

1 Human placental villi immune cells help maintain homeostasis *in utero*

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13 **Abstract**

14 Maintenance of healthy pregnancy is reliant on successful balance between the fetal and
15 maternal immune systems. Although maternal mechanisms responsible have been well studied,
16 those used by the fetal immune system remain poorly understood. Using suspension mass
17 cytometry and various imaging modalities, we report a complex immune system within the mid-
18 gestation (17-23 weeks) human placental villi (PV). Further, we identified immunosuppressive
19 signatures in innate immune cells and antigen presenting cells that potentially maintain immune
20 homeostasis in utero. Consistent with recent reports in other fetal organs, T cells with memory
21 phenotypes were detected within the PV tissue and vasculature. Moreover, we determined PV T
22 cells could be activated to upregulate CD69 and proliferate after T cell receptor (TCR)
23 stimulation and when exposed to maternal uterine antigens. Finally, we report that cytokine
24 production by PV T cells is sensitive to TCR stimulation and varies between mid-gestation,
25 preterm (26-35 weeks) and term deliveries (37-40 weeks). Collectively, we elucidated the
26 complexity and functional maturity of fetal immune cells within the PV and highlighted their
27 immunosuppressive potential.

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29 **Introduction**

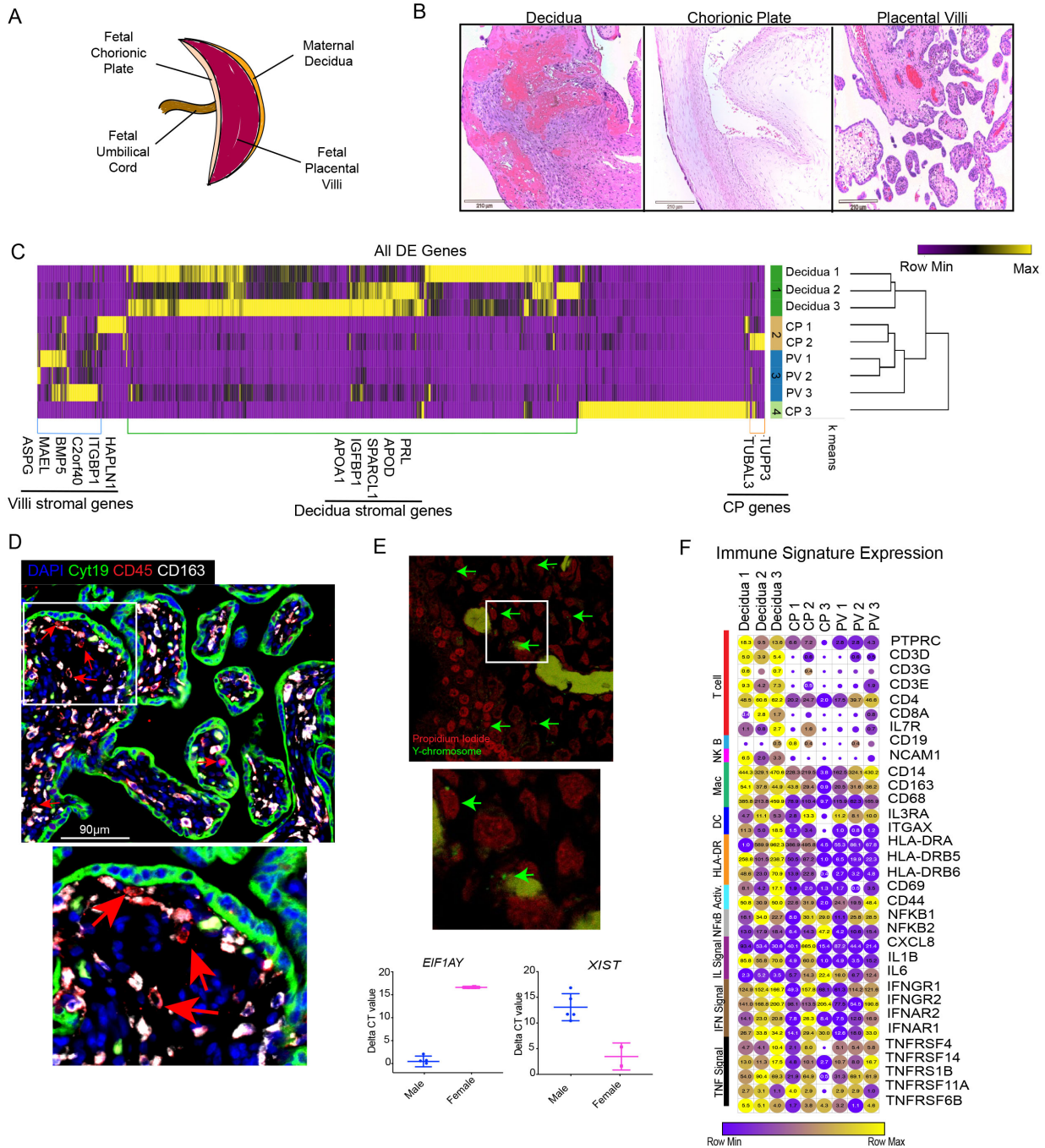
30 Successful pregnancy is dependent on a delicate immune homeostasis, yet many of the
31 factors required to maintain this homeostasis remain elusive. It is understood that the maternal
32 immune system must balance pathogen defense while also preventing rejection of the semi-
33 allogenic fetus for ~40 weeks in a healthy full term pregnancy (Erlebacher, 2013; PrabhuDas et
34 al., 2015). Adding to the complexity, the progression of pregnancy is mirrored by distinct
35 physiological states at many sites throughout the body requiring the maternal immune system to
36 be equally dynamic and adaptive. This point is clearly illustrated in the seminal study by
37 Agheepour and colleagues that tracked immunological responses in the periphery from early
38 gestation through delivery using mass cytometry (CyTOF) (Aghaeepour et al., 2017). Many
39 studies have identified numerous mechanisms by which the maternal immune system

40 accommodates the developing fetus at the fetal-maternal interface. These include: the detection
41 of suppressive uterine NK cells reviewed in (Gaynor and Colucci, 2017), presence of novel T
42 regulatory (Treg) populations (Salvany-Celades et al., 2019), detection of suppressive B cells
43 (Huang et al., 2017), restricted access to plasmacytoid dendritic cells (pDC) (Li et al., 2018) and
44 a predominance of type 2 helper T cells (Miyazaki et al., 2003). The importance of the maternal
45 immune system in pregnancy cannot be understated, however recent findings suggest that the
46 fetal immune system must also be considered to fully understand placental immune
47 homeostasis throughout gestation.

48 Historically, the fetal and neonatal immune systems were thought to be immature. This
49 hypothesis was supported by poor vaccine responses in neonates (Saso and Kampmann,
50 2017), high susceptibility to infection (Simonsen et al., 2014), and the predominance of naïve
51 lymphocytes in human cord blood (Palocz, 1999). However, novel insights suggest that the fetal
52 and neonatal immune systems are developed, though potentially have altered functions. Work
53 supporting this concept includes: the *in utero* maturation following education of fetal Tregs (Mold
54 et al., 2008), detection of novel immunosuppressive cell types present in neonatal blood (Elahi
55 et al., 2013; Halkias et al., 2019; Miller et al., 2018a), and the presence of *in utero* memory
56 lymphocytes in many fetal tissues (Li et al., 2019; Odorizzi et al., 2018; Schreurs et al., 2019;
57 Stras et al., 2019a; Zhang et al., 2014). However, our knowledge about the immunological
58 capabilities of the fetal cells at the fetal maternal interface is sparse.

59 A collection of recent single cell RNA-sequencing studies of the first trimester fetal-
60 maternal interface revealed multiple previously undocumented PV cell types and cell-cell
61 interactions (Suryawanshi et al., 2018; Vento-Tormo et al., 2018). Similarly, detection of novel
62 cell populations and interactions was observed in third trimester placental surveys (Pavličev et
63 al., 2017; Pique-Regi et al., 2019). Of interest, the work by Pique-Regi specifically identified PV-
64 specific immune cell signatures, notably the presence of both resting and activated T cells of
65 fetal origin in term PV (Pique-Regi et al., 2019). These data are in line with other work detecting
66 a previously undocumented complex and diverse PV immune system in third trimester preterm
67 rhesus macaques, which also contained T cells with activated phenotypes (Toothaker et al.,
68 2020).

69 Building on these studies we hypothesized that the active PV immune system detected
70 in the third trimester (Pique-Regi et al., 2019; Toothaker et al., 2020) must be present and
71 contribute to immune homeostasis at mid-gestation. Using a combination of RNA-sequencing,
72 CyTOF, imaging mass cytometry (IMC), and florescent microscopy, we investigated the PV
73 immune profile from healthy mid-gestation (17-23 weeks) placental tissues. With this unique
74 sample cohort, we detected multiple PV-specific immune signatures (absent in the decidua and
75 membranes from the chorionic plate). We also identify that PD-L1 expression on antigen
76 presenting cells is reduced in preterm placentas suggesting that PD-L1 expression may help
77 keeps this armed PV immune system homeostatic *in utero*. Furthermore, using functional
78 assays we uncovered that the PV T cells are poised to execute mature inflammatory functions
79 as early as 18 weeks' gestation, and that the cytokine secretion of PV T cells is variable in mid-
80 gestation and preterm pregnancies but consistent across term pregnancies after T cell receptor
81 (TCR) stimulation.



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Figure 1. Tissue specific signatures in the mid-gestation placenta. (A) Diagram of placental tissues. (B) Representative H&E staining of layers of placental tissues. (C) All differentially expressed genes between three placental tissues with p value <0.05, false-discovery rate <20%, and fold change > absolute value 2. (D) Representative image of CD45^{pos} CD163^{lo/h}eg cells within the intervillous space identified with immunofluorescence. (E) Representative image for fluorescent *in situ* hybridization of Y chromosome in PV (top). Delta CT values of Y and X chromosome genes in male and female PV (bottom). (F) Expression values of selected immune genes. Circle size indicative of expression value. Circle color indicative of relative expression across row. * = p value <0.05 upon post hoc analysis after Kruskal-Wallis (K-W) test. DE = differentially expressed. CP= chorionic plate.

94 Results

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96 *The human mid-gestation PV have tissue specific immune signatures*

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98 We collected placental specimens from 19 second trimester products of conception,
99 gestational age (GA) 17-23 weeks (**Table S1**). Maternal decidua and fetal chorionic/amniotic
100 membranes covering the chorionic plate (referred to hereafter as CP) were separated from the
101 PV (**Fig 1A**) with forceps under a dissecting microscope. Separation of layers was initially
102 confirmed by histology (**Fig 1B**). Tissue was then either cryopreserved for CyTOF analysis, as
103 previously described (Konnikova et al., 2018; Stras et al., 2019a) and validated in **Fig S1A**,
104 fixed with formalin prior to embedding in paraffin for imaging mass cytometry (IMC) and
105 immunofluorescence (IF) analysis or snap frozen for bulk RNA-sequencing (RNAseq). To verify
106 separation of placental layers, we used bulk RNAseq from 3 matched cases (**Table S1**).
107 Differential expression analysis (**Table S2**) and hierarchical clustering confirmed segregation of
108 layers based on transcription profiles with the exception of one outlier (CP3) sample which was
109 enriched for inflammatory signatures, likely upregulated during the D&E procedure or secondary
110 to undocumented *in utero* inflammation (**Fig 1C**). This segregation of samples was confirmed
111 with k-means clustering which grouped samples correctly by tissue with the exception of CP3
112 outlier (**Fig 1C**). Moreover, we confirmed the enrichment of decidua and PV specific stromal
113 genes previously reported in multiple studies (Pique-Regi et al., 2019; Suryawanshi et al., 2018;
114 Vento-Tormo et al., 2018) and detected two tubulin genes, TUPP3 and TUBAL3 enriched in all
115 three CP samples. To determine if immune cells in the mid-gestation PV were solely reflective
116 of the Hofbauer cell population we used immunofluorescence to co-stain for CD45, a marker of
117 all hematopoietic cells and CD163, a classical PV resident Hofbauer cell marker (Reyes and
118 Golos, 2018). Consistent with previous reports identifying non-Hofbauer immune subsets in the
119 first and third trimester PV (Bonney et al., 2000; Pique-Regi et al., 2019), we detected
120 CD45^{pos}CD163^{lo} cells within the mid-gestation PV (**Fig 1D**) ranging in abundance from 30-70%
121 of CD45^{pos} nuclei per high power field (**Fig S1B**). As the PV are bathed in maternal blood
122 (intervillous), we also confirmed that immune cells present in PV samples were reflective of cells
123 contained within the trophoblast layers PV itself (intravillous) and not simply contamination from
124 maternal blood cells (**Fig 1D, S1B**). Additionally, we detected the Y chromosome with *in situ*
125 hybridization in many intravillous cells and had enriched expression of Y chromosome derived
126 *EIF1AY* mRNA coupled with low expression of the X chromosome inactivation transcript *XIST* in
127 male PV samples indicating that the majority PV immune cells in our study were fetal in origin
128 (**Fig 1E, S1C**). To gain insight into what the CD45^{pos}CD163^{lo/neg} immune cell subsets could be,
129 we next assessed the expression of immune genes in our RNAseq dataset and confirmed the
130 presence of a diverse immune landscape in mid-gestation placental tissues. Though most
131 transcripts in the PV were expressed at lower levels than decidual counterparts (darker in color),
132 transcripts for most major immune subtypes analyzed were detected in PV samples (circle size)
133 including T cells, B cells, DCs and macrophages (Mφs) (**Fig 1F, Table S3**).

