Receptor patterns and function of γδT cells during healthy human pregnancy

Doctoral (PhD) Dissertation

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1 List of abbreviations

(m)PR-(A/B)	(membrane) Progesterone Receptor (A/B)
(m/p)DC	(myeloid/ plasmacytoid) Dendritic Cell
(m/s)HLA	(membrane bound/soluble) Human Leukocyte Antigen
(n/i)T_{REG}	(natural/ induced) regulatory T cell
AP-1	Activator Protein 1
APC	Antigen-Presenting Cell
APS	Anti-Phospholipid Syndrome
Bat3	HLA-B associated transcript 3
BTN	Butyrophilin
C	Constant domain
CDR	Complementarity-Determining-Region
CEACAM1	Carcinoembryonic Antigen 1
CT	Cytotrophoblast
CTLA-4	Cytotoxic T Lymphocyte-Associated protein 4
DALT	Decidua-Associated Lymphatic Tissue
DAP12	DNAX Activating Protein 12
DMC	Decidual Mononuclear Cell
DN	Double Negative
DNA-PKs	DNA-dependent Protein Kinases
EGF	Epidermal Growth Factor
ER(α/β)	Estrogen Receptor α/β
EV	Extracellular Vesicle
EVT	Extravillous Trophoblast
Fas(L)	Fragment apoptosis stimulating (-Ligand)
FCS	Fetal Calf Serum
FGF-2	Fibroblast Growth Factor-2
FMO	Fluorescence-Minus-One *controls*
FSH	Follicle-Stimulating Hormone

G/ GM-CSF	Granulocyte / Granulocyte - Macrophage Colony - Stimulating Factor
Gal-9	Galectin 9
gw	Gestational week
hCG	human Chorionic Gonadotropin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT	Immunoglobulin-Like Transcript
ILC	Innate Lymphoid Cells
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motive
IUGR	Intrauterine Growth Restriction
JAK/STAT	Janus Kinase/Signal Transduction and Transcription activation
KIR	Killer cell Immunoglobulin-like Receptor
Lck	Lymphocyte-specific protein Kinase
LH	Luteinizing Hormone
LILR(A/B)	Leukocyte Immunoglobulin-Like Receptor (subgroup A/B)
LIN	Lineage Marker
Mϕ	Macrophage
MCP-1	Monocyte Chemoattractant Protein-1
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MICA/B	MHC class I polypeptide-related sequence A/B
MIP	Macrophage Inflammatory Protein
MIS	Maternal Immune System
MR1	MHC-related protein 1
NCRs	natural cytotoxicity receptors
NFAT	Nuclear Factor of Activated T cells

NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NKG2	Natural Killer cell protein Group 2
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PD-1	Programmed cell Death protein 1
PD-L1/2	Programmed cell Death-Ligand 1/2
PGE/ F2α	Prostaglandin E/ F2α
PI3K	Phosphoinositide 3-Kinase
PIBF	Progesterone-Induced Blocking Factor
PKB	Protein Kinase B
RANK	Receptor Activator for Nuclear factor-κB
RSA	Recurrent Spontaneous Abortion
ST	Syncytiotrophoblast
TAK1	TGF-β-Activated Kinase 1
TCR	T Cell Receptor
TGF	Transforming Growth Factor
$T_{H/C/REG}$	helper/ cytotoxic regulatory T cell
TIM-3	T cell Immunoglobulin and Mucin domain-containing protein 3
TNF-α	Tumor Necrosis Factor-α
TRAF6	Tumor Necrosis Factor Receptor-Associated factor 6
TRAV	T cell Receptor Alpha Variable
u/dNK cells	uterine/ decidual Natural Killer cells
V	Variable domain
VEGF	Vascular Endothelial Growth Factor

2 Introduction

2.1 Immunological Aspects of Pregnancy

2.1.1 The paradox of pregnancy

In the mid-20th century, when fundamental mechanisms for transplantation were discovered, Nobel Prize Laureate Peter Medawar realized parallels between an implanted embryo in pregnancy and an allograft transplant. Like a foreign transplant, the embryo should be rejected by the mother. In his concept of the semi-allogenic fetus, the offspring was hidden from the maternal immune system (MIS). He proposed the absence of fetal antigens, anatomical separation by the placenta, or immune suppression as possible mechanisms to prevent rejection [1]. Since then, efforts have been made to explain the interplay of the MIS and the embryo.

Today, we know that the embryo is recognized by the MIS, as antibodies and T cells specific for paternal antigens could be identified during or after pregnancy [2,3]. In contrast to organ transplantations, it seems more beneficial if the conceptus' Human Leukocyte Antigen (HLA) signature differs from the mother one, and continuous general immunosuppression is unnecessary for normal pregnancy [4,5]. On the other hand, the placenta, or the trophoblast, provides a 'buffer zone' so that the child's antigens are not in direct contact with the maternal tissue (see 2.1.2). The original idea that the MIS would attack the conceptus shifts more and more to a concept where it supports implantation, ensures defense against pathogens, and contributes to the initiation of labor [6].

2.1.2 The trophoblast: An immunological 'buffer zone'

Considering the origin of reproductive immunology, it is essential to emphasize that the embryonic or fetal cells expressing class I and II HLA molecules are not in direct contact with maternal tissue. The classical or adult HLA class I molecules, namely HLA-A, HLA-B, and HLA-C, are crucial for the immune system to discriminate between self and foreign. Therefore, matching HLA genes is the main criterion in the case of an organ transplant. However, before the blastocyst implants into the decidua, it forms an outer layer called trophoblast, which mainly does not express classical but non-classical HLA molecules [7,8].

Among the classical HLA class I molecules, only HLA-C is expressed by trophoblast cells. Additionally, the trophoblast expresses the non-classical HLA class I molecules HLA-E, HLA-G, and HLA-F. This HLA expression pattern is unique in the human body and is assumed to influence the maternal immune system: Specific receptors of immune effector cells can recognize these HLA molecules (see 2.3.1). They could be the reason for tolerance towards the (semi-)allogeneic fetus and orchestrate the development of a microenvironment that supports implantation and fetal growth [9–11]. It is often assumed that the non-classical HLA molecules protect the trophoblast from being attacked. At the same time, paternal HLA-C expression might help recruit and activate local immune effector cells to promote tissue transformation and support placentation [12].

The placental barrier, typically considered regarding drug transfer or vertical infections, also has an immunological component. However, the term barrier might be misleading as fetal antigens might 'leak' into the maternal zone and trigger the formation of specific effector cell clones [2,3]. Hence, the term buffer zone seems to be more appropriate. This buffer zone is far from static:

The size and structure of the maternal-fetal interfaces change with the progression of pregnancy (see 2.1.3). Furthermore, from an immunological point of view, shifts in the embryonic and fetal expression patterns and intensities of the immunologically relevant HLA class I molecules have been reported [13–16] (see 2.3.1.1). The expression of HLA molecules changes not only with time but also specific to the type of trophoblast: The focus here is mainly on the extravillous trophoblast (EVT), which is in direct contact with the decidua. On the syncytiotrophoblast (ST), on the other hand, HLA molecules are absent (Table 1) [17–20].

Likewise, maternal decidual immune cell populations fluctuate. While the early decidua is dominated by decidual Natural Killer (dNK) cells, T cells accumulate over time and become the largest immune cell population shortly before term [21]. Therefore, it can be assumed that dNK cells are the critical immune effectors in implantation and placental formation and that T cells play their role later and at the end of pregnancy (see 2.1.5).

2.1.3 Maternal-fetal Interfaces

Maternal-fetal interfaces are defined locations where direct interactions of fetal antigens and maternal immune cells occur. The presented system here is based on the

publications of Sargent et al. and Tersigni et al. [22–24]. Respective interactions between maternal immune and fetal trophoblast cells are discussed in 2.1.5.

Before the embryo implants, it forms an outer cell mass called trophoectoderm, the origin of all types of trophoblasts (Table 1). At the time of implantation, the trophoectoderm can be divided into cytotrophoblast (CT) and ST, both being in contact with the decidua and local immune cells, creating the *maternal-fetal interface I*. At this early stage, the nutrient supply for the embryo is maintained via the ST engulfing apoptotic decidual cells and secretion products of uterine glands [25,26].

Table 1 Trophoblast tissue types *(summarized after [27,28])*

<table>
<tr><th colspan="4">Trophoblast tissue</th><th>HLA expression [13–16]</th><th>Maternal-fetal interface</th></tr>
<tr><td rowspan="7">Trophoectoderm</td><td colspan="3">Syncytiotrophoblast (ST)</td><td>negative</td><td>IV</td></tr>
<tr><td rowspan="6">Cytotrophoblast</td><td colspan="2">Villous cytotrophoblast</td><td rowspan="6">HLA-A^{-},-B^{-} HLA-C^{+},-E^{+},-F^{+},-G^{+} †</td><td>–</td></tr>
<tr><td rowspan="4">Extravillous cytotrophoblast (EVT)</td><td>Endovascular extravillous trophoblast</td><td rowspan="3">II</td></tr>
<tr><td>Interstitial extravillous trophoblast</td></tr>
<tr><td>Endoglanduar extravillous trophoblast</td></tr>
<tr><td>Smooth chorionic cytotrophoblast</td><td>III</td></tr>
</table>

In the 2nd gestational week (gw), a primitive uteroplacental circulation is established after the ST has formed lacunes and EVT invaded the first maternal vessels, replacing their inner lining (= endovascular EVT). Henceforth, the embryo can rely more and more on the maternal supply of nutrients and oxygen. However, until gw 6, only plasma and microcorpuscular particles will pass through into the lacunes [29]. This process evolves, and latest at gw 8, the first interface can be divided into three new interfaces. In the placental bed, only the EVT directly interacts with the immune and stroma cells of decidua basalis and forms the *maternal-fetal interface II*.

The *maternal-fetal interface III* is in the Amnion, where the chorion laeve cytotrophoblast meets with the decidua capsularis and later parietalis.

† Published results as expression of cytotrophoblast. The authors did not distinguish different subtypes.

Finally, the maternal blood bathes the ST-covered placental villi by which the *maternal-fetal interface IV* is formed [22,23] (Figure 1). At this surface, circulating immune cells may interact with the HLA^- ST.

Furthermore, extracellular vesicles (EVs) are secreted into the maternal circulation, and fetal cells can be detected in the maternal blood [30,31]. Although not commonly considered, these circulating particles could be seen as a different interface.

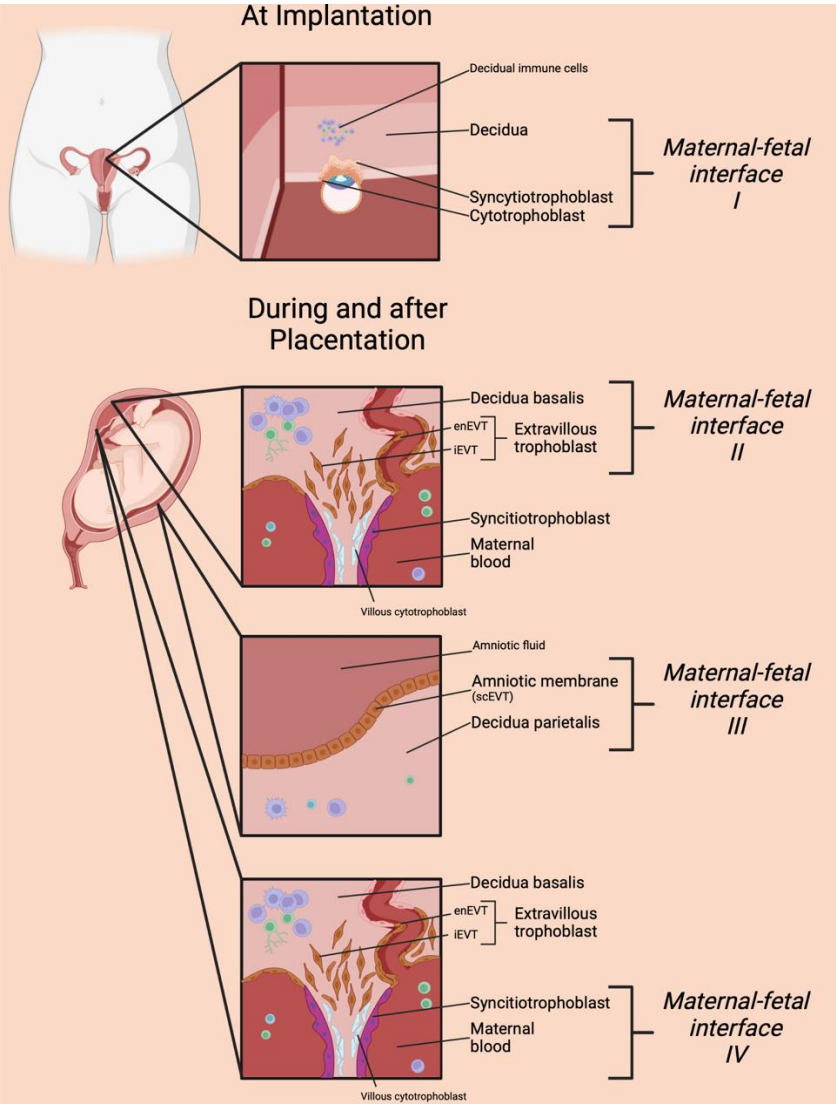

Figure 1 Maternal-fetal interfaces *(after the concepts of Sargent et al. and Tersigni et al. [22–24]) enEVT: endovascular extravillous trophoblast; iEVT: interstitial extravillous trophoblast; scEVT: smooth chorionic extravillous trophoblast*

Research in human reproductive immunology in the last decades mainly focused on maternal-fetal interfaces II and IV. Due to the nature of pregnancy detection and legal regulations regarding early pregnancy termination, human samples before gw 6 are

inaccessible, largely restricting the investigation of the maternal-fetal interface I. Amnionic tissue can be easily identified in samples from early pregnancy; however, data focusing on maternal-fetal interface III is scarce.

2.1.4 Eutherian Pregnancy - An evolutionary dichotomy of inflammation

When comparing eutherian and marsupialian reproduction, the uterine dwelling time catches the eye: While marsupialian pregnancies take only a few days or weeks, eutherian fetuses may grow in-utero for more than a year[‡]. This time allows the eutherian fetus to develop while being protected and nourished by the mother. On the other hand, the marsupialian conceptus attaches to the endometrium only for a short time and does not invade the maternal tissue [32,33]. This attachment induces acute inflammation within the endometrium, subsequently leading to parturition [34]. It is tempting to hypothesize that the attachment of the semi-allogeneic eutherian blastocyst initially also caused inflammation. However, this inflammation process changed during evolution and created three phases of eutherian pregnancy [6,34]:

1. After fertilization, the blastocyst attaches to the endometrium, initiating *implantation and placentation*. The attachment and invasion of the blastocyst trigger an inflammation-like environment, which is thought to be beneficial as it allows tissue remodeling and vascular invasion by the trophoblast. However, neutrophil infiltration, an essential aspect of inflammation and a potential risk for a non-selective attack of fetal-derived tissues, seems to be avoided during implantation [35,36]. Furthermore, tissue prostaglandin (PG) $F_{2\alpha}$ levels appear low during eutherian implantation [37,38]. High concentrations of $PGF_{2\alpha}$ would lead to uterine contractions and the disjunction of the maternal-fetal unit. However, PGE, which has similar uterine/ myometrial effects as PG $F_{2\alpha}$, is also an active mediator during implantation and is thought to support the vascular remodeling necessary for trophoblast invasion [6,37,38]. To prevent the disadvantageous effects of PGE, other signaling networks might influence these pathways during implantation [39]. Among Eutheria, three types of placentation[§], defined by their

[‡] Proportional correlation between neonatal brain size and gestational duration [511]
[§] epitheliochorial (no invasion); endotheliochorial (invasion of endometrial epithelium and connective tissue); hemochorial (invasion of endometrial epithelium, connective tissue, and endothelium)

invasion depth and placental shape, are known. In species with hemochorial placentation, like primates and humans, or rodents, three maternal tissue layers (endometrial epithelium, connective tissue, and endothelium) are penetrated by the fetal EVT, resulting in direct contact of fetal tissue to maternal blood [40,41]. However, this deep infiltration must be regulated. Too weak implantation most likely results in an early pregnancy loss, while too aggressive invasion leads to the placenta accreta spectrum [42]. A growing body of evidence emphasizes the importance of maternal immune effector cells in this invasive process. Over 40 % of decidual cells during implantation are leukocytes, and several studies demonstrated that these local immune cells actively contribute to the implantation process [40,41,43,44] (see 2.1.5).

2. Placentation is followed by a long *non-inflammatory period* [45,46]. At this stage of pregnancy, the fetus grows and matures while being nourished and protected by the mother.
3. At term, however, the *inflammation* flares again, initiating *parturition* [39,47,48]. This time, however, neutrophils invade the decidua, the fetal membranes, and the cervix. Furthermore, high levels of $PGF_{2\alpha}$ activate metalloproteinases and reduce progesterone sensitivity, contributing further to the progress of labor [47–53].

2.1.5 Immune cells and their contributions to implantation and pregnancy

Considering the (semi-)allogeneic nature of the conceptus and the inflammation-like processes connected to pregnancy, it is evident that immune cells' composition and contributions highly impact the outcome of this reproductive process. Due to medical and obvious ethical reasons, data on early human pregnancy's maternal-fetal interface I is unavailable. However, human endometrial biopsies during a normal menstrual cycle from non-pregnant women and implantation data from other species with hemochorial placenta allow modeling of this crucial stage of pregnancy. Most human data provide information about the maternal-fetal interfaces II (decidua basalis) or IV (peripheral blood) in the late first trimester (gw 6 - 14) or around parturition (gw 36 - 40). Only a few studies investigated immune cells at the maternal-fetal interface III (decidua parietalis) or between these time points [21,54,55].

In the hope of finding biomarkers for different pregnancy-associated pathologies, leukocyte populations in the blood have been extensively investigated. However, this data should only be interpreted with the knowledge about the initial local events. Therefore, this chapter focuses mainly on immune cells at the maternal-fetal interface II.

During a normal menstrual cycle, the prevalence of leukocytes increases so that they make up more than 20 % of all decidual cells in the late secretory phase [43,56,57]. In the case of fertilization and beginning implantation, this immune cell portion expands further to more than 40 % [43,58]. The major immune cell populations in the decidua are dNK cells, macrophages (Mϕ), dendritic cells (DC), and T cells, while neutrophil granulocytes only infiltrate this site at term and B cells are scarce [47,48,59–61].

To date, quantifiable data about the exact composition of the decidua-associated lymphatic tissue (DALT) at different stages of pregnancy are scarce. The available data for the major populations are summarized in Figure 2.

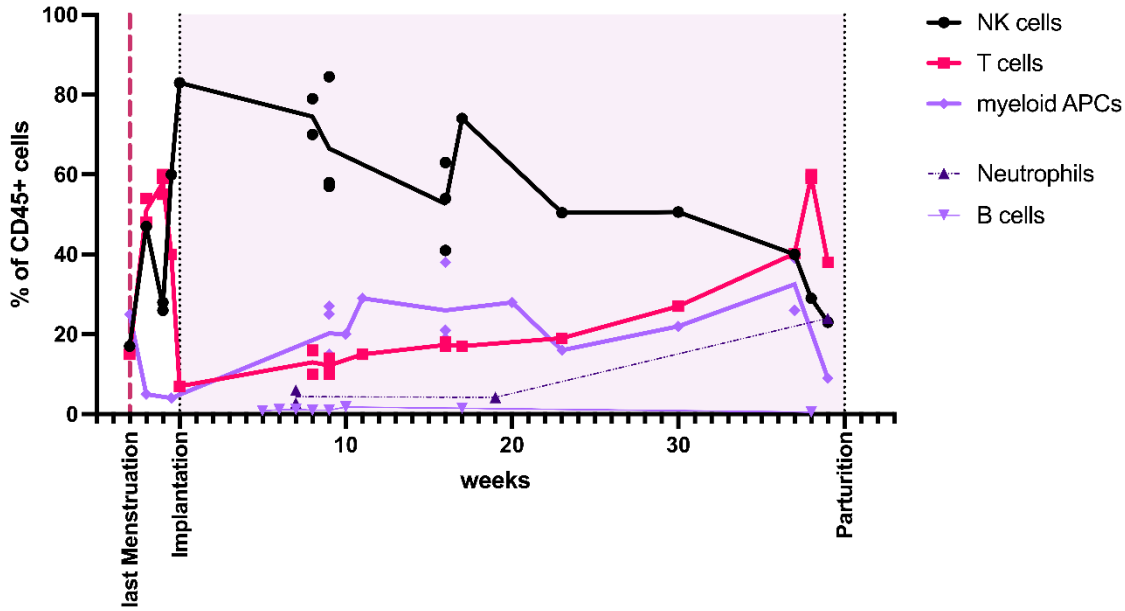

Figure 2 *Composition of immune cell population in the endometrium/ decidua [**] (summarized data from [21,60–68])*

Although incomplete, the available data shows the dominance of dNK cells during implantation, which is superseded by a growing T cell population at term. However,

[**] Defined as $CD45^+$ cells in the decidua; the here presented prevalence data refers only to comparable and quantifiable single-cell data from methods like flow cytometry, mass spectrometry, single-cell sequencing. Older data, only providing information like "cells/ field" were considered as not comparable.

myeloid antigen-presenting cells (Mϕ and DCs) were shown to remain relatively stable in their prevalence during pregnancy. Like decidual B cells themselves, data about them is rare. Therefore, decidual B cells will not be discussed further.

2.1.5.1 NK cells

As lineage marker (LIN) negative cells, $LIN^{-}/CD56^{+}$ NK cells are the most prominent group of innate lymphoid cells. These cells were first found in the peripheral blood and named due to their strong cytotoxic activity against tumors [69].

$LIN^{-}/CD56^{+}$ lymphoid cells were later found to be the most abundant cell population in the early DALT and accordingly named uterine or decidual NK cells [70]. The naming of these uterine $LIN^{-}/CD56^{+}$ cells strongly fed into the belief that these cells would attack the (semi-)allogeneic fetal-derived tissues. However, to date, no study could show the cytotoxic activity of dNK cells towards the (semi-)allogeneic fetus. Quite the contrary seems to be true. Decidual NK cells are likely to promote and regulate EVT invasion and placentation through angiogenesis and tissue remodeling [10,71–76]. It is essential to emphasize that u/dNK cells must not be confused with their peripheral counterpart. While peripheral blood NK cells are mainly $CD56^{dim}/CD16^{+}$ cytotoxic cells, dNK cells are $CD56^{bright}/CD16^{-}$ cytokine-secreting cells [10,77–79].

During a natural reproductive cycle, the local $LIN^{-}/CD56^{+}$ population proliferates and becomes the most dominant DALT population shortly before implantation. This expansion is induced by Interleukin (IL)-15 secreted by endometrial stroma cells influenced by the increasing progesterone level [63,80,81]. If implantation occurs, the prevalence of these cells further increases and remains high during the first trimester [21,63,65]. However, the T cell fraction overtakes the leading DALT population in the third trimester [21,55,66].

Within tissue-resident early decidual NK cells, at least three subclusters (dNK1, dNK2, and dNK3) can be defined [21,82]. All subclusters produce granzyme A, B, perforin, and granulysin. However, dNK1 cells are the strongest in producing these mediators of cytotoxicity. This subset has an active glucose metabolism and shows a higher expression of HLA-C and -G receptors (see 2.3.1.2 and 2.3.1.4, respectively) compared to dNK2 or dNK3 cells. However, HLA-E receptors (see 2.3.1.3) are similarly expressed by dNK2 cells but less by dNK3 cells [82]. The expression of HLA class I molecule receptors allows dNK cells to interact with the EVT directly.

Decidual NK cells affect spiral artery remodeling by producing angiogenic factors[††] after binding HLA-G with Killer Cell Immunoglobulin Like Receptor (KIR)2DL4 [71,72,74,83–85]. In addition, dNK cells exhibit specialized defense mechanisms to protect the maternal-fetal unit from pathogens: In the case of *Listeria monocytogenes*-infected EVTs, a well-known cause for spontaneous abortions and stillbirth, dNK cells transfer granulysin via nanotubes into the EVTs, targeting only the bacteria but not the EVT. Furthermore, dNK cells were shown to release granzymes, granulysin, and perforin when co-cultured with *Cytomegalovirus*-infected decidual stroma cells [86].

In summary, dNK cells are the most dominant early pregnancy DALT cell population. These large granulated lymphoid cells show a receptor repertoire that allows them to detect the EVT's unique HLA signature and support its invasion. Furthermore, they defend the microenvironment of this maternal-fetal interface against pathogens. As mentioned, these tissue-resident dNK cells must not be confused with peripheral NK cells as those are largely different in phenotype and function. Here, clinical trials failed to demonstrate any benefit of using NK cell counts for clinical diagnostics and infertility treatment, so most recent international guidelines do not recommend these practices [87,88].

2.1.5.2 Myeloid antigen-presenting cells

In addition to dNK cells, myeloid antigen-presenting cells (APC), like Mϕ and DCs, can be found in the decidua basalis. In contrast to decidual lymphoid cell clusters, the prevalence of these myeloid HLA class II^+ cells remains relatively stable throughout pregnancy. Notably, a phenotype-based distinction between Mϕ and DCs is controversial, and the data presented here is not necessarily exclusive to the respective myeloid cell type. Generally, $CD14^+$ cells are considered as Mϕ and $CD14^-$ as DCs. Both cell types are thought to arise from a common myeloid progenitor. However, recent research suggests alternative development pathways for decidual Mϕ and possible self-renewal in the tissue [89–91].

Decidual Mϕ are equipped with Leukocyte immunoglobulin-like receptors (LILRs, see 2.3.1.4), allowing them to recognize HLA-G expressed by the trophoblast [92,93]. Depending on environmental stimuli, Mϕ will show two different phenotypes after

[††] IL-1β, IL-8, CXCL10, IFN-γ, TNF-α, GM-CSF, angiopoietin 1 & 2, MMP-2 & -9, VEGF

activation. M1 Mϕ are inflammatory cells secreting pro-inflammatory cytokines (IL-1β, IL-6, IL-12, Tumor Necrosis Factor (TNF)-α) [94,95]. M2 Mϕ, however, are associated with tissue remodeling, immune tolerance, and angiogenesis [96]. These two subsets show remarkable plasticity and can transform from one to another [97–99]. They make up for 25 - 30 % of the DALT during human pregnancy [21,62,63,66,100]. In contrast to the data depicted in Figure 2, it can be assumed that Mϕ already infiltrated the decidua pre-implantation, as rodent data suggest a seminal fluid-triggered pre-implantation inflow [101,102]. It appears that decidual Mϕ change their polarization in the course of pregnancy. While Mϕ of the non-pregnant endometrium are rather M1, early decidual Mϕ are more heterogenous and show M1 and M2 characteristics [103–107]. At this time, decidual Mϕ can inhibit Interferon (IFN)-γ production by decidual T cells via Programmed cell death protein 1 (PD-1) – Programmed cell death – ligand-1 (PD-L1) interactions [108]. During the second trimester, this balance shifts towards an M2-dominance; at term, they express a DC-like phenotype [109].

With 1-5 % of decidual $CD45^+$, DCs are the smaller fraction of the myeloid APCs at the maternal-fetal interface II [21,110]. Like in the peripheral blood, decidual DCs can be subdivided into two myeloid subsets (mDC1: $CD1c^+CD19^-CD14^-$; mDC2: $CD141^+CD14^-$) and plasmacytoid DCs (pDCs: $CD303^+CD123^+$). Compared to the peripheral blood, the prevalence of mDC1 is similar, while pDCs are rarer. However, mDC2s are more common in the early decidua [111]. Decidual DCs express low levels of CD40, CD80, CD86, and CD205, indicating an immature phenotype [112]. To date, functional data about human decidual DCs are scarce, and results from murine models are hardly transferable due to different markers for DC populations [113]. However, murine models emphasized the importance of this cell population, as DC-depletion led to impaired dNK function and decreased IL-15 and IFN-γ levels at the site of implantation [114]. Human decidual Mϕ and DCs express CD209, a receptor, which has been associated with adhesion but also pattern recognition [115–117]. These $CD209^+$ DCs are considered immature and are more common in the decidua than the mature CD83-expressing DCs [67,118,119]. This aligns with the assumption that DC loaded with paternal antigens leave the decidua to present those to T cells in the draining lymph nodes [120,121]. Immature $CD209^+$ DCs, however, are thought to enhance the secretion of Granulocyte-

Macrophage Colony Stimulating Factor (GM-CSF) and IL-10 (see 2.3.4) by dNK cells [122].

In a nutshell, decidual APCs are thought to uptake fetal- and pathogen-derived antigens in the decidua. Loaded APCs migrate to the draining lymph nodes; consequently, the remaining resident APCs are skewed to an immature phenotype. APCs are known to influence the outcome of T cell priming, and the phenotypes of decidual Mϕ indicate an initial promotion of inflammation but facilitation of tolerance with the progression of pregnancy.

2.1.5.3 T cells

In contrast to the aforementioned immune cells, T cells are equipped with the antigen-specific T cell receptor (TCR). T cells only recognize processed antigens presented via Major Histocompatibility Complex (MHC) molecules (= MHC restriction). Therefore, T cell activation by the trophoblast is largely limited as the ST is void of HLA molecules and EVT's expression of classical HLA class I molecules is restricted to HLA-C.

When encountering their specific antigen, T cells undergo clonal expansion, becoming effector and memory cells. The type of their TCR subdivides T lymphocytes into two phenotypically and functionally different subsets. The majority of human peripheral blood and endometrial/ decidual T cells carry the αβTCR, whereas some express the γδTCR. Therefore, humans are considered as a 'γδT cell low' species, whereas ruminants or sharks were found to be 'γδT cell high' species [123]. However, in humans, γδT cells are more common at the interfaces between the organism and the environment, like the skin, intestinal mucosa, or the endometrium/ decidua, indicating a pronounced function at these frontiers [124,125] (see 2.2).

Classical αβT cells are well distributed over the body and are the largest lymphocyte population in the peripheral blood. These 'classical' T cells are further subdivided into $CD4^+$ helper T cells (T_H) and $CD8^+$ cytotoxic T cells (T_C). T_H recognize their specific antigen presented by professional APCs via MHC class II molecules. After activation, T_H cells differentiate into further subsets (Table 2), characterized by cytokine secretion patterns. On the other hand, antigens presented via MHC class I molecules can be detected by T_C cells. Activated T_C cells, like NK cells, can kill their target cells either by secreting cytotoxic molecules (perforin, granzymes, or granulysin) or via the Fragment apoptosis-stimulating (Fas) - Ligand (FasL) - Fas pathway.

Table 2 T_H cell subsets

	Key Cytokines	**Associated to**
T_{H1} cells	IFN-γ, IL-2	Cellular immune response
T_{H2} cells	IL-4, IL-5, IL-13	Humoral immune response
T_{H17} cells	IL-17, IL-22	Extracellular bacteria and fungi
regulatory T (T_{REG}) cells	TGF-β, IL-10	Tolerance

Maternal T cells populate the non-pregnant endometrium and are present in the decidua throughout pregnancy [21,63]. While they are abundant during the proliferative phase of the menstrual cycle, their numbers decrease shortly before implantation and remain low in early gestation [21,63,64,66,67]. However, as pregnancy progresses, the decidual T cell rate continuously increases so that T lymphocytes become the most dominant leukocyte type shortly before term [21,66,67]. Compared to the periphery, the proportion of antigen-primed memory T cells ($CD45RO^+$) in the decidua at term is significantly higher, and at least some of the $CD8^+$ T cells are specific for fetal antigens [126]. Fetus-specific T_C cells were shown to lyse fetal cells in vitro [3].

Furthermore, the development of fetus-specific Immunoglobulin (Ig)G antibodies during pregnancy indicates the presence of fetus-reactive T_H cells as well [127,128]. The existence of these cells raises the question of control mechanisms that prevent immune responses directed against the fetus. Over the last decades, several concepts for regulating immune cells during pregnancy have been developed. The most relevant hypotheses will be discussed in chapter 2.2.4.2.

In addition to fetus-reactive clones, T cells specific for pregnancy-harming pathogens are actively recruited to the maternal-fetal interface. Although to date only demonstrated in decidual NK cells, these virus-specific T cells might be capable of detecting intracellular pathogens in the trophoblast via HLA-C and selectively eliminating those without harming fetal cells [86,129].

All in all, T cells accumulate in the decidua with the progression of pregnancy. Some resident T cells are specific for fetal antigens, while others are likely to surveil this microenvironment and protect it from pathogens.

2.1.5.4 Neutrophils at term

While the previously mentioned decidual immune cells have been of primary interest in the field for the last decades, neutrophils were rarely the focus of investigations. In contrast to a 'classical' site of inflammation, neutrophils are scarce in the early decidua [21,82,130].

During pregnancy, low numbers of neutrophil granulocytes can be found in the decidua. However, pre-term, peripheral blood neutrophils activate and show an increased migratory potential [131]. Local cytokines, like IL-8, were demonstrated to attract these cells into the uterine tissue [131–133]. Consequently, they comprise ~60 % of all myeloid cells and ~25 % of $CD45^+$ cells in the term decidua [21]. Here, they were shown to secrete pro-inflammatory cytokines and matrix metalloproteinases and were thought to break down fetal membranes and initiate labor [131–133]. This is in line with the paradigm of eutherian pregnancy as an altered and separated inflammatory process, as mentioned previously (2.1.4).

2.1.5.5 Circulating Immune Cells in Pregnancy

While investigations of the maternal-fetal interfaces I, II, and III focus on local tissue interactions, interface IV allows the study of the relations between maternal blood and the ST, systemic adaptions to pregnancy, and potential spillover effects from the other interfaces. Compared to the dynamically changing composition of the DALT, however, changes in the peripheral blood immune cell populations are minor. Among the innate immune cells, Neutrophils and Monocytes are known to increase slightly in number, while the prevalence of Eosinophils, Basophils, and NK cells is not affected by pregnancy [134–138]. Furthermore, Neutrophils of pregnant women show lower phagocytic activity without changing their expression pattern for their activation markers (CD11b, CD15, CD18, or CD62L) [139–141]. Noteworthy, both NK cells and Monocytes express higher levels of the inhibitory receptor T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3; see 2.3.2.3), while plasma levels of Galectin-9 (Gal-9), its ligand, are also elevated [137,142].

The prevalence in the adaptive immune components does not change during pregnancy either [143–145]. However, even though the major T cell subsets do not alter numbers, a progressive shift to a T_{H2}-bias can be detected at the end of the first trimester [143,145–147]. Regulatory T cells have been discussed to prevent potentially harmful immune reactions

during pregnancy. Data regarding their prevalence in peripheral blood is inconclusive. This might be due to different defining criteria in the respective studies [148–151].

Although not necessarily circulating, B cells may influence pregnancy. Anti-paternal antibodies have been identified in the 1970s. These antibodies were demonstrated to have a protective in vitro effect on maternal lymphocyte-trophoblast co-culture [152,153]. During the same period, asymmetric antibody‡‡ titers were shown to be elevated in pregnant women while being low in patients with recurrent spontaneous abortions (RSA) [154,155]. These findings may have fed into the conclusion that these antibodies provide an essential protective contribution to the success of pregnancy by veiling crucial target antigens to the maternal immune system. Yet, it was demonstrated, partially by members of the same group, that "blocking antibodies" were not necessary for successful pregnancy [156–158].

On the other hand, autoantibodies in autoimmune diseases, like anti-phospholipid syndrome (APS), negatively affect pregnancy outcomes [159,160]. While the association of pregnancy loss and APS appears to be evident, the pathomechanisms connecting other antibody-associated autoimmune diseases, like autoimmune thyroiditis, to infertility remain unclear[161,162].

In addition to their antibody production, some B cells are known to secrete, among other cytokines, IL-10. These regulatory B cells were also implicated to contribute to a fetus-friendly environment [163]. However, causal connections and a relevant impact on pregnancy outcomes remain to be established.

In the attempt to find a practical immune biomarker for conditions like recurrent implantation failure, RSA, or pre-eclampsia, a large body of studies aimed to correlate them with the prevalence or activity of different leukocyte populations in the peripheral blood [149,164–168]. However, pregnancy is a dynamic process in which immune processes are far from being understood. Even though some data regarding the prevalence, activity, or function of different immune effector cells during pregnancy exists, it is fragmentary. To date, no study could provide convincing evidence. Therefore, official guidelines do not recommend immune testing/ profiling outside of clinical trials [87,88,169].

‡‡ High affinity antibodies, with post-transcriptional oligosaccharide-addition on one of the two antigen-binding Fragments leading to a univalent antibody.

The possibilities of high-dimension, high-throughput methods might allow a more realistic depiction of the acting immune networks during pregnancy.

2.2 Gamma/delta T cells

For approximately 500 million years, lymphocytes of vertebrates are divided into three compartments [170]. While B and αβT cells have been intensively studied, γδT remained enigmatic. Gamma/delta T cells are central players in the defense against microbes or tumors and control tissue and mucosal integrity [171–173].

The antigen receptor of γδT cells is a heterodimer composed of a γ and δ polypeptide chain, of which each has a variable (V) and constant © domain. Distinct molecular surfaces for antigen binding are located in the complementarity-determining region (CDR) within the V-domain of each chain. Gamma/delta T cells incorporate several subpopulations defined using different Vγ or Vδ domains. The major subpopulations, determined by the Vδ-usage, are $V\delta1^+$, $V\delta2^+$ and $V\delta3^+$ γδT cells. Noteworthy, genes encoding δ-chains are embedded in the T cell Receptor Alpha Variable (TRAV) gene locus (14q11). Within this locus, five further but seldomly expressed Vδ-gene segments exist, which also have a Vα-signature: TRAV14/Vδ4, TRAV29/Vδ5, TRAV23/Vδ6, TRAV36/Vδ7 and TRAV38/Vδ8 [174,175]. While those five associate with a β-chain in an αβTCR, Vδ1/2/3 will build γδTCRs in combination with Vγ-chains. The Vγ genes expressed in humans are Vγ2-5/8/9/11 [176,177].

The $V\delta2^+$ subset associates almost exclusively with Vγ9. These Vγ9Vδ2 T cells are the major γδT cell population in the blood and make up for 1-10 % of peripheral blood T cells [178]. $V\delta1^+$ and $V\delta3^+$ cells, on the other hand, can be found as tissue-resident cells and show some Vγ-chain preference depending on the respective location [179–181].

In contrast to most αβT cells, γδT cells do not depend on MHC molecules for antigen recognition [182]. However, direct cell-cell contact seems to be necessary for their activation [183,184]. This led to the hypothesis that either γδT cells require antigen-presentation via non-MHC but MHC-like molecules or depend on costimulatory factors from other cells [185]. Upon activation, γδT cells may show cytotoxicity, secrete different cytokines, or both [186–188].

2.2.1 Development and migration of γδT cell subsets

Both T cell types develop out of a common $CD4^{-}CD8^{-}$ double negative (DN) progenitor cell in the thymus through four stages (DN1-4). The determination of the αβT or γδT cell line occurs during DN2/3. Here, the rearrangement within γ- and δ-gene loci leads to the establishment of γδTCR complexes, which promote proliferation and differentiation into γδT cells, while cells determined for αβT cell development will begin to express pre-αβT complexes, which will silence the expression of γδTCR molecules and consequentially allows these cells to enter their positive selection process [189–193]. How γδT cells mature from this stage onwards is largely unknown. Most of the γδT cells leave the thymus after TCR gene rearrangement during fetal life and home to different tissues (Figure 3).

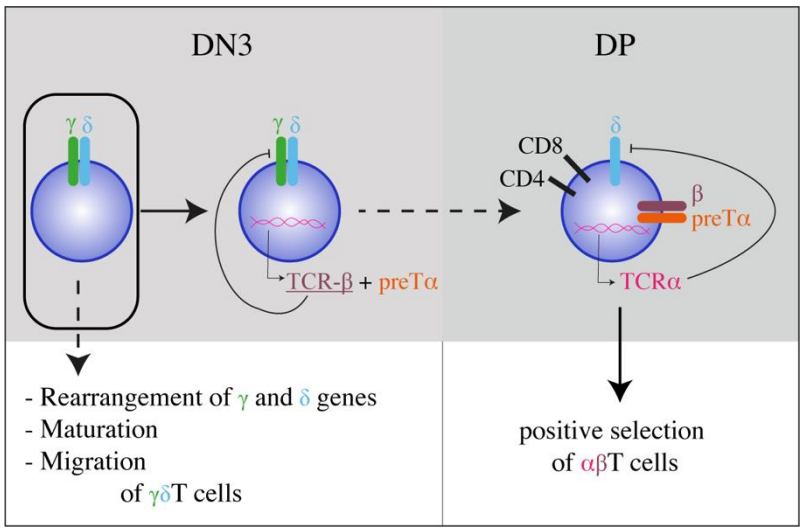

Figure 3 *Separation of γδT and αβT cell lines during thymic development.*

It was shown in mice that this migration process is organized in waves, each containing a specific γδT cell subset settling in a respective location. The first wave contains $Vγ5^{+}$ cells migrating to the skin's epidermis. Here, they secrete keratinocyte growth factor and change their appearance into a dendritic-like shape. Therefore, these cells are widely known as dendritic epidermal T cells [124,125]. The second wave is compromised out of $Vγ6^{+}$ cells, which will arrive in the reproductive tract, the lung, and the dermis. These cells were shown to produce IL-17, which is why they were also named Tγδ17 cells [124,125]. Hereafter, two more heterogenous waves containing $Vγ1^{+}$ and $Vγ4^{+}$, then $Vγ1^{+}$, $Vγ2^{+}$, $Vγ4^{+}$, and $Vγ7^{+}$ cells leave the thymus to inhabit the lung, spleen, lymph nodes,

liver, and intestinal mucosa [124,125,180]. However, in humans, Vγ1, Vγ6, and Vγ7 are pseudogenes and not expressed [177]. Nevertheless, a similar wave-like distribution of γδT appears to happen during human fetal life [194,195].

It is generally assumed that the γδT cells leaving the thymus are matured. However, studies demonstrated the plasticity of T cell development and could even transform γδT into αβT cells [196,197].

2.2.2 Antigen recognition of γδT cells

Despite several decades of research, antigen recognition of γδT cells remains enigmatic. Gamma/delta T cells are often seen as a bridge between the innate and adaptive immune systems. The rationale for this vague positioning is due to the expression of different innate immune receptors like natural cytotoxicity receptors or Natural Killer cell protein Group 2 (NKG2) D and the formation of a peripheral blood γδT cell population bearing the semi-invariant Vγ9Vδ2 TCR. At the same time, mainly the $V\delta1^+$ subset, with its diverse TCR repertoire, encompasses cells with naïve and mature effector cell phenotypes [198–201].

