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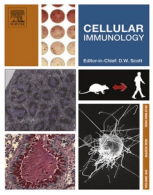

Human decidual gamma/delta T cells possess unique effector and TCR repertoire profiles during pregnancy

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ABSTRACT

Human γδ T cells are enriched at the maternal-fetal interface (MFI, *decidua basalis*) showing a highly differentiated phenotype. However, their functional potential is not well-known and it is not clear whether this decidua-enrichment is associated with specific γδ T cell receptors (TCR) as is observed in mice. Here we addressed these open questions by investigating decidual γδ T cells during early and late gestation, in comparison with paired blood samples, with flow cytometry (cytotoxic mediators, cytokines) and TCR high-throughput sequencing. While decidual γδ T cells expressed less perforin than their counterparts in the blood, they expressed significant more granulysin during early pregnancy. Strikingly, this high granulysin expression was limited to early pregnancy, as it was reduced at term pregnancy. In contrast to this granulysin expression pattern, decidual γδ T cells produced reduced levels of IFNγ and TNFα (compared to paired blood) in early pregnancy that then increased by term pregnancy. TCR repertoire analysis indicated that human decidual γδ T cells are not generated early in life as in the mouse. Despite this, a specific enrichment of the Vγ2 chain in the decidua in early pregnancy was observed that disappeared later onwards, reflecting dynamic changes in the decidual γδ TCR repertoire during human gestation. In conclusion, our data indicate that decidual γδ T cells express a specific and dynamic pattern of cytotoxic mediators, Th1 cytokines and TCR repertoire suggesting an important role for these unconventional T cells in assuring a healthy pregnancy in human.

1. Introduction

Mammalian pregnancy is an immune paradox because of the peaceful coexistence of two genetically different subjects - the mother and the fetus. This phenomenon of acceptance of "a mating product of non-histocompatible individuals" by the maternal immune system is still unsolved by the scientists [1]. Probably the piece of price for that unusual coexistence is the inefficiency of the human reproduction - only about 20–25 % of conceptive matings appear to result in a live birth [2,3]. Now it is well accepted that a pregnancy status requires a robust, dynamic and responsive maternal immune system and therefore the immunological milieu at maternal-fetal interface (MFI, *decidua basalis*) is unique, modulated and definitely not suppressed [4]. The contact between the mother and the fetus is dual: 1) between the embryo-derived extravillous trophoblast (EVT) and the maternal epithelial, stromal, endothelial and immune cells in *decidua basalis* and 2) between the maternal blood and syncytiotrophoblast cells of the chorionic villi of the placenta. *Decidua basalis* is the maternal part of the placenta at the implantation site, where the first contact between maternal cells and EVT occurs during early human pregnancy (1st trimester) [5]. The successful implantation is generally associated with downregulation of the adaptive immune system (conventional T-cell responses) and specific adaptation of the innate immune cell populations to the MFI [6 7–9]. Thus, the local maternal immune system provides an

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immunosuppression of specific responses towards the fetus without compromising massively the ability to fight infection and tumor transformation. The leukocytes are about 10 % of the endometrial cells into the proliferative phase of the cycle increasing to ~ 20 % in late secretory phase and > 40 % in early pregnancy [10–12]. The MFI during early pregnancy is mainly populated by uterine natural killer (uNK) cells (70 % of the leukocytes), M2 macrophages (10-20 %) and T cells (about 20 %) with predominance of CD8 αβ T cells [9–13]. Dendritic cells (DC) are about 1 %, B cells are thought to have a negligible amount at MFI [14] in contrast to B cells in mouse decidua [15]. We and others have previously shown that early in human pregnancy the decidual gamma delta T (γδ T) cells are enriched within the T-cell pool (15–50 % of T cells) [16–18] and have an end-stage-differentiated phenotype compared to their counterparts in the paired blood and to γδ T cells in term decidua [18,19]. TCRγδ cells belongs to the group of CTL and have anti-tumor and antimicrobial activity and share many of their receptors with NK cells - like activating and inhibitory NK receptors [20–23]. Although producing mainly Th1 inflammatory cytokines such as IFNγ and TNFα [24], Th17 producing γδ T cells have been also detected in terms of infections or autoimmune diseases [25–29]. Cytotoxic mechanisms have an important role at MFI by protecting the placenta against pathogens, controlling trophoblasts invasion and an elimination of fetus-reactive T cells [30]. The two major cytotoxic mechanisms – perforin- and FasL-mediated – performed by one common secretory pathway based on cytolytic granule exocytosis are operating at MFI in early pregnancy [30] and surprising observation has been made: plenty of cytotoxic cells present there (CD8 T cells, uNK) but their cytotoxicity against healthy trophoblasts is altered [31–33]. TCR γδ cells are T lymphocytes using γ and δ chains for their TCR, each composed of a variable (V), diversity (D) (only for δ), and joining (J) gene segment generated by "V(D)J recombination" [34]. The random rearrangement of different gene segments creates a high clonal diversity, which is particularly reflected in the complementarity-determining region 3 (CDR3) sequence of TCR chains. The process of V(D)J rearrangement in the thymus results in the expression of a unique TCR on each T cell [34]. The overall diversity is greatly amplified by junctional diversity, through the insertion of palindromic sequences (P nucleotides) and of non-templated nucleotides by the terminal deoxynucleotidyl transferase (TdT) enzyme (N nucleotides) at the V(D)J junction of CDR3 region [35]. However, after entering the periphery, clonal expansion of antigen-activated T cells is observed, leading to oligoclonal repertoires of TCR [36]. We have previously shown that in early/fetal life γδ T cells possess TCRs with very low number of N additions, due to low expression of TdT and that leads to production of public TCR sequences (shared among individuals) [37,38]. This is much rarer in the adult where more private repertoires (unique to each individual) are present in the blood [39]. While it is known that Vγ9Vδ2 γδ T cells are abundant in the blood and nonVγ9Vδ2, mainly Vδ1, γδ T cells are enriched in solid tissues, as in the decidua, there is scarce information on the features and origin of the CDR3 repertoire of the tissue-resident human γδ T cells [18,39]. In mice, it has been described that γδ T cells are generated in waves and migrate to tissues depending on their TCR and accordingly Vγ6 + γδ T cells home to the uterus [40,41]. These fetus-derived waves are highly enriched for invariant γδ TCR without N additions which is associated with the absence of TdT expression in fetal life [39]. It is not known whether there is a preferential TRGV usage in human, especially since there is no conservation of the TCR loci between mouse and human [42].