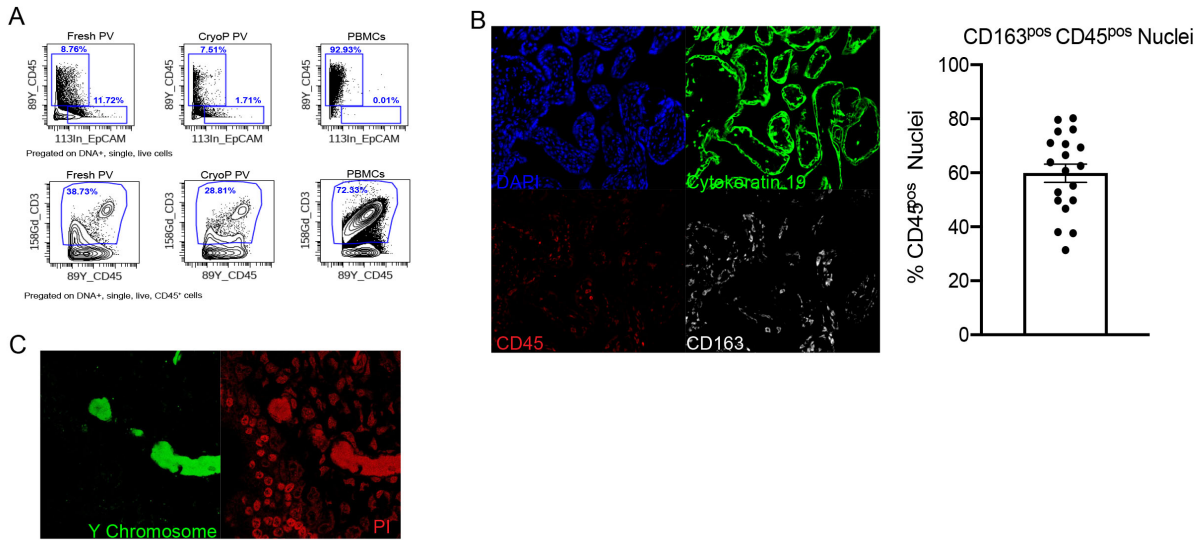
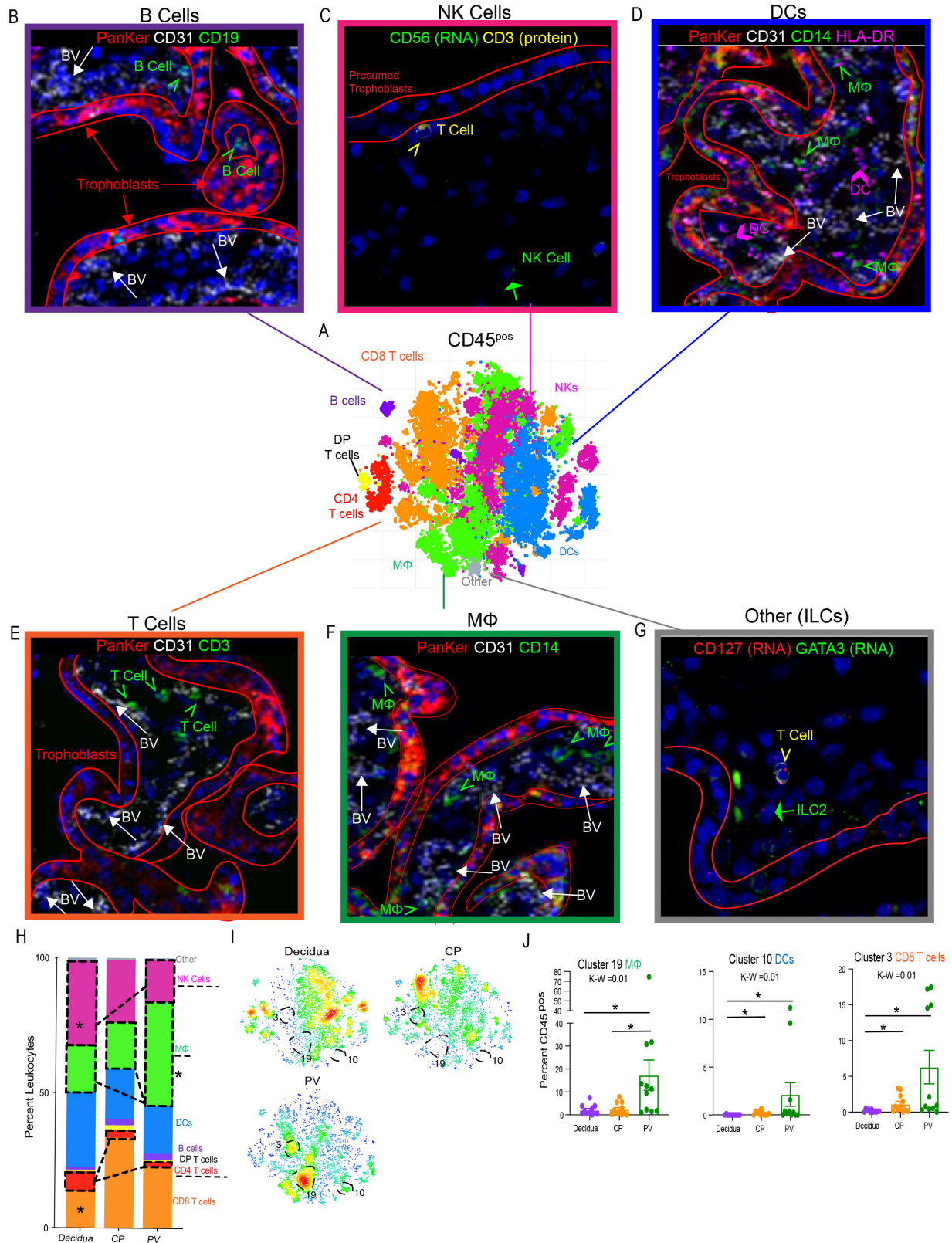


Figure S1. (A) Comparison of CyTOF analysis of CD45^{pos} and T cell abundances between fresh PV, cryopreserved PV and frozen Peripheral Blood Mononuclear Cells (PBMCs). (B) Splits of immunofluorescence for intravillous immune cell detection and quantification of CD163^{hi} immune cells. (C) Splits for Y-chromosome *in situ* hybridization.

To survey the CD45^{pos} populations in the PV, we used a panel of 38 metal conjugated antibodies (**Table S4**) and performed CyTOF analysis on 12 placenta-matched decidua, CP and PV samples (**Table S1**). Briefly, cryopreserved tissues were then batch thawed and digested to make single cell suspensions, stained with metal conjugated antibodies (**Table S4**) and analyzed using CyTOF (Konnikova et al., 2018) (**Fig S1A**). FCS files from CyTOF analysis were pre-gated for DNA^{pos}, single, live, non-bead, CD45^{pos} cells (**Fig S2A**). After omitting samples with insufficient cell numbers (>750 CD45^{pos} cells) we were left with 11 total samples for each tissue layer (**Table S5**). CD45^{pos} cells were clustered using an automated clustering algorithm, Phenograph (**Fig 2A**) and clusters identified based on mean metal intensities of the surface markers from Clustergrammer generated associated heatmaps (**Table S6, Fig S2B**).

To confirm that PV immune subsets identified were not solely reflective of blood leukocytes in the fetal circulation, we used immunofluorescence (IF) and imaging mass cytometry (IMC) with a panel that included 23 markers (**Table S7**) on 6 total regions of 2 individual PV cases (**Table S1**). Using IMC, we validated that B cells, DCs, T cells and Mφs were found outside the fetal vasculature (CD31) in the PV stroma (**Fig 2B-F**). Due to technical limitations in generating IMC antibodies to identify NK cells and ILCs, we used dual *in situ* hybridization and immunofluorescence to detect these populations in the PV as well (**Fig 2C,G**). Though it is likely that some PV immune cells detected with CyTOF represent blood leukocytes, we can conclude that a proportion of the PV immune cells represent stromal populations.

To further characterize these cells, we used CyTOF analysis consisting of 31 unique clusters of immune cells within the STP, belonging to Mφ, DC, NK, CD4 T cell, CD8 T cell, double positive (DP) T cell, B cell and other immune cell type subsets (**Fig 2H, S2B**). Each layer of the placenta housed a unique and complex immune profile (**Fig S2C**). When all clusters belonging to the same immune subset were combined, the decidua had a greater abundance of NK cells compared to PV (**Fig 2H**), consistent with previous studies (King et al., 1991; Koopman et al., 2003). Additionally, there was a higher proportion of CD4 T cells in the decidua than either of the fetal layers, likely attributed to the documented high abundance of decidual Tregs (**Fig 1H**). In contrast, the PV had a larger proportion of Mφ than either the decidua or CP, potentially representing the Hofbauer population (**Fig 2H**).

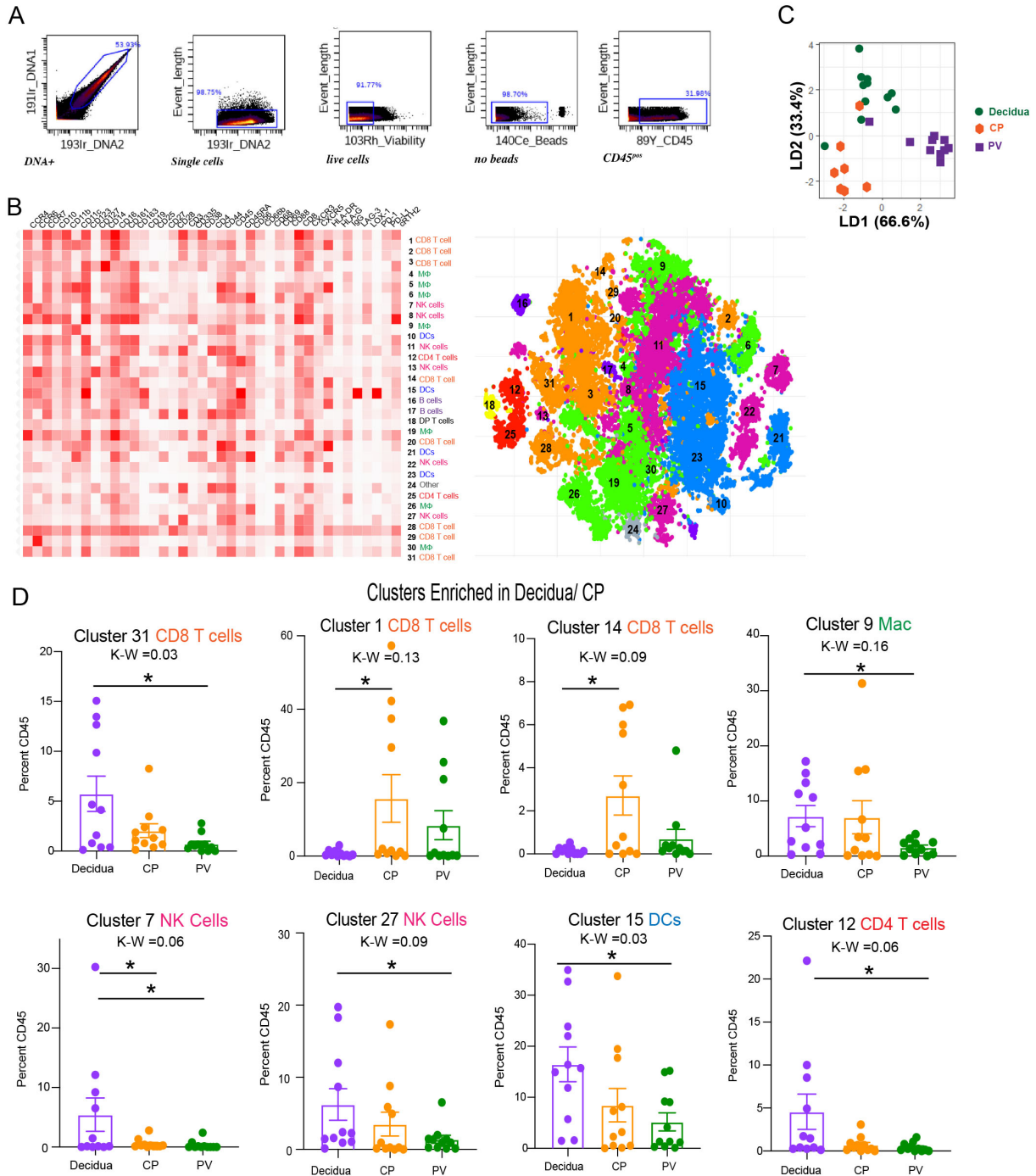


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Figure 2. Global Immune Landscape of Second Trimester Placenta. (A) Merged tSNE of CD45^{pos} cells from maternal decidua (n=11), CP (n=11) and PV (n=11) from Phenograph clustering of CyTOF data. (B) IMC identifying B cells (green) arrows located within the trophoblast bound (red) and outside the fetal

173 blood vessels (BV) (white arrows). (C) Dual in situ hybridization and immunofluorescence (IF) identifying
174 NK cells (green arrow) phenotypically distinct from T cell (yellow arrowhead) in PV. (D) IMC identifying
175 DCs (pink arrows) and HLA-DR^{pos} Mφs (green arrows). (E) IMC identifying T cells (green arrows). (F) IMC
176 identifying Mφs (green arrows). (G) Dual in situ hybridization and IF identifying ILC2s (green arrow)
177 phenotypically distinct from T cell (yellow arrowhead) in PV. (H) Stacked bar graph comparing summation
178 of all clusters belonging to same immune subsets from CyTOF data. (I) Density plot reflective of
179 populations in (A) segregated by tissue of origin. Clusters significantly enriched in PV outlined. (J)
180 Quantification of PV enriched cluster abundance. K-W = Kruskal-wallis test. * = p value <0.05 following
181 posthoc analysis.

182 When each cluster abundance was directly compared, 11/31 CD45^{pos} clusters were
183 differently distributed between the three layers of the placenta (**Fig 2I-J, S2D**). The PV was
184 uniquely enriched for cluster 19 CCR7^{neg} Mφs (**Fig 1I-J**). This robust cluster similarly suggests
185 the presence of Hofbauer cells (as prior reports show most Hofbauer cells are CCR7^{neg} (Joerink
186 et al., 2011)) in the PV and confirms the tissue-specificity of Hofbauer cells in our data set due
187 to this cluster being largely undetectable in decidua and CP samples (**Fig 2J**). Interestingly, we
188 also found cluster 10, CCR7^{neg} DCs, and cluster 3, CD69^{neg} CD8 T cells, to be enriched in the
189 PV over decidua (**Fig 2I-J**). CCR7 is highly expressed on DCs homing to secondary lymphoid
190 structures from peripheral tissues after antigen encounter (Ohl et al., 2004). CD69 is found to be
191 transiently upregulated on activated T cells (Cibrián and Sánchez-Madrid, 2017) and
192 constitutively upregulated on tissue resident memory T cells (Kumar et al., 2017). As PV
193 enriched clusters 9 and 3 lacked these respective markers, we hypothesize that PV are poised
194 (immune cell types are present) to execute mature immune function, such as antigen
195 presentation, but may not be actively performing such functions during a time of homeostasis
196 such as mid-gestation in healthy pregnancies. To further explore this idea, we next analyzed
197 each immune cell subset more thoroughly.



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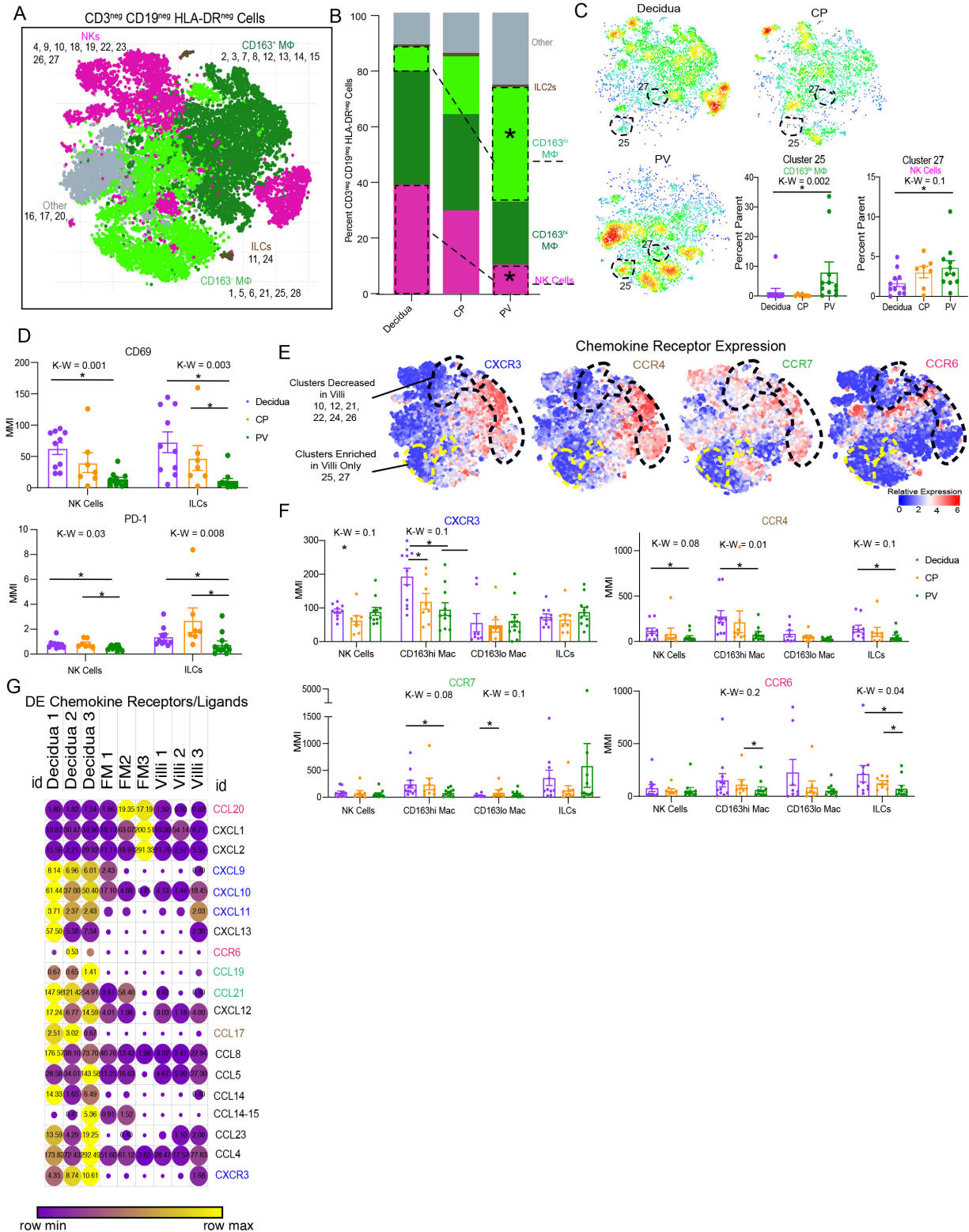
Figure S2. (A) Pre-gating strategy for CD45^{pos} population used in automated clustering. (B) Clustergram heatmap used for cluster identification (left) of tsne clusters mapped (right). (C) Linkage Disequilibrium (LD) plot confirming segregation of placental layer immune profile. (D) Abundance of decidua and CP enriched clusters. * = p value < 0.05 after posthoc analysis from Kruskal-Wallis (K-W) test.