In Innate-like γδT cells, the Vγ-chain appears to have a more prominent role. In this context, the Vγ9Vδ2 cells mainly detect phosphoantigens of microbial or cancerous origin. Even though the antigen recognition of these γδT does not underlie an MHC restriction, B7-like butyrophilins (BTN) on APCs play a pivotal role in the phosphoantigen reactivity. Here, the Vγ9 and Vγ4 bind the BTN molecules 2A1 or 3A1, which was proposed as one key aspect of phosphoantigen-dependent γδT cell activation [202–205].

In contrast, the MHC-like proteins CD1b/c/d and MHC-related protein 1 (MR1) have been established as ligands of $V\delta1^+$ T cells [206–209]. CD1 molecules are known to present lipid molecules to T cells. Among γδT cells, several clones have been identified showing reactivity against α-galactosylceramide, β-galactosylceramide, or β-glucosylceramide presented via CD1 molecules [207,210]. MR1, on the other hand, binds metabolites of bacterial riboflavin synthesis. MR1 molecules are physiologically located intracellularly. They associate with β2-microglobulin upon ligand binding and are then

expressed on the cell surface. Here, they present the bound ligand to immune effector cells [211–213].

When T cells interact with MHC-peptide complexes, the molecular buried surface area is equally shared by their α-/δ- and β-/γ-chain [214–217]. However, the mode of recognizing CD1- or MR1-presented antigens is distinct from this pattern. Here, the δ-chain dominates the buried surface area (75 – 100 %) and is therefore responsible for antigen-binding [201].

Even though still not completely understood, the mode of antigen recognition by γδT cells, in a nutshell, depends on the Vγ- and Vδ-usage of the respective subset. The Vγ-chain appears to be the antigen-determining factor for innate-like γδT cell subpopulations, while the adaptive-like subsets use Vδ-chains to identify antigens.

2.2.3 Similarities of γδT and NK cells

NK cells belong to the group of innate lymphoid cells (ILC), which morphologically appear like lymphocytes but are devoid of antigen receptors. Like T_H cell subsets, the groups of these cells are defined by their functional effects or cytokine profile rather than cell surface markers [218]. Peripheral NK cells are known for their strong cytotoxic effect against tumor cells, which they can execute either by the degranulation of cytotoxic mediators (perforin, granzymes, granulysin) or via the Fas-FasL pathway. Furthermore, NK cells are known to produce IFN-γ and TNF-α [71,219].

However, these functions are far from exclusive. The killing mechanisms of NK cells are also used by T_C cells or γδT cells, and circulating γδT cells are known to be potent producers of these cytokines [184,220,221]. In contrast to lymphocytes, NK cells recognize their targets not via an antigen-specific receptor but through a symphony of activating and inhibiting receptors on their cell surface. Several receptor groups have been first described on NK cells and named accordingly. However, they can also be found on γδT cells (Table 3). NKG2D, for example, was shown to be expressed by at least 40 % of γδT cells [222]

Table 3 Activating and Inhibiting receptors expressed by NK and γδT cells.

Group		Subgroup/ specific receptors	Functions	expressed by		
				pNK	u/dNK	γδT[§§]
HLA-specific receptors	Killer cell immunoglobulin-like receptors (KIR)	KIR2/3DLx	Inhib.	+ [225]	+ [82]	+ [226,227]
		KIR2/3DSx	Act.	+ [225]	+ [82]	+ [227]
		KIR2DL4	Act./Inhib., cytokine secretion	+ [225]	+ [82]	?
	Leukocyte immunoglobulin-like receptors (LILR/ ILT)	LILRA	Act.	+ [225]	?	?
		LILRB	Inhib.	+ [225]	+ [82]	+ [223]
	Natural killer group 2 (NKG2)	NKG2A/CD94	Inhib.	+ [225]	+ [82]	+ [228]
		NKG2C/CD94	Act.	+ [225]	+ [82]	+ [229]
		NKG2D	Act.	+ [225]	+ [230]	+ [222,231,232]
	LAG-3		Inhib.	+ [225]	+ [233]	+ [234]
Non-HLA-specific receptors	Natural Cytotoxicity receptors (NCR)	NKp30	Act.	+ [225]	+ [78]	+ [199,200]
		NKp44	Act.	+ [225,235]	– [21]	+ [236]
		NKp46	Act.	+ [225]	+ [21,78]	+ [237]
	Immune checkpoint molecules	PD-1	Inhib.	+ [225]	– [21,82]	+ [238]
		Siglec-7	Inhib.	+ [225]	?	?
		TIGIT	Inhib.	+ [225]	+ [82]	?
		TIM-3	Inhib.	+ [137,225]	– [82]	+ [239]

Phenotypically, NK cells were defined as $LIN^{-}CD56^{+}$ lymphoid cells. However, with the knowledge about the existence of other ILCs and the development of high-dimensional single-cell analyzing techniques, the usage of an adhesion molecule as a defining marker appears questionable. Furthermore, the expression of CD56 is not exclusive to NK cells. Subsets of both αβT and γδT cells (NKT-like cells), as well as DCs, Mϕ, muscle cells, or neurons, express CD56, and the knowledge of its function on hematopoietic cells remains vague [223,224].

Although this overlap in function and phenotype challenges the concept that γδT and NK cells are distinct populations, the expression of an antigen-receptor positions γδT

[§§] Circulating and decidual γδT are summarized in this table due to the limited availability of γδT cell-specific data

cells somewhere between innate and adaptive immunity. When focusing on pregnancy, these cells are of particular interest. As described in 2.1.5, during pregnancy, the composition of the DALT changes from innate- to adaptive-dominated cell types. In this context, γδT cells appear ideal candidates for regulating or supporting this transition and participating in local immunity throughout pregnancy.

2.2.4 γδT cells in pregnancy

In the context of pregnancy, circulating γδT cell subsets must not be confused with resident ones. Depending on their location, subsets of γδT cells differ in phenotype and functionality. Following the concept of the different maternal-fetal interfaces, circulating γδT cells may encounter the ST inside the placenta or interact on a systematic level, while decidual γδT cells will interact with the HLA class Ib expressing EVT. Therefore, decidual and circulating γδT cells will be discussed separately in this chapter.

2.2.4.1 γδT cells in the decidua

Pregnancy is a dynamic period in which immune reactions must be tightly orchestrated. While the early decidua is dominated by innate-like immune cells, adaptive immune effectors are the most prevalent DALT population at term (see 2.1.5) [21,63,64,66,67]. Gamma/delta T cells, with their duality, appear to be the ideal mediator in this transition. Furthermore, they are well known to surveil the tissue integrity of frontiers between the organism and the environment [198,201]. Studies reported a higher prevalence (10 % to 30 %) of γδT cells among decidual $CD3^+$ cells compared to the peripheral blood [222,227,240]. These decidual γδT cells, like dNK cells, are either clustered proximate to decidual glands or scattered as intraepithelial lymphocytes [222]. The association of decidual γδT cells and dNK cells to glands might be connected to the invasion of the EVT, as the EVT penetrates not only spiral arteries but also uterine glands [241]. A growing body of evidence attributes a central role to these glands, as they provide nutrients, growth factors, and cytokines during placentation [241–243]. In early human decidua (gw 6 - 12), studies reported between 43 % and 58 % of γδT cells as $V\delta1^+$, about 15 % as $V\delta2^+$, and 27 % to 45 % with unknown Vδ-usage. At term, 80 % of γδT cells are $V\delta1^+$ and 20 % $V\delta2^+$ [222,244]. One study, however, reported a surprisingly high prevalence of $V\delta2^+$ cells (~76 %) in second-trimester decidua [244]. $V\delta2^+$ cells are predominant in the peripheral

blood, and it might be possible that these peripheral cells contaminated the tissue samples. Further investigations are needed to confirm this drastic change in prevalence during mid-pregnancy.

Villous and extravillous trophoblast express CD1d, potentially allowing antigen recognition via the γδTCR [224,245,246]. However, other signals will determine the crosstalk between γδT cells and other participants in the microenvironment of the maternal-fetal interfaces. As described in 2.2.3, γδT cells share the expression of several receptors with NK cells. These receptors might allow decidual γδT cells to interact with the EVT. NKG2D, for example, is widely expressed by γδT cells regardless of their origin or pregnancy status and could recognize MHC class I polypeptide-related sequence A/B (MICA/B), which is expressed and secreted by the EVT [222,247]. To date, the expression of essential receptors targeting the unique HLA profile of the EVT (e.g., KIR, NKG2A/C, or LILR (see 2.3.1)) or other possible signaling pathways of the maternal-fetal interfaces (see 2.3.2) have not been investigated in decidual γδT cells. The expression pattern of these critical molecules would allow an insight into the interplay between decidual γδT cells, the EVT, and other immune cells.

Gamma/delta T cells are known for their cytotoxicity and capacity for strong cytokine production [248,249]. Both aspects are of relevance during pregnancy. Decidual γδT cells have granules positive for perforin, granzyme, granulysin, or FasL, which allow defense against pathogens during pregnancy [248]. On the other hand, their ability for cytokine production will enable them to direct and control implantation, placentation, or immune reactions. Tissue-resident γδT cells are primarily known for their T_{H1} or T_{H17} cytokine profile [124,125]. Murine decidual γδT cells produce TNF-α and IFN-γ at early implantation [250,251]. In the early human decidua (gw 8 - 14), γδT cells produce Transforming Growth Factor (TGF)-β1 and IL-10, also seen in mice at a similar stage of gestation [249–251]. The strong production of TGF-β1 and IL-10 is thought to promote immune tolerance towards the semi-allogeneic fetus by directly suppressing immune effector cells or promoting differentiation of regulatory T cells [249]. Furthermore, these decidual γδT cells also produce IL-6 and IL-1β, but in lower amounts compared to IL-10 or TGF-β1 [249]. These cytokines might have contributed to the almost completed implantation and placentation processes, as both were shown to enhance trophoblast invasion, and IL-6 to support M2 Mϕ polarization in the decidua [252–256].

2.2.4.2 Circulating γδT cells

Peripheral blood γδT cells comprise 1 - 10 % of circulating T cells and are mainly $V\delta2^+$ [222]. When circulating through the intervillous spaces of the placenta, these cells can be in direct contact with the ST. However, as the ST is void of MHC molecules, interactions between γδT and fetal tissues via the classical MHC-TCR pathway cannot occur. As γδT cells are not MHC-restricted, they might be capable of recognizing antigens or communicating with the trophoblast by facilitating other mechanisms. Since trophoblast cells were shown to express the known γδTCR ligand BTN2A1, antigen presentation to $V\delta2^+$ might be possible [257]. However, as the Human protein atlas does not distinguish between the different types of trophoblasts, it remains unclear if this pathway could be relevant for circulating γδT cells. In addition to antigen recognition, another juxtacrine signaling, like immune-checkpoint molecules, could allow γδT cells to interact directly with the ST (see 2.3.2).

During pregnancy, placental tissues release hormones, cytokines, and EV into the bloodstream. These communication pathways can explain the systematic effects of pregnancy on different organ systems, including the immune system. The influence of placental hormones on circulating γδT cells has only been investigated and published for the two steroid hormones progesterone and estrogen. In contrast, data regarding placenta-derived hormones, like human chorionic gonadotropin (hCG) or prolactin, is unavailable. As the name suggests, progesterone is essential for establishing pregnancy (see 2.3.4.1), and almost all peripheral γδT cells of pregnant women express progesterone receptors (PR) [258]. Under its influence, γδT cells produce Progesterone-induced inhibiting factor (PIBF), which was shown to inhibit peripheral NK cell activities. Degranulation of perforin and the secretion of arachidonic acid are blocked in the presence of PIBF [259–262]. The latter is of particular importance since the limited availability of arachidonic acid reduces the production of prostaglandins, which have a role in initiating labor [263]. However, arachidonic acid is the synthesis basis for all prostaglandins and leukotrienes, and as different prostaglandins may have opposing effects, conclusions should be formulated carefully. Furthermore, data suggest that migration of γδT cells is not only influenced by progesterone but also estrogen [263–265].

Different trophoblast tissues were demonstrated to secrete cytokines, like IL-1β, IL-8, IL12p70, IL-13, GM-CSF, M-CSF, Monocyte Chemoattractant Protein-1 (MCP-1), and

RANTES [266,267]. However, if and how these cytokines impact the circulating γδT cell populations is unknown. During pregnancy, the trophoblast releases EV into the maternal circulation. Subclasses of these vesicles are of different origins, functions, or sizes, but the specific definitions are used differently depending on the author and often overlap. Therefore, exosomes, apoptotic bodies, syncytial nuclear aggregates, microvesicles, or others are here summarized as EVs. EVs carry different molecules like microRNAs, proteins, or lipids with metabolic and signaling functions [268]. EVs expressing FasL and TNF-related apoptosis-inducing ligand might induce the apoptosis of specific T cell clones [269]. Furthermore, PD-L1$^+$ or MICA/B$^+$ EVs may affect the activities of PD-1$^+$ or NKG2D$^+$ peripheral NK or γδT cells [270,271]. The relevance of these pathways, however, remains unclear.

Like NK cells, circulating γδT cells differ strongly from decidual ones in phenotype and function. However, several channels allow intercellular communication over distance. Circulating cells may migrate into the decidua or execute systematic effects during pregnancy. Further investigations will be necessary to establish whether the mentioned pathways affect peripheral γδT cells and the potential consequences of these interactions.

2.3 Possible immuno-regulatory mechanisms at the feto-maternal interface

The immune system is often seen as the body's defense against external (bacteria, viruses, parasites) or internal (tumors) threats. However, it would be more precise to summarize its functions as organism-homeostasis, as in addition to defense mechanisms, it takes part in tissue repair, tissue remodeling, or the elimination of debris [75,272,273]. The outcome or direction of an immune reaction depends on several aspects. After target identification, co-stimulatory or -inhibitory factors (immune receptors, cytokines, hormones, etc.) determine whether the activation threshold will be reached. If activated, the signals' symphony directs the activated cells' fate.

Pregnancy creates a unique challenge for the immune system. In addition to the tasks mentioned previously, semi-allogenic tissue must not only be tolerated but supported and guided in growth. Therefore, immune effector cells must prepare the maternal tissue

and provide growth factors. Furthermore, the depth of trophoblast invasion must be controlled [42]. This concept requires precisely regulated activation of the correct immune cells. Since the initial paper of Peter Medawar, several models have been proposed to explain the unique physiological process of allopregnancy. However, several hypotheses (reviewed in [274]) lost relevance or were broadly rejected. This chapter will address the recently discussed possible regulatory mechanisms of the maternal immune system.

2.3.1 Human Leukocyte Antigen Molecules and their Receptors

Human Leukocyte Antigen molecules and their respective receptors control major functions of immune cells. Almost all human cells express organism-specific combinations of HLA class I molecules on their cell surface. These surface structures are essential for self-identification by the immune system.

While T cells will recognize peptides presented by HLA class I or II molecules, NK cells only detect the expression of HLA class I molecules via activating or inhibitory NK cell receptors. The organisms' own HLA signature normally inhibits NK cells, as inhibitory receptors show a higher affinity to HLA molecules [275,276]. However, NK cells will kill target cells that have lost or reduced their HLA surface expression [277]. This can happen in the case of malignancies or due to a viral infection. Consequently, based on the idea that a lost self-target causes NK cell activation, a 'missing self' hypothesis was established.

Although explaining the activity of NK cells against tumors or infected cells, this hypothesis finds its limitation in reproductive immunology. During pregnancy, the trophoblast is devoid of the main classical HLA class Ia molecules (HLA-A/-B), which reduces the chance of a possible attack by cytotoxic T cells. However, the alternative HLA expression pattern of the trophoblast does not lead to NK cell-mediated lysis. It is plausible that the unique HLA expression with dominating HLA class Ib (HLA-E/-F/-G) prevents lysis and further coordinates maternal tissue remodeling, placental growth, and trophoblast-specific immune tolerance. These class Ib molecules can be recognized by several classes of receptors (KIR, NKG2, and LILR), which will be discussed in the following sections.

2.3.1.1 Human Leukocyte Antigen Molecules in Human Reproduction

The pre-implantation blastocyst's trophoblast already expresses HLA-E and HLA-G, and HLA-F can be found on the trophoblast's surface from the second trimester onwards [14,15].

Henceforth, all three non-classical HLA molecules can be found on the cell surface of the EVT until term [14]. In addition to its membrane-bound form, HLA-G is also secreted into the maternal circulation. While its concentration is high during the first trimester, it decreases with the progression of pregnancy [13]. This placenta-specific expression pattern of MHC molecules indicates their relevance during pregnancy. This is further emphasized by murine data showing tolerance towards tumors with paternal MHC signature during pregnancy but post-partum tumor rejection [278]. HLA molecules are major immune regulators and can be divided into three classes. They are best known for presenting previously processed peptides to αβT cells, which rely on this mechanism in their priming and activation process (MHC restriction). Furthermore, they are critical organism identifiers, which allow the immune system to maintain the body's integrity. The gene cluster for human MHC molecules is located on the short arm of chromosome 6 (6p21) [279].

HLA class I molecules are encoded by several genes within the MHC class I locus. These genes are further divided into a classical (Ia) and a non-classical (Ib) group. MHC class Ia molecules are broadly expressed and show tremendous variability. In contrast, oligomorphic molecules of class Ib are expressed under specific circumstances [280]. Besides three HLA molecules (HLA-E, -F, and G), MHC class Ib genes encode for other proteins (MICA/B, CD1, UL16-binding proteins, MR1) with immune modulatory properties [207,224,281–288]. HLA class I molecules are heterodimers of a cell membrane-anchored α-chain containing three extracellular domains (α1, α2, α3) and β2-Microglobulin. These complexes present fragments of intracellular proteins to cytotoxic T cells ($CD3^+/CD8^+$). Although antigen presentation is the most prominent function of HLA class Ia molecules, they also serve as ligands for different groups of NK cell and Leukocyte receptors [289–291]. Especially, the recognition of HLA class Ia molecules by KIRs (see 2.3.1.3) led to the establishment of the 'missing self'-hypothesis [277].

Although the HLA molecules of class Ib also bind peptides and present them on the cell surface, this is not considered their primary function. These molecules instead serve as ligands for inhibitory and activating receptors on immune cells [292].

However, in the case of HLA-E, the peptide binding behavior is noteworthy. HLA-E presents specific signal sequences from other HLA class I molecules (HLA-A, -B, -C, -G) on the cell surface [293–295]. It is expressed in almost all tissues at the mRNA level, and it is assumed that HLA-E surface expression depends on the presence of the mentioned HLA molecules [296]. Although only limited data exists, in addition to its membrane-bound form, soluble HLA-E (sHLA-E) can be found in the serum [297]. HLA-E can be recognized by immune cells via the NKG2 receptors NKG2A, NKG2B, and NKG2C (see 2.3.1.3). While binding to NKG2A or NKG2B leads to inhibition, NKG2C-binding activates NK cells [298–300]. Although both inhibitory and activating receptors for HLA-E exist, its predominant effect seems to be the inhibition of effector cells. Therefore, blocking anti-NKG2A antibodies were developed to treat HLA-E^+ tumors [301,302].

HLA-F, on the other hand, is widely expressed intracellularly and can be detected on different immune cell lines on the cell surface upon activation [320]. HLA-F binds other HLA class I molecules and is assumed to support their transport within cells [321]. Furthermore, HLA-F can serve as a ligand of different KIRs and LILRs [304–306].

HLA-G is the most intensely investigated class Ib molecule. As a consequence of alternative splicing, it exists in membrane-bound or soluble forms. If a particular isoform is soluble or membrane-bound depends on the removal of intron 4 in the splicing process. If intron 4 remains in the mRNA, the anchoring transmembrane domain will not be translated, and an sHLA-G molecule will be translated [322,323]. These sHLA-G isoforms have been detected in the culture medium of embryos and the serum of pregnant women throughout pregnancy [324–327]. Besides presenting endogenous antigens, HLA-G can be bound via several immune cell receptors, like KIR2DL4 (see 2.3.1.2), LILR subfamily members B1 and B2 (see 2.3.1.4), as well as CD160 [313]. Depending on the cell type, receptor, or isoform of HLA-G, the consequence of the binding may alter.

Table 4 Major Histocompatibility complex classes

			expressed by			Ligand		known/assumed functions				known binding receptor
			maternal tissues	APCs	trophoblast	peptides	lipids	missing-self hypothesis	antigen presentation	secreted molecules	Tolerance promotion	
MHC class I	**Ia)**	HLA-A	X			X		X [289]	X			TCR, CD8, KIR [289]
		HLA-B	X			X		X [290,291]	X			TCR, CD8, KIR [290,291]
		HLA-C	X		X [303]	X		X [291]	X			TCR, CD8, KIR [291]
	Ib)	HLA-E			X [299]	X		(X)	(X)	X [297]	?	TCR, NKG2 [298,299]
		HLA-F			X [14]	X		(X)				TCR?, KIR [304], LILR [305,306]
		HLA-G			X [307]	X		(X)	X [308]	X [76,309,310]	X [109,311]	TCR, KIR2DL4 [166], LILR [312], CD160 [313]
		(HLA-K)[314]	*pseudogene*									
		(HLA-L) [314]	*pseudogene*									
		MICA/B				none						*NKG2D* [282]
		CD1		X [283]	X [224]	X [284]	X [207,208]		X [207,208,284]			*γδTCR* [207–209]*, unknown NK and invariant NKT cell receptor* [288]
		ULBP				none						*NKG2D* [315]
		MR1				X			X			*γδTCR* [206]*, TCRs of mucosa-associated invariant T cells* [316]
MHC class II		HLA-DP		X					X			TCR, CD4, NKp44 [235]
		HLA-DQ		X					X			TCR, CD4, LAG-3 [317]
		HLA-DR		X					X			TCR, CD4, LAG-3 [317], FCRL6 [318]
		HLA-DM		X								*major HLA class II molecules* [319]
		HLA-DO		X								*major HLA class II molecules* [319]
MHC class III [279]			· Tumor Necrosis Factor family members (TNF-α, Lymphotoxin α, Lymphotoxin β) · Heat shock proteins (HSP1L, HSP1A, HSP1B) · Complement factors (C2, C4, Complement Factor B) · Transmembrane receptors (RAGE, NOTCH4)									

HLA-G is almost exclusively expressed in the decidua by the EVT [328]. However, some decidual stromal cells are HLA-G^{+}, too. The HLA-G expression of these cells might be

induced by IFN-γ or IL-10 [329]. Due to the localization of HLA-G expression and its oligomorphism, it was seen as a good candidate for regulating immune tolerance toward the fetus. In short, three functions of HLA-G were suggested.

First, HLA-G supports trophoblast invasion and remodeling of spiral arteries via the production of angiogenic cytokines by decidual Mϕ and NK cells [73,75,330,331]. These functions are likely consequences of HLA-G binding KIR2DL4 leading to Nuclear Factor κ-light-chain-enhancer of activated B cells (NFκB) activation in NK cells, which promotes angiogenesis (see 2.3.1.2) [76,309,310,332].

Secondly, HLA-G promotes tolerance through regulatory T cell expansion (see 2.3.3) and inhibition of fetus-directed cytotoxicity [71,333,334]. However, it is unclear whether HLA-G directly promotes tolerance or enhances the surface expression of HLA-E, which inhibits NK cells' cytotoxicity [335–337]. Additionally, homozygotes with *HLA-G*1015*, a 'null'-allele, were reported. Their sole existence questions the importance of HLA-G for pregnancy success [338]. If HLA-G was the key to protecting the fetus from NK cell lysis, why is it not expressed by the ST? It remains unclear how the fetus or the fetus-derived trophoblast is protected from an attack of maternal immune effector cells. In this context, the role of HLA-G expression by the trophoblast seems to be more complex.

Third, augmented fetal growth through the secretion of growth factors by decidual immune cells [9,10].

In contrast, major HLA class II molecules (HLA-DP, -DQ, and -DR) are only expressed on the surface of APCs and T cells, and HLA-DM and -DO function intracellularly in APCs as chaperones [339–341]. These molecules present protein fragments of extracellular proteins to T helper cells ($CD3^+/CD4^+$). Although usually not expressed, in cases of chronic inflammation and recurrent spontaneous abortions, HLA class II molecules were found on the trophoblast [342,343].

The MHC class III gene locus was found between the loci of MHC class I and II and encodes for different proteins, some of which are connected to immunological functions (e.g., TNF-α, Heat shock proteins, or complement factors) [279].

In summary, fetal tissues express typical HLA molecules (HLA-A, HLA-B, HLA-C, HLA-E, HLA-DQ, HLA-DP, and HLA-DR), while trophoblast cells, which are in direct contact with maternal tissues, show a distinct expression dependent on the type of

trophoblast [17–19]. The ST and the villous CT are devoid of all HLA molecules. However, the EVT was shown to express HLA-C, HLA-E, HLA-G, and HLA-F, but not adult HLA-A/-B or HLA class II molecules. Additionally, the current data suggests dynamic alterations in the expression and secretion of HLA class Ib molecules. The membrane-bound form of HLA-G can be found on EVTs in all trimesters and even on preimplantation blastocytes [14,15].

As mentioned, sHLA-G can be detected in the serum of pregnant women with the highest concentration in the first trimester and the lowest at term [13,324,327,344]. Despite several studies associating low serum levels of sHLA-G with preeclampsia or intrauterine growth restriction (IUGR), it remains unclear if sHLA-G is eligible to predict these pregnancy disorders [13,326,327,345,346].

In contrast to HLA-G, just little data exists about HLA-E and HLA-F. The EVT expresses both in lower levels. However, their expression intensifies by the second trimester [14]. If and how soluble forms of HLA-E might have implications for pregnancy remains unclear.

2.3.1.2 Killer cell immunoglobulin-like receptors

KIRs (CD158) were initially defined as Killer cell inhibitory receptors. However, as this receptor family also includes activating receptors, the Human Genome Organization Gene Nomenclature Committee changed the meaning of this acronym. The individual subtypes' name indicates the amount of extracellular Ig-like domains by a number plus "D" and the length of the intracellular tail by "S" or "L" for short or long, respectively [347]. The genes encoding for KIRs are located on the long arm of chromosome 19 within the Leukocyte Receptor Complex (19q13.4) 214. Within this cluster, pseudogenes can be found, which are indicated by a "P". While complete sets of genes of other molecule families like HLA (see 2.3.1.1) or NKG2 (see 2.3.1.3) exist in all humans, possessing specific KIR genes is highly individual. So far, about 20 genotypes have been defined. However, it appears that the genes of KIR2DL4 and KIR3DL2/3 are present in all genotypes [349].

Having a short intracellular tail leads to the association with DNAX activating protein 12 (DAP12) and cell activation. In contrast, the long-tailed receptors have two immunoreceptor tyrosine-based inhibitory motives (ITIMs) in their intracellular domain [350].

This receptor family recognizes residues in α1 domain of HLA class I molecules. Although a few KIRs bind some alleles of HLA-A*** or HLA-B†††, among HLA class Ia, the main ligands are HLA-C molecules [290,291,351,352]. Due to their specific epitope for KIRs, these variable HLA-C alleles can be divided into groups HLA-C1‡‡‡ and HLA-C2§§§[349]. While HLA-C1 can be recognized via KIR2DL2/3 and KIR2DS2, HLA-C2 is bound via KIR2DL1 and KIR2DS1 (Figure 4) [275,353].

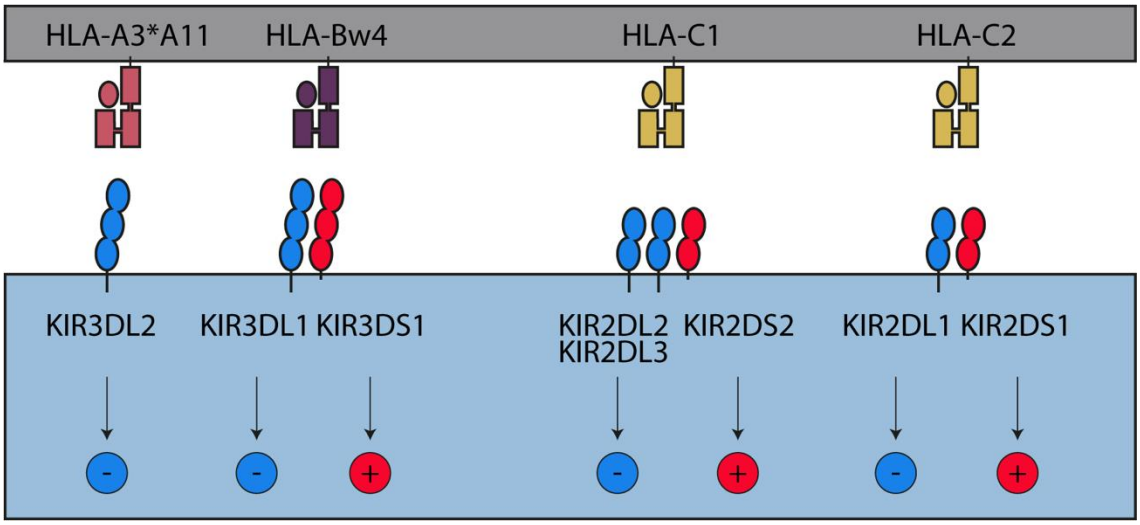

Figure 4 *MHC class Ia-binding KIRs*

KIR subtypes can bind the MHC class Ib molecules HLA-F and HLA-G but not HLA-E. HLA-F is a ligand for KIR3DL1/2, KIR2DS4, KIR3DS1, and KIR2DL4, whereas HLA-G will only be recognized by KIR2DL4 [73,304,309,354].

Among all KIRs, KIR2DL4 holds a unique position. As mentioned above, KIR2DL4 is present in all known KIR genotypes, and its long cytoplasmatic tail only contains one ITIM instead of two [74]. It binds the α1-domain of HLA-G and is, in contrast to all other KIRs, mainly expressed in intracellular $Rab5^+$ endosomes [309,354]. Rajagopalan et al. demonstrated the secretion of proinflammatory and angiogenic cytokines (IFN-γ, IL-1β, IL-6, IL-8, IL-23, Macrophage Inflammatory Protein (MIP)-1-α, and MIP-3-α) of peripheral NK cells after incubation with sHLA-G 177,181. For the activation of NFκB via endocytosed sHLA-G, TNF-receptor-associated-factor 6 (TRAF6) and DNA-

*** KIR3DL2 binds HLA-A*03 and HLA-A*11
††† KIR3DS1 and KIR3DL1 bind HLA-Bw4
‡‡‡ This group includes HLA-C alleles with Ser^{77} and Asn^{80} (Cw1, Cw3, Cw7, Cw8, Cw13, Cw14)
§§§ This group includes HLA-C alleles with Asn^{77} and Lys^{80} (Cw2, Cw4, Cw5, Cw6, Cw17, Cw18)

dependent protein kinases (DNA-PKs) interact with the cytoplasmic tail of KIR2DL4, leading to the phosphorylation of TGF-β-activated kinase 1 (TAK1) and Protein kinase B (PKB), respectively. When activated, both kinases will initiate the NFκB pathway [310,332]. The authors of these studies argue that the produced cytokines can promote remodeling of the spiral arteries in the uterus and support placental formation. These investigations are primarily based on peripheral NK cells, and the demonstration of this mechanism in decidual cells still needs to be verified. However, the intracellular expression of KIR2DL4 of decidual NK cells was already validated [10]. Furthermore, it is still unclear whether the consequences of HLA-F binding to KIR2DL4 are similar or different from those of HLA-G-binding (Figure 5).

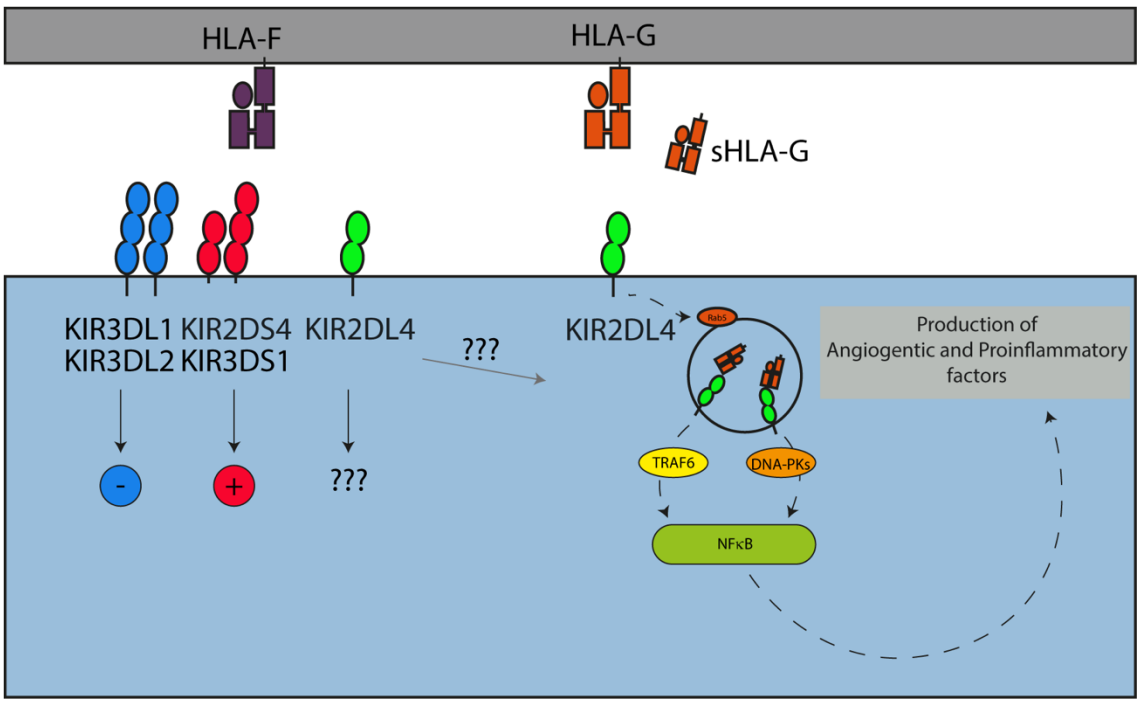

Figure 5 MHC class Ib-binding KIRs

Of note, the field of research regarding KIRs and HLA class I molecules as their ligands is constantly developing. Further KIRs have also been described, and they might be able to bind the HLA molecules mentioned above. However, only a little data about these further connections is available. Therefore, this chapter has focused only on those KIRs where the receptor-ligand relationship has already been thoroughly described.

Several studies aimed to associate KIR and HLA variations with RSA or pre-eclampsia. RSA is defined as the loss of three consecutive miscarriages. In the majority of these cases, an immunological background is assumed [88]. However, there is a disparity among

studies in this field. While some found an association between RSA and activating KIRs [355], others correlated RSA with inactivating KIRs [356–358], and still, others found no association at all [164]. Since not just KIRs themselves but also HLA-C, their main ligands, are highly variable, parental HLA-C haplotypes are obligatory in drawing a complete picture. Unfortunately, this was neglected by the majority of these investigations. The genomic presence of certain KIR type genes, however, does not ensure their expression on the cell surface. Specific KIRs are only expressed by subsets of NK cells. In this context, data about the precise surface expression of respective KIRs in decidual NK cells (-subsets) is insufficient. In the context of infertility diagnostics, the demand for KIR-typing increased in the last decade. Although in-practice reality, only weak evidence supports the relevance of NK cell-related diagnostics or KIR-typing [88].

The correlation between pre-eclampsia and KIR-HLA interactions is less controversial. Pre-eclampsia is characterized by high blood pressure, proteinuria, and edema during pregnancy and can lead to maternal and perinatal death. This condition most likely originates from poor placentation, which leads to endothelial stress and triggers systemic response [23]. Studies indicate that missing activating KIRs could lead to weak dNK cell activation and subsequently insufficient vascular remodeling in the decidua, whereas dNK activation aggravates trophoblast invasion [11,80,359]. However, possible diagnostic or treatment options are not even mentioned in the latest pre-eclampsia guidelines [169].

2.3.1.3 Natural Killer Cell Protein Group 2

The second group of receptors binding HLA class I molecules is NKG2. In contrast to the polymorphic KIR family, these receptors are highly conserved. Upon ligation, they can transmit activating or inhibiting signals. This receptor family contains seven members (NKG2A, B, C, D, E, F, H). While NKG2D (CD314) and NKG2F build homodimers, the other family members heterodimerize with CD94 [360,361]. This group of C-type lectin-like receptors is expressed by subsets of NK cells, $CD8^+$ αβT cells, and γδT cells [362,363]. The family members NKG2A and NKG2B (CD159a) are splicing variants of the same gene. Both variants transmit inhibiting signals via ITIMs localized within their cytoplasmic tail. NKG2C (CD159c) and NKG2E, on the other hand, associate with DAP-10 and transmit activating signals upon ligation [364]. While the family members discussed so far are homologous and recognize HLA-E, NKG2D's

molecular structure differs significantly [365–367]. Therefore, it is unsurprising that NKG2D binds different ligands, namely MICA and MICB. These members of the HLA class Ib gene cluster are typical signs of cell stress and are upregulated in the case of viral infection or DNA damage [368]. Little is known about NKG2F and NKG2H. Therefore, these molecules will not be further discussed in this chapter.

During the first trimester of pregnancy, about 95% of decidual NK cells express NKG2A, similar to the peripheral $CD56^{bright}$ NK cell subset [369]. Decidual immune cells expressing NKG2A could be inhibited by fetal HLA-E. However, recent data from mouse models suggest that the effect of NKG2A is highly determined by NK cell education via maternal HLA-E: The absence of NKG2A in dams leads to abnormal placentation followed by reduced fetal weight and impaired brain development. However, the application of blocking anti-NKG2A antibodies did not lead to an impaired development [370]. The activatory HLA-E-binding receptor NKG2C is only expressed by one-third of first-trimester decidual NK cells, of which all are $NKG2A^+$ [369]. Multigravid women have a five-fold increased rate of these $NKG2C^+$ decidual NK cells. These cells remain in the endometrium and have increased secretion and production of angiogenic factors like IFN-γ or Vascular Endothelial Growth Factor (VEGF)-α. In contrast, the prevalence of decidual T cells expressing NKG2C was constant [371]. Both NKG2A and NKG2C are known to be expressed by peripheral blood Vδ2 cells. However, the expression and effect of these receptors on decidual γδT cells is unknown. Further investigations will be necessary to determine whether and, if so, how interactions of fetal HLA-E and NKG2A/C influence the maternal-fetal microenvironment and their impact on decidual γδT cells.

While NKG2A/C-signaling is straightforward, the downstream pathway of NKG2D appears to be more complex. Although classically seen as an activating receptor on cytotoxic immune cells [372–374], NKG2D-ligation can also inhibit [375]. Recent studies suggest the ligation time as a determining factor, whereas short-term ligation leads to activation, while chronic signaling primes hyporesponsiveness [247,376,377]. As mentioned above, NKG2D binds stress-induced molecules MICA and MICB. Even though NKG2D is largely expressed on γδT cells, Groh et al. demonstrated that intestinal Vδ1 cells are also capable of recognizing MICA/B directly via their γδTCR [378]. This supports

a hypothesis that NKG2D influences the signaling of other receptors (e.g., TCR, Fas, or CD16) rather than acting on its own [375].

During pregnancy, the ST expresses MICA and MICB on its surface and releases $MICA^+$/$MICB^+$ microvesicles into the bloodstream. The high serum levels of MICA/B lead to a general downregulation of NKG2D on cytotoxic cells in the peripheral blood of pregnant women [247]. Today, however, considering the uncertain consequences of NKG2D-ligation, the biological relevance of this downregulation is not clear.

In the decidua, more than 50 % of NK and γδT cells express NKG2D in the first trimester [222,379]. From there, the prevalence of $NKG2D^+$ dNK cells increases from the first to the second trimester [379,380]. Whether the expression density of NKG2D on decidual immune effector cells changes has not been published yet, the precise impact of NKG2D on the maternal-fetal interface remains unclear.

2.3.1.4 Leukocyte immunoglobulin-like receptors (LILRs)

The LILR family (CD85, also known as Immunoglobulin-Like Transcript (ILT)) is encoded downstream of the KIR loci within the Leukocyte Receptor Complex (19q13.4) [381]. The molecular structure of LILRs consists, similar to KIRs, of extracellular Ig-like domains, a transmembrane region, and a cytoplasmatic tail. Inhibitory LILR members with an $ITIM^+$ long cytoplasmatic tail are grouped in LILR subgroup B (=LILRB), while LILR subgroup A (=LILRA) compromises short-tailed LILRs, which might have an activating function [93]. In contrast to their molecular similarities with KIRs, LILRs show low genetic and haplotypic variation [381,382].

LILRs recognize MHC class I molecules. In contrast to KIRs or TCRs, which bind the α1 and α2 domains, LILRs interact with the α3 domain and β2 microglobulin (Figure 6), which might explain their broad specificity to different MHC class I molecules [383,384].

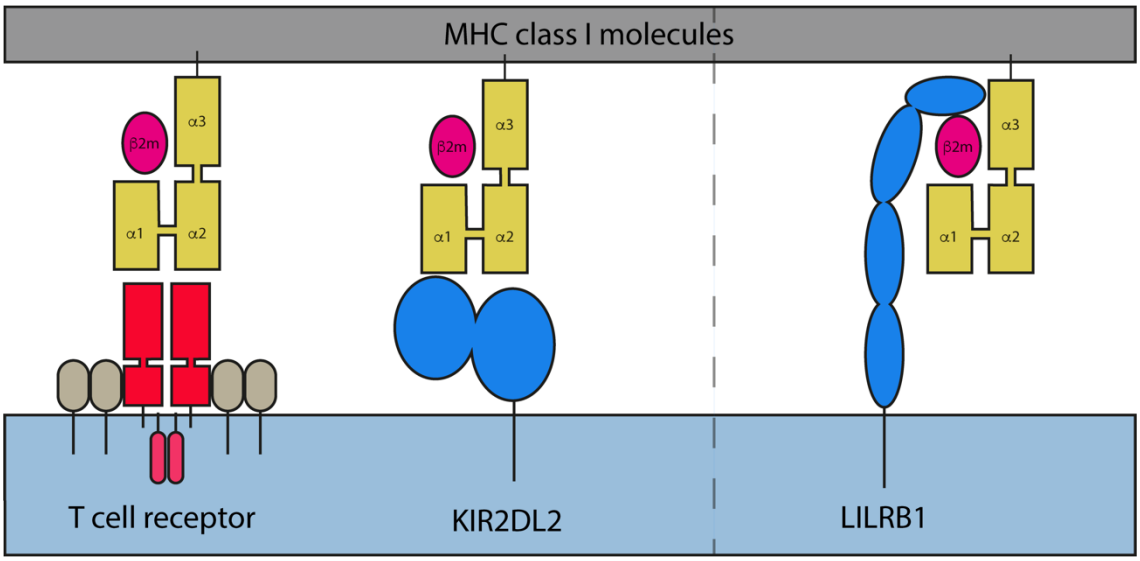

Figure 6 *LILRs bind MHC class I molecules in a distinct fashion.*

LILRs are mainly expressed by myeolomonocytic cells. However, one LILR-family member, LILRB1 (also known as ILT2), is also expressed by T cells, B cells, and NK cells [385–388]. Although the surface expression of ILT2 on T cells varies, it is continuously expressed intracellularly, and if expressed on the cell surface, ILT2-signaling inhibits TCR-mediated activation by dephosphorylating ITAMs of the TCR [386,389]. Furthermore, ILT2 cross-linking on T cells leads to lower IL-2 and IFN-γ but higher IL-10 and TGF-β production [390]. Whereas functional data about ILT2 on human B cells does not exist, it was shown that ILT2 inhibits cell lysis by peripheral NK cells [391].

