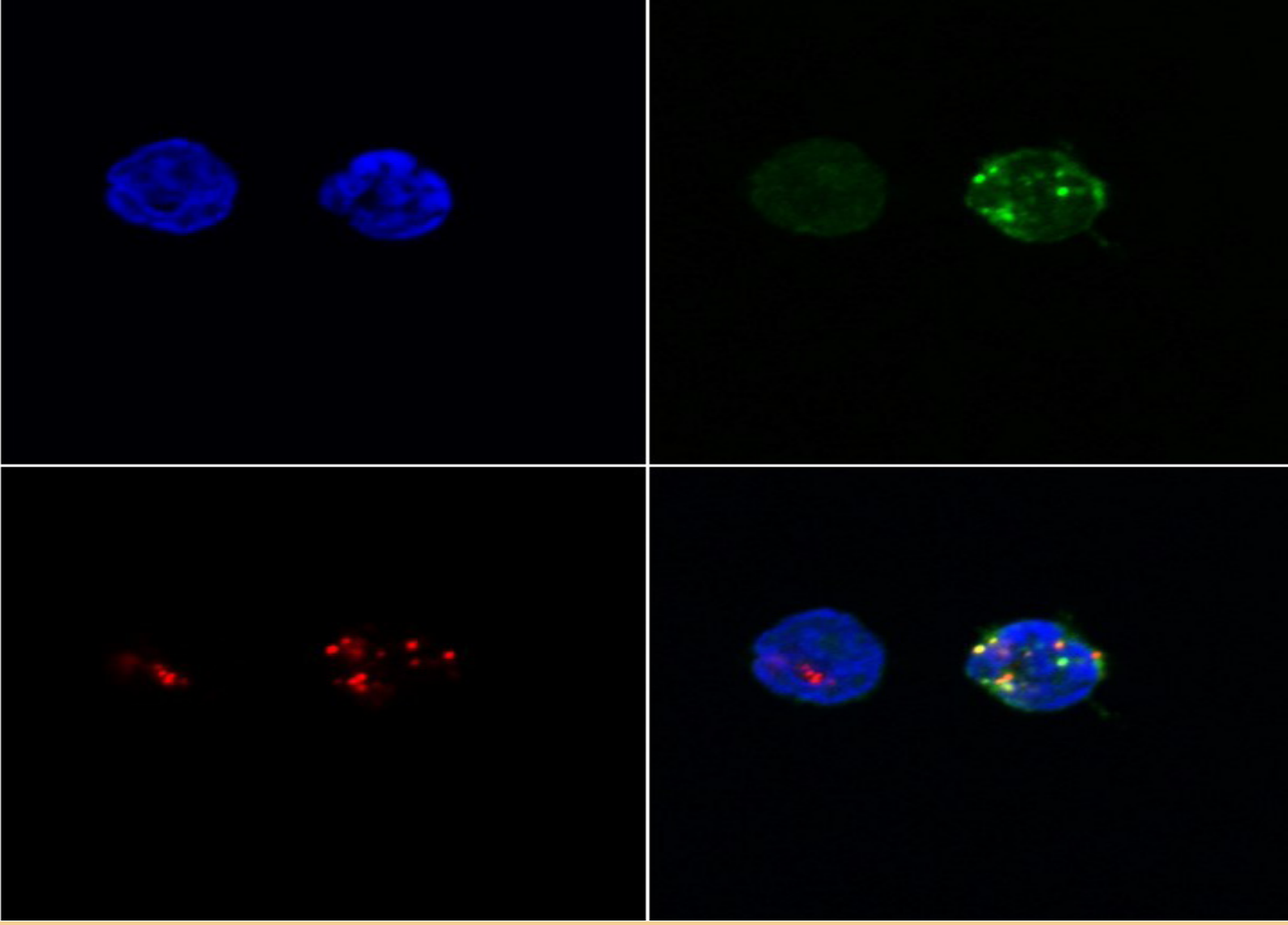

Ricardo de Mendonça Vieira

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF DECIDUAL NATURAL KILLER CELLS AT TERM PREGNANCY

Tese de Mestrado em Investigação Biomédica, especialização em Infeção e Imunidade,
sob orientação científica do Professor Doutor Jack Strominger (Universidade de Harvard, EUA) e do
Professor Doutor Manuel Amaro de Santos Rosa (Universidade de Coimbra, Portugal)
e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Dezembro 2017

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Universidade de Coimbra

Front cover: dNK cells culture stained with Diaminido-2-phenylindole (DAPI, blue). Granulysin stained with Alexa488 (green) and Perforin stained with Alexa647 (red).

HSCRB
HARVARD DEPARTMENT OF STEM CELL & REGENERATIVE BIOLOGY

FMUC FACULDADE DE MEDICINA
UNIVERSIDADE DE COIMBRA

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Dissertation presented to the Faculty of Medicine of University of Coimbra, Portugal, in partial fulfilment of the requirements for the Master degree in Biomedical Research, with specialty of Infection and Immunity. The research work presented was performed in the Department of Stem Cell and Regenerative Biology, Harvard University, under the supervision of Professor Jack Strominger and Professor Manuel Amaro de Matos Santos Rosa.

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para cumprimento dos requisitos necessários à atribuição do grau de Mestre em Investigação Biomédica, especialização em Infeção e Imunidade. O trabalho apresentado foi realizado no Departamento de Células Estaminais e Biologia Regenerativa, Universidade de Harvard, EUA, sob a supervisão do Professor Jack Strominger e do Professor Manuel Amaro de Matos Santos Rosa.

ACKNOWLEDGEMENTS

The welcoming attitude and friendship from the following people has contributed for this thesis success. For helping me overcome this incredible academic and personal journey, I want to show you all my gratitude.

Firstly, I would like to acknowledge Professor Jack Strominger for accepting me into his laboratory and so kindly show me that Science is an evolving field that always deserves to be explored. And also for being the living proof, at 92 years old, that we are never too old to come to work in the lab!

I express my gratitude to Dr. Tamara Tilburgs, for guiding me through experimental protocols, to contribute to my critical thinking and for encouraging me to be more independent and productive. Most importantly, for being so understanding and patient with me. I truly appreciated the inputs of Professor Manuel Santos Rosa together with Dr. Tamara on the results discussions and for their guidance throughout the thesis writing process. Dr. Ângela Crespo couldn't have been more welcoming since my first day in Boston. It is always comforting to see a familiar face and to be able to speak my native language when 5 131km away from home. Thank you all for leading me to become a better scientist and to learn to conduct rigorous experiments.

I cannot end this experience without mentioning all past and present SCRB Department research colleagues and friends: Ester Duran, Nadia Chanzu, Andrew Mazzanti, Theresa Bradstetter, Alana Allen, Tørsten Meiβner, Zhifen Chen, Celia Shneider, Bill McCullum, Mei-Ling Wong, Qin-Li, Kiwi Florido and Maria Selvany Celades. You made my stay in Boston a place to learn, grow but also have a lot of fun!

Finally, I wish to thank my amazing family for their unconditional support (including financial) and for never "cut my wings" and allow me to explore science around the world. And to my best friends Marta Santos and Vanessa Henriques: you are just the best chosen family possible!

I truly am grateful for this opportunity and excited about what comes next!

List of ABBREVIATIONS and ACRONYMS

BCR	B-cell Receptor
CD	Cluster of Differentiation
CD3	T-cell marker
CD14	Monocyte/Macrophage marker
CD45	Lymphocyte lineage marker
CD56	NK cell marker
CTLR	C-type lectin Receptor
DAP10	DNAX activation protein of 10kDa
DAP12	DNAX activation protein of 12kDa
dNK	decidual Natural Killer
EVT	Extravillious Trophoblast
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FGR	Fetal Growth Restriction
GNLY	Granulysin
GZMB	Granzyme B
HA	Hemagglutinin
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HLA	Human Leukocyte Antigen
I	Ionomycin
IC	Intracellular

IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cells
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KIR	Killer cell Immunoglobulin-like Receptor
MCH	Maternal Chronic Hypertension
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
NCR	Natural Cytotoxicity Receptor
NK	Natural Killer
PFN	Perforin
PAMP	Pathogen-Associated Molecular Patterns
pNK	peripheral Natural Killer
PBMC	Peripheral Blood Mononuclear Cell
PFA	Paraformaldehyde
PMA	Phorbol Myristate Acetate
PRR	Pattern Recognition Receptors
P/S	Penicillin/Streptomycin
RM	Recurrent Miscarriages
RPMI	Roswell Park Memorial Institute
TCR	T-cell Receptor
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory

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ABSTRACT

Immune tolerance during pregnancy is still a current challenging topic for the biomedical community. Natural Killer cells from decidua (dNK), as the main cell population in the uterine microenvironment, are known to be crucial for trophoblast implantation and protection against infections. But their function during the third trimester of pregnancy is largely unknown. For better understanding of the immune balance throughout the course of a pregnancy, a phenotypic and functional characterization of dNK cells at term pregnancy decidua basalis e decidua parietalis tissues from discarded human placenta is in this thesis assessed. The results obtained are innovative: dNK from decidua basalis and decidua parietalis show differences in their cell surface NK receptor profile, granule content and ability to degranulate in response to PMA/Ionomycin. Exploratory studies to determine the impact of KIR expression in relation to degranulation capacity of dNK cells was also studied and demonstrated that KIR2DL1+/S1+ dNK cells have increased levels of cytolytic granules. Future studies should expand functional analysis on term placenta dNK to increase scientific knowledge relevant to clinical practice, regarding the third trimester of pregnancy. This can contribute to predicting and preventing complications, such as viral and bacterial infections, during pregnancy.

Keywords: decidual NK cells; dNK receptors; KIR; dNK degranulation; term pregnancy.

RESUMO

A tolerância imunitária durante a gravidez é ainda um tema atual e desafiante para a comunidade biomédica. As células Natural Killer da decídua (dNK), a população celular predominante no microambiente uterino, são reconhecidas como cruciais para a implantação do trofoblasto e proteção contra infeções. Mas a sua função durante o terceiro trimestre da gravidez é ainda largamente desconhecida. Para um maior conhecimento do balanço imunitário durante a gravidez, uma caracterização fenotípica e funcional das células NK da decídua basalis e decídua parietalis de placentas humanas descartadas foi avaliada. Os resultados obtidos são inovadores: células NK da decídua basalis e decídua parietalis mostram diferenças no perfil de receptores membranares, conteúdo granular e capacidade de degranulação em resposta a PMA/Ionomicina. Estudos exploratórios para determinar o impacto da expressão de KIR na capacidade de degranulação das dNK também foi estudado e é demonstrado que células dNK KIR2DL1+/S1+ têm níveis mais elevados de grânulos citolíticos. Projectos futuros devem incluir análises funcionais das dNK de uma placenta de termo para aumentar o conhecimento científico sobre o terceiro trimestre da gravidez, sendo relevante para a prática clinica. Assim contribuímos para a previsão e prevenção de complicações, como infeções virais e bacterianas, durante a gravidez.

Palavras-chave: células NK da decidua; receptores de dNK; KIR; degranulação de dNK; gravidez de termo.

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INTRODUCTION

Immunology as a scientific discipline was raised upon observations that individuals whom had recovered from particular infections seemed to be protected from a second encounter caused by the same infectious agent, being, therefore, immune from the disease. Although the earliest reference to immunity can be found in Thucydides writings, a Greek historian from 430 BC, only in the 18th century the concept was converted into a successful medical practice: vaccination. Due to this medical procedure, where the patient is inoculated with an attenuated pathogen, some infectious diseases such as smallpox or paralytic polio were officially considered as eradicated worldwide [1, 2].

1.1 Immune System

The principles of the immune system are common to all vertebrates: to clear the disease-causing agent, to overcome the disease side effects and, consequently, to spare the host from death. But immunity can also be detrimental, especially, when it is activated in situations when the host does not need immune protection or, at least, not at an exacerbated level. Examples of immune dysfunctions include autoimmune diseases, asthma or allergy. Nevertheless, this remarkable defense system is able to distinguish the body's own cells and proteins, recognizing when an effector response should be triggered in order to neutralize or eliminate the foreign ones.

Since we can face an enormous variety of foreign invaders, the immune system evolved in order to generate a great variety of cells and molecules capable of recognizing them. Cellular immunity is divided in two major groups: myeloid and lymphoid cells. Although they evolve from different lineages and differ in their contribution to host defense, their actions are complementary for an effective immune response.

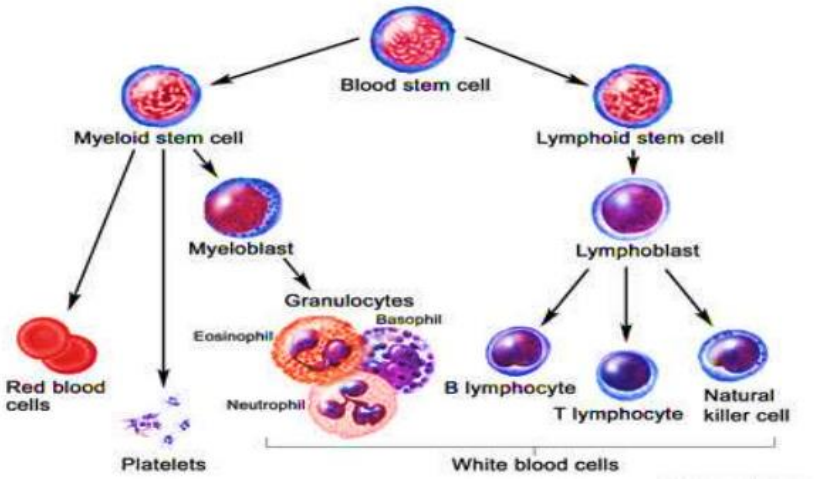

Figure 1. Immune cells differentiation. From a pluripotent hematopoietic stem cell, all human immune cells are generated comprising two cell lineages: myeloid and lymphoid. Adapted from reference 3.

1.1.1 Innate and Adaptive Immunity

Immunity, or the ability to protect the host from a disease agent, comprises two components that act at different stages of host defense: innate immunity and adaptive immunity. However, they do not act independently and the overall combined response is more effective than either would be alone.

The innate immune system is the first line of defense, a system non-specific to any particular type of invading pathogen, but still capable of rapidly clearing the infectious agent by recognizing common pathogenic structures not present in a healthy individual – the pathogen-associated molecular patterns (PAMPs) – and without the need for priming [4, 5]. PAMPs are identified by pattern recognition receptors (PRR) in the host cells and this recognition leads to activation of the immune response by producing bactericidal compounds and inducing chemokine secretion that controls cell migration. However, it does not confer long-term immunity against the infectious agent, since PRR-expressing cells do not generate memory to PAMPs across their life-time. Four types of innate defensive barriers are known (anatomic, physiologic, phagocytic and inflammatory) and when the pathogen escapes from all these innate mechanisms, a specific immune response is, then, triggered. At the cellular level, innate immunity is maintained by macrophages, neutrophils, dendritic cells and Natural Killer (NK).

Table 1. Types of innate immune defensive barriers. Four types of innate barriers are known with different mechanism of defense. Adapted from reference 2.

TYPE OF BARRIER	MECHANISM
ANATOMIC	Acidic environment (pH 3 – 5), mucus and cilia
PHYSIOLOGIC	High temperatures and chemical mediators (lysozymes, interferon, collectins)
PHAGOCYTIC	Endocytosis and phagocytosis
INFLAMMATORY	Serum proteins with antibacterial properties

In contrast with the innate mechanisms, the adaptive immunity is the specific component of the immune system, with high antigenic specificity and large diversity (since receptors are produced by somatic recombination of gene segments) which allows it to distinguish subtle differences among antigens.

Even though it requires more time to marshal, the adaptive immune system ensures a more "customized" immune response to any pathogen it has previously encountered. This important property - "immunological memory" - provides a quicker and stronger immune reactivity upon a second contact with the same foreign invader. This phenomenon is the principle behind many vaccines available nowadays [6].

The adaptive immune cells are special types of leukocytes – B-lymphocytes and T-lymphocytes – that originate from hematopoietic stem cells in the bone marrow and thymus, respectively. Whereas B-cells act in the humoral immune response (antibody production), T-cells are involved in cell-mediated immune response (cytotoxicity), but both types recognize antigen *via* a specialized receptor, BCR in B-cells and TCR in T-cells.