209 *PV innate cells have quiescent phenotypes*

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211 To evaluate PV non-antigen presenting innate cell subsets independent of their antigen
212 presentation abilities that represent the first cells to sense foreign antigens, we clustered on
213 CD3^{neg}CD19^{neg}HLA-DR^{neg} cells (**Fig S3A**). We identified Mφs, innate lymphoid cells (ILCs), NK
214 cells and multiple other immune cell populations that we were unable to fully phenotype with our
215 panel (**Table S4**), (**Fig 3A, S3B**). When comparing subtypes of immune cells, i.e. individual
216 clusters of the same subtype added together, we confirmed our findings from the CD45 level
217 (**Fig 1H**), with the decidua having a larger proportion of NK cells and the PV having a larger
218 proportion of Mφs (**Fig 2B**). The increased granularity of focusing on HLA-DR^{neg} innate cells
219 specifically, revealed that Mφs were both CD163^{hi} likely representing the Hofbauer cell
220 population, and also contained a significant proportion CD163^{lo} Mφs, presumably other non-
221 Hofbauer cell Mφs (**Fig 3B**). HLA-DR^{neg} Mφs in the decidua, in contrast were largely CD163^{hi},
222 consistent with previous data on the phenotype of decidual Mφs (Jiang and Wang, 2020) and
223 with the enriched CD163 gene signatures seen in the decidua over CP from RNAseq data (**Fig**
224 **1D**). The Mφ profile in the CP was more equally split between the two CD163^{lo}phenotypes (**Fig**
225 **3B**). At the individual cluster level, multiple clusters were enriched in either the decidua and/or
226 CP (**Fig S3C**). Within the PV there were enriched in cluster 25, CD163^{lo} Mφs, and cluster 27, NK
227 cells (**Fig 3C**). While cluster 27 NK cells were abundant in all three placental layers and only
228 slightly elevated in the PV, cluster 25 Mφs were specific to the PV and were only minimally
229 present in decidua and CP (**Fig 3C**).

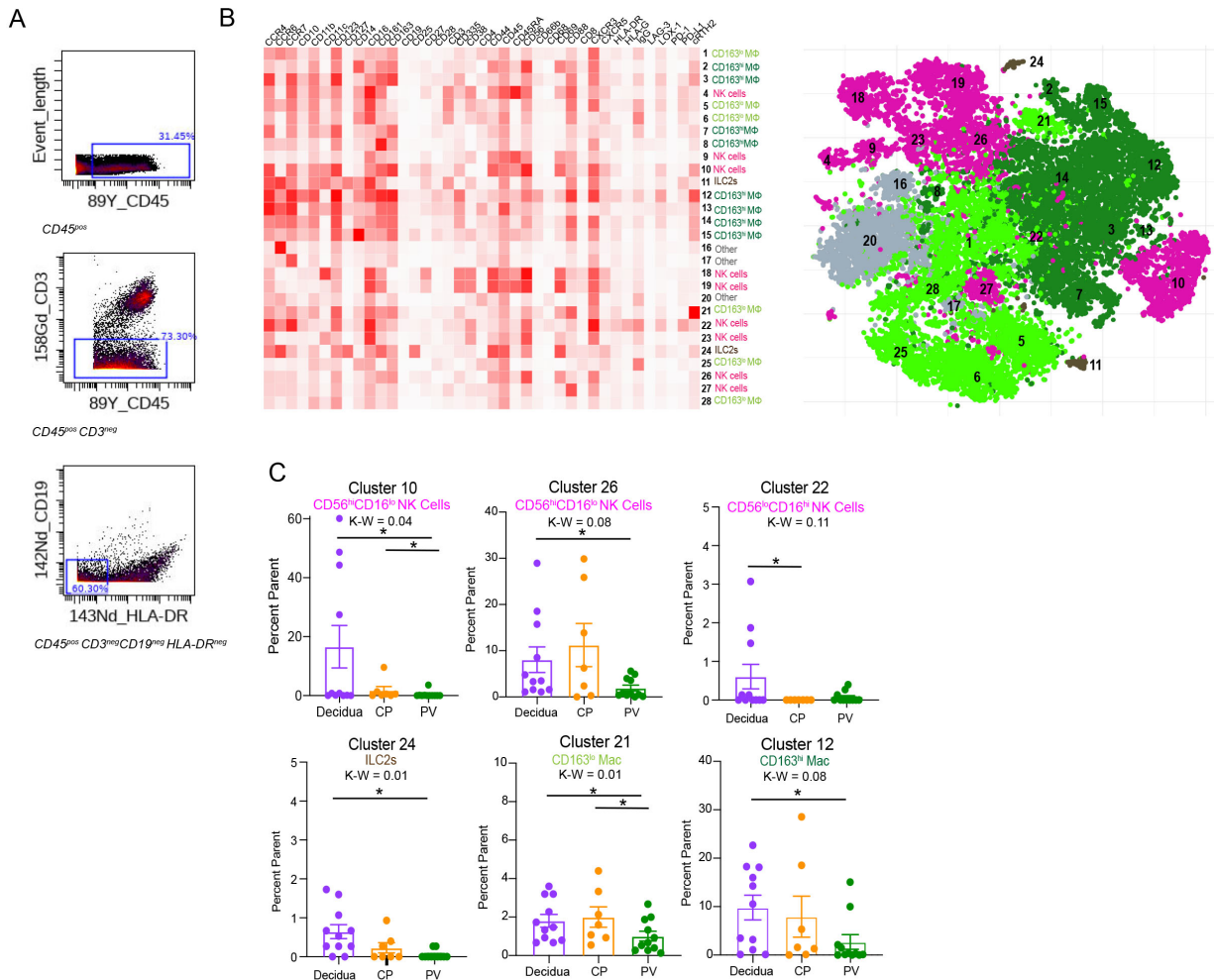
230 To determine if PV NK and ILCs were expressing markers consistent with activation, we
231 compared the mean metal intensities (MMIs) of CD69 and PD-1 on these cells and found that
232 PV NK cells and ILCs expressed significantly lower amounts of both CD69 and PD-1 than
233 decidual and CP counterparts (**Fig 3D**). Next, to examine if PV innate cells have migratory or
234 tissue-retentive phenotypes we compared chemokine receptor (CCR) expression among
235 subsets. Illustrated both visually (**Fig 3E**) and graphically (**Fig 3F**) we show that multiple
236 populations of PV innate cells including NK cells, ILC and CD163^{hi} Mφs had reduced expression
237 of four CCRs. Interestingly, the expression of these markers between the three compartments
238 was similar for the CD163^{lo} Mφs (**Fig 3F**). To determine if other chemokine receptor/ligand pairs
239 were also reduced in the PV, we identified 19 chemokine receptors/ligands that were
240 differentially expressed between PV, CP and decidua using bulk RNA-seq (**Fig 3G, Table S8**).
241 These results validated the CyTOF findings of reduced expression of CCR6 and CXCR3
242 specifically, as well as at least one ligand for CCR7 and CCR4. Nine other chemokine
243 ligand/receptors were implicated in this dataset (**Fig 3G**). These results suggest that PV innate
244 cells are either static or are migrating in a non-signal specific manner and in combination with
245 low expression of activation marker on PV immune cells, support a quiescent state of PV innate
246 cells at mid-gestation.



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Figure 3. Innate HLA-DR^{neg} cells in PV. (A) Combined CyTOF tSNE for CD45^{pos} CD3^{neg} CD19^{neg} HLA-DR^{neg} cells. (B) Stacked bar graph of abundance of major immune subtype. (C) Density plots separated by tissue of cell populations from (A). Statistically significantly abundant clusters in PV outlined. (right side) Cumulative data of PV abundant clusters outlined in density plots. (D) Mean metal intensities(MMI) of CD69 (top) and PD-1 (bottom) for 2D gated NK and ILC populations. (E) Expression heatmaps for

253 chemokine receptors mapped to cells identified in (A). (F) MMLs of chemokine receptors on innate cell
 254 subsets from 2D gating. (G) Expression from RNA-sequencing of differentially expressed chemokine
 255 ligand/receptor genes between tissues. Circle size indicative of expression value, circle color reflective of
 256 relative expression across row. Differentially expressed determined as: p value <0.05, false-discovery
 257 rate <20%, and fold change > absolute value 2. * = p value <0.05 upon post hoc analysis after Kruskal-
 258 Wallis(K-W) test. DE= differentially expressed.
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PV antigen presenting cells are diverse and phenotypically immunosuppressive

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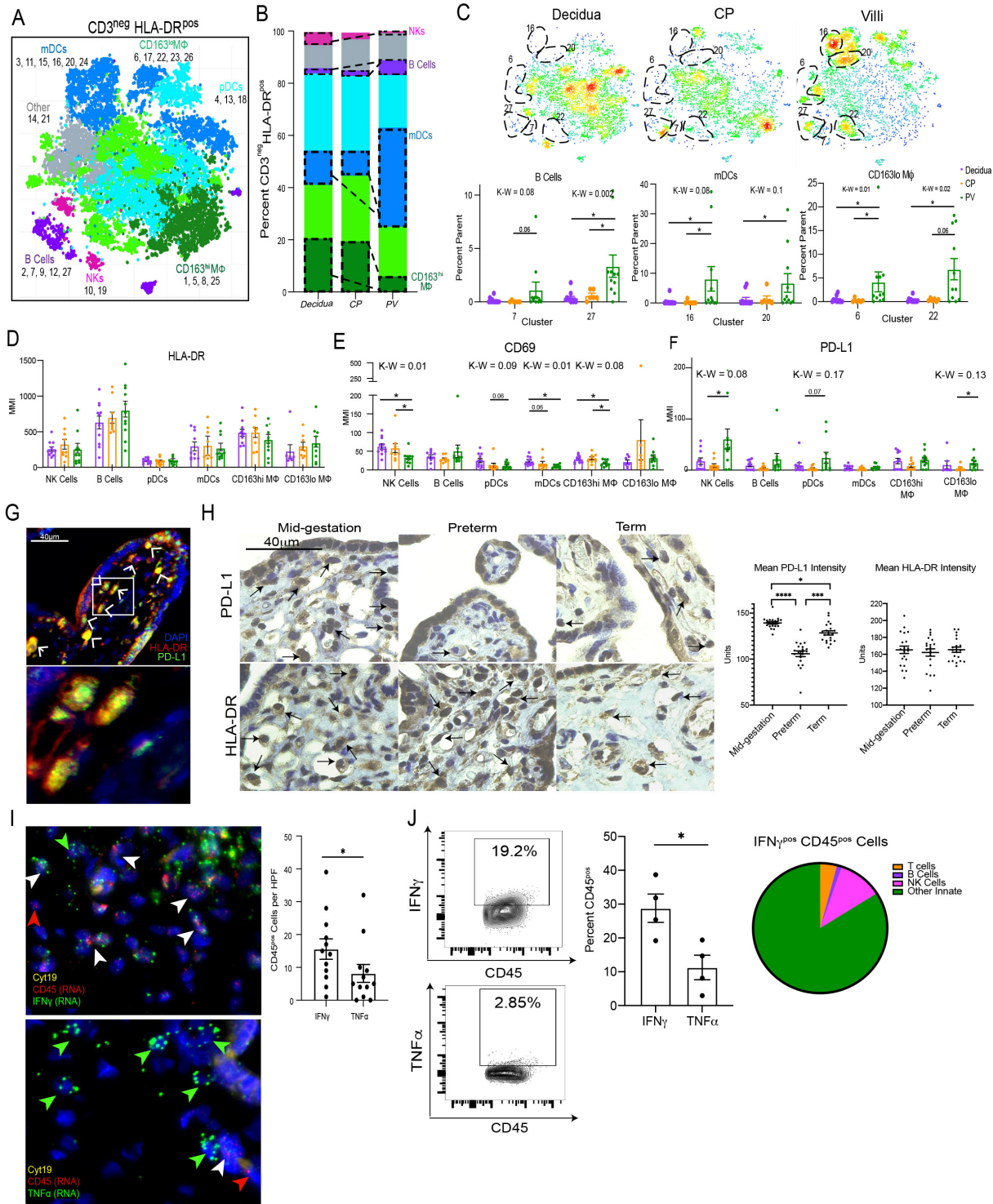
Next, we examined APC populations within each placental layer by clustering on CD45^{pos} CD3^{neg} HLA-DR^{pos} cells (**Fig S4A**). We identified seven sub-types of APCs including: myeloid DCs (mDCs), plasmacytoid DCs (pDCs), CD163^{hi} Mφs, CD163^{lo} Mφs, B cells, NK cells and other cell types that we could not identify based on the available markers (**Fig 4A, S4B**). In confirmation of our previous findings (**Fig 2-3**), NK cells were again more prevalent in the decidua compared to the PV (**Fig 4B**). HLA-DR^{pos} NK cells that are capable to independently present antigens to CD4 T cells have been described (Roncarolo et al., 1991). In contrast to

276 HLA-DR^{neg} innate cells (**Fig 3**), numerous individual APC clusters were enriched in the PV (**Fig**
277 **4C**). Of note, multiple populations were enriched in the decidua and CP as well (**Fig S4C**).
278 Specifically, B cell clusters 7 and 27, mDC clusters 16 and 20, and CD163^{lo} CD4^{neg} M ϕ clusters
279 6 and 22 were significantly more abundant in the PV than either decidua or CP (**Fig 4C**).
280 Complimenting this finding, CD4^{pos} CD163^{hi} M ϕ s (cluster 26) was reduced in the PV compared to
281 decidua and CP (**Fig S4C**). CD4^{pos} M ϕ s have been shown to be long lived tissue resident M ϕ s in
282 the intestine and perhaps they serve a similar role in the decidua (Shaw et al., 2018). The large
283 number of APC clusters (11 in total) differentially abundant between the PV and decidua/CP
284 suggests that antigen presentation in the PV may be functioning through non-classical
285 mechanisms at mid-gestation.