Although ILT2 interactions with some HLA-A, HLA-B, or HLA-C alleles were reported, it is mainly recognized for reacting with HLA-G. Due to the prominent HLA-G expression of the trophoblast and the high numbers of dNK cells expressing ILT2, it might be another relevant immunomodulatory receptor at the maternal-fetal interface [392,393].

2.3.2 Immune-checkpoint molecules

In addition to the HLA system, other immune molecules were suggested to allow discrimination between self and non-self. These ‘immune checkpoints’ compromise a heterogeneous group of molecules (mainly receptors), which also regulate immune cell activation in a stimulatory (CD27, CD28, CD122, OX40, ICOS, etc.) or inhibitory (PD-1, CTLA-4, TIM-3, SIGLEC7, LAG3, etc.) way.

Consequently, immune checkpoints could be involved in the regulation of immune effector cells at the maternal-fetal interfaces during pregnancy. In this context, PD-1, Cytotoxic T Lymphocyte-associated protein 4 (CTLA-4), and TIM-3 are the most thoroughly investigated of these molecules [168,394]. To date, data about other immune checkpoints or natural cytotoxicity receptors (NCRs) in pregnancy is scarce. Therefore, this chapter focuses only on PD-1, CTLA-4, and TIM-3.

2.3.2.1 Programmed cell death protein 1

The transmembrane receptor PD-1 (CD279) is expressed by lymphoid cells and antigen-presenting cells [168,395]. PD-1's ligands are PD-L1 and PD-L2, which can be expressed in various tissues, including the placenta. The expression of PD-L1 and PD-L2 is affected by the local environment. For example, GM-CSF, VEGF, IFN-γ or TNF-α upregulate PD-L1 expression [396,397]. Upon ligation, PD-1 transmits an inhibitory signal via its cytoplasmatic ITIM, which leads either to apoptosis in antigen-specific T cells or survival in regulatory T cells [398,399].

In human pregnancy, fetal PD-1's ligands could have systemic and local effects, as they are expressed by the ST and EVT, respectively [400,401]. Furthermore, PD-L1/2 are also expressed by maternal decidual stroma cells (PD-L1$^+$/PD-L2$^+$) and decidual Mϕ (PD-L1$^+$). In this context, it was demonstrated that decidual stroma cells might direct T cells to secrete a T_{H2}-like cytokine profile [402,403].

Mouse models generated more data about the impact of PD-1 in pregnancy. Several decidual lymphocyte subsets, including dNK and decidual γδT cells, express PD-1. The administration of anti-PD-L1 blocking antibodies resulted in an increased fetal resorption rate, reduced litter size, T cell infiltration, and complement deposits in the decidua [404]. These results would emphasize the relevance of this immune checkpoint during pregnancy, although investigations in PD-1- or PD-L1-knockout mice did not reproduce similar effects [405]. Further investigations will be necessary to establish the systemic and local impact of PD-1 during pregnancy.

2.3.2.2 Cytotoxic T-Lymphocyte-associated protein 4

The inhibitory receptor CTLA-4 (CD152) can be found intracellularly and extracellularly. Naïve T cells do not express CTLA-4. However, upon activation, T cells exhibit surface expression [406,407].

CTLA-4 binds to CD80 and CD86 (B7.1 and B7.2, respectively) on APCs. These two co-stimulatory ligands are also ligands for CD28, which is an essential co-stimulatory signal in T cells. The binding of CD28 to CD80 or CD86 leads in its consequence to the activation of the Nuclear Factor of activated T cells (NFAT), Activator protein 1 (AP-1), and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB) [408,409]. Together, NFAT, AP-1, and NFκB activate the transcription of several genes for cell growth, differentiation, and proliferation, including IL-2, which is the critical driver of T cell activation [408,409]. As CTLA-4 has a higher affinity for B7 molecules than CD28, it prevents T cell (over)activation by removing possible ligands for this central co-stimulatory molecule [410]. Further inhibition mechanisms via CTLA-4 have been promoted, like the production of indoleamine 2,3-dioxygenase by regulatory T cells or the direct activation of inhibitory pathways. However, these hypotheses would need further confirmation [410–412].

In pregnancy, decidual APCs express CD80 and CD86 similarly to their peripheral counterparts, and a single study demonstrated a correlation between low CTLA-4 expression levels and T_H1-like cytokine production in the decidua.

Several abortion-prone mouse models indicated CTLA-4's impact on pregnancy and demonstrated the importance of CD28-mediated T cell activation [413,414]. Nevertheless, mouse experiments focusing on the direct effects of CTLA-4 provided conflicting results [415,416]. This reflects the data collected from human pregnancy so far. Several attempts were made to correlate CTLA-4 expression or *CTLA-4* polymorphisms to pregnancy complications [417–419]. The data of these studies are conflicting, and concerns have been raised regarding sample size and selection [420]. Despite being a crucial modulator of T cell activation and a key target in other areas of immunological research or therapy, the relevance of CTLA-4 in reproduction remains unclear.

2.3.2.3 T-cell immunoglobulin and mucin-domain containing protein 3

TIM-3 is another inhibitory receptor expressed by multiple T cell subsets, NK cells, dendritic cells, and monocytes [421]. As its name indicates, TIM-3's extracellular section contains a distal IgV domain and a proximal mucin domain [422]. Without a ligand, the conserved cytoplasmic tail binds HLA-B associated transcript 3 (Bat3). Bat3 associates with the catalytically active form of Lymphocyte-specific protein kinase (Lck), which probably brings Lck close to the TCR complex, a key target for this enzyme in T cell

activation [423]. Upon ligand-binding, Bat3 is released, which abrogates the association of Lck to the TCR complex and consequently inhibits cell activation [423,424]. Alternatively, the protein kinase Fyn binds to the same binding site as Bat3. The potential competition between Bat3 and Fyn led to speculations of alternative signaling. Here, Fyn is often connected to T cell anergy [425,426].

With its extracellular IgV domain, TIM-3 binds several ligands. Interestingly, all ligands have their own binding site within the IgV domain. The C-type lectin Gal-9 binds to carbohydrate motifs and can trigger a Ca^{2+} influx and cell death in T_{H1} and T_{H17} cells [423,427]. Although with lower affinity, TIM-3, like the other human TIM molecules (TIM-1 and TIM-4), binds phosphatidylserine [428–430]. The consequences of this binding appear to depend on the cell type. Whereas Mϕ and DCs engulf apoptotic materials, T cells get activated but do not show any sign of phagocytotic activity [431]. Furthermore, high mobility group protein B1 has been identified as a TIM-3-ligand. This protein promotes the uptake of DNA by innate immune cells. Therefore, it was postulated that binding to TIM-3 might interrupt this process and suppresses the innate immune response [432]. Finally, Carcinoembryonic antigen 1 (CEACAM1) is recognized by TIM-3. Like Gal-9, binding this protein leads to the release of Bat3 and can inhibit TCR signaling [433].

During pregnancy, the prevalence of TIM-3^+ peripheral leukocyte populations increases, and decidual lymphocytes express TIM-3 [434–436]. Of the described TIM-3-ligands, only Gal-9 and CEACAM1 have been investigated in the context of pregnancy. Gal-9 is expressed intracellular (nucleus and cytoplasm) and on the cell surface. Furthermore, it is secreted into the extracellular matrix. The female reproductive tract shows a distinct pattern, where Gal-9 is expressed during the window of implantation by glandular cells of the endometrium [437–439]. After implantation, the ST and CT express Gal-9 as well, which would allow interactions with decidual TIM-3^+ cells via this pathway [142,439]. In addition to its expression at maternal-fetal interfaces I and II, Gal-9 levels increase in the peripheral blood during pregnancy. This made the TIM-3-Gal9-axis a target for potential immune therapy during pregnancy. In this context, the administration of recombinant Gal-9 in a rat model for pre-eclampsia improved trophoblast invasion and spiral artery remodeling [440]. CEACAM1, on the other hand, is known to promote TIM-3 surface expression and inhibit T cells [424]. This TIM-3-ligand is expressed by the EVT, dNK, and decidual T cells throughout pregnancy [441]. It has

been hypothesized that CEACAM1 could regulate spiral artery remodeling and trophoblast invasion [442]. As CEACAM1 can also interact with itself, it remains questionable if TIM-3-CEACAM1 or homotypic interaction is the dominant mode of action.

2.3.3 Regulatory T cells

Regulatory T cells (T_{REG}) are an antigen-specific tolerance-promoting T_H cell subpopulation. Their increased prevalence during pregnancy in the periphery and the decidua indicates their relevance in this unique physiological situation [278,443,444]. T_{REG} might recognize paternal antigens and promote selective tolerance allowing microchimerism during pregnancy.

During this time, most T_{REG} express an effector phenotype ($CD4+CD45RA^{-}CD127^{low}FoxP3^{bright}$). Among these effector T_{REG}, the frequency of clonally expanding cells increases locally during pregnancy in the decidua, and by the third semester, they comprise the majority of the resident T_{REG} population. Interestingly, identical clones can be found in the decidua during subsequent pregnancies [445]. In contrast, the peripheral clonal T_{REG} population remains relatively small [445,446]. When focusing on pregnancy-associated pathologies like miscarriage or preeclampsia, the share of decidual effector T_{REG} was found to be significantly reduced, indicating their relevance in healthy pregnancy [445].

Although found in murine models, identification of paternal antigen-specific T_{REG} clones in humans is still pending [447,448]. In this context, major and minor histocompatibility antigens have been considered potential targets [445,449]. In addition to the afore-discussed HLA molecules (see 2.3.1.1), the existence of minor histocompatibility H-Y-antigen-reactive T_C clones without rejection during male-fetus pregnancy indicates the presence of selective antigen-specific tolerance [3]. T_{REG} originate either "**n**aturally" from the thymus directly (nT_{REG}) or can be "**i**nduced" in the peripheral tissues (iT_{REG}). Here, the transcription factor FoxP3 is essential in T_{REG} development, and the conserved non-coding sequence 1, associated with its gene complex, enhances its transcription [450]. This sequence has been shown to be crucial for the formation of iT_{REG} but not nT_{REG}. As it can only be found in Eutheria, one could assume a particular role for iT_{REG} in placentation [451]. However, as nT_{REG} comprise the majority of T_{REG}

during the first trimester, this seems unlikely [446,452]. Nevertheless, taken together with the concept of eutherian pregnancy as a fragmented inflammatory process (see 2.1.4), iT_{REG} may influence the anti-inflammatory period when implantation and placentation are completed.

Although T_{REG} are usually considered as a subpopulation of $CD4^+$ αβT cells, γδT cells were shown to have regulatory capacities as well [453,454]. In 2019, Chang et al. demonstrated that a subset of decidual γδT cells expressing Receptor activator for nuclear factor-κB (RANK) could be transformed into regulatory-like γδT cells by decidual stroma cells expressing RANK-Ligand. These transformed regulatory-like $FoxP3^+$ γδT cells then also started producing TGF-β1. However, it remains unclear whether regulatory-like γδT cells function in an antigen-specific manner or are regulated via other pathways.

2.3.4 Hormones and Cytokine networks

Next to the already discussed – mainly juxtacrine-operating – regulatory mechanisms, both endocrine and paracrine mediators were suggested to influence MIS during pregnancy. These include hormones like progesterone, estrogen, hCG, follicle-stimulating hormone (FSH), luteinizing hormone (LH), or cytokines like Granulocyte-Colony Stimulating Factor (G-CSF), GM-CSF, TNF-α, IFN-γ, and TGF-β. To date, little data is available about further potentially relevant secreted mediators, like chemokines or angiogenic factors. These molecules, however, might impact implantation, maintenance of pregnancy, or labor initiation (see 2.1.4 and 2.1.5.4, respectively) and could gain more recognition in the future. Of note, the previously popular T_{H1}/T_{H2}-hypothesis (thoroughly reviewed in[274]) in pregnancy lost in significance and will not be discussed in this work. However, the basic concept of a complex balance (para-, juxta-, and endocrine) remains relevant when discussing pregnancy in the context of trophoblast invasion or vessel transformation.

2.3.4.1 Progesterone and PIBF

As the name suggests, the steroid hormone progesterone is a crucial regulator of pregnancy [455]. In non-pregnant women, it is produced by the corpus luteum and promotes decidualization by the growth and transformation of endometrial glands,

arteries, and stroma cells. In pregnant women, trophoblast cells start synthesizing progesterone, and the placental production will replace the ovarian one by the second month. Progesterone prevents potentially harmful uterine contractions during pregnancy by reducing the myometrial tonus [456].

In the context of the MIS, it affects gene expression via the nuclear PR-A or PR-B or influences intracellular signaling via membrane PRs (mPR) of immune cells [258,457]. Its relevance is supported by the significantly lower prevalence of PR^+ peripheral blood lymphocytes and lower PR and PIBF expression in the decidua in women with RSA [458,459]. Progesterone binding to its nuclear PRs leads to the expression of PIBF, which was not only shown to be produced by several immune cell types but also directly influences the MIS by binding to the IL-4 receptor [259–261,460]. PIBF inhibits degranulation of cytotoxic molecules and prostaglandin-synthesis in NK cells and favors a change from T_{H1} to T_{H2} cytokine production [259–261,461]. Next to its PIBF-mediated effects, progesterone promotes the generation of T_{REG} in mice, and with the progression of human pregnancy, the prevalence of peripheral mPR^+ T_{REG} increases until it suddenly drops directly before parturition [462,463]. Furthermore, colluding with estrogen and hCG, it influences the migration of immune cells into the decidua, the fetal membranes, and the myometrium.

Taken together, progesterone, partially via PIBF, influences the MIS fundamentally. It is considered to be one of the most crucial promoters of pregnancy. By acting via mPRs, it can rapidly influence immune cells' actions, and when binding to its nuclear receptors, it affects manifold genes programming cells for pregnancy.

2.3.4.2 Estrogens

Estrogens (E1, E2, E3, E4[****]) affect cells by binding cytoplasmatic estrogen receptors (ER) -α/-β, and they are generally known for their effects on endometrial growth and differentiation during the menstrual cycle [464,465]. Furthermore, they seem to promote embryo implantation and trophoblast invasion via angiogenesis [466]. Like with progesterone, trophoblast cells become estrogens' primary source in the first trimester, increasing their serum levels until term [467].

[****] E1: Estrone; E2: 17β-Estradiol; E3: Estriol; E4: Estetrol

Besides the effects on endometrial and vascular tissues, estrogens influence decidual immune cells. E1 and E2 increased the migration of uNK cells and stimulated endometrial cells to secrete chemokine ligand 2 in vitro, which is known to promote vascular formation [468]. Likewise, Mϕ migrate under the influence of estrogen into the decidua. In T cells, estrogen-signaling leads to a higher prevalence of $FoxP3^+$ cells, increases Indoleamine 2,3-dioxygenase expression, and the secretion of TGF-β [469,470]. Furthermore, although not demonstrated with decidual DC, estrogens were shown to inhibit antigen-specific T cell stimulation by DCs.

2.3.4.3 FSH, LH, and hCG

The production and secretion of progesterone and estrogens during the menstrual cycle are controlled by the pituitary hormones FSH and LH. LH is well known for its ovulation-initiating function. After fertilization, hCG is secreted by the blastocyst and after implantation by the ST. Human chorionic gonadotropin binds to the same receptor as LH and thus stimulates steroid synthesis in the ovaries.

Like estrogens, hCG has been shown to increase the prevalence of T_{REG} and NK cells in the endometrium or the early decidua, respectively, and promotes their functions [471–473]. However, as no immune cells express receptors for FSH, LH, or hCG, the described effects are rather due to the increased steroid hormone synthesis [257].

2.3.4.4 G-CSF and GM-CSF

The growth factors G-CSF or GM-CSF†††† are produced in the maternal-fetal unit and are both thought to be beneficial for pregnancy outcomes. While decidual GM-CSF appears to be synthesized by maternal and fetal cells, G-CSF production was only found in maternal ones [474–476].

G-CSF is considered to favor embryo implantation [477,478]. It has been proposed to support the invasion and migration of the G-CSF receptor-expressing trophoblast via the Phosphoinositide 3-kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK) pathways [479,480]. Furthermore, in vitro data show that G-CSF promotes upregulation of β-Integrin on the trophoblast and the disorganization of smooth muscle cells. In vivo, these effects are likely to facilitate trophoblast migration and vascular invasion,

†††† Also known as CSF1 or CSF2, respectively

respectively (Figure 7). Finally, G-CSF was suggested to initiate the local expansion of T_{REG} [481].

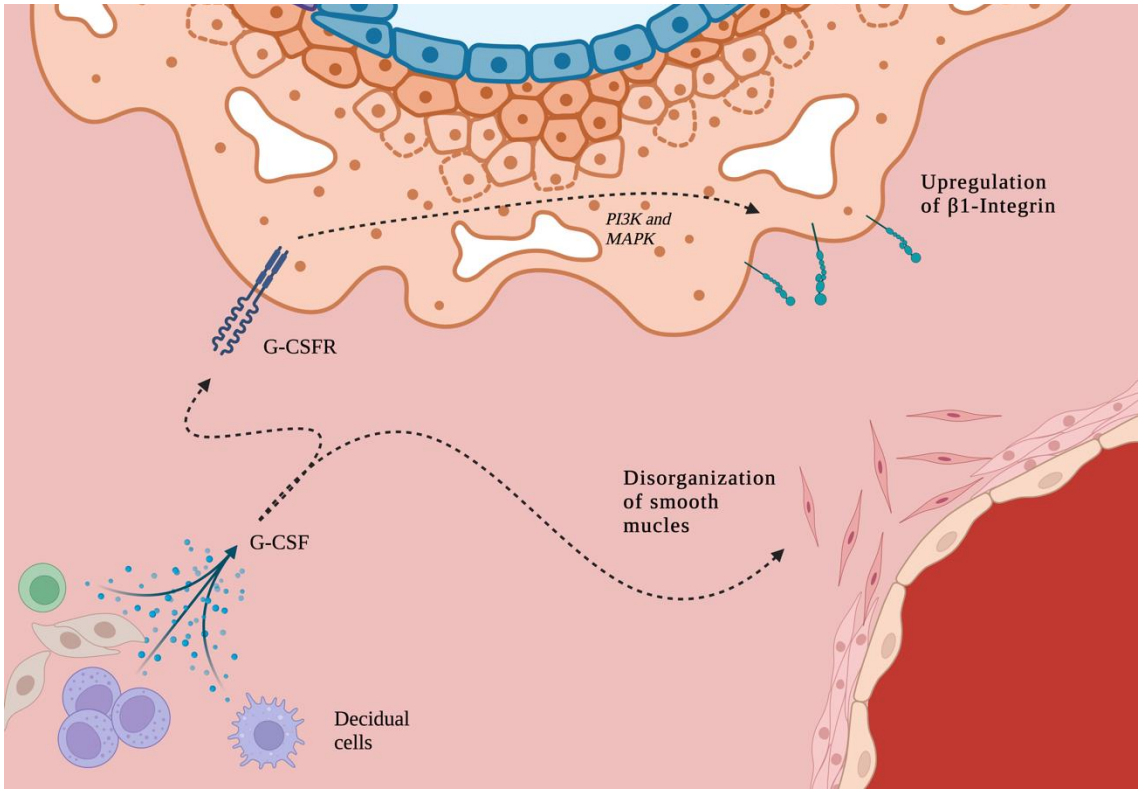

Figure 7 *Effects of G-CSF on trophoblast invasion*

GM-CSF is secreted by the endometrium before implantation and influences the pre-implantation embryo and the local immune network. GM-CSF is mainly produced by glandular cells, which increase their secretion after ovulation [482,483]. Pre-implantation embryos already express GM-CSF receptors, and in murine embryo experiments, GM-CSF was shown to improve embryo development and implantation rate [484,485]. After implantation, trophoblast tissue starts to produce GM-CSF on its own [486]. In addition, dNK cells are another source of GM-CSF in early decidual tissue [486]. Like G-CSF, binding to its receptor influences gene expression via the PI3K and MAPK pathways, but GM-CSF also activates the Janus Kinase/Signal Transduction and Transcription activation (JAK/STAT) pathway [487].

Animal-model experiments with GM-CSF null mutant mice demonstrated the relevance of GM-CSF for proper placentation during mammalian pregnancy [488]. Furthermore, it was shown that GM-CSF influences the maturation of dendritic cells and macrophages in human endometrium [489].

In contrast to the concept of an averted neutrophil invasion in the initial phase of pregnancy (described in 2.1.4), one group described a synergic effect of GM-CSF and IL-8 attracting neutrophils in a small number of primary endometrial cell culture experiments. However, this effect cannot be seen in vivo, as the rate of neutrophils at the maternal-fetal interfaces remains low until the initiation of labor [21]. To explain this contradiction, a deeper understanding of the local cytokine network is necessary.

Both the levels of G-CSF and GM-CSF have been considered factors in subfertility, and especially the mouse data indicates the relevance in placental development disorders, like pre-eclampsia or fetal growth restriction. However, both growth factors are mainly seen as juxtacrine-acting agents, and serum levels are also influenced by other factors like autoimmunity, infection, or lifestyle [490–492]. Few studies also investigated local endometrial GM-CSF in subfertile women but found no significant differences compared to endometrial samples from fertile women [493,494]. Nevertheless, could G-CSF or GM-CSF be a therapeutic option in cases of subfertility or pregnancy-associated disorders? Several clinical trials investigated the clinical potential of systemic or intra-uterine application of recombinant G-CSF or GM-CSF in assisted reproduction. So far, neither could be shown to improve the baby-take-home rate [495–497]. Further investigations will be necessary to understand local communication between fetal trophoblast and maternal decidual tissue. Then, with a correct application protocol and a precise selection of potential patients, influencing local growth factors or other mediators might become a suitable therapeutic option.

3 Aims and Organization of the Study

As mentioned above, next to mere scientific progress, improvement of clinical care by finding diagnostic biomarkers or therapeutical targets is a further goal. Therefore, a deep understanding of physiological processes is necessary. In the context of pregnancy, γδT cells remain under investigated. The objective of this project was to deepen our knowledge about this unique lymphocyte population.

Our study was divided into two phases, referred to as '**I**' or '**II**'. In the first phase (I), we aimed to investigate the cytotoxic potential of peripheral blood γδT cells during pregnancy and its correlation to the $CD56^+$ phenotype of γδT cells via flow cytometry. In the second phase (II), we focused on decidual γδT cells. We explored the capability to detect the trophoblast's unique HLA expression pattern and their potential functions regarding pathogen clearance and implantation. Therefore, we performed a multiparameter flow cytometric analysis and a functional assay with membrane-bound HLA-E/-G and soluble HLA-E/-G to determine the cytokine production of γδT cells.

4 Materials and Methods

4.1 Samples

4.1.1 Participants

I) Peripheral blood samples were obtained from healthy non-pregnant (n = 17) women between 18 and 40 years old from the Regional Blood Transfusion Service, Pécs, Hungary, and at the Department of Medical Microbiology and Immunology, University of Pécs, Medical School, Hungary. Samples from healthy pregnant women (age = 26.1 ± 0.6) in the first (gw = 10 - 12; n = 16), second (gw = 23 -27; n = 17), or third (gw = 34 – 37; n = 17) trimester were acquired at the Department of Obstetrics and Gynecology, University of Pécs, Medical School, Hungary.

II) Decidual tissue samples and matched peripheral blood were obtained from healthy pregnant women between 18 and 35 years old (n = 27, age = 25.9 ± 1.4) undertaking an elective pregnancy termination during the first trimester (gw 6 – 12; n = 27) at the Department of Obstetrics and Gynecology, University of Pécs, Medical School, Hungary.

Our study was reviewed and approved by the local Ethics Committee (5643-PTE 2019), and informed consent was obtained from all participants. The study adhered to the tenets of the most recent revision of the Declaration of Helsinki.

4.1.2 Isolation of Peripheral Blood Mononuclear Cells (I, II)

Peripheral Blood was diluted with phosphate-buffered saline (PBS), and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque™ (GE Healthcare) gradient centrifugation. Hereafter, the cells were washed and either resuspended in inactivated human serum (BIOWEST SAS) supplemented with 10 % dimethyl sulfoxide and frozen at -80 °C for later analysis (I and II) or used for Isolation of non-touched γδT cells (II).

4.1.3 Isolation of Decidual Mononuclear Cells (II)

First, the collected decidual pieces were macroscopically homogenized with scissors (max 1 mm^3) and then digested with type IV collagenase (1 mg/ ml) (Sigma-Aldrich) at

37°C for 1 hour. Hereafter, cells were collected through a 70 µm nylon cell strainer and washed in RPMI1640 medium (Lonza) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza). In the next step, the resuspended cells were filtered through a 40 µm nylon cell strainer, and decidual mononuclear cells (DMCs) were isolated by Ficoll-Paque™ (GE Healthcare) gradient centrifugation. The collected cells were filtered through a 30 µm nylon cell strainer, washed, and resuspended in RPMI1640 medium (Lonza) containing 10 % fetal calf serum (FCS) (Gibco®) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza). The resuspended cells were distributed onto cell culture dishes and incubated for at least 4 hours at 37°C and 5 % CO_2 to allow the remaining decidual stroma cells to settle. Finally, the non-adherent cells were aspirated, washed, and either cryopreserved as described in the previous paragraph or used to isolate non-touched γδT cells.

4.1.4 Isolation of non-touched γδT cells (II)

Untouched peripheral blood and decidual γδT cells were isolated using the 'TCRγ/δ^+ T cell Isolation Kit' (Miltenyi Biotech) according to the manufacturer's instructions (Accessible under the following link: https://static.miltenyibiotec.com/asset/150655405641/document_teq82ir4rt20n6rruhradt267j?content-disposition=inline or upon request by the author).

4.2 Functional Assays

4.2.1 Activation and CD107a Cytotoxic Assay (I)

To determine the cytotoxic potential of the investigated cell population, we analyzed the cell surface expression of CD107a, an essential protein of the lysosomal membrane that becomes externalized upon degranulation of cytotoxic granules. Cells were incubated for 4 h at 37 °C and 5 % CO_2 with the fluorochrome-conjugated anti-CD107a antibodies in RPMI 1640, supplemented with 10 % FCS, penicillin, streptomycin, ionomycin (1 µg/ ml) (Sigma-Aldrich), and phorbol myristate acetate (25 ng/ ml) (Sigma-Aldrich). Before labeling with the other monoclonal antibodies, the cells were

washed and resuspended in RPMI 1640 with 5 % FCS. Finally, the cells were fixed in 1 % paraformaldehyde and evaluated as described in 4.3.1.

4.2.2 Cell co-culture (II)

Three human choriocarcinoma cell lines (JAr) were used as model tissues: A standard JAr cell line (HLA class I^-) and the two transfectants JArE (HLA-E^+) and $JArG_{1m}$ (HLA-G^+).

The freshly isolated non-touched γδT cells were resuspended (10^6 γδT cells/ ml) in RPMI1640 medium (Lonza) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza), 0.1 % pyruvate (Gibco®), geneticin (300 µg/ ml) (Gibco®) and 10 % FCS. Hereafter, they were incubated separately with all cell lines, recombinant sHLA-E (0.5 µg/ mL) or recombinant sHLA-G (0.5 µg/ mL) (both from OriGene Technologies Inc.) or alone overnight. The cells were activated using ionomycin (1 µg/ ml) (Sigma-Aldrich) and phorbol myristate acetate (25 ng/ ml) (Sigma-Aldrich). The cells were centrifuged the next morning, and the supernatant was aspirated. The acquired supernatant was aliquoted and cryopreserved for Cytometric Bead Array or Enzyme-linked Immunosorbent Assay.

4.3 Measurements and Analysis

4.3.1 Flow cytometry (I)

Frozen PBMCs were thawed and washed twice in RPMI 1640 (Lonza) supplemented with penicillin (1 x 10^5 U/L) (Lonza), streptomycin (0.05 g/L) (Lonza), and 10 % FCS. The cells (10^6 cells per tube in 100 µl RPMI 1640 with 5 % FCS) were incubated with fluorochrome-conjugated antibodies (Table 5) for 30 min at 4 °C in the dark. Afterward, the cells were washed with PBS, resuspended with 1 % paraformaldehyde in PBS, and stored in the dark until flow cytometric measurement was performed with a Navios™ Ex (Beckman Coulter) and analyzed by using FlowJo™ version 10.6.1. Compensation matrices were calculated by FlowJo™ using CompBeads (BD™). All gates are based on fluorescence-minus-one controls (FMOs).

Table 5 Fluorochrome-conjugated antibodies used in Phase I

Laser	Target	Fluorophore	Dilution	Host Species	Clone	Company
405 nm	CD107a	Brilliant Violet 421™	1:200	Mouse	H4A3	Sony Biotechnology Inc.
405 nm	CD56	Brilliant Violet 510™	1:100	Mouse	HCD56	Sony Biotechnology Inc.
488 nm	TCRγ/δ	FITC (Fluorescein isothiocyanate)	1:50	Mouse	B1	Sony Biotechnology Inc.
488 nm	PD-1	PE (Phycoerythrin)	1:100	Mouse	EH12.2 H7	Sony Biotechnology Inc.
488 nm	CD4	PE-Dazzle™594	1:50	Mouse	RPA-T4	BioLegend®
488 nm	CD3	PE-Cyanine (Cy)7	1:100	Mouse	SK7	Sony Biotechnology Inc.
638 nm	CD8	Allophycocyanin (APC)-Cy7	1:100	Mouse	SK1	Sony Biotechnology Inc.

4.3.2 Flow cytometry (II)

Frozen PBMCs and DMCs were thawed and washed twice in RPMI 1640 (Lonza) supplemented with DNase (20 µg/mL), penicillin (1 x 10^5 U/L) (Lonza), streptomycin (0.05 g/L) (Lonza), and 10 % FCS. First, the cells (2x10^6 cells per tube) were washed in protein-free PBS, then incubated with ZombieNIR (BioLegend®) in protein-free PBS for 15 min at room temperature in the dark. Second, after washing in PBS supplemented with 5 % FCS (staining buffer), the cells were incubated with fluorochrome-conjugated antibodies for surface antigens in staining buffer for 30 min at room temperature in the dark. Third, after washing in staining buffer, cells were fixed, permeabilized, and stained for intracellular antigens using the Inside Stain Kit (Miltenyi Biotec) according to the manufacturer's instructions. Afterward, the cells were resuspended in 1 % paraformaldehyde in PBS and stored in the dark until flow cytometric measurement was performed with a Navios™ Ex (Beckman Coulter) and analyzed using FlowJo™ version 10.6.1. Compensation matrices were calculated by FlowJo™ using CompBeads (BD™) ™) and MACS® Comp Bead Kit. All gates are based on FMOs. The antibodies and dyes used in this measurement are summarized in Table 6 and Table 7. Due to day-to-day variability and the different fluorophores, we standardized fluorescence intensity data to the individual FMO:

$$Standardized\ Fluorescence\ Intensity\ = \frac{Median_{Population} - Median_{FMO}}{robust\ standard\ deviation_{FMO}}$$

Table 6 Fluorochrome-conjugated antibodies and dyes used in Phase II (Panel A)

Laser	Target	Fluorophore	Dilution	Host Species	Clone	Company
405 nm	Perforin	VioBlue®	1:50	Mouse	dG9	Miltenyi Biotec
405 nm	Vδ2	Brilliant Violet 510™	1:100	Mouse	B6	Sony Biotechnology Inc.
488 nm	TCRγ/δ	FITC (Fluorescein isothiocyanate)	1:50	Mouse	B1	Sony Biotechnology Inc.
488 nm	NKG2C	PE (Phycoerythrin)	1:200	Mouse	S19005E	Sony Biotechnology Inc.
488 nm	CD56	PE-Dazzle™594	1:200	Mouse	HCD56	Sony Biotechnology Inc.
488 nm	Vδ1	PerCP-Vio700	1:200	recombinant Human	REA173	Miltenyi Biotec
488 nm	CD69	PE-Cyanine (Cy)7	1:200	Mouse	FN50	Sony Biotechnology Inc.
638 nm	NKG2A	Allophycocyanin (APC)	1:200	Mouse	S19004C	Sony Biotechnology Inc.
638 nm	Dead cells	ZombieNIR™	1:2000	N/A	N/A	BioLegend®
638 nm	CD45	APC-Cy7	1:200	Mouse	2D1	Sony Biotechnology Inc.

Table 7 Fluorochrome-conjugated antibodies and dyes used in Phase II (Panel B)

Laser	Target	Fluorophore	Dilution	Host Species	Clone	Company
405 nm	Perforin	VioBlue®	1:50	Mouse	dG9	Miltenyi Biotec
405 nm	Vδ2	Brilliant Violet 510™	1:100	Mouse	B6	Sony Biotechnology Inc.
488 nm	TCRγ/δ	FITC (Fluorescein isothiocyanate)	1:50	Mouse	B1	Sony Biotechnology Inc.
488 nm	LILRB1 (CD85j)	PE (Phycoerythrin)	1:50	Mouse	GHI/75	Sony Biotechnology Inc.
488 nm	CD56	PE-Dazzle™594	1:200	Mouse	HCD56	Sony Biotechnology Inc.
488 nm	Vδ1	PerCP-Vio700	1:200	recombinant Human	REA173	Miltenyi Biotec
488 nm	CD69	PE-Cyanine (Cy)7	1:200	Mouse	FN50	Sony Biotechnology Inc.
638 nm	KIR2DL4 (CD158d)	Allophycocyanin (APC)	1:50	recombinant Human	REA768	Miltenyi Biotec
638 nm	Dead cells	ZombieNIR™	1:2000	N/A	N/A	BioLegend®
638 nm	CD45	APC-Cy7	1:200	Mouse	2D1	Sony Biotechnology Inc.

4.3.3 LegendPlex (II)

Diluted (1:10) cell culture supernatants were analyzed for IL-2 (6.5 – 20,000), IL-4 (9.04 – 14,000), IL-10 (5.06 – 14,000), IL-6 (11.58 – 15,000), IL-17A (8.51 – 18,000), TNF-α (12.68 – 12,000), sFas (4.53 – 81,000), sFasL (7.37 – 11,000), IFN-γ (57.13 – 20,000), granzyme A (62.96 – 15,000), granzyme B (24.99 – 52,000), perforin (60.18 – 12,000) and granulysin (175.95 – 57,000) utilizing the Human LEGENDplex™ CD8/NK Panel (Biolegend®, Catalog # 741187) on a Canto 2 flow cytometer (BD Bioscience) according to the manufacturer's instructions (Manufacturer's manual is accessible under

https://d1spbj2x7qk4bg.cloudfront.net/Files/Images/media_assets/pro_detail/datasheets/Hu-CD8-Panel-V02_FK.pdf?v=20240126073053 or upon request from the author).

4.3.4 Millipore Assay (II)

Undiluted cell culture supernatants were analyzed for Angiopoietin-2 (13.7 – 10,000), BMP-9 (2.7 – 2,000), EGF (2.7 – 2,000), Endoglin (13.7 – 20,000), Endothelin-1 (2.7 – 2,000), FGF-1 (13.7 – 10,000), FGF-2 (13.7 – 10,000), Follistatin (27.4 – 10,000), G-CSF (13.7 – 10,000), HB-EGF (1.4 – 1,000), HGF (27.4 – 20,000), IL-8 (1.4 – 1,000), Leptin (137.2 – 100,000), PLGF (6.9 – 1,000), VEGF-A (13.7 – 10,000), VEGF-C (6.9 – 5,000) and VEGF-D (6.9 – 5,000) utilizing the MILLIPLEX® Human Angiogenesis/Growth Factor Magnetic Bead Panel - Cancer Multiplex Assay Enzyme-linked Immunosorbent Assay (Millipore®) according to the manufacturer's instructions (Accessible under the following link: https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/173/950/protocol-hagp1mag12k-mk.pdf or upon request from the author). The parentheses' numbers indicate each cytokine's detection range (pg/ mL).

4.3.5 Statistics

For data comparison, we performed multiple types of statistical analysis using GraphPad Prism 6 (I) or GraphPad Prism 9 (II). We use the Shapiro-Wilk test to check for Gaussian distribution. We used an unpaired-samples t-test for normally distributed data to compare pregnant and non-pregnant donors. Analyses, including more than two groups, were tested using the Kruskal-Wallis Test. To evaluate the relation of two corresponding sets of data from the same donor, we used the paired-samples t-test. In cases where normal distribution could not be assumed, we performed the Mann-Whitney test for unpaired samples and the Wilcoxon test for paired samples. Differences were determined as significant if the *p*-value was equal to or less than 0.05. The level of significance is indicated in the text in parentheses.

5 Results

5.1 Phase I

The here presented results have been published as described in Frontiers in Immunology [238].

5.1.1 A small subset of γδT cells shows CD56 positivity.

In this study, we aimed to investigate peripheral blood γδT cells and demonstrate their expression of CD56 in the course of human pregnancy (Figure 8A1). We found a small population of $CD3^+$ lymphocytes, which was double-positive for γδTCR and CD56 but did not show any alterations in frequency during pregnancy or to the non-pregnant control (Figure 8A2). However, when gating for $CD3^+/CD56^+$ lymphocytes (a typical way to characterize NKT-like cells) (Figure 8B1), roughly half of the cells were $γδTCR^+$ in all groups (Figure 8B2). Finally, we wanted to define the rate of $CD56^+$ cells among γδT cells. Therefore, we defined γδT cells as $CD3^+/γδTCR^+$ lymphocytes and $CD3^+$ but $γδTCR^-$ cells as non-γδT cells. Thereafter, we determined the prevalence of $CD56^+$ cells within those T cell subsets (Figure 8C1). The rate of $CD56^+$ cells was significantly higher in γδT cells compared to non-γδT cells ($CD3^+/γδTCR^-$) in all four groups. This difference was most notably in the 2nd and 3rd trimester (non-pregnant: $p \leq 0.01$, 1st trimester: $p \leq 0.05$, 2nd trimester: $p \leq 0.01$, 3rd trimester: $p \leq 0.01$). Furthermore, $CD56^+$ cells among γδT cells and non-γδT cells were rare in non-pregnant women and during the 1st trimester. However, while the rate of $CD56^+$ cells in non-γδT cells significantly decline in the 2nd and 3rd trimester ($p \leq 0.05$), in γδT cells the percentage of $CD56^+$ cells spiked in the 2nd trimester ($p \leq 0.01$). From there, the rate of $CD56^+$ cells decreased marginally in the 3rd trimester (Figure 8C2).

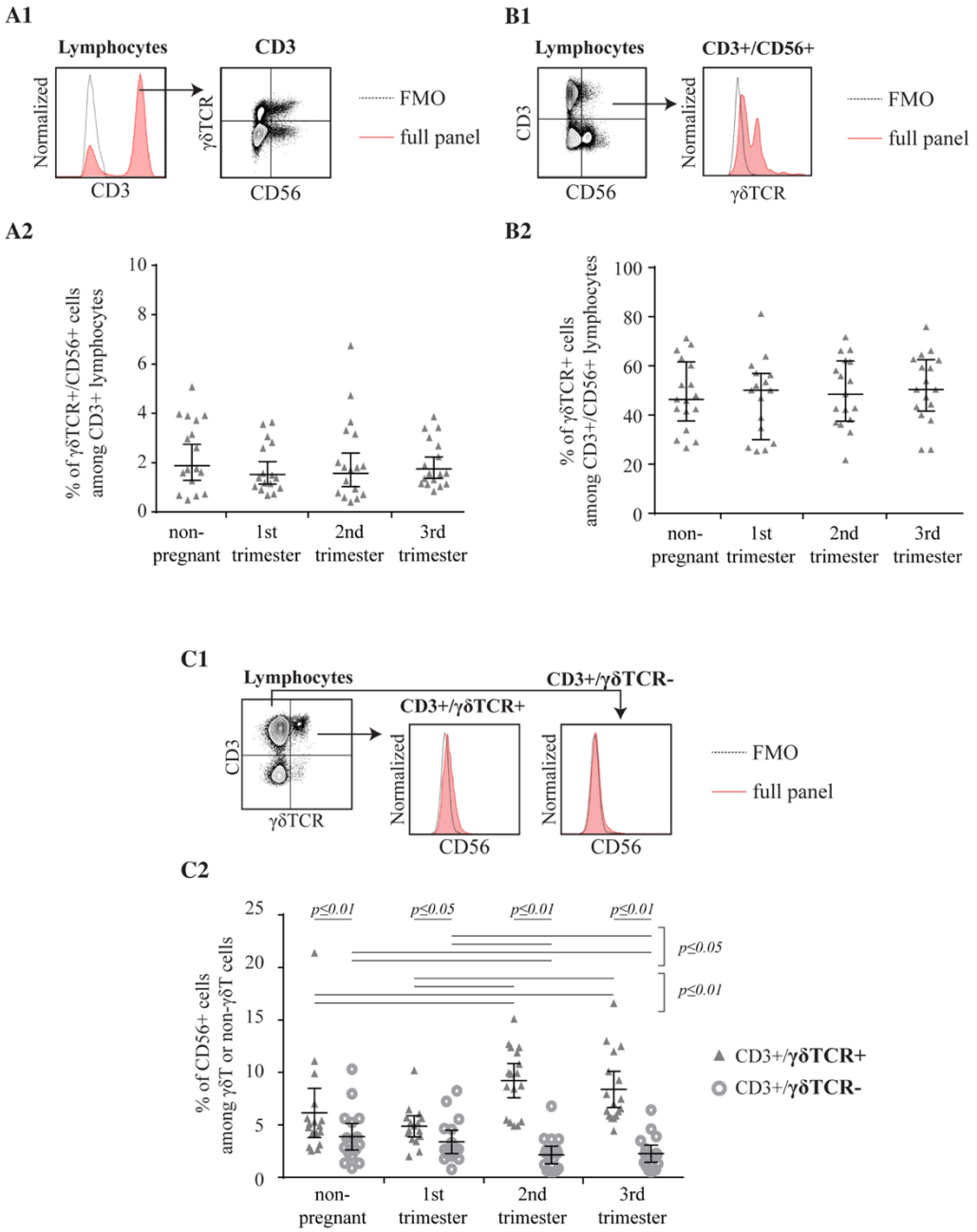

Figure 8 *Representative, shortened gating: First gated for CD3$^+$ cells in a histogram (FMO is depicted by the dotted line) then for CD56 and γδTCR (A1). The mean percentage of CD56$^+$/ γδTCR$^+$ cells among CD3$^+$ peripheral blood lymphocytes of non-pregnant (n=17) and pregnant (1st (n=16), 2nd (n=17) and 3rd (n=17) trimester) women is shown as horizontal line with the 95 % confidence interval represented by whiskers. The individual data points are shown as triangles. Statistical analysis with Kruskal-Wallis test showed no significant alterations between the different groups (A2). Representative, shortened gating: First gated for CD3$^+$/CD56$^+$ cells demonstrated as dot plot then for γδTCR$^+$ cells in a histogram. The FMO is depicted by the dotted line (B1). Mean percentage of γδTCR$^+$ cells among CD3$^+$/CD56$^+$ peripheral blood lymphocytes of non-pregnant (n=17) and pregnant (1st (n=16), 2nd (n=17) and 3rd (n=17) trimester) women depicted as horizontal line with the 95 % confidence interval represented by whiskers. The individual data points are shown as triangles. Statistical analysis with Kruskal-Wallis test showed no significant alterations between the different groups (B2). Representative,*

shortened gating: First gated for CD3$^+$/γδTCR$^+$ or CD3$^+$/γδTCR$^-$ cells demonstrated as dot plot then for CD56$^+$ cells in a histogram. The FMO is depicted by the dotted line (C1). Mean percentage of CD56$^+$ cells among CD3$^+$/γδTCR$^+$ and CD3$^+$/γδTCR$^-$ peripheral blood lymphocytes of non-pregnant (n=17) and pregnant (1st (n=16), 2nd (n=17) and 3rd (n=17) trimester) women depicted as horizontal line with the 95 % confidence interval represented by whiskers. The individual data points are shown as triangles (CD3$^+$/γδTCR$^+$) or circles (CD3$^+$/γδTCR$^-$). Statistical analysis was performed by using the Mann-Whitney test (non-pregnant vs 1st vs 2nd vs 3rd trimester) or the Wilcoxon test (CD3$^+$/γδTCR$^+$ vs CD3$^+$/γδTCR$^-$). Significant differences are depicted by a horizontal line above the respective data sets (C2).

