200 concentration for 1h, followed by washing and staining with goat anti-mouse – AlexaFluor 546 secondary antibody (1:500) for 1h. Cells were imaged in a Nikon Eclipse Ti fluorescence microscope.

Statistical analyses

All data was analyzed using GraphPad Prism (version 6.0) software. To determine differences between 2 unpaired groups, a non-parametric Mann-Whitney test was performed, whereas between 2 paired groups a non-parametric Wilcoxon Signed Rank test was performed. To determine differences among more than 2 unpaired groups, a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-test was performed. To determine differences among more than 2 paired groups, a non-parametric Friedman's test with Dunn's multiple comparison post-test was performed. For correlation analyses, Spearman correlation analysis was used. All data are depicted with median and interquartile range, except when otherwise indicated. p-values <0.05 were considered to reflect significant differences.*-$p<0.05$;**-$p<0.01$; *** $p<0.005$

TABLE I.

Epitope	Clone	Fluorochrome	Manufacturer
CD45	HI30	PerCP	Biolegend
	HI30	Alexa Fluor 700™	Biolegend
	HI30	Pacific Orange™	Life Technologies
	HI30	APC	Biolegend
CD56	HCD56	Alexa Fluor 488™	Biolegend
	HCD56	Alexa Fluor 700™	Biolegend
	HCD56	PE	BIolegend
	HCD56	PE-Cy7	Biolegend
	MEM-188	PE-Texas Red™	Life Technologies
CD14	M5E2	FITC	BD Biosciences
	TüK4	PE-Texas Red™	Life Technologies
	M5E2	PE-Cy7	Biolegend
	HCD14	Pacific Blue™	Biolegend
CD3	UCHT1	Pacific Blue™	Biolegend
	S4.1	PE-Texas Red™	Life Technologies
NKG2A	Z199	PE	Beckman Coulter
NKG2C	134591	PE	R&D Systems
KIR3DL1	DX9	PE	BD Biosciences
KIR2DL1	143211	APC	R&D Systems
KIR2DL1/S1	EB6	PE	Beckman Coulter
	EB6	PE-Cy7	Beckman Coulter
KIR2DL2/3	CH-L	FITC	BD Biosciences
	CH-L	PE	BD Biosciences
	DX27	PerCP	Miltenyi Biotec
Granzyme A	CB9	Alexa Fluor 488™	Biolegend
Granzyme B	GB11	Alexa Fluor 488™	Biolegend
	GB11	PE-Texas Red™	Life Technologies
Perforin	dG9	PE	Biolegend
	dG9	Pacific Blue™	Biolegend
Granulysin	DH2	PE	Biolegend
IgG1	MOPC-21	Alexa Fluor 488™	Biolegend
IgG1	MOPC-21	PE	Biolegend
IgG2b	MPC-11	Pacific Blue™	Biolegend
SYK	4D10	FITC	BD Biosciences
ZAP-70	1E7.2	PE	BD Biosciences
ZAP-70(PY319)/SYK(PY352)	17A/P-ZAP70	Alexa Fluor 488™	BD Biosciences
CD107a	H4A3	PerCP-Cy5.5	Biolegend
IgG1	MOPC-21	PerCP-Cy5.5	Biolegend
HLA-G	MEM-G/9	PE	Abcam
EGFR	ICR10	FITC	AbD Serotec

RESULTS

dNK degranulate in response to HCMV-infected DSC but not to infected JEG3 or EVT

dNK have recently been shown to be able to eliminate HCMV-infected placental fibroblasts (163). The target cells used in the referenced study were a mix of maternal and fetal fibroblasts. To discriminate the NK activity in response to HCMV-infected maternal or fetal tissue, maternal decidual stromal cells (DSC), the HLA-G^+ choriocarcinoma cell line JEG3 and primary fetal HLA-G^+ EVT were infected with HCMV-AD169-GFP. Both dNK and pNK showed significantly higher levels of degranulation in response to HCMV-infected DSC compared to healthy DSC (Figure 1B and C).

Interestingly, infection of JEG3 and primary fetal EVT with HCMV significantly increased degranulation by pNK compared to uninfected cells. However, dNK did not have an increased response to HCMV-infected JEG3 or EVT compared to uninfected cells. (Figure 1D-E). In addition, both dNK and pNK secreted significantly more IFN-γ, TNF-α and GM-CSF in response to HCMV-infected DSC compared to healthy DSC, whereas pNK but not dNK secreted more of these cytokines in response to HCMV-infected JEG3 and EVT (Figure 2A-C). In addition, HCMV infection of EVT induced higher IL-6 secretion by pNK but not dNK (Figure 2D). These data emphasize the difficulties of dNK in clearing placental viral infections, possibly due to the immune privileged status of the placenta.

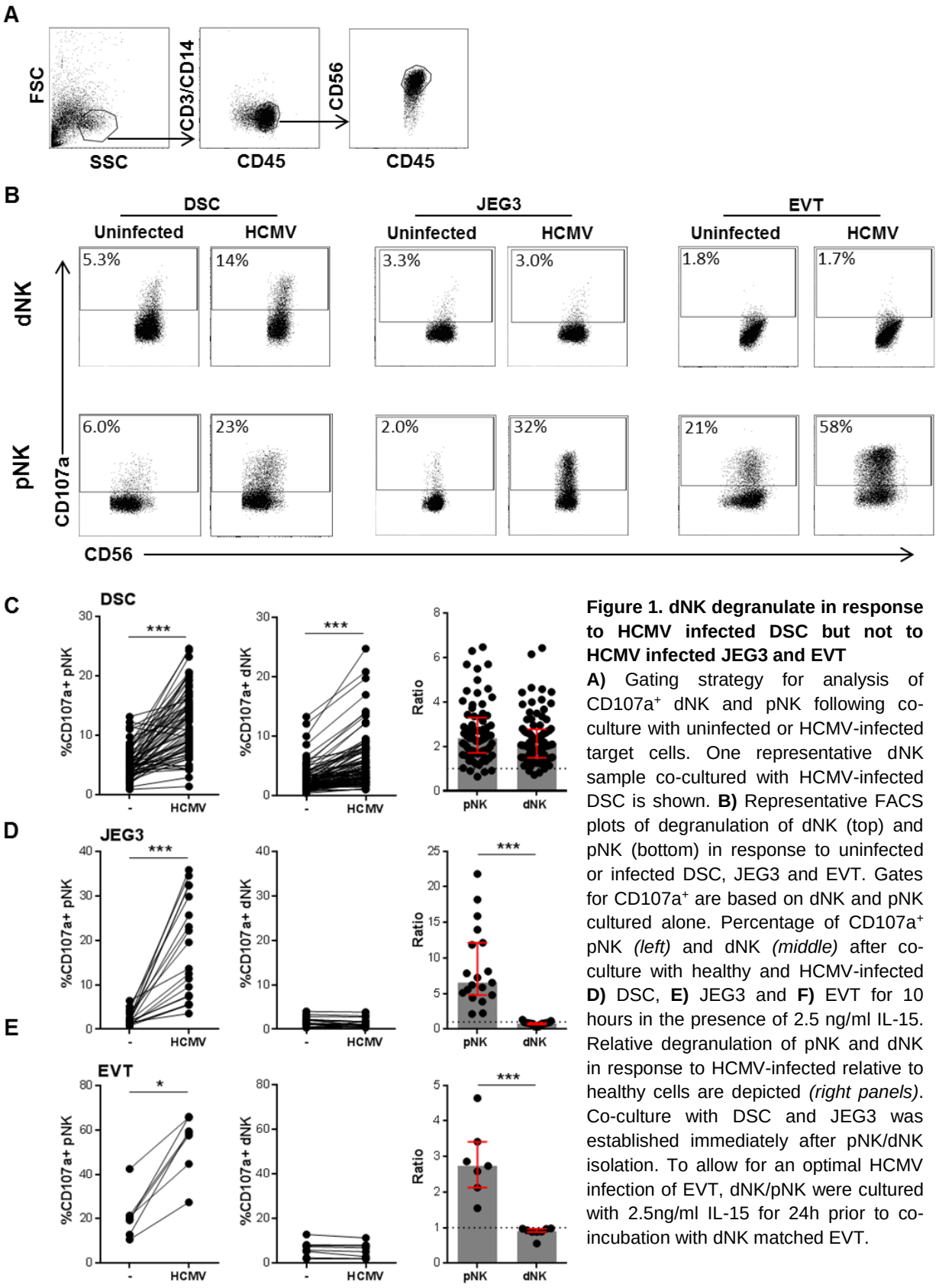

Figure 1. dNK degranulate in response to HCMV infected DSC but not to HCMV infected JEG3 and EVT

A) Gating strategy for analysis of CD107a$^+$ dNK and pNK following co-culture with uninfected or HCMV-infected target cells. One representative dNK sample co-cultured with HCMV-infected DSC is shown. **B)** Representative FACS plots of degranulation of dNK (top) and pNK (bottom) in response to uninfected or infected DSC, JEG3 and EVT. Gates for CD107a$^+$ are based on dNK and pNK cultured alone. Percentage of CD107a$^+$ pNK *(left)* and dNK *(middle)* after co-culture with healthy and HCMV-infected **D)** DSC, **E)** JEG3 and **F)** EVT for 10 hours in the presence of 2.5 ng/ml IL-15. Relative degranulation of pNK and dNK in response to HCMV-infected relative to healthy cells are depicted *(right panels)*. Co-culture with DSC and JEG3 was established immediately after pNK/dNK isolation. To allow for an optimal HCMV infection of EVT, dNK/pNK were cultured with 2.5ng/ml IL-15 for 24h prior to co-incubation with dNK matched EVT.

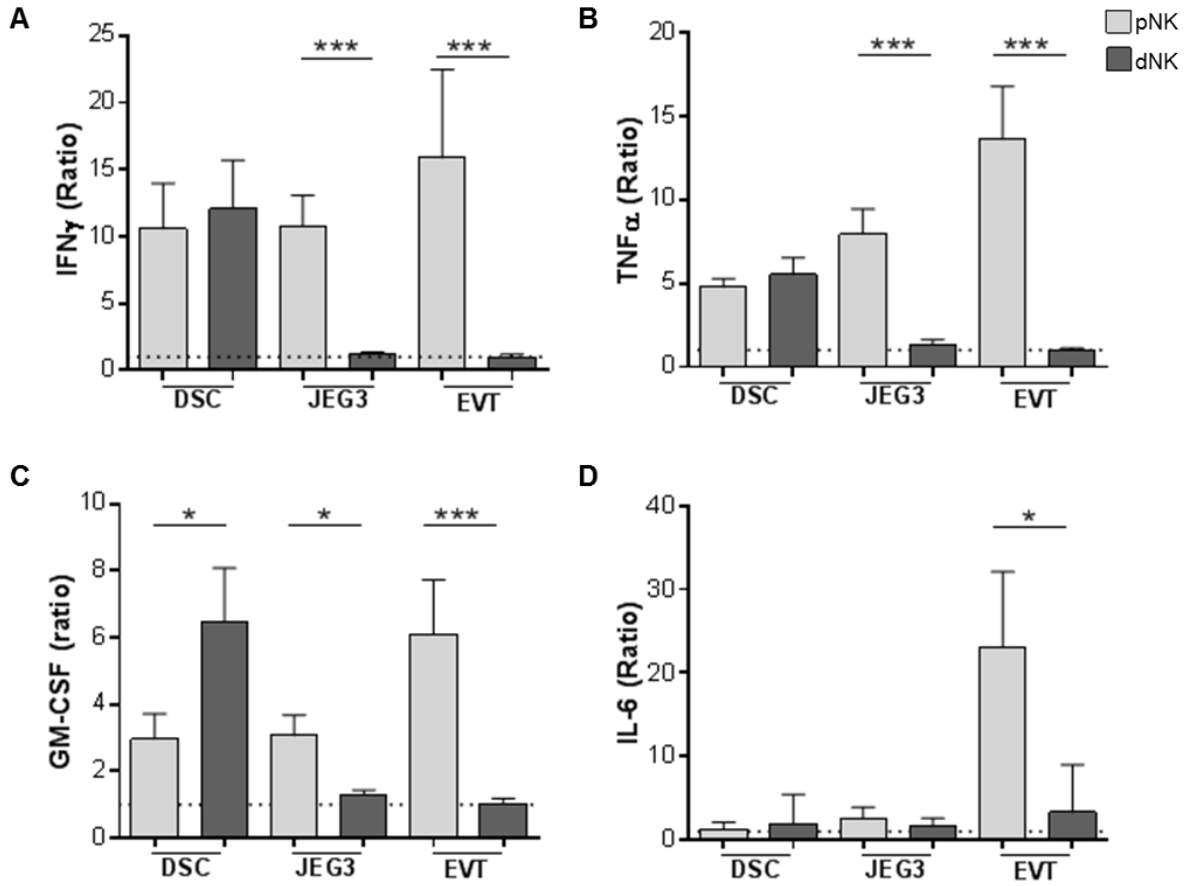

Figure 2. dNK produce cytokines in response to HCMV- infected DSC but not to HCMV-infected JEG3 and EVT. Cell culture supernatants of dNK and pNK incubated with healthy and HCMV infected DSC, JEG3 and EVT were analyzed for **A)** IFNγ **B)** TNFα, **C)** GM-CSF and **D)** IL-6. Cytokine production is depicted as the ratio of concentrations in HCMV infected cultures relative to uninfected cultures. (e. g. all ratios >1 indicate a significant increased response to HCMV infected versus uninfected cells).

