Pregnancy Imparts Distinct Systemic Adaptive Immune Function

Running title: Systemic adaptive immunity in pregnancy

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45 **Conflict of interest statement**

46 The authors declare no potential conflicts of interest.

47

48 **Ethics statement**

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49 The authors confirm that the ethical policies of the journal, as noted on the journal's
50 author guidelines page, have been adhered to and the appropriate ethical review committee
51 approval has been received from the Institutional Review Boards of Wayne State University
52 and the Detroit Medical Center. The study conformed to the US Federal Policy for the
53 Protection of Human Subjects.

54

55 **Author contributions**

56 NGL and RR conceived and designed the study. CDP, YX, MAH, DM, and LT
57 performed and analyzed experiments. CDP, YX, MAH, DM, JG, MFJ, GB, VGF, MS, and
58 ALT analyzed data, contributed to data visualization, and provided intellectual input. NGL,
59 RR, CDP, and YX interpreted the data. All authors revised and approved the final
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61 **ABSTRACT**

62 **Problem:** Pregnancy represents a state of systemic immune activation that is primarily driven
63 by alterations in circulating innate immune cells. Recent studies have suggested that cellular
64 adaptive immune components, T cells and B cells, also undergo changes throughout
65 gestation. However, the phenotypes and functions of such adaptive immune cells are poorly
66 understood. Herein, we utilized high-dimensional flow cytometry and functional assays to
67 characterize T-cell and B-cell responses in pregnant and non-pregnant women.

68 **Methods:** PBMCs from pregnant (n = 20) and non-pregnant (n = 25) women were used for
69 phenotyping of T-cell and B-cell subsets. T-cell proliferation and B-cell activation were
70 assessed by flow cytometry after *in vitro* stimulation, and lymphocyte cytotoxicity was
71 evaluated using a cell-based assay. Statistical comparisons were performed using linear
72 mixed effects models.

73 **Results:** Pregnancy was associated with modestly enhanced basal activation of peripheral
74 $CD4^+$ T cells. Both $CD4^+$ and $CD8^+$ T cells from pregnant women showed increased
75 activation-induced proliferation; yet, a reduced proportion of these cells expressed activation
76 markers compared to non-pregnant women. There were no differences in peripheral
77 lymphocyte cytotoxicity between study groups. A greater proportion of B cells from pregnant
78 women displayed memory-like and activated phenotypes, and such cells exhibited higher
79 activation following stimulation.

80 **Conclusions:** Maternal circulating T cells and B cells display distinct responses during
81 pregnancy. The former may reflect the unique capacity of T cells to respond to potential
82 threats without undergoing aberrant activation, thereby preventing systemic inflammatory
83 responses that can lead to adverse perinatal consequences.

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85 **Keywords:** T cell, B cell, maternal circulation, cytotoxicity, flow cytometry, adaptive
86 immunity

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87 **1. INTRODUCTION**

88 Pregnancy represents a state of mild intravascular inflammation that can be broadly
89 characterized by enhanced innate immune responses to defend against pathogenic threats[1-3].
90 Specifically, prior studies have indicated that the maternal circulation contains increased
91 numbers or frequencies of activated and functional myeloid cells (i.e., monocytes and
92 granulocytes)[4-11] as well as elevated concentrations of humoral innate immune components
93 such as complement[12-16]. More recently, the application of omics platforms to the maternal
94 circulation provided further evidence of innate immune activation and demonstrated
95 correlation between alterations in innate immune-related processes and advancing gestational
96 age[17-22]. Yet, these comprehensive studies also hinted at systemic alterations in adaptive
97 immune signatures, primarily T cells, during pregnancy and in particular prior to the onset of
98 physiologic or pathologic labor[18-21,23,24]. Such observations may have clinical implications for
99 the monitoring and prediction of the premature onset of labor leading to preterm birth.
100 Indeed, the aberrant activation of maternal T cells has also been associated with the
101 pathogenesis of preeclampsia[25-29]. Furthermore, changes in B-cell phenotypes have been
102 reported in the periphery[30] and at the maternal-fetal interface[31] throughout gestation and in
103 the pathology of preterm labor, respectively. Therefore, the cellular responses driven by the
104 adaptive limb of immunity during pregnancy warrant further investigation.

105 The conventional belief is that circulating T cells are skewed towards a Th2-like
106 phenotype throughout gestation[32-37]. Accordingly, a number of clinical investigations noted
107 that some autoimmune disorders (e.g., multiple sclerosis and rheumatoid arthritis) are
108 temporarily alleviated during pregnancy[38-46]. This suppression also seems to extend to the
109 maternal-fetal interface, where multiple protective mechanisms exist to prevent T-cell
110 activation such as exhaustion or senescence[47-49], local silencing of T-cell chemotactic signals
111 and trafficking[50-52], and expansion of regulatory T cells[53-64]. Importantly, single-cell RNA

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112 signatures derived from T cells infiltrating the maternal-fetal interface can be tracked in the
113 maternal circulation and may serve as biomarkers for obstetrical disease[21,23,65]. Hence,
114 investigating the functional status of circulating T cells during pregnancy may provide a
115 window in the events taking place at the maternal-fetal interface. Although a large body of
116 research has focused on examining the phenotypes and function of T cells and B cells
117 throughout gestation[3,31,66-69], little is known of the potential pregnancy-specific functional
118 differences in such adaptive immune cells. In the current study, we utilized high-dimensional
119 flow cytometry together with functional assays to characterize T-cell and B-cell cellular
120 responses in the periphery of pregnant and non-pregnant women.