5.1.2 CD56$^+$ γδT cells are predominantly double-negative for CD4 and CD8.

To determine, if the expression of CD56 on γδT cells has an impact on functional aspects of this cell population, we analyzed the expression of CD4 and CD8 on CD56$^+$ compared to CD56$^-$ γδT cells (Figure 9A). Here, we found significant differences in the prevalence of double negative (CD4$^-$/CD8$^-$) and CD4$^+$ cells between these two investigated subsets in all groups. The prevalence of double negative cells was permanently higher in CD56$^+$ γδT cells (all groups: $p \leq 0.01$) (Figure 9B) whereas the prevalence of CD4 was lower compared to CD56$^-$ γδT cells (all groups: $p \leq 0.01$). However, as there was no significant alteration during pregnancy in the CD56$^-$ γδT subset, among CD56$^+$ γδT cells, the prevalence of CD4$^+$ cells was significantly higher in the 1st and 3rd trimester compared to the 2nd trimester ($p \leq 0.01$ and $p \leq 0.05$, respectively) or in non-pregnant control group ($p \leq 0.01$ and $p \leq 0.02$, respectively) (Figure 9C). The prevalence of CD8$^+$ cells was significantly higher among CD56$^+$ compared to CD56$^-$ γδT cells in non-pregnant samples as well as in the 1st and 2nd trimester of pregnancy ($p \leq 0.01$, $p \leq 0.05$ and $p \leq 0.02$, respectively). Furthermore, in both γδT cell subsets, the rate of CD8$^+$ cells was significantly lower in the 1st trimester compared to the non-pregnant control (both: $p \leq 0.05$) (Figure 9D).

Further comparative analysis of the corresponding data of CD4 or CD8 positivity on CD56$^+$ and CD56$^-$ γδT cells revealed that, in each group, the rate of CD8-expressing cells among CD56$^+$ γδT cells was significantly higher than that of CD4$^+$-ones (all groups: $p \leq 0.01$; mean CD4/CD8-ratio of CD56$^+$ γδT cells in non-pregnant, 1st trimester, 2nd trimester and 3rd trimester group, respectively: 0.33, 0.63, 0.31, 0.58). Regarding the CD56$^-$ γδT cells we found the opposite, where in each sample group a significantly (all groups: $p \leq 0.01$) higher rate of CD56$^-$ γδT cells were CD4$^+$, compared with the rate of CD8$^+$ cells among CD56$^-$ γδT cells (mean CD4/CD8-ratio of CD56$^-$

γδT cells in non-pregnant, 1st trimester, 2nd trimester and 3rd trimester group, respectively: 1.3, 1.4, 1.28, 1.49).

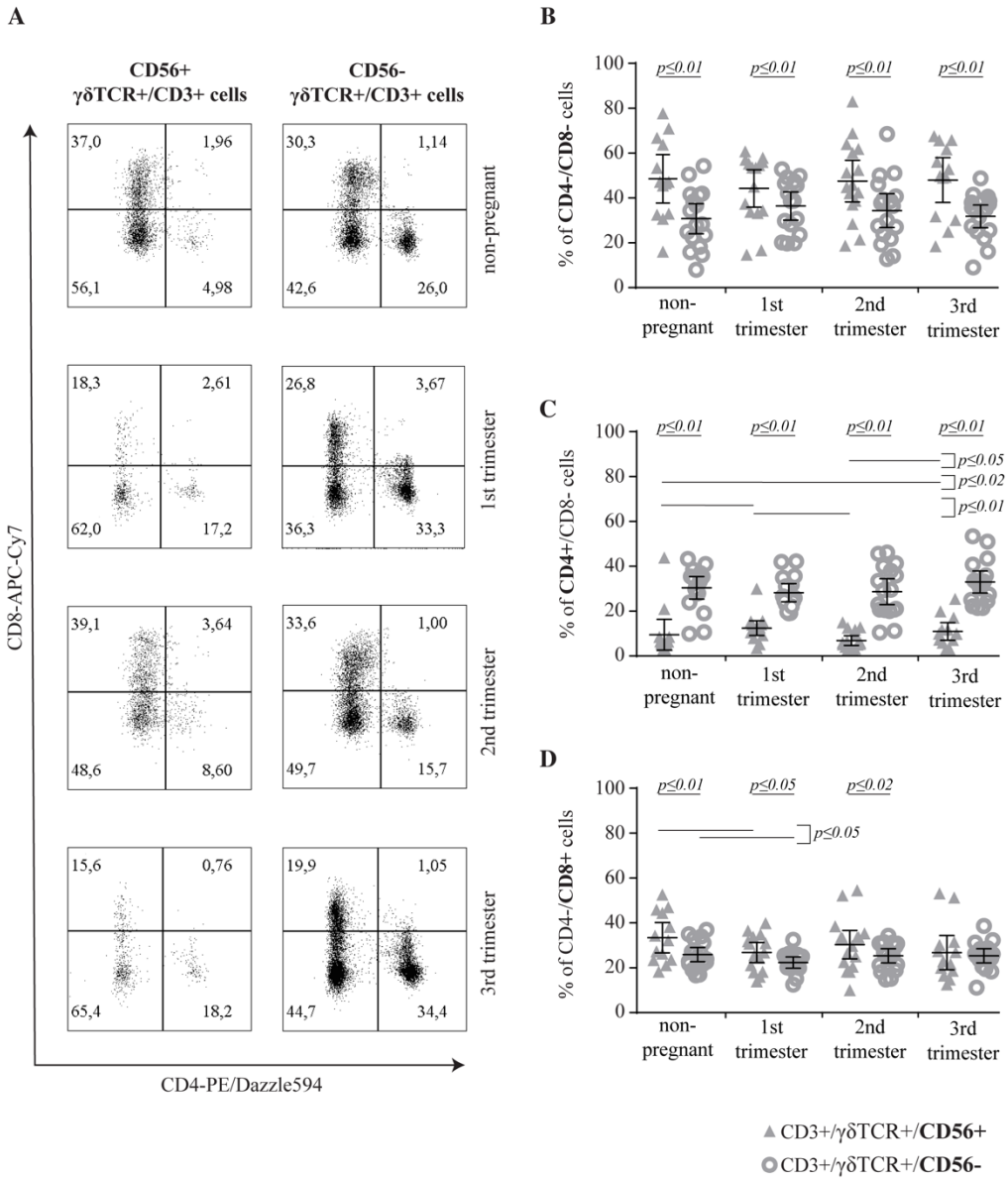

Figure 9 *Representative CD4/CD8 dot plots of peripheral blood CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$) in non-pregnant and pregnant (1st, 2nd and 3rd trimester) women (A) Percentage of CD4$^-$/CD8$^-$ (B), CD4$^+$ (C) and CD8$^+$ (D) cells among CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$) in peripheral blood of non-pregnant (n=17) and pregnant (1st (n=16), 2nd (n=17) and 3rd (n=17) trimester) women. The mean is depicted by a horizontal line with the 95 % confidence interval represented by whiskers. The individual data points are shown as triangles (CD3$^+$/γδTCR$^+$/CD56$^+$) or circles (CD3$^+$/γδTCR$^+$/CD56$^-$). Significant differences between the groups were determined as followed: Panel B: Differences between the CD56$^+$ and CD56$^-$ γδT cell subpopulation were tested by paired-samples-t-test; Panel C and D: Statistical analysis was performed by using the Mann-Whitney test (non-pregnant vs 1st vs 2nd vs 3rd trimester) or the Wilcoxon test (CD3$^+$/γδTCR$^+$/CD56$^+$ vs CD3$^+$/γδTCR$^+$/CD56$^-$). Significant differences are depicted by a horizontal line above the respective data sets.*

5.1.3 $CD56^+$ γδT cells show a strong cytotoxic potential.

The potential for cytotoxic activity of γδT cells was determined by the surface expression of CD107a upon activation (Figure 10A). Although, the rate of $CD107a^+$ cells was significantly higher among $CD56^+$ γδT cells compared to the $CD56^-$ subset in all groups (all groups: $p \leq 0.01$), this rate did not alter considerably during pregnancy. However, the percentage of $CD107a^+$ cells among $CD56^-$ γδT cells was significantly higher in pregnancy compared to the non-pregnant control ($p \leq 0.05$) (Figure 10B). For a better classification of the cytotoxic potential we determined the CD107a-mean fluorescens intensity (MFI) of $CD107a^+$ cells in both γδT subsets. Here, the MFI was significantly higher in the $CD56^+$ γδT subpopulation in all sampled groups (all groups: $p \leq 0.01$). In $CD56^+$ γδT cells, the CD107a-MFI was significantly lower during pregnancy compared to the non-pregnant control (all pregnant groups: $p \leq 0.01$). In pregnancy, the lowest CD107a-MFI was found in the 1^{st} trimester, from there it increased significantly to the 3^{rd} trimester ($p \leq 0.02$). The MFI of $CD56^-$ γδT cells did not alter (Figure 10C).

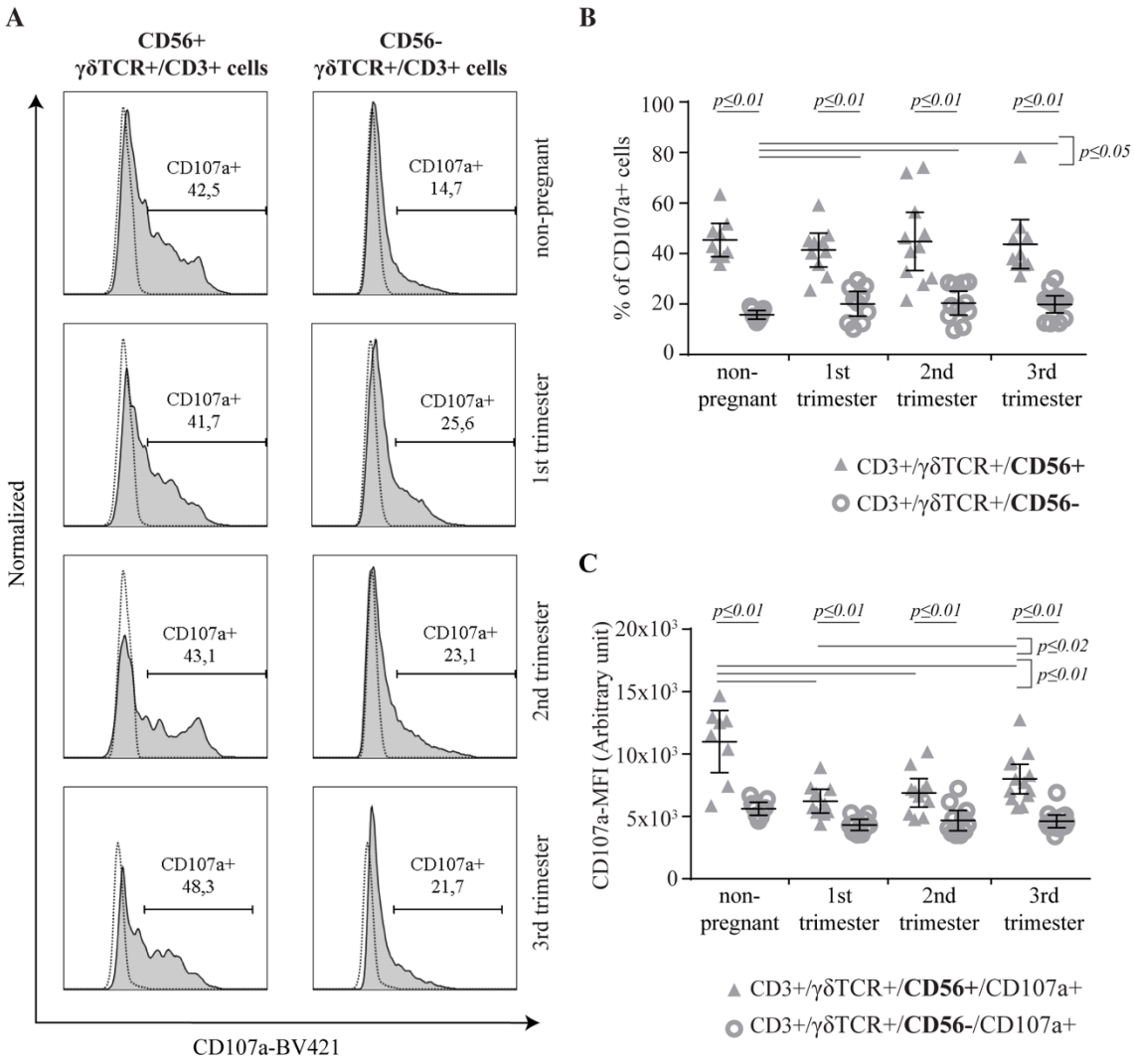

Figure 10 *Representative CD107a histograms (the FMO is depicted by the dotted line) of peripheral blood CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$) in non-pregnant and pregnant (1st, 2nd and 3rd trimester) women (A) Percentage (B) and Mean of Fluorescence Intensity (MFI) (C) of CD107a$^+$ cells within peripheral blood CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$) in non-pregnant (n=9) and pregnant (1st (n=10), 2nd (n=10) and 3rd (n=13) trimester) women. The mean is depicted by a horizontal line with the 95 % confidence interval represented by whiskers. The individual data points are shown as triangles (CD3$^+$/γδTCR$^+$/CD56$^+$ or CD3$^+$/γδTCR$^+$/CD56$^+$/CD107a$^+$, respectively) or circles (CD3$^+$/γδTCR$^+$/CD56$^-$ or CD3$^+$/γδTCR$^+$/CD56$^-$/CD107a$^+$, respectively). Statistical analysis was performed by using the Mann-Whitney test (non-pregnant vs 1st vs 2nd vs 3rd trimester) or the Wilcoxon test (CD3$^+$/γδTCR$^+$/CD56$^+$ vs CD3$^+$/γδTCR$^+$/CD56$^-$). Significant differences are depicted by a horizontal line above the respective data sets.*

5.1.4 The rate of PD-1$^+$ cells is higher in CD56$^+$ γδT cells.

The surface expression of PD-1 was measured on CD56$^+$ or CD56$^-$ γδT cells (Figure 11A). Compared to CD56$^-$ γδT cells, PD1$^+$ cells were significantly more common in

CD56$^+$ γδT cells at all measured timepoints (all groups: $p \leq 0.01$). Within the CD56$^+$ γδT subset, the prevalence of PD-1$^+$ cells increased significantly ($p \leq 0.01$) in the first trimester and fell back to non-pregnant-level in the 2nd and 3rd trimester (both: $p \leq 0.01$). Among CD56$^-$ γδT cells, the prevalence of PD-1$^+$ cells was significantly higher in the 1st and 2nd trimester compared to the non-pregnant control (both: $p \leq 0.01$) (Figure 11B). No significant difference was detected regarding the MFI of PD-1 (Figure 11C).

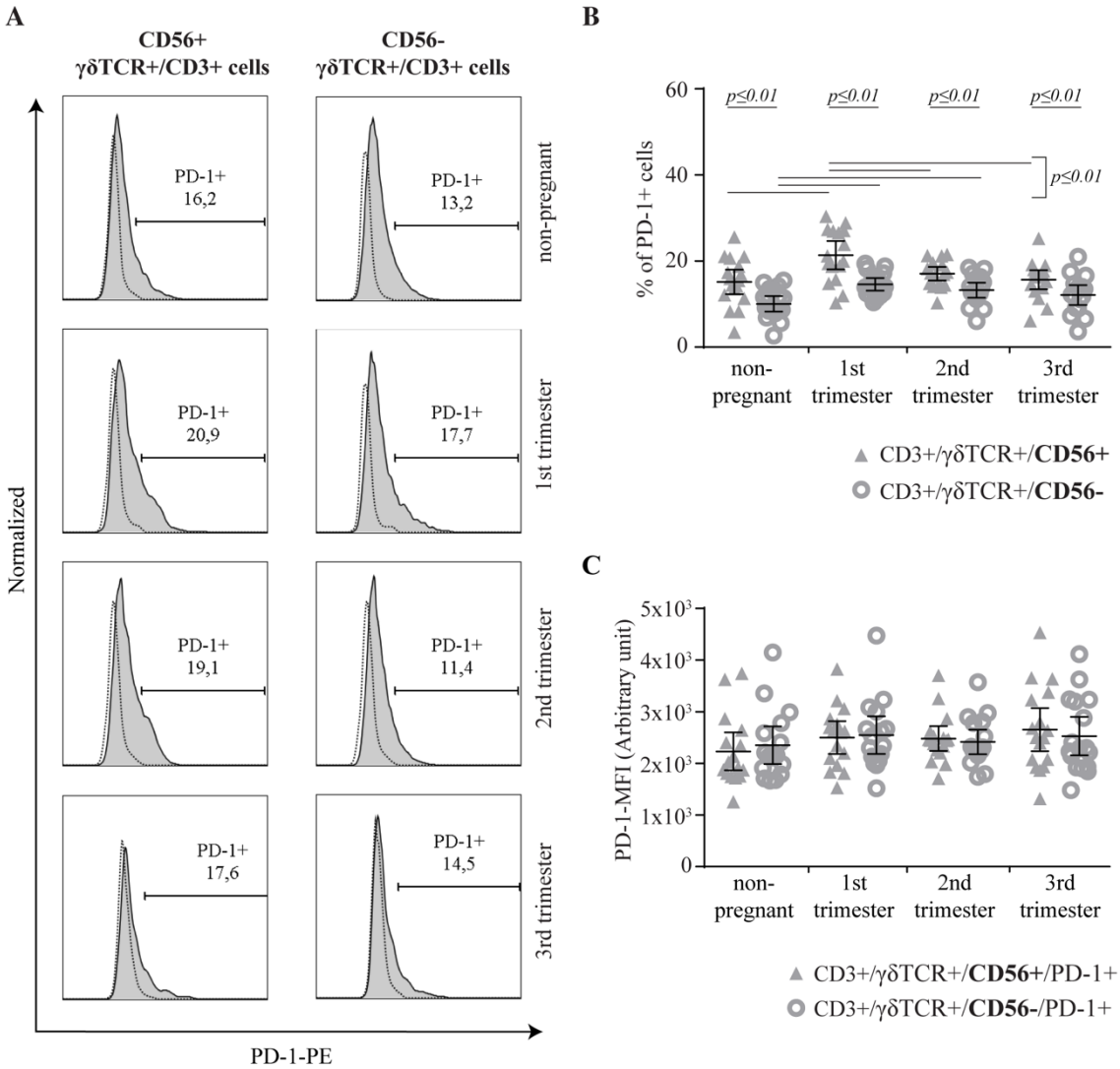

Figure 11 *Representative PD-1 histograms (the FMO is depicted by the dotted line) of peripheral blood CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$) in non-pregnant and pregnant (1st, 2nd and 3rd trimester) women (A)Percentage (B) and PD-1-MFI (C) of PD-1$^+$ cells among peripheral blood CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$) in non-pregnant (n=17) and pregnant (1st (n=16), 2nd (n=17) and 3rd (n=17) trimester) women. The mean is depicted by a horizontal line with the 95 % confidence interval represented by whiskers. The individual data points are shown as triangles (CD3$^+$/γδTCR$^+$/CD56$^+$ or CD3$^+$/γδTCR$^+$/CD56$^+$/PD-1$^+$, respectively) or circles (CD3$^+$/γδTCR$^+$/CD56$^-$ or CD3$^+$/γδTCR$^+$/CD56$^-$/PD-1$^+$, respectively). Statistical analysis was performed by using the paired-samples-t-test to compare data between*

the CD56$^+$ and CD56$^-$ γδT cell population and the unpaired-samples-t-test to compare within the same population but at different timepoints. Significant differences are depicted by a horizontal line above the respective data sets.

5.1.5 The co-expression of PD-1 and CD107a on γδT cells correlates with their CD56 expression.

Here, we aimed to investigate the co-expression of PD-1 and CD107a in CD56$^+$ or CD56$^-$ γδT cells (Figure 12A). No significant difference was observed in the prevalence of PD-1$^+$/CD107a$^-$ cells between CD56$^+$ and CD56$^-$ γδT subsets in any study group. Within both of these subsets, the prevalence of PD-1$^+$/CD107a$^-$ cells was significantly higher in the 1st and 2nd trimester than in the non-pregnant group (CD56$^+$: $p \leq 0.01$ and $p \leq 0.05$, respectively; CD56$^-$: $p \leq 0.01$ and $p \leq 0.02$, respectively). Furthermore, the highest rates were in the 1st trimester, which were also significantly higher compared to the 3rd trimester (both subsets: $p \leq 0.02$) (Figure 12B1). The prevalence of PD-1$^-$/CD107a$^+$ cells was significantly higher among CD56$^+$ than in CD56$^-$ γδT cells in all groups (all groups: $p \leq 0.01$). Among the CD56$^-$ γδT cells the prevalence of PD-1$^-$/CD107a$^+$ cells was higher in the 1st and 3rd trimester of pregnancy compared to the non-pregnant control (both: $p \leq 0.05$) (Figure 12B2). Compared to the CD56$^-$ subset, the prevalence of PD-1$^+$/CD107a$^+$ cells was significantly higher in the CD56$^+$ γδT subset at all timepoints (all groups: $p \leq 0.01$). Within this subset, the prevalence of double-positive cells was significantly lower in the 2nd trimester compared to the non-pregnant group ($p \leq 0.05$). In opposite, among the CD56$^-$ γδT cells, the rate of double-positive cells was the lowest in the non-pregnant group and significantly higher in the 2nd and 3rd trimester (both: $p \leq 0.05$) Figure 12B3).

Further comparative analysis of the corresponding data of PD-1$^+$/CD107a$^+$ and PD-1$^-$/CD107a$^+$ cells in the CD56$^+$ and CD56$^-$ γδT subsets revealed that in both subsets and in all groups γδT cells were rather PD-1$^-$/CD107a$^+$ than PD-1$^+$/CD107a$^+$ (all $p \leq 0.01$) We also calculated the ratio (R) of double- and single-positive cells:

$$R = \frac{PD\text{-}1+/CD107a+}{PD\text{-}1-/CD107a+}$$

When comparing the "R" values of CD56$^+$ and CD56$^-$ γδT subsets (mean R of CD56$^+$ γδT cells in non-pregnant, 1st trimester, 2nd trimester and 3rd trimester group,

respectively: 0.38, 0.37, 0.26, 0.27; mean R of $CD56^-$ γδT cells in non-pregnant, 1st trimester, 2nd trimester and 3rd trimester group, respectively: 0.22, 0.24, 0.26, 0.24) we found that the "R" value was significantly higher in the $CD56^+$ γδT subset in all groups, except in the 2nd trimester, where it was equal to the $CD56^-$ γδT subset (non-pregnant, 1st trimester: $p \leq 0.01$, 3rd trimester: $p \leq 0.02$). In the $CD56^+$ γδT subset, this ratio was significantly lower during the 2nd and 3rd trimester compared to the 1st trimester ($p \leq 0.02$, and $p \leq 0.05$, respectively) and the non-pregnant control ($p \leq 0.01$ and $p \leq 0.05$, respectively). In contrary, in the $CD56^-$ γδT subset, no significant alteration between the different timepoints was found.

For a better understanding of the impact of PD-1 within the cytotoxic γδT cells, we studied the expression of PD-1 on $CD56^+/CD107a^+$ and $CD56^-/CD107a^+$ γδT cells. Here, compared to $CD56^-$ γδT subset, significantly higher rate of cytotoxic $CD56^+$ γδT cells express PD-1 in non-pregnant group ($p \leq 0.01$) and in the 3rd trimester pregnancy ($p \leq 0.05$), whereas in the 1st trimester we found the opposite result ($p \leq 0.01$). The prevalence of PD-1^+ cells in the cytotoxic $CD56^+$ γδT subset was significantly lower in pregnancy than in non-pregnant state (1st trimester: $p \leq 0.01$, 2nd trimester: $p \leq 0.02$, 3rd trimester: $p \leq 0.05$). However, during pregnancy the rate of PD-1^+ cells among the cytotoxic $CD56^+$ γδT cell subset did not alter. Interestingly and in opposite to the cytotoxic $CD56^+$ γδT subset, the cytotoxic $CD56^-$ γδT subset showed a significant increase of PD-1^+ cells in the 1st trimester (non-pregnant: $p \leq 0.01$, 2nd and 3rd trimester: $p \leq 0.05$) (Figure 12C).

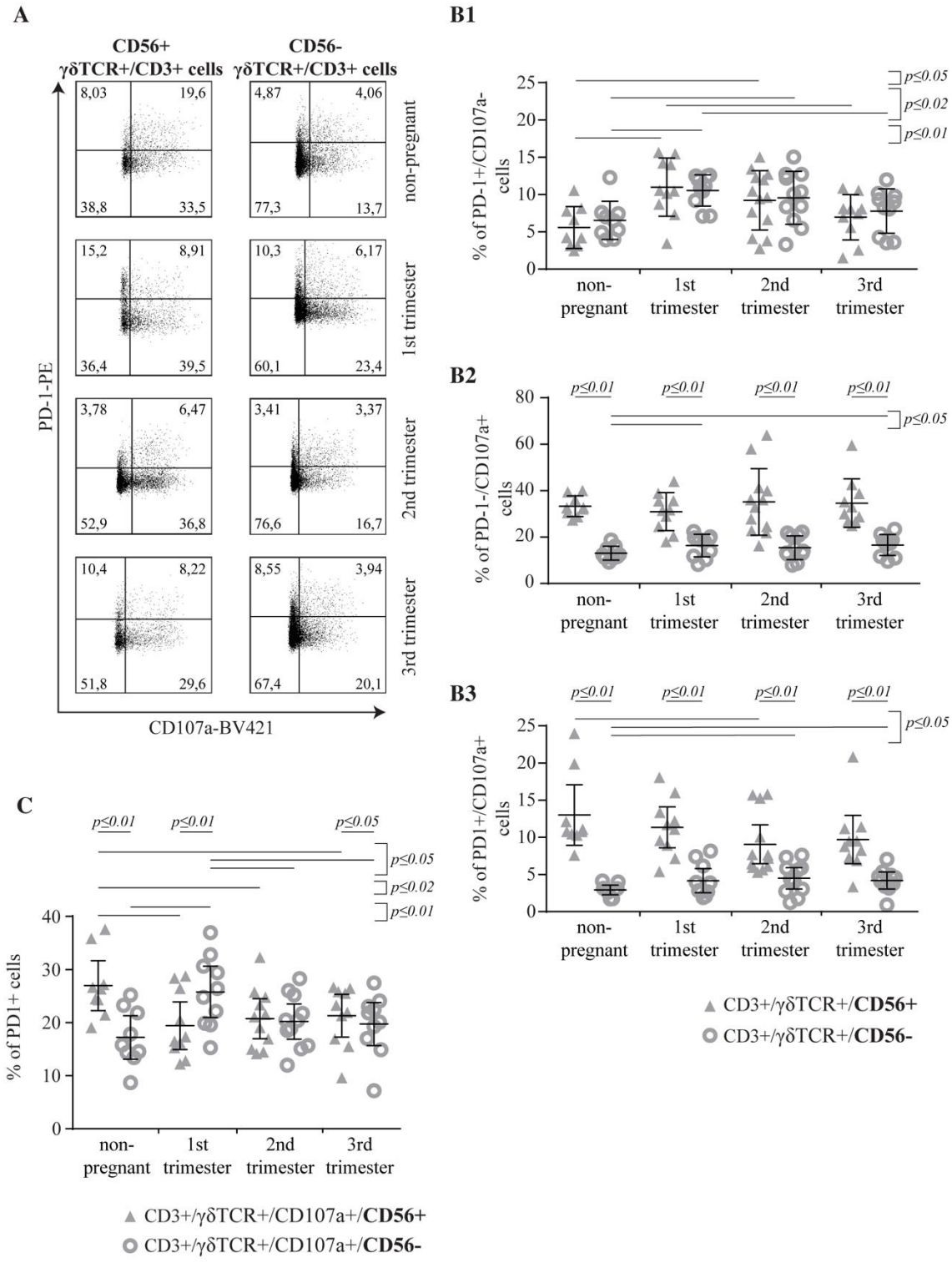

Figure 12 *Representative PD-1/CD107a dot plots of peripheral blood CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$) in non-pregnant and pregnant (1st, 2nd and 3rd trimester) women (A). Rates of PD-1$^+$/CD107a$^-$ (B1), PD-1$^-$/CD107a$^+$ (B2) and PD-1$^+$/CD107a$^+$ (B3) cells among peripheral blood CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$) in non-pregnant (n=9) and pregnant (1st (n=10), 2nd (n=10) and 3rd (n=13) trimester) women (B). Percentage of PD-1$^+$ cells among CD107a$^+$ cells in the peripheral blood CD56$^+$ or CD56$^-$ γδT cells (CD3$^+$/γδTCR$^+$)*

in non-pregnant (n=9) and pregnant (1st (n=10), 2nd (n=10) and 3rd (n=13) trimester) women (C). The means of the data presented in panels B and C are depicted as a horizontal line with the 95 % confidence interval represented by whiskers. The individual data points are shown as triangles ($CD3^+/\gamma\delta TCR^+/CD56^+$ or $CD3^+/\gamma\delta TCR^+/CD107a^+/CD56^+$, respectively) or circles ($CD3^+/\gamma\delta TCR^+/CD56^-$ or $CD3^+/\gamma\delta TCR^+/CD107a^+/CD56^-$, respectively). Statistical analysis was performed by using the paired-samples-t-test to compare data between the $CD56^+$ and $CD56^-$ γδT cell population and the unpaired-samples-t-test to compare within the same population but at different timepoints. Significant differences are depicted by a horizontal line above the respective data sets.

To determine, whether PD-1 expression is related to the intensity of the cytotoxic potential, we finally analyzed the CD107a-MFI on $PD\text{-}1^+$ versus $PD\text{-}1^-$ $CD56^+/CD107a^+$ and $CD56^-/CD107a^+$ γδT cells, respectively. Here, the CD107a-MFI-value of CD56+/CD107a+/PD-1+ γδT cells was significantly higher in all groups (non-pregnant, 1st trimester: $p \leq 0.02$; 2nd trimester, 3rd trimester: $p \leq 0.01$) (Figure 13A). This correlation was also significant in $CD56^-/CD107a^+/PD1^+$ γδT cells (all groups: $p \leq 0.01$), where among the $PD\text{-}1^+$ cells the CD107a-MFI was significantly lower in the 1st trimester compared to all other groups (non-pregnant, 3rd trimester: $p \leq 0.01$; 2nd trimester: $p \leq 0.05$) (Figure 13B). After statistical comparison of the corresponding data of Figure 13A and Figure 13B, we found a significant higher CD107a-MFI values among $CD56^+$ γδT cells in all groups (all $p \leq 0.01$).

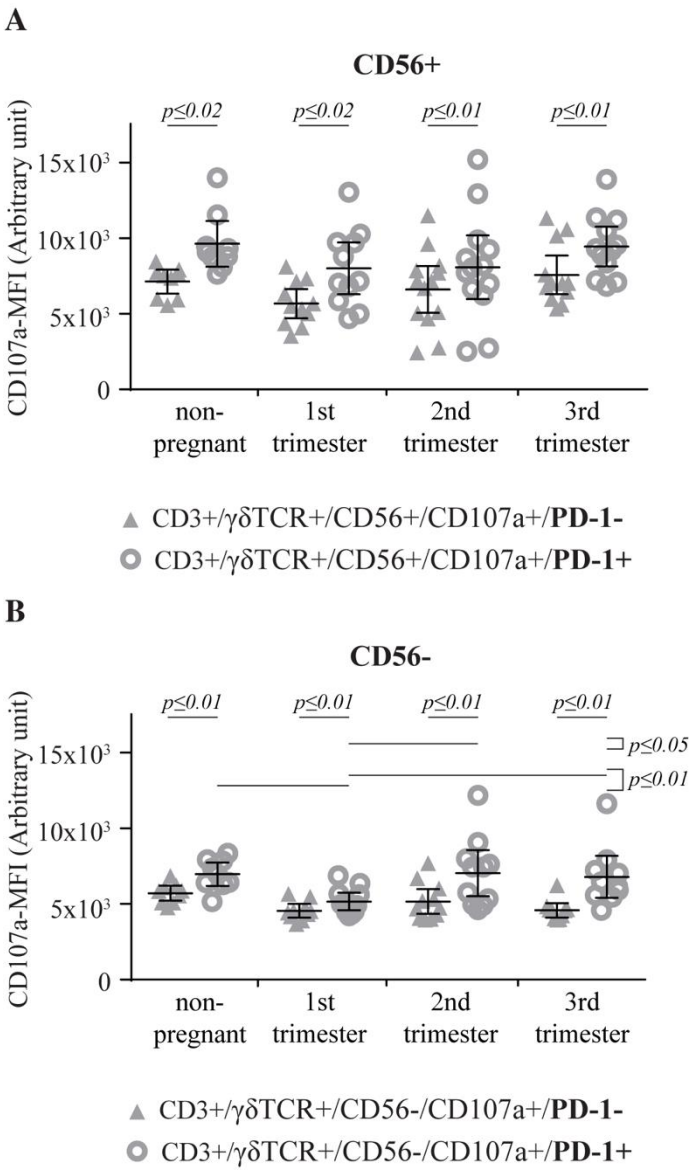

Figure 13 *CD107a-MFI in respect of PD-1 positivity in peripheral blood CD56$^+$(A) or CD56$^–$ (B) γδT cells (CD3$^+$/γδTCR$^+$) in non-pregnant (n=9) and pregnant (1st (n=10), 2nd (n=10) and 3rd (n=13) trimester) women. The means are depicted as a horizontal line with the 95 % confidence interval represented by whiskers. The individual data points are shown as triangles (CD3$^+$/γδTCR$^+$/CD56$^+$/CD107a$^+$/PD-1$^–$ or CD3$^+$/γδTCR$^+$/CD56$^–$/CD107a$^+$/PD-1$^–$, respectively) or circles (CD3$^+$/γδTCR$^+$/CD56$^+$/CD107a$^+$/PD-1$^+$ or CD3$^+$/γδTCR+/CD56–/CD107a$^+$/PD-1$^+$, respectively). Statistical analysis was performed by using the paired-samples-t-test to compare data between the PD-1$^+$ and PD-1$^–$ γδT cell population and the unpaired-samples-t-test to compare within the same population but at different timepoints. Significant differences are depicted by a horizontal line above the respective data sets.*

5.2 Phase II

The here presented results have been published as described in Frontiers in Immunology (Front Immunol), Q1 [498]

This second phase focuses on decidual γδT cells in the first trimester of pregnancy. For analyzing the γδT cell fraction of the decidual mononuclear cells we implemented CD45 as a pan-leukocyte marker and CD69 as a tissue-residency marker. (Figure 14).

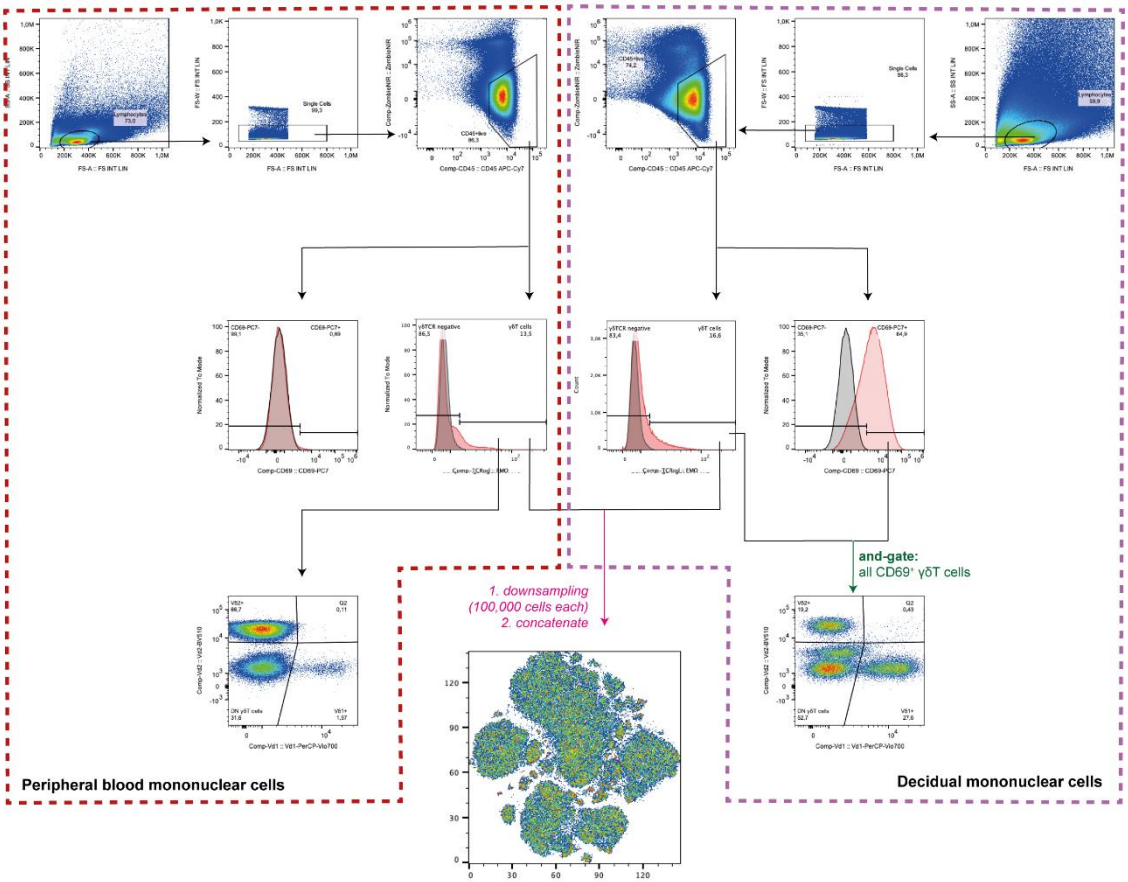

Figure 14 *Global gating strategy for peripheral blood and decidual mononuclear cells: Lymphocytes (FS-A/SS-A) → Single cells (FS-A/FS-W) → CD45+live (CD45-APC/Cy7/ZombieNIR) → γδT cells (TCRγδ-FITC). For dimension reduction, matched γδT cells population from the decidua and the peripheral blood were adjusted to the exact cell count utilizing the DownSampleV3 FlowJo plugin, then concatenated. Hereafter dimension reduction was performed with FlowJo's built-in tSNE algorithm. Gates are based on fluorescence-minus-one (FMO) controls. FMOs are depicted in the single-parameter histograms as a black line. Tissue-resident decidual γδT cells were defined by a Boolean "and"-gate (CD69+ and γδT cells).*

5.2.1 Heterogeneity of peripheral and decidual γδT cells during early pregnancy

To characterize decidual γδT cells and compare them to their circulating counterparts, we utilized the downsampling plugin of FlowJo™ and concatenated previously gated γδT cell populations from DMCs and PBMCs. The tSNE algorithm defined separate clusters with minimal overlap were assigned to decidual or peripheral blood γδT cells, respectively (Figure 15A, B, see also Appendix – Supplementary Figure 1A).

Due to the biological similarities between NK cells and γδT, we investigated the expression of CD56 on γδT cells. While $CD56^{dim}$ expression was detectable in several peripheral blood γδT cell clusters, decidual γδT cells exhibited both $CD56^{dim}$ and $CD5^{6bright}$ phenotypes (Figure 15C, see also Appendix – Supplementary Figure 1B). Nevertheless, $CD56^{+}$ γδT cells are more prevalent in the decidua than in the periphery (Figure 15D, see also Appendix – Supplementary Figure 1C).

Classical γδT cell subsets were associated with distinct clusters. $Vδ1^{+}$ ($CD45^{+}TCRγδ^{+}Vδ1^{+}Vδ2^{-}$) cells were more prevalent in the decidua, while $Vδ2^{+}$ ($CD45^{+}TCRγδ^{+}Vδ1^{-}Vδ2^{+}$) cells were more common among circulating γδT cells. However, double-negative (DN, $CD45^{+}TCRγδ^{+}Vδ1^{-}Vδ2^{-}$) γδT cells were the most common in both decidual and peripheral blood (Figure 15E, F, see also Appendix – Supplementary Figure 1D).