The immune system has the important ability to identify and recognize what is self and non-self. This property is crucial to respond to non-self-molecules and allow tolerance to self-ones. When this mechanism is not properly tuned, immune dysfunctions (as mentioned before) can emerge.

1.1.2 MHC: self *versus* non-self recognition

The major histocompatibility complex (MHC) is a region of multiple loci, on human chromosome 6 also known as human leukocyte antigens (HLA).

The genes are organized into regions that encode for different molecules. The class I MHC genes encode for classical (HLA-A, -B and –C), and non-classical (HLA-E, -F, -G) proteins. MHC class I plays a role in recognition of intracellular molecules, endogenous or foreign, and discrimination between self and non-self. T cells recognize antigens only when they are combined with MHC molecules.

The classical genes are constitutively expressed on the surface of nucleated cells. Due to thymic deletion of self-reactive CD8+ T-cells and natural immune suppressive regulatory T-cells with specificity for self-antigens, presentation of self-antigen leads to inhibition of the immune system, sparing healthy cells. In contrast, when a cell is infected by an intracellular pathogen (e.g viruses, *Listeria*, Protozoa), it presents foreign antigenic peptides derived from the pathogen in MHC class I molecules in its membrane. CD8+ T lymphocytes can recognize the foreign peptide/MHC complex and elicit an immune response. The non-classical gene HLA-E has a similar main function (antigen presenting), while HLA-F function is still unknown, and HLA-G is tissue restricted and seems to be involved in immune modulation, namely during pregnancy [7 - 9]. All 3 non-classical genes can also interact with NK cells (HLA-E and -G are inhibitory whereas HLA-F can activate NK cells) [10 - 12].

While MHC class I molecules are composed by a large α chain anchored in the plasma membrane and a smaller β2-microglobulin molecule, MHC class II are heterodimeric α and β glycoproteins chains with extracellular domains, transmembrane segments and cytoplasmic tails. These MHC class I and II molecules are highly polymorphic, and their alleles are co-dominantly expressed. This is particular relevant for transplantation and also in pregnancy since, in both scenarios, rejection of allogeneic (genetically non-identical) tissues can be elicited. For instance, after fertilization, the fetal cells will express both maternal and paternal MHC molecules challenging the maternal

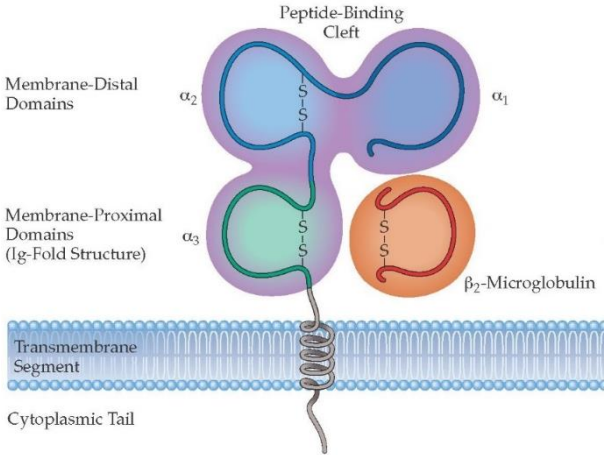

Figure 2. MHC class I molecular structure. MHC class I molecules comprise a large α chain anchored in the plasma membrane and a smaller β2-microglobulin molecule with a peptide binding cleft on top. Adapted from reference 2.

immune system to tune their activity into tolerance of paternal (and foreign) peptides – an extreme reactivity will lead to fetal rejection and miscarriages [13].

1.1.3 Natural Killer cells

Lymphocytes can be broadly subdivided into three populations: B-cells, T-cells and Natural Killer (NK) cells. Bone marrow-derived stem cells are the precursors of the three lymphocyte population. From the bone marrow, mature and precursor cells migrate to tissues and lymphoid organs and enter into the circulation to defend against invading pathogens and to contribute for tissue repair [14, 15]. To become functional, all NK cells undergo a process of licensing during their development through the engagement of their inhibitory and/or activating molecular receptors [16]. *In vitro*, NK cells showed the ability to kill a variety of tumor cells, virus- and intracellular bacteria-infected cells in a nonspecific cytotoxicity manner – they do not need specific epitopes or antigens to be activated [17] – and are tasked to act over a rapid time scale [18]. They recognize cells that express reduced levels of MHC I (the founding feature of the "missing-self" hypothesis) and the unusual profile of antigens at the surface of tumor/infected transformed cells. When this happens, NK cells secrete cytolytic granules (e.g perforin) or engage death of receptors in target cells (e.g Fas receptor) [19] that will mediate cell lysis and/or induce apoptosis of target cells [20].

This ability to recognize "stressed" cells is, then, directly related to low MHC expression which works as a danger signal for NK cells [21]. As mentioned before, healthy tissues constitutively express MHC molecules, flagging the cells to be spared by NK cells cytotoxicity.

1.1.3.1 Peripheral Natural Killer cells

Peripheral blood NK (pNK) cells represent up to 10% of the recirculating lymphocyte population and they can be, traditionally, divided into two main subsets based on the expression of two surface density markers: CD56 and CD16 (or FcγRIII). The cytolytic, fully mature and primed to kill subset is $CD56^{low}CD16^{+}$ cells, representing about 90% of pNK. The remaining subset is comprised by relatively immature non-cytotoxic $CD56^{high}CD16^{-}$ cells, which secrete cytokines, such as IFN-γ or TNF-α [22, 23]. The differentiation from $CD56^{high}$ into $CD56^{low}$ cells with cytotoxic capability occurs during the maturation process in lymph nodes or inflamed tissues [24]. Mature NK

cells also express a specific surface marker NKp46 which is present in humans, several strains of mice and in three common monkey species [25].

NK cells can express an impressive variety of activating and inhibitory receptors, in different combinations [26]. Being so, the combination of receptors in an individual NK cell is unique and modulates the outcome of each NK cell reactivity when in contact to the ligands in target cells [27]. Of notice, in healthy conditions, when both inhibitory and activating receptors are simultaneously engaged, the inhibitory signals predominate and NK cells are inhibited from killing and releasing cytokines [28].

1.1.3.2 Decidual Natural Killer cells

This thesis focusses on a distinctive NK cell population only found at the maternal-fetal interface during pregnancy: decidual NK (dNK) cells. This NK population are found in decidua (a name used to refer to endometrium during pregnancy). The size of this particular NK cell population increases in the mid-secretory phase of menstrual cycle until the early stages of pregnancy in the human uterine decidua, and its immunophenotypic characterization, cytokine secretion and cytolytic ability differs from pNK cells [29]. In contrast with the high cytotoxicity ability of recirculating NK cells ($CD56^{low}CD16^{+}$), dNK are poorly cytotoxic ($CD56^{high}CD16^{-}$) but are major cytokine and growth factor producers [30, 31]. Nevertheless, NK cells are the most important decidual immune cells representing up to 80% of total immune cell population in 1st trimester decidua (with the remaining 30% being macrophages and T cells) [32].

dNK are associated with 1) promotion of placental development, 2) support of trophoblast implantation and invasion, 3) vascular remodelling while still providing 4) immune protection to the pregnant woman and fetus against infections [30].

Their relevance for the trophoblast implantation was confirmed by elegant work by Leon L, *et al*) where the authors generated IL-15 knockout female mice that were devoid of NK cells. When pregnant, these mice developed pregnancy complications, such as intrauterine growth restriction. The nature and origin of dNK is still elusive – the literature suggests they may infiltrate from peripheral blood or differentiate from immature endometrial NK cells [30].

Since the discovery of dNK, the immune cell dynamics at the maternal-fetal interface in early pregnancy become better understood. However, very limited data is available regarding dNK populations at term pregnancy (> 37 weeks). The dNK population is known to decrease to

just 20-40% of the immune cell population by the time of delivery but the phenotype and function of dNK at term pregnancy tissues are largely unknown.

1.2 Activating and Inhibitory Receptors in NK cells

NK cells, including dNK, express several surface receptors with different functions (activating and inhibitory). These receptors control NK cell function in response to the target cells, either directly or through associated adaptor proteins [33 - 35]. This section introduces the families of receptors studied in this thesis: Killer cell Immunoglobulin-like Receptor, Natural Cytotoxicity Receptor and C-type lectins, among others. While some of these families express either activating or inhibitory receptors, others include both types. NK cells also express others receptors (adhesion molecules or integrins, like LFA-1) related to NK cell activation [36] but for clarity purposes they won´t be addressed here.

1.2.1 Killer cell Immunoglobulin-like Receptor

Killer-cell Immunoglobulin-like Receptors (KIRs) comprise both inhibitory and activating receptors, the common ITIM and ITAM-containing adaptor DAP-12 signaling pathways [37], respectively. ITIM and ITAM stand for **I**mmunoreceptor **T**yrosine-based **I**nhibitory (or **A**ctivation) **M**otifs and provide the basis for the two opposed signaling modules [38]. The KIR gene loci are located on human chromosome 19q13.4 [39]. These receptors are type I transmembrane glycoproteins with two or three extracellular immunoglobulin-like domains, therefore named KIR2D or KIR3D receptors, respectively [40, 41]. Moreover, they also include either a short (S) or long (L) cytoplasmic tail, indicated in their acronym – KIR2DS or KIR3DL, for example. KIRs with long cytoplasmic tails contain an ITIM that initiates the inhibitory signaling pathway, while KIRs with short cytoplasmic tails signal through an adapter molecule containing an ITAM, leading to activation. The combination of different KIR functions, number of domains and the length of the cytoplasmic tails generates an extensive variety of KIR expression on NK, and KIR genotype adds an extra layer of polymorphism. The overall KIR repertoire of an individual can vary with intrinsically allele type and frequency, and each NK cell expresses a unique assortment of KIRs with high degree of diversity [42]. In addition, it has been reported that KIR expression is regulated by the methylation of KIR gene loci [43] and can also be influenced by the MHC Class I alleles of the

individual [44, 45]. In sum, each individual inherits a different combination of genes, and they are expressed randomly in different subsets of NK cells.

This receptor specifically bind HLA-A, -B and –C molecules recognizing polymorphisms [46]. If sufficient levels of HLA class I molecules are present on cell surface, inhibitory KIRs will trigger ITIM, which blocks the signaling cascade induced by activating receptors, generating tolerance [47]. When cells fail to express self-MHC molecules, NK cells are no longer inhibited and eliminate the target.

Within HLA class I molecules, HLA-C may be the most important in NK cell regulation since all individuals carry HLA-C alleles [39] that acts as primary ligands for KIRs. KIR/HLA-C allele recognition is based on dimorphic residues present in the HLA-C α-helix at position 80 [36]. If an asparagine (HLA-C^{asn80}) is present at this position, the allele is classified as HLA-C1 and binds inhibitory KIR2DL2 and -3. If position 80 contains a lysine (HLA-C^{lys80}) the allele is classified as HLA-C2 and binds inhibitory KIR2DL1 and activating KIR2DS1 [36, 39].

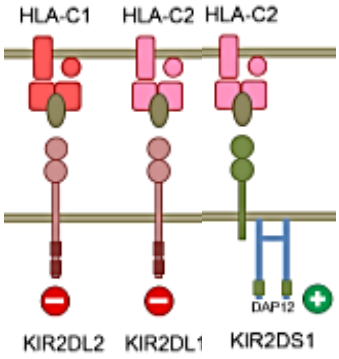

Figure 3. Killer Immunoglobulin-like Receptors. Different types of KIRs, both activating and inhibitory, with their known HLA ligand are shown. Image courtesy of Ângela Crespo. Adapted from reference 36.

The specificity of HLA class I activating receptors has been unequivocally demonstrated only for KIR2DS1 [48]. Recent studies have demonstrated the clinical importance of KIR2DS1 and KIR2DS5 for a successful pregnancy. The KIR2DS1 receptor, expressed by maternal dNK and pNK, has an extracellular domain very similar to inhibitory KIR2DL1, binding HLA-C2 ligand. However, its cytoplasmic tail is short and elicits an activating signal, in contrast with its KIR2DL1 counterpart. Interestingly, NK cells expressing KIR2DS1 were revealed to be hyporesponsive in HLA-$C2^+$ individuals [49, 50] which may be beneficial in the context of pregnancy. The ligand for KIR2DS5 is still not established but the presence of the *KIR2DS5* gene in Uganda pregnant women with HLA-$C2^+$ fetuses was associated with lower risk of developing preeclampsia, leading researchers to assume that its ligand might also be HLA-C2. Preeclampsia is a pregnancy complication characterized by

maternal high blood pressure, presence of protein in urine and signs of damage in kidneys and liver. The effect of *KIR2DS1* seems to be characteristic of European populations [51]. Their roles in protection from pregnancy complications, as well as their role in viral clearance, will be address later in this thesis.

1.2.2 Natural Cytotoxicity Receptor

The Natural Cytotoxicity Receptors (briefly, NCRs) belong to the Ig-superfamily [52] and include NKp46, NKp44 and NKp30 receptors, in humans, which are encoded by *NCR1, NCR2* and *NCR3* genes, respectively [53]. *NCR1* is localized near the leukocyte regulatory complex in human chromosome 19 and *NCR3* in the MHC class III locus in chromosome 6. All these receptors are known to be activating type I transmembrane glycoproteins with a positively charged arginine in the transmembrane region and one (in NKp30) or two (in the case of NKp46) extracellular domains responsible for ligand binding to TCR and/or FcεRγ [54]. These receptors are expressed in nearly all human NK cells [55] and some studies reported that they interact with viral hemagglutinin from avian Newcastle disease virus [56], B7-H6 protein or pp65, the main tegument protein of Human Citomegalovirus (HCMV) [57]. For optimal recognition and clearance of target cells, the NCRs work best as a team when identifying potential targets [58]. Other studies on NCR expression suggested they each play discreet roles: only NKp46 was able to induce dNK cytotoxicity, while NKp30 was shown to promote cytokine secretion, and NKp44 was found to have a predominantly inhibitory function in dNK [30, 59 - 61]. Moreover, in a pregnancy context, increased mRNA encoding for activating NKp30 was found in the placentas of women who had experienced sporadic or recurrent miscarriages within the first trimester. This phenomenon showcases NKp30 contribution to failed pregnancies, mainly due to dysregulated cytokine production [62].

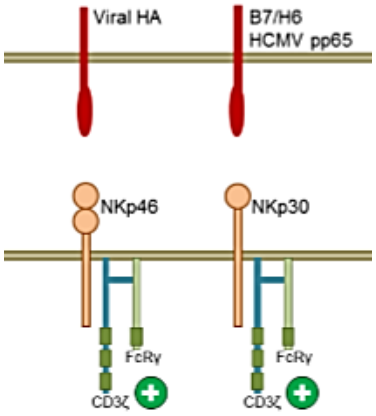

Figure 4. Natural Cytotoxicity Receptors. NKp46 and NKp30 receptors with their viral ligands. Both are activating receptors. Image courtesy of Ângela Crespo. Adapted from reference 36.

Although many NCR ligands remain unidentified, NCRs undoubtedly recognize several ligands that are believed not to be expressed on healthy cells [63]. This underlines the NCR importance in cancer therapy as we learn their roles in the anti-tumor immune response [46]. Interestingly, there have been some reports of differentially spliced isoforms of NCRs that exhibit inhibitory functions, which are linked to poor prognosis in cancer, but may translates into a healthy outcome in the context of pregnancy [53].

1.2.3 C – type lectin Receptors

In contrast with NCRs, C-type lectin receptors (CTLRs) include both activating and inhibitory NK receptors. This type of receptors are PRRs, which are able to recognize a variety of endogenous and exogenous ligands such as the evolutionary conserved PAMPs ligands. This way, CTLRs are essential for innate responses but also contribute to induction of adaptive immune responses [65] as well as the maintenance of homeostasis [66]. The C-type lectin receptor family has been classified into 17 groups according to their domain organization and phylogenetic features [67, 68] and can be found in soluble form in mucosal fluids (collectins) or in transmembrane form in cell surfaces. The transmembrane CTLRs, which are expressed mainly in myeloid cells, are divided according to the activating (ITAM) or inhibitory (ITIM) signaling they induce [67, 69]. The most studied CTLR is the NKG2D receptor, an activating receptor that recognizes the induced-self ligands MICA/B highly expressed by unhealthy cells as a response to pathways induced by viruses or stress [70-72]. Activating C-type lectin receptors use either DAP-10 or DAP-12 as an adaptor for their signaling mechanism resulting in cytokine secretion and cytotoxicity [73, 74]. Activating CTLRs have also been shown to be important in the NK cell-mediated control of some cancers [75, 76].