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121 **2. METHODS**

122 *2.1 Human subjects and clinical specimens*

123 Peripheral blood samples were collected from healthy pregnant and non-pregnant women
124 under research protocols at the Perinatology Research Branch, an intramural program of the
125 *Eunice Kennedy Shriver* National Institute of Child Health and Human Development
126 (NICHD), National Institutes of Health (NIH), U. S. Department of Health and Human
127 Services (DHHS), Wayne State University (Detroit, MI, USA), and the Detroit Medical
128 Center (Detroit, MI, USA). The collection and use of biological specimens for research
129 purposes were approved by the Institutional Review Boards of Wayne State University and
130 the Detroit Medical Center. All patients provided written informed consent prior to sample
131 collection. The present study included pregnant women (n = 20), predominantly African-
132 American, whose peripheral blood was collected in the third trimester prior to the
133 administration of any medication, with a median gestational age of 39.1 weeks at sampling,
134 prior to the onset of labor. The control study group was comprised of healthy non-pregnant
135 women (n = 27) of reproductive age from the same community.

136

137 *2.2 Stimulation of T-cell proliferation*

138 Peripheral blood was obtained by venipuncture and collected in Vacutainer K3 EDTA
139 tubes (BD Biosciences, San Jose, CA, USA). Peripheral blood mononuclear cells (PBMCs)
140 were isolated by Lymphoprep density gradient (Axis Shield, Oslo, Norway), per
141 manufacturer instructions. Isolated PBMCs were centrifuged at 300 x *g* for 5 min and
142 resuspended in phosphate-buffered saline (PBS) at a density of $1x10^6$ cells/mL. Next, PBMCs
143 were stained with 1 µL/mL CellTrace™ Violet dye (Thermo Fisher Scientific, Life
144 Technologies Corporation, Carlsbad, CA, USA) for 20 min at 37°C. The staining reaction
145 was quenched by adding complete RPMI 1640 medium (Thermo Fisher Scientific, Life

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146 Technologies Limited, Paisley, UK) [enriched with 5% human serum (Sigma-Aldrich, St
147 Louis, MO, USA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific)] and allowing
148 the suspension to incubate at room temperature (RT) for 2 min. The PBMCs were then
149 centrifuged at 300 x *g* for 5 min, resuspended in complete RPMI 1640 medium, and counted
150 using ViaStain AOPI Staining Solution (Nexcelom Bioscience, Lawrence, MA, USA) and a
151 Nexcelom Bioscience Cellometer Auto 2000. An aliquot containing 1 x 10^6 cells was set
152 aside for basal (day 0) immunophenotyping. The remaining cell suspension volume was
153 divided into control and stimulated samples. Control suspensions were treated with 55µM 2-
154 mercaptoethanol (Life Technologies Corporation, Grand Island, NY, USA); stimulated
155 solutions were treated with 55µM 2-mercaptoethanol, Dynabeads™ Human T-activator
156 CD3/CD28 (Thermo Fisher Scientific) at a ratio of 1:1 cells:beads, and 2000 U/mL
157 recombinant human IL-2 (BD Biosciences). Each sample was seeded in triplicate, for both
158 control and stimulated cells, at a density of 1 x 10^5 cells per well in a 96-well U bottom plate.
159 The plate was incubated at 37°C with 5% CO_2 for six days.

160 *2.3 T-cell phenotyping for basal and proliferated samples*

161 Following six days of incubation, PBMCs were collected, washed, and resuspended in
162 PBS. For basal immunophenotyping, 1 x 10^6 cells were resuspended in PBS. Cell suspensions
163 were incubated with 0.5 µL/mL Fixable Viability Stain 575V (BD Biosciences) for 15 min in
164 the dark at RT. Next, PBMCs were washed and incubated with extracellular fluorochrome-
165 conjugated anti-human mAbs (Supplemental Table 1) for 30 min in the dark at 4°C. Cells
166 were washed in stain buffer (BD Biosciences), then fixed and permeabilized using the
167 Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific), per manufacturer
168 instructions. Intranuclear staining was performed with fluorochrome-conjugated anti-human
169 mAbs (Supplemental Table 1), which were added to cell suspensions and then incubated for
170 30 min in the dark at 4°C. Finally, cells were washed in Foxp3 Permeabilization Buffer

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171 (Thermo Fisher Scientific) and resuspended in 0.5 mL of stain buffer for analysis by flow
172 cytometry.

173 CountBright absolute counting beads (Thermo Fisher Scientific) were added prior to
174 analysis. Flow cytometry acquisition was performed on a BD LSRFortessa flow cytometer
175 using FACSDiva software version 6.0. The analysis and figures were performed and created
176 using FlowJo software version 10 (FlowJo, Ashland, OR, USA). T cell subsets were
177 identified based on the gating strategy presented in Supplemental Fig. 1.

178

179 *2.4 Peripheral lymphocyte cytotoxicity assay*

180 Target K-562 cells (ATCC, Manassas, VA, USA) – myelogenous leukemia cells that
181 lack MHC class I and II expression[70-72] – were cultured in complete RPMI 1640 medium
182 [enriched with 10% fetal bovine serum and 1% Penicillin-Streptomycin], collected,
183 centrifuged at 300 x *g* for 5 min, and resuspended in PBS. Next, cells were incubated with 1
184 µL/mL carboxyfluorescein diacetate succinimidyl ester (CFSE; Thermo Fisher Scientific) at
185 37°C with 5% CO_2 for 20 min. To stop the reaction, complete RPMI 1640 medium was
186 added and the suspension was incubated at RT for 2 min. The cells were resuspended in
187 complete RPMI 1640 medium and counted using ViaStain AOPI Staining Solution and a
188 Nexcelom Bioscience Cellometer Auto 2000.