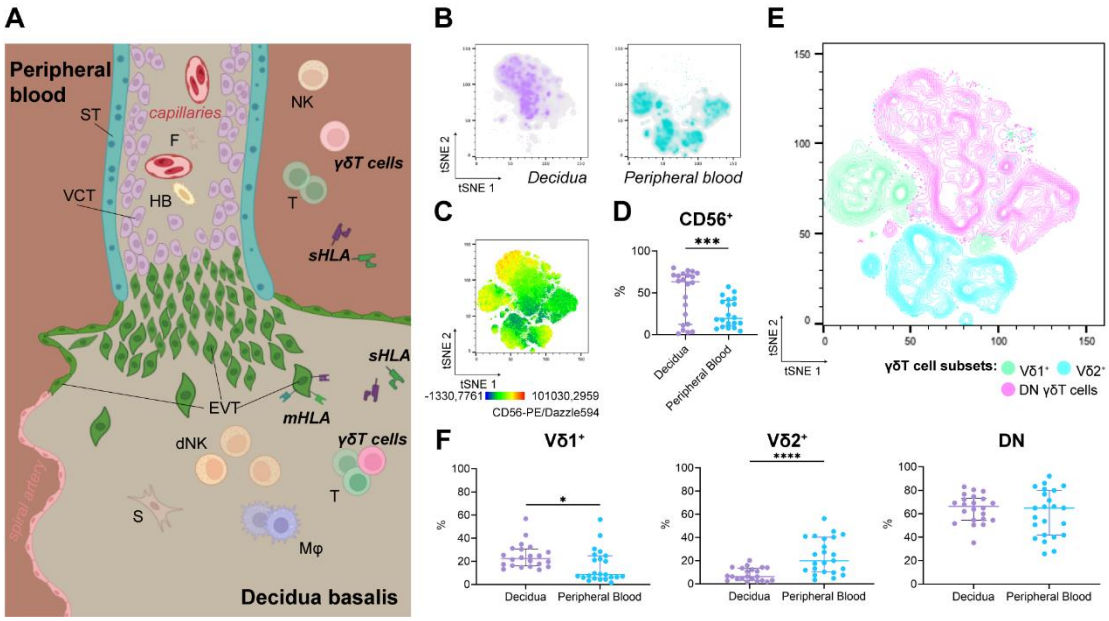

Figure 15 *Gamma/delta T cells at the early feto-maternal interface: Illustration of anatomical structure and spatial relations at the feto-maternal interface (A). Representative isolated depiction of tSNE-clustered decidual (left) and maternal peripheral blood (right) γδT cells as*

*density plots (n=1; Panel A, see also Appendix – Supplementary Figure 1A) (B). Representative tSNE-plot depicting fluorescence intensity of CD56-PE/Dazzle594™ γδT cells from peripheral blood and decidua (n=1; Panel A, see also Appendix – Supplementary Figure 1B) (C). Statistical comparison of CD56+ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23) (see also Appendix – Supplementary Figure 1C) (D). Representative contour plot overlay of Vδ1+, Vδ2+, and DN γδT cells on clustered γδT cells from peripheral blood and decidua (n=1; Panel A, see also Appendix – Supplementary Figure 1D) (E). Statistical comparison of Vδ1+, Vδ2+, and DN γδT cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23) (F). Testing for significance was performed with the Wilcoxon test. *: $p \leq 0.05$, ***: $p \leq 0.001$****: $p \leq 0.0001$; ST: syncytiotrophoblast, VCT: villous trophoblast, F: fibroblast, HB: Hofbauer cell, (d)NK: (decidual) Natural killer cell, T: T cell, s/mHLA: soluble/ membrane-bound Human Leukocyte Antigen class I, EVT: extravillous trophoblast, S: stroma cell, Mφ: Macrophage.*

5.2.2 Decidual γδT subsets express receptors that bind to HLA-E or HLA-G molecules

Using two flow cytometric panels (Figure 14, Table 6 and Table 7), we investigated the prevalence and expression of HLA-E and HLA-G-binding receptors (NKG2C, NKG2A, and ILT2, KIR2DL4, respectively) on γδT cell subsets in the decidua and the matched peripheral blood (Figure 16A). To estimate the expression intensity, we compared the median fluorescence intensity (normalized to the respective FMO) of all investigated receptors. While decidual DN γδT cells exhibited relatively high expression levels for all investigated receptors, decidual Vδ1+ cells showed a more focused expression of the activating NKG2C and the inhibiting ILT2. In contrast, decidual Vδ2+ cells expressed significantly more NKG2A on their cell surface (Figure 16B, see also Appendix – Supplementary Figure 2).

The Prevalence of $NKG2C^+$ cells was generally higher among decidual γδT cells. However, this difference reached the level of significance only in the $V\delta2^+$ and DN subsets. Furthermore, NKG2C positivity was significantly more common in the $V\delta1^+$ subset compared to the $V\delta2^+$ subset (Figure 16C). Likewise, cells expressing the inhibitory counterpart NKG2A were more prevalent in the decidua. While the percentage of $NKG2A^+$ $V\delta2^+$ cells did not differ between decidua and peripheral blood, a significantly higher proportion of DN γδT cells expresses NKG2A and NKG2C in the decidua (Figure 16D).

The inhibitory HLA-G-binding ILT2 was commonly expressed by γδT cells independently of their origin. Generally, ILT2^{+} cells were less prevalent in the Vδ2^{+} subsets. However, when focusing on the prevalence of ILT2^{+} cells within each γδT cell subset, significantly fewer decidual DN γδT expressed ILT2 than their peripheral blood counterpart (Figure 16E). The HLA-G-binding KIR2DL4 was expressed by the majority of γδT cells (Figure 16F).

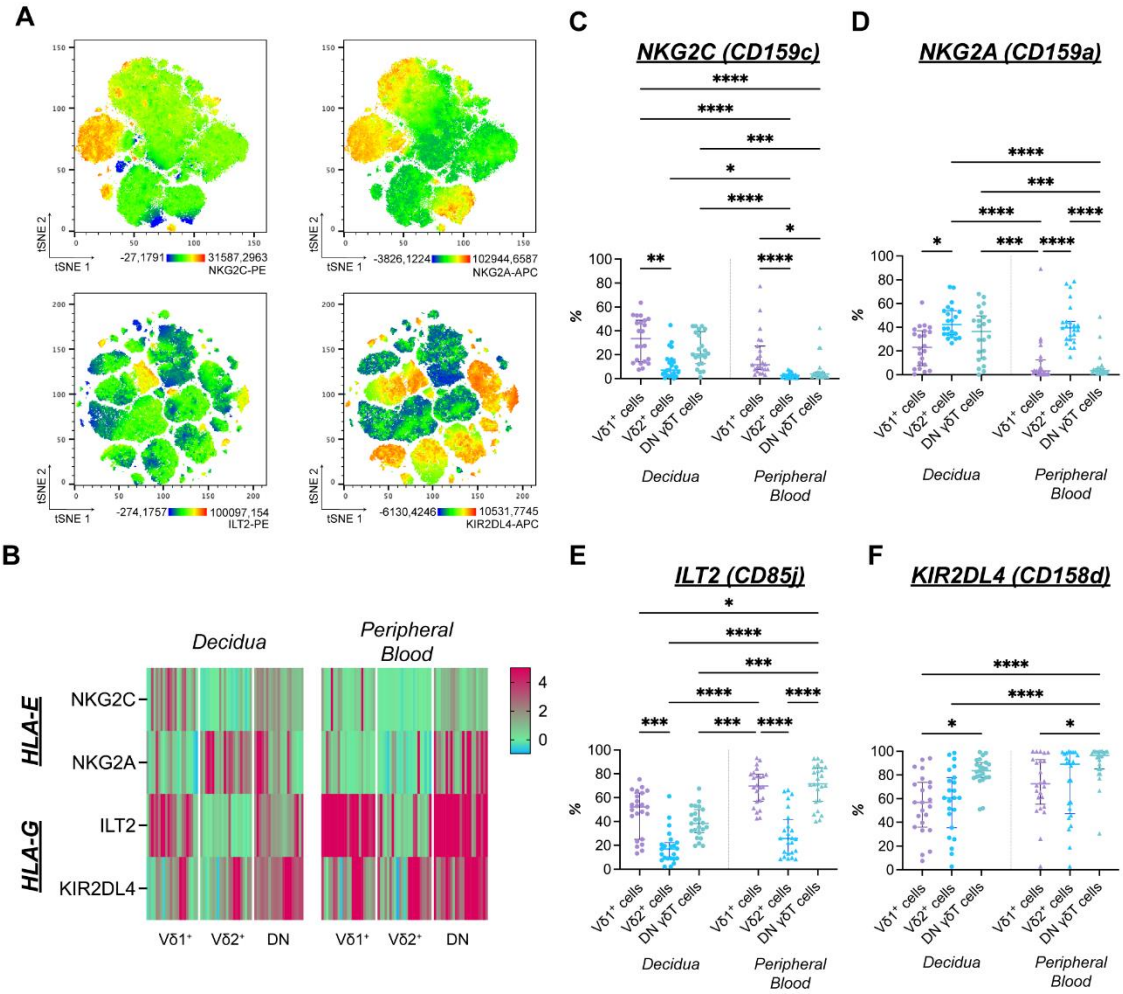

Figure 16 *Expression of HLA-E or HLA-G-binding receptors on γδT cells at the early feto-maternal interface: Expression distribution of NKG2C-PE (upper left), NKG2A-APC (upper right) of flow cytometric Panel A, ILT2-PE (lower left, see also Appendix – Supplementary Figure 1A/D), and KIR2DL4-APC (lower right, see also Appendix – Supplementary Figure 1A/D) of flow cytometric panel B depicted as representative tSNE plots of paired, concatenated decidual and peripheral blood γδT cells (n=1) (A). Heatmap of standardized median fluorescence intensity ([MedianSubset-MedianFMO]/rSDFMO) of NKG2C-PE, NKG2A-APC, ILT2-PE, KIR2DL4-APC on matched decidual and peripheral blood γδT cell subsets (n=22; see also Appendix – Supplementary Figure 2) (B). Statistical comparison of NKG2C^{+} cells'*

*prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23) (C). Statistical comparison of NKG2A$^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23) (D). Statistical comparison of ILT2$^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23) (E). Statistical comparison of KIR2DL4$^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23) (F). Testing for significance was performed with the Wilcoxon test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$****: $p \leq 0.0001$.*

5.2.3 Decidual γδT cells secrete trophoblastotropic cytokines

To determine the functional consequences of the HLA-E or -G recognition by γδT cells, we incubated purified γδT cells with soluble HLA-E or -G (sHLA-E/-G). Furthermore, we utilized human choriocarcinoma cell lines (JAr) transfected with HLA-E or HLA-G1m to investigate more complex interactions of membrane-bound HLA-E or -G (mHLA-E/-G) (Figure 17A).

Vascular transformation by the trophoblast and the local immune environment is crucial to establish a healthy placenta during early pregnancy. Therefore, we analyzed the collected cell co-culture supernatants for potential angiogenic cytokines (Figure 17B, see also Appendix – Supplementary Figure 3). When comparing peripheral blood to decidual γδT cells without HLA molecules ("Control (sHLA)" in Figure 17B), we found significantly higher levels of G-CSF produced by the decidual ones (Figure 17C). Furthermore, decidual γδT cells produced Fibroblast Growth Factor (FGF)-2, whereas no FGF-2 was detected in the wells of peripheral γδT cells (Figure 17D). On the other hand, peripheral blood γδT cells produce small amounts of Epidermal growth factor (EGF), which was not detected in the wells of decidual samples (Figure 17E). While the production of most measured cytokines was not influenced by the presence or absence of HLA-E or -G molecules, incubating mHLA-G with γδT cells, independently from their origin, increased the measured Leptin concentrations (Figure 17F). Additionally, we detected elevated concentrations of Follistatin when incubating peripheral blood γδT cells with mHLA-E. However, compared to all other co-culture wells, the level of significance was not reached (Figure 17G).

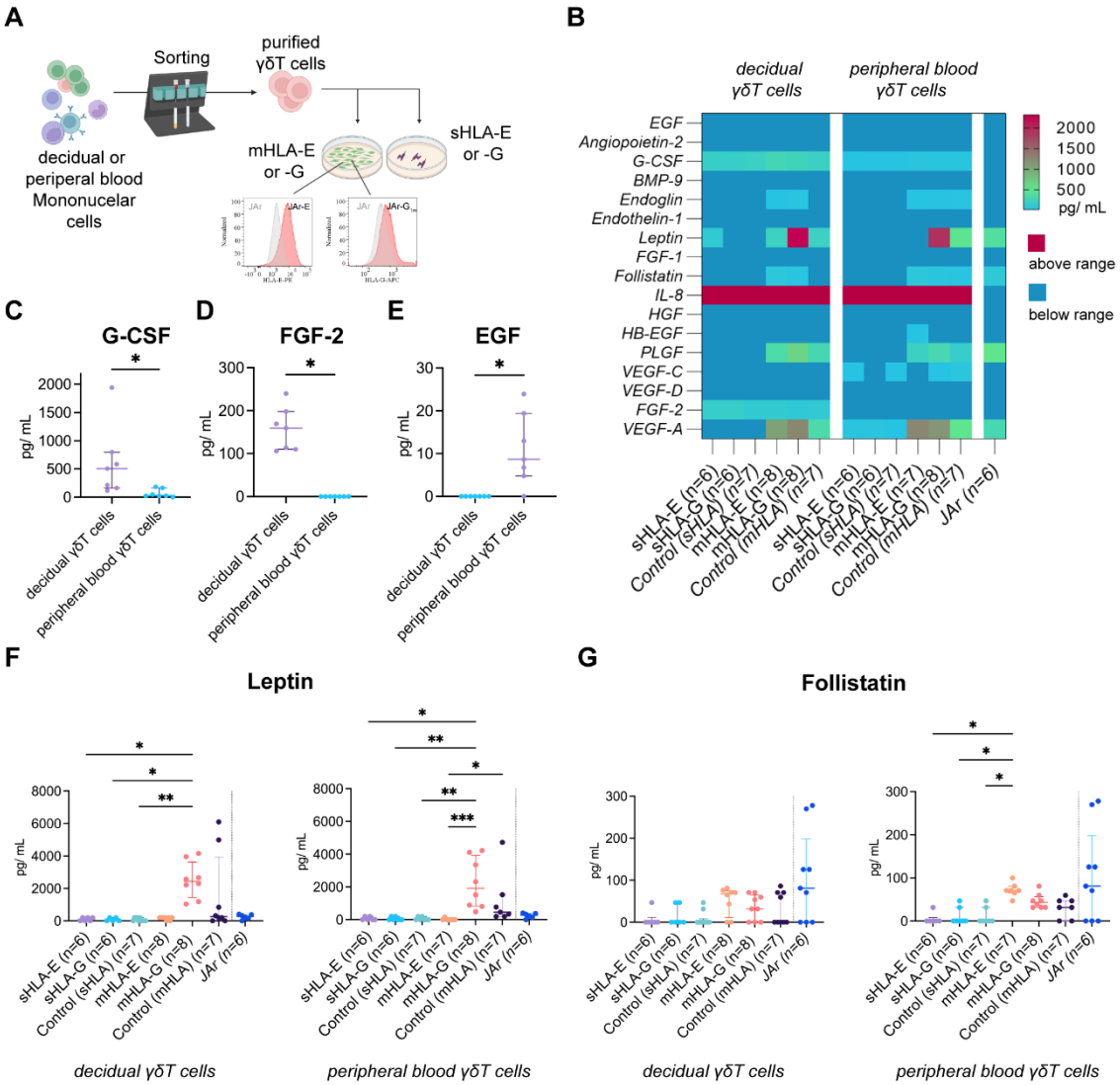

Figure 17 *Angiogenic factor secretion profile of early decidual γδT cells: Illustration of the experimental setup (A). Heatmap depicting the median measured concentration of the respective angiogenic factor after incubating peripheral blood (left) or decidual (right) γδT cells with soluble (s) or membrane-bound (m)HLA-E or -G. The last column depicts measurements from the human choriocarcinoma cell line JAr without γδT cells as an additional control (B, also see SI3). Statistical comparison of G-CSF concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test (C). Statistical comparison of FGF-2 concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test (D). Statistical comparison of EGF concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. (E) Statistical comparison of Leptin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test. (F) Statistical comparison of Follistatin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test (G). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.*

5.2.4 Decidual γδT cells are strong producers of cytotoxic mediators

γδT cells act as first responders in the mucosal defense against pathogens and many frontiers between the body and its environment. Therefore, we also analyzed the intracellular perforin expression to determine each γδT cell subset's cytotoxic potential in the decidua (Figure 18, see also Appendix – Supplementary Figure 4A,B).

Upon interaction with HLA-E or HLA-G, NKG2C, NKG2A, ILT2, and KIR2DL4 are potential regulators of the cytotoxic capability of immune cells. Investigating the perforin content of the different NK receptor-expressing decidual γδT cell populations, we found that the expression of NKG2C and ILT2 was associated with significantly higher levels of intracellular perforin in all decidual subsets. The expression of NKG2A, however, correlated only in the Vδ1+ and DN γδT subset with higher levels of intracellular perforin. A similar, significant relation between KIR2DL4 expression and perforin content was only detectable in the DN γδT cell subset (Figure 18B).

To determine if this hypothetical relationship between cytotoxicity and the expression of HLA class I binding receptors has functional consequences, we analyzed the secretion of typical NK cell cytokines and cytotoxicity-related soluble molecules after exposure to sHLA-E/-G or mHLA-E/-G (Figure 18C, see also Appendix Supplementary Figure 4C-N). However, the measured perforin concentration did not differ significantly (Figure 18D). In addition, we found that decidual γδT cells secrete excessive amounts of granulysin and high levels of interferon-γ (IFN-γ) (Figure 18E).

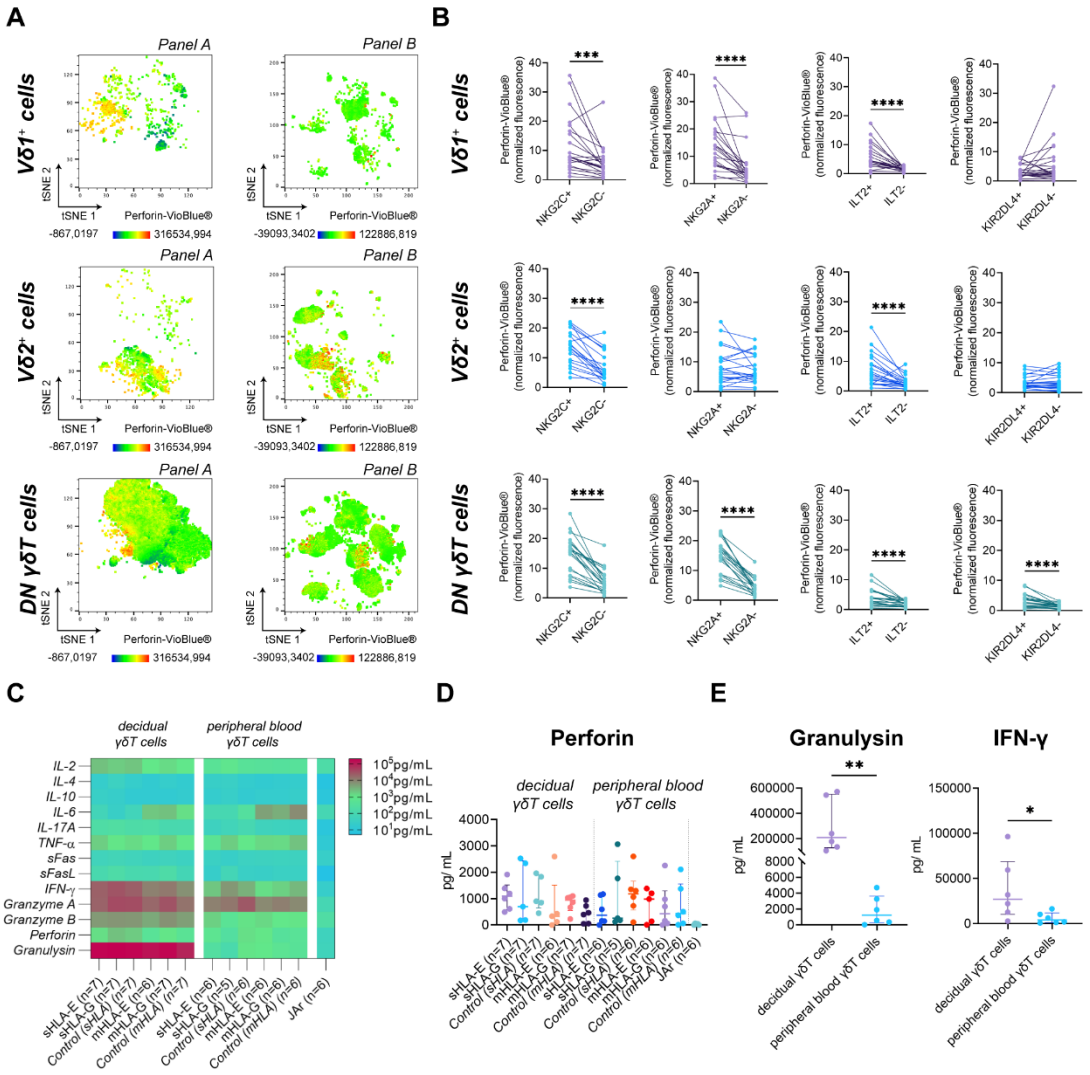

Figure 18 *Cytotoxic potential of early decidual γδT cells:Fluorescence intensity of Perforin-VioBlue® on gated γδT cell subsets from the decidua for the two flow cytometric panels depicted as representative tSNE plots (n=1)(A). Statistical association of standardized Perforin-VioBlue® median fluorescence intensity ([MedianSubset-MedianFMO]/rSDFMO) to the expression of NKG2C, NKG2A, ILT2, and KIR2DL4 on decidual γδT cell subsets (n=22). Testing for significance was performed with the Wilcoxon matched-pairs signed rank test (B). Heatmap depicting the median measured concentration of the respective soluble factor after incubating decidual (left) or peripheral blood (right) γδT cells with soluble (s) or membrane-bound (m) HLA-E or -G (C). Statistical comparison of Perforin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test (D). Statistical comparison of Granulysin (left) and INF-γ (right) concentrations secreted from decidual (n=6) or peripheral blood (n=6) γδT cells in the absence of HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test (E). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.*

6 Theses

1. About half of NKT-like ($CD3^+/CD56^+$) cells express a γδT cell receptor.
2. The $CD56^+$ population of γδT cells expands during the 2nd and 3rd trimesters of pregnancy.
3. $CD56^+$ γδT cells maintain predominantly $CD4^-/CD8^-$ or $CD8^+$ phenotypes during pregnancy.
4. Peripheral blood $CD56^+$ cells represent the cytotoxic fraction of γδT cells.
5. Regarding the potential regulation of γδT cells' cytotoxicity, the prevalence of PD-1^+ peripheral blood γδT cells is increased during the first trimester. This trend is more dominant in more cytotoxic $CD56^+$ γδT cells.
6. Interestingly, in the whole peripheral blood γδT cell population, cytotoxic potential and PD-1 expression are not correlated.
7. $V\delta1^+$, $V\delta2^+$ and DN γδT cells are present in the decidua during early pregnancy. Previous flow cytometric studies neglected the largest DN γδT cell population.
8. All decidual γδT cell subsets harbor cells expressing receptors for HLA-E or HLA-G.
9. Decidual γδT cells secrete G-CSF and FGF-2 independently of HLA-E and HLA-G.
10. The presence of mHLA-G and γδT cells increases measured leptin concentrations.

7 Discussion

Seeing viviparity with the establishment of a hemochorial placenta as a modified inflammatory process allows an understanding of the importance of immune regulation for a successful pregnancy. This project aimed to shed light on γδT cells and their role during human pregnancy, a special T cell fraction showing different phenotypes and functions depending on the location. During pregnancy, these aspects become even more relevant since different maternal-fetal interfaces develop, and interactions between fetal tissues and maternal immune cells depend on the participating cells of both sides. During the first phase of our research, we investigated peripheral blood γδT cells. As these cells circulate through the vascular system of the body, they can encounter the fetal HLA class I^- syncytiotrophoblast in the placenta's intervillous spaces. Due to the absence of HLA class I molecules but the expression of PD-L1/2 on the syncytiotrophoblast, we explored the PD-1 – PD-L1/2 pathway as an alternative communication pathway between fetal tissue and maternal peripheral blood γδT cells. Previous studies linked the dysregulation of immune checkpoints, like PD-1 or CTLA-4, to cases of recurrent spontaneous abortions [165,167,499]. We demonstrated an increased prevalence of PD-1+ γδT cells during the first trimester, which normalized in the 2nd and 3rd trimester. This trend was even more dominant in the NK phenotype $CD56^+$ γδT cell population. Especially in the context of tumor immunology, γδT cells are known for their cytotoxic potential. Here, we demonstrate its association with this NKT-like γδT cell population. In this context, we assumed that PD-1 expression would influence the cytotoxic potential. However, our data did not support this hypothesis. Taken together, our data supports the assumption that immunoregulation via PD-1 can be relevant during the time of implantation and placentation but not necessarily in the context of cytotoxicity. In the second and third trimesters of pregnancy, when the maternal blood shows an elevated presence of peripheral $CD56^+$ γδT cells, there is a concurrent strong expression of PD-L1 on fetal cyto- and syncytiotrophoblast cells within the placenta. This suggests a potential correlation between these phenomena. As pregnancy advances, the blood flow within the maternal placenta can rise significantly, reaching up to 600 ml/min, while the surface area of the syncytiotrophoblast also expands. This dynamic feto-maternal interface becomes a site where maternal $CD56^+$

γδT cells in circulation can encounter fetal antigens. In this context, the interaction between PD-1 and PD-L1 could serve as a crucial regulatory mechanism. Notably, fetal trophoblast cells expressing PD-L1 have the ability to exit the placental structure and enter the maternal bloodstream. Consequently, these fetal cells, which carry the potential to influence immune responses, might have further implications for the maternal immune system.

Practicing medical reproductive specialists are in need of easy-access biomarkers, which provide a clear insight into the immunoregulatory processes at different stages of pregnancy. However, with the understanding of pregnancy as a modified inflammatory process with three distinct phases, it becomes evident that in each phase, specific features are of importance. The first phase of implantation and placentation, controlled trophoblast invasion and vascular remodeling, can be considered as the most important aspects influenced by the immune system. Errors in this phase will impact fertility or lead to pregnancy complications like preeclampsia or IUGR. However, to date, our understanding is still limited. Systemic and local processes must be fully understood before we can aim to use immunomodulatory treatment for infertility. Therefore, we executed the second phase of this study, in which we focused on decidual γδT cells during this early time in pregnancy. Evidently, due to ethical and legal limitations, true early human samples (gw 1-6) are not available. Therefore, we focused on samples from healthy pregnancies during the first trimester.

Decidual γδT cells serve multiple functions within the microenvironment of the maternal-fetal interface located between the decidua basalis and the extravillous trophoblast. They can impact the implantation and placentation process through the secretion of cytokines or by eliminating pathogens. Due to exhibiting innate and adaptive immunity characteristics, γδT cells are often regarded as a link between these two aspects of the immune system. Although there is a more didactic categorization, γδT cells display considerable plasticity, and some subtypes (such as Vγ9Vδ2) exhibit innate behavior, while others (such as Vδ1) manifest adaptive features [202–209]. Because of this, when studying γδT cells in any context, it is crucial to determine the dominant subpopulations before drawing further conclusions.

Our multidimensional analysis demonstrates the difference between tissue-resident and circulating peripheral blood γδT cells. We described three major γδT cell populations: Vδ1+, Vδ2+, and DN γδT cells.

The $Vδ1^+$ subset is known to increase in all tissues that interact with the external environment and is typically considered the "first line defense" [198,378]. Our findings regarding the prevalence of $Vδ1^+$ and $Vδ2^+$ subsets align with previously published flow cytometric data [244]. However, these studies overlooked the largest decidual γδT cell population, which expresses neither the Vδ1 nor the Vδ2 chain. DN γδT cells are a diverse population due to the existence of different variants of Vδ-chains. Nevertheless, a recent study revealed that $Vδ1^+$, $Vδ2^+$, and $Vδ3^+$ γδT cell subsets are the only inhabitants of early human decidua [500,501]. Therefore, DN γδT cells can be considered as $Vδ3^+$ cells in the context of the decidua. The $Vδ3^+$ subset is generally believed to induce antigen-presenting cell maturation, which is crucial for correct antigen presentation during pregnancy [501]. Further investigations are necessary to determine whether decidual $Vδ3^+$ cells can direct antigen-presenting cell maturation toward a tolerance-promoting phenotype. Regrettably, anti-Vδ3 antibodies are not commercially available, and we cannot confirm that the DN subset represents the $Vδ3^+$ one. Consequently, we will refer to this population as DN γδT cells. All three populations of γδT cells contain clusters of CD56-expressing cells, which are more abundant in the decidua. It is worth noting that although these decidual 'NKγδT-like' cells may be associated with dNK cells. However, the expression of CD56 on lymphoid cells serves only as a phenotypical marker. Therefore, any conclusions drawn from this should be made with caution. At present, investigations into the physiological similarities between these cells are still ongoing.

During pregnancy, the trophoblast expresses HLA class I molecules in a unique pattern that allows temporal chimerism. At the maternal-fetal interface between maternal blood and fetal syncytiotrophoblast in the intervillous spaces, HLA molecules are absent. This absence of HLA molecules is believed to prevent adaptive immune cells from driving an immune response at this location. However, the decidua interacts directly with the extravillous trophoblast, which expresses HLA-C, HLA-E, and HLA-G. HLA-G is uniquely expressed at this interface and is believed to influence the maternal immune system to support viviparity. On the other hand, HLA-E expression is connected to the

expression of other HLA class I molecules. It is loaded with their leader sequence peptides, and its expression level on the cell surface is usually proportional to a given cell's HLA class I expression level [293,294]. In instances of viral infections or malignancies, alterations in HLA class I molecule expression may impact HLA-E expression. This alteration can be identified by NK or cytotoxic T cells through the utilization of either the activating receptor NKG2C or the inhibitory receptor NKG2A.

Among all decidual γδT cell subsets, cells expressing receptors for HLA-E or HLA-G are prevalent, suggesting a possible influence of these non-classical HLA molecules. Especially the decidual $V\delta1^+$ subset shows high expression levels of NKG2C and co-expresses NKG2A with lower intensity. However, considering the higher affinity of NKG2A to HLA-E, this difference in surface expression may be irrelevant [502].

Both ILT2 and KIR2DL4 have been shown to bind various ligands, including HLA-G [354,391]. Next to its physiological expression and secretion by the EVT during pregnancy, malignancies are known to express and secret HLA-G. In this context, its ITL2-mediated inhibitory effects on Vγ9Vδ2 cells are well known (reviewed in [503]), and similar effects can be expected in the decidual microenvironment. KIR2DL4 is a unique Killer cell immunoglobulin-like receptor family member, as it is reportedly expressed on the cell surface or intracellularly by activated or non-activated NK cells. According to our findings, both decidual and peripheral γδT cells only express KIR2DL4 intracellularly. Considering its location, interactions with sHLA-G are more likely. However, intercellular HLA-G transfer through mechanisms like trogocytosis, nanotube transfer, or exosome provide alternative possibilities for KIR2DL4-HLA-G interactions [504].

Despite the molecular structure of KIR2DL4 suggesting an inhibitory function, recent studies have shown that the effect of its ligation is context-dependent. Rajagopalan et al., for example, demonstrated that peripheral blood NK cells produce angiogenic factors upon KIR2DL4 ligation [71,76].

To investigate the role of HLA-E and HLA-G in controlling different subsets of γδT cells in the decidua, we analyzed the proportions of HLA class I-binding receptor expressing decidual γδT subpopulations. We found that decidual Vδ2 cells have reduced potential for activation and increased potential for inhibition through HLA-E. Therefore,

we suggest that HLA-E is responsible for controlling the function of Vδ2 cells in the placenta, among other factors.

Regarding HLA-G, our data suggest that this non-classical HLA molecule preferentially regulates not only decidual γδT cells but also peripheral Vδ1 and DN γδT cells in its soluble form. These effects can be mediated through ILT2 and KIR2DL4 receptor functions. In addition, because of the high ratio of KIR2DL4 expressing peripheral Vδ2 cells, sHLA-G can potentially inhibit Vδ2 cells as well. This peripheral inhibition of γδT cell subsets could be part of the known systemic immunological adaptation during pregnancy, which could also be measured in the peripheral blood.

A further interesting issue is whether peripheral and decidual γδT cells differ in their NK cell receptor expression patterns. Regarding the proportion of NK receptor-positive cells, decidual Vδ1 or Vδ2 cells do not differ significantly in their inhibitory NK receptor expression pattern from their peripheral counterpart; however, decidual and peripheral DN γδT cells are phenotypically different and, therefore, show presumably distinct regulation by the non-classical HLA molecules. Here, in contrast to the peripheral DN γδT cells, the binding of the HLA-E molecule can result in both inhibitory and activating signals in the decidua. Whereas HLA-G primarily inhibits decidual DN γδT cells via KIR2DL4 rather than ILT2.

Although decidual γδT cells produce some angiogenic factors (G-CSF, FGF-2) in our experiments, the presence of HLA-G did not affect their production in vitro. G-CSF is also secreted by decidual NK cells and promotes the disorganization of vascular muscles. This, in turn, aids the invasion of the extravillous trophoblast into the spiral arteries, increasing the blood supply in the placental bed [479,480].

FGF-2, on the other hand, activates MAPK signaling and was assumed to improve endometrial receptivity [505]. Furthermore, it was demonstrated that FGF-2 enhances the proliferation and survival of trophoblast organoid cultures in vitro [506]. Although the concentrations of FGF-2 in our experiments were significantly lower than in the trophoblast culture experiments, it demonstrates that decidual γδT cells contribute to creating a nursing environment for the invading trophoblast.

Furthermore, Leptin is produced by trophoblast tissue and is known to support trophoblast invasion [507]. Administration of leptin increases HLA-G expression on the EVT in vitro [508]. In this context, our experiments demonstrate that higher leptin

concentrations are connected to the expression of HLA-G itself, indicating an interdependent relationship between HLA-G expression and leptin secretion. However, in our experiments, leptin concentrations increased only in the presence of γδT cells, emphasizing the relevance of immune cells for leptin secretion. Other angiogenic factors like endoglin, placenta growth factor, or vascular endothelial growth factor, which were detected in our experiments, are known to be secreted by trophoblast tissue [509].

Regarding potential defense mechanisms, decidual γδT cells' intracellular perforin level correlates positively with NKG2C, NKG2A, and ILT2 expression on the $V\delta1^+$ and DN subsets. However, our experiments do not show altered secretion of perforin in the presence of HLA-E or -G. While innate lymphoid cells utilize various activating and inhibiting receptors for their activity, γδT cells can use their TCR for antigen recognition. Our results suggest no immediate consequences for the isolated presence of either HLA-E or -G. However, long-term consequences are possible and likely. In peripheral blood NK cells, these receptors are not just associated with immediate cell reaction but also with a process of activation threshold alteration called NK cell education [510]. We suggest a similar process might also be possible for γδT cells.

Decidual γδT cells secrete high levels of IFN-γ and impressive levels of granulysin. This emphasizes their importance in pathogen defense, as granulysin allows pathogen eradication from virus-infected trophoblast cells without harming the trophoblast itself [86].

Our study consisted of two phases aimed at exploring the role of γδT cells in human pregnancy. In the first phase, we observed the dynamic expression of PD-1 on γδT cells and the high cytotoxic potential of their $CD56^+$ subpopulation in the peripheral blood during the course of pregnancy.

In the second phase, we focused on γδT cells in decidua during the first trimester and discovered their multifaceted functions and interactions. Notably, we observed that non-classical HLA molecules expressed by the EVT, such as HLA-G and HLA-E, may influence decidual γδT cells through receptors like NKG2C, NKG2A, ILT2, and KIR2DL4. These interactions may modulate immune responses and angiogenic factor production, thereby adding another layer of complexity to the maternal-fetal interface.

8 Publications

8.1 Contributions to Scientific Journals

Total cumulative impact factor: ***44.759***

Total citations: ***28***, H-index: ***3***

Cumulative impact factor of articles associated with this PhD thesis (**8.1.1**): ***16.087***

8.1.1 Publications related to the Thesis

1. **Nörenberg J**, Vida L, Bösmeier I, Forro B, Nörenberg A, Buda A, Simon D, Erdo-Bonyar S, Jakso P, Kovacs K, Miko E, Berki T, Mezosi E, Barakonyi A. Decidual γδT cells of early human pregnancy produce angiogenic and immunomodulatory proteins while also possessing cytotoxic potential. *Front. Immunol.* 2024 Mar 27;15:1382424. Doi: 10.3389/fimmu.2024.1382424
   Impact factor: 7.300, Q1
2. **Nörenberg J**, Jakso P, Barakonyi A. Gamma/Delta T Cells in the Course of Healthy Human Pregnancy: Cytotoxic Potential and the Tendency of CD8 Expression Make CD56+ γδT Cells a Unique Lymphocyte Subset. *Front. Immunol.* 2021 Feb 2;11:596489. doi: 10.3389/fimmu.2020.596489. eCollection 2020. PMID: 33603738
   Impact Factor: 8.787, Q1

8.1.2 Publications not related to the Thesis

1. Simon D, Erdo-Bonyar S, Böröcz K, Balazs N, Badawy A, Bajnok A, **Nörenberg J**, Sereny-Litvai T, Varnagy A, Kovacs K, Hantosi E, Mezosi E, Nemeth P, Berki T. Altered Levels of Natural Autoantibodies against Heat Shock Proteins in Pregnant Women with Hashimoto's Thyroiditis *Int. J. Mol. Sci.* 2024 Jan, 25(3), 1423; doi: 10.3390/ijms25031423
   Impact factor 5.600, Q1
2. Erdo-Bonyar S, Simon D, Bajnok A, **Nörenberg J**, Litvai T, Varnagy A, Kovacs K, Hantosi E, Mezosi E, Berki T. Physiological Changes in the Levels of Anti-Cytokine Autoantibodies in Early Pregnancy Are Missing in Pregnant Women with Hashimoto's Thyroiditis. *J. Immunol. Res.* 2023 Aug 25:2023:5221658. doi: 10.1155/2023/5221658. eCollection 2023. PMID: 37663050

Impact factor: 4.100, Q1

3. Bajnok A, Sereny-Litvai T, Temesfoi V, **Nörenberg J**, Herczeg R, Kaposi A, Berki T, Mezosi E. An Optimized Flow Cytometric Method to Demonstrate the Differentiation Stage-Dependent Ca2+ Flux Responses of Peripheral Human B Cells. *Int. J. Mol. Sci.* 2023 May 22;24(10):9107. doi: 10.3390/ijms24109107. PMID: 37240453
   Impact Factor: 5.600, Q1
4. Sereny-Litvai T, Bajnok A, Temesfoi V, **Nörenberg J**, Pham-Dobor G, Kaposi A, Varnagy A, Kovacs K, Pentek S, Koszegi T, Mezosi E, Berki T. B cells from anti-thyroid antibody positive, infertile women show hyper-reactivity to BCR stimulation. *Front Immunol.* 2022 Oct 25:13:1039166. doi: 10.3389/fimmu.2022.1039166. eCollection 2022. PMID: 36389812
   Impact Factor: 7.300, Q1
5. **Nörenberg J**, Meggyes M, Jakso P, Miko E, Barakonyi A. TIM-3 and TIM-1 Could Regulate Decidual gamma-delta TCR Bright T Cells during Murine Pregnancy. *J. Immunol. Res.* 2019 May 20:2019:3836942. doi: 10.1155/2019/3836942. eCollection 2019. PMID: 31236420
   Impact Factor: 3.327, Q1
6. Meggyes M, Szereday L, Jakso P, Bogar B, Bogdan A, **Nörenberg J**, Miko E, Barakonyi A. Expansion of CD4 phenotype among CD160 receptor-expressing lymphocytes in murine pregnancy. *Am. J. Reprod. Immunol.* 2017 Dec;78(6). doi: 10.1111/aji.12745. Epub 2017 Sep 16. PMID: 28921767
   Impact Factor: 2.745, Q1

8.2 Contributions to Scientific Conferences

1. Poster presentation: Nörenberg J. et al. Exploring the interactions between decidual γδT cells and non-classical HLA molecules expressed bz the extravillous trophoblast. 18th International Congress of Immunology (Cape Town, South Africa, 2023)
2. Invited speaker: Nörenberg J. et al. Different γδT cell population and their possible role in the maintenance of pregnancy. 16th International Medical

Postgraduate Conference at Charles University (Hradec Králové, Czech Republic, 2019)

3. Poster presentation: Nörenberg J. et al. Flow cytometric analysis of gamma-delta T cells in spleen and placenta during murine pregnancy. 34th Congress of the International Society for Advancement of Cytometry (Vancouver, Canada, 2019)
4. Poster presentation: Nörenberg, J. et al. Characteristics of gamma/delta T cells at the feto-maternal interface of murine pregnancy. European Congress of Immunology (Amsterdam, Netherlands, 2018)