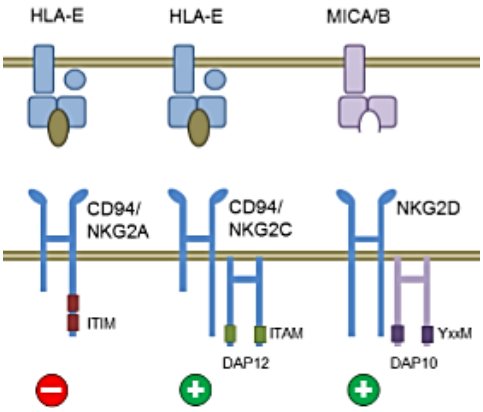

Figure 5. C-type Lectins Receptors. CD94/NKG2A, CD94/NKG2C and NKG2D receptors with their ligands. Both NKG2C and NKG2D are activating receptors while NKG2A is inhibitory. Image courtesy of Ângela Crespo. Adapted from reference 36.

Another important group of C-type lectin family receptors are the CD94-NKG2A/C heterodimers, which are addressed in this thesis. These receptors react to non-classical HLA-E molecules on the surface of potential target cells [77] and reveal antagonist effects: while CD94/NKG2A has an ITIM receptor that disrupts the activating pathways, CD94/NKG2C

heterodimer regulates activation *via* the association with the ITAM-bearing molecule DAP-12 signaling pathway [78, 79].

In humans, the levels of NKG2A and NKG2C receptors are up-regulated in preeclamptic women, which highlight their importance in pregnancy complications [80].

Table 2. Ligands for NK cell receptors. All receptors studied in this thesis are shown, together with their ligands and immune function. Adapted from reference 36.

FAMILY	RECEPTOR	LIGAND	FUNCTION
Killer cell Ig-like Receptors	KIR2DL1	HLA-C2 (HLA-C^{lys80})	Inhibitory
	KIR2DL2	HLA-C1 (HLA-C^{asn80})	Inhibitory
	KIR2DS1	HLA-C2 (HLA-C^{lys80})	Activating
Natural Cytotoxicity Receptors	NKp30	Viral HA	Activating
	NKp46		
C-type Lectins Receptors	CD94/NKG2A	HLA-E	Inhibitory
	CD94/NKG2C		Activating
	NKG2D	MICA/B	Activating
Nectin-like Receptor	DNAM-1	CD112/5	Activating
SLAM	2B4	CD48	Activating
Lectin-like Receptor	CD57	Lectin	?

1.2.4 DNAM-1, 2B4 and CD57 receptors

The DNAM-1 receptor (CD226) is part of the Ig-superfamily and is constitutively expressed in up to 50% of all NK cells [81]. There are many ligands associated with this receptor, like Polio virus receptor or Nectin-2 receptor showcasing its relevance in NK cell-mediated anti-tumor responses [82, 83] even though some studies suggest this is merely a co-stimulatory receptor [84]. Moreover, the DNAM-1 receptor has been associated with monocyte movement through endothelial cell junctions [85], and it may permit stable interactions between NK and target cells [86]. Overall, this receptor is believed to be mainly involved in tumor cell recognition and migration of NK cells [46].

The 2B4 receptor (CD224), in contrast, is present in all NK cells [87, 88]. Its activating *versus* inhibitory function is still up to discussion, but in humans, it has been described to induce rejection of melanoma cells that express CD48 [89].

Lastly, the CD57 receptor is a carbohydrate antigen expressed on highly mature cells within the $CD56^{low}CD16^{+}$ NK cell compartment. While the CD57 receptor is expressed in many blood NK cells, it is absent in dNK. Most $CD57^{+}$ cells in the endometrium are T-cells [90]. This receptor was shown to be significantly increased in NK cells of $HCMV^{+}$ individuals', co-expressed with $NKG2C^{+}$, years after the primary infection [38].

1.3 Pregnancy: an Immunological Paradox

The ability of a woman to tolerate a nine-month pregnancy has always been intriguing. Sir Peter Medawar was one of the first biologists to dig deep into the (apparently) acquired immune tolerance from the maternal immune system towards the semi-allogeneic fetus. He proposed three possible explanations for such a phenomenon on one of his dissertations [91]:

1) Lower immune reactivity of the mother;
2) Antigenic immaturity of the fetal tissues;
3) Physical separation of the maternal and fetal tissues.

This topic is still being discussed by the research community, which aims to fully understand the mechanisms behind the maternal-fetal tolerance and immunological modulation during the course of pregnancy.

1.3.1 Maternal and fetal interactions

The miracle of life, in humans, requires one of the most aggressive (and invasive) types of placentation. The human uterine mucosa changes deeply to allow embryonic implantation and placental invasion: this newly-arised tissue is called decidua. Decidualization is accompanied by the recruitment of immune cells: 60-80% NK cells, 15-20% macrophages and the remaining 5-10% T cells [92]. When implantation is successful, the blastocyst attaches to the decidua, originating the inner cell mass that will develop into the fetus, and the trophectoderm that will originate placental structures like the syncytiotrophoblast and the cytotrophoblast. When these structures

are stablished, the blastocyst invasion is facilitated. At gestacional day 8 – 9, both placental and fetal layers are determined. Among the cells that form the placental layers and column-like structures (villi), known as trophoblasts, there is a highly specialized cell type – extravillous trophoblast (EVT). EVT are the most invasive placental cells and contact directly with the maternal blood vessels and the enriched environment of immune cells in decidua. When EVT reach the uterine spinal arteries, they actively remodel the maternal vessels, substituting the endothelial cells [92]. The remodeling of the vessels lowers their resistance and allows for a high flow of maternal blood that surrounds the trophoblast villous structures and irrigates the placenta, providing nutrient and gas exchanges with the fetus [93].

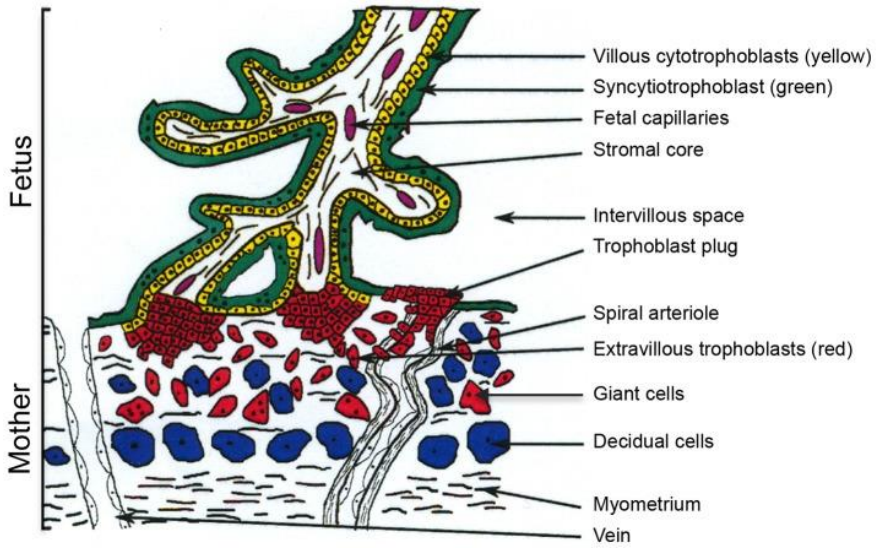

Figure 6. Trophoblast's layers differentiation. During implantation, the blastocyst cells proliferate to form multilayered columns of cells which will invade the decidua. The maternal and fetal-originated cells are identified in distinctive areas. Adapted from reference 94.

Two distinctive decidual layers are identified: decidua basalis and decidua parietalis. Decidua basalis is the maternal layer of the placenta and lies under the implanted ovum and interact with the trophoblast. Decidua parietalis exclusively surrounds the fetal membranes (chorion and amnion) [95]. dNK at term are isolated from the decidual layers, which represent one of the main focus of this thesis.

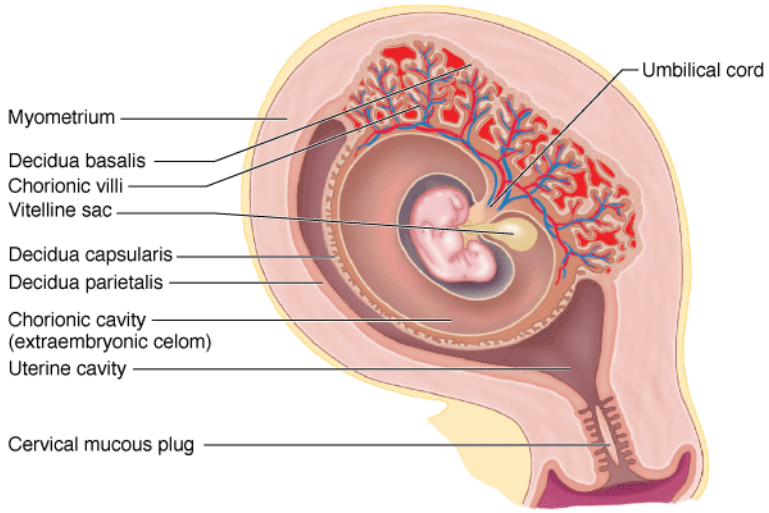

Figure 7. Decidua basalis and decidua parietalis membranes. After embryonic implantation, different regions of decidual layers are established. The diagram depicts the location of decidua basalis and decidua parietalis. Adapted from reference 96.

Fetal cells (EVT) and maternal dNK are, then, in direct contact since the early stages of pregnancy, and dNK have been shown to be important for the fine-tuning that regulates placentation. Syncytiotrophoblast and villious cytotrophoblast layers do not express MHC molecules or NK activation ligands, and do not elicit maternal immune activation [97, 98].

However, EVT express a classical polymorphic MHC Class I molecule - HLA-C - , and the non-classical MHC class I HLA-E and HLA–G molecules. All human nucleated cells express HLA-C and HLA-E, but HLA-G expression is exclusive to the trophoblast and, therefore, it is assumed to enhance NK modulation and tolerance throughout the pregnancy [99]. dNK express both inhibitory and activating receptors (as seen in Chapter 1.2) some of them in higher levels than blood NK cells [100]. HLA-C is expressed by both fetal and maternal tissues, and its polymorphism is responsible for a potential immunological paternal mismatch, and consequently, possible dNK immune activation and tissue rejection [101].

1.3.2 Clinical Complications

With such a complex and invasive placentation, immunological factors are implicated in different reproductive complications. Failure in placentation with defective trophoblast invasion and vessel remodeling can lead to spontaneous abortions, and other pregnancy disorders may arise, such as preeclampsia, recurrent miscarriages (RM) or fetal growth restriction (FGR). The etiology of these pregnancy complications is still largely unknown.

Maternal chronic hypertension (MCH) is a severe condition that may occur during pregnancy [102], whose impact is not only restricted to the pregnancy period: MCH predisposes the development of preeclampsia [103] which leads to an increased incidence of cardiovascular disease in the newborn later in life [104]. FGR is another pathology established in the first trimester of human pregnancy as a result of insufficient spiral artery remodeling [105]. Newborns with this pathology also have life-long consequences including metabolic disorders [106]. Both pathologies affect 5-8% of human pregnancies worldwide and contribute for neonatal morbidity and mortality [92].

Throughout the course of the pregnancy, the mother might also acquire viral and/or bacterial infections. Understanding these infections is extremely important because they may also contribute to some congenital defects or fetal loss, and increase the complexity of the immunological paradox of pregnancy. On the one hand, the maternal immune system needs to be tuned down to allow fetal tolerance and development. On the other hand, it still needs to remain active and responsive while facing HCMV or *Listeria monocytogenes* infections, for example. This protective immunity is essential to defend the mother from the nefarious effects of the infection, *per se*, but also to avoid the transmission to the fetus. Infections contribute to an increased rate of spontaneous abortions, but their real effect may be underestimated because, more frequently than not, the causes for miscarriage are not clinically determined [107].

How dNK allow for a pregnancy to progress without complications is still an unanswered question. The education, licensing and regulation of dNK in the uterine microenvironment, both in a context of health and diseased pregnancies, are still largely unexplored topics.

1.3.3 KIRs and HLA-C in pregnancy tolerance and infection clearance

In all of the above-mentioned scenarios, NK cells are present. For instance, dNK functional responsiveness is regulated by the presence of inhibitory and activating receptors which interact

with trophoblast HLA class I molecules. The strength of these interactions will determine the NK cell education process towards both tolerance of self and cell activation upon encounter with activating ligands.

There is evidence in the literature that particular combinations of highly polymorphic KIRs in dNK and HLA-C variants on the invasive trophoblast impacts the overall pregnancy success [92]. This finely tuned interaction between maternal KIR and fetal HLA-C is not only important for the trophoblast placentation or fetal birth weight [108, 109]. KIR and HLA-C combinations also correlates with the clinical outcome of some human diseases like leukemia [110], inflammatory diseases [111], and certain tumors [112, 113].

On the Faridi and Agrawal study (2010), the authors made a first attempt to characterize the KIR-HLA-C allorecognition pattern on recurrent miscarriages, taking into account the contribution of the paternal antigens for the pregnancy outcome [16]. And the results showed a higher prevalence of $2DL1^{-}$ $2DS1^{+}$ individuals in the patients group, putting these couples at higher risk of RM if the fetus carries HLA-C2 [16] – the main ligand for KIR2DS1. These findings are in accordance with the 15th International Histocompatibility Workshop Reproductive Immunology Component [114] outlines where the inhibiting combination of KIR and HLA is found to be lower in RM couples.

In contrast, more recently, Crespo *et al* (2016), contributed to show the clinical importance of the activating KIR2DS1 receptor and its ligand HLA-C2 (expressed by EVT) to avoid pregnancy complications, even though this engagement leads to NK cell activation [115, 116]. The concept of NK cell activation being important for a successful pregnancy is difficult to understand, because immune activation is expected to be detrimental to the fetus. Nevertheless, activation of dNK may not lead to cytotoxicity, but instead to the secretion of beneficial cytokines and growth factors, which are essential for trophoblast invasion and placental growth [108].

Although the carrier frequency of *KIR2DS1* ranges from 13% to 63% throughout different world populations [117], its presence has been shown to improve the outcome of viral infections [118, 119]. In human papilloma virus or HCMV infections, for example, activating KIRs specific for C2 allotype are suggested to play a key role in NK-mediated viral clearance [120, 121].

HCMV is the most common congenital viral infection and contributes for placental underdevelopment. The Crespo *et al* study demonstrated that expression of KIR2DS1 by dNK increases their ability to respond to placental HCMV infection, preventing viral spread and limiting the virus-induced pathology effects [115]. A summary of epidemiological studies regarding different KIRs and HLA-C is shown in the following Table.

Table 3. Impact of KIR/HLA-C in diseases prognosis. The combination of KIR genes and HLA-C molecules impact disease outcomes, with protective or detrimental effects. Adapted from reference 36.

DISEASE	KIR GENES	HLA-C GROUP	EFFECT	REFERENCE
HCV	KIR2DL2	C1	Protection	122
Multiple Sclerosis	KIR2DS1	C2	Protection	123
Psoriasis	KIR2DS1	C2	Susceptibility	124
Chronic Myeloid Leukemia	KIR2DL2	C1	Protection	110
Recurrent Miscarriages	Absence of KIR2DS1	C2	Susceptibility	125

1.3.4 Immune Activation: parallelism with transplantation

Pregnancy can be viewed, from an immunological point of view, as analogous to transplantation, since both involve allorecognition. In fact, considering the paternal alleles inherited, at least half of the genetic information present in the fetal cells is exogenous to the maternal body. And, as it happens in transplantation, if an individual receives a graft from a non-genetically identical donor, an immune response is elicited, mainly due to the mismatched MHC molecules from the donor that are recognized as foreign antigens by the host's immune system. This immune reaction elevates the cytokines levels in the serum and involves inflammation in multiple organs [126]. Although inflammation can be associated with deleterious effects, in 2004, Redman and Sargent suggested that a state of controlled inflammation must exist for a successful pregnancy [127].