HLA-C type of DSC has no influence in total dNK response to HCMV infection

To evaluate the importance of HLA-C allotype in the response of dNK and pNK from *KIR2DS1*- and *KIR2DS1*$^+$ donors, two DSC lines were used: one homozygous for HLA-C1 (DSC.C1) and one expressing both HLA-C1 and C2 (DSC.C2). No difference was found in the ratio of degranulation in response to HCMV-infected cells versus uninfected cells between dNK from *KIR2DS1*$^-$ or *KIR2DS1*$^+$ donors, or in dNK response to *HLA-C2*$^+$ vs *HLA-C2*$^-$ DSC (Figure 3A). In contrast, the ratio of degranulation in response to HCMV-infected cells vs uninfected cells of pNK was higher in response to HLA-C2$^+$ DSC, particularly in *KIR2DS1*$^-$ donors (Figure 3B). These results indicate the presence of activating KIR (KIR2DS1) does not enhance the responses of total dNK population to HCMV infection.

Increased degranulation of KIR2DS1$^+$ dNK in response to HCMV-infected HLA-C2$^+$ DSC

To determine the degranulation capacity of each of the four L1/S1 NK subtypes, degranulation assays for the response to DSC.C1 and DSC.C2 were performed as described previously (Chapter 2). dNK that express KIR2DS1 (L1$^-$S1$^+$ and L1$^+$S1$^+$)

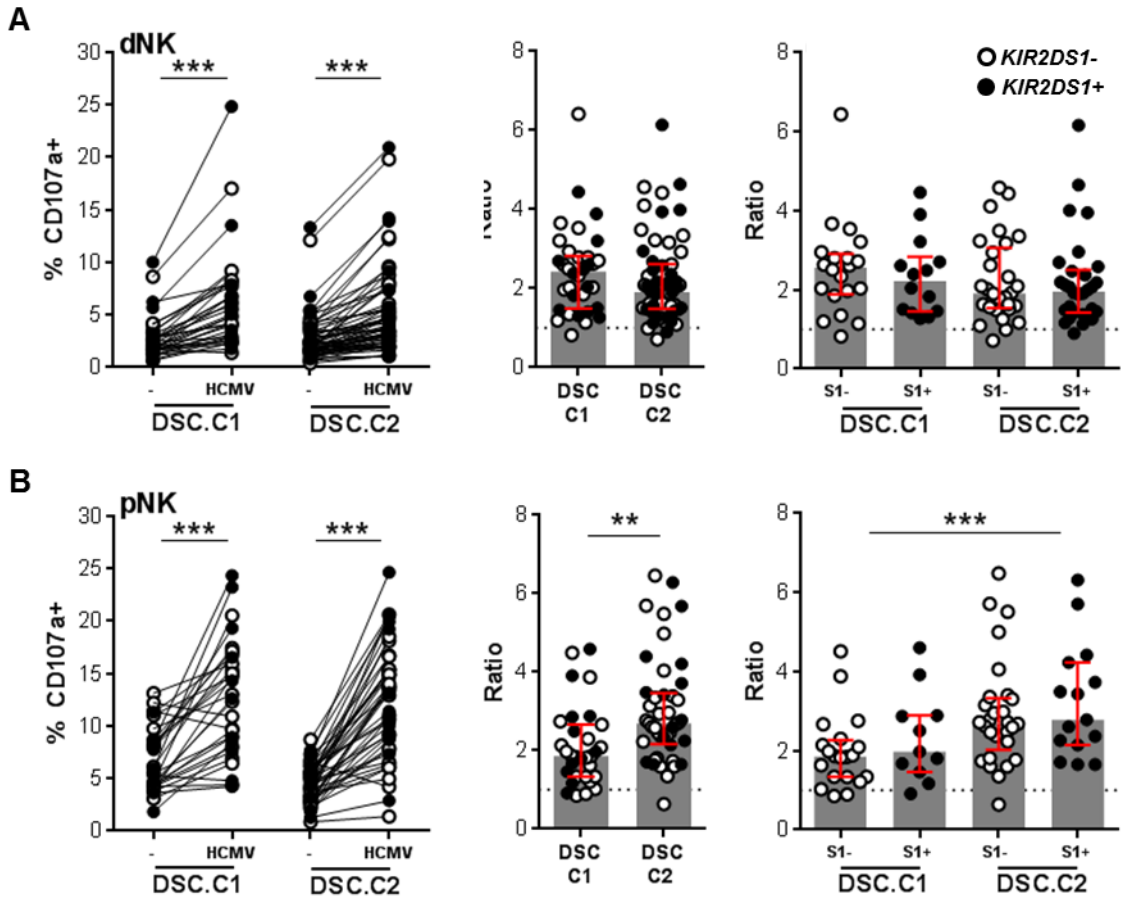

Figure 3. dNK *KIR2DS1* genotype and DSC HLA-C genotype have no influence in the degranulation response to HCMV infected targets (*Left*) Percentage of CD107a$^+$ dNK **(A)** and pNK **(B)** after co-culture with uninfected or HCMV-infected DSC. The response to HLA-C2$^-$ and HLA-C2$^+$ DSC is compared. Relative degranulation of pNK and dNK in response to HCMV-infected relative to healthy HLA-C2$^-$ or HLA-C2$^+$ DSC are depicted (*right panels*), comparing *KIR2DS1*$^-$ with *KIR2DS1*$^+$ donors.

demonstrated a significantly increased response to HCMV-infected DSC.C2 compared to L1$^-$S1$^-$ dNK. A similar increase was also showed in response to infected DSC.C1 but it was not significant (Figure 4A-B). Moreover, the percentage of total S1$^+$ dNK (both L1$^-$S1$^+$ and L1$^+$S1$^+$) positively correlated with the relative degranulation in response to infected DSC.C2 (Figure 4C). In pNK, the L1$^+$S1$^+$ double positive subset also showed an increased response to HCMV-infected DSC but this was independent of HLA-C2 expression on DSC (Figure 4 D-E). Furthermore S1$^+$ pNK did not correlate with the degranulation level in response to infected DSC.C2 (Figure 4F). These data demonstrate the combination of KIR2DS1 expression on dNK and the presence of HLA-C2 on DSC increased the ability of dNK to clear decidual HCMV infection.

Early HCMV infection reduced HLA-C on DSC, JEG3 and EVT while late infection restored HLA-C expression

HCMV has many molecular strategies to circumvent immune recognition and avoid lysis of infected cells (128). Immune evasion strategies by HCMV include the blockade of peptide presentation and downregulation of cell surface expression of MHC class I molecules (165).

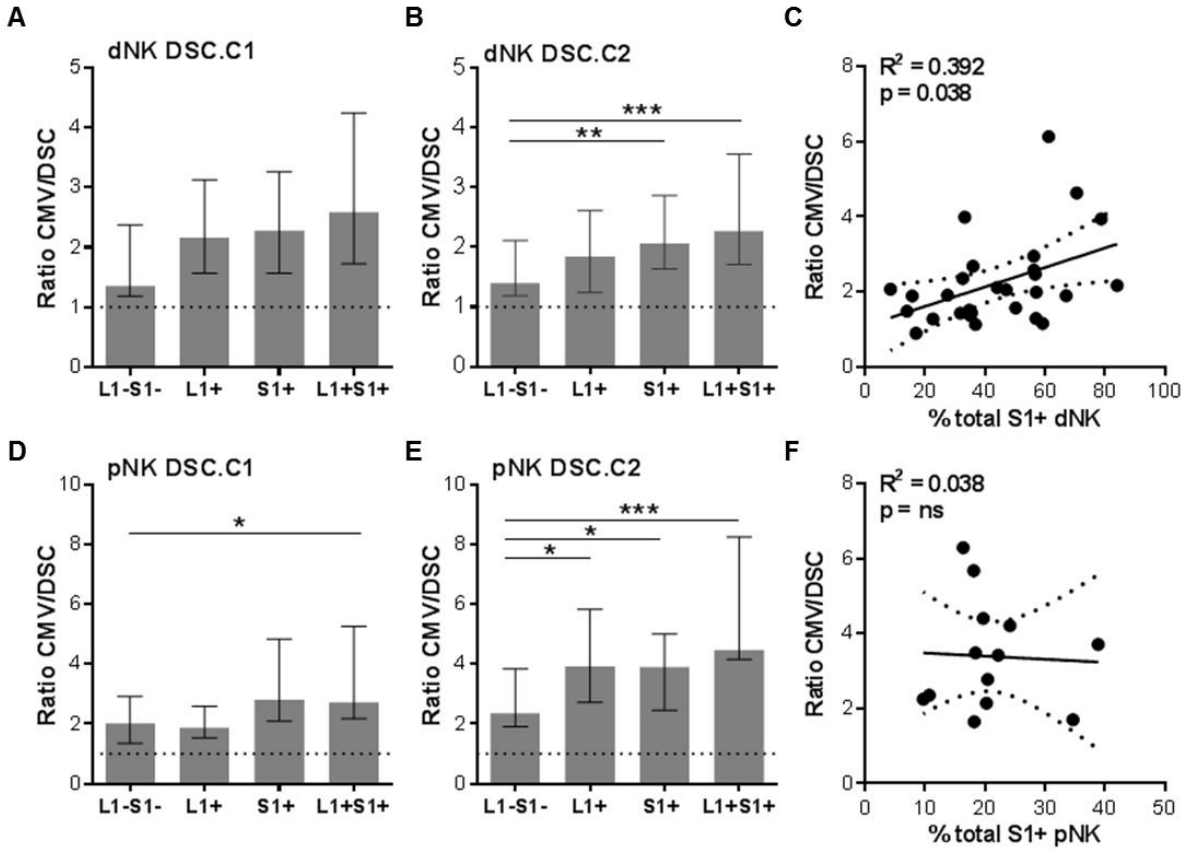

Figure 4. S1⁺ and L1⁺S1⁺ dNK respond strongly to HCMV infected HLA-C2⁺ DSC. Specific degranulation of the four L1/S1 NK subsets in response to HCMV infected DSC depicted as the ratio of %CD107a⁺ NK to HCMV-infected versus uninfected DSC. **A)** dNK HLA-C2⁺ DSC (n=24) **B)** dNK to DSC.C1 (n=13) **C)** pNK to DSC.C2 (n=13) and **D)** pNK to DSC.C1.C2 (n=10) **E)** Correlation plot between percentage of S1 positive dNK and relative degranulation of total dNK population in response to HCMV-infected or healthy DSC.C2. **F)** Correlation plot between percentage of S1 positive pNK and relative degranulation of total pNK population in response to HCMV-infected or healthy DSC.C2.

This prevents cytolysis by CD8⁺ effector T cells that require MHC/peptide-TCR binding. However downregulation of MHC class I molecules increases the susceptibility of HCMV-infected cells to lysis by NK cells through missing self-recognition (165). Downregulation of MHC, including HLA-C, may also prevent activation of NK cells through KIR2DS1.