189 Peripheral blood was obtained by venipuncture and collected in Vacutainer K3 EDTA
190 tubes. PBMCs were isolated by Lymphoprep density gradient, per manufacturer instructions.
191 Target (K-562) cells and PBMCs were mixed in sterile FACS tubes in the following ratios
192 (PBMCs:target cells): 0:1 6:1, 12:1, 25:1, and 50:1. The resulting cell suspensions were
193 centrifuged at 300 x *g* for 5 min, resuspended in complete RPMI 1640 culture medium, and
194 transferred to a 96-well U-bottom plate. The plate was centrifuged at 100 x *g* for 2 min and
195 then incubated at 37°C with 5% CO_2 for 4 h. Following incubation, cell suspensions were

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196 transferred to FACS tubes, diluted with PBS, and centrifuged at 300 x *g* for 5 min. Cell
197 pellets were resuspended in PBS and incubated with 1 μL/mL 7-aminoactinomycin D (7-
198 AAD; Thermo Fisher Scientific) in the dark at 4°C for 15 min. Cell suspensions were
199 centrifuged at 300 x *g* for 5 min and resuspended in 0.5 mL of stain buffer for analysis by
200 flow cytometry.

201 CountBright absolute counting beads (Thermo Fisher Scientific) were added prior to
202 analysis. Flow cytometry acquisition was performed on a BD LSRFortessa flow cytometer
203 using FACSDiva software version 6.0. Viable target cells were classified as $CFSE^+7AAD^-$,
204 while killed target cells were $CFSE^+7AAD^+$. Viable and dead lymphocytes were classified as
205 $CFSE^-7AAD^-$ and $CFSE^-7AAD^+$, respectively. The percentage of killed target cells was
206 calculated as follows: # of $CFSE^+7AAD^+$ cells / (# of $CFSE^+7AAD^+$ cells + # of
207 $CFSE^+7AAD^-$ cells). The analysis and figures were performed and created using FlowJo
208 software version 10.

209

210 *2.5 B-cell phenotyping*

211 PBMCs were isolated and counted as described above. An aliquot of 1 x 10^6 cells was
212 used for phenotyping. The cells were incubated with 1.0 μL/mL Fixable Viability Stain 510
213 (BD Biosciences) for 15 min in the dark at RT. Next, PBMCs were washed and incubated
214 with extracellular fluorochrome-conjugated anti-human mAbs (Supplemental Table 2) for 30
215 min in the dark at 4°C. The cells were then washed once with stain buffer and resuspended in
216 0.5 mL of stain buffer for analysis by flow cytometry.

217 CountBright absolute counting beads were added prior to analysis. Flow cytometry
218 acquisition was performed on a BD LSRFortessa flow cytometer using FACSDiva software
219 version 6.0. The analysis and figures were performed and created using FlowJo software

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220 version 10. B-cell subsets were identified based on the gating strategy presented in
221 Supplemental Fig. 2.

222

223 *2.6 B-cell activation assay*

224 PBMCs were isolated and counted as described above. For both control and
225 stimulated arms of the B-cell activation assay, PBMCs were seeded in sterile FACS tubes
226 with 2.5 x 10^5 cells. The control suspension received no treatment; the stimulated suspension
227 was treated with 10 µg/mL F (ab') 2-goat anti-human IgG, IgM (H^+L) (Functional grade, Life
228 Technologies Corporation, Carlsbad, CA, USA). The cells were incubated at 37°C for 30
229 min. Next, an equivalent volume of Phosflow Fix Buffer I (BD Biosciences) was added and
230 the cells were incubated at 37°C for 10 min. Cells were washed twice with Permeabilization
231 Solution I (BD Biosciences), per manufacturer instructions. After resuspension in
232 Permeabilization Solution I, anti-human fluorophore-conjugated mAb Phospho-BKT
233 (Supplemental Table 1) was added and incubated in the dark at 4°C for 30 min. After 15 min,
234 the fluorophore-conjugated anti-human CD19 mAb (Supplemental Table 1) was added, and
235 the incubation was resumed under the same conditions. Next, the cells were washed twice
236 with Permeabilization Solution I. Finally, the cell pellets were resuspended in 0.5 mL stain
237 buffer for analysis via flow cytometry.

238 CountBright absolute counting beads were added prior to analysis. Flow cytometry
239 acquisition was performed on a BD LSRFortessa flow cytometer using FACSDiva software
240 version 6.0. The analysis and figures were performed and created using FlowJo software
241 version 10. Fold change in B-cell activation was calculated as follows: [Stimulated (MFI_{mAb} –
242 $MFI_{Isotype}$)] / [Control (MFI_{mAb} – $MFI_{Isotype}$)]. Any fold changes < 1 were considered to be "no
243 change" and assigned a value of 1.0, which did not impact the significance of the results. B-
244 cell activation was determined based on the gating strategy presented in Supplemental Fig. 3.

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245

246 *2.7 Statistical analysis*

247 Statistical analyses for baseline T-cell phenotyping, stimulated and control
248 proliferated T-cell phenotyping, and B-cell phenotyping were performed using the R
249 statistical programming language. Linear mixed effects models[73] were fit for the comparison
250 of stimulated and control T-cell flow cytometry data and between study groups to account for
251 repeated measurements. The data obtained by flow cytometry were modeled as proportions.
252 For T-cell baseline (day 0) phenotyping and B-cell phenotyping, the proportion of cells with
253 a given phenotype was compared between pregnant and non-pregnant study groups, and a p-
254 value <0.05 was considered statistically significant. For T-cell proliferated (day 6)
255 phenotyping, involving interactions between control and stimulated samples within both
256 study groups, a false discovery rate-adjusted p-value[74] (q-value) <0.05 was considered
257 statistically significant. For heatmap representations of immunophenotyping results, flow
258 cytometry data were transformed into Z-scores by subtracting the mean and dividing by the
259 standard deviation. Of note, the heatmaps were generated to display the proportion of cells
260 with a given phenotype in pregnant vs. non-pregnant women, which included control and
261 stimulated samples for the T-cell proliferation analyses. Phenotypes listed in the heatmap
262 were thus not statistically compared among each other. Statistical analyses for PBMC
263 cytotoxicity and B-cell activation were performed using the Shapiro-Wilk test for normality
264 followed by the Mann-Whitney *U*-test and GraphPad Prism software version 9.0.0 for
265 Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). A p-value <0.05
266 was considered statistically significant.