286 To investigate potential functional distinctions in PV APCs, we examined the expression
287 of both activation and immunosuppressive markers on each APC subset identified in **Figure 4B**.
288 In contrast to the hypothesis that PV APCs have altered ability to function as APCs compared to
289 decidua and CP, we found no difference in HLA-DR expression among APC subsets (**Fig 4D**).
290 However, consistent with PV APCs being more inhibited than decidua and CP counterparts, we
291 identified significantly reduced expression of the activation maker CD69 in the PV CD163^{hi} M ϕ s,
292 NK cells, pDCs and mDCs (**Fig 4E**). Furthermore, when we examined the inhibitory ligand, PD-
293 L1, we documented its increased expression on multiple APC subsets, significantly so on
294 CD163^{lo} M ϕ s and HLA-DR^{pos} NK cells (**Fig 4F**). This observation of high PD-L1 expression on
295 PV APCs was confirmed by IF staining, where almost every observable PV HLA-DR^{pos} cell co-
296 expressed PD-L1 (**Fig 4G, S4D**).

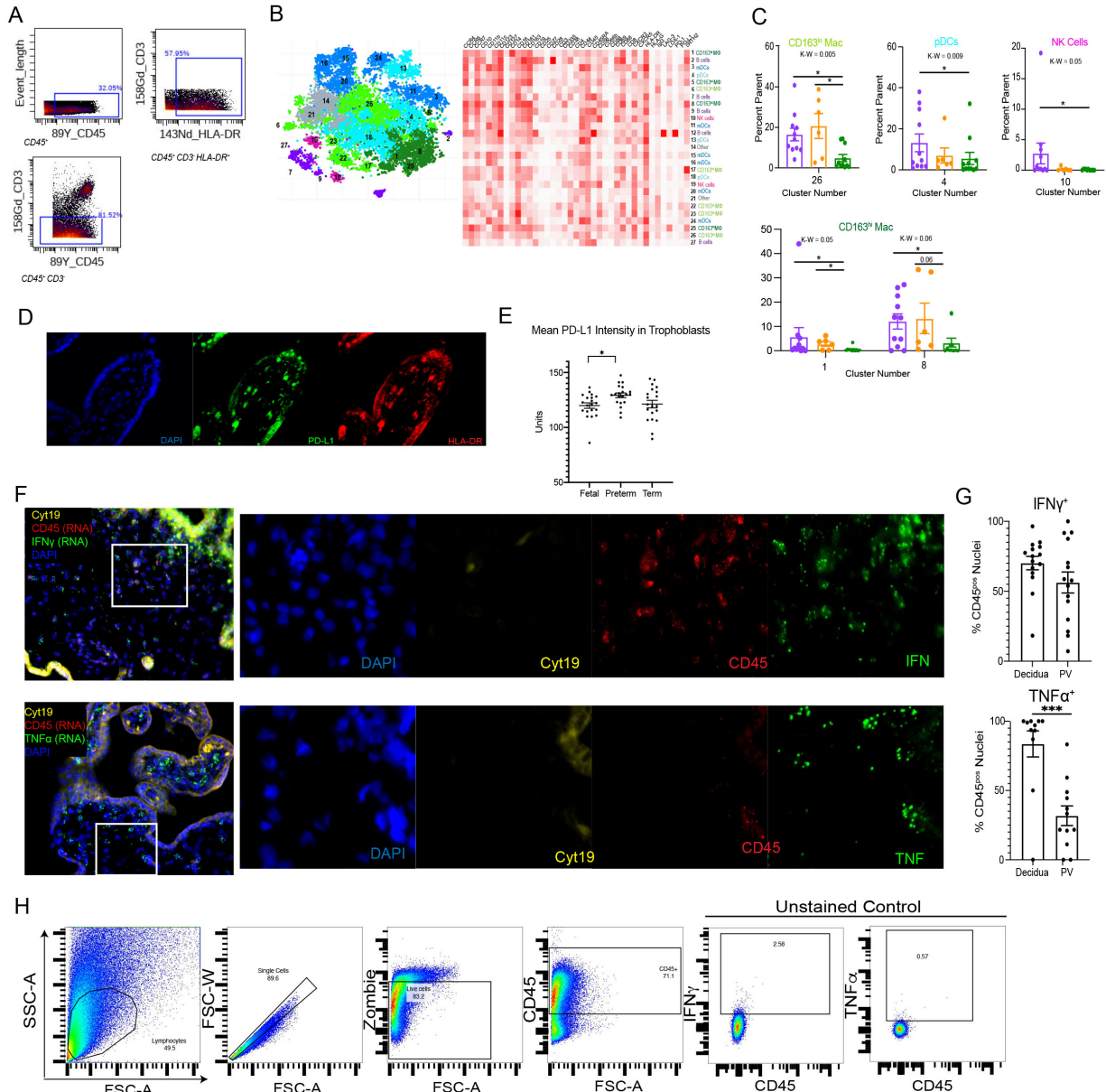
297 To determine if PD-L1 expression on PV APCs is important for maintaining healthy
298 pregnancy we compared both PD-L1 and HLA-DR expression in the PV stroma with
299 immunohistochemistry (IHC) between healthy mid-gestation placentas (21-23 weeks' gestation),
300 preterm placentas from complicated pregnancies (29-35 weeks' gestation) and healthy
301 placentas delivered at full-term (39 weeks' gestation) (**Fig 4H**). Consistent with our CyTOF
302 findings, mid-gestation stromal cells have high expression of PD-L1 per nuclei and PD-L1
303 staining patterns are congruent with stromal HLA-DR staining indicating that the PD-L1^{pos} cells
304 analyzed were likely APCs (**Fig 4H**). Of note, we discovered that stromal PD-L1 expression in
305 preterm PV is significantly lower than that of mid-gestation and term PV. Furthermore, term PV
306 also have significantly reduced PD-L1 expression on stromal cells than mid-gestation PV (**Fig**
307 **4H**). In contrast, we observed increased PD-L1 expression on preterm trophoblasts compared
308 to mid-gestation trophoblasts (**Fig S4E**). To confirm that the reduction of stromal PD-L1 was not
309 an artifact of reduced APC abundance in preterm and term placentas we compared mean HLA-
310 DR expression between all three groups and found no differences (**Fig 4H**). These results
311 suggest PD-L1 expression on PV APCs helps maintain homeostasis during pregnancy
312 illustrated by the highest expression during the mid-gestation window and significantly reduced
313 expression in complicated (preterm) pregnancies.

314 To investigate the regulation of constitutive PD-L1 expression by PV APCs we measured
315 the expression of IFN γ , a well-documented regulator of PD-L1 (Garcia-Diaz et al., 2017). We
316 hypothesized constitutive expression of PD-L1 on PV APCs may be resultant from IFN γ (a
317 regulator of PD-L1) production. Consistent with this hypothesis we found preferential
318 transcription of IFN γ over TNF α at baseline by PV immune cells (**Fig 4I, S4F**). Additionally, PV
319 immune cells transcribe comparable IFN γ , but transcribe decreased TNF α than decidual
320 immune cells (**Fig S4G**). The preferential production of IFN γ to TNF α was confirmed in PV
321 immune cells with flow cytometry. We report this IFN γ was mostly derived from non-NK cell
322 innate immune cells (**Fig 4J**). As such, it is possible that PV immune cells produce IFN γ during
323 homeostasis to drive expression of PD-L1 on APCs.



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 325 **Figure 4. Antigen Presenting Cells in the PV.** (A) Cumulative CyTOF tSNE for CD45^{pos} CD3^{neg} HLA-
 326 DR^{pos} cells. (B) Stacked bar graph of abundance of major immune subtype. (C) Density plot separated by
 327 tissue of origin from (A). Statistically significantly abundant clusters in PV outlined. Cumulative data of PV
 328 abundant clusters outlined in density plots. MMI of HLA-DR (D), CD69 (E) and PD-L1 (F) for 2D gated
 329 populations. * = p value < 0.05 upon post hoc analysis after Kruskal-Wallis test. (G) Representative image
 330 of PD-L1^{pos} APC populations in PV. (H) Representative images (left) and quantification of average stain
 331 intensity per stromal nuclei (right) for PD-L1 and HLA-DR IHC. (I) Representative images (left) and

332 quantification of automated image analysis with CellProfiler (right) for dual RNA *in situ* hybridization and
 333 immunofluorescence in PV. (J) Representative flow plots and quantification for cytokine positive PV
 334 immune cells via flow cytometry. Pie-chart representing major immune subset abundance of IFN γ ^{pos}
 335 immune cells from flow cytometry. * = p-value <0.05 in Mann-Whitney two-tailed test. K-W = Kruskal
 336 Wallis test p value. MMI = mean metal intensity.
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338 **Figure S4.** (A) Pregating strategy for APC population. (B) Clustergram used for cluster
 339 identification (left) of tsn clusters mapped (right). (C) Cumulative data on abundance of decidua and CP
 340 enriched clusters. * = p value < 0.05 after posthoc analysis from Kruskal-Wallis (K-W) test. (D) Splits for
 341 PD-L1^{pos} PV APCs representative image. (E) Quantification of trophoblast expression of PD-L1 via IHC.
 342 (F) Splits for dual RNA *in situ* hybridization and IF. Representative images in main figure taken from
 343 region identified in white rectangle. (F) Quantification of staining for cytokine positive immune cells in PV
 344 and decidua. (H) Pregating for CD45^{pos} population with flow cytometry. *** = p-value < 0.001 in Mann-
 345 Whitney two tailed test.
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348 *The second trimester placenta is dominated by memory CD8 T cells*

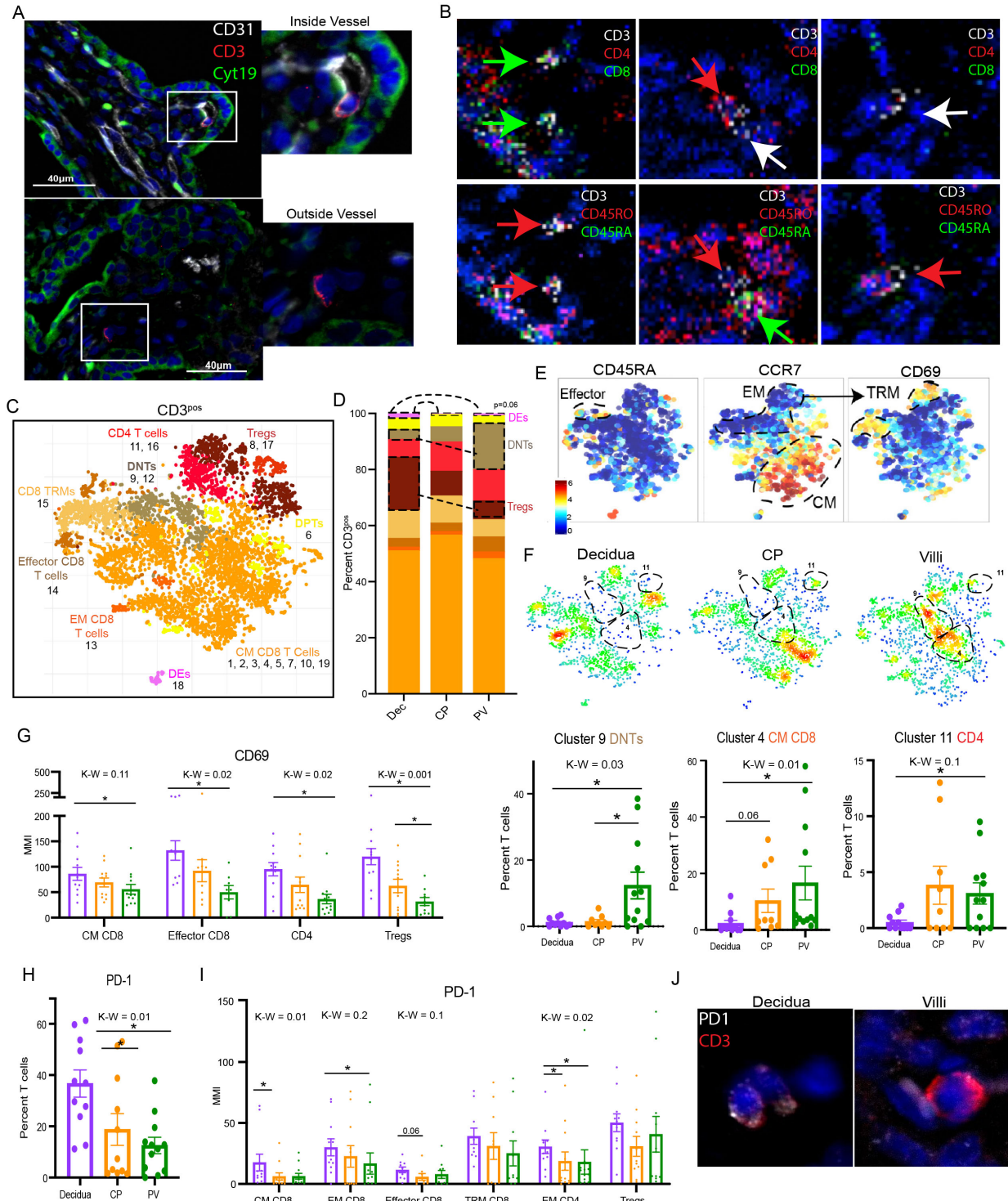
349 As we observed high PD-L1 expression on APCs, we next explored if there were T cells
350 present in the PV that would be inhibited by immunosuppressive APCs. We identified both
351 circulating (within blood vessel) and potentially tissue resident T cells in the PV (**Fig 5A, S5A**).
352 Moreover, using IMC, we surprisingly identified non-circulating T cells of CD4, CD8 and double
353 negative (DN) phenotypes that expressed CD45RO, a marker upregulated after antigen
354 experience and absent on naïve T cells (**Fig 5B, S5B**). Turning to our CyTOF data, when
355 clustering specifically on T cells (**Fig S5C**), we found that all three layers of the placenta had a T
356 cell profiles dominated by CD8 T Cells (**Fig 5C-D, S5D**). Building off the initial detection of
357 CD45RO by IMC, we found that the majority of T cells in the PV were of memory phenotypes,
358 delineated based on expression of CCR7 and CD45RA (**Fig 5E**). Additionally, we found CD8 T
359 cells with tissue-resident memory (TRM) phenotype in all three layers (**Fig 5E**). We detected
360 CD69^{pos} T cells both as a marker of TRMs on CCR7^{neg} CD45RA^{neg} T cells and also among other
361 T cell subsets (**Fig 5E**). The expression of CD69 on multiple T cell populations strongly
362 suggests that some populations of PV T cells are stromal and not reflective of fetal blood T
363 cells, because recent work has shown that blood CD8 T cells do not express CD69 (Buggert et
364 al., 2020). The detection of both CD69^{neg} and CD69^{pos} CD8 T cells in the PV is consistent with
365 the enrichment of one cluster of CD69^{neg} CD8 T cells from our initial CD45^{pos} clustering (**Fig**
366 **2**). CD8 and CD4 non-Treg cell subtypes were evenly distributed between all three layers (**Fig**
367 **5D**). However, CD4 Tregs were enriched in the decidua compared to the PV (**Fig 5D**). The
368 abundance and importance of Tregs throughout pregnancy in the decidua is well documented
369 (Mjösberg et al., 2010; Salvany-Celades et al., 2019), but the role of Tregs in the CP and the PV
370 is unclear, though we have shown PV Tregs function abnormally during intraamniotic
371 inflammation (Toothaker et al., 2020). Moreover, there was an enrichment of CD4^{neg} CD8^{neg} that
372 were also CD56^{neg} and CD16^{neg} double negative (DN) T cells in the PV (**Fig 5D, S5D**). These
373 DN T cells were likely $\gamma\delta$ T cells whose presence in the first trimester PV has been described
374 (Bonney et al., 2000).