Here, we aimed to characterize the killing potential and cytokine production of decidual γδ T cells during early and late human pregnancy, in comparison with paired blood samples. Furthermore, we analyzed the γδ TCR repertoire by high-throughput sequencing in order to assess possible unique human decidua-associated γδ TCR characteristics, including their potential origin from mother's early life hemopoiesis.

2. Materials and Methods

2.1. Study Populations and Samples

Healthy pregnant women in early pregnancy, directed to elective pregnancy termination (6–12 gw, $n = 39$) and in term pregnancy, directed to delivery (38–40 gw, $n = 28$) as well as healthy non-pregnant women (volunteers, control group, $n = 31$) were involved in the study. Pregnancies complicated by clinical evidence of infection, steroid treatment, AIDS, alcohol abuse, and/or drug abuse and immune-associated diseases were excluded. This study was carried out in accordance with the Declaration of Helsinki and was approved by Human Research Ethics Committee at the University Obstetrics and Gynecology Hospital "Maichin Dom" and the Medical University, Sofia, Bulgaria (No 250569/2018). Written informed consent was taken from all subjects for the use of blood and tissue samples. Paired samples blood and decidua from women in early pregnancy, term decidua/placental tissue from women in term pregnancy and blood from non-pregnant women were subjected to investigation. Samples were processed within one hour after blood withdrawal and tissue collection. To exclude the effects of labor or vaginal delivery on the decidual cells, only decidua from pregnant women delivered by the elective cesarean section prior to the onset of labor were selected.

2.2. Mononuclear Cells Isolation (PBMC, DMC)

Blood samples were obtained in heparin anti-coagulated vacutainer tubes (BD Biosciences, San Jose, CA, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples diluted with PBS (1:2) by Lymphoprep density gradient centrifugation method (20 min/800 × *g*, density: 1.077 g/ml, Sigma–Aldrich). The aliquots of PBMCs were used immediately for FACS staining or stocked frozen in fetal calf serum (FCS) containing 10 % dimethylsulfoxide (DMSO) at − 80 °C until the RNA extraction. For early pregnancy decidual tissue (1st trimester), only *decidua basalis* connected to villous tissue was used and processed after careful separation from trophoblasts. Third trimester *decidua basalis* (term decidua, placenta) was dissected from the maternal-facing surface of the basal plate, covered by *decidua basalis*. To avoid selective cell death or selective loss of surface proteins, mechanical disintegration rather than enzymatic digestion was used to process the decidual tissue and to isolate the decidual leukocytes. Separation of decidual mononuclear cells (DMC) from early and term decidual tissues were prepared by mechanical disruption of tissue in sterile PBS (5 g/50 mL) followed by sequential filtrations of resultant suspension with a 100 μm metal sieve and a 60 μm strainer (Becton Dickinson, San Jose, CA, USA), and centrifugation at 1500 rpm for 15 min. The pellet was resuspended in sterile PBS, layered on Lymphoprep and spun at 800 × *g* for 20 min (without break). The mononuclear cells were removed from the interface, washed, assessed for viability with trypan blue exclusion (always achieving a purity greater than 95 %) and then were used for FACS staining or were frozen in FCS, containing 10 % DMSO at − 80 °C until RNA extraction. The yield was usually 0.5 -1 × 10e6 cells per gram tissue.

2.3. Flow cytometry

Freshly separated untouched PBMCs and DMCs adjusted to 1 × 10e6 cells per sample were used for surface staining of T cell markers. For subset identification, the suspensions were incubated with the following monoclonal antibodies (mAbs) in different combinations: CD3 − FITC (clone UCHT1, ImmunoTools, Friesoythe, Germany), CD3 APC (clone UCHT-1, BD Biosciences, San Jose, CA, USA), γδ TCR-PE (clone F11; BD Biosciences), perforin-FITC (clone dG9, BD Biosciences), granzyme A–FITC (clone CB9, BD Biosciences), Granulysin-Alexa Fluor 488 (clone RB1, BD Biosciences), TNFα-PE/Cy7 (clone Mab11, BD Pharmingen), IFNγ-v 450 (clone B27, BD Horizon), IFNγ-APC (clone B27, Elabscience),

isotype IgG2-FITC, IgG1-FITC and IgG1-AlFl488 (BD Biosciences). The cells were washed with 3 ml ice-cold FACS buffer (PBS containing 0.1 % bovine serum albumin, Sigma-Aldrich, Munich, Germany) and incubated with the mAbs for 20 min at 4 °C in dark. After washing with FACS buffer, the cells were fixed in 300 μl 1 % paraformaldehyde (Sigma-Aldrich). For the detection of cytokines PBMC and DMC after polyclonal stimulation with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 2 μM ionomycin in the presence of 2 μM monensin (Sigma) for 4 hours were subjected to FACS staining. For intracellular staining (granzyme A, granulysin, perforin, IFNγ, TNFα) the Cytofix/Cytoperm kit (eBioscience) was used. Flow cytometric analyses were performed on an FACS Calibur or BD™ LSR II instruments and data were processed using FloJo software (Treestar, San Carlos, CA, USA). A real-time gate was set around the viable lymphocytes based on their forward scatter/side scatter profile. Approximately 50,000 cells per sample were acquired for analysis. Compensation controls were prepared simultaneously with sample processing using cells stained with a single mAb. Fluorescence minus one (FMO) and isotype-matched immunoglobulins were used as controls for nonspecific immunofluorescence and to set gates (Fig. S1). T lymphocytes were selected based on forward and side scatter plots and staining for CD3. Within the CD3 + cell population, γδ T cells were distinguished by staining with anti-γδTCR mAb. Data acquisition and FACS analysis of all markers are not available on all samples due to limitations on lymphocyte yield of some of the isolates. The exact numbers of samples for each analysis are shown in figure legends.