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267 **3. RESULTS**

268 **3.1 Pregnancy is associated with a modest increase in activated CD4$^+$ T cells**

269 Pregnancy includes the selective modulation of the adaptive immune system at the
270 maternal-fetal interface[19,48,49,52,63,75-79] and in the periphery[18,20,21,23,80]. Therefore, we first
271 sought to uncover differences in systemic baseline (day 0) T-cell subset composition as a
272 function of pregnancy. Peripheral blood mononuclear cells (PBMCs) were isolated from non-
273 pregnant and pregnant women for phenotyping via flow cytometry (Fig. 1A) using the gating
274 strategy presented in Supplemental Fig. 1. The relative proportions of CD4$^+$ and CD8$^+$ T cells
275 with the characterized phenotypes in each patient are presented in Fig. 1B. While there were
276 no pregnancy-specific differences in the proportions of total CD4$^+$ or CD8$^+$ T cells,
277 pregnancy was associated with a significantly higher basal proportion of CD4$^+$ T cells
278 expressing the early activation marker, CD69 (Fig. 1C)[81,82], although the effect size was
279 small. In addition, a significantly higher proportion of cells co-expressed CD69 and the co-
280 inhibitory receptor, PD-1[83-85], among CD4$^+$ T cells isolated from pregnant compared to non-
281 pregnant women (Fig. 1D). Importantly, the co-expression of CD69 and PD-1 is likely to be
282 indicative of prolonged T-cell activation[86,87]. These data suggest that pregnancy is associated
283 with a modest enhancement in the baseline activation of peripheral CD4$^+$ T cells.

284

285 **3.2 Circulating T cells display a pregnancy-specific increase in proliferative capacity**
286 **with diminished susceptibility to activation**

287 Given the baseline differences in peripheral T-cell activation between non-pregnant
288 and pregnant women, we next considered whether pregnancy was associated with altered
289 function, including proliferative capacity, of such cells. Accordingly, PBMCs isolated from
290 pregnant and non-pregnant women were stimulated with anti-CD3/anti-CD28 and rhIL-2 to
291 assess pregnancy-specific differences in CD4$^+$ (Fig. 2A) and CD8$^+$ (Fig. 3A) T-cell

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292 proliferation. As expected, significant changes in subset proportions (Fig. 2B&3B) and
293 absolute numbers (Fig. 2C&3C) were observed in T cells derived from non-pregnant and
294 pregnant women following stimulation (Extended Dataset 1); here, we focused on the
295 phenotypic and functional differences between study groups. Both CD4$^+$ (Fig. 2C) and CD8$^+$
296 T cells (Fig. 3C) had a significantly higher proliferative capacity, as determined by absolute
297 cell counts, in pregnant compared to non-pregnant women. Next, we analyzed the
298 proliferated CD4$^+$ (Fig. 2B) and CD8$^+$ (Fig. 3B) T-cell subset composition of the two study
299 groups relative to controls that were cultured under identical conditions without stimulation.
300 Strikingly, we found that the proportion of T cells expressing the activation marker CD69
301 was significantly reduced in stimulated CD4$^+$ (Fig. 2D) and CD8$^+$ (Fig. 3D) T cells from
302 pregnant compared to non-pregnant women. Furthermore, a significantly decreased
303 proportion of CD4$^+$ (Fig. 2E) and CD8$^+$ (Fig. 3E) T cells expressed PD-1 in pregnant
304 compared to non-pregnant women following stimulation. The proportion of cells co-
305 expressing CD69 and PD-1 was also found to be significantly lower in CD4$^+$ (Fig. 2F) and
306 CD8$^+$ (Fig. 3F) T cells from pregnant women. Finally, a significantly lower proportion of
307 CD8$^+$ terminally differentiated effector memory T cells (CD45RA$^+$CCR7$^-$), which are
308 characterized by low proliferative capacity and rapid effector function[88,89], was observed in
309 pregnant compared to non-pregnant women (Fig. 3G). Taken together, these results
310 demonstrate that CD4$^+$ and CD8$^+$ T cells isolated from pregnant women have an increased
311 capacity for proliferation; however, when proliferated, pregnancy-derived T cells show a
312 reduced proportion of cells expressing CD69 and PD-1, suggesting that pregnancy modulates
313 T-cell responses.
314

315 **3.3 Pregnancy does not alter peripheral lymphocyte cytotoxicity**

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316 Having observed pregnancy-specific differences in lymphocyte activation status, we
317 wondered whether the cytotoxicity of circulating lymphocytes would differ based on
318 pregnancy status. Cytotoxic lymphocytes directly kill target cells through the release of
319 granules, which represents an important mechanism of defense against viruses and
320 intracellular bacteria[90,91]. Hence, we isolated PBMCs from pregnant and non-pregnant
321 women and incubated them with CFSE-labeled target cells (Fig. 4A). Flow cytometry was
322 used to quantify the number of killed target cells (Fig. 4B). No significant differences were
323 found between the pregnant and non-pregnant study groups among the various ratios of
324 PBMCs:target cells evaluated (Fig. 4C), indicating that peripheral lymphocytes from both
325 study groups were able to display comparable cytotoxic activity.