DSC, JEG3 and EVT were infected with HCMV-AD169-GFP. This HCMV strain has GFP tagged to an HCMV-Immediate Early (IE) gene. Infected cells were analyzed 48h post infection for expression of IE-GFP and stained for pp65 (a late expression gene) for fluorescence microscopy (Figure 5). HCMV infection of DSC reached nearly 100% with ~70% IE-GFP⁺ and ~30 % pp65⁺GFP⁻ cells. HCMV infection levels for JEG3 reached closer to 40%, with 10% IE-GFP⁺ cells and ~30% pp65⁺GFP⁻ cells (Figure 5 and 6A). At 48h, 100% of EVT expressed IE-GFP and also pp65, although different levels of expression could be observed for the 2 proteins. HCMV-infected and uninfected DSC, JEG3 and EVT were analyzed for expression MHC class I molecules. HLA-A2, HLA-E and especially HLA-C expression in HCMV-infected DSC was significantly reduced on all infected DSC that expressed the IE-GFP protein (Figure 6A, C, D, F). However, when HCMV-infected DSC lost

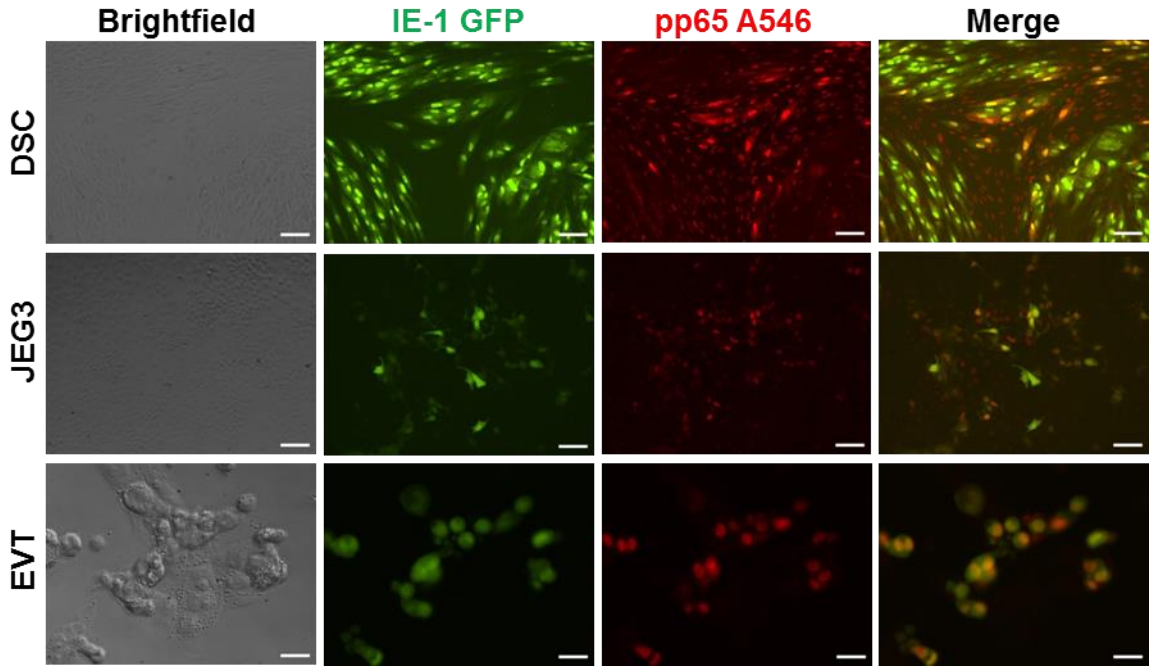

Figure 5. HCMV infection of DSC, JEG3 and EVT. Fluorescence microscopy of DSC, JEG3 and EVT infected with GFP-tagged AD169 HCMV strain. HCMV infection is represented by expression of viral IE-1-GFP (immediate early) or pp65 (late infection) after 48 hours of incubation. Scale bars: DSC and JEG3 - 100µm; EVT - 25µm.

expression of IE-GFP protein and only expressed pp65, HLA-E, HLA-A2 and HLA-C expression were restored and HLA-C expression increased in comparison to uninfected DSC (Figure 6A, C, D, G). In JEG3 and EVT, HLA-C expression was significantly reduced on about ~50% of IE-GFP^{+} cells compared to uninfected cells (Figure 6A, F). Similarly to DSC, when HCMV-infected JEG3 and EVT lost expression of IE-GFP protein, HLA-C expression was increased in comparison to uninfected cells (Figure 6F). HCMV infection did not change HLA-E and HLA-G expression on JEG3 and EVT. Furthermore, HCMV infection did not induce MICA or MICB, two potent activating ligands that bind the activating NK receptor NKG2D, on any of the HCMV-infected cells (data not shown). This data demonstrate HCMV infection has distinct effects on DSC, JEG3 and EVT

CD57 expression increases pNK responses to HCMV, but not dNK

pNK expressing high levels of NKG2C and CD57 have been found to be expanded in HCMV seropositive individuals (166, 167). NKG2C is an activating receptor for HLA-E, while CD57 is a marker of maturation in NK cells. To analyze if expression of NKG2C and CD57 influence pNK and dNK responses to HCMV-infected cells, degranulation by NKG2C$^{+/-}$ and CD57$^{+/-}$ NK subsets were analyzed. Only 2 out of 15 blood donors analyzed had an expanded subset of more than 20% NKG2C-expressing pNK, which may indicate HCMV seropositivity. The majority of the remaining donors have very low (<5%) percentages of NKG2C^{+} pNK. In contrast, decidua contained high frequencies of NKG2C^{+} dNK (Figure 7A,

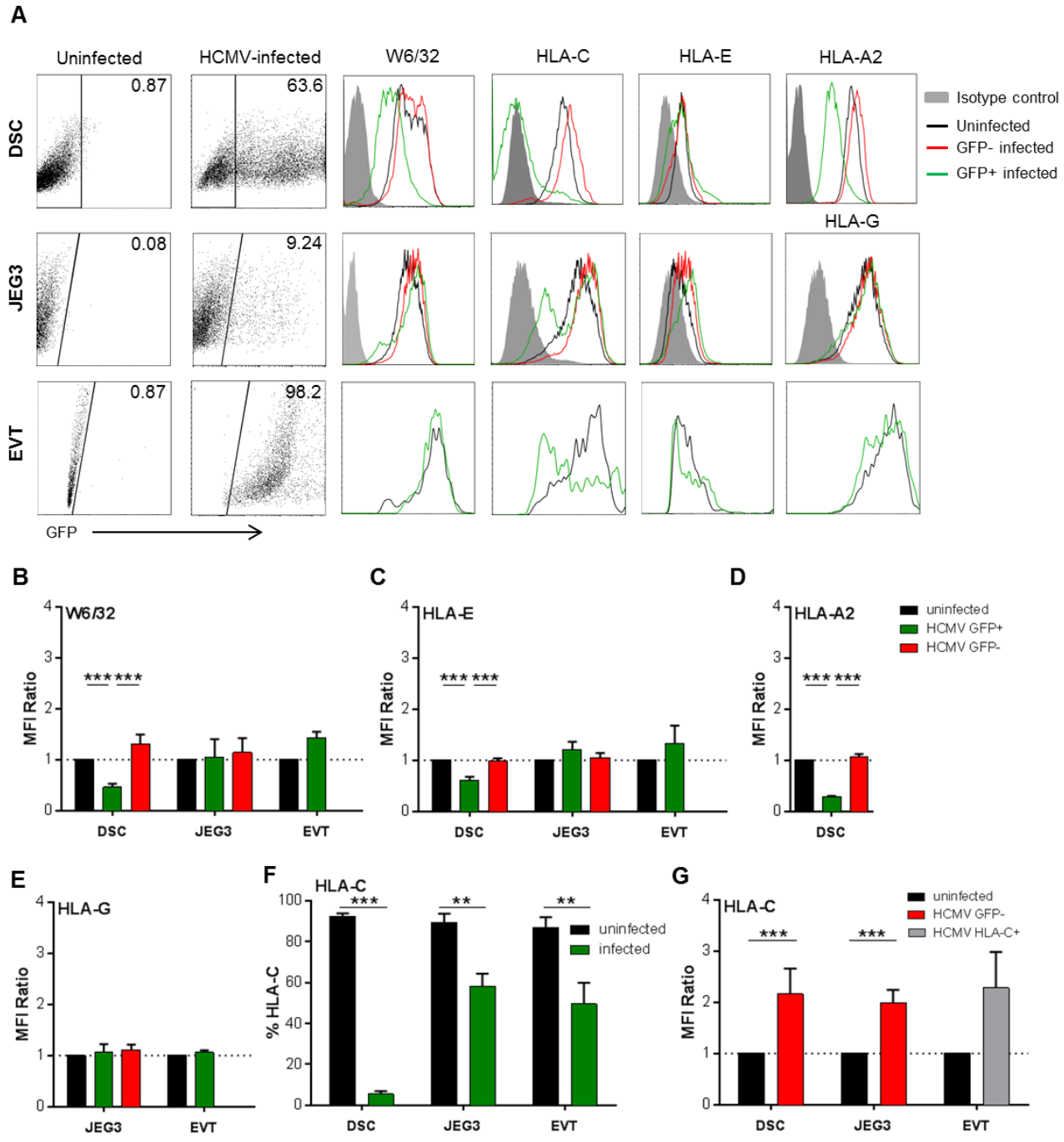

Figure 6. HCMV early infection reduces expression of HLA-C in DSC, JEG3 and EVT.
A) Representative FACS plots of percentage of infection and expression of total MHC Class I (W6/32), HLA-C, HLA-E, HLA-A2 and HLA-G in healthy or GFP^-/GFP^+ HCMV-infected DSC, JEG3 and EVT. Graphs depict the ratio between the expression levels (MFI) of the MHC Class I molecules **(B)**, HLA-E **(C)**, HLA-A2 **(D)** and HLA-G **(E)** in GFP^-/GFP^+ HCMV-infected and healthy DSC (n=10), JEG3 (n=3) and EVT (n=5).. **F)** Percentage of $HLA\text{-}C^+$ DSC (n=10), JEG3 (n=3) and EVT (n=3) after infection with HCMV. **G)** Ratio between expression levels (MFI) of HLA-C in GFP^- HCMV-infected and healthy DSC and JEG3. For HCMV-infected EVT, no GFP^- cells were detected. The grey bar refers to the ratio of the MFI of $HLA\text{-}C^+$ population in GFP^+ HCMV-infected EVT and MFI of HLA-C expression in healthy EVT. Bars indicate mean ±SE.

B). CD57 expression followed the opposite pattern of NKG2C: while ~70% of pNK expressed CD57 (with some variation), virtually no dNK expressed CD57 (Figure 7A, C).

To evaluate the possible role of NKG2C in HCMV clearance, the ratio of degranulation in response to HCMV-infected versus uninfected DSC of NKG2C$^-$ and NKG2C$^+$ dNK and pNK were compared. No differences were found between the two subsets in dNK or pNK, and no correlation was found between percentage of NK cells expressing NKG2C and the relative degranulation in response to HCMV-infected versus uninfected DSC (Figure 7D-G). However, in pNK, CD57$^+$ cells had higher ratios of degranulation to HCMV-infected versus uninfected DSC than CD57$^-$ (Figure 7H), although no correlation between the strength of the response and the percentage of CD57-expressing pNK was found (Figure 7I). These results indicate mature CD57$^+$ pNK display higher capacity to eliminate HCMV infections.

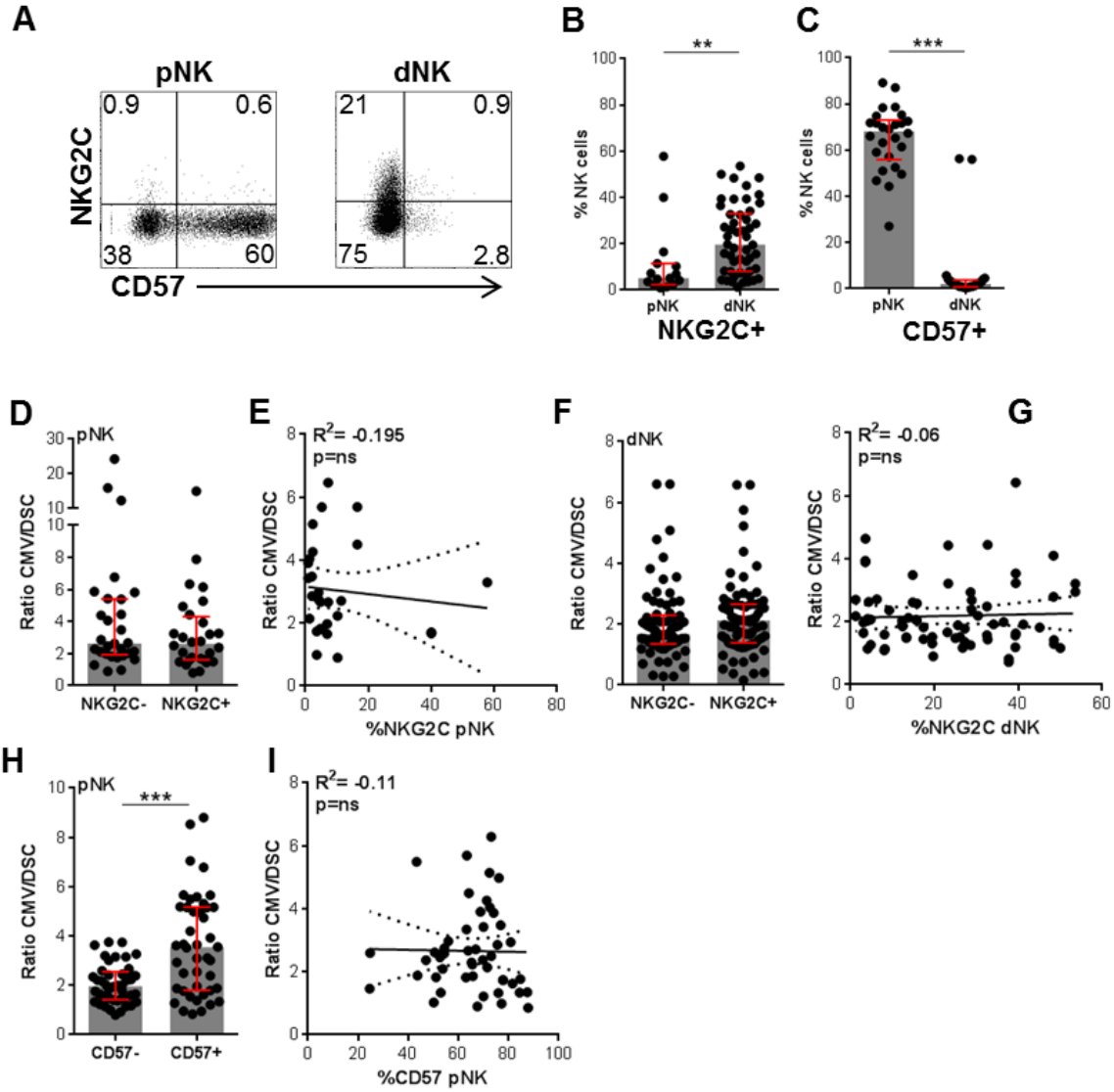

Figure 7. CD57 expression increases pNK degranulation response to HCMV infected DSC. A) Representative FACS plots of NKG2C and CD57 expression in pNK and dNK. Graphs depict the percentages of NKG2C **(B)** and CD57 **(C)** in pNK and dNK. Ratios of degranulation of NKG2C$^-$ and NKG2C$^+$ pNK **(D)** or dNK **(F)** to HCMV infected vs uninfected DSC. Correlation plot between percentage of NKG2C$^+$ pNK **(E)** or dNK **(G)** and relative degranulation of total pNK/dNK population in response to HCMV-infected or healthy DSC. **H)** Ratios of degranulation of CD57$^-$ and CD57$^+$ pNK to HCMV infected vs uninfected DSC. **I)** Correlation plot between percentage of CD57$^+$ pNK and relative degranulation of total pNK population in response to HCMV-infected or healthy DSC.