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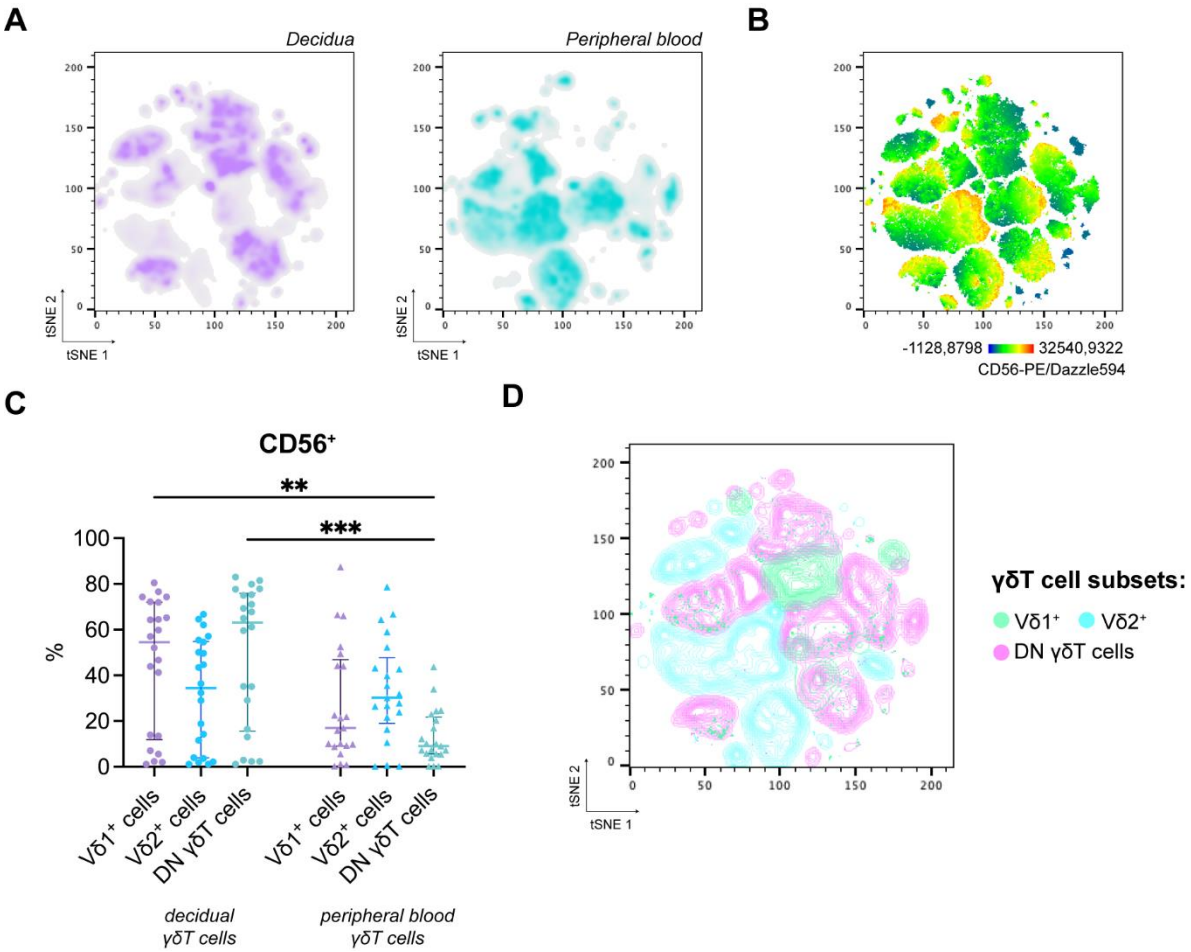
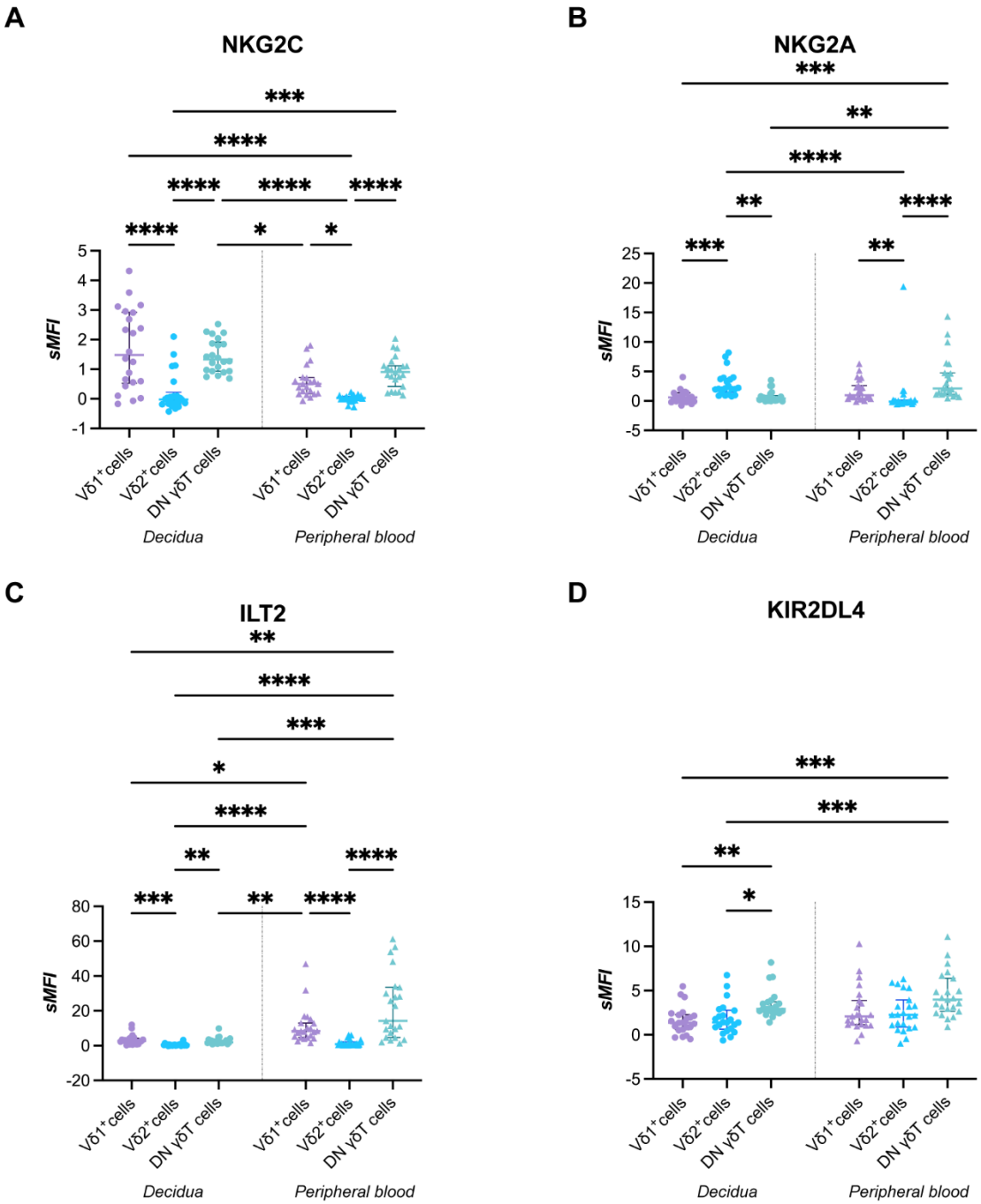
11 Appendix

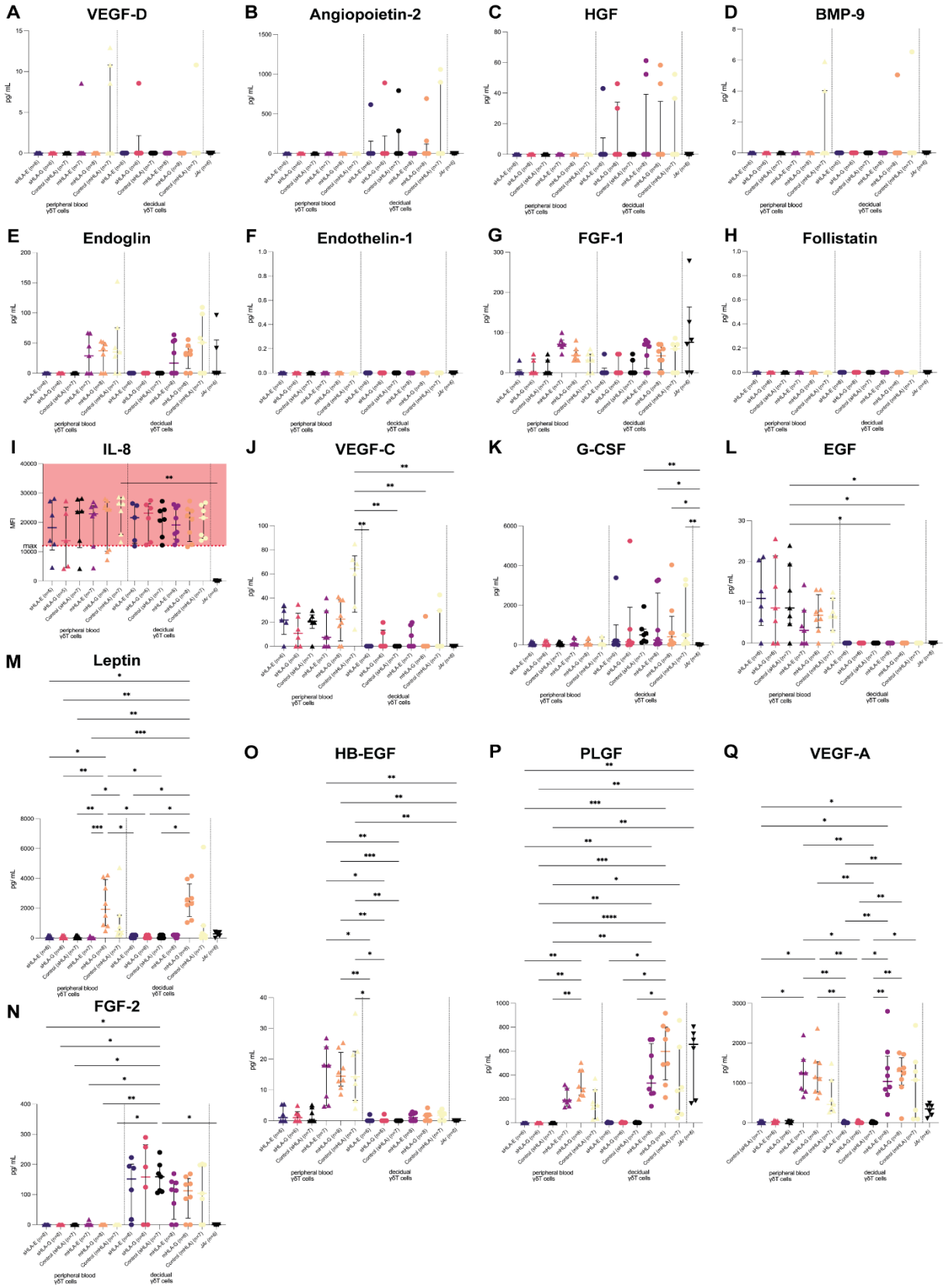
11.1 Supplementary Figures



Supplementary Figure 1. Gamma/delta T cells at the early feto-maternal interface: *(A) Isolated depiction of decidual (left) and maternal peripheral blood (right) γδT cells as tSNE density plots (n=1; Panel B). (B) Fluorescence intensity of CD56-PE/Dazzle594™ on clustered γδT cells from peripheral blood and decidua (n=1; Panel B). (C) Statistical comparison of CD56⁺ cells' prevalence among γδT cell subsets from the decidua (n=22) and peripheral blood (n=23). Testing for significance was performed by the Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test. (D) Contour tSNE plot overlay of Vδ1⁺, Vδ2⁺, and DN γδT cells on γδT cells from peripheral blood and decidua (n=1; Panel B). **: p ≤ 0.01, ***: p ≤ 0.001.*

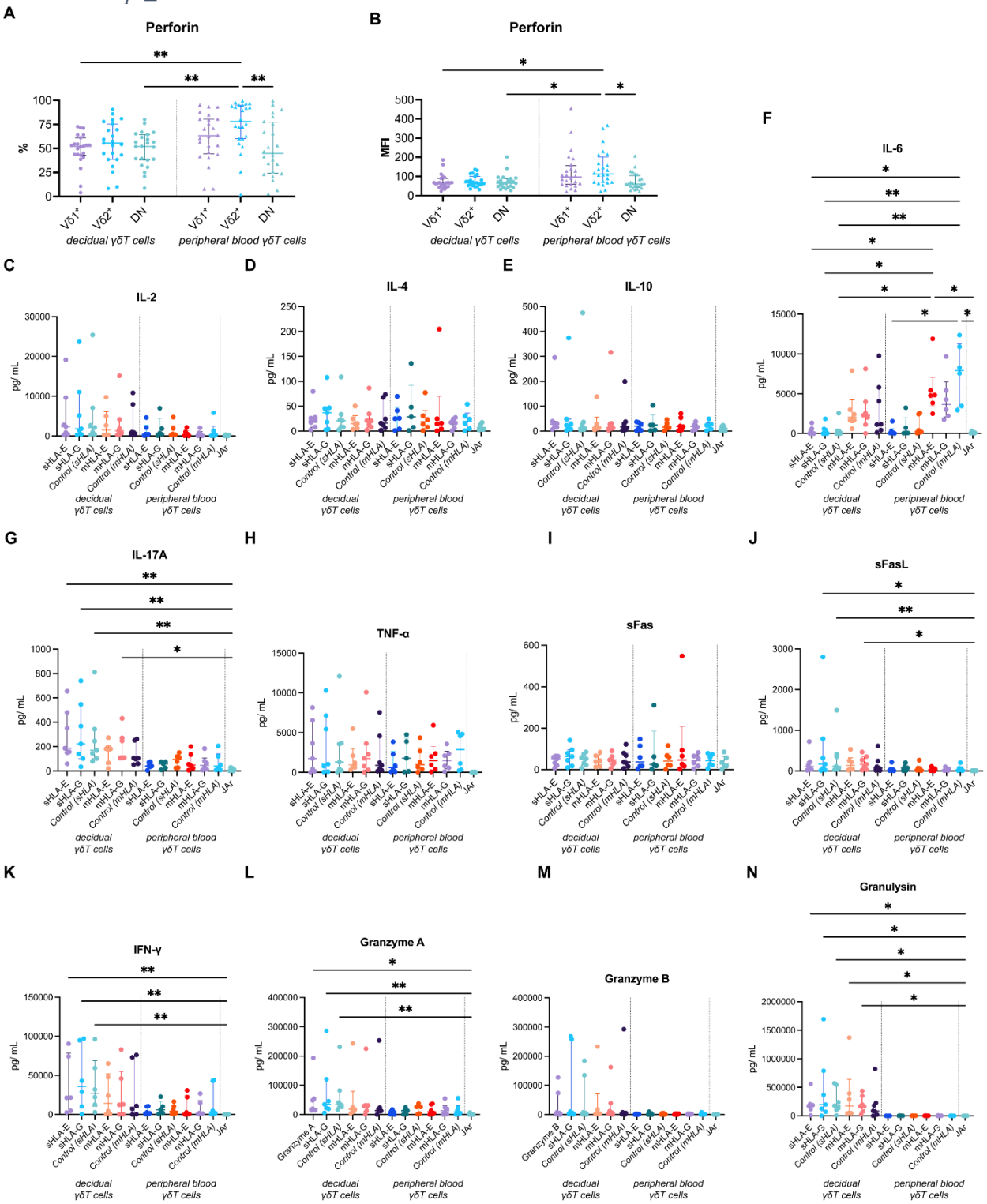


Supplementary Figure 2. Expression of HLA-E or HLA-G-binding receptors on γδT cells at the early feto-maternal interface: *Statistical comparison of standardized median fluorescence intensity (sMFI=[MedianSubset-MedianFMO]/rSDFMO) of (A) NKG2C-PE, (B) NKG2A-APC, (C) ILT2-PE, (D) KIR2DL4-APC on decidual (n=22) and peripheral blood γδT cell subsets (n=23). Testing for significance was performed by the Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.*



Supplementary Figure 3. Angiogenic factor secretion profile of early decidual γδT cells: *Statistical comparison of measured cytokine concentrations of (A) VEGF-D, (B) Angiopoietin-*

*2, (C) HGF, (D) BMP-9, (E) Endoglin, (F) Endothelin-1, (G) FGF-1, (H) Follistatin, (I) IL-8, (J) VEGF-C, (K) G-CSF, (L) EGF, (M) Leptin, (N) FGF-2, (O) HB-EGF, (P) PLGF, and (Q) VEGF-A in all experimental settings. All statistical comparisons were performed by the Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.*



Supplementary Figure 4. Cytotoxic potential of early decidual γδT cells: *(A) Prevalence of Perforin-VioBlue®$^{+}$ cells among all γδT subpopulations of decidual (n=22) and peripheral blood (n=23) samples. Median fluorescence intensity (MFI) of Perforin-VioBlue® of all γδT subpopulations of decidual (n=22) and peripheral blood (n=23) samples. Statistical comparison of measured cytokine concentrations of (C) IL-2, (D) IL-4, (E) IL-10, (F) IL-6, (G) IL-17A, (H) TNF-α, (I) sFas, (J) sFasL, (K) IFN-γ, (L) Granyzme A, (M) Granzyme B, (N) Granulysin in all experimental settings. All statistical comparisons were performed by the Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test. *: $p \leq 0.05$, **: $p \leq 0.01$.*

11.2 Scientific publications associated with this doctoral thesis

See the following pages.

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Decidual γδT cells of early human pregnancy produce angiogenic and immunomodulatory proteins while also possessing cytotoxic potential

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During pregnancy, the maternal immune system must allow and support the growth of the developing placenta while maintaining the integrity of the mother's body. The trophoblast's unique HLA signature is a key factor in this physiological process. This study focuses on decidual γδT cell populations and examines their expression of receptors that bind to non-classical HLA molecules, HLA-E and HLA-G. We demonstrate that decidual γδT cell subsets, including Vδ1, Vδ2, and double-negative (DN) Vδ1-/Vδ2- cells express HLA-specific regulatory receptors, such as NKG2C, NKG2A, ILT2, and KIR2DL4, each with varying dominance. Furthermore, decidual γδT cells produce cytokines (G-CSF, FGF2) and cytotoxic mediators (Granulysin, IFN-γ), suggesting functions in placental growth and pathogen defense. However, these processes seem to be controlled by factors other than trophoblast-derived non-classical HLA molecules. These findings indicate that decidual γδT cells have the potential to actively contribute to the maintenance of healthy human pregnancy.

KEYWORDS

decidua, γδT cells, HLA-E, HLA-G, NK receptors, cytokines, angiogenic factors, cytotoxic mediators

1 Introduction

During pregnancy, the coexistence of two genetically and immunologically different individuals within one body challenges primary transplantation and tumor physiology concepts. In their context, the maternal immune system's task is to ensure the integrity of the mother's body and remove foreign or dysplastic tissues. However, the maternal immune system does not attack embryonal or fetal tissues but supports implantation, placentation, and fetal growth (1–3).

In human pregnancy, the trophoblast infiltrates deeply into the decidua and spiral arteries, allowing the establishment of a hemochorial placenta. This type of placentation, in which maternal blood is in direct contact with fetal tissues, ensures a sufficient supply of oxygen and nutrients. Insufficient supply, caused by weak trophoblast invasion during the first trimester, may lead to human pregnancy disorders, like fetal growth restriction or preeclampsia (4–6). Furthermore, early pregnancy loss or infertility cases might be connected to even weaker implantation and invasion. Research of the last decades emphasizes the importance of the interaction of trophoblast and decidua for controlling invasion depth and establishing a healthy placenta (7–9). Cases of the placenta accreta spectrum, in which the trophoblast might even invade neighboring organs, highlight the role of the decidua in this process as they commonly occur when the blastocyte implants at the site of a uterine scar, where decidua is absent (9–11).

At the time of receptivity (window of implantation), leukocytes accumulate in the decidua, dominated by a unique CD56^{bright} innate lymphoid cell population, commonly known as uterine/decidual NK (u/dNK) cells (12). Decidual NK cells have an array of activating and inhibiting receptors, which bind specific classical and non-classical HLA class I molecules (13–17). The extravillous cytotrophoblast (EVT), which is in direct contact with the decidua and invades uterine spiral arteries (Figure 1A), is unique in its HLA class I expression pattern: The highly variable HLA-A and -B are not expressed by the EVT. Instead, its cells express HLA-C and the oligomorphic HLA-E and -G on their cell surface. Under physiological circumstances, HLA-G is exclusively known to be expressed by the EVT (18, 19). Next to the membrane-bound form of HLA-E and HLA-G (mHLA-E/-G), soluble forms (sHLA-E/-G) have been found in the sera of pregnant women (20–23).

Next to dNK cells, γδT cells and their potential roles during pregnancy have attracted interest. They are well known to surveil the tissue integrity of frontiers between the organism and the environment (24, 25). Studies reported a higher prevalence of γδT cells among decidual CD3$^+$ cells compared to the peripheral blood (26–28). These decidual γδT cells, like dNK cells, are either clustered proximate to decidual glands or scattered as intraepithelial lymphocytes (26). The association of decidual γδT cells and dNK cells to glands might be connected to the invasion of the EVT, as the EVT penetrates not only spiral arteries but uterine glands (29). A growing body of evidence attributes a central role to

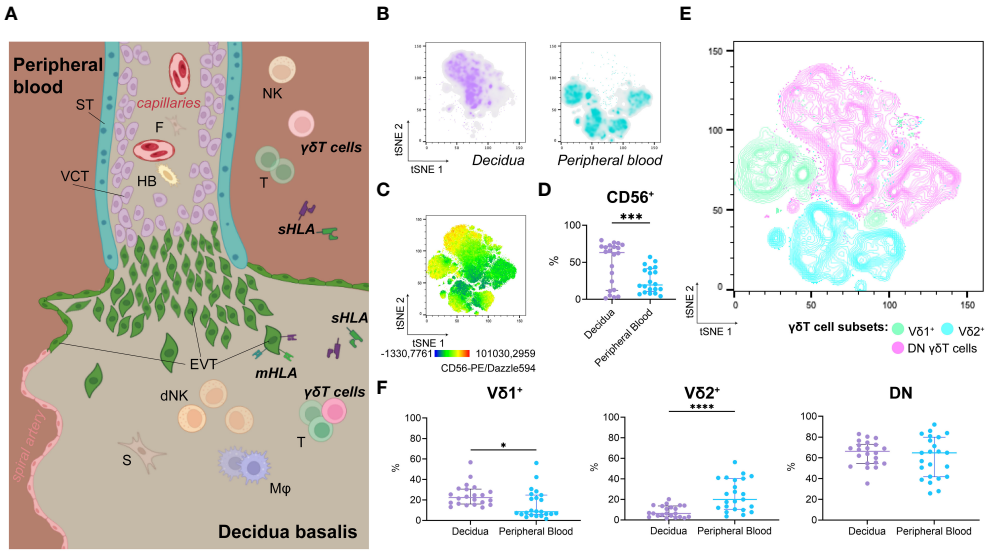

FIGURE 1
(A) Illustration of anatomical structure and spatial relations at the feto-maternal interface. **(B)** Representative isolated depiction of tSNE-clustered decidual (left) and maternal peripheral blood (right) γδT cells as density plots (n=1; Panel A, also see Supplementary Information 1B). **(C)** Representative tSNE-plot depicting fluorescence intensity of CD56-PE/Dazzle594^{TM} on γδT cells from peripheral blood and decidua (n=1; Panel A, also see Supplementary Information 1C). **(D)** Statistical comparison of CD56$^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23) (see also Supplementary Information 1D). **(E)** Representative contour plot overlay of Vδ1$^+$, Vδ2$^+$, and DN γδT cells on clustered γδT cells from peripheral blood and decidua (n=1; Panel A, also see Supplementary Information 1E). **(F)** Statistical comparison of Vδ1$^+$, Vδ2$^+$, and DN γδT cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). Testing for significance was performed with the Wilcoxon test. *: $p \leq 0.05$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$; ST, syncytiotrophoblast; VCT, villous cytotrophoblast; F, fibroblast; HB, Hofbauer cell; (d) NK, (decidual) Natural killer cell; T, T cell; s/mHLA, soluble/membrane-bound Human Leukocyte Antigen class I; EVT, extravillous cytotrophoblast; S, stroma cell; Mφ, Macrophage.

these glands, providing nutrients, growth factors, and cytokines during placentation (29–31).

Innate lymphoid cells, like dNK cells, and γδT are closely related. Although γδT cells have an antigen-recognition receptor, they rely on an arsenal of cytotoxicity receptors for their activity. Furthermore, in contrast to αβT cells, they are not MHC-restricted for their antigen recognition. However, some of these cytotoxicity-related receptors bind HLA class I molecules and transmit activating or inhibiting signaling upon ligation. Consequently, HLA expression is likely to influence γδT cell behavior.

Considering the EVT's unique HLA class I expression profile, three receptor groups come into focus at the maternal-fetal interface. The NKG2 receptor family, with a particular emphasis on activating NKG2C and inhibitory NKG2A, plays a pivotal role in recognizing HLA-E expression (32, 33). HLA-G, conversely, can be bound by KIR2DL4, a receptor from the Killer cell Immunoglobulin-like Receptor (KIR) family (CD158). This family is mainly known for HLA-C binding. However, KIR2DL4 stands out among the KIR receptor family due to its unique ligand preference, location, and function. Unlike other KIR receptors, KIR2DL4 is predominantly located intracellularly and is only expressed on the cell surface during activation states. Although its molecular structure suggests an inhibitory function, KIR2DL4-ligation was shown to trigger cytokine release (34–36). Last, Immunoglobulin-like transcript (ILT2), a member of the leukocyte immunoglobulin-like receptor subfamily B, binds HLA class I molecules, including HLA-G, and transmits an inhibitory signal upon ligation (17, 33).

In addition, the non-classical MHC molecule CD1d, which presents lipid antigens, has the potential to facilitate antigen recognition through the γδTCR (37–39). However, further signals will influence the crosstalk between maternal γδT cells and the fetal EVT in the decidua. Although, investigations in tumor immunology have already demonstrated the expression of different non-classical HLA receptors (40), a detailed expression profile of these receptors on decidual γδT cells has not been published.

Here, we present an expression profile for HLA-E- or HLA-G-binding receptors of decidual γδT cells during early pregnancy. Furthermore, we investigated the potential consequences of respective receptor-ligand interactions. In this context, we focused on γδT cells' secretion of mediators, which may influence vascular transformation or pathogen defense.

2 Results

2.1 Heterogeneity of peripheral and decidual γδT cells during early pregnancy

We found no significant difference between decidual and peripheral blood γδT cells' prevalence among $CD45^+$/live cells (Supplementary Information 1A). To characterize decidual γδT cells and compare them to their circulating counterparts, we utilized the downsampling plugin of FlowJo™ and concatenated previously gated γδT cell populations from decidual mononuclear cells (DMCs) and peripheral blood mononuclear cells (PBMCs). Defined separate clusters with minimal overlap were assigned to decidual or peripheral blood γδT cells, respectively (Figures 1A, B, see also Supplementary Information 1B).

Due to the biological similarities between NK cells and γδT, we investigated the expression of CD56 on γδT cells. While $CD56^{dim}$ expression was detectable in several peripheral blood γδT cell clusters, decidual γδT cells exhibited both $CD56^{dim}$ and $CD56^{bright}$ phenotypes (Figure 1C, see also Supplementary Information 1C). Nevertheless, $CD56^+$ γδT cells are more prevalent in the decidua than in the periphery (Figure 1D, see also Supplementary Information 1D).

Classical γδT cell subsets were associated with distinct clusters. $V\delta1^+$ ($CD45^+TCR\gamma\delta^+V\delta1^+V\delta2^-$) cells were more prevalent in the decidua, while $V\delta2^+$ ($CD45^+TCR\gamma\delta^+V\delta1^-V\delta2^+$) cells were more common among circulating γδT cells. However, double-negative (DN, $CD45^+TCR\gamma\delta^+V\delta1^-V\delta2^-$) γδT cells were the most common in both decidual and peripheral blood (Figures 1E, F, see also Supplementary Information 1F).

2.2 Decidual γδT subsets express receptors that bind to HLA-E or HLA-G molecules

Using two flow cytometric panels (Supplementary Information 6), we investigated the prevalence and expression of HLA-E and HLA-G-binding receptors (NKG2C, NKG2A, and ILT2, KIR2DL4, respectively) on γδT cell subsets in the decidua and the matched peripheral blood (Figure 2A, see also Supplementary Information 1B, E). To estimate the expression intensity, we compared the median fluorescence intensity (normalized to the respective FMO) of all investigated receptors. Peripheral γδT cells show higher expression intensity for KIR2DL4 or ILT2 receptors than that for NKG2C and NKG2A. Within the different decidual γδT cell subpopulations, decidual DN γδT cells exhibited relatively high expression levels for all investigated receptors, while decidual $V\delta1^+$ cells showed a more focused expression of the activating NKG2C and the inhibiting ILT2. In contrast, decidual $V\delta2^+$ cells expressed significantly more NKG2A on their cell surface (Figure 2B, see also Supplementary Information 2A, B).

The prevalence of $NKG2C^+$ cells was generally higher among decidual γδT cells compared to the periphery. However, this difference reached the level of significance only in the $V\delta2^+$ and DN subsets. Furthermore, NKG2C positivity was significantly more common in the $V\delta1^+$ subset compared to the $V\delta2^+$ one (Figure 2C). Likewise, cells expressing the inhibitory counterpart NKG2A were more prevalent in the decidua. While the percentage of $NKG2A^+$ cells among $V\delta2^+$ cells did not differ between decidua and peripheral blood, a significantly higher proportion of DN γδT cells expresses NKG2A and NKG2C in the decidua compared to the periphery (Figure 2D).

The inhibitory HLA-G-binding ILT2 was commonly expressed by γδT cells independently of their origin. Generally, $ILT2^+$ cells were less prevalent in the $V\delta2^+$ subsets than in other γδT cell populations. However, when focusing on the prevalence of $ILT2^+$ cells within each γδT cell subset, significantly fewer decidual DN γδT cells expressed ILT2 than their peripheral blood counterpart (Figure 2E). The HLA-G-binding KIR2DL4 was expressed by the majority of γδT cells (Figure 2F).

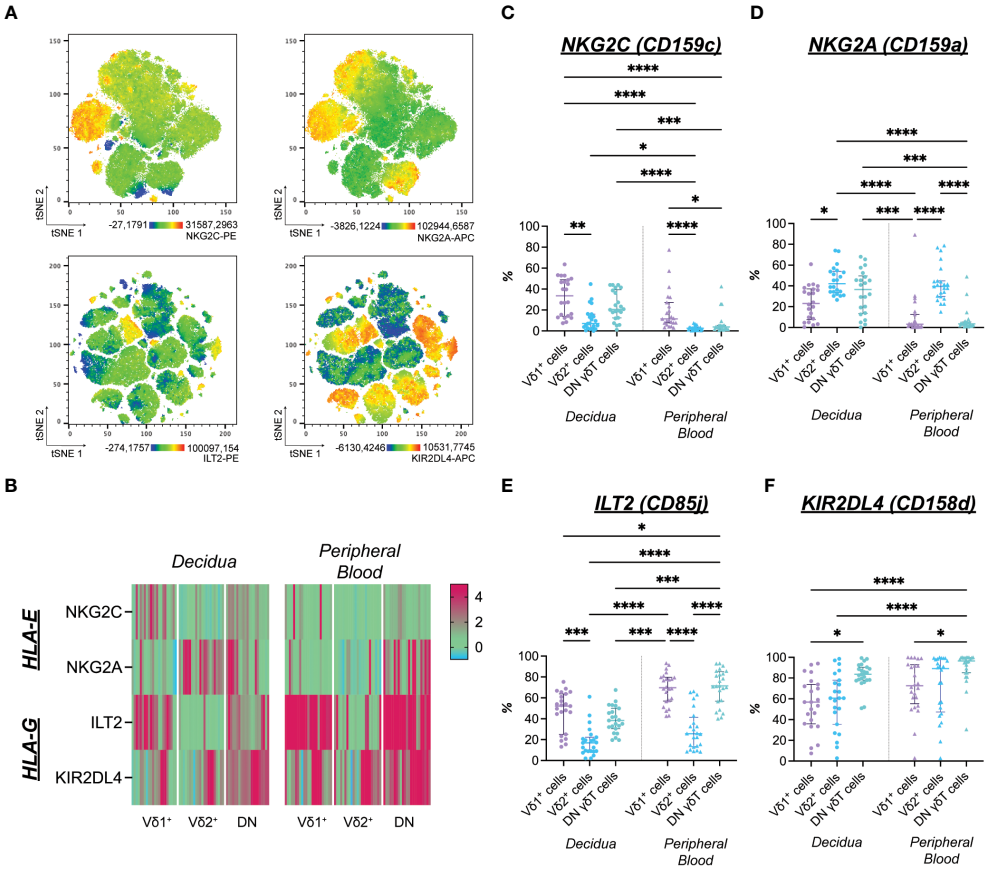

FIGURE 2
(A) Expression distribution of NKG2C-PE (upper left), NKG2A-APC (upper right) of flow cytometric Panel A, ILT2-PE (lower left, see also Supplementary Information A/D), and KIR2DL4-APC (lower right, see also Supplementary Information A/D) of flow cytometric panel B depicted as representative tSNE plots of paired, concatenated decidual and peripheral blood γδT cells (n=1). **(B)** Heatmap of standardized median fluorescence intensity ($[Median_{Subset}\text{-}Median_{FMO}]/rSD_{FMO}$) of NKG2C-PE, NKG2A-APC, ILT2-PE, KIR2DL4-APC on matched decidual and peripheral blood γδT cell subsets (n=22; also see Supplementary Information 2). **(C)** Statistical comparison of $NKG2C^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). **(D)** Statistical comparison of $NKG2A^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). **(E)** Statistical comparison of $ILT2^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). **(F)** Statistical comparison of $KIR2DL4^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). Testing for significance was performed with the Kruskal-Wallis test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$****: $p \leq 0.0001$.

2.3 Decidual γδT cells secrete trophoblastotropic cytokines

To determine the functional consequences of the HLA-E or -G recognition by γδT cells, we incubated purified γδT cells with soluble HLA-E or -G (sHLA-E/-G). Furthermore, we utilized human choriocarcinoma cell lines (JAr) transfected with HLA-E or $HLA\text{-}G_{1m}$ to investigate more complex interactions of membrane-bound HLA-E or -G (mHLA-E/-G) (Figure 3A).

Vascular transformation by the trophoblast and the local immune environment is crucial to establishing a healthy placenta during early pregnancy. Therefore, we analyzed the collected cell co-culture supernatants for potential angiogenic cytokines (Figure 3B). When comparing peripheral blood to decidual γδT cells without HLA molecules ("Control (sHLA)" in Figure 3B), we found significantly higher levels of G-CSF produced by the decidual ones (Figure 3C). Furthermore, decidual γδT cells produced FGF-2, whereas no FGF-2 was detected in the wells of peripheral γδT cells (Figure 3D). On the other hand, peripheral blood γδT cells produce small amounts of EGF, which was not detected in the wells of decidual samples (Figure 3E). While the production of most measured cytokines was not influenced by the presence or absence of HLA-E or -G molecules in our experimental model, incubating mHLA-G with γδT cells, independently from their origin, increased the measured Leptin concentrations (Figure 3F). Additionally, we detected elevated concentrations of Follistatin when incubating peripheral blood γδT cells with mHLA-E. However, compared to all other co-culture wells, the level of significance was not reached (Figure 3G).

2.4 Decidual γδT cells are strong producers of cytotoxic mediators

γδT cells act as first responders in the mucosal defense against pathogens and many frontiers between the body and its environment. Therefore, we also analyzed the intracellular perforin content to determine each γδT cell subset's cytotoxic potential in the decidua.

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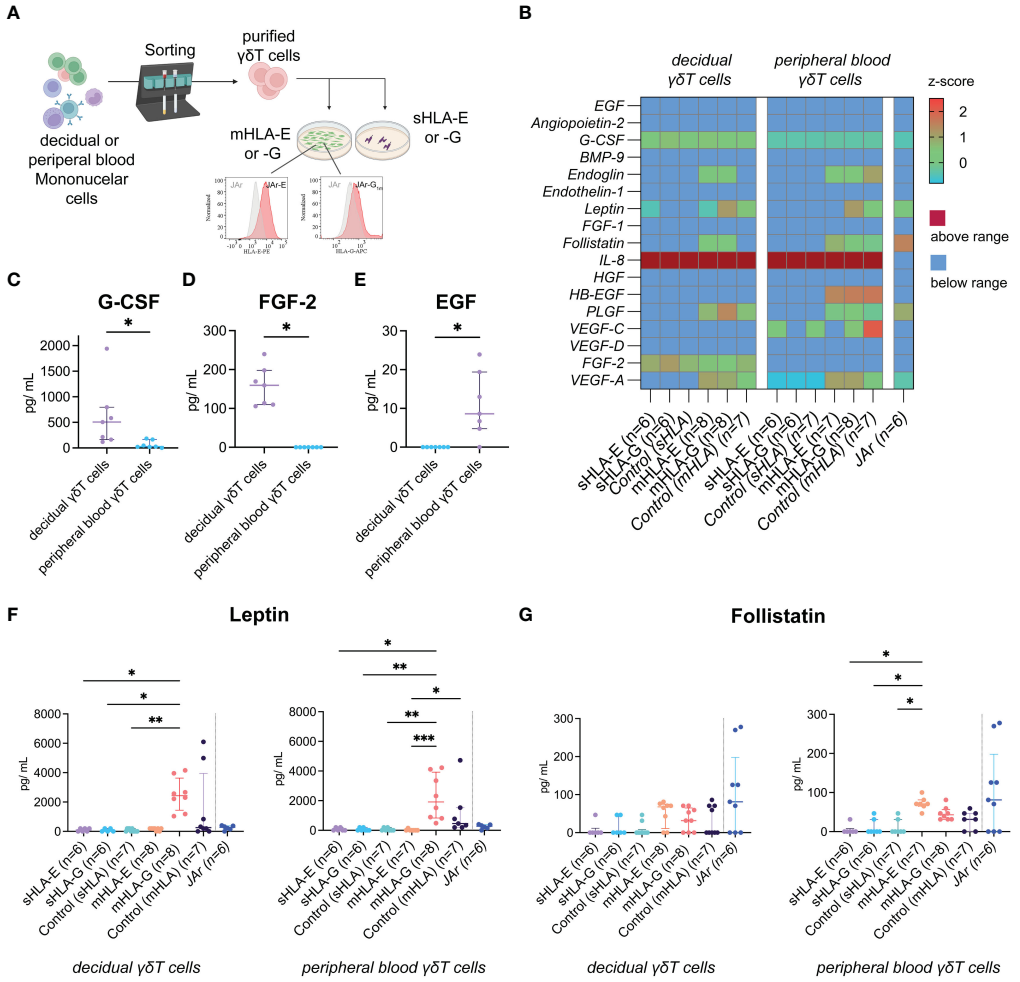

FIGURE 3
(A) Illustration of the experimental setup. **(B)** Heatmap depicting the z-score of the respective angiogenic factor after incubating peripheral blood (left) or decidual (right) γδT cells with soluble (s) or membrane-bound (m)HLA-E or -G. The last column depicts measurements from the human choriocarcinoma cell line JAr without γδT cells as an additional control (also see Supplementary Information 3). **(C)** Statistical comparison of G-CSF concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. **(D)** Statistical comparison of FGF-2 concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. **(E)** Statistical comparison of EGF concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. **(F)** Statistical comparison of Leptin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test. **(G)** Statistical comparison of Follistatin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

Although in the periphery significantly less intracellular Perforin was measured in DN γδT cells compared to the Vδ2 population (Supplementary Information 4A, B), the Perforin content of the different γδT cell subsets did not differ in the decidua. Figure 4A confirms the distinct features of Perforin-positive decidual γδT cell subpopulations, as analyzed in our two flow cytometric antibody panels. (Figure 4A, see also Supplementary Information 4A, B).

Upon interaction with HLA-E or HLA-G, NKG2C, NKG2A, ILT2, and KIR2DL4 are potential regulators of the cytotoxic capability of immune cells. Investigating the perforin content of the different NK receptor-expressing decidual γδT cell populations, we found that the expression of NKG2C and ILT2 was associated with significantly higher levels of intracellular perforin in all decidual γδT cell subsets. The expression of NKG2A, however, correlated only in the $V\delta1^+$ and DN γδT subset with higher levels of intracellular perforin. A similar, significant relation between KIR2DL4 expression and perforin content was only detectable in the DN γδT cell subset (Figure 4B).

To determine if this hypothetical relationship between cytotoxicity and the expression of HLA class I binding receptors has functional consequences, we analyzed the secretion of typical NK cell cytokines and cytotoxicity-related soluble molecules after exposure to sHLA-E/-G or mHLA-E/-G (Figure 4C, see also Supplementary Information 4C-N). However, the measured perforin concentration did not differ significantly (Figure 4D). In addition, we found that decidual γδT cells secrete excessive amounts of granulysin and high levels of interferon-γ (IFN-γ) (Figure 4E).

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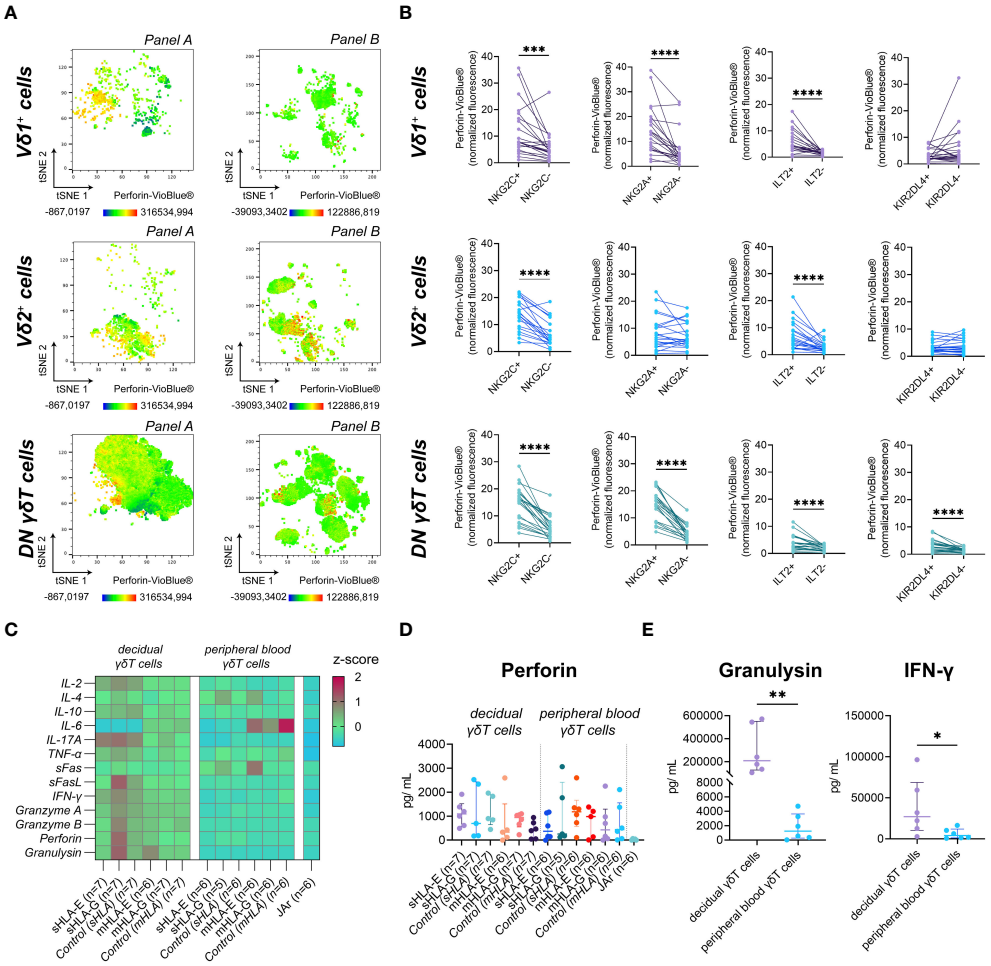

FIGURE 4
(A) Fluorescence intensity of Perforin-VioBlue® on gated γδT cell subsets from the decidua for the two flow cytometric panels depicted as representative tSNE plots (n=1). **(B)** Statistical association of standardized Perforin-VioBlue® median fluorescence intensity ([$Median_{Subset}$-$Median_{FMO}$]/rSD_{FMO}) to the expression of NKG2C, NKG2A, ILT2, and KIR2DL4 on decidual γδT cell subsets (n=22). Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. **(C)** Heatmap depicting the z-score of the respective soluble factor after incubating decidual (left) or peripheral blood (right) γδT cells with soluble (s) or membrane-bound (m) HLA-E or -G. **(D)** Statistical comparison of Perforin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test. **(E)** Statistical comparison of Granulysin (left) and INF-γ (right) concentrations secreted from decidual (n=6) or peripheral blood (n=6) γδT cells in the absence of HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

3 Discussion

Decidual γδT cells may have several functions in the microenvironment of the maternal-fetal interface between the decidua basalis and the extravillous trophoblast. They could influence the process of implantation and placentation via cytokine secretion or clear out pathogens.