326

327 **3.4 B-cell activation following IgM/IgG stimulation is increased in pregnancy**

328 After evaluating functional differences in peripheral T cells in the context of
329 pregnancy, we next focused on the second cellular component of adaptive immunity, B
330 cells[66]. During gestation, B cells are necessary for immune regulation and the promotion of
331 humoral immunity[66], including the production of protective antibodies against paternal
332 antigens[92,93]. However, the pregnancy-specific cellular responses exhibited by circulating B
333 cells require further investigation. Hence, PBMCs were isolated from pregnant and non-
334 pregnant women to evaluate differences in B-cell phenotypes as well as B-cell functionality
335 (Fig. 5A). First, flow cytometry was used to evaluate differences in B-cell phenotypes
336 following the gating strategy presented in Supplemental Fig. 2. Several B-cell subsets
337 displayed distinct modulation in pregnant compared to non-pregnant women (Fig. 5B). The
338 proportion of memory-like $CD27^{+}IgG^{+}$ B cells (Fig. 5C) was found to be elevated during
339 pregnancy, as was the proportion of B cells with an activated $CD38^{+}CD24^{-}$ phenotype (Fig.
340 5D). In contrast, the proportion of B cells displaying a $CD40^{+}CD138^{-}$ phenotype was found

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341 to be increased in non-pregnant women compared to the pregnant study group (Fig. 5E).
342 Next, PBMCs were stimulated with anti-human IgM and IgG, and then flow cytometry was
343 utilized to quantify downstream B-cell receptor activation (Fig. 5F). Consistent with the
344 increased proportion of B cells displaying a $CD38^+$ activated phenotype, we found that there
345 was a significantly higher fold-change in activation following anti-human IgM/IgG
346 stimulation by B cells isolated from pregnant compared to non-pregnant women (Fig. 5G).
347 This finding suggests that pregnancy enhances circulating B-cell responses.

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348 **4. DISCUSSION**

349 Herein, we evaluated the phenotypes and functions of peripheral T and B cells in
350 pregnant compared to non-pregnant women, as these adaptive immune cells play a critical
351 role in maintaining maternal-fetal tolerance[63,94-99]. First, we showed that pregnancy is
352 associated with modestly enhanced basal activation of peripheral $CD4^+$ T cells. Interestingly,
353 both $CD4^+$ and $CD8^+$ T cells derived from pregnant women showed increased activation-
354 induced proliferation; yet, a reduced proportion of these cells expressed markers of activation
355 compared to T cells from non-pregnant women. No differences were observed in peripheral
356 lymphocyte cytotoxicity between the study groups. Finally, a greater proportion of B cells
357 from pregnant women displayed memory-like and activated phenotypes, and such cells
358 exhibited higher activation following stimulation. Taken together, these data may reflect
359 generalized T- and B-cell activation in pregnancy, with a restricted T-cell responsiveness to
360 stimulation that can foster systemic maternal-fetal tolerance.

361 We observed a pregnancy-specific increase in the basal proportion of activated
362 peripheral $CD4^+$ T cells, as indicated by expression of the early activation marker, CD69. In
363 line with this finding, a higher baseline proportion of $CD4^+CD69^+$, but not $CD8^+CD69^+$, T
364 cells has been reported in C57BL/6 mice in late pregnancy relative to non-pregnant mice[100].
365 We also detected a modest pregnancy-specific increase in the proportion of peripheral $CD4^+$
366 T cells co-expressing CD69 and PD-1, which is typically regarded as a co-inhibitory
367 receptor[83,101,102]. Yet, PD-1 expression is upregulated within 24 - 48 hours of T-cell
368 activation[86], potentially as a mechanism to limit excessive responses and tissue damage[87].
369 Thus, the co-expression of CD69 and PD-1 likely indicates prolonged T-cell activation, as
370 would be expected following chronic antigen exposure. Considering the presence of fetal
371 antigens in the maternal circulation[53,103,104], it is tempting to suggest that the increased
372 proportion of $CD4^+CD69^+PD\text{-}1^+$ T cells in pregnant women may reflect repeated exposure to

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373 such fetal antigens[103-106]. Indeed, prior studies in mice have demonstrated that innate immune
374 cells in the periphery interact with fetal antigens throughout pregnancy, which was replicated
375 *in vitro* using human innate immune cells from the second and third trimester[104].
376 Furthermore, cell-free fetal DNA (cffDNA) concentrations have been shown to increase in
377 the maternal circulation in late gestation, which coincides with a pro-inflammatory shift in
378 maternal immunity prior to parturition[107-110]. Specifically, cffDNA has been demonstrated to
379 stimulate a monocyte response in the third trimester that is capable of activating bystander T
380 cells[110]. Moreover, phenotyping and omics studies have provided evidence of T-cell
381 activation that occurs during labor[19,21,23,111], and T-cell responses in late pregnancy have been
382 associated with the increased expression of activation markers[112-114]. In support of these
383 concepts, the samples herein were obtained from pregnant women in late gestation and close
384 to delivery. Of note, parity information was not available for the control/non-pregnant study
385 participants, so analyses accounting for both pregnancy status and parity were not performed.
386 Collectively, these data suggest the possibility that the presence of or increases in the
387 circulating concentrations of fetal antigens and cffDNA may contribute to the modest
388 increase in basal activation of peripheral $CD4^+$ T cells observed in pregnancy.

389 Herein, we also found that both $CD4^+$ and $CD8^+$ T cells from pregnant women
390 displayed greater proliferation in response to *in vitro* stimulation than those from non-
391 pregnant women. In support of this finding, increased proliferation of $CD4^+$ and $CD8^+$ T cells
392 as a function of pregnancy has also been reported in mice[115]. One of the most prominent
393 findings in the current study was that, in contrast to the baseline differences in T-cell subset
394 composition, the proportions of proliferated $CD4^+$ and $CD8^+$ T-cells expressing populations
395 the activation markers CD69 and PD-1 were reduced in pregnant women. In mice, an
396 increase in the proliferation of both $CD4^+$ and $CD8^+$ pregnancy-derived T cells following the
397 blockade of PD-1 has been reported[115]; therefore, the reduced proportion of peripheral T cells

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398 expressing PD-1 in pregnant women may have contributed to the higher proliferation of
399 pregnancy-derived $CD4^+$ and $CD8^+$ T cells observed in this study. PD-1 is well-studied for its
400 role in cancer and the therapeutic potential of inhibiting this pathway[116,117], and the co-
401 expression of PD-1 and CD69 has been reported in activated $CD4^+$ and $CD8^+$ T[118] and NK[119]
402 cells isolated from cancer patients. Of note, CD69 has also been demonstrated to play a role
403 in immune[120,121] and metabolic[122,123] regulation, indicating it may be more than just a marker
404 of activation[124]. Yet, additional experiments are needed to define the functional implications
405 of this phenotype in the context of pregnancy.