2.4. TCRγ (TRG) and TCRδ (TRD) high-throughput sequencing

RNA was isolated from PBMC or DMC with the RNeasy Mini Kit (Qiagen). cDNA was generated performing a template switch anchored RTPCR. RNA was reverse transcribed via a template-switch cDNA reaction using TRGC (5'- CAAGAAGACAAAGGTATGTTCCAG) and TRDC (5'- GTAGAATTCCTTCACCAGACAAG) specific primers in the same reaction tube, a template-switch adaptor (5'-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG) and the Superscript II RT enzyme (Invitrogen). The TRGC primer binds both TRGC1 and TRGC2. The cDNA was then purified using AMPure XP Beads (Agencourt). Amplification of the TRG and TRD region was achieved using a specific TRGC primer (binding also both TRGC1 and TRGC2 5'- *GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*AATAGTGGGCTTGGGGGAA ACATCTGCAT, adapter and a specific TRDC primer (5'- *GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*ACGGATGGTTTGGTATGAGG CTGACTTCT, adapter in italic) and a primer complementary to the template-switch adapter (5'-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACA*-GAAGCAGTGGTATCAACGCAG, adapter in italic) with the KAPA Real-Time Library Amplification Kit (Kapa Biosystems). Adapters were required for subsequent sequencing reactions. After purification with AMPure XP beads, an index PCR with Illumina sequencing adapters was performed using the Nextera XT Index Kit. This second PCR product was again purified with AMPure XP beads. High-throughput sequencing of the generated amplicon products containing the TRG and TRD sequences was performed on an Illumina MiSeq platform using the V2 300 kit, with 150 base pairs (bp) at the 3′ end (read 2) and 150 bp at the 5′ end (read 1) [at the GIGA center, University of Liège, Belgium]. Raw sequencing reads from fastq files (read 1 and read 2) were aligned to reference V, D and J genes from GenBank database specifically for 'TRG' or 'TRD' to build CDR3 sequences using the MiXCR software version 3.0.13 [43]. Default parameters were used except to assemble TRDD gene segment where 3 instead of 5 consecutive nucleotides were applied as assemble parameter. CDR3 sequences were then exported and analyzed using VDJtools software version 1.2.1 using default settings [44]. Sequences out of frame and containing stop codons were excluded from the analysis. The degree of TCR repertoire overlap between two different samples was analyzed using the overlap F metrics calculated with the software package VDJtools [45]

2.5. Statistical analysis

Statistical analyses were performed with Prism Version 5.0 software (GraphPad Software Inc.). For comparisons of independent groups, a Student t-test or the Mann–Whitney test was performed. For comparisons of matched groups, a paired Student t-test or Wilcoxon matched test was performed. If the p-value was greater than 0.05, we considered the difference as not statistically significant.

3. Results

It is important to emphasize that our study was designed in a way that allowed to perform comparisons of human γδ T cells in several ways1) paired decidua and blood during early pregnancy, 2) early and term decidua (placenta) and 3) blood of pregnant and non-pregnant women. This allowed to assess the specificity of the observations in decidual γδ T cells compared to the blood compartment and allowed to identify possible changes between early and late pregnancy.

3.1. High cytotoxic potential of decidual γδ T cells despite low perforin expression

While more than half of γδ T cells expressed perforin, regardless of their location (decidual or blood) and regardless of the stages of pregnancy (early or full-term), paired decidua-blood comparison revealed that decidual γδ T cells expressed significantly less perforin than their counterparts in the blood ($p = 0.0012$, Fig. 1C and D, top rows). This decidua-blood difference was specific as no differential perforin expression was found between early and late-stage pregnancy decidual γδ T cells ($p = 0.2428$) and not between peripheral blood γδ T cells of pregnant versus non-pregnant women ($p = 0.0841$, Fig. 1C, top row). Note that the specific pattern of perforin expression of γδT cells was similar to that of NK cells: like dNK cells γδ T cells were enriched for perforin bright (perforin ++) cells, while αβ T cells were enriched for perforin dim (perforin +) cells (Fig. 1 A and B). The vast majority of γδ T cells during human pregnancy (80 %) expressed granzyme A regardless of their localization (blood-decidua) and stage of pregnancy (early-term) (Fig. 1C and D, middle rows). In striking contrast, granulysin showed a different expression pattern during human pregnancy (Fig. 1C and D, bottom rows): the granulysin was highly expressed among decidual γδ T cells compared to paired blood γδ T cells during early pregnancy ($p = 0.0095$). Furthermore, this high granulysin expression decreased almost 2.5 times in term pregnancy ($p = 0.0013$, Fig. 1C and D, bottom rows). In contrast to these dynamic changes within the decidua compartment, no difference could be observed within the blood γδT cell pool during early pregnancy for none of the cytotoxic mediators (Fig. 1D).