T cells are mainly responsible for rejection of organ grafts. However, they represent only 10-30% of the leukocytes in the decidua, and so far, there is no evidence that they are responsible for the fetal "rejection" in humans [128].

NK cells, in contrast, represent up to 80% of immune cells in the decidua. NK cells have a high spontaneous lytic activity which is tuned down in the presence of self-MHC class I molecules on the cell surface that inhibit the killing pathway *per se*. When these molecules are not present, NK cells find themselves in a "missing-self" setting, inhibition is abrogated and immune rejection can be induced. In fact, in transplantation, NK cells are responsible for the graft acute rejection

[46]. Of notice, other cell subsets have been associated, in contrast, with fetal tolerance. In Ana Areia's studies, Treg cells seemed to prevent fetal rejection by the maternal immune system under the influence of progesterone [129].

If the missing-self scenario were the norm in all pregnancies, the Human race wouldn't have prevailed (nor would have other mammalian species). Thus, the maternal immune system developed immune tolerance to the foreignness of semi-allogeneic fetal trophoblastic cells without attacking it.

1.4 Granules and degranulation

NK cytotoxicity is mediated by the release of cytotoxic molecules, expressed in specialized vesicles known as cytotoxic granules. Three main proteins are commonly identified in these granules: Perforin (PFN), Granzyme B (GZMB) and Granulysin (GNLY), which is expressed as a 15kDa precursor and an active isoform GNLY 9kDa [130].

PFN is a cytolytic glycoprotein responsible for the formation of membrane pores in a Ca2+ dependent manner. These large transmembrane pores (5-20 nm in diameter) [97] promote cytolysis and apoptosis of target cells by facilitating the uptake of cytotoxic granzymes [98]. PFN is found to be present in cytoplasmic granules of T-lymphocytes and NK cells [99].

Granzymes are pro-apoptotic proteases. GZMB is a serine protease found in the same granules as PFN (and secreted along with it) and it is responsible for induction of apoptosis in target cells. GZMB is the most abundant granzyme among five different types (A, B, H, K and M), in humans [100, 101]. Human GZMB-mediated apoptosis is in part mediated by mitochondria and caspases in a well-studied pathway [102 - 104].

Lastly, GNLY is a 15kDa antimicrobial molecule, also present in granules of cytotoxic T cells and NK cells. When cleaved, it originates a 9kDa isoform with cytolytic and proinflammatory potential. GNLY 15kDa also acts as a chemoattractant for proinflammatory cells, as part of the immune system.

Degranulation of these proteins is an important defense mechanism against infections. The bulk of secreted proteins by the immune cells is vital in determining whether there is a net pro or anti-inflammatory balance in the tissue, including the placenta.

AIMS

The aim of this thesis is to provide a detailed phenotypic and functional characterization of the NK cell population present in healthy term decidual tissues: decidua basalis and decidua parietalis, in comparison with peripheral blood NK cells (control samples) and 1st trimester (6-12 weeks) decidua NK cells.

For the phenotypic analysis, emphasis will be given to:

1) Expression of cytolytic granules: perforin (*PFN*), granzyme B (*GZMB*) and granulysin (*GNLY*);
2) Expression of killer cell immunoglobulin-like receptor (KIR) with specificity for HLA-C;
3) Influence of KIR on the expression of cytolytic granules.

For functional analysis the specific goal is to:

1) Investigate the capacity of term pregnancy dNK, from decidua basalis and decidua parietalis, to degranulate in response to MHC negative targets cells (K562 and 721.221) as well as upon stimulation by PMA/Ionomycin.

The idea for this project was born from the little attention dNK phenotype and function a human term pregnancy have received. The data presented in this thesis therefore contributes to a novel understanding of the immune impact of decidua NK cells at term gestational age. Decidual NK cells in first trimester (6-12 weeks) were shown to be important in the production of growth factor and cytokines to facilitate the trophoblast invasion as well as responses to virus infected placental cells. In contrast, term dNK function is not well understood.

MATERIALS AND METHODS

3.1 Clinical Samples

Discarded human placenta (villous trophoblast tissue) and maternal decidua were obtained from women undergoing delivery after a healthy term pregnancy (gestational age > 37 weeks) or undergoing voluntary surgical pregnancy termination (gestational age 6-12 weeks) at a local reproductive health clinic. Gestational age of the surgical abortion samples was calculated from the last menstrual period and confirmed by ultrasound measurements of crown-rump length. These last samples were obtained by vaginal suction curettage and collected in aseptic flasks with RPMI 1640 medium completed with 10% FBS and 1% P/S (Gibco™). Women with anatomical, chromosomal, hormonal and/or autoimmune abnormalities were excluded from our study. All human tissue used in this project was de-identified and the Committee on the Use of Human Subjects (the Harvard IRB) approved the protocol and determined this use of placental and decidual material is Not Human Subjects Research.

3.2 Decidual Lymphocyte Isolation

Decidual and villous trophoblast were macroscopically identified and separated. Decidual tissues were washed with PBS, minced and therefore gently digested with 0.1% collagenase type IV and 0.01% DNAse I (Sigma-Aldrich®) in a shaking water bath for 60 – 75mins at 37°C. Released lymphocytes were washed for 7min, 1800rpm with RPMI 10% FBS (Gibco™) and filtered through 100µm, 70µm and 40µm nylon cell strainer (Corning Incorporated – Life Sciences, FALCON®). Lymphocytes were then dissolved in 20ml 0.830 g/ml Percoll™ (GE Healthcare Bio-Sciences) and layered on a Percoll gradient – 10ml 1.085 g/ml; 15ml 1.054 g/ml – for density gradient centrifugation for 30min at 2000rpm, no brake. Lymphocytes were isolated from the 1.085 – 1.054 g/ml gradient interface, washed twice with RPMI 1% P/S and stained for flow cytometry analysis or flow cytometry sort.

3.3 Peripheral Blood Natural Killer cell Isolation

Peripheral Blood Mononuclear cells (PBMC's) were obtained from discarded LeukoPak® from healthy volunteer blood donors. All blood donations used in this project were de-identified. The leukocyte enriched fractions were incubated with RosetteSep™ Human NK Cell enrichment cocktail (STEMCELL™ Technologies) for 20min and subsequently layered on top of 12.5ml Ficoll-Plaque™ Plus (GE Healthcare Bio-Sciences) density gradient. The mix was centrifuged for 25min, 2000rpm, no brake. Natural Killer cells were isolated from the gradient interface, washed twice with RPMI 1% P/S and used for further experiments.

3.4 Cell Culture

pNK and dNK cells were cultured in X-VIVO™10 media (Lonza) supplemented with gentamicin, 5% Human AB Serum (Corning) and 20ng/ml IL-15 (Biolegend®). Target cell lines - 721.221 (221) and K562 - were cultured with RPMI 10% FBS, 5% P/S and 5% L-glutamine (Gibco™).

3.5 Degranulation Assay

Freshly isolated pNK and dNK were plated and incubated overnight at a concentration of $2x10^6$ cells/ml in X-VIVO™10 media (Lonza) supplemented with 5% Human AB Serum (Corning) and 20ng/ml IL-15 (Biolegend®). The next day, pNK and dNK (effectors cells) were harvested and $7.5x10^4$ NK cells were co-cultured with $2.25x10^5$ 721.221 (221) and/or K562 targets (E:T 1:3) in a 96 well plate for 2 hours. As a positive control, PMA/Ionomycin (Sigma-Aldrich®) was used at a concentration of 2.5µg/ml (PMA) and 1 µg/ml (Ionomycin), respectively. Moreover, 250ng/ml of CD107a PerCP-Cy5.5 antibody was added to all co-cultures. After the incubation period, cells were collected and fixed for 10min in 1% PFA (Sigma-Aldrich®) and therefore stained for surface markers for flow cytometry analysis.

3.6 Flow Cytometry

Antibodies used for flow cytometry analysis and FACS are listed in Supplemental Material. For surface staining, cells were stained for 30min on ice, in the dark, in PBS 1% FBS. For KIR2DS1 staining, NK were first stained with 1.5µg/ml KIR2DL1 (clone 143211, R&D Systems®) for 20min and thereafter with 0.5µl KIR2DL1/S1 (clone EB6B, Beckman Coulter) for additional 20min, as described previously [115]. For intracellular staining, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). Analysis was performed on LSR II (BD) and FACS was done using a BD FACS Aria™ II.

3.7 Statistical Analyses

All data was analysed using GraphPad Prism version 6.0 software. To determined differences among more than 2 unpaired groups, a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-test was performed. All data points were lined with median and interquartile range. P-values < 0.05 were considered to reflect significant differences.

RESULTS

The following results represent a novel and exploratory understanding of the phenotype and function of term decidual NK cells (dNK) at the maternal-fetal interface during pregnancy.

This thesis is mainly focused on healthy term pregnancy dNK, present in decidua basalis and decidua parietalis tissues of > 37 weeks of gestation. The phenotype and function of term dNK were compared with both pNK and 1st trimester dNK. Term pregnancy is a gestational period largely ignored by Immunological research community, and this thesis represents an effort to bridge the gap.

4.1 Percentage of CD56+ NK cells is decreased in term pregnancy

A phenotypic analysis of healthy term pregnancy NK cells, from both decidua basalis and decidua parietalis, was performed. The results obtained from these samples were compared to pNK and to dNK from 1st trimester tissue (gestational age 6 to 12 weeks). pNK and dNK were isolated as described in reference 36.

To identify NK cells, a flow cytometry based strategy was used, as shown (Fig. 8A). The first hallmark noticed was the significant reduction in the percentage of dNK within the term pregnancy tissues analysed - decidua basalis and decidua parietalis, compared to 1st trimester dNK (Fig. 8C). The percentage of pNK was not determined since pNK were isolated through a purification kit that yielded close to 100% purity. The actual pNK population present in peripheral blood comprises between 5%-10% [36].

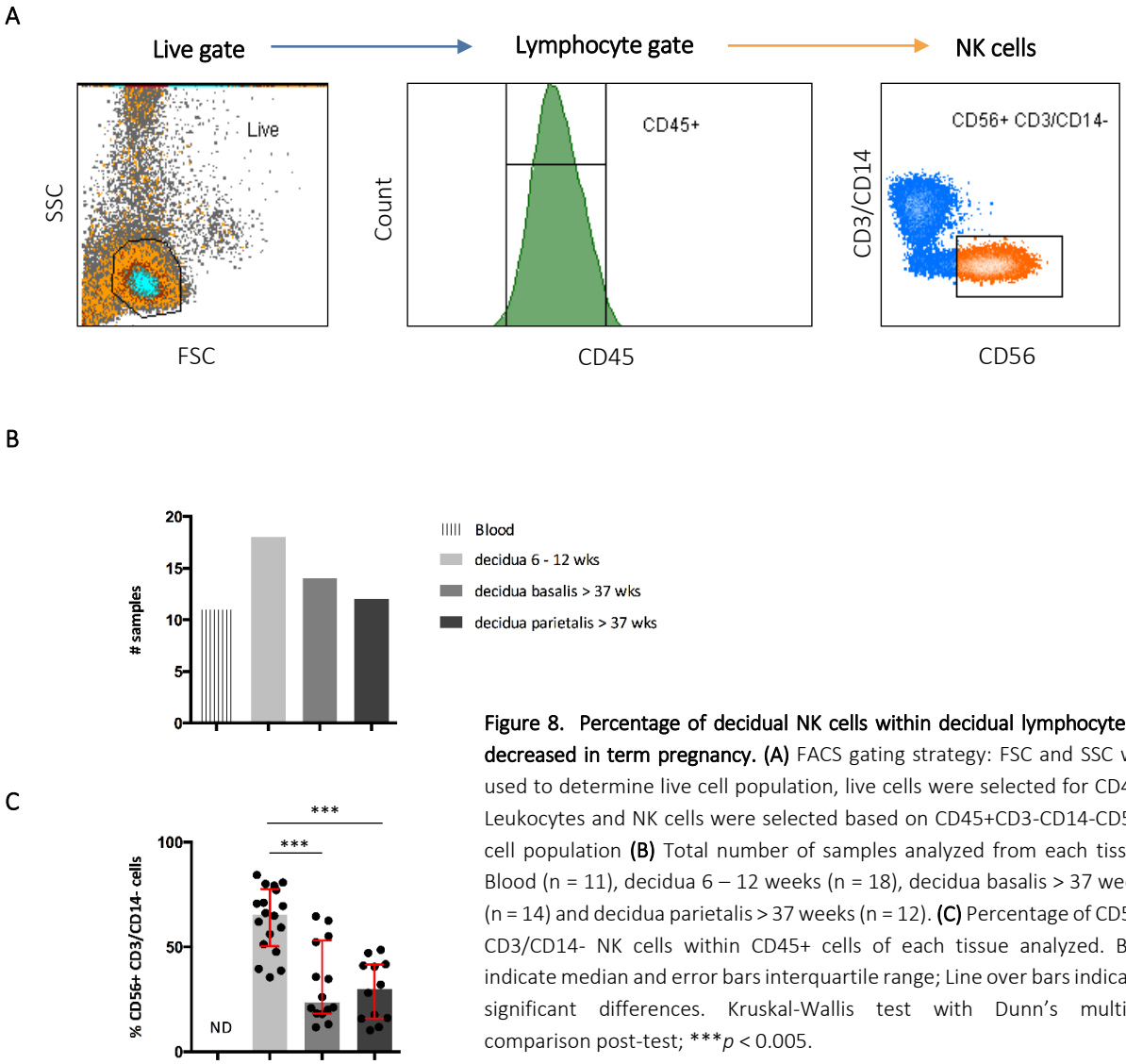

Figure 8. Percentage of decidual NK cells within decidual lymphocytes is decreased in term pregnancy. (A) FACS gating strategy: FSC and SSC was used to determine live cell population, live cells were selected for CD45+ Leukocytes and NK cells were selected based on CD45+CD3-CD14-CD56+ cell population **(B)** Total number of samples analyzed from each tissue: Blood (n = 11), decidua 6 – 12 weeks (n = 18), decidua basalis > 37 weeks (n = 14) and decidua parietalis > 37 weeks (n = 12). **(C)** Percentage of CD56+ CD3/CD14- NK cells within CD45+ cells of each tissue analyzed. Bars indicate median and error bars interquartile range; Line over bars indicates significant differences. Kruskal-Wallis test with Dunn's multiple comparison post-test; ***$p < 0.005$.

4.2 Intracellular cytolytic granule expression is lower in term NK cells

After identifying the NK cell populations, their intracellular expression of cytolytic molecules - Perforin (PFN), Granzyme B (GZMB) and Granulysin (GNLY) - both 9kDa (active form) and 15kDa (GNLY Total isoform) - was determined. Isotype controls and histogram plots were used for better reliability of the results obtained (Fig. 9A).

The percentage of GNLY 9kDa positive cells was the most variable among the tissues studied. 1st trimester dNK has the highest percentage of GNLY 9kDa+ cells as well as the highest level of GNLY 9kDa expression (MFI) when compared to blood and term decidua basalis and decidua parietalis (Fig. 9B and C). Similar results were observed regarding the total GNLY, particularly MFI levels (Fig. 9C). The percentage of PFN and GZMB positive NK cells were similar in pNK and dNK from all tissues. However, the MFI for PFN and GZMB were significantly reduced in dNK from all three decidual tissues compared to pNK (Fig. 9B and C). Term decidua basalis and decidua parietalis revealed the lowest MFI levels for PFN and GZMB (Fig. 9C).

4.3 Activating receptors expression is reduced in term NK cells

Cell surface expression of nine NK cells receptors, both activating and inhibitory, was determined on pNK and dNK by flow cytometry. The percentage of CD94+ NK cells was significantly increased in 1st trimester decidua compared to pNK (Fig. 10B). In addition, dNK from decidua basalis had significantly higher MFI levels of CD94 compared to pNK (Fig. 10C). Within the CTLR, NKG2A expression was increased in all decidual tissues, with significant differences in 1st trimester decidua and term decidua basalis compared to pNK. NKG2C and NKG2D receptors did not show significant differences in MFI levels, between dNK and pNK (Fig. 10D).