DISCUSSION

HLA-C expressed by fetal EVT has a unique role in pregnancy, as the only polymorphic molecule that can express viral peptides to maternal T cells and as the main molecule to which immune tolerance needs to be established (161). The importance of HLA-C was further demonstrated by the observation that the combination of the HLA-C allotype HLA-C2 expressed by fetal trophoblasts and the presence of its receptor KIR2DS1 expressed by maternal NK cells reduces the risk for pregnancy complications and are related to fetal birth weight (71, 76, 118). Here we present an exciting and novel mechanism to explain how expression of KIR2DS1 by dNK increases their ability to control placental HCMV infection and thus may limit subsequent virus-induced placental inflammation and pathology.

dNK and in particular KIR2DS1$^+$ dNK degranulated in response to HCMV-infected decidual stromal cells, but failed to respond (degranulate and produce cytokines) to HCMV-infected JEG3 and EVT. Thus EVT are tolerant for dNK lysis even when infected. Many molecules, in particular HLA-G, but also others (e. g. surface expression of PDL1, CTRAM and secretion of anti-inflammatory cytokines such as TGFβ and EBI3) have been shown to be expressed on EVT and may explain how EVT are protected (95).

Interestingly pNK degranulated and produced pro-inflammatory cytokines (IFN-γ, TNF-α and GM-CSF) in response to HCMV-infected JEG3 and EVT. pNK are not in direct contact with EVT *in vivo* and whether influx of pNK to HCMV-infected decidua contributes to clearance of HCMV-infected EVT requires further investigation. Furthermore, degranulation and cytokine production of pNK in response to HCMV-infected EVT demonstrates EVT are not completely resistant to killing by immune cells. Additional activation of dNK during virus-induced inflammation may be required for dNK to clear infected EVT. Further investigation is needed to determine if there are conditions under which dNK are able to kill infected EVT. In the present study, dNK were activated with a low dose of IL-15 (2.5ng/ml). However during a placental viral infection other decidual immune cells, most importantly macrophages can secrete pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12) that can enhance dNK activation.

dNK and in particular KIR2DS1$^+$ dNK may limit the spread of HCMV infection by lysis of DSC. DSC require a MOI of 0.5 to be infected and infection reaches 100% after 48 hours. In contrast EVT and especially JEG3 require an MOI of 3-4 for only 30-50% of the cells to become infected. Previously, miRNAs produced and secreted by EVT exosomes were shown to limit HCMV infection of trophoblast cells (168), but HCMV-infected EVT can be found in placental tissue (169). Other pathogens such as *Listeria monocytogenes* have previously shown to preferentially infect EVT (170).

HLA-C expression was downregulated in HCMV-infected IE-GFP$^+$ DSC, JEG3 and EVT, and upregulated during late infection. Other MHC molecules such as HLA-A2 and HLA-E were downregulated during early infection of DSC. Interestingly, HLA-G expression in EVT and JEG3 was not affected, contrarily to what was claimed in an earlier study (171). Park *et al* demonstrated HCMV infection of HLA-G-transfected human foreskin fibroblasts (HFF) downregulated HLA-G expression and identified the HCMV protein US10 as the main factor involved in HLA-G degradation. In addition, transfection of JEG3 with US10 also led to decrease of HLA-G expression (171). In our study, JEG3 were directly infected with HCMV and the efficiency of infection is much lower than HFF. HCMV infection of HFF and US10 transfection of JEG3 potentially yield high levels of US10 that are not observed in direct JEG3 infection. Thus, HCMV infection of JEG3 does not produce sufficient levels of US10 to downregulate HLA-G. This hypothesis is reinforced by the observation that HCMV early infection of DSC reduces HLA-C expression in ~100% of IE-1-GFP$^+$ cells, compared to ~50% of JEG3 and EVT. The modulation of HLA-C expression by HCMV potentially leads to different responses by dNK in early and late infection. While early infected cells may become susceptible to missing self-recognition through inhibitory KIR (KIR2DL1/2/3), late infection and the increase in HLA-C expression levels may provide more potent activation through activating KIR such as KIR2DS1. The presence of KIR2DS1$^+$ cells among the total sample of dNK does not provide an advantage on viral clearance over dNK from *KIR2DS1*- individuals (Figure 3). However, expression of KIR2DS1 in individual dNK increases their cytotoxicity to HCMV-infected cells and may be important in clearing local foci of infections (Figure 4).

The increased activation state of S1$^+$ dNK was previously linked to increased ability to secrete cytokines and growth factors that facilitate trophoblast invasion and placental growth (118). However co-culture of dNK with primary EVT did not enhance cytokine secretion in dNK and no differences were found in cytokine responses between dNK or pNK from *KIR2DS1*$^+$ and *KIR2DS1*$^-$ individuals toward any of the target cells, including 221, 221.C2, DSC, JEG3 and EVT (Chapter 2) (95). Our results suggest KIR2DS1 provides protection from pregnancy complications due to early elimination of local infections in decidua by KIR2DS1$^+$ dNK. This may avoid the spread of the infection in the placenta, which is known to inhibit trophoblast invasion that can lead to fetal growth restriction and miscarriage (123). Improvement of the diagnosis of congenital infections is crucial to study associations of KIR/HLA with susceptibility to pregnancy related HCMV infection or reactivation.

HCMV reactivation leads to long-term expansion of NKG2C$^+$ pNK, an effect particularly observed in recipients of hematopoietic stem cell transplantation. The expanded NKG2C$^+$ population was long lived and also expressed the maturation marker CD57. NKG2C$^+$CD57$^+$ pNK population expressed several other markers of activation and displayed potent cytolytic

function in response to classical NK targets and NKG2C antibody stimulation (172). In this study, high percentages of NKG2C$^+$ dNK were present in the decidua, whereas only two blood donors had an expanded population of NKG2C$^+$ pNK. Furthermore no CD57$^+$ dNK were found in decidua, while the majority of pNK were CD57$^+$. NKG2C expression was not associated with increased degranulation to HCMV-infected DSC in pNK or dNK, but CD57 expression in pNK increased the response to HCMV. This indicates the maturation status of pNK is an important determinant of their anti-viral activity.

NKG2C expression was associated with lack of FcRγ in NK cells from HCMV-seropositive individuals. NKG2C$^+$FcRγ$^-$ cells were found primarily in individuals infected with HCMV and showed an increased response to HCMV-infected cells in the presence of anti-HCMV antibodies or serum from seropositive individuals. Thus, the potent response of NKG2C$^+$ pNK is ADCC-dependent (173, 174). NK cells are not able to rearrange somatic receptor genes as T or B cells and their ability to generate an immunological memory to antigens is controversial. However, several studies of murine models demonstrated that subsets of NK cells can proliferate in response to a pathogen and mount a stronger response upon a second infection with the same pathogen. An example is mouse cytomegalovirus (MCMV) which upon infection produces a MHC-like protein (m157) that is expressed in the surface of infected cells and functions as a ligand for the activating NK receptor Ly49H. Ly49H$^+$ mouse NK cells proliferate and mount stronger responses upon second MCMV infection (23). In humans, more research is required to identify memory NK cells, but NKG2C may serve as a marker for memory NK cells formed during virus infections. The existence of NKG2C$^+$FcRγ$^-$ dNK has not been studied. It will be important to identify and characterize this subset and to find associations with the previous HCMV history of the individual.

Many pregnancy complications are associated with insufficient trophoblast invasion, inadequate spiral artery remodeling and underdeveloped placentas. The interaction of maternal immune cells with fetal extravillous trophoblast at the maternal-fetal interface regulates these processes. Here we demonstrate that maternal immune cells, especially dNK, are able to fight infections in the maternal fetal interface, reducing the risk of infection-related pregnancy disorders. Moreover, the expression of KIR2DS1 in dNK, in association with HLA-C2 expression in decidual stromal cells, increases the response of dNK to HCMV infections and contributes to explain the protective effect of KIR2DS1 in pregnancy. These results open new pathways for the development of early diagnosis and preventative therapies to fight congenital infections, by identifying high-risk pregnancies and enhancing dNK immune responses.

CHAPTER 4

CONTROL OF BACTERIAL INFECTIONS BY DECIDUAL NATURAL KILLER CELL – SECRETED GRANULYSIN

INTRODUCTION

Decidual NK cells (dNK) are the most abundant leukocyte type in the maternal fetal interface, but their cytotoxic response to MHC Class I – negative cells and virus-infected cells is lower compared to peripheral NK (pNK) (106). dNK contain cytolytic molecules (granzymes and perforin) in similar amounts to pNK, and in particular, express increased levels of the active form of the anti-bacterial granulysin (Chapter 2). These observations suggest dNK may require distinct signals to display their full cytotoxicity and, particularly, granulysin may be important in bacterial infection clearance in the placenta.

Granulysin, an unusual lytic peptide

Cytotoxic T cells (CTL) and NK cells have multiple ways to eliminate target cells. The most extensively studied is the directional release of cytolytic granules into the targets during the immune synapse. Cytolytic granules contain several types of molecules involved in target cell death. Two families of these molecules have been extensively studied: Granzymes and Perforin. The granzyme family of serine proteases is involved in the cell death (apoptosis): Granzyme B has a role in caspase-dependent apoptosis, while Granzyme A leads to cell death independently of caspases. Cell membranes are not permeable to granzymes and for these proteins to reach the cytoplasm they require perforin. Perforin polymerizes in the plasma membrane of the target cells and forms a pore through which granzymes can enter the cytoplasm (14).

A third cytolytic molecule, granulysin, was identified by Alan Krensky's lab. The expression pattern of granulysin falls in the category of "late" after T cell activation and is similar to that of perforin and granzymes (175). Granulysin was found to be in cytoplasmic granules and to be exocytosed following stimulation of cytotoxic T lymphocytes (CTL) through the TCR and was also identified in NK cells (176). Granulysin is synthesized as a propeptide of 15kDa that can be proteolytically cleaved to generate a 9kDa peptide, which in high concentrations has lytic activity to tumor cell lines. The carboxyl-terminal region of granulysin shares structural homology with a family of proteins named saposin-like proteins (SAPLIP), which are generally small peptides that interact with lipids. The most similar member of the SAPLIP family is NK-lysin, a porcine NK and T cell granule protein, known to associate with the membrane of target cells and for having cytolytic and antibacterial activity (177). The proteins of the SAPLIP family share a common structure based primarily on the conserved positioning of cysteines and a series of hydrophobic residues. The predicted structure of SAPLIP family members, including granulysin, suggests the presence of four relatively amphipathic helices held together as a bundle by disulfide bonds (Figure 1) (178).