3.2. Low number of decidual γδ T cells producing pro-inflammatory and cytotoxic Th1 cytokines IFNγ and TNFα during early pregnancy

As the effector γδT cells can be rapidly activated to produce effector Th1 cytokines such as IFNγ and TNFα [46] we explored this capacity with strong short-term PMA and ionomycin stimulation. The lowest IFNγ and TNFa production was detected by γδ T cells into decidua early in pregnancy (Fig. 2B). As shown, the number of IFNγ + γδ + T cells was higher in the paired blood of pregnant women ($p = 0.005$) and in term decidua ($p = 0.0151$). Similarly, the number of γδT cells producing TNFα was twice as high in the corresponding blood of pregnant women ($p = 0.0003$), as well as in the place of maternal-fetal contact at the end of pregnancy ($p = 0.0343$, Fig. 2B). Of note, while cytotoxic mediators showed equal expression by the peripheral blood γδT cells of pregnant and non-pregnant women (Fig. 1), non-pregnant women showed the highest expression of IFNγ and TNFα (Fig. 2B). Co-staining for IFNγ and TNFα revealed a large population of polyfunctional IFNγ + TNFα + γδ T cells and a small single TNFα + population while single IFNγ positive cells were virtually absent (Fig. 2A). In early pregnancy the number of