The prevalence of γδT cells among $CD45^+$/live cells was not different between the decidua and the peripheral blood. However, it is important to note that literature data shows that while the prevalence of T cells ($CD3^+$) among all lymphocytes is around 70% in the peripheral blood, it is only around 15% in the early human decidua (41). Furthermore, according to previously published data, the population of γδT cells is markedly expanded in the decidua compared to the periphery (28, 41). Thus, due to the lack of CD3 staining, our prevalence data must be interpreted carefully.

As γδT cells exhibit innate and adaptive immunity aspects, they are often described as a bridge between these two parts of the immune system. Although a more didactic subdivision, γδT cells show quite some plasticity, and some subsets (e.g., Vγ9Vδ2) behave more in an innate manner, while others (e.g., Vδ1) show rather adaptive features (42–49). Therefore, when investigating γδT cells in any context, determining the prevalent subpopulations is a crucial first step before further conclusions.

The increase in the Vδ1 subset is typical for all tissues that frontier the environment and is often understood as a "first line defense" (24, 50). Our data on the prevalence of Vδ1 and Vδ2 subsets are in line with previously published flow cytometric data (51). However, these publications ignored the largest decidual γδT cell population, which expresses neither the Vδ1 nor the Vδ2 chain. Different variants of Vδ-chains exist, which makes DN γδT cells a heterogeneous population. However, a recent study used

sequencing to show that the early human decidua is only inhabited by the Vδ1$^+$, Vδ2$^+$, and Vδ3$^+$ γδT cell subsets (52). Therefore, DN γδT cells of the decidua can be considered as Vδ3$^+$ cells. The Vδ3 subset is generally assumed to induce antigen-presenting cell maturation (53). In the context of pregnancy, the correct presentation of antigens is essential. Further investigations will be necessary to determine if decidual Vδ3 cells could direct antigen-presenting cell maturation toward a tolerance-promoting phenotype. Currently, anti-Vδ3 antibodies are not commercially available. Therefore, we cannot finally confirm that the DN subset represents the Vδ3-one, so henceforward, we refer to this population as DN γδT cells.

Interestingly, the majority of decidual γδT cells are CD56$^+$, suggesting a special characteristic for decidual γδT cells. In addition, all three γδT cell populations contain cell clusters of CD56-expressing cells, which are more prevalent in the decidua. Although these decidual 'NKγδT-like' cells may be associated with dNK cells, as the expression of CD56 on lymphoid cells serves rather as a phenotypical marker, conclusions must be made carefully. To date, investigations indicating physiological similarities are still outstanding.

As mentioned previously, the trophoblast's unique expression pattern of HLA class I molecules is crucial in allowing temporal chimerism during pregnancy. HLA molecules are absent at the maternal-fetal interface between the circulating maternal blood and the fetal syncytiotrophoblast in the intervillous spaces, which may explain why circulating adaptive immune cells do not drive an immune response at this location. However, the decidua directly interacts with the extravillous trophoblast expressing HLA-C, HLA-E, and HLA-G. Under physiological circumstances, HLA-G is uniquely expressed at this interface and is believed to influence the maternal immune system to accept and support viviparity. The expression of HLA-E, on the other hand, is connected to the expression of other HLA class I molecules, as it is loaded with their leader sequence peptides. Therefore, its expression on the cell surface usually is proportional to a given cell's HLA class I expression level (54, 55). In cases of viral infection or malignancies, the expression of HLA class I molecules may alter, which consequently will be reflected in the expression of HLA-E. This can be detected by NK or cytotoxic T cells via the activating receptor NKG2C or the inhibitory receptor NKG2A. However, tumors may utilize upregulated HLA-E expression or secretion as an escape mechanism. Therefore, the extravillous trophoblast's HLA-E positivity might simply result from its HLA-G expression.

Control of γδT cell function is essential, where trophoblast-expressed non-classical HLA molecules could serve as potent mediators. Among all decidual γδT cell subsets, cells expressing receptors for HLA-E or HLA-G are prevalent, suggesting an efficient influence of these non-classical HLA molecules. Especially the decidual Vδ1$^+$ subset shows high expression levels of NKG2C and co-expresses NKG2A with lower intensity. However, considering the higher affinity of NKG2A to HLA-E, this difference in surface expression may be irrelevant (32). Both ILT2 and KIR2DL4 have been shown to bind various ligands, including HLA-G (33, 34). Next to its physiological expression and secretion by the EVT during pregnancy, malignancies are known to express and secret HLA-G. In this context, its ITL2-mediated inhibitory effects on Vγ9Vδ2 cells are well known [reviewed in (56)], and similar effects can be expected in the decidual microenvironment. KIR2DL4 is a unique Killer cell immunoglobulin-like receptor family member, as it is reportedly expressed on the cell surface or intracellularly by activated or non-activated NK cells. According to our findings, both decidual and peripheral γδT cells only express KIR2DL4 intracellularly. Considering its location, interactions with sHLA-G are more likely. However, intercellular HLA-G transfer through mechanisms like trogocytosis, nanotube transfer, or exosome provides an alternative possibility for KIR2DL4-HLA-G interactions (57). Despite its molecular structure suggesting an inhibitory function, it has been shown that the consequence of its ligation depends on the context. Rajagopalan et al., for example, demonstrated the production of angiogenic factors by peripheral blood NK cells due to KIR2DL4 ligation (36, 58).

For deeper investigation of the role of HLA-E and HLA-G in the functional control of different γδT cell subpopulations in the decidua, we analyzed the proportions of HLA class I-binding receptor expressing decidual γδT subsets. Decidual Vδ2 cells showed a reduced potential for activation and an increased potential for inhibition via HLA-E. Therefore, we suppose that HLA-E could be responsible, among others, for the control of Vδ2 cell function in the placenta. Regarding HLA-G, our data suggest that this non-classical HLA molecule preferentially regulates not only decidual γδT cells but also peripheral Vδ1 and DN γδT cells in its soluble form. These effects are mediated through ILT2 and KIR2DL4 receptor functions. In addition, because of the high ratio of KIR2DL4 expressing peripheral Vδ2 cells, soluble HLA-G is potentially able to inhibit Vδ2 cells also. This peripheral inhibition of γδT cell subsets could be part of the known systemic immunological adaptation during pregnancy, which could be measured in the peripheral blood also.

A further interesting issue is whether peripheral and decidual γδT cells differ in their NK cell receptor expression patterns. Regarding the proportion of NK receptor-positive cells, decidual Vδ1 or Vδ2 cells do not differ significantly in their inhibitory NK receptor expression pattern from their peripheral counterpart; however, decidual and peripheral DN γδT cells are phenotypically different and therefore show presumably distinct regulation by the non-classical HLA molecules. Here, in contrast to the peripheral DN γδT cells, the binding of the HLA-E molecule can result in both inhibitory and activating signals in the decidua. Whereas HLA-G primarily inhibits decidual DN γδT cells via KIR2DL4 rather than ILT2.

Although activated decidual γδT cells produce angiogenic factors (G-CSF, FGF-2), the presence of HLA-G did not affect their production *in vitro*. G-CSF is also secreted by decidual NK cells and promotes the disorganization of vascular muscles. This, in turn, aids the invasion of the extravillous trophoblast into the spiral arteries, increasing the blood supply in the placental bed (59, 60). FGF-2, on the other hand, activates MAPK signaling and was assumed to improve endometrial receptivity (61). Furthermore, it was demonstrated that FGF-2 improves proliferation and survival of trophoblast organoid cultures *in vitro* (62). Although the

concentrations of FGF-2 in our experiments were significantly lower than in the the trophoblast culture experiments, it demonstrates that decidual γδT cells contribute to the creation of a nursing environment for the invading trophoblast. Leptin is produced by trophoblast tissue and is known to support trophoblast invasion (63). Administration of leptin increases HLA-G expression on the EVT *in vitro* (64). In this context, we hypothesize that higher leptin concentrations could be connected to the expression of HLA-G itself, indicating an interdependent relationship between HLA-G expression and leptin secretion. In our experiments, leptin concentrations increased only in the presence of decidual but also peripheral blood γδT cells, without a significant difference between the two experimental settings. This generally emphasizes the relevance of immune cells for leptin secretion. Other angiogenic factors like endoglin, placenta growth factor, or vascular endothelial growth factor, which were detected in our experiments, are known to be secreted by trophoblast tissue (65).

Regarding potential defense mechanisms, decidual γδT cells' intracellular perforin level correlates positively with NKG2C and ILT2 expression on all γδT subsets and with NKG2A for Vδ1 and DN subsets. However, our experiments do not show altered secretion of perforin in the presence of HLA-E or -G. While innate lymphoid cells utilize various activating and inhibiting receptors for their activity, γδT cells can use their TCR for antigen recognition. Our results suggest no immediate consequences for the isolated presence of either HLA-E or -G. However, long-term consequences are possible and likely. In peripheral blood NK cells, these receptors are not just associated with immediate cell reaction but also with a process of activation threshold alteration called NK cell education (66). We suggest a similar process might also be possible for γδT cells.

Decidual γδT cells secrete high levels of IFN-γ and impressive levels of granulysin. This emphasizes their importance in pathogen defense, as granulysin allows pathogen eradication from virus-infected trophoblast cells without harming the trophoblast itself (67).

Our findings highlight the multifaceted functions and interactions of γδT cells in decidua during the first trimester, confirming the concept that γδT cells are potential effector immune cells at the feto-maternal interface, contributing to healthy pregnancy. The presented data provide further evidence that decidual γδT lymphocytes significantly differ from peripheral γδT cells - they produce angiogenic and immunomodulatory proteins, have conserved or even increased cytotoxic potential, and they could be controlled by non-classical HLA molecules. Accordingly, HLA-G and HLA-E, expressed by EVT can influence decidual γδT cell function through receptors like NKG2C, NKG2A, ILT2, and KIR2DL4, which interactions may modulate immune responses, adding another layer of complexity to the maternal-fetal interface. However, the detailed consequences of the cross-linking of the non-classical HLA molecules and their receptors on the different γδT cell subpopulations remain to be elucidated in the future. Moreover, our study also reveals the potential research interest of the under-researched DN γδT cell population, which could be a promising target for further investigations in reproductive immunology.

4 Materials and methods

4.1 Human samples

Decidual tissue samples and matched peripheral blood were obtained from healthy pregnant women (n = 27, age *(mean ± SD)* = 25.9 ± 1.4) undertaking an elective pregnancy termination during the first trimester (gestational age *(mean ± SD)*= 9.3 ± 0.3) in the Department of Obstetrics and Gynecology, University of Pécs, Medical School, Hungary.

4.2 Isolation of decidual mononuclear cells

The pregnancy was terminated by vacuum aspiration, and the collected tissue was immediately processed. First, the collected decidual pieces were macroscopically homogenized with scissors (approximately 2 mm^3). Hereafter, the tissue was resuspended with prewarmed (37°C) collagenase type IV (1 mg/mL, Gibco®) and transferred to C-tubes (Miltenyi Biotec). To create a single-cell solution, the samples were then further dissected using a gentleMACS™ dissociator (Miltenyi Biotec) with three fast contrarotating cycles (800 rpm/25 sec/cycle) and slow agitation (40 rpm) for one hour at 37°C. After that, the cells were collected through successive 100 µm, 70 µm, and 40 µm nylon cell strainers (Miltenyi Biotec) and washed in RPMI1640 medium (Lonza) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza). In the next step, decidual mononuclear cells (DMCs) were isolated by Ficoll-Paque™ (GE Healthcare) gradient centrifugation. The collected cells were washed and resuspended in RPMI1640 medium (Lonza) containing 20% fetal calf serum (Gibco®) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza). The resuspended cells were distributed onto cell culture dishes and incubated overnight at 37°C and 5% CO_2 to allow the remaining decidual stroma cells to settle and adhere. The next morning, the non-adherent cells were aspirated, washed, controlled for viability with trypan blue, and split for cryopreservation and isolation of γδT cells. Cryopreserved DMCs were used for flow cytometric measurements, while isolated decidual γδT cells were co-cultured with choriocarcinoma cell lines or soluble HLA proteins.

4.3 Isolation of peripheral blood mononuclear cells

Heparinized peripheral blood was diluted with phosphate-buffered saline (PBS), and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque™ (GE Healthcare) gradient centrifugation. Hereafter, the collected cells were washed and resuspended in RPMI1640 medium (Lonza) containing 20% fetal calf serum (Gibco®) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza), then incubated overnight at 37°C and 5% CO_2. The next morning cells were controlled for viability and split cryopreservation and isolation of γδT cells. Cryopreserved PBMCs were used for flow cytometric

measurements, while isolated peripheral γδT cells were co-cultured with choriocarcinoma cell lines or soluble HLA proteins.

4.4 Cryopreservation

The washed cells were resuspended in heat-inactivated human serum containing 10% dimethyl sulfoxide and frozen at -80°C utilizing MrFrosty™ Freezing Container (Thermo Scientific™) for later analysis.

4.5 Isolation of γδT cells

Decidual and peripheral blood γδT cells were isolated using the 'TCRγ/δ$^+$ T cell Isolation Kit' (Miltenyi Biotech) according to the manufacturer's instructions. The purity of the yielded γδT cells was determined by flow cytometry, and samples showing more than 90% γδT cells (γδTCR$^+$/all living cells) were used for cell co-culture experiments (n=8). Although the precise composition of the antibody cocktail used in this kit is not publicly available, in-house testing confirmed the efficient elimination of CD56$^+$ cells.

4.6 Flow cytometry

Matched decidual and peripheral blood cryopreserved cells of 27 participants were thawed and transferred into prewarmed (37°C) RPMI1640 medium (Lonza) supplemented with 10% fetal calf serum (Gibco®), penicillin (1 x 10^5 U/L) (Lonza) streptomycin (0.05 g/L) (Lonza), and DNase (20µg/mL) (Sigma). Then, the cells were washed at 400x*g* for 7 min, resuspended in protein-free PBS, distributed into round-bottom polystyrene tubes (2 x 10^6/tube), and washed at 400x*g* for 7 min in protein-free PBS. Consecutively, the cells were stained for viability (according to the manufacturer's instructions) and surface antigens (30 min at RT in the dark). Then, the cells were fixed and permeabilized for intracellular target (Perforin, KIR2DL4) staining utilizing the InsideStain Kit (according to the manufacturer's instructions) (Miltenyi Biotec). The fluorochrome-conjugated antibodies used in each panel are summarized in Supplementary Table 1. Finally, the cells were resuspended in PBS with 1% paraformaldehyde and stored in the dark at 4°C until measurement on a Navios™ flow cytometer (Beckman Coulter). Due to low live cell count or poor sample quality, five decidual and four peripheral blood samples were excluded during preanalytical quality control in FlowJo™. Compensation matrices were calculated by FlowJo™ using CompBeads (BD™) and MACS® Comp Bead Kit, anti-REA (Miltenyi Biotec) for fluorochrome-labeled antibodies, and PBMCs for the viability dye. Gamma/delta T cells were defined as lymphocytes → single cells → ZombieNIR$^-$CD45$^+$TCRγδ$^+$ events. Decidual cells were further defined as residency marker (CD69) positive to exclude peripheral blood-derived cells in the decidual sample (68). All gates are based on fluorescence-minus-one controls (FMO; also see Supplementary Information 5). Due to day-to-day variability and the different fluorophores, we standardized fluorescence intensity data to the individual FMO:

$$Standardized\ Fluorescence\ Intensity = \frac{Median_{Population} - Median_{FMO}}{robust\ standard\ deviation_{FMO}}$$

4.7 Cell (co-)culture

Three human choriocarcinoma cell lines (JAr) were used as model tissues: A standard JAr cell line (HLA class I$^-$) and the two JAr lines transfected with either HLA-E or HLA-G$_{1m}$ (JArE and JArG$_{1m}$, respectively).

The cell lines were donated by P. Le Bouteiller (INSERM UMR 1043, Toulouse, France). JArG$_{1m}$ was produced by transfection of the pCDNA3/*HLA-G1m* plasmid, a gift of Dr. M. Lopez-Botet (Department of Immunology, University Hospital la Princesa, Madrid, Spain), in which the HLA-G leader sequence was modified as follows: the methionyl residue at position 2 was mutated to threonine; therefore, it could not provide a functional signal peptide for the expression of HLA-E ensuring the exclusive expression of HLA-G (69). JAR-E was transfected with a cd3.14 cosmid encoding HLA-E, a gift of M. Ulbrecht (Institute of Anthropology and Human Genetics, Munich, Germany) (70), in which the HLA-E leader sequence was replaced by that of HLA-A2, providing stable peptides for the expression of HLA-E, as described by Lee et al. (71). Upon arrival, aliquots of all cell lines were stored in our liquid nitrogen biobank. Low passage-count aliquots were thawed for our experiments. The cell lines were cultured in RPMI1640 medium (Lonza) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza), pyruvate (100mM) (Gibco®), geneticin (300 mg/mL) (Gibco®) for all transfectants and 10% fetal calf serum (Gibco®) at 37°C and 5% CO_2. The expression of HLA-E or HLA-G was regularly confirmed via flow cytometry (Figure 3).

On the day of sample acquisition, cells of all three cell lines were seeded onto 96-well plates (30,000 cells/well). After that, cells were incubated at 37°C and 5% CO_2 overnight for confluent growth.

On the next day, the old culture medium was carefully aspirated. Then, 100 µL of freshly isolated, matched decidual and peripheral blood γδT cells (10^6 γδT cells/mL), resuspended in cell line culture medium, were pipetted into the wells. In addition to membrane-bound HLA-E and G$_{1m}$, decidual and peripheral blood γδT cells were incubated with soluble HLA-E (0.5 µg/mL) or HLA-G (0.5 µg/mL) (both from OriGene Technologies) in independent wells. All tests were performed as biological duplicates. All wells were activated using ionomycin (1 µg/mL) (Sigma-Aldrich) and phorbol myristate acetate (25 ng/mL) (Sigma-Aldrich) for 18h. Hereafter, the 96-well plates were centrifuged, and aliquots of the supernatants were cryopreserved at -80°C for batched analysis.

4.8 Measurement of cell-(co-) culture supernatants

Diluted (1:10) cell culture supernatants were analyzed for IL-2 (6.5 – 20,000), IL-4 (9.04 – 14,000), IL-10 (5.06 – 14,000), IL-6

(11.58 – 15,000), IL-17A (8.51 – 18,000), TNF-α (12.68 – 12,000), sFas (4.53 – 81,000), sFasL (7.37 – 11,000), IFN-γ (57.13 – 20,000), granzyme A (62.96 – 15,000), granzyme B (24.99 – 52,000), perforin (60.18 – 12,000) and granulysin (175.95 – 57,000) utilizing the Human LegendPlex™ CD8/NK Panel (BioLegend) on a Canto 2 flow cytometer (BD Bioscience) according to the manufacturer's instructions.

Undiluted cell culture supernatants were analyzed for Angiopoietin-2 (13.7 – 10,000), BMP-9 (2.7 – 2,000), EGF (2.7 – 2,000), Endoglin (13.7 – 20,000), Endothelin-1 (2.7 – 2,000), FGF-1 (13.7 – 10,000), FGF-2 (13.7 – 10,000), Follistatin (27.4 – 10,000), G-CSF (13.7 – 10,000), HB-EGF (1.4 – 1,000), HGF (27.4 – 20,000), IL-8 (1.4 – 1,000), Leptin (137.2 – 100,000), PLGF (6.9 – 1,000), VEGF-A (13.7 – 10,000), VEGF-C (6.9 – 5,000) and VEGF-D (6.9 – 5,000) utilizing the MILLIPLEX® Human Angiogenesis/Growth Factor Magnetic Bead Panel - Cancer Multiplex Assay Enzyme-linked Immunosorbent Assay (Millipore) according to the manufacturer's instructions. The parentheses' numbers indicate each cytokine's detection range (pg/mL).

4.9 Statistics and data presentation

All statistical tests were performed in GraphPad Prism 9. Datasets were checked for Gaussian distribution by the D'Agostino-Pearson omnibus normality test. The test used in each comparison is indicated in the respective figure legend. Generally, p-values ≤ 0.05 were considered significant. Illustrations were produced using BioRender and Adobe Illustrator 23.0.4. Plots of flow cytometric data were exported from FlowJo™. Diagrams and Heatmaps were created using GraphPad Prism 9.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by University of Pecs - Medical School, Ethics Committee (5643-PTE 2019, 5643-PTE 2023). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. PV: Data curation, Investigation, Project administration, Writing – review & editing. IB: Data curation, Investigation, Methodology, Writing – review & editing. BF: Data curation, Formal analysis, Methodology, Validation, Writing – review & editing. AN: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing. ÁB: Data curation, Investigation, Validation, Writing – review & editing. DS: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. SE-B: Data curation, Investigation, Methodology, Writing – review & editing. PJ: Data curation, Methodology, Resources, Supervision, Validation, Writing – review & editing. KK: Investigation, Resources, Supervision, Writing – review & editing. ÉM: Writing – review & editing. TB: Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. EM: Validation, Writing – review & editing, Funding acquisition, Project administration, Resources, Supervision. AB: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1382424/full#supplementary-material

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Gamma/Delta T Cells in the Course of Healthy Human Pregnancy: Cytotoxic Potential and the Tendency of CD8 Expression Make CD56+ γδT Cells a Unique Lymphocyte Subset

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To date, pregnancy is an immunological paradox. The semi-allogenic fetus must be accepted by the maternal immune system, while defense against pathogens and immune surveillance cannot be compromised. Gamma/delta T cells are believed to play an important role in this immunological puzzle. In this study, we analyzed peripheral blood CD56+ γδT cells from pregnant women (1st, 2nd, and 3rd trimester) and non-pregnant women by multicolor flow cytometry. Interestingly, γδT cells represent almost half of CD3+ /CD56+ cells. Among γδT cells, the CD56+ population expands in the 2nd and 3rd trimester. CD56+ γδT cells maintained a predominantly CD4–/CD8– or CD8+ phenotype, while CD56– γδT cells were in similar rates CD4–/CD8– or CD4+ during pregnancy. Investigation of the lysosomal degranulation marker CD107a revealed a preserved elevated rate of potentially cytotoxic CD56+ γδT cells in pregnancy, while their cytotoxic strength was reduced. Furthermore, CD56+ γδT cells continuously showed a higher prevalence of PD-1 expression. CD56+ γδT cells' rate of PD-1 increased in the 1st trimester and decreased hereafter back to normal level. We correlated the cytotoxic potential and the expression of the inhibitory immune checkpoint PD-1 and were able to demonstrate that highly cytotoxic cells within this CD56+ γδT population tend to express PD-1, which might allow the inhibition of these cells after binding its ligand in the placenta. These findings should support the understanding of the complex processes, which ensure the maintenance of pregnancy.

Keywords: gamma/delta T cells, human pregnancy, CD4, CD8, CD56, PD-1, cytotoxicity, flow cytometry

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INTRODUCTION

Gamma/delta T cells and their possible functions in pregnancy have been the scope of many investigations over the last decades. This T cell population has a unique physiology, as it does not underlie MHC-restriction nor requires antigen processing (1, 2). Gamma/delta T cells occur at early stages of fetal development where they are probably already capable of executing defense

mechanisms against intrauterine viral infections (3). To date, the exact origin and development of γδT cells is uncertain. Although, data suggests the thymus as main source of γδT cells (4), experiments with athymic mice suggested that further development sites must exist (5). Their Vδ usage divides them into two major subsets, of which one is the Vδ2 population whereas the other is often named as non-Vδ2 population. The non-Vδ2 of adult humans consists mainly of the Vδ1 phenotype and is tissue associated. The Vδ2 phenotype is strictly associated to the usage of Vγ9 and the dominant subset in peripheral blood of adult humans (6). It was shown that the Vγ9Vδ2 subset in 1-year-old humans mostly contains non-naïve cells producing INFγ (7) and it was assumed that the dominance of the Vγ9Vδ2 subset occurs due to a pathogen-derived antigen-dependent selection process. Yet, current data suggests that pathogen-reactive effector γδT cells develop before they encounter pathogens (8, 9). Although, γδT cells are mainly double-negative for the classical T cell marker CD4 and CD8, small fractions express CD4 or CD8. As research is mainly focused on the TCR repertoire of γδT cells, the importance of these major co-receptors on γδT cells has not been investigated thoroughly.

Human γδT cells are believed to play an active role in the tolerance of paternal antigens (10–13). Nevertheless, it was not possible to describe the full picture of the immunology of pregnancy and the role of γδT cells in this complex puzzle. It can be assumed, that γδT cells have a regulatory function, which supports tolerance towards the semi-allogenic fetus on the one hand and intact defense against pathogens on the other (14). In our previous work we demonstrated the possible duality of γδT cell function in murine pregnancy: Decidual γδT cells have a strong cytotoxic potential, while expressing a tolerance promoting receptor profile (15).

As with γδT cells, the role of CD56+ cells in pregnancy, especially in human early decidua, was intensely studied. CD56 or neural cell adhesion molecule is largely known as a phenotypical marker for natural killer (NK) cells, which are often defined as CD3–/CD56+ cells. However, the expression of CD56 was also demonstrated on CD3+ αβT and γδT cells as well as on dendritic cells, monocytes and non-hematopoietic cells (16–18). CD3+/CD56+ cells were often named NKT or NKT-like cells, but with growing knowledge about the immune system and the advanced opportunities of multiparameter cytometry, the borders between cell populations became more and more vague. So far, it was not possible to demonstrate the physiological function of CD56 on any lymphoid population. However, the expression of CD56 can be correlated to the state of activation of lymphoid cells (18–20). CD56+ γδT cells show increased cytotoxicity against tumors (21) and are strong in Interferon-γ production (22). Therefore, on γδT cells, CD56 should rather be considered as a marker of activation than as a lineage marker. To date, no data about CD56-expressing γδT cells in pregnancy is available.

The basic setting of the immune system changes during pregnancy. In the first trimester, during implantation, trophoblast invasion and placentation, the maternal immune system shows a strong pro-inflammatory response (23), which is followed by a long anti-inflammatory period, where CD4+CD25+ FOXP3+ regulatory T cells and CD56bright/CD16– uterine NK cells are dominant at the feto-maternal interface (24). At the end of the third trimester, due to the lower progesterone level, pro-inflammatory cytokines (IL-8, IL-1b, IL-6, TNFα) are produced (25), recruiting neutrophils and macrophages (26) secreting proteinase (27). Furthermore, the percentages of tolerance promoting regulatory T cells and decidual NK cells are decreasing. The elevated levels of IL-6 lead to high expression of oxytocin-receptor, which allows oxytocin to induce contractions and labor (28).

Here, we provide first data about CD56+ γδT cells in the course of pregnancy regarding cytotoxic potential and a possible regulation mechanism. Cytotoxicity is believed to be important in the defense against pathogens, tumor surveillance as well as the regulation of immune responses and tolerance (29–31). Those functions must be ensured during the whole time of pregnancy, while cytotoxicity must be regulated to avoid rejection of the fetus.

The B7-CD28 superfamily member programmed cell death protein 1 (PD-1) is a transmembrane receptor, which transmits co-inhibitory signal upon binding to its ligands PD-L1 or PD-L2 (32, 33). So far, the role of PD-1 and its ligands have been investigated in murine models of pregnancy. During murine pregnancy the expression of PD-1 in T lymphocytes did not change (34). However, according to the results of *Guleria et al.*, anti-PD-L1 or -L2 application to pregnant CBA/CaJ mice showed an increased fetal resorption rate and a reduction in litter size for anti-PD-L1 treatment, while no effect was observed in subject treated with anti-PD-L2 (35). These findings are further supported by the results of *D'Addio et al*, where blocking PD-L1 lead to an increase in the prevalence of Th17 cells and a decrease in the prevalence of regulatory T cells (36). In human pregnancy, both, PD-L1 and PD-L2, are expressed on all trophoblast cells (37, 38) as well as on decidual stroma cells and macrophages (39, 40). Compared to the periphery, higher rates of PD-1+ T cells have been described in the decidua. However, the prevalence of PD-1+ T cells was similar to that found in non-pregnant endometrium (40).

In this context, cytotoxicity and immune checkpoint molecule PD-1 are of particular interest on CD56+ γδT cells during pregnancy. We hypothesized that CD56+ γδT cells represent a cytotoxic subpopulation of γδT cells and assumed that PD-1—similarly as in αβT cells (32, 41)—could be a possible regulator of their cytotoxicity in the course of healthy human pregnancy. Furthermore, we anticipated that more functional aspects of γδT cells depend on their CD56-expression. Therefore, we investigated the expression of the co-receptors CD4 and CD8 to provide a first insight into CD56+ γδT cells' characteristics.

MATERIALS AND METHODS

Human Samples

Peripheral blood samples were obtained from healthy non-pregnant women between 18 and 40 years old (n = 17) from the Regional Blood Transfusion Service, Pecs, Hungary and in the

Department of Medical Microbiology and Immunology, University of Pécs, Medical School, Hungary. Samples from different healthy pregnant women in the first [blood draw in gestational week (gw) 10–12; n = 16], second (blood draw in gw 23–27; n = 17) or third trimester (blood draw in gw 36 - 37; n = 17) were acquired in the Department of Obstetrics and Gynecology, University of Pécs, Medical School, Hungary. Our study was reviewed and approved by the local Ethics Committee (5643-PTE 2019) and informed consent was obtained from all participants. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral Blood was diluted with phosphate buffered saline (PBS) and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque™ (GE Healthcare) gradient centrifugation. Hereafter, the cells were washed, resuspended in inactivated human serum (BIOWEST SAS) supplemented with 10% dimethyl sulfoxide and frozen at -80°C for later analysis.

Fluorochrome Labeling and Flow Cytometric Analysis

Frozen PBMCs were thawed and washed twice in RPMI 1640 (Lonza) supplemented with penicillin (1 x 10^5 U/L) (Lonza), streptomycin (0.05 g/L) (Lonza) and 10% fetal bovine serum (FCS) (Gibco®). The cells (10^6 cells per tube in 100 µl RPMI 1640 with 5% FCS were incubated with fluorochrome-conjugated monoclonal antibodies (**Table 1**) for 30 min at 4°C. Afterwards, the cells were washed with PBS and resuspended with 1% paraformaldehyde in PBS and stored in the dark until flow cytometric measurement, performed with a Navios™ Ex (Beckman Coulter) and analyzed by using FlowJo™ version 10.6.1. Compensation matrices were calculated by FlowJo™ using CompBeads (BD™). All gates are based on fluorescence-minus-one controls (FMO) (gating strategy in **Supplementary Material** demonstrates data of one representative sample in the 3rd trimester pregnancy).

Activation and CD107a Cytotoxic Assay

To determine the cytotoxic potential of the investigated cell population we analyzed the cell surface expression of CD107a, which is an essential protein of the lysosomal membrane and becomes externalized upon degranulation of cytotoxic granules. We performed this well-established assay (42–45) as described by Andzelm et al. and previously presented in our publications (15, 46, 47): Cells were incubated for 4 h at 37°C and 5% CO_2 with the fluorochrome-conjugated anti-CD107a antibodies in RPMI 1640, supplemented with 10% FCS, penicillin, streptomycin, ionomycin (1 µg/ml) (Sigma-Aldrich) and phorbol myristate acetate (25 ng/ml) (Sigma-Aldrich). Before labeling with the other monoclonal antibodies the cells were washed and resuspended in RPMI 1640 with 5% FCS. Finally, the cells were fixed in 1% paraformaldehyde and evaluated as described in the previous paragraph.

Statistics

For comparison of data we performed multiple types of statistical analysis performed using GraphPad Prism 6. We use the Shapiro-Wilk test to check for gaussian distribution. For normal distributed data we used unpaired-samples-t-test to compare pregnant and non-pregnant donors. Analyses, including more than two groups were tested using Kruskal Wallis Test. To evaluate the relation of two corresponding sets of data from the same donor we used paired-samples-t-test. In cases, where normal distribution could not be assumed, we performed the Mann-Whitney test for unpaired samples and the Wilcoxon test for paired samples. Differences were determined as significant, if the *p*-value was equal to or less than 0.05. The level of significance is indicated in the text in parentheses. The respective mean ± SEM of the presented results and figures are provided in the **Supplementary Material**.

RESULTS

A Small Subset of γδT Cells Shows CD56 Positivity

In this study, we aimed to investigate peripheral blood γδT cells and demonstrate their expression of CD56 in the course of human pregnancy. We found a small population of CD3+ lymphocytes, which was double-positive for γδTCR and CD56 but did not show any alterations in frequency during pregnancy or to the non-pregnant control (**Figure 1A**). However, when gating for CD3+ /CD56+ lymphocytes (a typical way to characterize NKT-like cells, **Figure 1B1**), roughly half of the cells were γδTCR+ in all groups (**Figure 1B2**). Finally, we wanted to define the rate of CD56+ cells among γδT cells. Therefore, we defined γδT cells as CD3+/γδTCR+ lymphocytes and CD3+ but γδTCR− cells as non-

TABLE 1 | Used antibodies with conjugated fluorophore, used dilution, host species, clone, and the providing company.

Antibody	Fluorophore	Dilution	Host Species	Clone	Company
Anti-human-TCRγ/δ	FITC (Fluorescein isothiocyanate)	1:50	Mouse	B1	Sony Biotechnology Inc.
Anti-human-PD-1	PE (Phycoerythrin)	1:100	Mouse	EH12.2H7	Sony Biotechnology Inc.
Anti-human-CD4	Alexa Fluor® 700	1:100	Mouse	SK3	Sony Biotechnology Inc.
Anti-human-CD4	PE-Dazzle™594	1:50	Mouse	RPA-T4	BioLegend®
Anti-human-CD3	PE-Cy7	1:100	Mouse	SK7	Sony Biotechnology Inc.
Anti-human-CD8	APC-Cy7	1:100	Mouse	SK1	Sony Biotechnology Inc.
Anti-human-CD107a	Brilliant Violet 421™	1:200	Mouse	H4A3	Sony Biotechnology Inc.
Anti-human-CD56	Brilliant Violet 510™	1:100	Mouse	HCD56	Sony Biotechnology Inc.

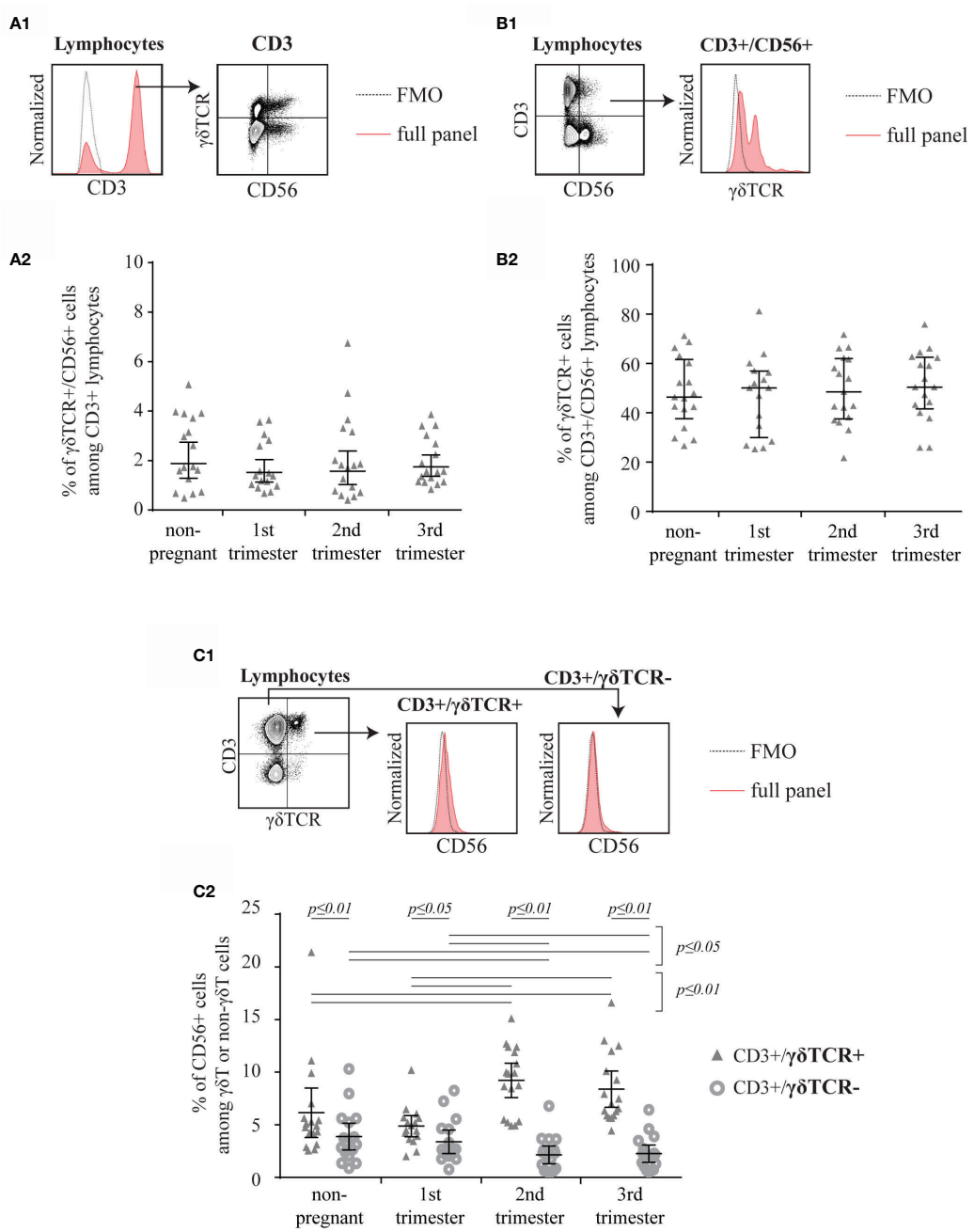

FIGURE 1 | Representative, shortened gating: First gated for CD3+ cells in a histogram (the fluorescence-minus-one control (FMO) is depicted by the dotted line) then for CD56 and γδTCR **(A1)**. The mean percentage of CD56+/γδTCR+ cells among CD3+ peripheral blood lymphocytes of non-pregnant (n = 17) and pregnant [1st (n = 16), 2nd (n = 17), and 3rd (n = 17) trimester] women is shown as horizontal line with the 95% confidence interval represented by whiskers. The individual data points are shown as triangles. Statistical analysis with Kruskal-Wallis test showed no significant alterations between the different groups **(A2)**. Representative, shortened gating: First gated for CD3+/CD56+ cells demonstrated as dot plot then for γδTCR+ cells in a histogram. The FMO is depicted by the dotted line **(B1)**. Mean percentage of γδTCR+ cells among CD3+/CD56+ peripheral blood lymphocytes of non-pregnant (n = 17) and pregnant [1st (n = 16), 2nd (n = 17), and 3rd (n = 17) trimester] women depicted as horizontal line with the 95% confidence interval represented by whiskers. The individual data points are shown as triangles. Statistical analysis with Kruskal-Wallis test showed no significant alterations between the different groups **(B2)**. Representative, shortened gating: First gated for CD3+/γδTCR+ or CD3+/γδTCR– cells demonstrated as dot plot then for CD56+ cells in a histogram. The FMO is depicted by the dotted line **(C1)**. Mean percentage of CD56+ cells among CD3+/γδTCR+ and CD3+/γδTCR– peripheral blood lymphocytes of non-pregnant (n = 17) and pregnant [1st (n = 16), 2nd (n = 17), and 3rd (n = 17) trimester] women depicted as horizontal line with the 95% confidence interval represented by whiskers. The individual data points are shown as triangles (CD3+/γδTCR+) or circles (CD3+/γδTCR–). Statistical analysis was performed by using the Mann-Whitney test (non-pregnant vs 1st trimester vs 2nd trimester vs 3rd trimester) or the Wilcoxon test (CD3+/γδTCR+ vs CD3+/γδTCR–). Significant differences are depicted by a horizontal line above the respective data sets **(C2)**.

γδT cells (**Figure 1C1**). Thereafter, we determined the prevalence of CD56+ cells within those T cell subsets. The rate of CD56+ cells was significantly higher in γδT cells compared to non-γδT cells (CD3+/γδTCR−) in all four groups. This difference was most notably in the 2nd and 3rd trimester (non-pregnant: $p \leq 0.01$, 1st trimester: $p \leq 0.05$, 2nd trimester: $p \leq 0.01$, 3rd trimester: $p \leq 0.01$). Furthermore, CD56+ cells among γδT cells and non-γδT cells were rare in non-pregnant women and during the 1st trimester. However, while the rate of CD56+ cells in non-γδT cells significantly decline in the 2nd and 3rd trimester ($p \leq 0.05$), in γδT cells the percentage of CD56+ cells spiked in the 2nd trimester ($p \leq 0.01$). From there, the rate of CD56+ cells decreased marginally in the 3rd trimester (**Figure 1C2**).