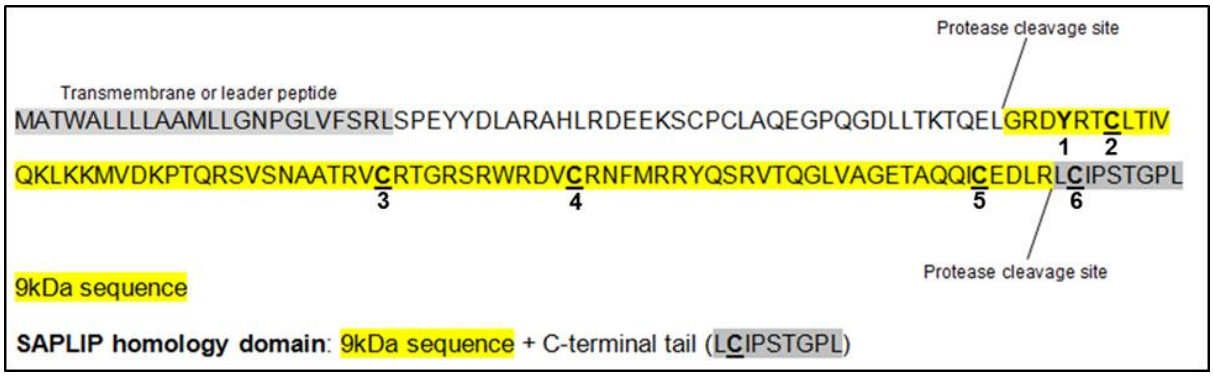

Figure 1. Sequence of the granulysin protein. After cleavage of the predicted hydrophobic leader sequence, a protein of 15 kDa is produced. The carboxyl-terminal region of the molecule contains a SAPLIP domain that is retained in the 9 kDa granulysin protein. Five cysteines important for protein folding in SAPLIP are shown in bold, underlined and numbered . The first tyrosine in granulysin-9 kDa (Tyr 1), which normally corresponds to Cys 1 in SAPLIP, is also numbered. Disulfide bonding patterns in NK-lysin and PSP-B are: Cys 1-Cys 6, Cys 2-Cys 5, and Cys 3-Cys 4. Adapted from reference (178).

Granulysin has antimicrobial activity

Although 9 kDa granulysin shows lytic activity to mammalian cells, the activity is dose dependent and only very high concentrations of recombinant granulysin are able to yield significant lysis (10-100 μM) (177). 9kDa granulysin was subsequently found to be able to lyse mycobacteria and parasites when used in much lower concentrations (1-5 μM) (136), showing antimicrobial activity similar to NK-lysin. Bacterial death is due to granulysin-induced lesions in the membrane, an activity similar to that of perforin in mammalian cell membranes – creation of pores in bacterial membranes that alter permeability (179). The higher affinity of granulysin to bacterial membranes versus mammalian cell membranes is due to the cholesterol content – granulysin preferentially lyses negatively charged cholesterol-poor membranes(180).

Low micromolar concentrations of 9kDa granulysin are enough to lyse extracellular mycobacteria, but not to kill intracellular mycobacteria in macrophages. For lysis of intracellular bacteria, the addition of recombinant perforin is necessary. Thus CTL must respond to bacteria-infected cells and release both perforin (which creates a pore in the mammalian cell through which granulysin can enter) and granulysin. In general, formation of an immune synapse and granule exocytosis also involves the transfer of granzymes, which will then cause the infected cell to die by apoptosis (136).

Concerted action of low-dose granulysin and granzymes lyses bacteria

Granulysin has lytic activity to mammalian cells, but only at irrelevantly high doses. The Lieberman lab recently described that at nanomolar concentrations (~25 fold less than is needed by granulysin on its own to kill bacteria), granulysin delivers granzymes into bacteria. The granzymes then rapidly kill the bacteria by cleaving components of bacterial electron

transport chain (ETC) complex-I to disrupt electron transport and generate superoxide anions. At the same time, granzymes cleave and disrupt key oxidative stress defense enzymes that detoxify superoxide anion, rendering the bacteria defenseless to superoxide anion-induced death. Killer cell lysis of intracellular bacteria relies on the concerted action of all three cytotoxic effector molecules – granzymes, perforin and granulysin, in which perforin delivers granzymes and granulysin into the infected mammalian cells and then granulysin binds to bacterial membranes to deliver granzymes into bacteria to kill them (135). Furthermore these cytolytic molecules activate a similar death pathway in intracellular protozoan pathogens (*T. cruzi*, *L. major*, *T. gondii*) (F. Dotiwala *et al*, Nature Med, provisionally accepted).

Decidual NK cells express higher levels of 9kDa granulysin than peripheral NK cells

dNK at the maternal fetal interface have been shown by various labs, including ours, to express cytolytic molecules, even though their cytotoxicity is lower than that of pNK (104, 105). In 2008, granulysin expression was shown to be increased in spontaneous abortion tissue compared to healthy placental tissue, and transfection with granulysin gene was able to induce apoptosis in the trophoblast cell lines HTR8/SV40neo and JEG3 (181). In 2011, the Rukavina lab reported that dNK contained more granulysin than pNK. dNK also constitutively secreted granulysin independently of *in vitro* activation, while pNK did not (138). The secretion of granulysin may be due to previous activation *in situ* (at the maternal fetal interface) to which pNKs are not subjected.

Although the only functional observation of granulysin in the maternal-fetal interface was related to tissue apoptosis during spontaneous abortion, its striking abundance raises the question of its function in healthy pregnancies. Given its antimicrobial properties, the analysis of granulysin role in the clearance of placental bacterial infections is imperative.

In utero infection with viruses (diverse herpesviruses, rubella, B19 parvovirus), bacteria (*L. monocytogenes*, GBS, syphilis), and parasites (*T. gondii*) can lead to fetal loss, premature labor, congenital anomalies and fetal distress. For example, *Listeria monocytogenes* infection among pregnant women is 17-fold higher than among the general population (12 vs. 0.7 per 100,000) (131). *L. monocytogenes* is a Gram-positive rod associated with ingestion of raw meats, unwashed raw vegetables, and soft unpasteurized cheeses; fetal sequelae are severe with spontaneous abortion in 10–20%, intrauterine fetal death in 11%, and preterm birth in 50% (132). *L. monocytogenes* may spread to the uterus via either an ascending infection or a hematogenous route. EVT may control the spread of *L. monocytogenes* at the maternal–fetal interface by confining the bacteria within vacuolar compartments destined for lysosome degradation (170). Infection by *L. monocytogenes* during pregnancy can result in miscarriage, stillbirth, preterm delivery, and maternal and

neonatal sepsis (134). The mechanism of fetal death may result from impaired suppression of maternal T cells to fetal antigens by maternal $Foxp3^+$ T regulatory cells (133).

Understanding the immune response to infection in the pregnant uterus is critical to protecting the fetus and the mother from infection to improve pregnancy outcome. In this study, the main aim is to characterize the antimicrobial effect of dNK and pNK on a trophoblast-like cell line, JEG3, and the role of granulysin in this process. The abundance of dNK in the maternal fetal interface and their potential role in bacterial infection clearance are key points that can be targeted for prenatal therapy.

MATERIALS AND METHODS

Isolation of dNK and pNK

Discarded human placental and decidual material (gestational age 6-12 weeks) were obtained from women undergoing elective pregnancy termination at a local reproductive health clinic. Peripheral blood leukocytes were isolated from discarded Leukopacks® from healthy volunteer blood donors at the Massachusetts General Hospital in Boston, MA. All of the human tissue used for this research was de-identified, discarded clinical material. The Committee on the Use of Human Subjects (the Harvard IRB) determined this use of placental and decidual material is Not Human Subjects Research. The procedure to isolate dNK and pNK was described in Chapter 2.

Cell culture

pNK and dNK were cultured in X-VIVO 10™ media (Lonza) supplemented with 5% human AB serum (Corning) and 2.5 ng/ml recombinant IL-15 (Biolegend). The choriocarcinoma cell line JEG-3 was cultured in RPMI 10% FCS supplemented with 1% Pen/Strep (Gibco). 721.221 (221) immortalized cell line was cultured in RPMI 10% FCS supplemented with 1%Pen/Strep (Gibco). Whenever target cells (721.221 or JEG3) were plated for bacterial infection, cells were washed three times in antibiotic-free media and seeded for overnight culture in antibiotic-free media.

Flow Cytometry

Antibodies used for flow cytometric analysis and FACS sort were as follows: CD45 Pacific Orange (HI30), CD14 PE-Texas Red (TüK4), CD3 PE-Texas Red™ (S4.1) (Invitrogen), CD14 FITC (ME52), CD56 PE (HCD56), CD56 AlexaFluor 700™ (HCD56), Granulysin AlexaFluor 647™ (DH2), Granulysin PE (DH2), CD107a PerCP-Cy5.5 (H4A3) (Biolegend), Granulysin AlexaFluor 488™ (RB1) (BD Pharmingen). For surface staining, cells were stained for 30 minutes on ice in the dark in PBS 1% FCS. Cells were analyzed immediately or fixed in 1% paraformaldehyde (PFA) for 10 minutes for next day analysis. For intracellular staining, cells were fixed and permeabilized using the BD CytoFix/CytoPerm kit (BD Biosciences). Analysis was performed on a FACSCalibur (BD) or LSR II (BD) and FACS sort was done using a BD Aria III, MoFlo XDP or MoFlo Astrios (Beckman Coulter).

Cytolytic granule secretion

dNK and pNK were cultured overnight in antibiotic-free X-vivo 10™ (Lonza) at a concentration of $2x10^6$ cells/ml with 2.5ng/ml IL-15 (Biolegend). Supernatants from these cultures were collected and snap frozen at -80ºC till analysis. Granulysin concentration was

measured by ELISA (LEGENDMAX, Biolegend). Granzyme A, Granzyme B and Perforin concentration were measured by Milliplex MAX (EMD Millipore) in a Luminex instrument.

Colony forming unit assays

dNK and pNK were cultured overnight in antibiotic-free X-vivo 10™ (Lonza) at a concentration of $2x10^6$ cells/ml with 2.5ng/ml IL-15 (Biolegend). Supernatants were collected and cells were harvested. JEG3 were seeded overnight in 96 well plates (Corning) at a concentration of 10.000 cells/well, and then infected with *L. monocytogenes* at MOI=5 for 15 minutes. JEG3 were washed 3 times with RPMI 10% FCS (Gibco) and cultured with 100.000 dNK/pNK cells or 100 µl of dNK/pNK supernatant in duplicate for 3 hours. 10.000 free *L. monocytogenes* were also cultured in 100 µl of supernatant for 3 hours in duplicate. At endpoint, media was removed from cells and replaced with 200µl of deionized water for hypotonic lysis. Released bacteria were plated in LB plates at 10^{-1}, 10^{-2} and 10^{-3} concentrations. For free bacteria cultured with dNK/pNK supernatant, aliquots of the cultures were plated in LB plates in the same 3 concentrations. LB plates were incubated overnight and colonies were counted.

51Cr release assays

10.000 JEG3 plated on 96 well plates were incubated with 100 µCi of ^{51}Cr for 1h. Half of the wells were infected with *L. monocytogenes* at MOI=5 for the last 15 minutes. Uninfected and infected target cells were incubated with 100.000 dNK/pNK or 100 µl of dNK/pNK supernatant from the overnight cultures for 4h. Controls for spontaneous release and total release (lysed with 0.1%SDS) were also prepared. 50ul of supernatant was transferred to a Luma Plate, dried overnight and read in a scintillation counter. Percentage of specific lysis was calculated as follows: $\frac{(Sample\ release\ counts)-(Spontaneous\ release\ counts)}{(Total\ release\ counts)-(Spontaneous\ release\ counts)} \times 100$

Degranulation assays

For degranulation assays, dNK and pNK were cultured overnight in antibiotic-free X-VIVO 10™ (Lonza) supplemented with 2.5ng/ml IL-15 (Biolegend). The dNK and pNK (effectors) were harvested, counted and co-cultured with 721.221 (221) in a 1:3 E:T ratio and with uninfected or *L. monocytogenes*-infected confluent JEG3 in antibiotic free RPMI 10% FCS (Gibco) for 4 hours. 250ng/ml CD107a PerCP-Cy5.5 antibody (Biolegend) was added to all co-cultures. NK cells were collected and fixed for 10min in 1% PFA and subsequently stained for all relevant surface markers for FACS analysis.

Confocal microscopy

Fresh dNK and pNK were plated in 10mm 1.5 coverslips coated with 0.01% poly-L-lysine (Sigma) and allowed to adhere for 1 hour. Cells were fixed with 4% paraformaldehyde for 10 minutes and then permeabilized with PBS/0.01% Triton-X for 10 minutes. Primary conjugated antibodies (Perforin AlexaFluor^{TM} 488 (dG9), Perforin AlexaFluor 647^{TM} (dG9), Granulysin AlexaFluor 647^{TM} (DH2) (Biolegend) Granulysin AlexaFluor 488 (RB1) (BD) were added for 1h. Cells were washed and stained with Hoechst (Immunochemistry Technologies) for 10 minutes, and coverslips were mounted in slides for confocal microscopy using ProLong Gold Antifade mounting media (Life Technologies). Cells were imaged in a Zeiss LSM510 microscope.

Statistical analysis

All data was analyzed using GraphPad Prism (version 6.0) software. To determine differences between 2 unpaired groups, a non-parametric Mann-Whitney test was performed. To determine differences among more than 2 unpaired groups, a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-test was performed. To determine differences among more than 2 paired groups, a non-parametric Friedman's test with Dunn's multiple comparison post-test was performed. P-values <0.05 were considered to reflect significant differences.*-$p<0.05$;**-$p<0.01$; *** $p<0.005$

RESULTS

dNK express higher levels of 9kDa and 15kDa granulysin than pNK

To characterize granulysin expression, dNK and pNK were intracellularly stained for total granulysin (15kDa and 9kDa) and the 9kDa isoform separately and analyzed by flow cytometry. dNK express significantly higher levels of total granulysin and the 9kDa isoform than pNK. pNK do not express the 9kDa isoform and thus the low expression levels as found with the total granulysin stain must identify the 15kDa isoform (Figure 2 A and B).