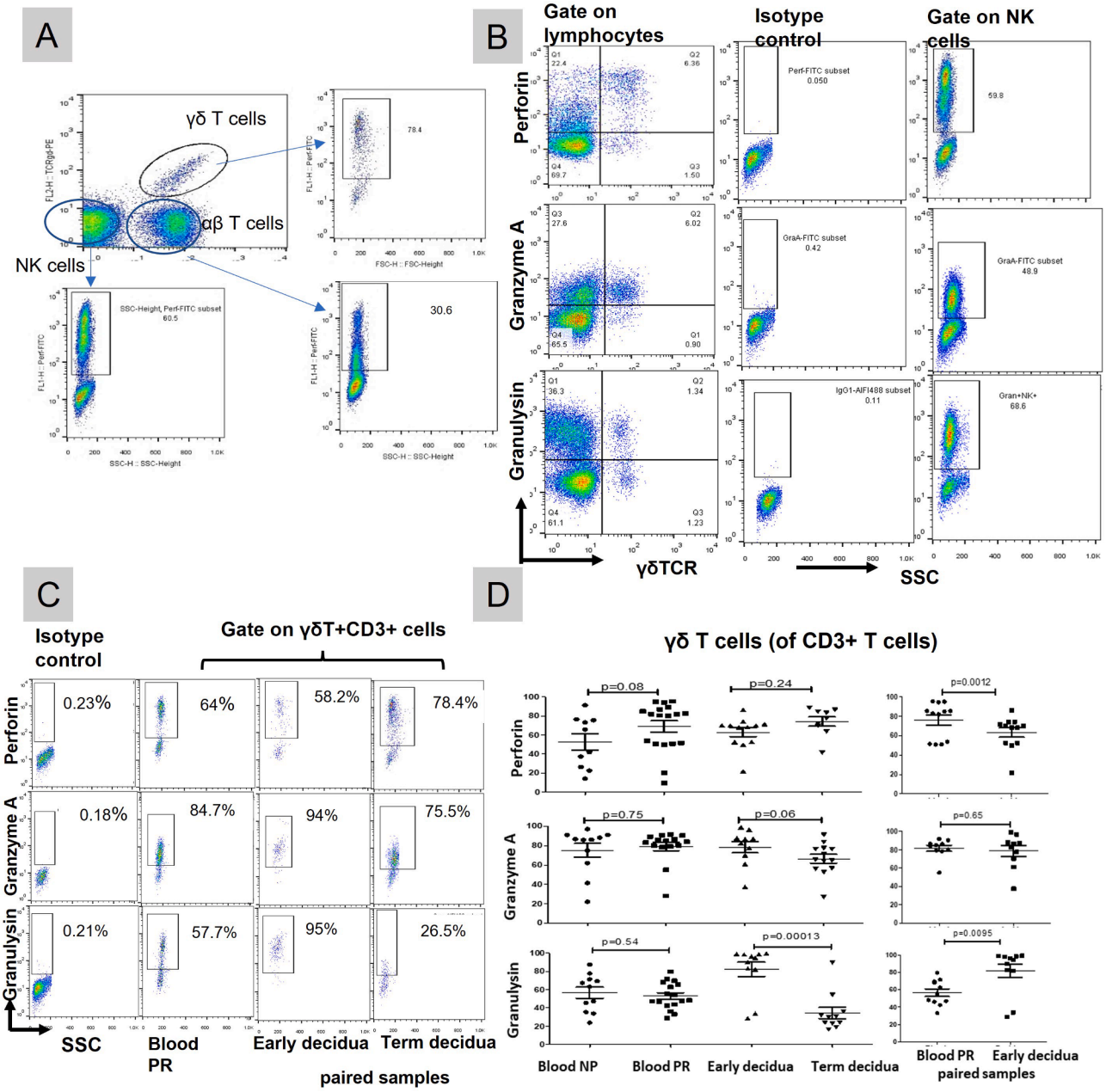

Fig. 1. Perforin, granzyme A and granulysin expression by γδ T cells during human pregnancy. A) Staining with mAbs against CD3 and γδ TCR (gate on lymphocytes) and gate strategy in order to define γδT cells and αβT cells (both CD3-positive) and NK cells (CD3-negative). Specific pattern of perforin staining was observed: both NK cells (positive control) and γδ T cells contained mostly perforin bright expressing (perforin++) subset whereas within αβT cells bright and dim (perforin +) subsets could be detected. **B)** FACS plots showing staining of term placenta sample for the cytotoxic molecules and the strategy to properly define positive and negative populations. Perforin-FITC, or granzyme A-FITC or granulysin-AlFl488 vs γδTCR-PE plotting, staining with matching isotype controls in parallel and NK cells staining as positive control were used. Pattern of perforin (top row), granzyme A (middle row) and granulysin (bottom row) staining. Gate was set on lymphocytes. **C)** Representative FACS plots, showing the percentage of γδT cells positive for perforin (top row), granzyme A (middle row) and granulysin (bottom row) of mononuclear cell suspensions isolated from blood and decidua (paired samples, early pregnancy) and from term placenta, stained with specific mAbs and isotype IgGs. **D, top row)** Proportion of perforin-positive γδT cells (of CD3 + T cells) in all tested groups (left) and in paired decidua-blood samples of women in early pregnancy (right). Left – comparable numbers of perforin + γδT cells in the blood of pregnant vs. non-pregnant women and into decidua in early vs term pregnancy; Right – lower number of perforin + γδT cells into early decidua compared to the paired blood of pregnant women; **D, middle row)** Granzyme A expression by γδ T cells (of CD3 + T cells) in all tested groups (left) and in paired decidua-blood samples of women in early pregnancy (right). Left - High and comparable numbers of granzyme A + γδT cells in the blood of pregnant and non-pregnant women and at MFI in early and term pregnancy; Right – No difference was detected of γδ T cells containing granzyme A when paired decidua-blood samples of women in early pregnancy were compared; **D, bottom row)** proportion of granulysin-positive γδT cells (of CD3 + T cells) in all tested groups (left) and in paired decidua-blood samples of women in early pregnancy (right). Left – comparable numbers of granulysin + γδT cells in the blood of pregnant and non-pregnant women and higher number of granulysin + γδT cells at MFI in early compared to the full-term pregnancy; Right – higher number of granulysin + γδT cells at MFI compared to that of their counterparts in the paired blood of pregnant women. Data in the graphs are presented as mean ± SEM and analyzed using Mann–Whitney and Wilcoxon matched pairs tests, p-value greater than 0.05 reflects a difference which is not statistically significant. PR – pregnant women, NP – nonpregnant women.