Expression of the NCR, NKp46 was increased in all decidual tissues, mainly in decidua 6 – 12 weeks and term decidua basalis, which were significantly different compared to blood. In contrast, the NKp30 receptor didn't show any significant differences in expression levels among tissues, being very lowly expressed in term tissues (Fig. 10E).

Lastly, DNAM-1 receptor was reduced in all decidual tissues, especially in decidua basalis and decidua parietalis (Fig. 10F). 2B4 receptor expression was similar in all tissues analysed (Fig. 10F). CD57 receptor expression was diminished in all decidual tissue groups, with significant differences when compared to the expression of pNK (Fig. 10F).

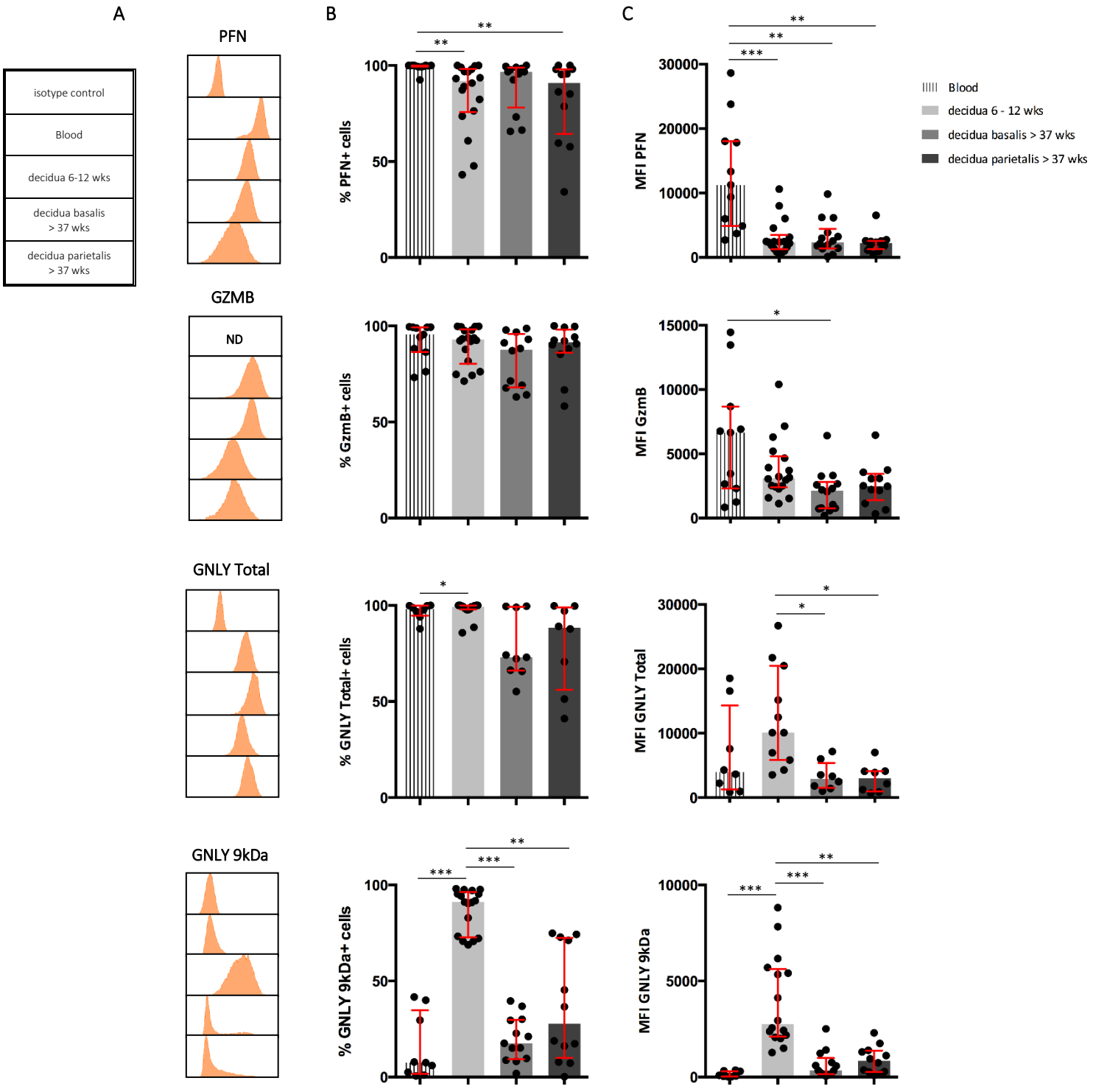

Figure 9. Intracellular granule expression is reduced in term dNK. **(A)** Representative histogram plots of granule expression in blood, 1st trimester decidua (6 – 12 weeks), decidua basalis (> 37 weeks) and decidua parietalis (> 37 weeks) NK cells *(from top to bottom)* **(B)** Percentage of granule positive NK cells in all tissues analysed. **(C)** MFI of granule expression NK cells of all tissues analysed. Bars indicate median and error bars interquartile range; Line over bars indicates significant differences; Kruskal-Wallis test with Dunn's multiple comparison post-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

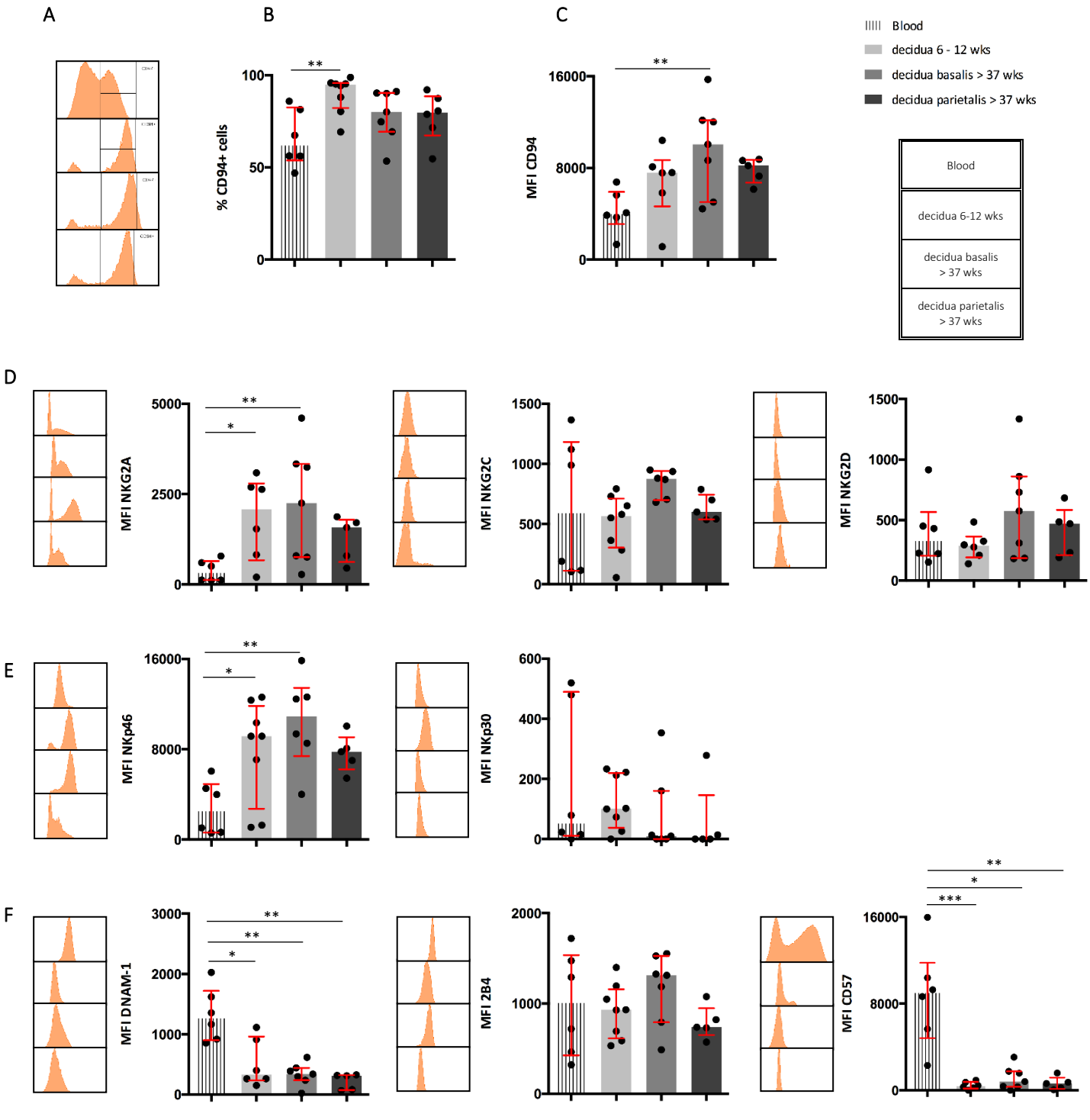

Figure 10. NK-cell receptor expression is lower in dNK than pNK. (A) Representative histograms of CD94 in blood, 1st trimester decidua (6 – 12 weeks), decidua basalis (> 37 weeks) and decidua parietalis (> 37 weeks) *(from top to bottom)* **(B)** Percentage of CD94+ cells within the NK cells population in all tissues analysed. **(C)** MFI of CD94+ NK cells of all tissues analysed. **(D)** Representative histograms and MFI of expression of C-type lectins (NKG2A, NKG2C and NKG2D) in all tissues analysed. **(E)** Representative histograms and MFI of expression of NCR (NKp46 and NKp30) in all tissues analysed. **(F)** Representative histograms and MFI of expression of DNAM-1, 2B4 and CD57 receptors in all tissues analysed. Bars indicate median and error bars interquartile range; Line over bars indicates significant differences; The mean rank of 1st trimester decidua (6 – 12 weeks), term decidua basalis (> 37 weeks) and term decidua parietalis (> 37 weeks) were compared with the mean rank of the blood control group; Kruskal-Wallis test with Dunn's multiple comparison post-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

4.4 KIRs expression is lower in term NK cells

To identify *KIR2DL1, KIR2DL2* and *KIR2DS1* gene carriers, the flow cytometry strategy shown in Fig. 11A was used. Three different NK subpopulations were discriminated based on KIR2DL1 and KIR2DS1 staining: L1+S1-, L1-S1+ and L1+S1+ (Fig. 11A). The percentages of KIR2DL1+ and KIR2DL2+ NK cells were higher in 1st trimester decidua (6 – 12 weeks) samples than in the other tissues confirming previous studies (Xiong *et al* and Crespo *et al*) (Fig. 11B). Interestingly, decidua basalis and decidua parietalis at term pregnancy had significantly reduced percentages of KIR+ dNK cells compared to 1st trimester. Term dNK showed the lowest percentage of KIR2DL1 and KIR2DS1 expression in all three combinations studied (L1+S1-, L1-S1+ and L1+S1+) (Fig. 11B).

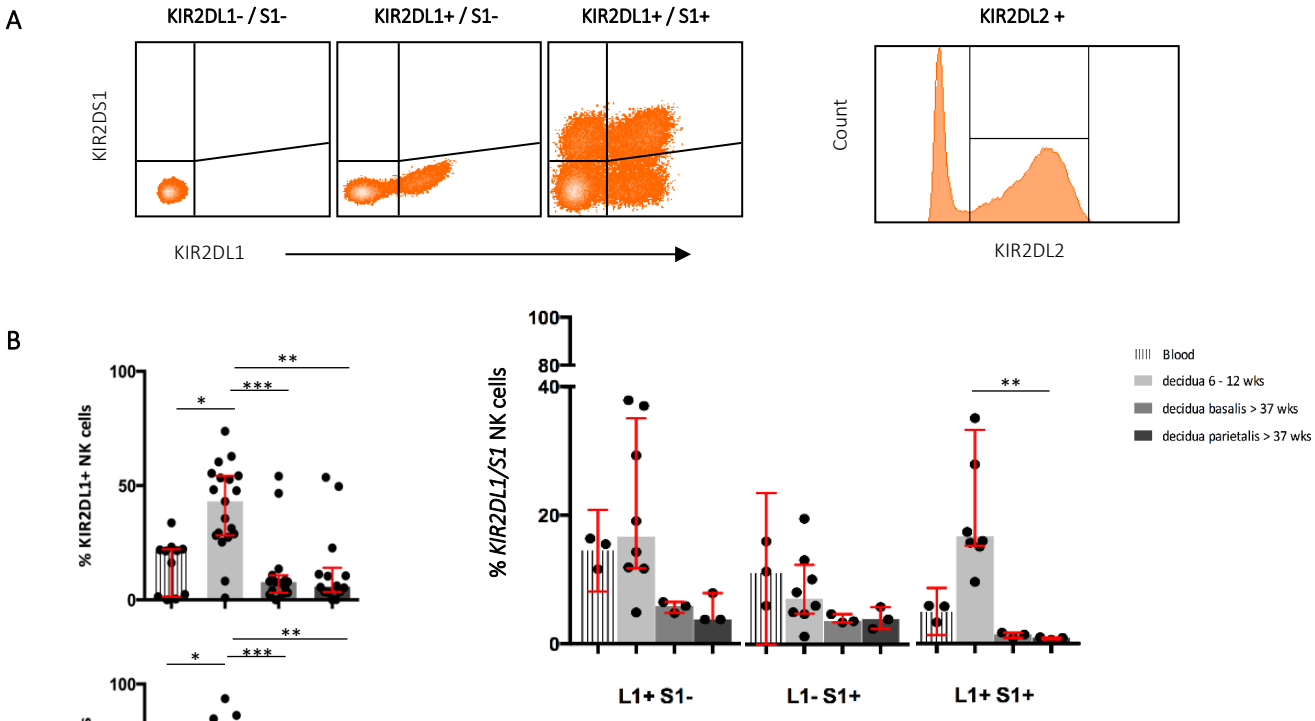

Figure 11. KIRs expression differs between 1st trimester dNK, term dNK and pNK cells. **(A)** Representative contour and histogram plots showing KIR2DL1, KIR2DS1 and KIR2DL2 expression in *KIR2DL1-/S1-*, *KIR2DL1+/S1-*, *KIR2DL1+/S1+* and *KIR2DL2+* individuals. **(B)** Percentage of KIR positive NK cells in all tissues analysed, highlighting of the different subpopulations of *KIR2DL1/S1*. Bars indicate median and error bars interquartile range; Line over bars indicates significant differences; Kruskal-Wallis test with Dunn's multiple comparison post-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