375 The detection of PV T cells expressing memory markers was recently reported in the
376 third trimester rhesus macaque (Toothaker et al., 2020). However, their identification in human
377 mid-gestation PV is novel. To investigate T cell signatures unique to the PV, we next compared
378 the abundance of individual T cell clusters. While two clusters were enriched in the decidua (**Fig**
379 **S5E**), cluster 9 CD4^{neg} CD8^{neg} T cells, cluster 4 CM CD8 T cells, and cluster 11 CD4 T cells
380 were enriched in the PV (**Fig 5F**). Cluster 11 T cells were CCR4^{pos} CXCR3^{neg} CCR6^{neg} (**Fig 5F,**
381 **S5D**), surface marker expression pattern suggestive of a TH2 phenotype, however further
382 analysis for detection of TH2 specific transcription factors (GATA3) is needed for confirmation.

383
384 *Resting signatures define PV T cell subsets*

385 Since there was a resting, homeostatic trend in PV innate cells (**Fig 2**) and a
386 resting/coinhibitory profile among PV APCs (**Fig 4**), we next explored if a resting phenotype was
387 consistent among PV T cell subsets identified in **Fig 5D**. Among non-TRM T cells, PV T cell
388 subsets exhibit reduced expression of CD69 (**Fig 5G**). Moreover, the PV housed fewer PD-1^{pos}
389 cells (**Fig 5H**) and reduced PD-1 per T cell compared to decidual counterparts (**Fig 5I-J**).
390 Though PD-1 is a marker of T cell exhaustion, it is also upregulated upon activation of the T cell
391 receptor (summarized in (Xu-Monette et al., 2017)). We propose this is the more likely role of
392 observed down-regulation of PD-1 in PV T cells as it is consistent with the downregulation of
393 CD69.

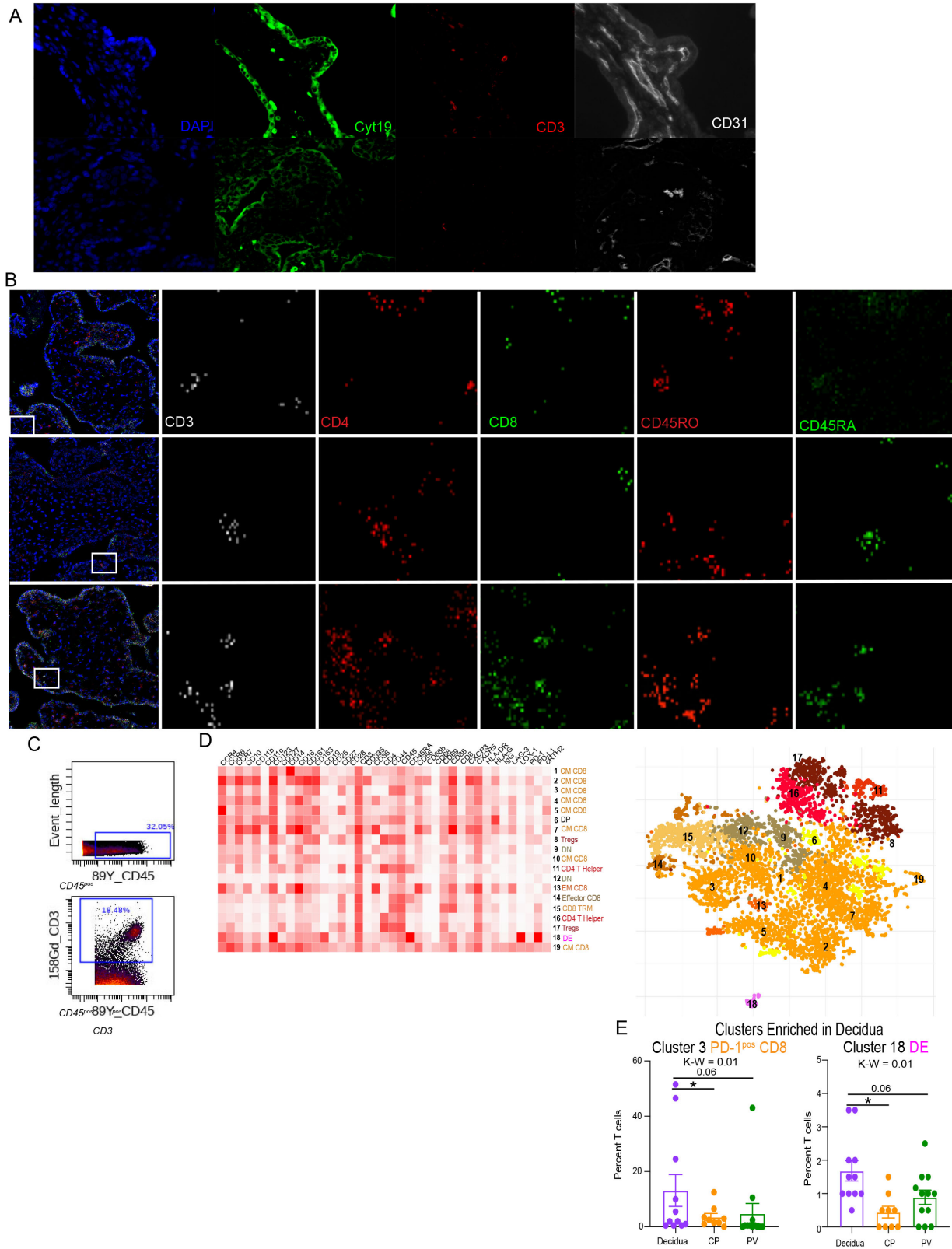
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Figure 5. T cell subsets in placental tissues. Representative images of T cells inside (top) and outside (bottom) fetal vasculature (CD31) in PV. (B) IMC images of T cell subtypes in PV. (C) Cumulative CyTOF tSNE for PV, CP and decidua CD45^{pos} CD3^{neg} HLA-DR^{pos} cells. (D) Stacked bar graph of abundance of major immune subtype. (E) Relative expression of memory T cell markers in PV cell populations from (C). (F) Density plot separated by tissue of origin (C). Statistically significantly abundant clusters in PV outlined. Cumulative data of PV abundant clusters outlined in density plots. (G) MMI of CD69 from 2D gating of populations. (H) Abundance of PD-1^{pos} T cells by 2-D gating. (I) PD-1 MMI from 2D gated

403 subsets. (J) Representative image of PD-1 on T cells in PV and decidua. * = p value <0.05 upon post hoc
404 analysis after Kruskal-Wallis (K-W) test. MMI = mean metal intensity. CM = central memory, EM = effector
405 memory, TRM = tissue-resident memory.
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411 identification (left) of tsne clusters mapped (right). (E) Cumulative data on abundance of decidua and CP
412 enriched clusters. * = p value < 0.05 after posthoc analysis from Kruskal-Wallis (K-W) test.

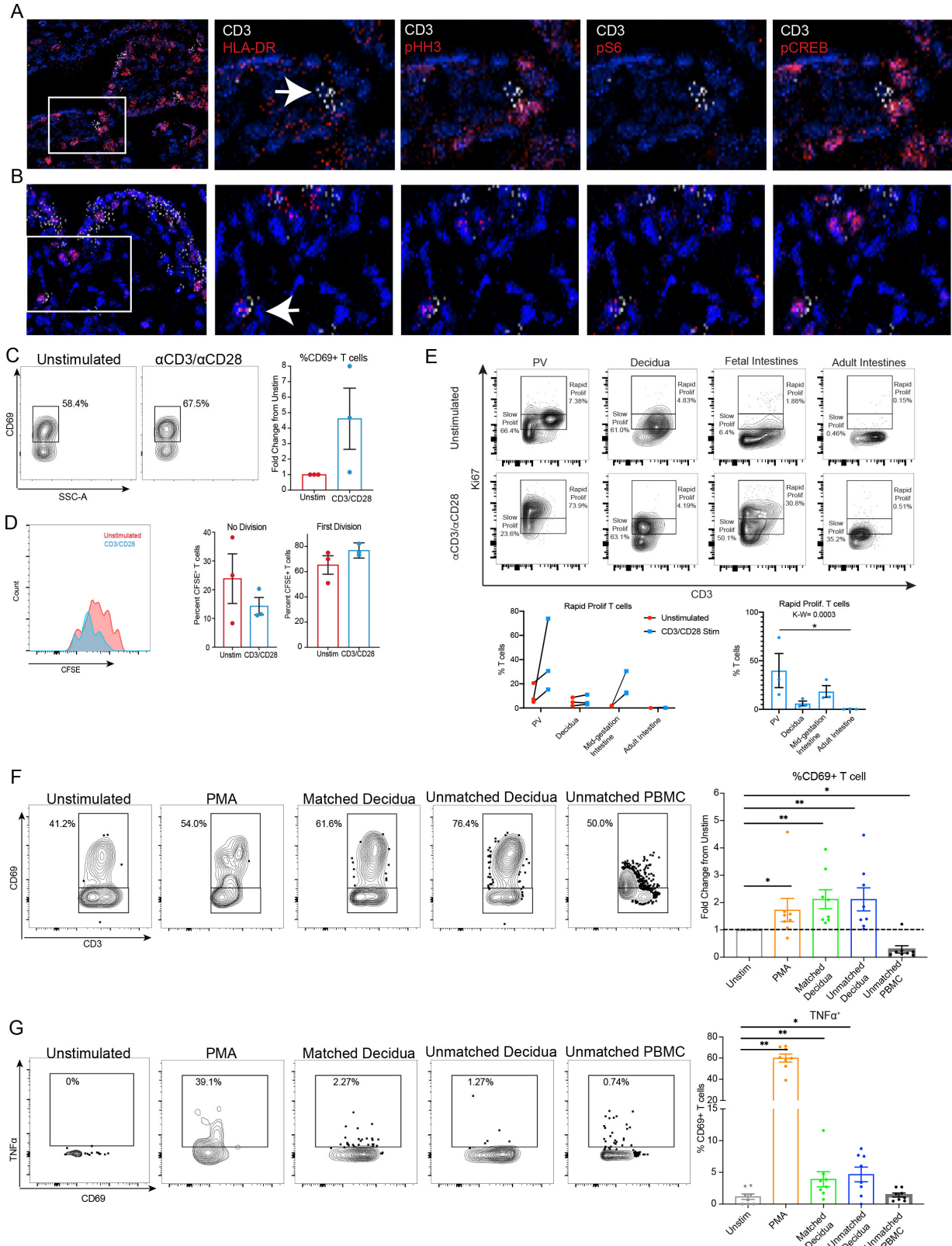
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414 *Maternal antigens can activate PV T cells*

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416 To determine if PV T cells have a reduced activation profile due to functional
417 abnormalities or immaturity, we scanned for the presence of activated T cells via the detection
418 of HLA-DR, phosphorylated Histone H3, phosphorylated S6 and phosphorylated CREB in T
419 cells using IMC. Based on the above markers we were able to identify both resting (**Fig 6A**) and
420 activated T cells in the PV (**Fig 6B**). These results suggest that PV T cells are capable of being
421 activated, consistent with our previous study in non-human primate which demonstrated TCR
422 activation during *in utero* inflammation (Toothaker et al., 2020). As such we questioned if mid-
423 gestation PV T cells could be activated in a TCR dependent manner. To test the functionality of
424 the TCR pathway in PV T cells, we stimulated single cell suspensions from PV with soluble
425 α CD3 and α CD28 antibodies for 72 hours. Consistent with normal TCR pathway functionality,
426 we observed increased CD69 expression after stimulation (**Fig 6C**) and an increased proportion
427 of cells proliferating (**Fig 6D, S6B-C**) with stimulation. Validating our previous findings that PV T
428 cells are fetal in origin and unique from the decidua and other fetal organs, we observed an
429 increase in abundance of rapidly proliferating T cells identified by high Ki67 expression (Miller et
430 al., 2018b) after 4 hours of stimulation with α CD3 and α CD28 antibodies in fetal T cells
431 (placenta and intestine) compared to adult T cells (decidua and intestine) (**Fig 6E, S6D**). Of
432 note, the placenta had the highest proportion of rapidly proliferating cells across all organs and
433 ages following stimulation (**Fig 6**).

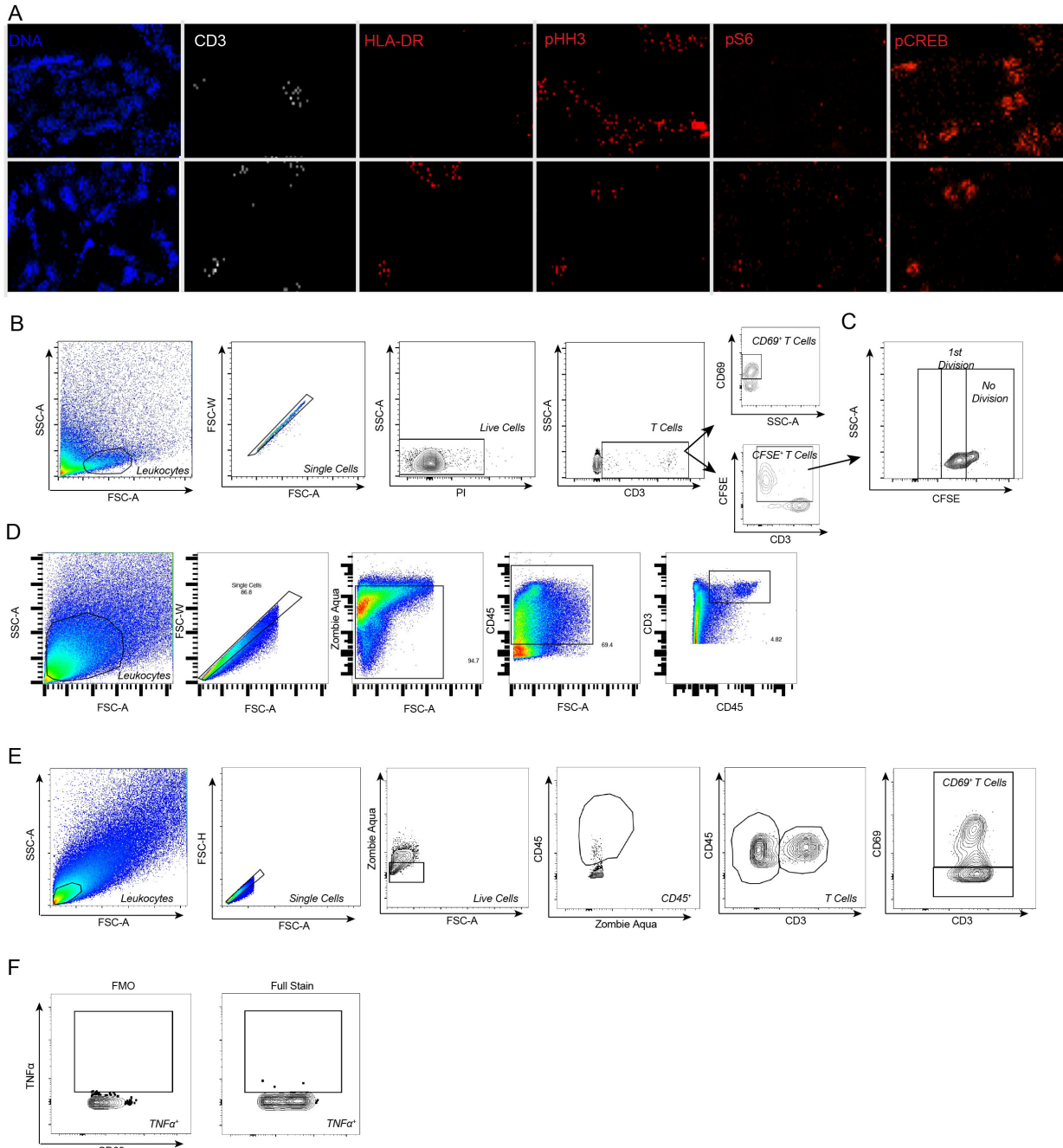
434 To discern what antigens could activate PV T cells, we stimulated isolated PV cells with
435 either PMA/Ionomycin as a positive control or lysed cellular components from either: pregnancy-
436 matched decidua, unmatched decidua or unmatched pooled donor PBMCs. Consistent with
437 activation of PV T cells, we saw elevated CD69 expression in the PMA/Ionomycin, matched and
438 unmatched decidual components conditions and but not with unmatched PBMC components
439 (**Fig 6F**), suggesting that some antigens present in the decidua are capable of activating PV T
440 cells. Validating the activation of PV T cells by decidual antigens, we also observed increased
441 production of TNF α in a significant (though minor) proportion of CD69^{pos} T cells (**Fig 6G, S6E-
442 F**). These functional assays show that PV T cells have the potential to be activated by PV APCs
443 through the TCR pathway and may become proinflammatory when stimulated in a TCR-
444 dependent manner when exposed to particular antigens present in the uterine environment.