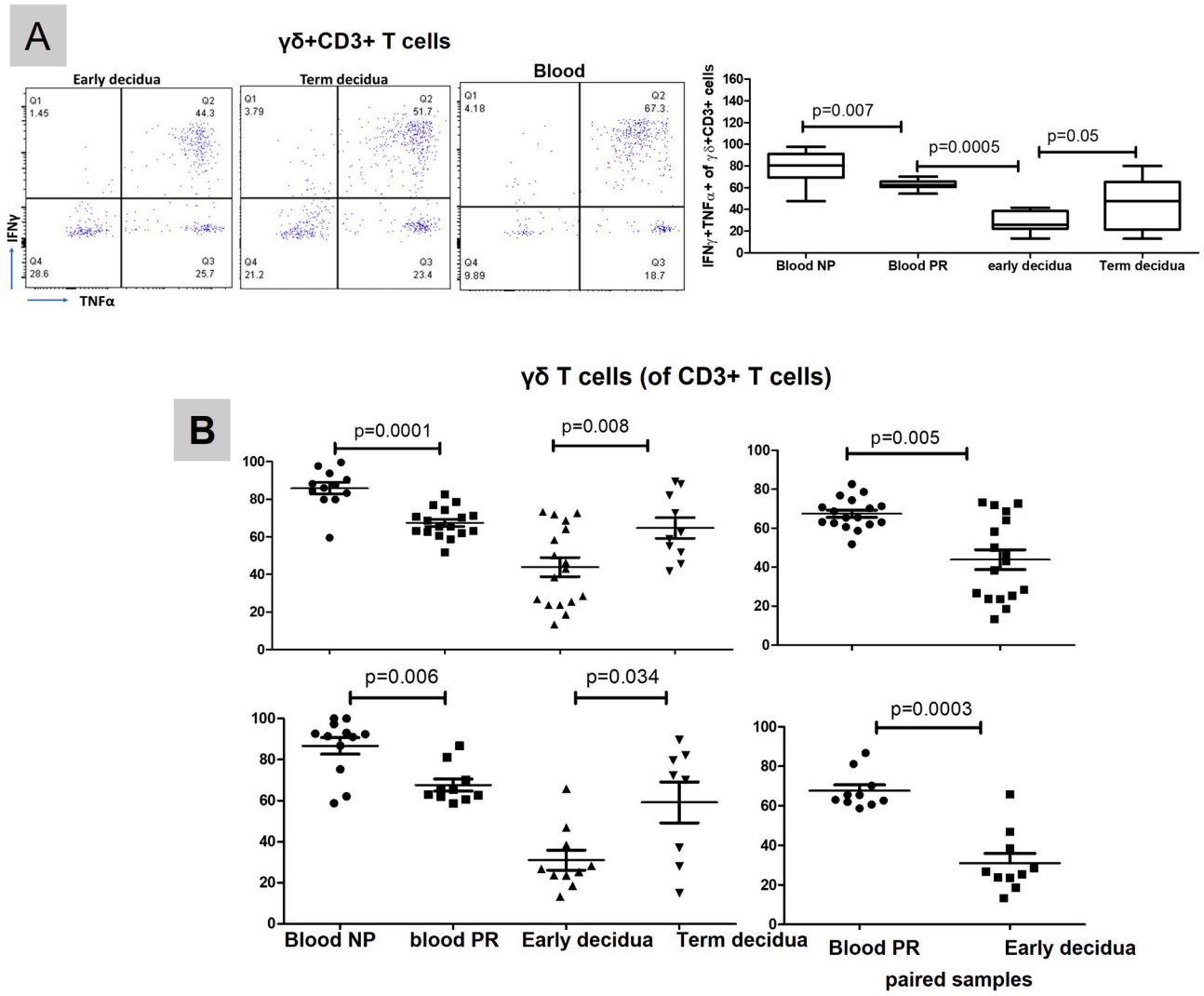

Fig. 2. Numbers of IFNγ- and TNFα-positive γδ T cells during human pregnancy. A) Representative FACS plots showing the presence of single IFNγ, TNFα and polyfunctional γδ T cells (IFNγ + TNFα +) in blood and early and term decidua and their quantity dynamics during human pregnancy, (n = 3-12). **B, top row)** Proportion of IFNγ + γδ + T cells (of CD3 + T cells) in all tested groups (left) and in paired decidua-blood samples of women in early pregnancy (right). Left – higher amount of IFNγ-positive γδ T cells in the blood of non-pregnant vs pregnant women. Lower number of IFNγ + γδ T cells at MFI in early as compared to the term pregnancy; Right – lower number of IFNγ + γδ T cells at MFI compared to the paired blood of women in early pregnancy. **B, bottom row)** Proportion of TNFα + γδ + T cells (of CD3 + T cells) in all tested groups (left) and in paired decidua-blood samples of women in early pregnancy (right). Left – higher amount of TNFα-positive γδ T cells in the blood of non-pregnant vs pregnant women. Decreased number of TNFα + γδ T cells at MFI early in pregnancy as compared to the term pregnancy; Right – lower number of TNFα + γδ T cells at MFI compared to that of their counterparts in the paired blood of early pregnant women. Data in the graphs are presented as mean ± SEM and analyzed using Mann–Whitney and Wilcoxon matched pairs tests, p-value greater than 0.05 reflects a difference which is not statistically significant. PR – pregnant women, NP – nonpregnant women.

the polyfunctional γδ T cells was three times lower at MFI than in the paired blood (p = 0.0005) and remained stable until the end of pregnancy (p = 0.1457, Fig. 2A). The blood of pregnant women contained less polyfunctional γδ T cells than the blood of nonpregnant ones (p = 0.0074, Fig. 2A).

Summing up the data showed that the expression of cytotoxic mediators and cytokines by decidual γδ T cells follow a differential, specific (decidua vs blood) and dynamic (early versus late pregnancy) pattern.

3.3. The decidua CDR3 repertoire is private and enriched for TRGV2 in early pregnancy

In the mouse, the uterus is populated with highly invariant fetus-derived γδ TCR repertoire [41,47]. Whether this is also the case in human is not known. Furthermore, we wanted to investigate whether dynamic changes observed at the effector function level during pregnancy (Fig. 1, 2) could be associated with changes at the γδ TCR repertoire. To address these questions, we compared in detail the CDR3 repertoire of gamma (TRG) and delta (TRD) chains in the blood, decidua and term placenta by NGS. We first examined the distribution of the V segment usage for a possible specific enrichment as has been described in mice, where the uterus is inhabited specifically by invariant Vγ6 + γδ T cells [48]. The TRGV2 segment was enriched in the decidua which was associated with a decrease of the blood abundant TRGV9 segment (Fig. 3A**).** The TRGV2 was specifically enriched in early decidua as in the term decidua/placenta the levels were similar to peripheral blood (of pregnant or non-pregnant women). Regarding the CDR3 TRD repertoire, the TRDV1 segment was increased in pregnancy, confirming our previous flow data (Fig. S1 A) [18]. We then investigated the possibility the decidual γδT cells originate from the fetal period of the pregnant/non-pregnant women by using the number of N additions employed in the TCR as a 'signature' that is associated with the absence of TdT