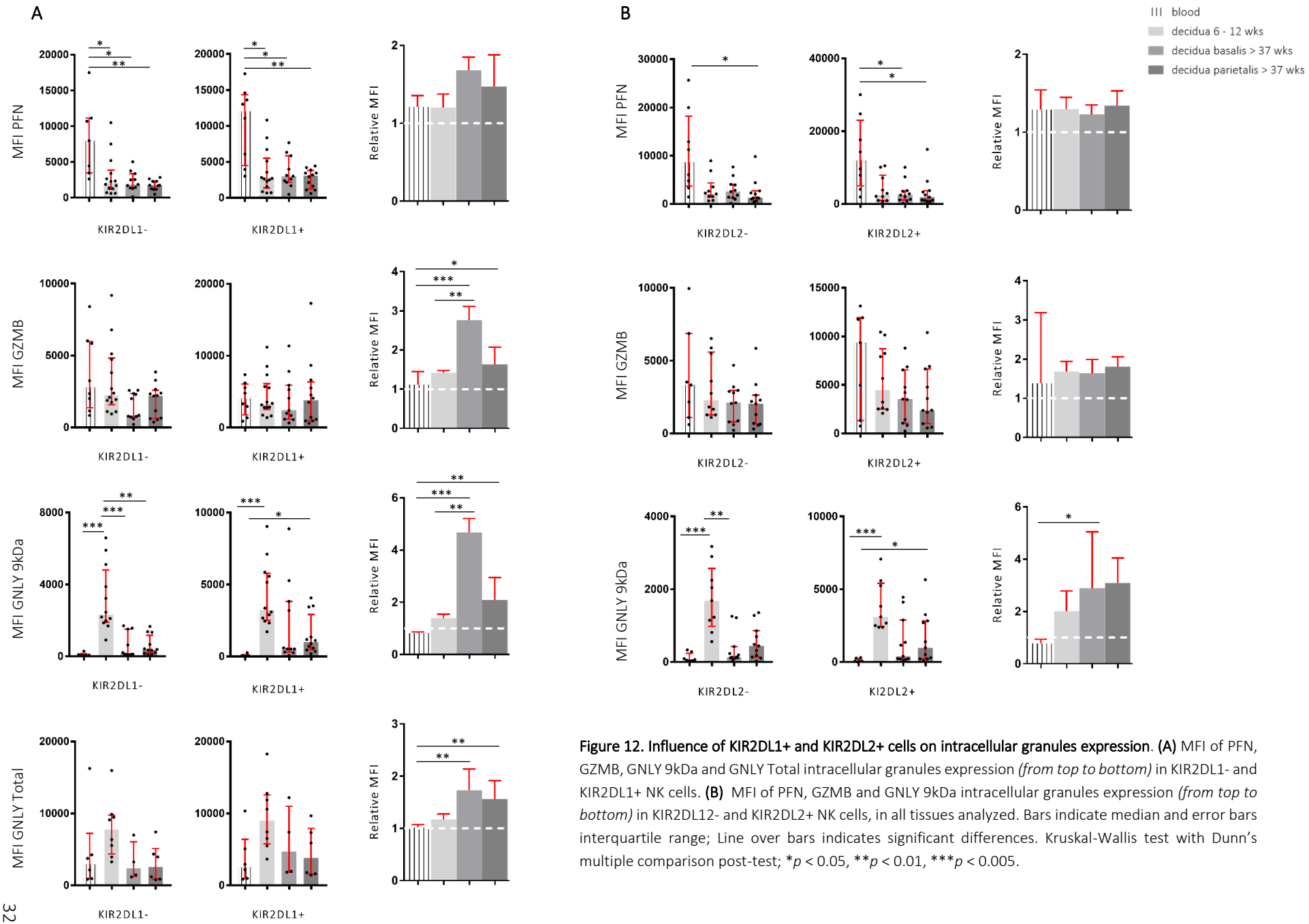

Figure 12. Influence of KIR2DL1+ and KIR2DL2+ cells on intracellular granules expression. **(A)** MFI of PFN, GZMB, GNLY 9kDa and GNLY Total intracellular granules expression *(from top to bottom)* in KIR2DL1- and KIR2DL1+ NK cells. **(B)** MFI of PFN, GZMB and GNLY 9kDa intracellular granules expression *(from top to bottom)* in KIR2DL12- and KIR2DL2+ NK cells, in all tissues analyzed. Bars indicate median and error bars interquartile range; Line over bars indicates significant differences. Kruskal-Wallis test with Dunn's multiple comparison post-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

4.5 KIR expression correlates with increased intracellular granule expression

Previous studies demonstrated that expression of the activating receptor KIR2DS1 correlates with significantly increased granule content of 1st trimester dNK cells. To evaluate this phenomenon in term dNK, all dNK and pNK were stained for KIR2DL1, KIR2DL2 and KIR2DS1 in combination with PFN, GZMB, GNLY Total and GNLY 9kDa.

In *KIR2DL1* and *KIR2DL2* gene carriers, two populations for each receptor were determined: KIR2DL1- and KIR2DL1+, KIR2DL2- and KIR2DL2+, respectively. The relative MFI was calculated by dividing the granule MFI of the KIR+ subset by the granule MFI of the KIR- subset to establish the influence of the KIR expression in granule expression.

All intracellular granules studied – PFN, GZMB, GNLY Total and GNLY 9kDa – were more expressed in KIR2DL1+ than KIR2DL1- NK cells, particularly in the term decidua basalis tissue. Exceptions were the expression of GNLY 9kDa and GNLY Total in pNK that were not significantly different between KIR2DL1+ and KIR2DL1- NK cells. Significant differences in relative MFI were noticed while analysing GZMB, GNLY 9kDa and GNLY Total (Fig. 12A).

KIR2DL2 did not impact PFN and GZMB expression levels, but relative GNLY 9kDa MFI levels were higher in KIR2DL2+ NK cells of decidual tissues, mainly at term pregnancy (Fig. 12B) GNLY Total was not assessed.

In *KIR2DL1* and *KIR2DS1* gene carriers, four NK populations were identified: double-negatives (L1-S1-), KIR2DL1 single-positive (L1+S1-), KIR2DS1 single-positive (L1-S1+) and double-positives (L1+S1+), based on the flow cytometry strategy depicted in Fig. 11A. Expression of both receptors in the same cell (L1+S1+) was associated with enhanced granule expression in pNK, dNK from 1st trimester decidua and term decidua (decidua basalis combined with decidua parietalis, Fig. 13), with exception of GNLY Total and GNLY 9kDa which are unaltered and decreased, respectively, in pNK (Fig. 13A). The L1+S1+ NK cells from term deciduas had the highest enhancement of granule expression among the tissues studied (Fig. 13C), with significant on the expression of all granules compared to the L1-S1- population.

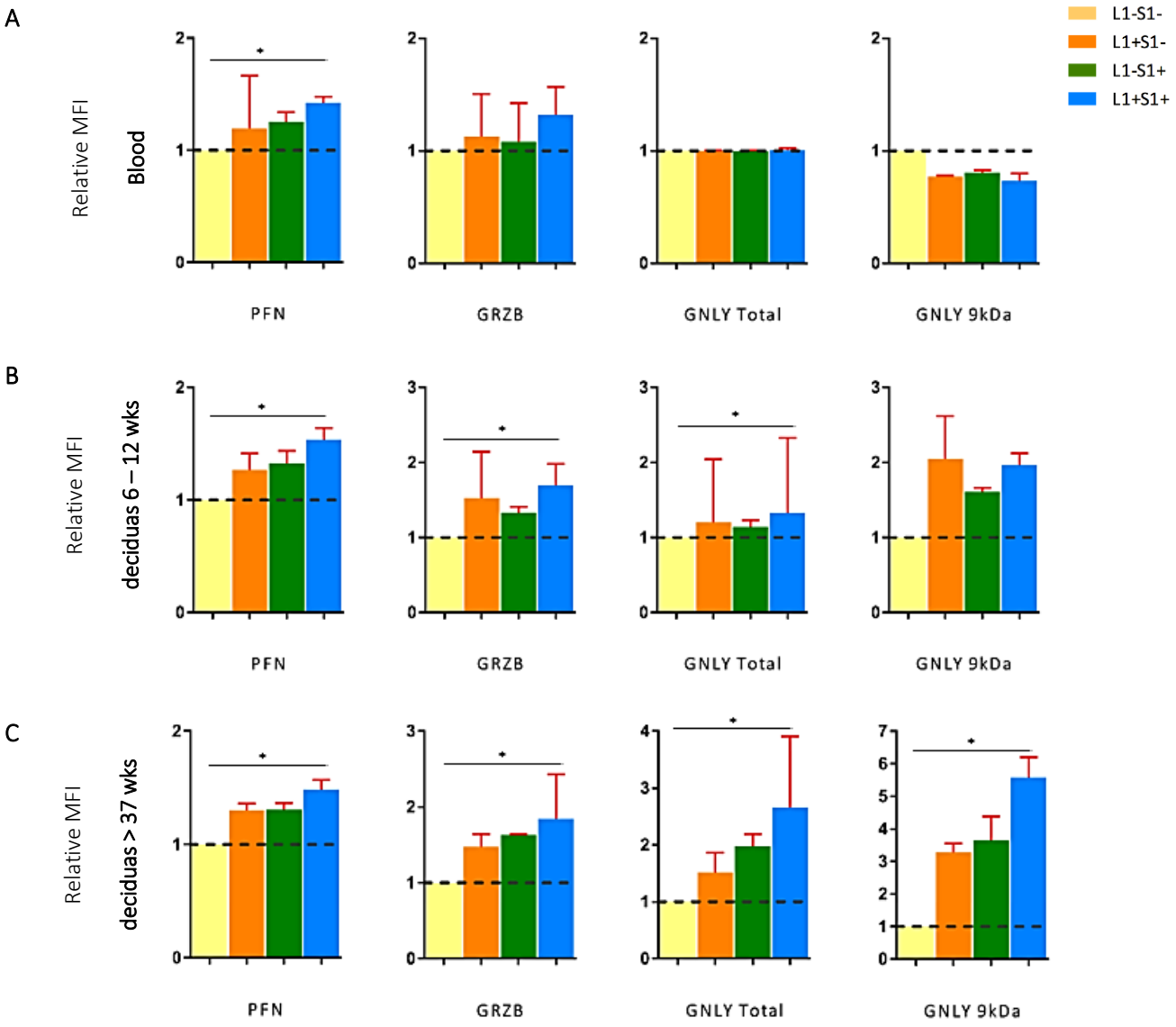

Figure 13. KIR2DL1+/S1+ expression correlates with intracellular granule expression. Relative expression of cytolytic granules in the four L1+S1-, L1-S1+ and L1+S1+ subsets of NK cells from **(A)** Blood (n = 3), **(B)** deciduas 6 – 12 weeks (n = 7) and **(C)** deciduas > 37 weeks (decidua basalis and decidua parietalis, n = 4) compared to the L1-S1- subset. The expression level was depicted as the relative MFI compared with the L1-S1- subset, in all tissues analyzed. Bars indicate median and error bars interquartile range; Line over bars indicates significant differences; Kruskal-Wallis test with Dunn's multiple comparison post-test; The mean rank of L1+S1-, L1-S1+ and L1+S1+ were compared with the mean rank of L1-S1- group. *$p < 0.05$.

4.6 Term pregnancy dNK degranulation capacity differs from 1st trimester dNK

pNK and dNK from all tissues were co-cultured with PMA/Ionomycin and/or K562 and 721.221 target cell lines in the presence of anti-CD107a antibody to measure degranulation.

Representative dot plots of CD107a staining on dNK cultures with PMA/I, K562 and 721.221 are shown (Fig. 14A).

When unstimulated, decidua basalis and decidua parietalis NK cells showed no CD107a dependent degranulation, while pNK and 1st trimester dNK had some background (up to ~18 %). PMA/I induced high levels of degranulation in pNK while 1st trimester dNK degranulation levels in response to the same stimulus was significantly reduced. Interestingly, dNK from decidua basalis and decidua parietalis had similar degranulation levels to pNK, confirming their ability to elicit an immune response and degranulate upon stimulation. When in contact with the target K562 cell line, degranulation of term pregnancy dNK was significantly lower, in comparison with pNK and dNK in 1st trimester. During co-culture with 721.221 cells degranulation levels of pNK and dNK were similar (Fig. 14B). However, the number of decidua basalis samples co-cultured with 721.221 was low and the absence of decidua parietalis samples contribute to a limited interpretation of these results.

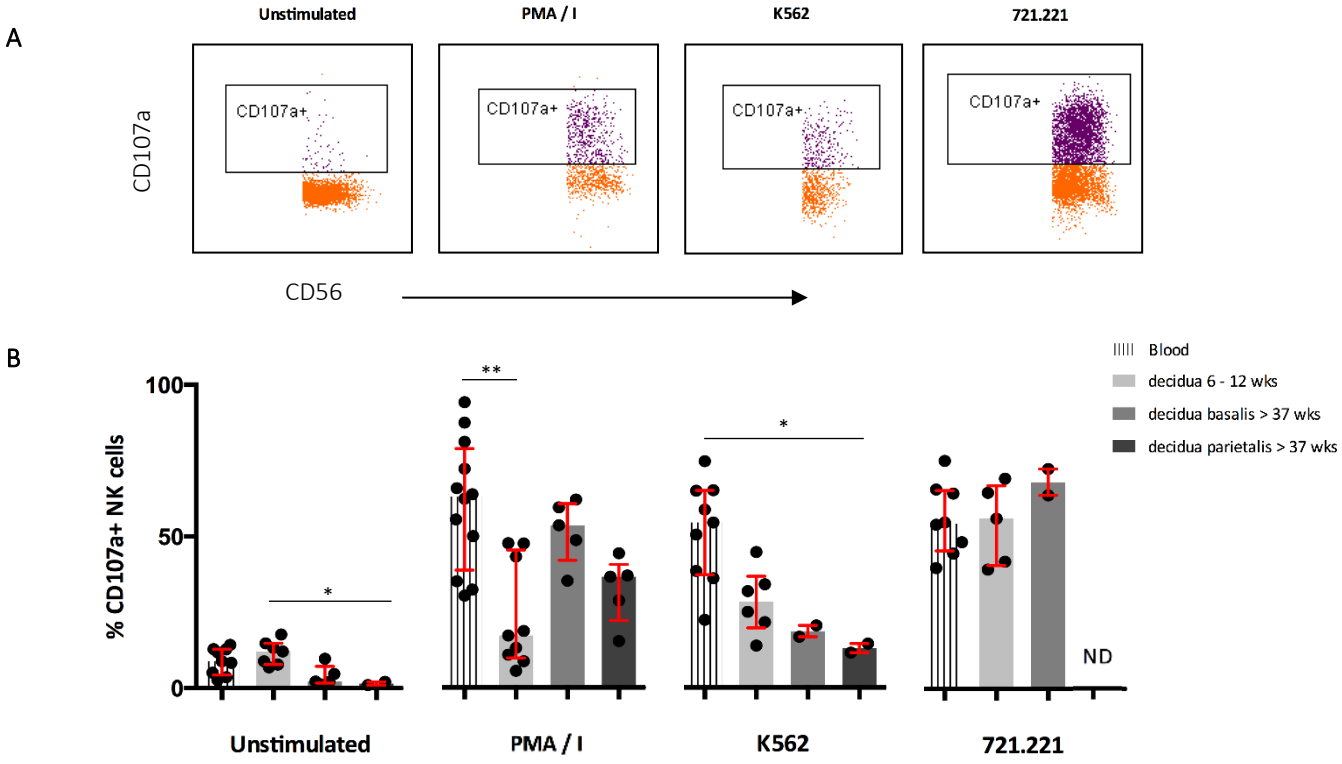

Figure 14. CD107a degranulation of term dNK is different from pNK and 1st trimester dNK cells. (A) Representative dot plots of CD107a expression in + NK cells after various stimuli. **(B)** Percentage of CD107a degranulation in NK cells of all tissues analysed after each stimuli. Four conditions were addressed: unstimulated dNK, dNK upon stimulation with 2.5µg/ml PMA and 1µg/ml Ionomycin, dNK co-cultured with target cell line K562 and 721.221 (E:T ratio 1:3). No decidua parietalis tissue sample was co-cultured with the 721.221 target cell line. Bars indicate median and error bars interquartile range; Line over bars indicates significant differences; Kruskal-Wallis test with Dunn's multiple comparison post-test; *$p < 0.05$, **$p < 0.01$.

DISCUSSION

While plenty of studies were performed using primary dNK from first trimester decidua, no major study has been published on the phenotypic and functional characterization of dNK from the last trimester of pregnancy. In fact, just three studies were published regarding the topic "term dNK cells + human pregnancy", after 2014! [138 – 141]

The multiple functions of dNK are crucial for the success of a healthy pregnancy, and the interaction of maternal NK receptors and fetal ligands is important to maintain fetal tolerance. While the role of dNK and its activation has been well explored at the start of pregnancy, once placentation is well established in the 3rd trimester, the role of dNK is not clear.

In this thesis, term pregnancy dNK phenotype and function were compared to pNK and dNK cells from 1st trimester (gestational age 6 - 12 weeks). Moreover, term pregnancy dNK from two sites of the placenta - decidua basalis and decidua parietalis - were investigated. The results were surprising: dNK cells from term pregnancy decidua basalis and decidua decidua parietalis have many differences in expression of cell surface NK receptors profile, granule content and ability to degranulate from 1st trimester dNK.

In contrast to reports suggesting that dNK completely disappear toward the end of pregnancy, we demonstrate that by the 3rd trimester of a pregnancy, the overall percentage of CD56+ dNK cells decreases to a maximum of 40% in term decidua basalis and decidua parietalis samples. An inversion on the frequency of cell populations from 1st trimesters occurs, with T cells being more frequent at term. Key questions arising are whether the decreased dNK population is responsible for the fetal membrane rupture necessary for labor, and if dNK at term pregnancy contributes to the clearance of infections?

The dNK receptor profile in decidua basalis and decidua parietalis tissues were shown to be quite different from the pNK and dNK 1st trimester.