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Figure 6. T cell activation in PV. (A) IMC images of inactive T cell in PV. (B) IMC images of activated T cell in PV. (C) Representative flow plots (left) and quantification (right) of CD69^{pos} T cell population. (D) Histogram showing proliferation as tracked by CFSE dye (left). Quantification of cells in proliferative

449 subsets identified. (E) Representative flow plots (top) and quantification (bottom) for proliferating (Ki67^{hi})
 450 T cells after stimulation across tissues indicated. (F) Representative flow plots (left) and quantification
 451 (right) of CD69^{pos} T cell population. (F) Representative flow plots (left) and quantification (right) of TNF α ^{pos}
 452 CD69^{pos} T cell population. * = p-value <0.05 in Mann-Whitney test.

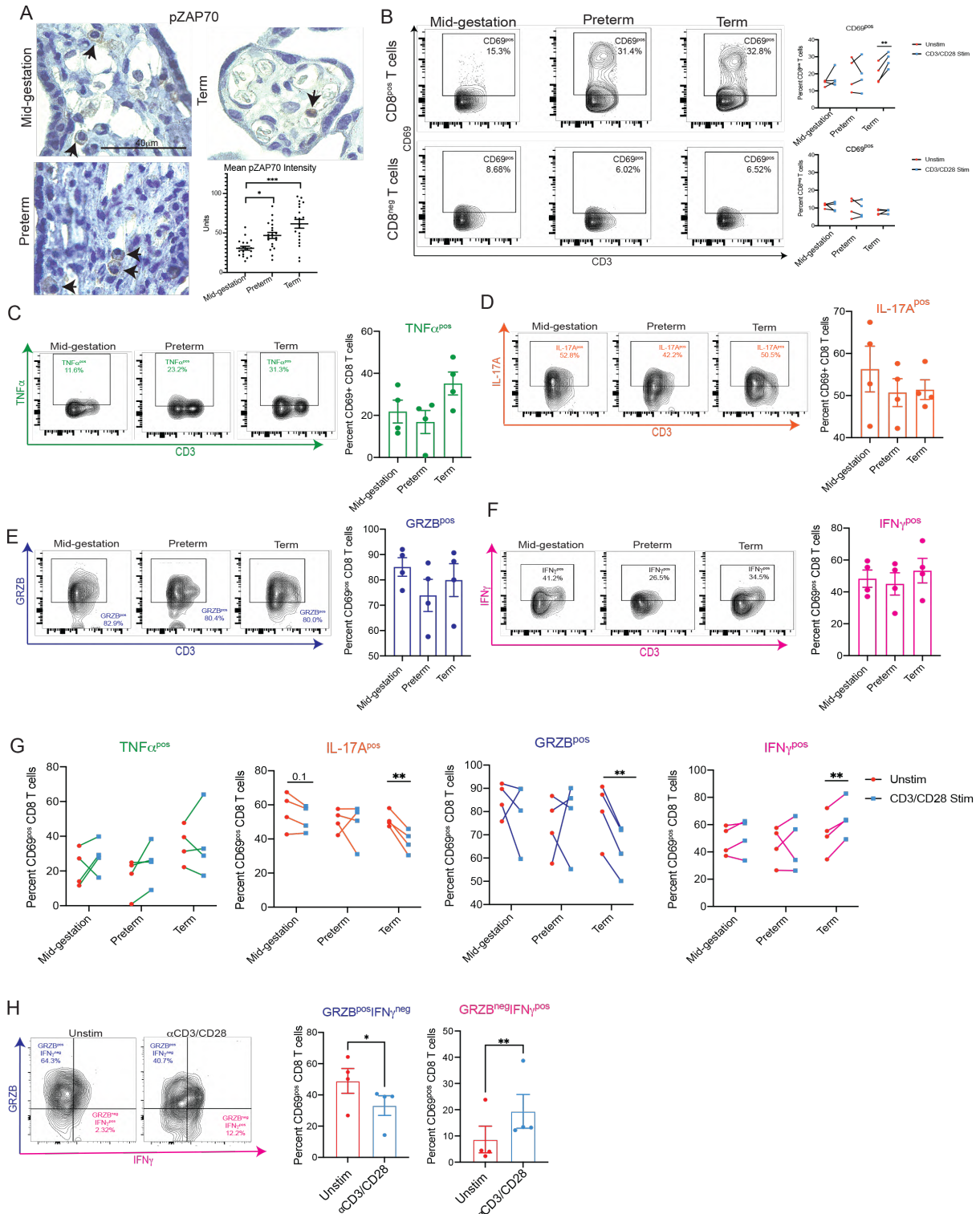


453 **Figure S6.** (A) Splits from IMC images showing inactive and active T cells in PV. (B) Pregating strategy
 454 for CD69^{pos} and CFSE^{pos} T cells. (C) Gating for proliferative populations quantified in main text. (D) Gating
 455 for T cells in Ki67 proliferation experiment in main text. (E) Pregating strategy for CD69^{pos} T cells used in
 456 decidual stimulation. (F) Gating of TNF α ^{pos} population.

457
 458 *Cytokine profile of PV T cells differentiates between mid-gestation, preterm, and term*
 459 *pregnancies*
 460

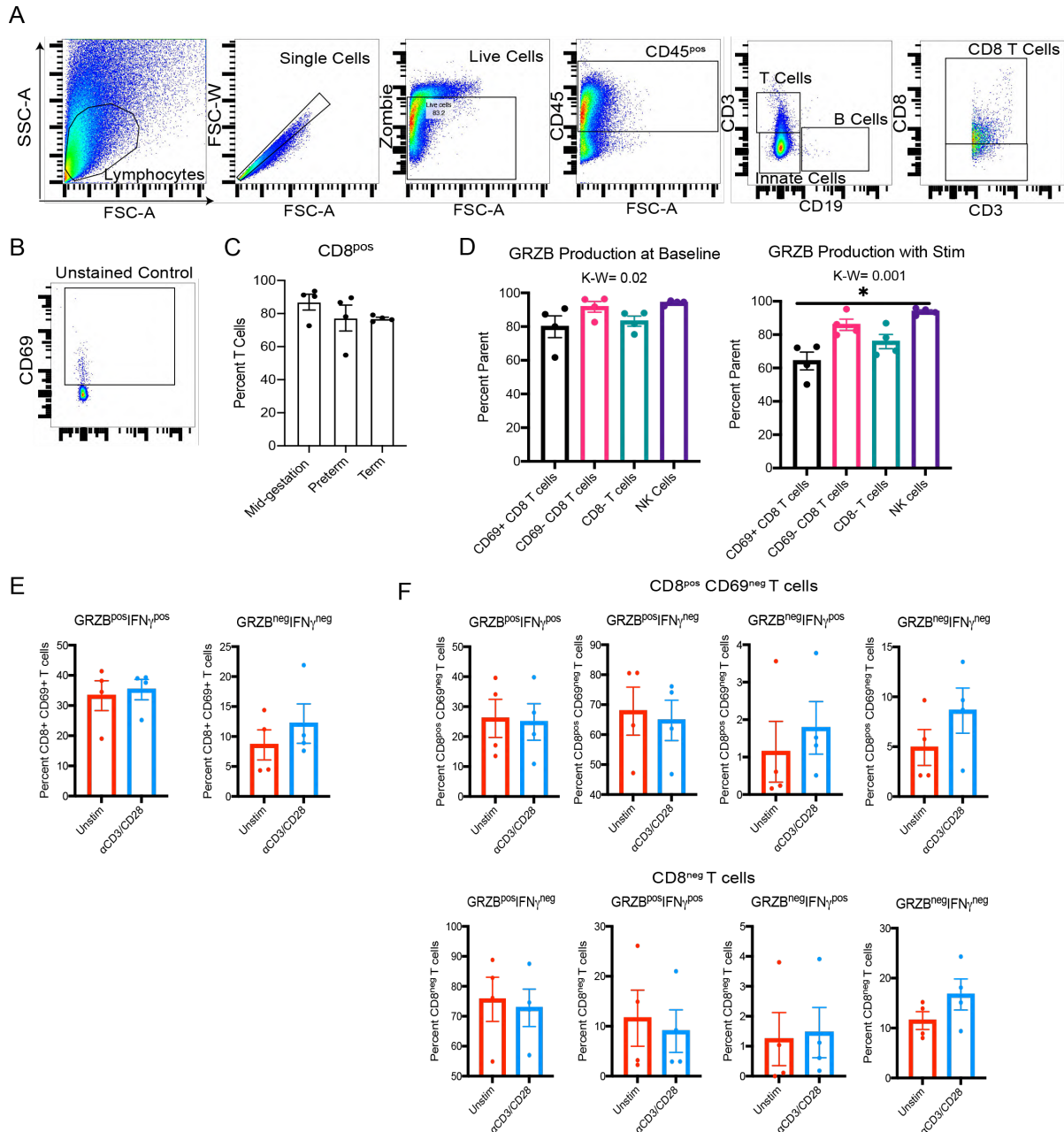
461 As we observed activation potential for mid-gestation PV T cells (**Fig 6**), we next
462 explored if PV T cell activation was consistent across gestation and between healthy and
463 complicated pregnancies. To assess T cell activation at baseline in the absence of any ex-vivo
464 T cell activation as consequence to tissue digestion and cell isolation, we measured
465 phosphorylated (p)ZAP70 expression with IHC. ZAP70 is phosphorylated following engagement
466 of the TCR. Interestingly, we observed increased pZAP70 intensity per ZAP70^{pos} stromal cell
467 with increasing gestation (Term >preterm >mid-gestation) (**Fig 7A**). Building upon this
468 observation that TCR activation is variable between groups, we next assessed T cell function
469 following a 4-hour stimulation with α CD3 and α CD28 antibodies. In confirmation of our CyTOF
470 findings (**Fig 6**), we report that the majority of PV T cells are CD8 T cells (**Fig S7A-C**). We
471 observed activation of CD8^{pos} T cells and very little activation of CD8^{neg} T cells via CD69
472 expression following stimulation in all three groups (**Fig 7B, S7A-B**). We therefore chose to
473 analyze activated (CD69^{pos}) CD8 T cells in subsequent analysis. Surprisingly, we found that the
474 production of four cytokines, TNF α , IL-17A, GranzymeB (GRZB), IFN γ , and did not vary
475 between the three groups at baseline (**Fig 7C-F**). Interestingly, the potential for CD4 PV T cells
476 to make GRZB has been reported in the non-human primate PV (Toothaker et al., 2020).
477 Consistent with this finding we identified high production with of GRZB from CD8 CD69^{pos/neg} T
478 cells, CD8^{neg} T cells and NK cells (**Fig S7D**). This finding Perhaps indicates an intrinsic function
479 of PV T cells and NK cells as a second layer of antipathogen protection if the trophoblast layer
480 is breached. In contrast to GRZB (**Fig 7E**) production of IL-17A, IFN γ , and TNF α was observed
481 by ~30-60% of CD69^{pos} CD8 T cells (**Fig 7C-D,F**).

482 Although baseline production of cytokines was consistent between mid-gestation,
483 preterm, and term PV T cells, alterations in cytokine production occurred following stimulation.
484 Across all four cytokines PV T cells isolated four distinct term placentas responded consistently.
485 In contrast, mid-gestation and preterm PV T cells had high patient-to-patient variability (**Fig 7G**).
486 When tracking cytokine production of term PV T cells specifically, we observed no change in
487 TNF α production. Additionally, we observed significant downregulation of IL-17A and GRZB
488 following stimulation complimented with an increase in IFN γ production (**Fig 7G**). To determine if
489 the CD69^{pos} CD8 T cells were downregulating GRZB in favor of IFN γ we gated the two cytokines
490 against one another (**Fig 7H**). Consistent with skewing of cytokine production from GRZB to
491 IFN γ , there was a significant decrease of GRZB single positive cells and a concurrent increase
492 of IFN γ single positive cells with stimulation (**Fig 7H**). No significant differences were observed
493 in either double positive or double negative populations (**Fig S7E**). Importantly, this alteration
494 from GRZB to IFN γ production was specifically observed in CD69^{pos} CD8 T cells and was not
495 observed in other T cell subsets (**Fig S7F**). Collectively, this experiment revealed that CD69^{pos}
496 CD8 T cells in the PV are capable of producing a variety of cytokines at baseline and in full term
497 healthy pregnancies a unique subset of CD69^{pos} CD8 T cells skews its cytokine production to
498 favor IFN γ production upon stimulation of the TCR pathway.
499



500
 501 **Figure 7. Gestational variation in PV T cell response.** (A) Representative images and quantification of
 502 pZAP70 expression via IHC. (B) Representative flow plots and quantification of CD69 expression in
 503 CD8^{pos} and CD8^{neg} T cells following stimulation. (C-F) Representative flow plots and quantification of
 504 TNF α (C), IL-17A (D), GranzymeB (GRZB) (E), and IFN γ (F) at baseline. (G) Quantification of cytokine
 505 production before and after stimulation by CD69^{pos} CD8^{pos} T cells* = p-value < 0.05 in Mann-Whitney test.