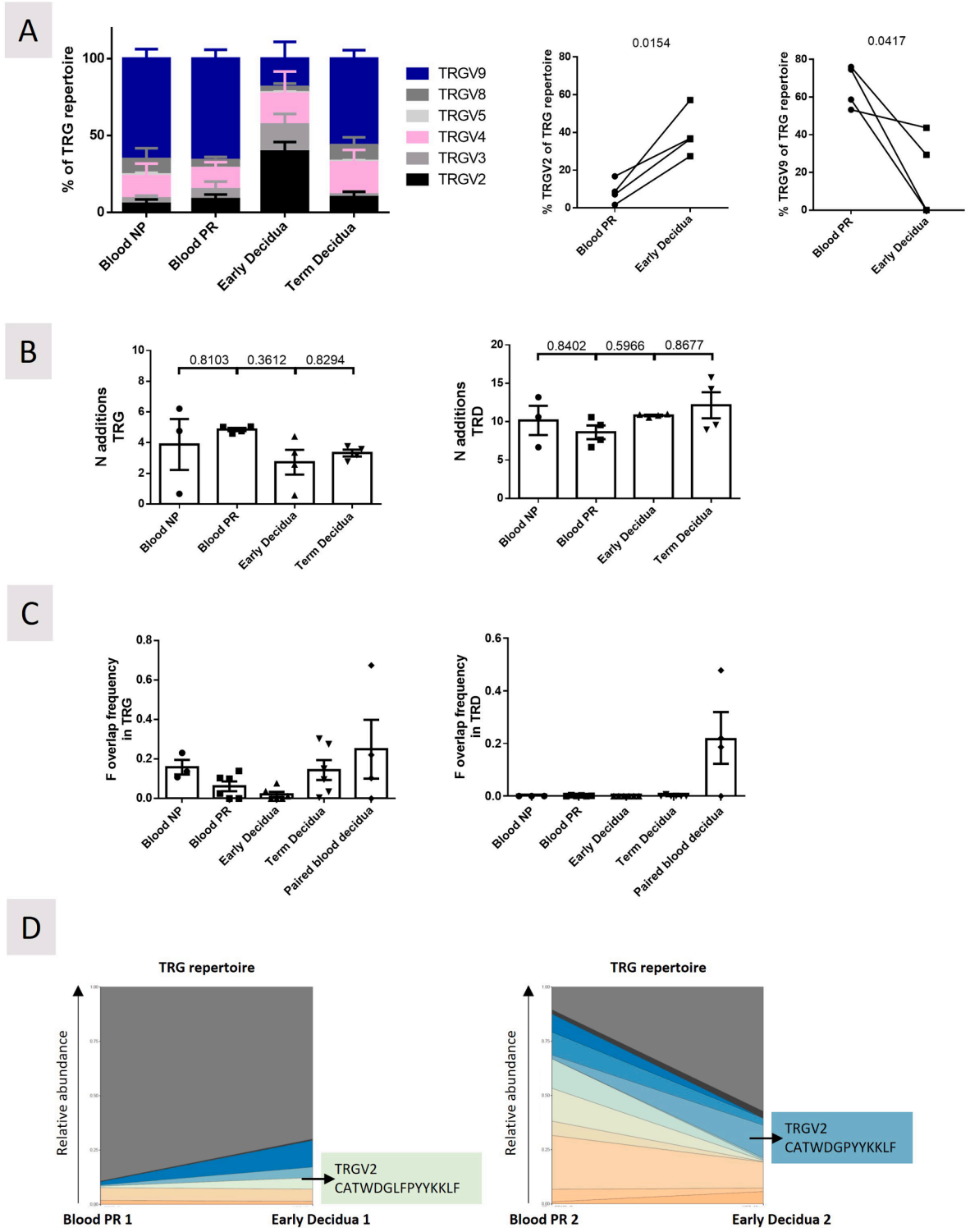

Fig. 3. Decidual TCR CDR3 repertoire has unique and shared features compared to blood. TRG and TRD CDR3 repertoire analysis on PBMC of non-pregnant women (blood NP, n = 3) and pregnant women (blood PR, n = 4), and decidual mononuclear cells from early gestation (early decidua, paired samples, n = 4) and term gestation (term decidua, n = 4). **A)** TRGV gene segment usage distribution (mean ± SEM, left panel). Prevalence of TRGV2 (middle panel) and TRGV9 (right panel) in paired blood and decidual samples; **B)** Number of N additions in TRG (left panel) and TRD repertoire (right panel); each dot represents the weighted mean of an individual sample; **C)** Overlapping/shared repertoire among different individuals (column 1-4) or within paired blood and decidua (column 5). Each dot represents the overlap of a pair of samples. Overlap is defined as the geometric mean of relative overlap frequencies (F metrics by VDJ tools) within pairs of indicated group, in TRG (left panel) and TRD repertoire (right panel). **D)** Shared clonotype abundance plots for the TRG repertoire of two pregnant women between blood and corresponding decidua. The shared top 20 clonotypes between two samples are each represented in a distinct colour. The frequency of these clonotypes in each sample is represented (left) for Blood PR and (right) for paired Early Decidua. The other shared clonotypes are represented in dark grey. The rest of the repertoire (nonoverlapping) is represented in light grey.

expression in fetal life [37,39]. The level of N additions at the MFI was comparable to the blood for both TRD and TRG CDR3 repertoire, although variable between subjects (Fig. 3B), thus arguing against an origin in early life of decidual γδT cells. Furthermore, no decidua-specific TRG or TRD clonotype could be detected (Fig. 3C). The public TRGV9 clonotype (CALWEVQELGKKIKVF) present in blood from fetal until adult life [39,49,50] was detected in several decidual samples (Fig. S1 B). The baseline sharing observed in the TRG CDR3 repertoire was not decidua- or pregnancy-specific, while the TRD CDR3 repertoire was highly private, as shown by the minimal sharing between individuals (Fig 3C, first four columns). In contrast, there was a substantial sharing between decidua and blood paired samples in three out of four subjects (Fig. 3C, 5th columns; Fig. 3D, pair overlap plots of two subjects, blood on the left, decidua on the right). The level and pattern of shared sequences were variable from individual to individual, with either increase or decrease of frequency of sequences in the decidua compared to the paired blood. Even so, in all cases we detect tissue specific sequences (in grey, not shared with the blood). Interestingly, the sharing in the TRG CDR3 repertoire was due to TRGV9- and less to TRGV2-associated clonotypes (Fig. S1 C). Nonetheless, enrichment of individual TRGV2 clonotypes in the decidua were detected, as shown in Fig. 3D (sequence indicated with arrow). Thus, our data indicate that human decidual γδ T cells are not generated in early life of the mother and probably originate from a late thymic or uterine output. Despite this, we did observe a specific enrichment of the Vγ2 chain in the early decidua that disappeared later in gestation, reflecting dynamic changes in the decidual γδ TCR repertoire during human gestation.

4. Discussion

Unlike well-studied uNK cells as major constituents of *decidua basalis* during early human pregnancy [10,12,51,52] the data on γδ T cells is scarce and their role is far from understood.

Here we found that decidual γδ T cells during normal human pregnancy express a specific and dynamic pattern of cytotoxic mediators and cytokines. We extended earlier findings about the expression of the cytotoxic molecules [30] by analyzing the matched decidua and blood specimens from early and term pregnancy. While high number of γδ T lymphocytes produce granzyme A regardless localization and stage of pregnancy, a relatively low and constant number of perforin-containing γδ T cells into decidua was observed suggesting "impaired" cytotoxicity of γδ T cells at MFI as the function of granzymes in the cytotoxic granules is perforin-dependent [53]. Compatible with our data, Tilburgs T et al. have found that decidual tissue is dominated by effector memory CD8 + T cells, which are potentially cytotoxic but with reduced expression of perforin and granzyme B, compared to peripheral counterparts [54]. High granzyme expression with high perforin expression in CTL and NK cells is a hallmark for effector cytotoxic cells ready to kill [53]. However, γδ T cells producing granzyme A without perforin suggests a pro-inflammatory function of this molecule than a pure cytotoxic function [53]. In contrast to these dynamic changes within the decidua compartment, none of the cytotoxic mediators of the blood γδ T cells changed significantly between pregnant and nonpregnant women. In line with our results, high and comparable perforin and granzyme A expression was detected by peripheral γδ T cells in both nonpregnant and pregnant women and in RSA patients as well [23]. The authors did not provide data about cytotoxic potency of decidual γδ T cells. Intriguing data in our study was decidua-specific predominance of granulysin-positive γδ T cells at MFI in early pregnancy (80 %) which number halved later at birth. Such high level of granulysin expression might be a more general feature of decidua-associated lymphocytes since the Rukavina's group has shown that more than 85 % of the uNK cells and more than half of the conventional T lymphocytes into decidua express granulysin compared to few percent in peripheral blood [55,56]. Unlike granzymes, granulysin can act independently of perforin [57]. Its action is not expected to be compromised by the low perforin expression that we observed in decidual γδ T cells and thus, the granulysin-producing γδ T cells might confer protection to intracellular pathogens [58,59]. Moreover, the beneficial effect of granulysin has been shown in angiogenesis during early gestation [60] and we hypothesize that granulysin-producing γδ T cells might contribute to that as well. However, a detrimental role for granulysin was also suggested at MFI as contributor of apoptosis of EVTs in spontaneous abortions [61]. The human γδ T cells comprise a heterogeneous population at MFI: double positive γδ+/CD56 + dim cells and γδ T single positive cells [62]. Decidual CD56neg γδ T cells express CTL4 and show mRNA for IL-10 and TGF-β during healthy human pregnancy compared with CD56pos ones, suggesting the immunoregulatory potential of the CD56neg γδT cell population [63]. Recently has been published that the rate of potentially cytotoxic cells is consistently higher among CD56 + γδ T cells [64] but did not differ between pregnant and non-pregnant groups [65]. The modulation of the maternal immune cell cytotoxicity locally at the site of implantation is probably a key issue of accepting and tolerating the semi-allogeneic fetus. It has been shown that the nonclassical HLA-E molecule, expressed by the trophoblast, could inhibit the potential cytotoxicity of Vδ2 γδT cells in vitro via binding to CD94/NKG2A receptor and this interaction is disturbed in threatened premature pregnancy termination [66]. Such a modulation works at MFI via KIRs-HLA axis in normal [67] and compromised [68] pregnancy.