CD56+ γδT Cells Are Predominantly Double-Negative for CD4 and CD8

To determine, if the expression of CD56 on γδT cells has an impact on functional aspects of this cell population, we analyzed the expression of CD4 and CD8 on CD56+ compared to CD56− γδT cells (**Figure 2A**). Here, we found significant differences in the prevalence of double negative (CD4−/CD8−) and CD4+ cells between these two investigated subsets in all groups. The prevalence of double negative cells was permanently higher in CD56+ γδT cells (all groups: $p \leq 0.01$) (**Figure 2B**) whereas the prevalence of CD4 was lower compared to CD56− γδT cells (all groups: $p \leq 0.01$). However, as there was no significant alteration during pregnancy in the CD56− γδT subset, among CD56+ γδT cells, the prevalence of CD4+ cells was significantly higher in the 1st and 3rd trimester compared to the 2nd trimester ($p \leq 0.01$ and $p \leq 0.05$, respectively) or in non-pregnant control group ($p \leq 0.01$ and $p \leq 0.02$, respectively) (**Figure 2C**). The prevalence of CD8+ cells was significantly higher among CD56+ compared to CD56− γδT cells in non-pregnant samples as well as in the 1st and 2nd trimester of pregnancy ($p \leq 0.01$, $p \leq 0.05$ and $p \leq 0.02$, respectively). Furthermore, in both γδT cell subsets, the rate of CD8+ cells was significantly lower in the 1st trimester compared to the non-pregnant control (both: $p \leq 0.05$) (**Figure 2D**).

Further comparative analysis of the corresponding data of CD4 or CD8 positivity on CD56+ and CD56− γδT cells revealed that, in each group, the rate of CD8-expressing cells among CD56+ γδT cells was significantly higher than that of CD4+-ones (all groups: $p \leq 0.01$; mean CD4/CD8-ratio of CD56+ γδT cells in non-pregnant, 1st trimester, 2nd trimester and 3rd trimester group, respectively: 0.33, 0.63, 0.31, 0.58). Regarding the CD56− γδT cells we found the opposite, where in each sample group a significantly (all groups: $p \leq 0.01$) higher rate of CD56− γδT cells were CD4+, compared with the rate of CD8+ cells among CD56− γδT cells (mean CD4/CD8-ratio of CD56− γδT cells in non-pregnant, 1st trimester, 2nd trimester and 3rd trimester group, respectively: 1.3, 1.4, 1.28, 1.49).

CD56+ γδT Cells Show a Strong Cytotoxic Potential

The potential for cytotoxic activity of γδT cells was determined by the surface expression of CD107a upon activation (**Figure 3A**). Although, the rate of CD107a+ cells was significantly higher among CD56+ γδT cells compared to the CD56− subset in all groups (all groups: $p \leq 0.01$), this rate did not alter considerably during pregnancy. However, the percentage of CD107a+ cells among CD56− γδT cells was significantly higher in pregnancy compared to the non-pregnant control ($p \leq 0.05$) (**Figure 3B**). For a better classification of the cytotoxic potential we determined the CD107a-mean fluorescens intensity (MFI) of CD107a+ cells in both γδT subsets. Here, the MFI was significantly higher in the CD56+ γδT subpopulation in all sampled groups (all groups: $p \leq 0.01$). In CD56+ γδT cells, the CD107a-MFI was significantly lower during pregnancy compared to the non-pregnant control (all pregnant groups: $p \leq 0.01$). In pregnancy, the lowest CD107a-MFI was found in the 1st trimester, from there it increased significantly to the 3rd trimester ($p \leq 0.02$). The MFI of CD56− γδT cells did not alter (**Figure 3C**).

The Rate of PD-1+ Cells Is Higher in CD56+ γδT Cells

The surface expression of PD-1 was measured on CD56+ or CD56− γδT cells (**Figure 4A**). Compared to CD56− γδT cells, PD1+ cells were significantly more common in CD56+ γδT cells at all measured timepoints (all groups: $p \leq 0.01$). Within the CD56+ γδT subset, the prevalence of PD-1+ cells increased significantly ($p \leq 0.01$) in the first trimester and fell back to non-pregnant-level in the 2nd and 3rd trimester (both: $p \leq 0.01$). Among CD56− γδT cells, the prevalence of PD-1+ cells was significantly higher in the 1st and 2nd trimester compared to the non-pregnant control (both: $p \leq 0.01$) (**Figure 4B**). No significant difference was detected regarding the MFI of PD-1 (**Figure 4C**).

The Co-Expression of PD-1 and CD107a on γδT Cells Correlates With Their CD56 Expression

Here, we aimed to investigate the co-expression of PD-1 and CD107a in CD56+ or CD56− γδT cells (**Figure 5A**). No significant difference was observed in the prevalence of PD-1+/CD107a− cells between CD56+ and CD56− γδT subsets in any study group. Within both of these subsets, the prevalence of PD-1+/CD107a− cells was significantly higher in the 1st and 2nd trimester than in the non-pregnant group (CD56+: $p \leq 0.01$ and $p \leq 0.05$, respectively; CD56−: $p \leq 0.01$ and $p \leq 0.02$, respectively). Furthermore, the highest rates were in the 1st trimester, which were also significantly higher compared to the 3rd trimester (both subsets: $p \leq 0.02$) (**Figure 5B1**). The prevalence of PD-1−/CD107a+ cells was significantly higher among CD56+ than in CD56− γδT cells in all groups (all groups: $p \leq 0.01$). Among the CD56− γδT cells the prevalence of PD-1−/CD107a+ cells was higher in the 1st and 3rd trimester of pregnancy compared to the non-pregnant control (both: $p \leq 0.05$) (**Figure 5B2**). Compared to the CD56− subset, the prevalence of PD-1+/CD107a+ cells was significantly higher in the CD56+ γδT subset at all timepoints (all groups: $p \leq 0.01$). Within this subset, the prevalence of double-positive cells was significantly lower in the 2nd trimester compared to the non-pregnant group ($p \leq 0.05$). In opposite, among the CD56− γδT cells, the rate of double-positive cells was the lowest in

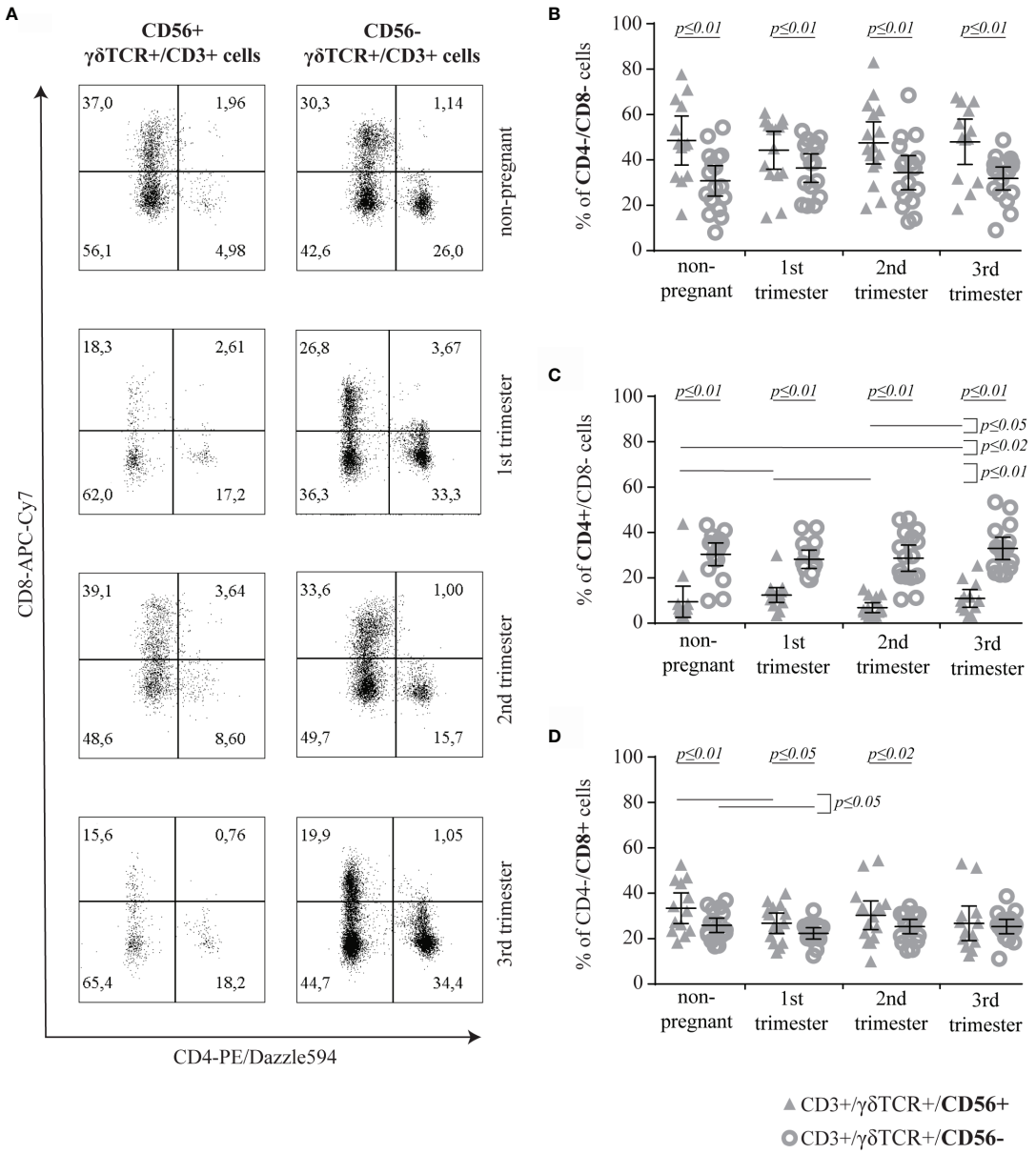

FIGURE 2 | Representative CD4/CD8 dot plots of peripheral blood CD56+ or CD56– γδT cells (CD3+/γδTCR+) in non-pregnant and pregnant (1st, 2nd, and 3rd trimester) women **(A)** Percentage of CD4–/CD8– **(B)**, CD4+ **(C)** and CD8+ **(D)** cells among CD56+ or CD56– γδT cells (CD3+/γδTCR+) in peripheral blood of non-pregnant (n = 17) and pregnant [1st (n = 16), 2nd (n = 17), and 3rd (n = 17) trimester] women. The mean is depicted by a horizontal line with the 95% confidence interval represented by whiskers. The individual data points are shown as triangles (CD3+/γδTCR+/CD56+) or circles (CD3+/γδTCR+/CD56–). Significant differences between the groups were determined as followed: Panel B: Differences between the CD56+ and CD56– γδT cell subpopulation were tested by paired-samples-t-test; Panels C and D: Statistical analysis was performed by using the Mann-Whitney test (non-pregnant vs 1st trimester vs 2nd trimester vs 3rd trimester) or the Wilcoxon test (CD3+/γδTCR+/CD56+ vs CD3+/γδTCR+/CD56–). Significant differences are depicted by a horizontal line above the respective data sets.

the non-pregnant group and significantly higher in the 2nd and 3rd trimester (both: $p \leq 0.05$) (**Figure 5B3**).

Further comparative analysis of the corresponding data of PD-1+/CD107a+ and PD-1–/CD107a+ cells in the CD56+ and CD56– γδT subsets revealed that in both subsets and in all groups γδT cells were rather PD-1–/CD107a+ than PD-1+/CD107a+ (all $p \leq 0.01$)

We also calculated the ratio (R) of double- and single-positive cells:

$$R = \frac{PD-1+/CD107a+}{PD-1-/CD107a+}$$

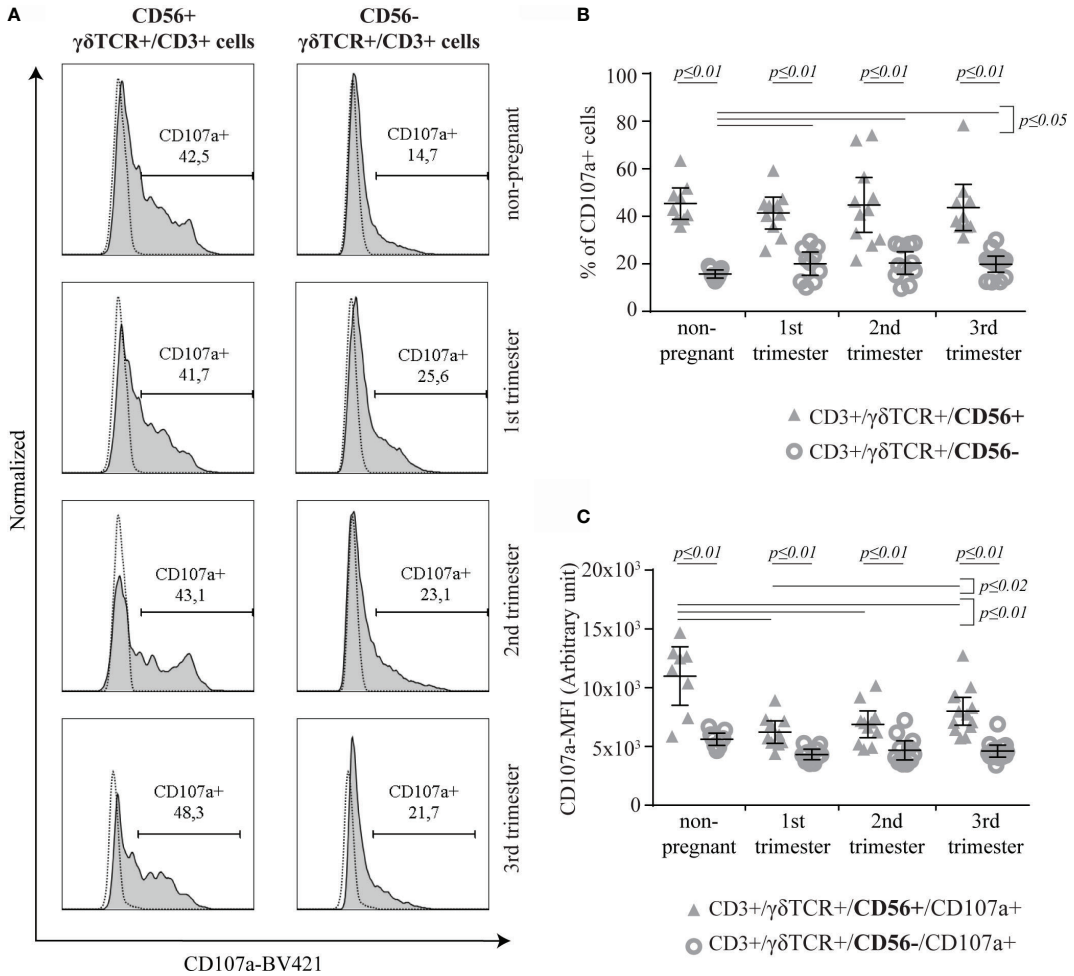

FIGURE 3 | Representative CD107a histograms (the FMO is depicted by the dotted line) of peripheral blood CD56+ or CD56– γδT cells (CD3+/γδTCR+) in non-pregnant and pregnant (1st, 2nd, and 3rd trimester) women **(A)** Percentage **(B)** and Mean of Fluorescence Intensity (MFI) **(C)** of CD107a+ cells within peripheral blood CD56+ or CD56– γδT cells (CD3+/γδTCR+) in non-pregnant (n = 9) and pregnant [1st (n = 10), 2nd (n = 10), and 3rd (n = 13) trimester] women. The mean is depicted by a horizontal line with the 95% confidence interval represented by whiskers. The individual data points are shown as triangles (CD3+/γδTCR+/CD56+ or CD3+/γδTCR+/CD56+/CD107a+, respectively) or circles (CD3+/γδTCR+/CD56– or CD3+/γδTCR+/CD56–/CD107a+, respectively). Statistical analysis was performed by using the Mann-Whitney test (non-pregnant vs 1st trimester vs 2nd trimester vs 3rd trimester) or the Wilcoxon test (CD3+/γδTCR+/CD56+ vs CD3+/γδTCR+/CD56–). Significant differences are depicted by a horizontal line above the respective data sets.

When comparing the "R" values of CD56+ and CD56– γδT subsets (mean R of CD56+ γδT cells in non-pregnant, 1st trimester, 2nd trimester and 3rd trimester group, respectively: 0.38, 0.37, 0.26, 0.27; mean R of CD56– γδT cells in non-pregnant, 1st trimester, 2nd trimester and 3rd trimester group, respectively: 0.22, 0.24, 0.26, 0.24) we found that the "R" value was significantly higher in the CD56+ γδT subset in all groups, except in the 2nd trimester, where it was equal to the CD56– γδT subset (non-pregnant, 1st trimester: $p \leq 0.01$, 3rd trimester: $p \leq 0.02$). In the CD56+ γδT subset, this ratio was significantly lower during the 2nd and 3rd trimester compared to the 1st trimester ($p \leq 0.02$, and $p \leq 0.05$, respectively) and the non-pregnant control ($p \leq 0.01$ and $p \leq 0.05$, respectively). In contrary, in the CD56– γδT subset, no significant alteration between the different timepoints was found.

For a better understanding of the impact of PD-1 within the cytotoxic γδT cells, we studied the expression of PD-1 on CD56+/CD107a+ and CD56–/CD107a+ γδT cells. Here, compared to CD56– γδT subset, significantly higher rate of cytotoxic CD56+ γδT cells express PD-1 in non-pregnant group ($p \leq 0.01$) and in the 3rd trimester pregnancy ($p \leq 0.05$), whereas in the 1st trimester we found the opposite result ($p \leq 0.01$). The prevalence of PD-1+ cells in the cytotoxic CD56+ γδT subset was significantly lower in pregnancy than in non-pregnant state (1st trimester: $p \leq 0.01$, 2nd trimester: $p \leq 0.02$, 3rd trimester: $p \leq 0.05$). However, during pregnancy the rate of PD-1+ cells among the cytotoxic CD56+ γδT cell subset did not alter. Interestingly and in opposite to the cytotoxic CD56+ γδT subset, the cytotoxic CD56– γδT subset showed a significant increase of PD-1+ cells in the 1st trimester (non-pregnant: $p \leq 0.01$, 2nd and 3rd trimester: $p \leq 0.05$) (**Figure 5C**).

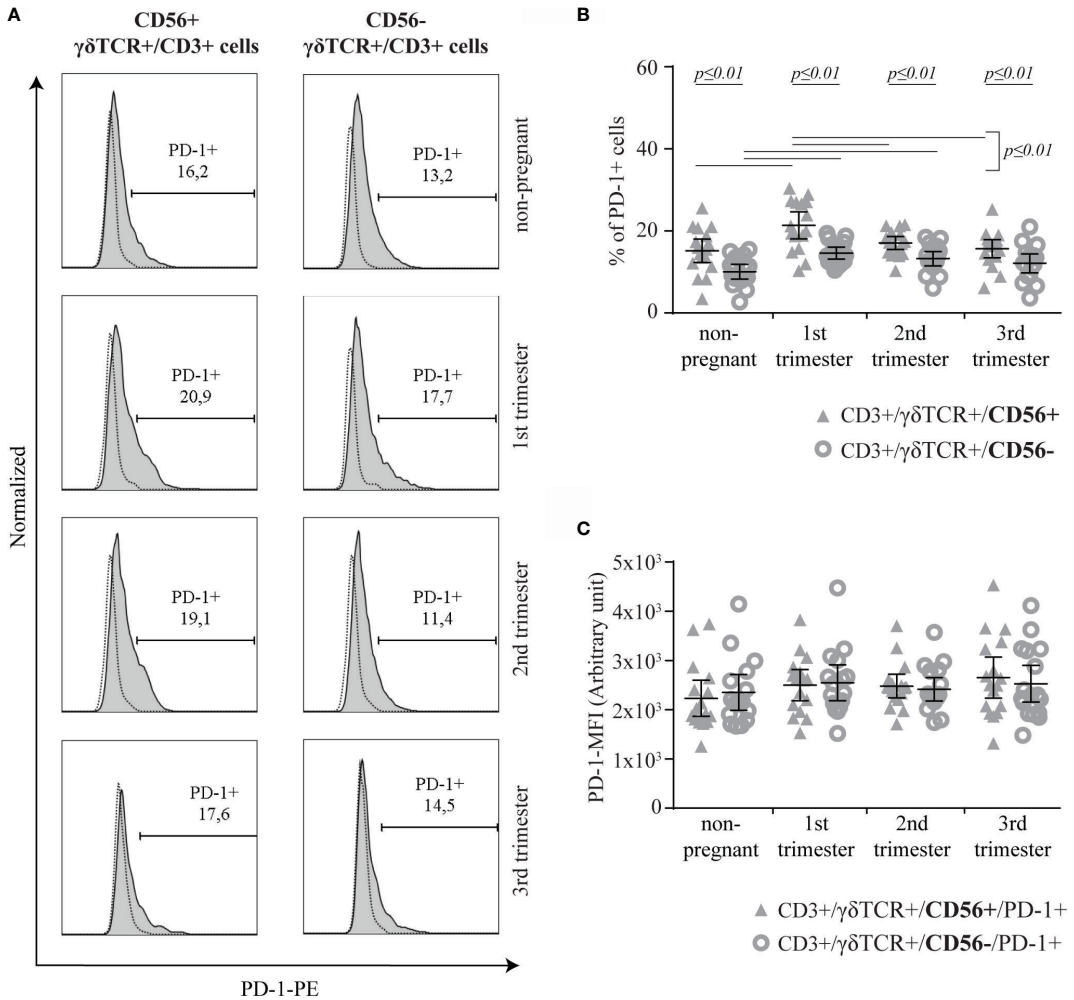

FIGURE 4 | Representative PD-1 histograms (the FMO is depicted by the dotted line) of peripheral blood CD56+ or CD56– γδT cells (CD3+/γδTCR+) in non-pregnant and pregnant (1st, 2nd, and 3rd trimester) women **(A)** Percentage **(B)** and PD-1-MFI **(C)** of PD-1+ cells among peripheral blood CD56+ or CD56– γδT cells (CD3+/γδTCR+) in non-pregnant (n = 17) and pregnant [1st (n = 16), 2nd (n = 17), and 3rd (n = 17) trimester] women. The mean is depicted by a horizontal line with the 95% confidence interval represented by whiskers. The individual data points are shown as triangles (CD3+/γδTCR+/CD56+ or CD3+/γδTCR+/CD56+/PD-1+, respectively) or circles (CD3+/γδTCR+/CD56– or CD3+/γδTCR+/CD56–/PD-1+, respectively). Statistical analysis was performed by using the paired-samples-t-test to compare data between the CD56+ and CD56– γδT cell population and the unpaired-samples-t-test to compare within the same population but at different timepoints. Significant differences are depicted by a horizontal line above the respective data sets.

To determine, if PD-1 expression is related to the intensity of the cytotoxic potential, we finally analyzed the CD107a-MFI on PD-1+ versus PD-1– CD56+/CD107a+ and CD56–/CD107a+ γδT cells, respectively. Here, the CD107a-MFI-value of CD56+/CD107a+/PD-1+ γδT cells was significantly higher in all groups (non-pregnant, 1st trimester: $p \leq 0.02$; 2nd trimester, 3rd trimester: $p \leq 0.01$) (**Figure 6A**). This correlation was also significant in CD56–/CD107a+/PD1+ γδT cells (all groups: $p \leq 0.01$), where among the PD-1+ cells the CD107a-MFI was significantly lower in the 1st trimester compared to all other groups (non-pregnant, 3rd trimester: $p \leq 0.01$; 2nd trimester: $p \leq 0.05$) (**Figure 6B**). After statistical comparison of the corresponding data of **Figures 6A, B**, we found a significant higher CD107a-MFI values among CD56+ γδT cells in all groups (all $p \leq 0.01$).

DISCUSSION

While αβT cells are traditionally divided into CD4+ and CD8+ T cells, γδT cells are mainly classified based on their Vδ-chain-usage into circulating Vδ2+ and resident Vδ1+ cells. Although, there is a number of reports describing CD8 and/or CD4 positive γδT subtypes or clones (15, 48–50), γδT cells are mainly considered to be double negative and therefore these classical phenotype markers are rarely used in γδT cell research. According to our phenotypic characterization of peripheral blood γδT cells from pregnant and non-pregnant women, it is important to note that γδT cells can express CD4 and CD8. However, we did not find any notable shifts in the rate of CD4 or CD8 expressing cells in the course of pregnancy.

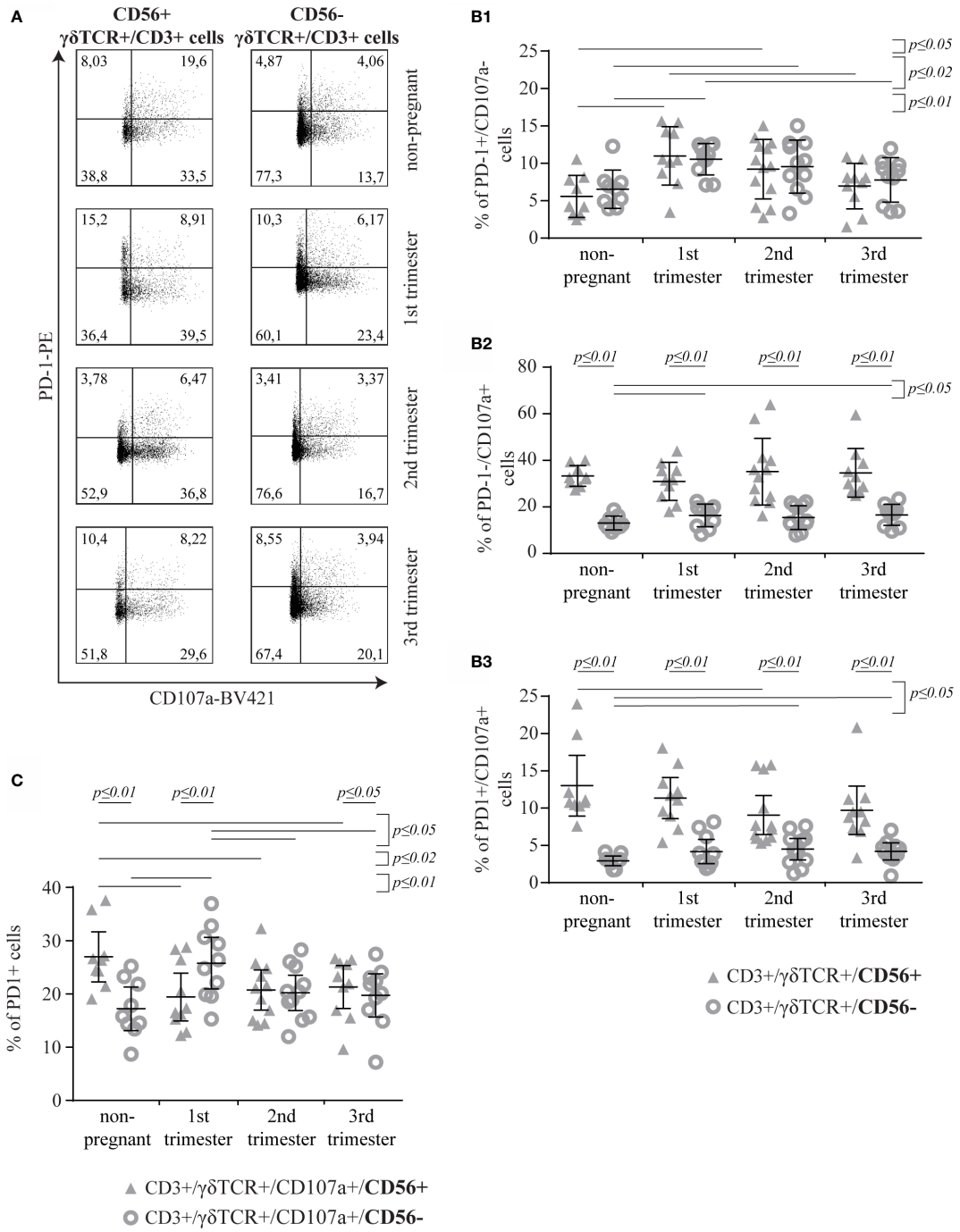

FIGURE 5 | Representative PD-1/CD107a dot plots of peripheral blood CD56+ or CD56– γδT cells (CD3+/γδTCR+) in non-pregnant and pregnant (1st, 2nd, and 3rd trimester) women **(A)**. Rates of PD-1+/CD107a– **(B1)**, PD-1–/CD107a+ **(B2)** and PD-1+/CD107a+ **(B3)** cells among peripheral blood CD56+ or CD56– γδT cells (CD3+/γδTCR+) in non-pregnant (n = 9) and pregnant [1st (n = 10), 2nd (n = 10), and 3rd (n = 13) trimester] women **(B)**. Percentage of PD-1+ cells among CD107a+ cells in the peripheral blood CD56+ or CD56– γδT cells (CD3+/γδTCR+) in non-pregnant (n = 9) and pregnant [1st (n = 10), 2nd (n = 10), and 3rd (n = 13) trimester] women **(C)**. The means of the data presented in Panels B and C are depicted as a horizontal line with the 95% confidence interval represented by whiskers. The individual data points are shown as triangles (CD3+/γδTCR+/CD56+ or CD3+/γδTCR+/CD107a+/CD56+, respectively) or circles (CD3+/γδTCR+/CD56– or CD3+/γδTCR+/CD107a+/CD56–, respectively). Statistical analysis was performed by using the paired-samples-t-test to compare data between the CD56+ and CD56– γδT cell population and the unpaired-samples-t-test to compare within the same population but at different timepoints. Significant differences are depicted by a horizontal line above the respective data sets.

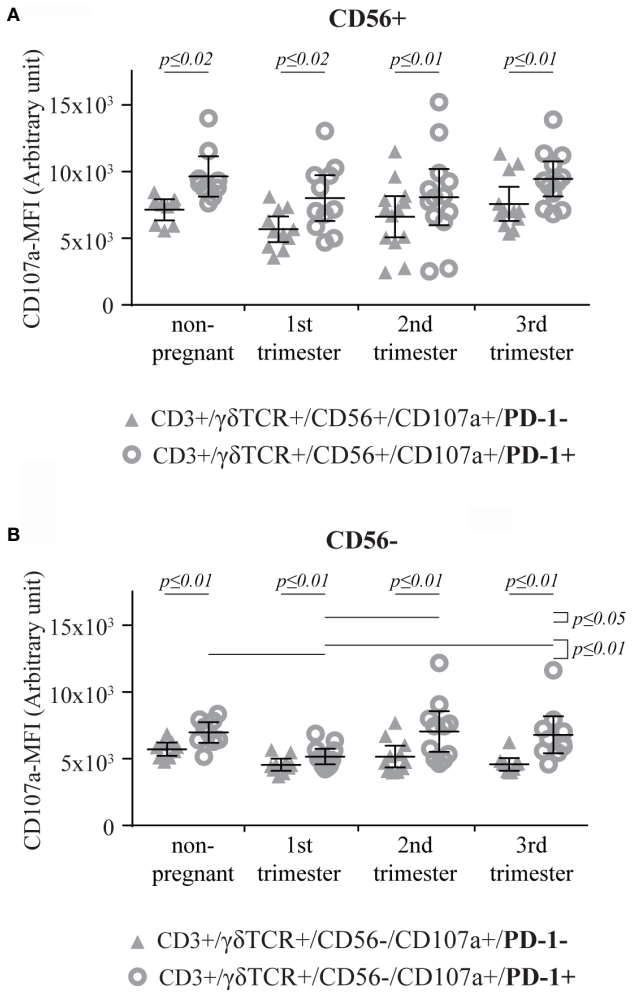

FIGURE 6 | CD107a-MFI in respect of PD-1 positivity in peripheral blood CD56+ **(A)** or CD56– **(B)** γδT cells (CD3+/γδTCR+) in non-pregnant (n = 9) and pregnant [1st (n = 10), 2nd (n = 10), and 3rd (n = 13) trimester] women. The means are depicted as a horizontal line with the 95% confidence interval represented by whiskers. The individual data points are shown as triangles (CD3+/γδTCR+/CD56+/CD107a+/PD-1– or CD3+/γδTCR+/CD56–/CD107a+/PD-1–, respectively) or circles (CD3+/γδTCR+/CD56+/CD107a+/PD-1+ or CD3+/γδTCR+/CD56–/CD107a+/PD-1+, respectively). Statistical analysis was performed by using the paired-samples-t-test to compare data between the PD-1+ and PD-1– γδT cell population and the unpaired-samples-t-test to compare within the same population but at different timepoints. Significant differences are depicted by a horizontal line above the respective data sets.

During human pregnancy, a main interface where circulating immune cells can encounter fetal antigens is the syncytiotrophoblast, which covers the embryonic villi of the placenta and is bathed by the maternal blood in the intervillous space. The main characteristic of the syncytiotrophoblast is the lack of HLA Class I and Class II molecule expression (51). Here, γδT and NK cells - as they do not underlie MHC-restriction - are of particular interest. Gamma/delta T cells express several typical NK cell associated receptors, such as NK cell phenotype marker CD56 (52), activating [e.g. NKG2D (53) or NKp44 (54)] or inhibitory NK cell receptors [CD94/NKG2A (55) or p58 (56)]. Therefore, γδT cells are often considered to be a bridge between the innate and the adaptive immune system. So far, CD56+ γδT cells and their functions have mainly been investigated in the aspect of anti-tumor activity, as CD56+ γδT cells show much stronger cytotoxicity following stimulation with IL-2 and IL-12 than CD56– γδT cells (57). It seems that this cytotoxicity is executed *via* the perforin/granzyme pathway and is mainly γδTCR/NKG2D dependent. However, CD56+ γδT cells are resistant to Fas ligand-mediated apoptosis (21). Interestingly, according to our results, half of all CD3+/CD56+ cells were γδTCR+ regardless of existence or stage of pregnancy. In our view, this is a highly important information for all fellow researchers investigating CD56+ T cells. Under physiological circumstances, one to ten percent of peripheral blood T cells are γδT cells (58), which might have led to some negligence of this highly differing subset in T cell research. Due to this high rate of γδT cells among CD3+/CD56+ lymphocytes, γδT cells must be considered in any further "NKT-like" cell research as well. Hereafter, the focus of this study was narrowed to this poorly reviewed T cell subpopulation, CD56+ γδT cells.

To date, the precise role of CD56+ γδT cells in pregnancy has not been described, therefore we aimed to investigate this topic. In our study CD56+ γδT cells were much more common during the 2nd and 3rd trimester, suggesting that they could be especially important in the second half of pregnancy. Regarding the expression of classical T cell phenotype markers CD4 and CD8, the majority of CD56+ γδT cells were double negative, a third of them were CD8+ and just a small percent expressed CD4. Therefore, we assume, that the function of CD56+ γδT lymphocytes could be more cytotoxic than regulatory. However, since the rate of CD4+ cells among CD56+ γδT cells was higher in the 1st and 3rd trimester, these cells might play a role at those specific time points. Further investigation with much higher cell counts are necessary to determine the function of this rare CD4 +/CD56+ γδT subpopulation. Compared to CD56– γδT cells, CD56+ γδT cells show a smaller rate of CD4+ cells, while the prevalence of CD8+ cells was higher in CD56+ γδT subset. This relation was prominent in non-pregnant women as well as during pregnancy, with the exception of the 3rd trimester, where both γδT subsets had a similar low CD8-expression.

The CD4/CD8 ratio of CD3+ cells is used as a clinical indicator for the health or functionality of the immune system, whereas a ratio of 1.5 to 2.5 is regarded as normal (59–61). Interestingly and in opposite to CD56+ γδT cells, where we found an inverted CD4/CD8 ratio, CD56– γδT cells' CD4/CD8 ratio was almost in normal range. Thus, CD56– γδT cells express rather CD4 than CD8. Mincheva–Nilsson described that decidual CD56– γδT cells show an enhanced IL-10 and TGF-β transcription during healthy human pregnancy compared with CD56+ ones, suggesting the immunoregulatory potential of the CD56– γδT cell population (12). It is possible that the CD4+ CD56– γδT cells described in the present study could produce IL-10 and TGF-β.

Besides the regulatory aspect of γδT cells, their cytotoxic potential and its regulation could also be essential for heathy pregnancy. A basic level of cytotoxicity seems necessary for defense against pathogens and tissue remodeling (62). On the contrary, cytotoxicity against the fetus must be prevented. Here we show that the rate of potentially cytotoxic cells is consistently

higher among CD56+ γδT cells compared to CD56− γδT but did not differ within the studied non-pregnant or pregnant groups. This general connection of cytotoxic potential and CD56 expression has already been described for CD8+ αβT cells (63) and for anti-tumor γδT cells (21). Interestingly, the potential cytotoxic power (indicated by the CD107a-MFI) of cytotoxic CD56+ γδT cells was low during pregnancy, although it was increasing from the 1st to the 3rd trimester. This finding suggests a weaker cytotoxic burst of CD56+ γδT cells during pregnancy, which might promote fetal survival. This is in line with the previously conducted research in reproductive immunology, that the majority of pregnancy is dominated by a Th2-mediated immune tolerance, which is replaced by a pro-inflammatory period shortly before term (24).

During pregnancy, fetal HLA-G seems to be a major regulator of resident cytotoxic T cells and NK cells (64, 65). However, as HLA-G is expressed by the extravillous cytotrophoblast but not by the syncytiotrophoblast (51), the regulation of circulating cytotoxic cells must be mediated *via* different mechanisms. During the 2nd and 3rd trimester, when the rate of peripheral CD56+ γδT cells is higher in the maternal blood, PD-L1 is also strongly expressed on fetal cyto- and syncytiotrophoblast in the placenta, suggesting a possible association between those (38, 40). With the progression of pregnancy, the maternal placental blood flow increases up to 600 ml/min and the surface area of the syncytiotrophoblast becomes larger (51). At this growing feto-maternal interface, where circulating maternal CD56+ γδT cells can encounter fetal antigens, inhibition *via* PD-1–PD-L1 interactions might be an important control mechanism. Additionally, since PD-L1+ fetal trophoblast cells could leave the placental structure and enter the maternal blood stream (51), these circulating and potentially immunoreactive fetal cells could trigger further effects in the maternal immune system. Altered immunological parameters, which can be detected in the peripheral blood are important in clinical practice, as blood draws are easy, safe and part of standard pregnancy care protocols anyway. To explore the possibility of systemic PD-1-mediated inhibition, we investigated the expression of PD-1 on peripheral CD56+ γδT cells. The rate of PD-1+ cells was generally higher among CD56+ γδT cells compared to CD56− γδT cells. This could imply that CD56+ γδT cells underlie a stronger PD-1-mediated regulation. The prevalence of PD-1+ cells among CD56+ γδT cells is higher in the 1st trimester compared to the non-pregnant group but decreases by the 2nd trimester, which disfavors PD-1 as a major regulator of CD56+ γδT cells at the feto-maternal interface in the 2nd half of pregnancy. For a better understanding of the influence of PD-1 on the cytotoxicity of CD56+ γδT cells during pregnancy, we explored the co-expression of PD-1 and CD107a. This analysis revealed that the previously described 1st trimester increase of PD-1+ cells, however, is only present in PD-1+/CD107a − cells, whereas PD-1+/CD107a+ cells show a decrease in pregnancy (2nd trimester) compared to non-pregnant samples. As previously described, the PD-1 expression is higher in CD56+ than in CD56− γδT cells, and the significance of this difference seems to originate from the CD107a+ subgroup, as the PD-1 co-expression in the CD107a− subgroup is comparable in CD56+ and CD56− cells. This means that whereas the PD-1+/CD107a+ population strongly correlates with CD56 expression, the PD1+/CD107a− phenotype is not dependent on it. Accordingly, we hypothesized that PD-1-mediated regulation should be of higher importance in CD56+ cytotoxic γδT cells. When analyzing the expression of PD-1 within potentially cytotoxic CD56+ γδT cells, just about a quarter of these cells were PD1+ and the prevalence of PD-1+ cells decreased in the 1st trimester and stayed low during pregnancy. On the other hand, cytotoxic CD56− γδT cells showed a peak in PD1 expression only in the 1st trimester. These results suggest, that the PD1 receptor alone might not be able to ensure the entire control of cytotoxicity of peripheral γδT cells during pregnancy. However, when thinking about cytotoxicity, beside the rate of potentially cytotoxic cells, the strength of the potential cytotoxicity (indicated by the CD107a-MFI) of these cells could be more informative. Here, PD-1 +/CD107a+ CD56+ γδT cells show a more powerful cytotoxic potential than PD1−/CD107a+ ones during all stages of human pregnancy. This indicates that a PD-1–PD-L1 receptor-ligand interaction could result in an effective inhibition of the threatened high cytotoxicity caused by CD56+ γδT cells. Nevertheless, since a considerable proportion of CD56+ γδT cells are PD1−, it could be supposed that there must be additional mechanisms controlling potential cellular aggression of cytotoxic CD56+ γδT cells. Typical cytotoxicity-inhibiting receptors are NK receptors, like KIR and NKG2A, have HLA molecules as ligands (66, 67), therefore they are possibly not implicated in this regulation. NKG2D, although an activating receptor, could be an appropriate candidate in pregnancy, since the results of Hedlund et al. show that NKG2D could inhibit cytotoxic activity of peripheral blood mononuclear cells after binding syncytiotrophoblast-derived circulating exosomes containing soluble NKG2D-ligands (68).

Nevertheless, pregnancy is a highly dynamic physiological situation, where a higher number of sampling timepoints, especially in the 1st trimester would draw clearer picture. However, studies with a thorough surveillance of immune changes in the course of healthy human pregnancy are rare and as the majority of healthy human pregnancies are not detected before gestation week 5, the extremely interesting phase of implantation and placentation is hard to study.

In this study we demonstrated that half of CD3+/CD56+ lymphocytes showed a γδTCR-phenotype, which was also prevalent over the course of pregnancy. Due to the unique physiology of γδT cells, this observation should be taken in consideration in any further research regarding "NKT-like" cells. Additionally, we demonstrated again the CD4+ and CD8+ phenotype of γδT lymphocytes. The expression of CD4 and CD8 on γδT cells appears to be dependent on the expression of CD56. Although largely double-negative, CD56+ γδT cells express rather CD8+ than CD4+. However, CD4-expressing γδT cells are mainly CD56−. Furthermore, our results indicate that the often-described cytotoxic capacity of γδT cells seems to be mainly represented by the small subset of CD56+ γδT cells, which expands during the 2nd and 3rd trimester of pregnancy. Although the intensity of a potentially cytotoxic burst is low in pregnancy, a small PD-1+/CD107a+ population of CD56+ γδT cells maintain a relatively strong cytotoxic capacity. Evidently, a good immuno-balance is generally important, but for a successful pregnancy it is crucial. Among others, PD-1 expression might prevent potentially harmful overreaction of these highly cytotoxic

CD56+ γδT cells at the feto-maternal interface. We hypothesize that conventional CD56– but CD4+ γδT cells, on the other hand, could have an immunoregulatory function, which should be investigated in further studies. Our present findings about γδT cells might help for a deeper understanding of the complex puzzle of immune tolerance in pregnancy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Pecs (5643-PTE 2015 and 5643-PTE 2019). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The project was conceived by AB and JN and realized by JN. AB provided all critical reagents, experimental support, and critical discussion. PJ ensured the quality of the cytometric data acquisition. The manuscript was written by JN and edited by AB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.596489/full#supplementary-material

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