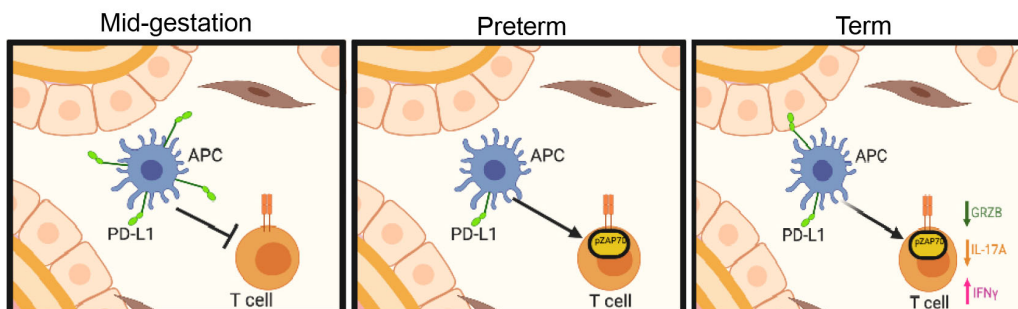
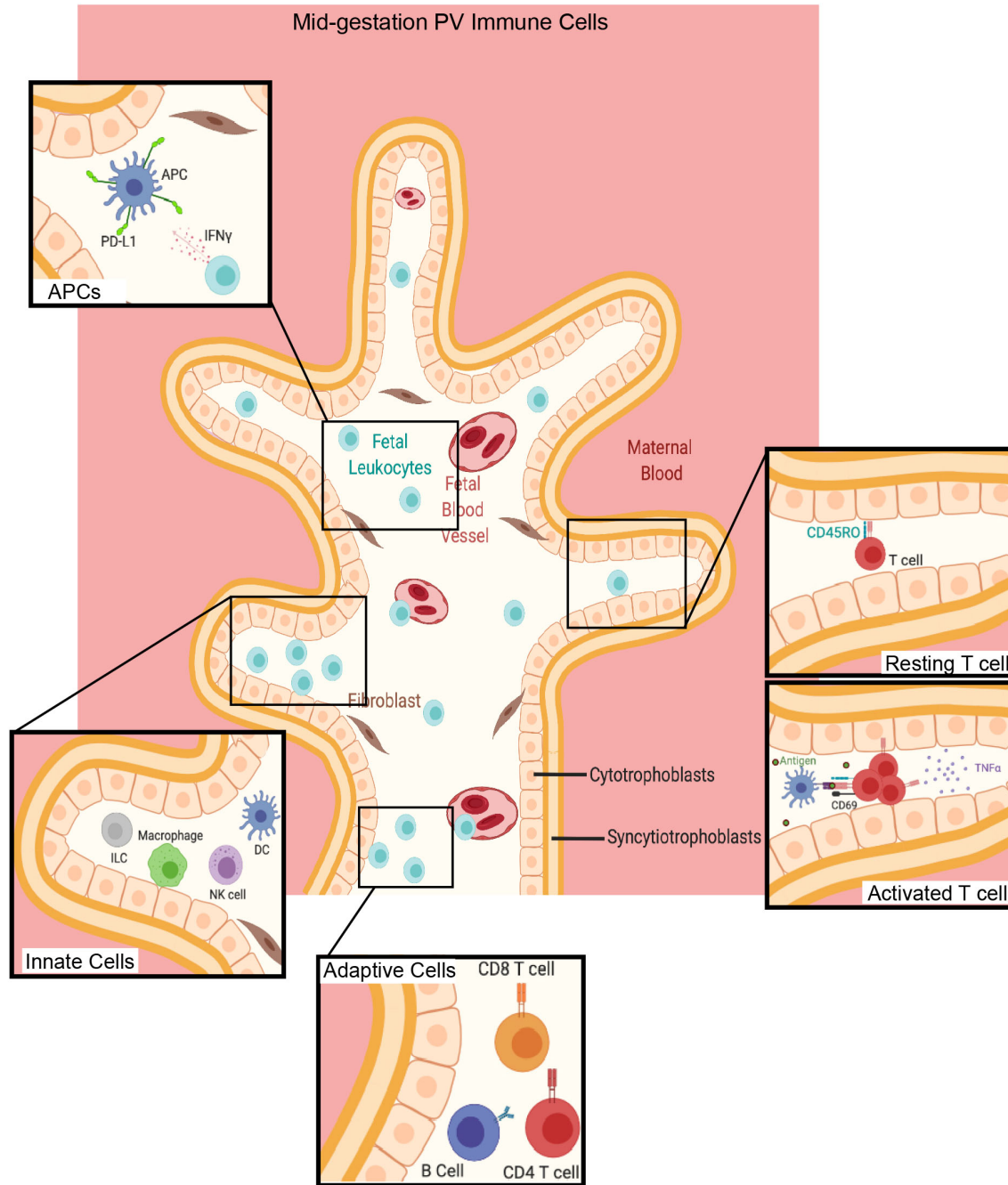
506 (H) Representative flow plots of GRZB and IFN γ production by CD69^{pos} CD8^{pos} T cells* = p-value <0.05 in
 507 Mann-Whitney test.
 508



509 **Figure S7.** (A) Pregating of flow cytometry to CD8 T cells. (B) Unstained control for CD69 and cytokine
 510 stains in flow cytometry. (C) Quantification of CD8 T cell abundance in flow cytometry. (D) Quantification
 511 of GRZB by T cell and NK subsets from flow cytometry. (E) Quantification of GRZB and IFN γ populations
 512 in CD8^{pos} CD69^{pos} T cells. (F) Quantification of GRZB and IFN γ populations in T cell subsets.
 513
 514

515 In summation (**Fig 8**), we demonstrate that the intravillous compartment of the healthy
 516 second trimester PV contains a diverse immune landscape comprised of innate cells such as
 517 M ϕ s, NK cells and ILCs, as well as APCs including B cells, and memory T cells (**Fig 2**).
 518 Moreover, we determined that the immune cells in the PV are likely capable of eliciting an
 519 inflammatory response, but instead maintain immune homeostasis at baseline through a variety

520 of mechanisms. The mechanisms described include reducing chemotaxis by reduced
521 expression of chemokine receptors on innate cells and low transcription of chemokine ligands in
522 the PV overall (**Fig 3**). Moreover, PV APCs constitutively express PD-L1, possibly regulated by
523 the high expression of IFN γ by PV immune cells (**Fig 4**). This PD-L1 expression by APCs is
524 potentially needed to prevent the activation of antigen-experienced PV T cells (**Fig 5**) which can
525 be activated through the TCR pathway by antigens present in the uterine environment (**Fig 6**)
526 and result in the secretion of a variety of cytokines (**Fig 7**).
527



529 **Figure 8. Summary of major findings in study.** Image was generated with Biorender.com.

530

531 Discussion

532 Preserving tolerance at the fetal-maternal interface is critical for maintaining a healthy
533 pregnancy. Studies of the roles of maternal immunity within the decidua and maternal peripheral
534 blood have resulted in the discovery of important immunological tolerance mechanisms
535 including: tolerogenic uterine NK cells (Erlebacher, 2013), unique populations of Tregs
536 (Salvany-Celades et al., 2019) restriction of DC migration to the uterus (Tagliani and
537 Erlebacher, 2011) and suppressive B cells (Huang et al., 2017). Until recently however, the
538 contribution of leukocytes within the PV beyond Hofbauer cells (PV resident M ϕ s) has been far
539 less explored.

540 Single cell studies within the past three years of the first trimester (Suryawanshi et al.,
541 2018; Vento-Tormo et al., 2018) and full term (Pavličev et al., 2017; Pique-Regi et al., 2019)
542 placenta highlighted the diversity of immune cells within the PV. Interestingly Pique-Rige and
543 colleagues identified leukocyte signatures specific to the PV and highlighted a potential role for
544 activated PV T cells in post-delivery PV (Pique-Regi et al., 2019). A finding also reported in
545 preterm macaque PV (Toothaker et al., 2020). With these first and third trimester studies in
546 mind, we hypothesized that the activated leukocytes detected by Pique-Regi (Pique-Regi et al.,
547 2019) in humans and our group in non-human primates (Toothaker et al., 2020) may be present
548 and poised to be activated in the PV earlier in gestation and subject to immunosuppressive
549 mechanisms which maintain immune homeostasis within the PV in utero.

550 To answer these questions, we analyzed placental tissue (including decidua, PV and
551 CP) and confirmed that there are diverse immune cells in second trimester PV samples that are
552 located within the intravillous space and largely fetal in origin as suggested by the presence of
553 the Y chromosome in majority of intravillous cells via FISH and the enrichment of Y genes in
554 male fetuses. Although it is still possible that our analysis of PV immune cells included a
555 proportion of maternal cells, ethical limitations precluded us from including maternal peripheral
556 blood in our study. Future studies employing dual HLA-haplotyping/Y chromosome detection
557 with staining for various immune populations on the same PV sections is needed to distinctly
558 determine the fetal versus maternal origin of individual immune cell populations within the PV
559 vasculature and stroma.

560 Previous studies have suggested that PV fetal immune cells are limited to Hofbauer cells
561 (Thomas et al., 2021) However, the increased granularity provided by single cell methods, have
562 allowed for the detection of T cells, B cells and NK cells of fetal origin in healthy term placentas
563 (Pique-Regi et al., 2019). Moreover, a recent study identified infiltrating cells in the PV to be
564 largely of fetal origin in cases of infectious villitis (Enninga et al., 2020) and Erbach et al.
565 isolated T cells from single cell suspension at 18-24 weeks' gestation (Erbach et al., 1993). It is
566 important to note that studies using single cell suspensions make the delineation of immune
567 cells from the PV vasculature indistinguishable from those in the PV stroma. In the current
568 study, by combining CyTOF to capture the diversity of PV immune cells and multiple imaging
569 modalities, we identified a diverse immune landscape in the PV that was distinct from both the
570 decidua and CP populations consisting of both innate and adaptive immune cells, including T
571 cells, present outside the fetal vasculature in the intravillous space.

572 Focusing specifically on the innate non-APC population we identified NK cells, ILCs and
573 M ϕ s to be present in the PV. Thomas et al. recently reported that Hofbauer cells in the first
574 trimester are HLA-DR^{neg} (Thomas et al., 2021), consistent with this we found an increased
575 abundance of M ϕ s in the PV compared to the decidua in the innate HLA-DR^{neg} compartment.
576 Surprisingly, we report that many of these HLA-DR^{neg} M ϕ s in the PV lacked the expression of
577 CD163, a marker reported to be expressed in all Hofbauer cells (Reyes and Golos, 2018;
578 Schliefssteiner et al., 2017). It is possible that CD163^{lo} M ϕ s reflect downregulation of CD163 by

579 Hofbauer cells during cell isolation as has been reported in the presence of collagenase (Tang
580 et al., 2011). However, it is also possible that Hofbauer cell populations are more diverse than
581 previously thought and CD163 should be used in combination with other M ϕ s markers such as
582 CD14, CD68 and DC-SIGN (Yang et al., 2017) in future studies. It is also possible that some of
583 these cells represent non-Hofbauer macrophages within the PV.

584 In addition to reporting novel immune cell phenotypes, we identified mechanisms of
585 immunosuppression and nonclassical function for multiple subsets of PV immune cells likely
586 involved in preventing inflammation *in utero*. Specifically, we found that innate non-APCs
587 expressed lower levels of multiple chemokine receptors including: CXCR3, CCR6, CCR4 and
588 CCR7 than decidual counterparts. This finding is consistent with histologic evaluation of
589 Hofbauer cells showing a lack of CCR7 and CX3CR1 staining (Joerink et al., 2011). This
590 coupled with reduced expression of multiple chemokine ligands for these and other chemokine
591 receptors we observed in the bulk RNAseq data, could suggest that innate cells in the PV are
592 either more static or are mobile in a non-targeted manner at baseline.

593 We also report a diversity of HLA-DR^{pos} cells present in the PV, where we identified
594 mDCs, pDCs, B cells, M ϕ s and a population of HLA-DR^{pos} NK cells. An antigen-presenting role
595 for NK cells has been previously described (Roncarolo et al., 1991). The identification of fetal
596 HLA-DR^{pos} M ϕ s contrasts Thomas et al's recent findings showing no HLA-DR^{pos} cells in the PV
597 core up to the 10th week of gestation (Thomas et al., 2021). It should be noted that some of
598 these HLA-DR^{pos} M ϕ s in our study may reflect the presence of contaminate maternal M ϕ s
599 (termed PAMMs) repairing breaks in the trophoblast layer. However, the location of HLA-DR^{pos}
600 cells in our study by both immunofluorescent and IMC show that some cells are located distant
601 from the trophoblast layer and suggest that HLA-DR^{pos} M ϕ s appear in the stroma after the time
602 period studied by Thomas et al or between 10-18 weeks' gestation. It would be interesting to
603 determine the fetal versus maternal origin of these second trimester HLA-DR^{pos} M ϕ s and
604 evaluate if the Hofbauer cell population from 18-23 weeks is transcriptionally distinct from those
605 detected in prior studies (Vento-Tormo et al., 2018).

606 Similar to other innate population in the PV, we detected novel immunosuppressive
607 mechanisms in APC subsets observed within the PV. Irrespective of PV APC ontogeny, we
608 determined that PV APCs express more PD-L1 per cell than decidual counterparts. PD-L1's
609 function as a coinhibitory molecule has been extensively studied (Sun et al., 2018). Moreover,
610 PD-L1 expression can be mediated through interferon (IFN γ) signaling (Garcia-Diaz et al.,
611 2017). Interestingly we showed that PV immune cells produce IFN γ preferentially to TNF α ,
612 another proinflammatory cytokine. These findings insinuate that PV APCs mediate in utero
613 homeostasis by controlling T cell activation through the expression of coinhibitory ligands.
614 Further validating the possibility of PV APC mediation of homeostasis via PD-L1 expression, we
615 found that preterm PV have significantly reduced expression of PD-L1 on PV stromal cells
616 compared to mid-gestation PV. Though it is not possible to determine if PD-L1 reduction
617 precedes or is the consequence of preterm delivery, we concurrently observed increased
618 pZAP70 in preterm PV over mid-gestation PV, consistent with increased T cell activation. Thus,
619 it is possible that loss of PD-L1 on PV APCs results in elevated T cell activation leading to
620 increased inflammation that is a well-documented phenotype in preterm deliveries (Romero et
621 al., 2006). With the recent advancements in immunotherapy in multiple disease contexts it
622 would be very interesting to study the local effects in the placenta and pregnancy outcomes of
623 check-point blockade therapies throughout gestation.

624 The detection of memory T cells within the PV justifies the need for PV APCs to limit T
625 cell activation. Memory T cells have been detected in multiple fetal human organs (Angelo et al.,
626 2019; Halkias et al., 2019; Li et al., 2019; Schreurs et al., 2019; Stras et al., 2019b), and with in
627 the non-human primate PV (Toothaker et al., 2020). Additionally activated and resting T cells
628 have been detected in PV samples post-delivery (Pique-Regi et al., 2019) and central memory T

629 cell can be found in human cord blood from preterm infants (Frascoli et al., 2018). Here we
630 report with both CyTOF and flow cytometry that PV T cells are enriched for CD8 and DN T cells,
631 consistent with previous findings in term placentas (Erbach et al., 1993; Kim et al., 2008).
632 Moreover, we found that at baseline PV T cells express low activation signatures (CD69 and
633 PD-1) potentially suggesting that PV T cells have been previously educated (hence the memory
634 marker expression) but remain quiescent due to either the lack of antigens or direct inhibition
635 from PD-L1^{pos} APCs. Moreover, we found no difference in baseline cytokine production by PV T
636 cells between mid-gestation, preterm and term pregnancies, and increased proliferation of PV T
637 cells compared to adult T cells in multiple organs further suggesting that PV T cell machinery
638 and functionality is established early in pregnancy (prior to 17weeks' gestation). Interestingly,
639 we observed high production of GRZB (>75%) among all T cell subsets and NK cells at baseline
640 in term PV. GRZB production in CD4 and CD8 T cell subsets has been previously observed in
641 non-human primate PV T cells in the third trimester, however its direct implications for placental
642 health remain unclear (Toothaker et al., 2020). Interestingly, we observed downregulation of
643 GRZB in CD69^{pos} CD8 T cells and an increase in IFN γ in these same cells upon stimulation of
644 the TCR pathway. One explanation for this phenomenon could be that PV T cells have altered
645 cytokine production between TCR-independent and TCR-dependent activation pathways. We
646 propose that TCR-independent activation, such as by cytokines allows for consistent production
647 of GRZB by CD69^{pos} CD8 T cells at baseline. This TCR-independent high level of GRZB may
648 allow PV T cells to act as a secondary layer of viral defense if the trophoblast layer is breached
649 *in utero*; aide in the clearance of dying cells during cellular turnover as the placenta grows; or
650 have a novel function specific to the placenta that remains to be elucidated. However, if PV T
651 cells are activated through the TCR (experimentally measured with α CD3/CD28 antibodies),
652 they respond by changing cytokine production from a cytotoxic predominance to favor IFN γ
653 production. It is also possible then that PV T cells increase IFN γ upon TCR stimulation to
654 increase PD-L1 on APCs to promote homeostasis and prevent *in utero* inflammation.
655 Alternatively, IFN γ production in activated PV T cells from term placentas participates in the
656 induction of the labor cascade that would be consistent with data showing that both IFN γ and
657 TNF α from preterm cord blood memory T cells can induce uterine contractility (ref).