Pro-inflammatory Th1-immune response is necessary at the time of implantation to promote tissue remodeling and angiogenesis [69]. Quickly after placenta formation, the Th2 shift is critical for the maintenance and development of normal fetus and placenta [70]. Later, at the end of pregnancy the re-shifting of Th2 immunity to inflammatory responses may be associated with the preparation of parturition [71]. Th1 cells at MFI secrete various cytokines, typically TNFα, and IFNγ, which participate in immune surveillance and prevent excessive trophoblast invasion [72]. TNFα has been reported to protect the feto-placental unit [72] and have a regulatory role in trophoblast invasion by altering trophoblast cell adhesion and inhibiting trophoblast cell mobility *in vitro* [73] but excessive TNFα has been also associated with the immunopathology of various obstetrical complications [74]. Although IFNγ has an essential role in vascular remodeling during the peri-implantation period [75] its overproduction has been related to implantation failures, early pregnancy losses, and repeated implantation failures [71]. In concordance with above-mentioned data, here we show that decidual γδ T cells produce low levels of pro-inflammatory and cytotoxic cytokines IFNγ and TNFα when the placenta is already established (6-12gw). The vast majority of the human decidual γδ T cells are Vδ1+ [18,62,76] and this subset generally exhibited lower IFNγ and TNFα production than "blood" Vδ2 subset, which higher IL-17A and lower IL-4 secretion is specific for RSA patients [23]. Similarly, circulating Vδ2 T cells in third trimester preeclampsia showed strong Th1 phenotype with higher perforin and IFNγ expression [77].

Here, we show for the first time a specific enrichment for the TRGV2 segment in human decidua. The transient enrichment of TRGV2 bearing γδT cells in the decidua might be linked to the ephemeral cytotoxic profiles observed at the MFI. The current absence of a specific antibody for this Vγ chain hinders the direct investigation by flow cytometry. Combined single-cell gene expression and TCR sequencing analysis could help elucidate its role at the transcriptomic level. This could also address the question of whether the TRGV2 chain preferentially pairs with the decidual enriched TRDV1 chain. The existence of such a population could constitute an example of attributing a specific function to a γδ T-cell subset for a specific place- and period- of time. γδT cells which are generated early in embryonic development are characterized by low number of N additions involved in their CDR3, making it an easy predictor for the origin and publicity of the TCR repertoire [37,39]. Here we observe that the γδT cells at MFI employed a relatively high number of N additions, comparable to the blood, indicating their post-natal output, in contrast to the early fetal wave of Vγ6 γδT cells in mice [40]. Although there is an enrichment of a specific TRGV segment in human decidua

(TRGV2), reminiscing of the TRGV6 predominance in the mouse, there is no detection of invariant human γδ TCRs in the human decidua as in the mouse [40,41]. The complexity of the human biology including pregnancy is undoubtedly higher than the mouse. The sharing of the TRG repertoire between individuals in pregnancy is not increased compared to blood of non-pregnant women, indicating that there is some selection of private TCRs over the more prevalent public ones usually shared among blood samples. At the same time, the TRD repertoire is extremely private which could reflect the absence of a pregnancy-associated antigen/pattern. It would be interesting to investigate the TCR repertoire in pregnancy disorders and recurrent abortions and explore the possible pathogenic role of a skewed repertoire or its association to a public TCR repertoire. Finally, the expression of specific butyrophilin (BTN) molecules at the MFI might be driving the repertoire shaping. It has recently gained increased attention that, in human as well as in mouse, different butyrophilin and butyrophilin-like (BTNL) molecules are important in the activation/antigen recognition by γδ T cells. Examples in the human are the BTN2A1 and BTN3A1 for phosphoantigens by Vγ9Vδ2 γδ T cells as well as BTNL3/8 by resident Vγ4 γδ T cells in the gut epithelium [78–81]. It would be worth investigating the abundance of various BTN and BTNL molecules at the MFI which could prompt the TRGV2 enrichment detected in early decidua thanks to the expected interaction between the γδ TCR the BTN/BTNL and their antigen/ligand(s).

In summary, the specific predominance of TRGV2 + perforin low/granulysin high γδ T cell effectors with reduced levels of Th1 activity in early pregnancy decidua suggest a peculiar role for γδ T cells at MFI during this pregnancy period.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2022.104634.

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