406 In this regard, increased expression of CD69 by peripheral T cells has been described
407 in patients with a history of recurrent spontaneous abortion[125,126], and both basal and
408 stimulated CD69 expression were higher in women with miscarriage than in those with
409 normal pregnancy[126]. Furthermore, increased CD69 expression by peripheral $CD8^+$ T cells
410 has been reported in patients with cardiac[127] and renal[128] allograft rejection, and thus
411 proposed as a biomarker for transplant rejection. Collectively, these studies suggest that the
412 strong upregulation of this activation marker in response to a stimulus can indicate adverse
413 consequences for pregnancy. Indeed, the *in vivo* activation of T cells using an anti-CD3ε
414 antibody in late and mid pregnancy has been shown to cause systemic inflammation and
415 preterm labor and birth[19] as well as pregnancy loss (Gomez-Lopez et al., unpublished data),
416 respectively. In this murine model, the systemic inflammatory response also extended to the
417 amniotic cavity and resulted in fetal growth restriction[19], indicating that the systemic over-
418 activation of maternal T cells in pregnancy can be detrimental to the fetus. Thus, the lower
419 proportion of pregnancy-derived $CD69^+PD\text{-}1^+$ peripheral T cells following stimulation
420 observed herein may indicate a higher threshold for T-cell activation as a mechanism to
421 preserve systemic immune homeostasis.

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422 In addition to the protective mechanism proposed above, the diminished activation of
423 circulating maternal T cells observed in the current study may also allow them to retain
424 memory and proliferative functions for a longer duration[49,129]. This concept is in line with our
425 finding that a lower proportion of terminally differentiated effector memory cells was
426 observed in proliferated T cells from pregnant compared to non-pregnant women. Terminally
427 differentiated $CD8^+$ effector memory T cells display greater effector functions but lower
428 memory and proliferative capabilities and are considered to be short-lived[130-133]. The reduced
429 proportion of T cells expressing activation and terminal effector memory phenotypes
430 following stimulation may reflect a more stringent use of effector functions by T cells during
431 pregnancy. That is, we observed fewer T cells to be activated or terminally differentiated
432 following stimulation in the context of pregnancy, which could reflect a diminished tendency
433 to display effector functions by this peripheral T cell population.

434 In line with the above concept, it is reasonable to propose that maternal peripheral T-
435 cell responses are controlled in an antigen-specific manner[68], which could be a useful feature
436 for avoiding unnecessary T-cell activation that could adversely affect pregnancy. Herein, we
437 utilized a form of T-cell stimulation that bypasses antigen recognition to directly stimulate
438 the T-cell and costimulatory receptors. Yet, prior in vitro studies evaluating the response to
439 influenza A viral stimulation in PBMCs have demonstrated a pregnancy-specific attenuation
440 of the release of pro-inflammatory cytokines such as IFNα[134,135] and IL-2[135]. On the other
441 hand, we have recently shown an increase in the proportions of pro-inflammatory T-cell
442 subsets, such as Th1 and Tc17, in pregnant women with SARS-CoV-2 infection relative to
443 healthy controls[136]. Together, these data suggest that stimulation with antigens of differing
444 pathogenicity can elicit distinct T-cell responses in the maternal circulation.

445 In this study, we observed comparable cytotoxic activity by PBMCs from pregnant
446 and non-pregnant women, as has been reported previously[137]. In the periphery, both T and

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447 NK cells are capable of cytotoxic activity[138], and T cells have been reported to be more
448 prevalent in the maternal periphery[139]. However, the assay used herein relies on C-type
449 lectin-like receptor NKG2D-mediated cytotoxicity[140]; although both $CD8^+$ T and NK cells
450 express this activation receptor[141], NKG2D signaling in isolation is only sufficient to activate
451 NK cells, as $CD8^+$ T cells require simultaneous stimulation of the T-cell receptor and by
452 cytokines[140]. Despite this limitation, the data demonstrate comparable peripheral lymphocyte
453 cytotoxicity upon exposure to non-self-antigens between pregnant and non-pregnant women.

454 While a large body of work has considered the role of T cells in establishing and
455 maintaining maternal-fetal tolerance, the second cellular component of adaptive immunity –
456 B cells – is also critical to establishing and maintaining tolerance through
457 gestation[31,66,93,142,143]. Prior studies have demonstrated that IgG immunoglobulins contained in
458 maternal serum prevent maternal lymphocytes from mounting a cytotoxic response against
459 cultured trophoblasts[92,144]. Indeed, spontaneous recurrent abortions are characterized by a
460 lack of protective maternal antibodies directed towards paternal HLA antigens[145-148].
461 Protective antibodies bind their antigens with high affinity but are unable to initiate
462 downstream immune responses such as complement activation and cytotoxicity[149]. In
463 contrast, natural or autoantibodies, which are produced by B1a cells[66,150,151], are associated
464 with a range of obstetrical complications including intrauterine fetal demise and
465 preeclampsia[152-155]. Accordingly, the circulating proportion of B1, but not B2, cells has been
466 reported to decrease throughout gestation[30], and B-cell subset composition at the maternal-
467 fetal interface is altered by the process of labor, preterm birth, or chronic histologic
468 chorioamnionitis[31]. Herein, we considered alterations in peripheral B-cell subset composition
469 as a function of pregnancy itself, and report increased frequencies of memory-like
470 $CD27^+IgG^+$ B cells and activated $CD38^+CD24^-$ B cells in pregnant women. Notably, the latter
471 finding is consistent with the observed greater responses to *in vitro* IgM/IgG stimulation in

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472 pregnancy-derived B cells, given that CD38 ligation has been linked to Bruton tyrosine
473 kinase (BTK) phosphorylation[156]. Higher median peripheral concentrations of B cell
474 activating factor (BAFF) have been reported in pregnant compared to non-pregnant women,
475 suggesting that BAFF may prime B cells during pregnancy and thus contribute to the
476 pregnancy-specific increase in activation displayed by these cells[157]. In this study, we showed
477 that peripheral B cells display a heightened response to stimulation during gestation, which
478 could provide a more efficient cellular immune response to insults.