9kDa granulysin has been described to be confined to the same granular vesicles and perforin and granzymes in pNK, while 15kDa was localized in different vesicles (182). To determine the localization of granulysin in dNK, freshly isolated dNK and pNK were stained intracellularly for total granulysin, 9kDa granulysin and perforin and analyzed by confocal microscopy. Granulysin was expressed in granules both with and without perforin, while some of the granulysin was diffusely distributed in the cytoplasm of dNK (Figure 2C, upper panels). Staining with the antibody specific for 9kDa granulysin combined with the total granulysin antibody revealed 9kDa granulysin is confined in granules, which identifies the diffuse cytoplasmic granulysin as the 15kDa form (Figure 2C, middle panels). Furthermore, pNK did not stain for 9 kDa granulysin (confirming the flow cytometry data), while 15 kDa granulysin was found confined to granules that were either perforin-positive or perforin-negative (Figure 2C, lower panels).

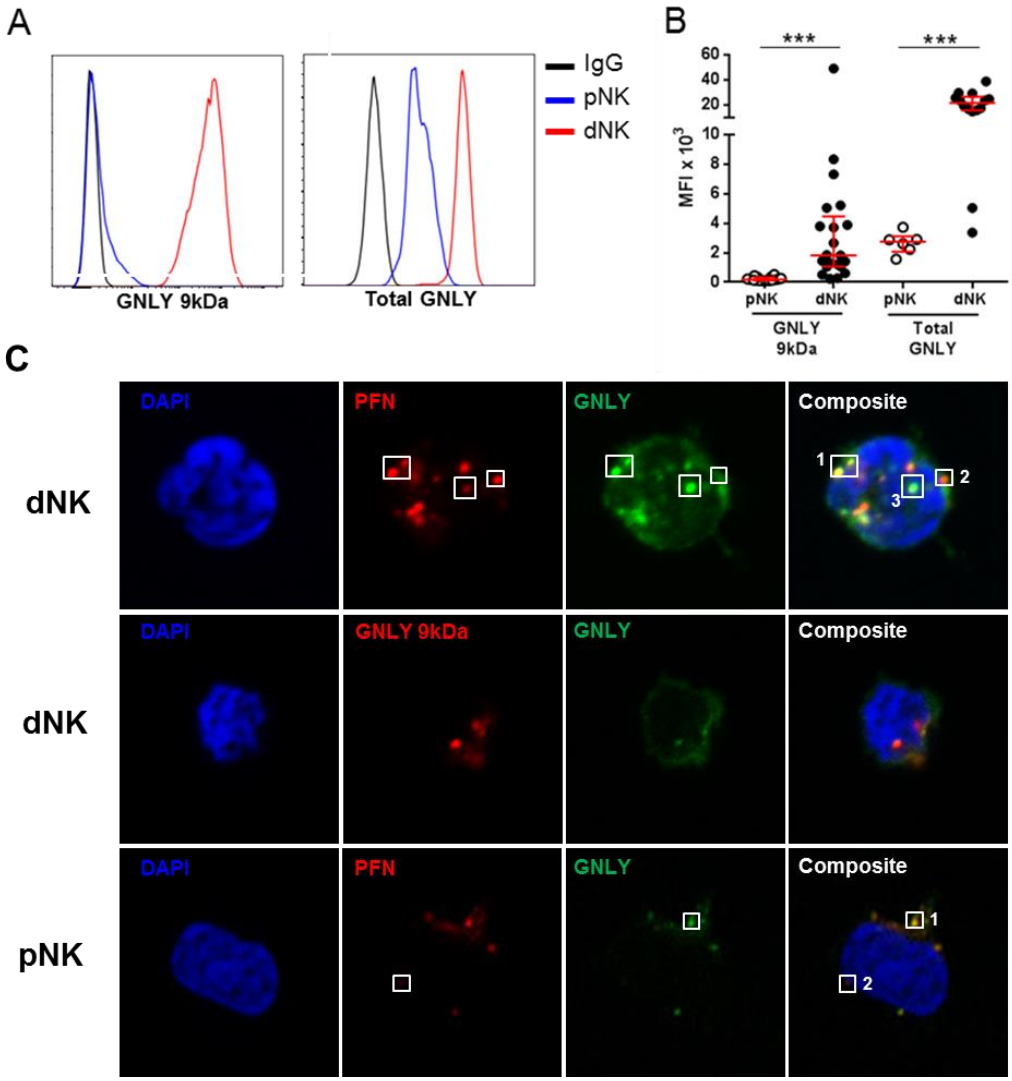

Figure 2. dNK express higher levels of granulysin than pNK and granulysin has distinct localization in intracellular granules. **A)** Representative FACS plots of 9kDa and total granulysin in pNK and dNK. **B)** Graph depicts expression level (MFI) of 9kDa isoform and total granulysin in pNK and dNK. **C)** Confocal microscopy of dNK and pNK showing the localization of perforin, total granulysin and 9kDa granulysin. Granules containing 1. perforin and granulysin; 2. perforin only; 3. granulysin only.

dNK spontaneously release high levels of granulysin

dNK have been shown to spontaneously secrete granulysin, while pNK do not have that capacity (138). dNK secreted granulysin in the absence of stimulation (e. g. cytokine stimulation IL-15 or IL-2, or stimulation with target cells). To confirm this observation, freshly isolated dNK and pNK were cultured for 12 hours in the presence or absence of IL-15 and the culture supernatants were analyzed for the presence of cytolytic molecules by ELISA (granulysin) and Bioplex assay (granzyme A, B and perforin). The levels of granzyme A, granzyme B and perforin found in the supernatant were negligible (<0.5 nM). In contrast, granulysin levels in dNK supernatants were variable, (mean 1.42 ± SD 2.27 nM), while pNK supernatants contained virtually no granulysin (0.067 ± 0.064 nM, $p<0.001$) (Figure 3A). The concentrations of all cytolytic molecules found in the supernatants of dNK and pNK cultured

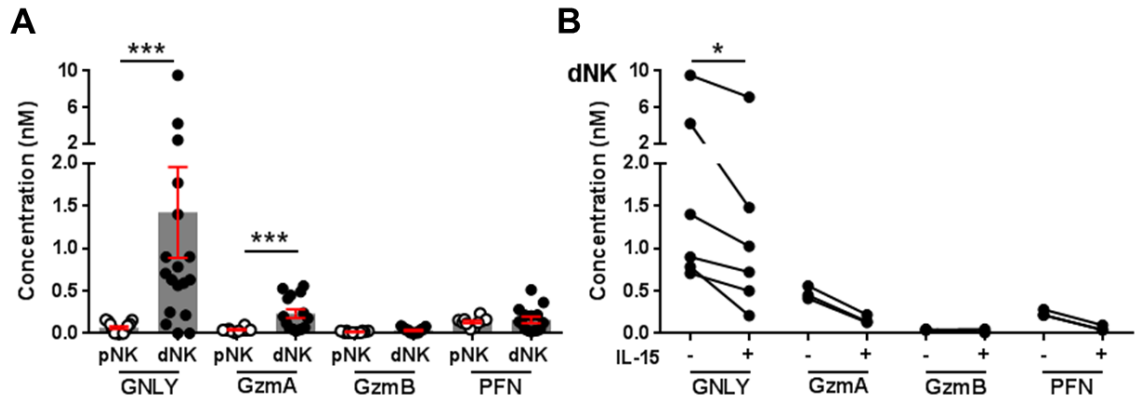

Figure 3. dNK secrete granulysin and granzyme A in higher levels than pNK. A) pNK and dNK were cultured for 12h in the presence of 2.5 ng/ml IL-15. Granulysin levels analyzed by ELISA. Granzyme A, Granzyme B and Perforin levels in the culture supernatant were analyzed by Luminex **B)** Cytolytic granule concentrations in the supernatant of dNK cultured in the presence or absence of IL-15. Bars represent mean±SE.

in the absence of IL-15 were lower, but only granulysin secretion was significantly decreased by lack of IL-15 (Figure 3B). Thus, granulysin release by dNK was independent from perforin and granzymes and may point to the existence of special granulysin-exclusive vesicles.

dNK and pNK cells and dNK supernatants eliminate intracellular bacteria without lysing the host cell

Given the high levels of granulysin in dNK and the antimicrobial functions of granulysin, the next step was to determine if dNK have a role in eliminating bacterial infections in placenta. JEG3 cells (a choriocarcinoma cell line with the same MHC pattern of EVT) were infected with *Listeria monocytogenes* and co-cultured with dNK or pNK at a ratio of 10:1 (effector:target) for 3 hours. Bacterial and target cell death were analyzed by colony forming unit (CFU) assay and ^{51}Cr release assay, respectively. Both dNK and pNK were able to kill a significant fraction of the intracellular bacteria compared to control. However, none were able to kill infected or uninfected JEG3 (Fig 4 A and C).

pNK and dNK cell culture supernatants obtained were added to free *L. monocytogenes* or to

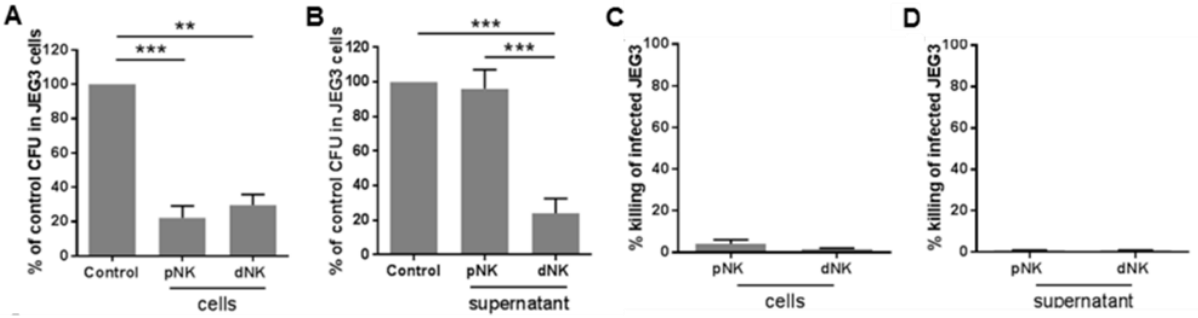

Figure 4. dNK and their supernatants kill intracellular *L. monocytogenes* but spare infected cells. A) Relative colony forming units (CFU) of intracellular *L. monocytogenes* (MOI 5) in JEG3 co-cultured with dNK or pNK (E:T 10:1) for 3h. **B)** Relative CFU of intracellular *L. monocytogenes* (MOI 5) in JEG3 cultured with dNK or pNK supernatant for 3h **C)** Killing of *L. monocytogenes* -infected JEG3 by dNK or pNK (E:T 10:1, 4h) **D)** Killing of *L. monocytogenes*-infected JEG3 4h by supernatants from dNK or pNK. Bars represent mean±SE.

JEG3 infected with *L. monocytogenes*. dNK supernatants were able to kill both extra and intracellular *L. monocytogenes,* while pNK supernatant did not (Figure 4B). In addition, neither of the supernatants were able to kill JEG3 (Figure 4D).

At sites of immune privilege (e. g. eye, testes, and placenta), cell types such as EVT are resistant to killer cells by expression of serine protease inhibitors called serpins (183): SERPINB9 inhibits granzyme B (and also caspase-1 and caspase-8) (184–186); SERPINB4 and possibly SERPINB9B inhibit granzyme M (187); SERPINB1 inhibits granzyme H (188). Inhibitors of apoptosis (IAP), namely c-IAP1(*BIRC2*), c-IAP2 (*BIRC3*), livin (*BIRC7*), and/or survivin (*BIRC5*) (189–191), Bcl-xL (*BCL2L1*) (192) and Bim (*BCL2L11*) (193) may also be involved. As a preliminary study of anti-apoptotic protein expression by JEG3, the available microarray developed in our lab and published in (95) was analyzed. Indeed, EVT and JEG3 shows high level expression of several of the serpins, IAP and BCL family genes, which may explain the failure of dNK and pNK to kill JEG3 (Figure 5).

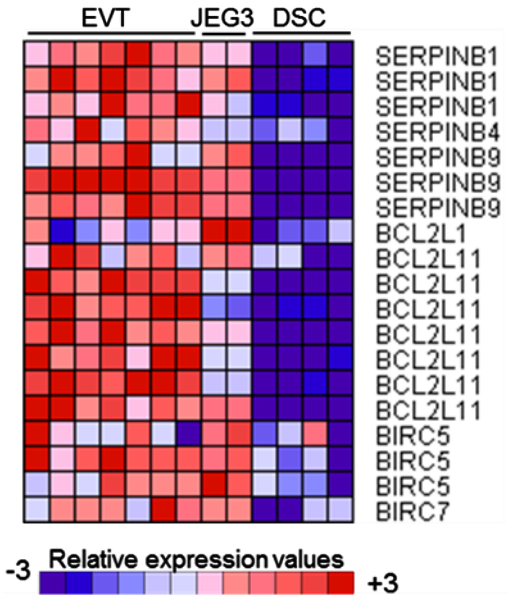

Figure 5. – Trophoblasts express high levels of apoptosis inhibitors. Heatmap depicts the expression of apopotosis inhibitors' genes [Serpin B1, B4 and B9, Bcl-xl (BCL2L1) , Bim (BCL2L11), survivin (BIRC5) and livin (BIRC7)] in EVT, JEG3 and maternal decidual stromal cells (DSC).