In order to maintain a healthy pregnancy, there is an expectation that activating receptors known to trigger cytotoxic immune responses need to be "tuned down". This expectation was confirmed, since the expression of some of the activation receptors was lower in term decidual NK samples (such as NKp30 and DNAM-1 receptors). It would be interesting to explore if this is this a dynamic change throughout the pregnancy, with activating receptor expression decreasing and inhibitory receptors increasing from the 1st trimester to term.

In this thesis, KIRs were also studied, with special attention to the KIR2DS1 receptor which is suggested to enhance placentation [108].

The KIR results were intriguing: inhibitory KIR2DL1 and KIR2DL2 were shown to be present in a lower frequency of NK cells on term decidual samples. This tendency was also observed on KIR2DL1 and KIR2DS1 combinations, mainly KIR2DL1+/S1+ dNK cells whose percentage is significantly lower when compared with 1st trimester ones. These results do not support the hypothesis that inhibitory receptors, even from different gene families, would be more expressed on term. Thus, all receptors, activating or inhibitory, seem to be lower expressed in term tissues. The cytolytic ability of term cells is also downregulated, a fact observed in Figure 14 of this thesis. This can interpreted as a preventive measure to avoid excessive or aberrant production of proinflammatory cytokines or direct cytotoxicity against fetal tissues, which are harmful to a successful pregnancy.

However, the basic function of immune cells, such as dNK, is still to react to foreign molecules, ensuring the clearance of pathogens and to spare the host from the side effects of infections. Some of the receptors studied in this thesis are directly linked to bacterial and viral clearance such as KIR2DS1 and NKG2C. Previously observations that 1st trimester dNK (6 - 12 weeks) express higher levels of KIR2DL1 and S1, compared to pNK, were confirmed [100, 108, 115]. This observation is also valid for term dNK cells from decidua basalis and decidua parietalis.

The expression of cytotoxic granules was correlated to the KIR combination expressed in dNK cells. Although intracellular granules were expressed in both KIR- and KIR+ dNK cells, the intracellular granule expression in KIR+ dNK was enhanced when compared with the negative counterpart. This difference is more noticeable while analysing GNLY Total and GNLY 9kDa expression. For instance, KIR2DL1+ expression has the highest impact in granule expression on

decidua basalis dNK, with a 4- fold increase in GNLY 9kDa expression. Since decidua basalis is the mucosal lining of the uterine wall where the trophoblasts adhere, the immune system in this layer is in tight contact with the fetal tissues. The higher reactivity of KIR2DL1+ dNK cells at this site can be explained by the location of this tissue and the direct maternal-fetal interaction – KIR2DL1 interaction with its HLA-C ligand may stimulate the production of granules. Moreover, in all KIR/granules expression analysis performed, GNLY 9kDa revealed to be the granule most positively impacted by KIR+ cell subsets, suggesting it may be crutial for pregnancy homeostasis.

When analysing *KIR2DL1/KIR2DS1* gene carriers the results were similar: KIR2DL1+/S1+ term dNK cells have higher granule expression than any other repertoire combination.

Additional studies evaluating the capacity of term pregnancy dNK to degranulate were performed. dNK cells from decidua basalis and decidua parietalis were shown to have a significantly increased ability to degranulate upon stimulation with PMA/I compared to 1st trimester dNK. PMA/I induce degranulation, independently of receptor engagement, reflecting the total degranulation capacity of dNK cells. Interestingly, in contact with target MHC Class I negative cells, their degranulation ability was decreased compared to 1st trimester, especially when co-cultured with K562. The type of stimulation, using target cells, depends on how many inhibitory KIRs or activating receptors are expressed by the human dNK samples, and therefore elicits a higher variability of responses.

While the results of dNK co-cultured with K562 are debatable, mainly due to the small sample size, the results with PMA/I stimulation lead to an interesting rationale: term dNK, having an enhanced ability to degranulate, compared with dNK 1st trimester, can be involved in the labor induction through immunological fetal "rejection". Alternatively, term dNK could provide immunity to infections at the maternal fetal-interface. Further experiments to determine if term dNK can degranulate to virus-infected cells (e.g. HCMV and/or Zika) should be carried out to address this question.

For the pregnant woman to carry on a pregnancy for nine months, her immune system has to have reached a local hyporesponsive state with inhibition of the cytolytic granule release, which, in other circumstances, could lead to miscarriages or pregnancy maladies. However, at term, the maternal-fetal tolerance is potentially broken. Labor is an inflammatory process that includes secretion of cytokine by the resident and infiltrated immune cells into the uterine microenvironment [143].

Many studies in humans and mice have reported the presence of pro-inflammatory immune cells in the reproductive tissues involved in the activation of parturition [144-150], NK cells

included. A possible pathway for the induction of labor was presented by Gomez-Lopez *et al* (2014).

dNK is truly intriguing and its specific role on term pregnancy is an interesting problem for further exploration. In addition, other important roles of dNK in anti-viral and bacterial defense mechanisms were recently described by our group for 1st trimester dNK and should be expanded for term pregnancy dNK.

CONCLUSIONS

The aims proposed at the beginning of this project were achieved during the nine months of work that culminated in this thesis. The main conclusions are the following:

For the phenotypic analysis:

1) The percentage of CD56+ CD3/CD14- NK cells, in term decidua basalis and decidua parietalis tissue, is lower when compared with 1st trimester;
2) The expression of cytolytic granules – PFN, GZMB and GNLY (including the 9kDa isoform) decreases in dNK from term pregnancy decidua when compared with dNK from 1st trimester;
3) The expression of Killer immunoglobulin-like receptors (KIRs) studied – KIR2DL1, KIR2DL2 and KIR2DL1/S1 decreases in dNK from term pregnancy decidua when compared with dNK from 1st trimester;
4) The expression of KIR2DL1, KIR2DL2 and KIR2DS1 positively impacts the expression of cytolytic granules in dNK off all tissues.

For the functional analysis:

1) Term pregnancy dNK, from decidua basalis and decidua parietalis, revealed a lower capacity to degranulate in response to MHC negative targets cells (K562 cell line) when compared with dNK from 1st trimester, but higher capacity to degranulate after stimulation by PMA/I.

FUTURE PERSPECTIVES

The development of an integrated picture of the overall events occurring at the maternal-fetal interface during pregnancy is the ultimate goal to benefit parenthood and child development worldwide.

More phenotypic analyses should be performed to increase sample size and statistical relevance, especially the sample size of decidua basalis and decidua parietalis. Furthermore, additional phenotypic markers like CD16 should be investigated. CD16 surface expression is associated with NK cell activation, which eliminate target cells through direct killing (cytotoxic granule release) and cytokine production. Interestingly, with immune activation, CD16 expression decreases [151]. This surface marker is present in almost 90% of CD56low+ pNK cells but rare in 1st trimester dNK (6 -12 weeks) samples. By investigating its expression on dNK > 37 weeks samples we can reach stronger conclusions regarding cytoxicity in term tissues.

The mechanisms through which term dNK (> 37 weeks) cells act are still largely unknown. Infections in the third trimester are responsible for preterm birth and can be transmitted to the new-borns during labor, with consequences to their mortality/morbidity. Through degranulation assays of dNKs from decidua basalis and decidua parietalis we can assess their antiviral response when contact with infectious agents, like HCMV or Zika. Additional functional analysis should be performed on cytokine secretion to determine their role in placental remodelling (GM-CSF, VEGF, IL-8) and antiviral responses (IFN-γ).

Lastly, the analysis of term dNK from pregnancy pathology is also important. This will allow us to determine if dNK has the ability to remodel placental tissue, if anti-viral responses are impaired, and, by direct association, if anti-fetal/placental allo-responses are increased.

REFERENCES

1 Orenstein W.A, *et al*. (2005). Immunizations in the United States: success, structure, and stress. *Health Affair;* 24(3): 599-610.

2 Goldsby RA, *et al*. (2000). Immunology, 4th Edition. W.H. Freeman and Company, New York.

3 Winslow T. (2008). The genetics of cancer. NIH: National Cancer Institute, Visuals Online.

4 Herberman RB, Nunn ME, Lavrin DH. (1975). Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors: distribution of reactivity and specificity. *Int J Cancer;* 16(2): 216-229.

5 Kiessling R, Klein E, Wigzell H. (1975). "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol*; 5(2): 112-117.

6 Goldsby RA, *et al*. (2003). Immunology, 5th Edition. W.H. Freeman and Company, New York.

7 Bogunia-Kubik K, Iwaszko M. (2011). Clinical Significance of the HLA-E and CD94 / NKG2 Interaction. *Arch. Immunol. Ther*. *Exp*; 59: 353-367.

8 Amiot L., N. Vu, and M. Samson. (2014). Immunomodulatory Properties of HLA-G in Infectious Diseases. *J. Immunol. Res*; 2014: 298569.

9 Petroff, MG. (2005). Immune interactions at the maternal–fetal interface. *J Reprod Immunol;* 68: 1-13.

10 Tomasec P, Braud VM. (2000). Surface Expression of HLA-E, an Inhibitor of Natural Killer Cells, Enhanced by Human Cytomegalovirus gpUL40. *Science;* 287(5455): 1031-1033.

11 Rajagopalan S, *et al*. (2014). HLA-G-mediated NK cell senescence promotes vascular remodeling: implications for reproduction. *Cell Mol Immunol*; 11(5): 460-466.

12 Wilfredo F. (2016). Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1. *Nature Immunology;* 17: 1067-1074.

13 Rankin L, *et al*. (2013). Diversity, function, and transcriptional regulation of gut innate lymphocytes. *Front Immunol*; 4: 22.

14 Artis D, Spits H. (2015). The biology of innate lymphoid cells. *Nature*; 517: 293-301.

15 G. Trinchieri, *et al*. (1986). Biology of natural killer cells. *Adv Immunol.*; 47: 187-376.

16 Faridi RM, Agrawal S. (2011). Killer immunoglobulin-like receptors (KIRs) and HLA-C allorecognition patterns implicative of dominant activation of natural killer cells contribute to recurrent miscarriages. *Human Reproduction;* 26(2): 491-497.

17 Bellora F, *et al*. (2014). Human NK cells and NK receptors. *Immunol. Lett;* 161: 168-173.

18 Vanherberghen B, *et al*. (2013). Classification of human natural killer cells based on migration behavior and cytotoxic response. *Blood*; 121(8): 1326-1334.

19 Littwitz-Saloman, *et al*. (2016). Insufficient natural killer cell responses against retroviruses: how to improve NK cell killing of retrovirus-infected cells. *Retrovirology;* 13(1): 77.

20 Leavy, O. (2012). Maturation and function of NK cells. *Nat. Rev. Immunol*; 12: 150.

21 Moretta, L., *et al*. (2014). Human Natural Killer Cells: Origin, Receptors, Function, and Clinical Applications. *Int. Arch. Allergy Immunol;* 164: 253-264.

22 Blish C. (2016). Natural killer cell diversity in viral Infection: why and how much? *Pathog Immun*; 1(1): 165-192.

23 Poli A, Michel T. (2009). CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology;* 126: 458-465.

24 Vivier, E. (2006). What is natural in natural killer cells? *Immunol. Lett;* 107: 1-7.

25 Walzer T, Bléry M, *et al.* (2007). Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proceedings of the National Academy of Sciences.* 104(9): 3384-3389.

26 Horowitz A, *et al*. (2013). Genetic and Environmental determinants of human NK cell Diversity Revealed by Mass Cytometry. *Sci. Transl. Med;* 5: 208.

27 Biassoni R. (2009). Human natural killer receptors, co-receptors, and their ligands. *Curr. Protoc. Immunol;* Chapter 14 Unit 14.10.

28 Long EO, Kim HS *et al*. (2013). Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol;* 31: 227-58.

29 Fu B, Tian Z *et al*. (2014). Substes of human natural killer cells and their regulatory effects. *Immunology;* 141: 483-489.

30 Hanna J, *et al*. (2006). Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat. Med;* 12: 1065-1074.

31 Koopman LA, *et al.* (2003). Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J. Exp. Med*; 198(8): 1201-1212.

32 Fu B, Wei H. (2016). Decidual natural killer cells and the immune microenvironment at the maternal-fetal interface. *Sci China Life Sci;* 59(12): 1224-1231.

33 MacFarlane AW 4th, Campbell KS. (2006). Signal transduction in natural killer cells. *Curr Top Microbiol Immunol;* 298: 23-57.

34 Lanier LL. (2008). Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol*; 9: 495-502.

35 Vivier E, *et al*. (2008). Functions of natural killer cells. *Nat Immunol*; 9: 503-510.

36 Crespo A. (2015). Characterization of KIR2DS1+ decidual natural killer cells in healthy and viral/bacterial – infected human pregnancy. Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

37 Barten R, *et al*. (2001). Divergent and convergent evolution of NK-cell receptors. *Trends Immunol;* 22: 52-57.

38 Billadeau D, Leibson P. (2002). ITAMs versus ITIMs: striking a balance during cell regulation. *J Clin. Invest*; 109: 161-168.

39 Middleton D, Gonzelez F. (2010). The extensive polymorphisms of KIR genes. *Immunology;* 129: 8-19.

40 Colonna M, Samaridis J. (1995) Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science;* 268: 405-408.

41 Wagtmann N, *et al*. (1995). Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra and intracellular domains. *Immunity*; 2: 439-449.

42 Gardiner CM. (2008). Killer cell immunoglobulin-like receptors on NK cells: the how, where and why. *Int J Immunogenet;* 35: 1-8.

43 Chan HW, *et al*. (2003). DNA methylation maintains allele-specific KIR gene expression in human natural killer cells. *J Exp Med*; 197: 245-255.

44 Yawata M, *et al*. (2006) Roles for HLA and KIR polymorphism in natural killer cell repertoire selection and modulation of effector function. *J Exp Med*; 203: 633-645.

45 Andersson S, *et al*. (2009). KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression. *Blood*; 114: 95-104.

46 Pegram HJ, *et al*. (2011). Review: activating and inhibitory receptors of natural killer cells. Immunol. *Cell Biol*; 89: 216-224.

47 Alari-pahissa E, et al. (2014). Inhibitory receptor-mediated regulation of natural killer cells. *Crit Rev. Immunol*; 34: 455-465.

48 Chiesa MD, *et al*. (2015). Activating KIRs and NKG2C in viral infections: towards NK cell memory? *Front Immunol*; 6: 573.

49 Fauriat C, *et al*. (2010). Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood*; 115(6): 1166-1174.

50 Ivarsson MA, *et al*. (2014). Activating killer cell Ig-like receptors inn healthy and disease. *Front Immunol*; 5: 184.

51 Nakimuli A, *et al*. (2015). A KIR B centromeric region present in Africans but not Europeans protects pregnant women from pre-eclampsia. *Proc Natl Acad Sci USA*; 112(3): 845-850.

52 McQueen KL, Parham P. (2002). Variable receptors controlling activation and inhibition of NK cells. *Curr Opin Immunol*; 14: 615-621.

53 Pazina T, *et al*. (2017). Regulation of the functions of natural cytotoxicity receptors by interactions with diverse ligands and alterations in splice variant expression. *Front Immunol*; 8: 369.

54 Pessino A, *et al*. (1998). Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med*; 188: 953-960.

55 Pende D, *et al*. (1999). Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J Exp Med*; 190: 1505-1516.

56 Jarahian M, *et al*. (2009). Activation of natural killer cells by Newcastle disease virus hemagglutinin-neuraminidase. *J Virol*; 83: 8108-8121.

57 Arnon TI, *et al*. (2005). Inhibition of the NKp30activating receptor by pp65 of human cytomegalovirus. *Nat Immunol*; 6: 515-523.

58 Augugliaro R, *et al*. (2003). Selective cross-talk among natural cytotoxicity receptors in human natural killer cells. *Eur J Immunol*; 33: 1235-1241.

59 El Costa H. *et al.* (2008). Critical and differential roles of NKp46- and NKp30-activating receptors expressed by uterine NK cells in early pregnancy. *J. Immunol*; 181: 3009-3017.

60 Siewiera J. *et al.* (2013). Human cytomegalovirus infection elicits new decidual natural killer cell effector functions. *PLoS Pathog*; 9: e100325.

61 Siewiera J. *et al.* (2015). Natural cytotoxicity receptor splice variants orchestrate the distinct function of human natural killer cell subtypes. *Nature Communication*; 6: 10183.