479 Collectively, the results presented herein indicate that maternal circulating T cells and
480 B cells display specific responses during pregnancy. Pregnancy-derived T cells show higher
481 basal activation and greatly increased proliferative capacity; yet, such proliferated T cells
482 resist signs of prolonged activation displayed by their non-pregnant counterparts. Moreover,
483 B cells isolated from pregnant women display greater basal proportions of memory-like and
484 activated phenotypes and exhibit higher activation following stimulation. These findings
485 indicate that maternal circulating T cells and B cells display distinct responses during
486 pregnancy, and suggest that maternal peripheral T cells are capable of responding to potential
487 threats but are more resistant to aberrant activation, thereby preventing a systemic
488 inflammatory response that can lead to adverse perinatal consequences.

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915 **FIGURE LEGENDS**

916 **Fig. 1. Comparison of basal T-cell subset composition between non-pregnant and**
917 **pregnant women. (A)** Peripheral blood samples were collected from non-pregnant (n = 25,
918 indicated in blue) and pregnant (n = 18, indicated in red) women to isolate peripheral blood
919 mononuclear cells (PBMCs) for T-cell phenotyping at baseline (day 0). **(B)** Heatmap
920 representation showing the basal proportion of T cells with various immunophenotypes from
921 non-pregnant (indicated in blue) and pregnant (indicated in red) women. The color key
922 indicates the relative proportion of T cells with the various immunophenotypes considered,
923 which were not compared among each other. **(C)** Proportion of $CD4^+$ T cells expressing
924 CD69 and **(D)** co-expressing CD69 and PD-1 at baseline from non-pregnant (blue circles)
925 and pregnant (red circles) women. Data are presented as box-and-whisker plots where
926 midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate
927 minimum/maximum ranges. *$p < 0.05$.

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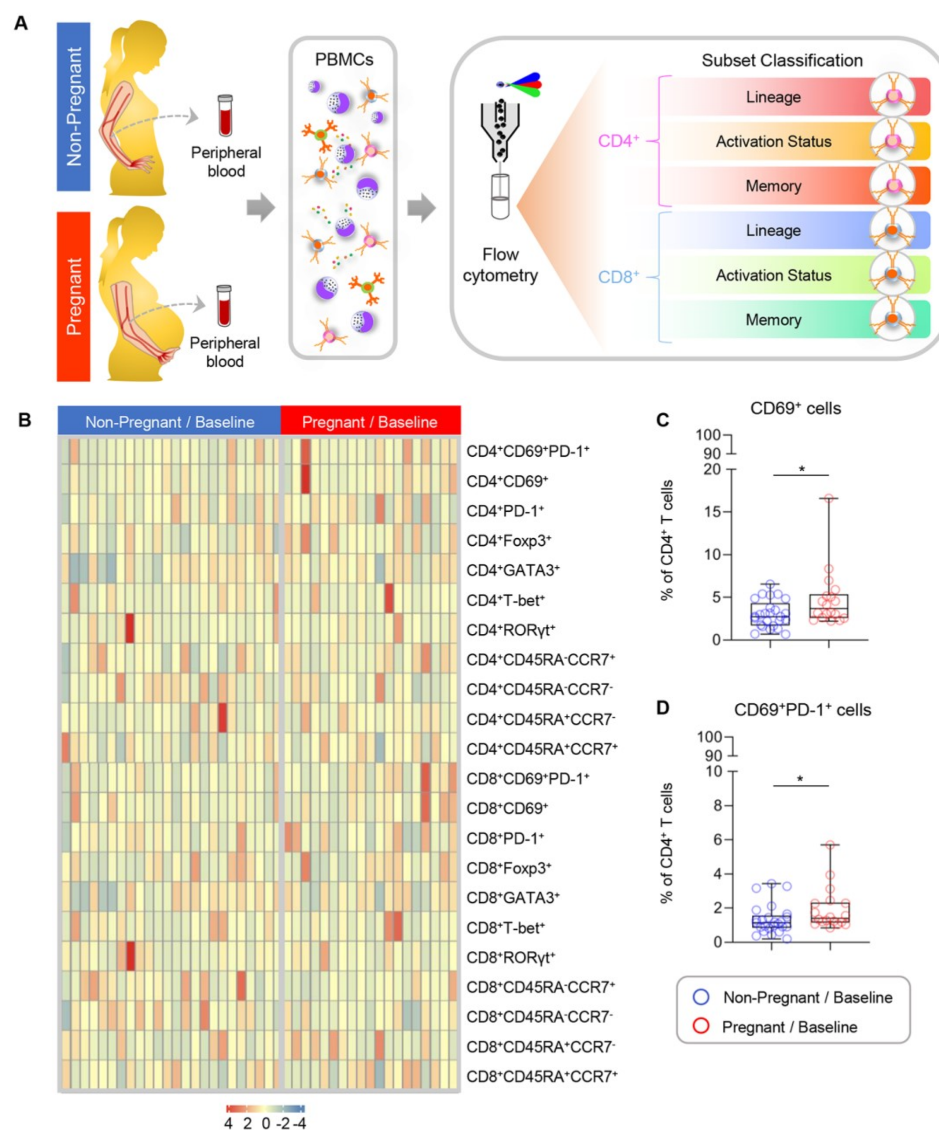

928

929 **Fig. 2. Comparison of CD4$^+$ T-cell proliferation between non-pregnant and pregnant**

930 **women. (A)** Peripheral blood samples were collected from non-pregnant (n = 25, indicated in

931 blue) and pregnant (n = 20, indicated in red) women to isolate peripheral blood mononuclear

932 cells (PBMCs) for *in vitro* stimulation with anti-CD3/anti-CD28 and recombinant human IL-