Bacterial killing is not associated with CD107a$^+$ granule exocytosis

NK mediated target cell death or intracellular bacterial death depends on granule exocytosis and the release of granzymes and perforin to the immune synapse. To verify if contact with bacteria-infected cells would lead to CD107a mobilization to the cell surface (an indication of granzyme and perforin release), dNK and pNK were cultured with *L. monocytogenes*-infected or uninfected JEG (and 721.221 for control). While co-culture with 721.221 led to CD107a-dependent degranulation by dNK and pNK, co-culture with JEG3 did not, even when infected with *L. monocytogenes* (Figure 6A and B). Thus, bacterial clearance by NK in placental cells is independent of degranulation, suggesting perforin and granzymes may not be involved in the process.

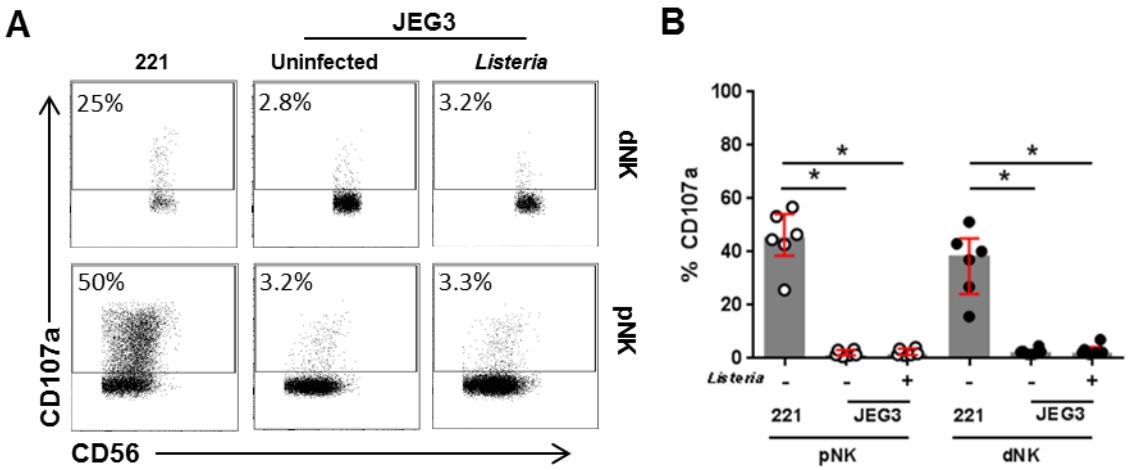

Figure 6. *L. monocytogenes* infection of JEG3 does not elicit degranulation of dNK and pNK. A) Representative FACS plots of degranulation of dNK (top) and pNK (bottom) in response to 221 and uninfected or *L. monocytogenes* -infected JEG3. **B)** Percentage of $CD107a^{+}$ pNK and dNK after co-culture with 221, healthy and *L. monocytogenes* -infected JEG3.

DISCUSSION

The present work describes a new and exciting phenomenon – NK cells are able to eliminate microbes within infected cells without killing the host cell. The killing of intracellular microbes does not rely on CD107a-dependent degranulation and is possibly due to granulysin that is constitutively secreted by dNK. In dNK, granulysin was found to be diffusely distributed in the cytoplasm as well as contained in vesicles. Although some of the granulysin-positive vesicles also contained perforin, granulysin-exclusive vesicles were identified. In addition, the granulysin secretion was independent of the release of granzymes and perforin. Thus granulysin exclusive vesicles may be responsible for the constitutive secretion of granulysin. The ability to secrete granulysin constitutively may be due to a high activation state of dNK (e.g. constitutive expression of CD69, high levels of cytotoxic granules) (105).

The concentration of granulysin in the dNK supernatants do not fall within the range considered to be microbicidal by itself (1-3μM) (136). In addition, which isoform of granulysin is found in dNK supernatants is not known. Previous studies of pNK demonstrated the 15kDa isoform of granulysin is preferentially secreted. The 15kDa form has not been studied extensively, and until recently it was not clear whether it serves only as a precursor of the 9kDa isoform or if it has a separate function by itself (177). In activated CTLs and pNK, 9kDa granulysin co-localizes with granzymes and perforin in cytolytic granules, while 15kDa is localized in its own granules that can exocytose upon activation without target cell contact and synapse formation(182). Also, the 15kDa form has no cytolytic activity against mammalian cells or extracellular microbes. However, it can stimulate monocytes to differentiate into dendritic cells, and work as a chemoattractant for leukocytes (182). An interesting hypothesis concerns the possibility of the 15kDa form being taken up by bacteria-infected JEG3 and then proteolytically cleaved within the cells to release 9kDa form, leading to intracellular bacteria death. These questions are currently being addressed by performing detailed analysis of the contents of the dNK cell culture supernatant.

The levels of granulysin in dNK supernatants may contribute to the extracellular bacterial killing, in combination with other putative antimicrobial substances present in the supernatant. In fact, dNK constitutively secrete other molecules, such as galectin-1, which may have a role in bacteria elimination (105). Galectins are β-galactoside-binding lectins that accumulate in the cytosol before being secreted via a leader-peptide-independent pathway. Extracellular galectins bind glycans on cell membranes and modulate cellular behavior. However, they can also occur in the cytosol and can act as danger and pattern-recognition receptors (194). Galectin 3, for example, binds bacterial lipopolysaccharide (LPS) and acts as a negative regulator of LPS functions (195), and Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion (194). pNK supernatants have negligible

levels of granulysin and were not able to kill extracellular bacteria, and have also been shown to not secrete Galectin-1 (105).

The ability of dNK supernatants to kill intracellular bacteria may lie in the capacity of granulysin to enter JEG3 by binding lipid rafts and subsequently being transported to the intracellular space. This mechanism is independent of perforin. Recombinant granulysin was shown to bind lipid rafts of *Listeria innocua*-infected DCs. Subsequently the granulysin/lipid rafts were endocytosed and transported to bacteria-containing vesicles, leading to microbe killing (196).

Although pNK supernatants do not kill bacteria, pNK and dNK cells can kill intracellular *L. monocytogenes*. In this case, an immune synapse may be formed where all three granules are transferred to the infected cells and act together to eliminate bacteria. dNK have been shown to form immature synapses with EVT, where perforin was not polarized to the synapse (Tilburgs *et al*, provisionally accepted). In our study, no degranulation was observed during dNK and pNK co-culture with infected JEG3, which indicates perforin and granzymes were not released. Thus, bacterial death may be due to granulysin in combination with other antibacterial peptides. dNK contact with infected cells may increase secretion or dNK may form a new kind of synapse with JEG3 where only granulysin-containing vesicles are polarized, resulting in exclusive transfer of granulysin between cells. This mechanism may apply to pNK, which are able to kill intracellular bacteria when in contact with infected cells, but do not secrete granulysin without activating stimuli.

Uninfected or infected JEG3 were not killed during interaction with NK, at least during the time of assay (4h), which was sufficient to inhibit bacterial growth. A longer exposure of infected JEG3 to NK may result in cell death; indeed, a previous study on HCMV-infected primary decidual stromal cells (DSC) demonstrated efficient lysis of infected DSC only occurs after 18h of contact (163). In addition, dNK do not degranulate against HCMV-infected JEG3 or EVT even after 10-12 hours of contact, while pNK are able to degranulate in response to infected JEG3 and EVT (chapter 3). pNK do not contact EVT *in vivo*, but whether there is influx of pNK to the decidua during infection has not been explored. dNK may form synapses with infected JEG3 that lead to bacterial death. JEG3 may be resistant to NK killing due to expression of anti-apoptotic proteins such as serpins ((95) and figure 5).

The findings reported in this chapter point to an important role of dNK in clearance of bacterial infection through the action of granulysin. These observations reveal a novel function of dNK as true immune effector cells besides their role in secretion of cytokines and growth factors that facilitates trophoblast invasion. Further experiments are necessary to study the mechanism of action of dNK-secreted granulysin in the elimination of placental infections. Current studies in include the detailed analysis of dNK supernatants and potential binding of granulysin to lipid rafts for internalization in infected JEG3. Furthermore, the

cytolytic potential of NK in response to bacteria infected-JEG3 and JEG3 resistance to death will be studied, in addition to immune synapse formation between dNK and JEG3. The influence of cytokines in the expression and secretion of granulysin by dNK and pNK will also be analyzed.

In summary, these data unveil a new target for pre-natal therapies. Bacterial infections in early gestations can be treated by increasing the dNK potential to secrete granulysin, or by administration of granulysin in microbicidal doses that do not affect the placental cells. The treatment of placental infections in an early stage may avoid complications later in pregnancy such as miscarriage, congenital malformations or fetal growth restriction.

CHAPTER 5

GENERAL DISCUSSION

The discovery of the presence of high numbers of large granular lymphocytes (LGL) in human decidua, later identified as decidual Natural Killer (dNK) cells (197), led to the hypothesis that fetal placental cells actively inhibit maternal dNK to avoid immunologic rejection (198). The subsequent characterization of dNK as poor cytotoxic lymphocytes and major cytokine and growth factor producers distanced dNK from true immune effector cells. The main role for dNK was established as agents that facilitate implantation, trophoblast invasion and vascular remodeling. These processes are of key importance for placental development and enhance the exchange of gases and nutrients from mother to fetus (94, 107, 199). Until recently the role of dNK in clearance of infections, a key function of pNK, was largely ignored. In 2013, Siewiera *et al* demonstrated the ability of dNK to clear HCMV-infected cells (163). The results presented in this thesis build upon this observation and highlight the dual role of dNK, capable to mount immune effector responses during viral and bacterial infections as well as provide immune tolerance to the fetus and facilitate placental growth. The expression of the activating MHC Class I receptor KIR2DS1 by dNK was associated with improved clearance of HCMV-infected decidual stromal cells (DSC). In addition, dNK eliminated placental bacterial infections without affecting the host cells, possibly though secretion of the anti-microbial peptide granulysin. These results emphasize the importance of dNK to provide both immunity to infections and tolerance to fetal cells.

KIR2DS1, HLA-C2 and HCMV

The MHC Class I molecule HLA-C expressed by fetal EVT is the main factor that can cause allogeneic recognition and possible immune rejection by maternal immune cells. This immune response can be driven by T cells through direct allo-recognition (e.g. fetal EVT express a foreign HLA-C molecule that directly activates maternal T cells) or by NK cells due to missing-self (e.g. EVT lack a MHC molecule and fail to inhibit NK cytotoxicity through inhibitory receptors) (91, 94). dNK express high levels of receptors for MHC class I [of which the killer cell immunoglobulin-like receptors (KIR) are most important for HLA-C]. Particular combinations of KIR and MHC haplotypes influence reproductive success, clearance of viral infections and development of auto-immune diseases. In particular, women with KIR-AA haplotypes (lacking most activating KIR) are in high risk of developing complications such as pre-eclampsia, recurrent miscarriage or fetal growth restriction. The effect is enhanced when the fetus expresses group 2 HLA-C alleles (71, 112, 114). This observation led to the hypothesis that the lack of activating KIR (KIR2DS1) allows for a strong inhibition of dNK through KIR2DL1 (inhibitory receptor), reducing dNK secretion of cytokines that enhance trophoblast invasion and placental development (117, 118). In this thesis, KIR2DS1^{+} dNK did not respond directly to EVT by degranulation or secretion of growth factors such as GM-CSF or VEGF. In addition, degranulation of KIR2DS1^{+} dNK in response to HLA-C2^{+} target cells

did not correlate with the HLA-C epitope of the fetus, indicating *de novo* education fetal HLA-C does not occur (Chapter 2). Instead, KIR2DS1$^+$ dNK were shown to have increased cytolytic granule content (granzymes, perforin and granulysin) and to have higher levels of degranulation in response to 221.C2 (Chapter 2). Most importantly, KIR2DS1$^+$ dNK displayed higher capacity to clear human cytomegalovirus (HCMV)-infected decidual stromal cells (DSC), in particular when HLA-C2 molecules were present (Chapter 3). These observations were specific for dNK and not observed in pNK.

HCMV employs many strategies for immune evasion and prevent the lysis of infected cells. These strategies include interference with MHC Class I processing and surface expression (128). As presented in chapter 3, HCMV infection downregulated HLA-C in DSC in early stages, but upregulated HLA-C expression in late infection. Consequently, cells in early infection stages are not able to inhibit dNK through KIR2DL1, while cells in late infection stages can increase activation of single-positive KIR2DS1$^+$ dNK. Thus the presence of KIR2DS1$^+$ dNK can enhance elimination of decidual HCMV-infection and limit virus-induced placental pathology. These observations may lead to novel pathways for development of immune therapies that prevent congenital infections and virus-induced pregnancy complications.