658 The presence of a placental and/or fetal microbiome as a source of potential antigens for
659 PV T cells remains highly contested (Aagaard et al., 2014; de Goffau et al., 2019; Kuperman et
660 al., 2020; Leiby et al., 2018; Mishra et al., 2021; Rackaityte et al., 2020; Theis et al., 2020).
661 However, our group recently showed that xenobiotic metabolites including bacterial metabolites
662 are present in the fetal intestine at 14 weeks' gestation (Li et al., 2020). As such, it is possible
663 that maternal bacteria derived peptides similarly cross the placenta and educate fetal T cells.
664 Fetal T cell activation by maternal antigens has also been implicated in preterm birth where T
665 cells from cord exposed to maternal antigens delivered on cord blood APCs showed increased
666 proliferation and secretion of TNF α and IFN γ (Frascoli et al., 2018). As previously mentioned,
667 our data indicates that T cells obtained from mid-gestation PV can be stimulated through the
668 activation of the TCR signaling (α CD3/CD28 antibodies) and when exposed to decidual
669 antigens but not to unmatched PBMC antigens. Of note, we found many more T cells
670 upregulating CD69 compared to those secreting TNF α upon decidual stimulation. It is likely that
671 there is a differential response to maternal antigens among PV T cells. Further validating
672 differential response to stimulation by mid-gestation PV T cells, we observed high patient to
673 patient variability in cytokine secretion following stimulation. This was also observed in preterm
674 PV T cells. Yet, term PV T cells behaved in an orchestrated fashion across all patients. This
675 suggests that prior term PV T cells responses have not been fully established, but by the end of
676 healthy pregnancy PV T cells mature enough to have similar responses upon activation. This
677 variation in PV T cells responses to stimulation could be attributed to either incomplete T cell
678 development or incomplete exposure to an array of antigens. This variability could alternatively

679 be attributed to PV T cells at term having a well-defined role whereas prior to complete
680 gestation, the PV T cell response to stimulation is variable depending on the overall state of
681 individual pregnancies. Collectively, these findings have identified unique functions of PV T cells
682 and suggest that antigens could stimulate a proinflammatory PV T cell response upon crossing
683 the placental barrier, however the multiple immunosuppressive mechanisms are in place as
684 early as 17 weeks within the placenta to prevent inappropriate T cell activation including: limited
685 chemotaxis of innate sensor cells and high expression of coinhibitory molecules by PV APCs.

686 Our study had several limitations of note the lack of genetic information to segregate
687 fetal from maternal cells. It would be very interesting for a future cohort to definitively determine
688 the origin of each PV immune cell subset identified in this work using dual *in situ* hybridization
689 and immunodetection techniques. Furthermore, legal limitations prevented the collection of
690 maternal and fetal blood to use for comparison.

691 The ability of fetal immune cells to execute mature functions has recently been validated
692 in multiple cell types and organs throughout the fetus (Angelo et al., 2019; Frascoli et al., 2018;
693 Halkias et al., 2019; McGovern et al., 2017; Schreurs et al., 2019; Stras et al., 2019a). As such
694 the detection of immunosuppressive mechanisms to control this fetal immune response and
695 prevent *in utero* inflammation is critical, particularly so at the point of contact and potential
696 antigen exchange between mother and fetus. Throughout this study we have identified
697 previously understudied immune cell populations within the mid-gestation placental villi.
698 Moreover, we detected multiple mechanisms of immunosuppression utilized by these PV
699 immune cells to help maintain homeostasis and prevent inflammation *in utero*. This work has
700 implications for future studies to better understand the complex roles of fetal and maternal
701 immune cells within the placenta and potentially contribute to a better understanding of immune
702 tolerance in multiple disease contexts.

703

704 **Methods**

705

706 **Placental Tissue Collection**

707 Human products of conception were obtained through the University of Pittsburgh Biospecimen
708 core after IRB approval (IRB# PRO18010491). Preterm placentas resultant from a variety of
709 obstetric complications were obtained from the University of Pittsburgh MOMI Biobank. Term
710 placentas were collected both at the University of Pittsburgh through the MOMI biobank and
711 through the Yale University YURS Biobank from C-section deliveries devoid of obstetric
712 complications (**Table 1**). Placental villi were separated using forceps under a light dissection
713 microscope (Fisherbrand #420430PHF10) from the chorionic and amniotic membranes lining
714 the chorionic plate (CP) and from the decidua basalis (referred to as decidua throughout
715 manuscript) on the basal plate side of the placenta. Tissue was thoroughly washed with sterile
716 PBS prior to cryopreservation and subsequent single cell isolation as previously described
717 (Konnikova et al., 2018).

718

719 **RNA sequencing:** Snap frozen placental tissues were shipped on dry ice to MedGenome for
720 mRNA extraction and library preparation. RNA extractions were completed with the Qiagen All
721 Prep Kit (#80204). cDNA synthesis was prepped with the Takara SMART-seq kit (#634894) and
722 NexteraXT (FC-131-1024, Illumina) was used to fragment and add sequencing adaptors. Quality
723 control was completed by MedGenome via Qubit Fluorometric Quantitation and TapeStation
724 BioAnalyzer. Libraries were sequenced on the NovaSeq6000 for Paired End 150 base pairs for
725 90 million reads per sample.

726

727 **RNA sequencing analysis:** FASTQ files were imported and subsequently analyzed with CLC
728 Genomics Workbench 20.0 (<https://digitalinsights.qiagen.com>). Briefly, paired reads were first
729 trimmed with a quality limit of 0.05, ambiguous limit of 2 with automated read through adapter

730 trimming from the 3'-end with a maximum length of 150. Trimmed reads were then mapped to
731 the homo_sapiens_sequence_hg38 reference sequence. Differential gene expression was
732 computed in CLC Genomics with an Across groups ANOVA-like comparison. Significantly
733 differentially expressed genes were delineated as those with a p-value <0.05, False-Discovery
734 Rate <20% and fold-change > absolute value of 2. Heatmaps for gene expression were created
735 with Morpheus (<https://software.broadinstitute.org/morpheus>).

736
737 **RISH:** Formalin fixed samples were sectioned and embedded in paraffin by the Pitt
738 Biospecimen Core. Staining was completed per manufacturer's instructions for RNAScope®
739 multiplex V2 detection kit (ACD Bio) coupled with immunofluorescent protein staining for either
740 Cytokeratin19 (ab52625 Abcam) at 1:250 dilution. Echo® Revolve microscope at 20x was used
741 to image sections. All images were batch processed using FIJI (Schindelin et al., 2012), and all
742 edits were made to every pixel in an image identically across all patients per experiment.
743 Quantification of cell populations was done using a custom pipeline in CellProfiler (McQuin et
744 al., 2018).

745
746 **FISH:** In situ hybridization for the Y chromosome was adapted from the protocol outlined
747 in(Enninga et al., 2020). Briefly, slides were deparaffinized with a series of xylene and ethanol
748 washes. Target retrieval was done at 95°C for 10 minutes, slides were placed in 70% ethanol,
749 85% ethanol and 100% ethanol for 2 minutes each. DYZ3 probe (D5J10-034, Abbott
750 Laboratories) was diluted 1:10 in LSI/WCP hybridization buffer (D6J67-011, Abbott
751 Laboratories) and incubated for 5 minutes at 83°C prior to overnight hybridization at 37°C. Slides
752 were soaked in SSC/0.1% NP-40 (ab142227, Abcam) to remove cover slips and placed in 2X
753 SSC/0.1% NP-40 for 2 minutes at 74°C before mounting with antifade plus Propidium Iodide
754 (p36935, Invitrogen). Slides were imaged on the LSM 710 (Leica Biosystems) confocal at the
755 Yale Center for Cellular and Molecular Imaging.

756
757 **RNA extraction and qPCR:** RNA was extracted from snap-frozen villi samples using the
758 RNAEasy Minikit (#217004, Qiagen) RNA was converted to cDNA using iScript (#1708891,
759 BioRad) reagents according to manufacturer protocol. Samples were run on the Taqman
760 StepOnePlus Real-Time PCR System (Applied Biosciences) machine with probes for ACTB
761 (Hs01060665_g1) as housekeeping gene and with either XIST (Hs01079824_m1) or EIF3AY
762 (Hs01040047) all from Qiagen. Values undeterminable were given cycle values of 40 for
763 quantification purposes.

764
765 **Immunofluorescent staining:** Slides with 10um sections of FFPE tissue were deparaffinized
766 with a series of xylene and ethanol washes. Antigen retrieval was performed in the Biocare
767 Medical LLC decloaking chamber (NC0436641) for 1 hour with citrate-based antigen retrieval
768 buffer (H-3300, Vector Laboratories) and washed with PBS. Slides were then blocked for 30
769 minutes with 10% horse serum prior to overnight incubation at 4°C with primary antibodies.
770 Slides were washed with PBS and incubated with secondary antibodies for 45 minutes at RT.
771 Slides were mounted with Antifade mounting media + DAPI (H-1300, Vectashield).

772
773 **Imaging mass cytometry:** Slides with 4um sections of FFPE tissue were deparaffinized with a
774 series of xylene and ethanol washes. Antigen retrieval was performed at 95°C for 20 minutes
775 using 1X Antigen Retrieval Buffer (#CTS013 R&D) and washed with water and dPBS. Slides
776 were then blocked for 30 minutes with 3% BSA in dPBS prior to overnight incubation at 4°C with
777 a primary antibody cocktail (**Table S8**). Slides were rinsed and co-stained with 191/193 DNA-
778 intercalator (Fluidigm), rinsed and air dried for >20 minutes prior to analysis. Slides were
779 analyzed on the Hyperion Mass Cytometer with an ablation energy of 4 and frequency of 100Hz

780 for ~30 minutes per section. Representative images were generated using Histocat++
781 software(Catena et al., 2018).

782
783 **CyTOF staining:** Samples were stained with antibody cocktail (**Table S4**) per previously
784 published protocol(Stras et al., 2019a) and incubated with 191Ir/193Ir DNA intercalator
785 (Fluidigm) and shipped overnight to the Longwood Medical Area CyTOF Core. Data was
786 normalized and exported as FCS files, downloaded and uploaded to Premium Cytobank®
787 platform. Any files with insufficient cell number were excluded from analysis (**Table S5**). Gating
788 and analysis was completed with cytofkit(Chen et al., 2016) as published (Stras et al., 2019a).
789 Cluster abundance was extracted, and statistically analyzed using R.

790
791 **Stimulation of PV T cells: α CD3/ α CD28 with CFSE:** Cells were isolated from cryopreserved
792 PV samples as described throughout manuscript. Dead cells were removed prior to stimulation
793 using Millitenyl dead cell removal kit (130-090-101 Millitenyl Biotec). Cells were incubated with
794 CFSE (65-0850-85) alone or with α CD3 (clone HIT3a, #300302, Biolegend) and α CD28 (clone
795 CD28.2, 302902, Biolegend) soluble antibodies for 72 hours rotating at 37°C + 5% CO₂.
796 GolgiPlug (51-2301K2, BD Biosciences) and GolgiStop (51-2092K2, BD Biosciences) were
797 added for the last 4 hours of stimulation. **α CD3/ α CD28 with Ki67:** Cells were isolated as
798 described above and incubated with aforementioned α CD3 and α CD28 antibodies for 4 hours
799 rotating at 37°C + 5% CO₂.

800
801 **Stimulation of PV T cells lysed decidual cells:** Single cells from PV and decidua were
802 isolated from cryopreserved tissue as previously described(Konnikova et al., 2018). PBMCs
803 were thawed and DMSO was washed out. PV tissue was thawed and made into single-cell
804 suspensions (as described above). Cells were incubated in 5mLs of media with GolgiPlug (51-
805 2301K2, BD Biosciences) and GolgiStop (51-2092K2, BD Biosciences) and designated stimuli.
806 For PMA condition, PMA (1:2000) (Sigma-Aldrich) and Ionomycin (1:1000) (Sigma-Aldrich) were
807 added. Decidua and PBMC cells were lysed via ultracentrifugation at max speed for 7 minutes
808 and 1mL of lysed components was added to appropriate conditions. PV cells were exposed to
809 stimuli for 4 hours at 37°C with 5% CO₂.

810
811 **Flow cytometry:** Post stimulation cells were washed with PBS and incubated with either
812 Propidium Iodide or Zombie Aqua live/dead stain. Viability marker was washed out and cells
813 were resuspended and spun down in FACS buffer then incubated with Human TruStain FcX
814 (Biolegend) for 10 minutes prior to the addition of a surface antibody cocktail (**Table S9**) Cells
815 were washed with FACS buffer and permeabilized with FoxP3 fix/perm (Invitrogen) overnight.
816 Cells were washed with 1X FoxP3 Wash Buffer (Invitrogen) and incubated with intracellular
817 antibodies. Cells were washed, fixed for 10 minutes with 4% PFA and resuspended in FACS
818 buffer. All samples were run either on BD LSRFortessa
819 (BDBiosciences) at the University of Pittsburgh Department of Pediatrics Flow Cytometry core
820 (decidual cell stimulation) or on BD LSRII (BDBiosciences) at the Yale University Flow
821 Cytometry core (all other stimulations). Output FCS files were analyzed with FlowJo®.

822
823
824 **Statistics:** R version 3.6.1 with Kruskal-Wallis analysis and Dunn's multiple comparison test for
825 post-hoc analysis among groups. One-tailed t-test was used to compare groups of two.
826 Comparisons of mean expression values corrected using the Bonferroni method. P-values of
827 0.05 or less were significant.

828

829 **Plot generation:** Plots comparing multiple groups were generated using Prism GraphPad 8. In
830 each plot, each data point represents one subject as per figure description.

831
832 **Data and code availability:** Data analyzed in this study has been stored according to IRB
833 guidelines and is subject to institutional regulations. Requests can be directed to Lead Contact,
834 Liza Konnikova liza.konnikova@yale.edu.

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844
845 **Author Contributions:** JMT and LK conceived the work. CyTOF and flow cytometry
846 procedures and analysis and drafting of manuscript were done by JMT supervised by LK.
847 Tissue collection and preparation was done by JMT, RMC, CCM and OOO. IMC analysis was
848 done by OOO. Immunocytochemistry was completed by BTM; RISH was done by RMC; RNA
849 extraction/qPCR was performed by CCM. Cytometry bioinformatics consultation was done with
850 PL supervised by GT. RNA sequencing assistance provided by DY. Figure construction was
851 done by JMT and BTM. All authors contributed to the editing and compilation of the manuscript.

852
853 **Declaration of Interests:** The authors have declared that no conflict of interest exists.

854
855 **Supplemental Tables included at end of document**

856 Table S1. Patient Cohort
857 Table S2. Differentially Expressed Genes Between Decidua, CP and PV
858 Table S3. Selected Immune Genes
859 Table S4. CyTOF Panel
860 Table S5. Files Omitted from CyTOF Analyses
861 Table S6. Cell Type Identification
862 Table S7. IMC Panel
863 Table S8. Differentially Expressed Chemokines
864 Table S9. Flow Cytometry Antibodies

865
866
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