62 Shemesh A, *et al*. (2015). First trimester pregnancy loss and the expression of alternatively spliced NKp30 isoforms in maternal blood and placental tissue. *Front Immunol*; **6:** 189.

63 Bottino C, *et al*. (2015). Cellular ligands of activating NK receptors. Trends Immunol; **26:** 221-226.

64 Akira S, *et al*. (2006). Pathogen recognition and innate immunity. *Cell*; **124:** 783-801.

65 Chaplin DD. (2010). Overview of the immune response. *J Allergy Clin. Immunol*; **140:** S3-S23.

66 Goyal S, *et al*. (2016). C-type lectin receptors in tuberculosis. What we know. *Med Microbiol Immunol*; **205:** 513-535.

67 Zelensky AN, Gready JE. (2005). The C-type lectin-like domain superfamily. *FEBS J*; **272(24):** 6179-6217.

68 Drickamer K. (1999). C-type lectin-like domains. *Curr Opin Struct Biol*; **9(5):** 585-590.

69 Sancho D, Reis e Sousa C. (2012). Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu Rev Immunol*; **30:** 491-529.

70 Bauer S, *et al*. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science*; **285:** 727-729.

71 Steinie A, *et al*. (2008). Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse REA-1 protein family. *Immunogenetics*; **53:** 279-287.

72 Cosman D, *et al*. (2001). ULBPs, novel MHC class-I related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity*; **14:** 123-133.

73 Zompi S, *et al*. (2003). NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases. *Nat Immunol*; **4:** 565-572.

74 Billadeau DD, *et al*. (2003). NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol*; **4:** 557-564.

75 Upshaw JL, Leibson PJ. (2006). NKG2D-mediated activation of cytotoxic lymphocytes: unique signalling pathways and distinct functional outcomes. *Semin. Immunol*; **18:** 167-175.

76 Guerra N, *et al*. (2008). NKG2D-deficient mice are defective in tumour surveillance in models of spontaneous malignancy. *Immunity*; **28:** 571-580.

77 Borrego F, *et al*. (1998). Recognition off human histocompatibility leukocyte antigen (HLA) – E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J Exp Med*; **187:** 813-818.

78 Borrego F, *et al*. (2006). The CD94/NKG2 family of receptors. *Immunol Res*; **35:** 263-277.

79 Braud VM, *et al*. (1998). HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*; **391:** 795-799.

80 Bachmayer N, *et al*. (2009). Women with pre-eclampsia have an altered NKG2A and NKG2C receptor expression on peripheral blood natural killer cells. *Am J Reprod Immunol*; **62(3):** 147-57.

81 Shibuya A, *et al*. (1996). DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity*; **4:** 573-581.

82 Bottico C, *et al*. (2003). Identification of PVR (CD155) and Nectn-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med*; **198:** 557-567.

83 Tabara-Hanaoka S, *et al*. (2004). Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and necin-2 (PRR-2/CD112). *Int. Immunol*; **16:** 533-538.

84 Gilfillan S, *et al*. (2008). DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *J Exp Med*; **205:** 2965-2973.

85 Reymond N, *et al*. (2004). DNAM-1 and PVR regulate monocyte migration through endothelial junctions. *J Exp Med*; **199:** 1331-1341.

86 Tassi I, *et al*. (2006). Dissecting natural killer cell activation pathways through analysis of genetic mutations in human and mouse. *Immunol Rev*; **175:** 1558-1565.

87 Brown MH, *et al*. (1998). 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med*; **188:** 2083-2090.

88 Latchman Y, *et al*. (1998). Identification of the 2B4 molecule as a counter-receptor for CD48. *J Immunol*; **161:** 5809-5812.

89 Vaidya SV, *et al*. (2005). Targeted disruption of the 2B4 gene in mice reveals an in vivo role of 2B4 (CD224) in the rejection of B16 melanoma cells. *J Immunol*; **174:** 800-807.

90 Mor G. (2006). Immunology of pregnancy: Medical Intelligence Unit. Springer Science & Bussiness of Media.

91 Billington, WD. (2003). The immunological problem of pregnancy: 50 years with the hope of progress. A tribute to Peter Medawar. *J. Reprod. Immunol*; **60:** 1-11.

92 Cartright JE, *et al*. (2017). The role of decidual NK cells in pregnancies with the impaired vascular remodeling. *J. Reprod. Immunol*; **119:** 81-84.

93 Moffett A, Loke C. (2006). Immunology of placentation in eutherian mammals. *Nat. Rev. Immunol*; **6:** 584-594.

94 Tarrade A, *et al*. (2001). PPARgamma/RXNalpha heterodimers control human trophoblast invasion. *J Clin Endocrinol Metab*; **86:** 5017-5024.

95 Miller-Keane Encyclopedia and Dictionary of Medicine, Nursing, and Allied Health, Seventh Edition. © 2003 by Saunders, an imprint of Elsevier, Inc.

96 Mescher AL. Junqueira's basic histology: text and atlas, 12th edition. The McGraw-Hill Companies.

97 Guleria I, Sayegh MH. (2007). Maternal acceptance of the fetus: true human tolerance. *J. Immunol*; **178:** 3345-3351.

98 Shorter SC, *et al*. (1993). Antigenic heterogeneity of human cytotrophoblast and evidence for the transient expression of MHC class I antigens distinct from HLA-G. *Placenta*; **14(5):** 571-582.

99 R, *et al*. (2008). A critical look at HLA-G. *Trends Immunol*; **29:** 313-321.

100 Male V, *et al*. (2011). The effect of pregnancy on the uterine NK cell KIR repertoire. *Eur. J. Immunol*; **41:** 3017-3027.

101 Hiby, SE, *et al*. (2008). Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriages. *Hum. Reprod*; **23:** 972-976.

102 Mammaro A, *et al*. (2009). Hypertensive disorders of pregnancy. *J. Prenat. Med*; **3:** 1-5.

103 Bramham K, *et al*. (2014). Chronic hypertension and pregnancy outcomes: systematic review and meta-analysis. *BMJ*; **348:** g2301.

104 Sattar N, Greexiongr IA. (2002). Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening? *Br. Med. J*; **325:** 157-160.

105 Pijnenborg R, *et al*. (2011) Deep placentation. *Best Pract. Res. Clin. Obstet. Gynaecol*; **25:** 273-285.

106 Davis EF, *et al*. (2012). Pre-eclampsia and offspring cardiovascular health: mechanism insights from experimental studies. *Clin. Sci*; **123:** 53-72.

107 Nigro G, *et al*. (2011). Role of the infections in recurrent spontaneous abortion. *J Matern Fetal Neonatal Med*; **24(8):** 983-989.

108 Xiong S, *et al*. (2013). Maternal uterine NK cell-activating receptor KIR2DS1 enhances placentation. *J Clin Invest*; **133(10):** 4264-4272.

109 Hiby SE, *et al*. (2014). Maternal KIR in combination with paternal HLA-C2 regulate human birth weight. *J Immunol*; **192(11):** 5069-5073.

110 Middleton D, *et al*. (2009). Killer immunoglobulin-like receptors (KIR2DL2 and/or KIR2DS2) in presence of their ligand (HLA-C1 group) protect against chronic myeloid leukemia. *Tissue Antigens*; **73:** 553-560.

111 Boyton RJ, *et al*. (2006). HLA-C and killer cell immunoglobulin-like receptor genes in idiopathic bronchiectasis. *Am J Respir Crit Care Med*; **173:** 327-333.

112 Jamil KM, Khakoo SI. (2011). KIR/HLA interactions and pathogen immunity. *J Biomed Biotechnol*; 2011: 298348.

113 Zambello R, *et al*. (2014). Activating KIRs in chronic lymphoproliferative disorder on NK cells: protection from viruses and disease induction? *Front Immunol*; **45:** 72.

114 Varla-Leftherioti M, *et al*. (2010). HLA-DQAI*0505 sharing and killer immunoglobulin-like receptors in sub fertile couples: report from the 15th International Histocompatibility Workshop. *Tissue Antigens*; **75:** 668-672.

115 Crespo A, *et al*. (2016). Expression of KIR2DS1 by decidual natural killer cells increases their ability to control placental HCMV infection. *PNAS*; **113(52):** 15072-15077.

116 Foley B, *et al*. (2008). KIR2DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C C2 negative individuals. *Int Immunol*; **20(4):** 555-563.

117 Gonzalez-Galarza FF, *et al*. (2011). Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. *Nucleic Acids Res*; 39: D913-D919.

118 Korner C, Altfeld M. (2012). Role of KIR3DS1 in human diseases. *Front Immunol*; 3: 326.

119 De Re V, *et al*. (2015). Genetic diversity of the KIR/HLA system and susceptibility to hepatitis C virus-related diseases. *PLoS One*; 10(2): e0117420.

120 Chazara O, *et al*. (2016). Maternal KIR and fetal HLA-C: a fine balance. *J Leuk Biol*; 90(4): 703-716.

121 Stern M, *et al*. (2008). The number of activating KIR genes inversely correlates with the rate of CMV infection/reactivation in kidney transplant recipients. *Am J Transplant*; 8(6): 1312-1317.

122 Zuniga J, *et al*. (2009). Protective KIR-HLA interactions for HCV infection in intravenous users. *Mol. Immunol*; 46: 2723-2727.

123 Fusco C, *et al.* (2010). KIRs and their HLA ligands in remitting-relapsing multiple sclerosis. *J Neuroimmunol*; 229: 232-237.

124 Luszczek W, *et al*. (2004). Gene for the activating natural killer cell receptor is associated with susceptibility to *Psoriasis vulgaris*. *Hum Immunol*; 65: 758-766.

125 Hiby SE, *et al*. (2010). Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest*; 120: 4102-4110.

126 Jiao Y, *et al*. (2016). Type 1 innate lymphoid cell biology: lessons learnt from natural killer cells. *Front Immunol*; 7: 426.

127 Redman C, Sargent I. (2004). Preeclampsia and the systemic inflammatory response. *Semin Nephrol*. 24: 565-70.

128 Moffett A, Colucci F. (2015). Co-evolution of NK receptors and HLA ligands in humans is driven by reproduction. *Immunol. Rev*; 267: 283-297.

129 Areia A, *et al*. (2015). Membrane progesterone receptors in human regulatory T cells: a reality in pregnancy. *BJOG*; 122: 1544-1550.

130 Pitabut N, et al. (2013). Potential function of granulysin, other related effector molecules and lymphocyte subsets in patients with TB and HIV/TB coinfection. *Int J Med Sci*.; 10(8): 1003-14.

131 Liu CC, *et al*. (1996). Lymphocyte-mediated cytolysis and disease. *N Engl J Med*; 335: 1651-1659.

132 Law R.H *et al*. (2010). The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature*; 468: 445-451.

133 Farlex Partner Medical Dictionary. © 2012 by McGraw-Hill Companies, Inc.

134 Afonina IS. (2010). Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. *Immunol. Rev;* 235(1): 105-16.

135 Grossman WJ, *et al*. (2003). The orphan granzymes of humans and mice. *Curr. Opin. Immunol;* 15: 544-552.

136 Bots M, Medema JP. (2006). Granzymes at a glance. *Journal of Cell Science*; 119: 5011-5014.

137 Trapani JA, Sutton VR. (2003). Granzyme B: pro-apoptotic, antiviral and antitumor functions. *Curr. Opin. Immunol;* 15: 533-543.

138 Waterhouse NJ, *et al*. (2006). Role of Bid-induced mitochondrial outer membrane permeabilization in granzyme B-induced apoptosis. *Immunol. Cell Biol*; 84: 72-78.

139 Sindram-Trujillo AP *et al*. (2004). Comparison of decidual leukocytes following spontaneous vaginal delivery and elective cesarean section in uncomplicated human term pregnancy. *J Reprod Immunol*; 62(1-2): 125-37.

140 Kwan M, *et al*. (2014). Dynamic changes in maternal decidual leukocyte populations from first to second trimester gestation. *Placenta*; 35(12): 1027-34.

141 Laban M, *et al*. (2014). Placenta accreta is associated with decreased decidual natural killer (dNK) cells population: a comparative pilot study. *Eur J Obstet Gynecol Reprod Biol*; 181: 284-8.

142 Sotnikova N, *et al*. (2014). Interaction of decidual CD56+ NK with trophoblast cells during normal pregnancy and recurrent spontaneous abortion at early term of gestation. *Scand J Immunol*.; 80(3): 198-208.

143 Gomez-Lopez N, *et al*. (2014). Immune cells in term and preterm labor. *Cellular and Molecular Immunol*; 11: 571-581.

144 Thomson AJ, *et al*. (1999). Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. *Hum Reprod*; 14: 229-236.

145 Osman I, *et al*. (2003). Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Mol Hum Reprod*; 9: 41-45.

146 Bokstrom H, *et al*. (1997). Leukocyte subpopulations in the human uterine cervical stroma at early and term pregnancy. *Hum Reprod*; 12: 586-590.

147 Payne KJ, *et al*. (2012). Residency and activation of myeloid cells during remodeling of the prepartum murine cervix. *Biol Reprod*; 87: 106.

148 Young A, *et al*. (2002). Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term. *Biol Reprod*; 66: 445-449.

149 Mackler AM, *et al*. (1999). Macrophage trafficking in the uterus and cervix precedes parturition in the mouse. *Biol Reprod*; 61: 879-883.

150 Hamilton S, *et al*. (2012). Macrophages infiltrate the human and rat decidua during term and preterm labor: evidence that decidual inflammation precedes labor. *Biol Reprod*; 86: 39.

151 Romee R, *et al*. (2013). NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood*; 121(18): 3599-608.

SUPPLEMENTARY MATERIAL

ANTIBODY EPITOPE	CLONE	FLUOROCHROME	MANUFACTURER
IgG1	MOPC-21	FITC	BioLegend®
IgG1	MOPC-21	PE	BioLegend®
IgG2b	MOPC-11	Pacific Blue	BioLegend®
IgG1	MOPC-21	PerCP-Cy5.5	BioLegend®
CD3	UCHT1	PerCP	BioLegend®
CD3	S4.1	PE-Texas Red®	Life Technologies™
CD14	HCD14	PerCP	BioLegend®
CD45	HI30	Pacific Orange™	Life Technologies™
CD56	HCD56	A700®	BioLegend®
CD56	HCD56	PE	BioLegend®
CD57	HNK-1	Pacific Blue	BioLegend®
CD94	DX22	PE-Cy7	BioLegend®
CD16	B73.1	PerCP	BioLegend®
GRZB	GB11	PE-Texas Red®	Invitrogen™
PRF	dG9	Pacific Blue	BioLegend®
GNLY	DH2	PE	BioLegend®
GNLY	RB1	A488®	BD Biosciences
CD107a	H4A3	PerCP-Cy5.5	BioLegend®
DNAM-1	11A8	FITC	BioLegend®
NKG2A	Z199	PE	Beckman Coulter
NKG2D	1D11	APC	BioLegend®
NKp46	9E2	FITC	BioLegend®
NKG2C	134591	PE	R&D Systems®
NKp30	P30-15	APC	BioLegend®
2B4	C1.7	Pacific Blue	BioLegend®
KIR2DL1	143211	APC	R&D Systems®
KIR2DS1	EB6B	PE-Cy7	Beckman Coulter
KIR2DL2	HP-MA4	FITC	BioLegend®
KIR2DL2	CH-L	PE	BD Biosciences

PRIMARY CELLS used in this thesis	Description
Decidual NK cells (dNK)	▪ Defined as CD45+CD14-CD3-CD56high ▪ Isolated from discarded decidual tissue from elective pregnancy terminations and healthy deliveries ▪ No proliferation in culture; cultured in suspension (1-2 days survival)
Peripheral NK cells (pNK)	▪ Defined as CD45+CD14-CD3-CD56lowCD16+ ▪ Isolated from Leukopacks® processed from healthy blood donations ▪ Proliferation in culture ▪ Growth in suspension

CELL LINES used in this thesis	Description
721.221 cell line (221)	▪ EBV-transformed B cell line ▪ HLA-A, B and C negative ▪ MHC Class II positive ▪ Immortalized cell line ▪ Growth in suspension
Erythroleukemia cell line (K562)	▪ Immortalized myelogenous leukemia line ▪ MHC Class I negative ▪ Growth in suspension