Interaction of EVT did not induce secretion of growth factors by dNK. Whether or not interaction with other cell types in decidua (e.g. decidual macrophages, T cells and stromal cells) and modulation by the decidual environment induce cytokine secretion by dNK remains to be investigated. Several lines of evidence indicate DSC as conditioners of dNK phenotype and function, via direct interaction or production of cytokines such as IL-33 and TGF-β (156, 200). DSC decrease pNK cytotoxicity and upregulate the expression of inhibitory receptors in dNK (200, 201). Other sources of anti-inflammatory cytokines that can influence dNK behavior are decidual macrophages (103). dMϕs have been shown to secrete TGF-β and to inhibit dNK cytotoxicity *in vitro* (202). Crosstalk between dNK and dMϕs or dendritic cells in the maternal fetal interface is also involved in decidualization in mice (203).

The detrimental association of the maternal KIR-AA haplotype with fetal HLA-C2 may have been a strong evolutionary force for the distribution of KIR and MHC haplotypes in human populations. Frequency of AA haplotype and HLA-C2 epitope is inversely correlated and this reduces the frequency of potentially complicated pregnancies: women who are homozygous for the group A KIR haplotype paired with men who carry the HLA-C2 epitope (204). Infections are also known to be strong forces of evolution and natural selection. Combinations of KIR and HLA are associated with increased clearance of viral infections (59, 64, 164). For example, KIR2DL2/3 and HLA-C1 are associated with control of HCV infection, due to the low affinity interaction of KIR2DL2/3 with HLA-C1 (compared with the high affinity interaction of KIR2DL1 and HLA-C2). This leads to a less effective inhibition of

NK and consequently higher activation (70). Although the evidence is correlative, it raises the hypothesis that KIR-A haplotypes are mainly selected for their role in immune defense, whereas KIR-B haplotypes are selected for their role in placental reproduction. An episode of viral infection would select for KIR-A haplotypes, which will be enriched in the survivors, but in subsequent expansion of the surviving population there will be selection for KIR-B haplotypes and against KIR-A. Thus, long-term survival of human populations have been selected for retain both KIR-A and KIR-B haplotypes (205, 206). In this thesis, the KIR-B haplotype correlates with improved clearance of viral infection in pregnancy, and not KIR-A. However, elimination of viral infection leads to reproductive success and contributes to the maintenance of KIR B haplotypes in the population. These results suggest a more complex model for KIR haplotype influence in evolution and demand further investigation.

dNK, infections and pregnancy complications

Viral infections in pregnancy, in particular HCMV, are frequent and lead to pregnancy complications that include miscarriage and pre-term birth (122, 132). HCMV persists as a latent infection and, in the absence of high levels of protective antibodies, HCMV can reactivate in the placenta. Placental HCMV infection interferes with trophoblast invasion and placental development and can give rise to placental pathology and congenital syndromes (123, 126) Due to the high variety of pathogens involved and subclinical nature of some infections, occurrence of infections during pregnancy is underdiagnosed. $CD8^+$ T cells represent only 2-7% of $CD45^+$ lymphocytes in decidua and thus dNK are the prime candidates to respond to viral infections in decidua (162). Siewiera *et al* provided the first evidence that dNK are able to clear HCMV infected DSC (163). In this thesis, we expanded these studies and showed while dNK can clear HCMV-infection of maternal DSC, HCMV-infected trophoblast cannot be cleared (Chapter 3). While dNK degranulated and secreted pro-inflammatory cytokines in response to HCMV-infected DSC, no response was observed to HCMV-infected JEG3 or primary trophoblast (Chapter 3). The clearance of infected DSC limits the viral spread to trophoblast, which are naturally more resistant (168). The failure of dNK to kill EVT, even when infected with HCMV, may be due to the immunomodulatory properties of EVT, to avoid detrimental consequences to the fetus caused by excessive inflammation. Virus, bacteria and parasites may take advantage of the immune privileged status of the placenta and EVT to avoid immune attack. However, pNK are able to kill infected trophoblasts *in vitro*, although pNK and EVT do not interact *in vivo*. These observations underline the importance of early diagnosis of infections during pregnancy to avoid the spread of pathogens to the placenta and fetus. Understanding the mechanisms that regulate the switch in dNK immune tolerance and immunity contribute to discover key

factors involved in the immunopathology of placental HCMV infection and lead to novel strategies to limit congenital infection.

dNK, granulysin and the control of bacterial infections

Similarly to viral infections, bacterial (e.g. Listeria monocytogenes, Group B streptococcus) and parasite infections (e.g. *Toxoplasma gondii, Plasmodium spp*) can also cause major complications during pregnancy and this includes miscarriage, preterm delivery and maternal and fetal sepsis (139, 140). In this thesis, the importance of the high level of antimicrobial peptide granulysin in dNK was demonstrated in the clearance of Listeria monocytogenes. dNK were shown for the first time to clear intracellular Listeria monocytogenes in the trophoblast cell line JEG3 without killing the JEG3 cells (Chapter 4). The failure of dNK to lyse infected JEG3 may be due to the expression of anti-apoptotic proteins that neutralize the effect of granzymes by JEG3 (95). This reveals a novel function of dNK as immune effectors able to clear bacterial infections while maintaining tolerance to the trophoblast.

Killing of intracellular bacteria by NK in most cell types is dependent on the concerted action of granzyme, perforin and granulysin (135). However, the elimination of *L. monocytogenes* by dNK in trophoblast lines was independent of degranulation (Chapter 4). This suggests the existence of other pathways such as specialized immune synapses that may transfer granulysin and other antimicrobial substances to target cells. Furthermore dNK may target bacteria through constitutive secretion of granulysin or other antimicrobial peptides that enter the infected host cells without killing them (138).

The stimuli that induce the high granulysin expression and constitutive secretion of granulysin by dNK are unknown and may be related to the contact of dNK with the placenta bacterial flora. Until recently, the placenta was thought to be sterile, but a multi-center study reported the existence of a unique microbiome (207). pNK may require activating signals (e.g. of placental cells or the placental microbiome) to increase the expression and secretion of granulysin.

The data presented in Chapter 4 is preliminary and raises several questions related to the regulation of granulysin expression and secretion by dNK, the mechanisms involved in the bacterial elimination and trophoblast protection, as well as other microbial compounds that may be involved in bacterial elimination. Nevertheless, these novel findings provide promising new possibilities for congenital therapies and for the elimination of bacterial and parasite infections. This includes a focus on the enhancement of dNK function and granulysin activity.

Mice are not humans

Although mouse placentation is not as invasive as human placentation, both mouse and human placentation are haemochorial. These arguments have been used to support the claim that mouse are both a good and not a good model to study human reproduction. Other differences between mouse and humans are gestational length (3 versus 40 weeks), exposure to pathogens (mouse are kept in pathogen free environments) and MHC differences (mouse are mainly inbred strains with limited polymorphisms) (208). Most importantly, the NK receptor repertoire for MHC Class I of mice and humans have evolved separately (mouse have Ly49 genes instead of KIR genes) (204). Despite these differences, mice are useful to study placental immunology and development of pregnancy complications as long as these differences are taken in consideration.

In particular, Anne Croy and others have shown VEGF and IFN-γ produced by uterine NK (uNK) is crucial for an effective vascularization of mouse decidua (108, 209, 210). Mice that lack both T and NK cells ($Rag2^{-/-}Il2rg^{-/-}$) or only NK cells (IL-15 KO), although still bearing successful semi-allogeneic pregnancies, have delayed vascularization compared to WT and the litter sizes are smaller (211). Although both KO mice were capable to successful semi-allogeneic pregnancies, the fact that these mice were kept in a pathogen-free environment should be considered. This does not address the crucial role of uNK cells in the resolution of infection. Interestingly, no interactions occurred between uNK cells and trophoblasts in the live tissues studied at early gestation. This is in stark contrast with the claims that interaction of dNK with EVT in humans is the main event that triggers cytokine and growth factor secretion by dNK (107, 117, 118). In fact, our lab demonstrated, both in early publications and in this thesis, that direct co-culture of dNK with EVT does not elicit cytokine or growth factor secretion such as IFN-γ, VEGF or GM-CSF (Chapter 2) (95).

The maternal KIR AA/fetal HLA-C2 detrimental association has been hypothesized to be due to strong inhibition of $KIR2DS1^-$ dNK cytokine secretion by $HLA\text{-}C2^+$ EVT. This theory was explored with the use of a mouse pregnancy model in which the fetus expressed an extra MHC Class I molecule to increase the inhibition of maternal NK cells. The litter sizes in this model were smaller and the secretion of IFN-γ by uNK cells was significantly lower than in pregnancies without the extra MHC. However, the effects were independent of the paternal or maternal origin of the extra MHC and the results did not account for the presence of NK activating receptors (117). These results leave open the possibility that maternal $HLA\text{-}C2^+$ DSC, and not only fetal EVT, can be involved in the inhibitory interactions in $KIR2DS1^-$ women and dNK activation in $KIR2DS1^+$ women. Chapter 3 demonstrated maternal HCMV-infected $HLA\text{-}C2^+$ DSC did increase the cytotoxicity of S1+ dNK – showing the influence of maternal HLA-C.

Other notes and final conclusion

All the work presented in this thesis was performed using primary NK cells and trophoblasts, isolated from human tissue. The use of primary cells yields results relevant for human clinical applications However, primary cells have several limitations, such as short culture life, lack of proliferation, limited cell numbers, and a great variability in phenotype and activity that is inherent to human populations. In addition, it was not possible to obtain peripheral blood cells from the same women that donated decidual tissue. All the pNK were obtained from unrelated blood donors, and the results may be different from pNK from pregnant women.

The data presented in this thesis on dNK and EVT in healthy and infected pregnancies opens exciting new possibilities for therapies that can improve maternal and fetal health, with special focus in enhancing dNK function in viral and bacterial infections. Identifying high risk pregnancies (e.g. women with KIR-AA genotypes) at early stages for monitoring viral and bacterial infections will be crucial to avoid disorders that affect both mother and fetus. The preliminary data on the possible role of dNK-derived granulysin will need further development, but it presents a promising strategy to eliminate bacteria without disturbing the delicate placental tissue. Finally, this work represents a step further on the understanding of Peter Medawar's proposed problem – the balance between immunity and tolerance in the maternal-fetal interface.

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APPENDIX I. PRIMARY CELLS AND LINES USED IN THE PRESENT WORK

Primary cells	Description
pNK	**Peripheral NK cells** Defined as $CD45^{+}CD14^{-}CD3^{-}CD56^{low}CD16^{+}$ Isolated from Leukopacks® processed from healthy blood donations Proliferation in culture; growth in suspension
dNK	**Decidual NK cells** Defined as $CD45^{+}CD14^{-}CD3^{-}CD56^{high}CD16^{-}$ Isolated from discarded decidual tissue from elective pregnancy terminations No proliferation in culture; cultured in suspension (1-2 days survival)
VT	**Villous trophoblast** Defined as $CD45^{-}EGFR^{+}HLA\text{-}G^{-}$ Isolated from discarded placental tissue from elective pregnancy terminations MHC Class I-negative No proliferation in culture, growth in suspension
EVT	**Extravillous trophoblast** Defined as $CD45^{-}EGFR^{low}HLA\text{-}G^{+}$ Isolated from discarded placental tissue from elective pregnancy terminations. Express HLA-C, HLA-E and HLA-G No proliferation in culture. Cultured in attachment in fibronectin-coated surfaces (3-4 days of survival)
DSC	**Decidual Stromal Cells** Defined as $CD45^{-}CD10^{+}CD29^{+}$ Isolated from discarded decidual tissue from elective pregnancy terminations Express HLA-A, HLA-B, HLA-C, HLA-E Proliferation in culture; growth in attachment; lines survive up to 15 passages

Cell Line	Description
221	**721.221 cell line** EBV-transformed B cell line HLA-A, B and C negative MHC Class II positive Immortalized cell line; growth in suspension
221.C1	**721.221 cell line constitutively expressing HLA-C1 group alleles** Retrovirally transfected with HLA-Cw3 or HLA-Cw7
221.C2	**721.221 cell line constitutively expressing HLA-C2 group alleles** Retrovirally transfected with HLA-Cw6 or HLA-Cw4
K562	**Erythroleukemia cell line** Immortalized myelogenous leukemia line MHC Class I negative Growth in suspension
K562.C1	**K562 cell line constitutively expressing HLA-C1 group alleles** Retrovirally transfected with HLA-Cw1
K562.C2	**K562 cell line constitutively expressing HLA-C2 group alleles** Retrovirally transfected with HLA-Cw6
JEG3	**Choriocarcinoma cell line** Immortalized cell line isolated from placenta affected with choriocarcinoma Expresses HLA-C, HLA-E, HLA-G (same pattern as EVT) Growth in attachment