933 2. Cells were cultured for 6 days prior to phenotyping. Controls were cultured in parallel

934 without stimulation. **(B)** Heatmap representation showing the proportion of CD4$^+$ T cells with

935 various immunophenotypes from non-pregnant (indicated in blue) and pregnant (indicated in

936 red) women with (stimulated) or without (control) stimulation. The color key indicates the

937 relative proportion of T cells with the various immunophenotypes considered, which were not

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938 compared among each other. **(C)** Absolute number of $CD4^+$ T cells in control and
939 proliferated samples from non-pregnant (blue symbols) and pregnant (red symbols) women.
940 **(D-F)** Proportion of proliferated $CD4^+$ T cells with the phenotype of **(D)** CD4+CD69+, **(E)**
941 $CD4^+PD\text{-}1^+$, and **(F)** $CD4^+CD69^+PD\text{-}1^+$. Data are presented as box-and-whisker plots where
942 midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate
943 minimum/maximum ranges. Each dot represents the mean of three biological replicates per
944 sample. Grey lines and asterisks represent within-group differences between control and
945 stimulated samples (i.e., pregnant control vs. pregnant stimulated), while black lines and
946 asterisks represent significant differences between groups after stimulation (i.e., pregnant
947 stimulated vs. non-pregnant stimulated). *$p < 0.05$; ***$p < 0.001$.

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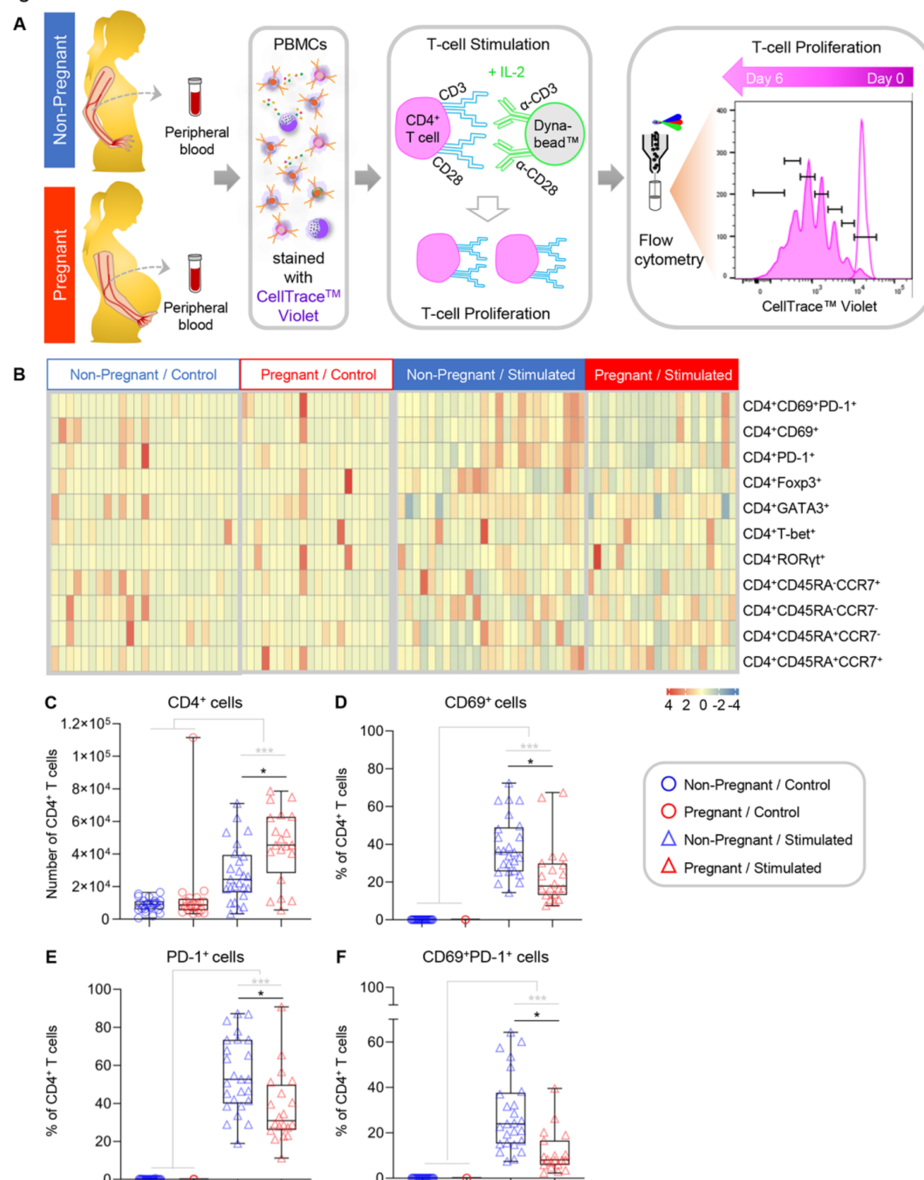

948

949 **Fig. 3. Comparison of CD8^{+} T-cell proliferation between non-pregnant and pregnant**

950 **women. (A)** Peripheral blood samples were collected from non-pregnant (n = 25, indicated in

951 blue) and pregnant (n = 20, indicated in red) women to isolate peripheral blood mononuclear

952 cells (PBMCs) for *in vitro* stimulation with anti-CD3/anti-CD28 and recombinant human IL-

953 2. Cells were cultured for 6 days prior to phenotyping. Controls were cultured in parallel

954 without stimulation. **(B)** Heatmap representation showing the proportion of CD8^{+} T cells with

955 various immunophenotypes from non-pregnant (indicated in blue) and pregnant (indicated in

956 red) women with (stimulated) or without (control) stimulation. The color key indicates the

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957 relative proportion of T cells with the various immunophenotypes considered, which were not
958 compared among each other. **(C)** Absolute number of $CD8^+$ T cells in control and
959 proliferated samples from non-pregnant (blue symbols) and pregnant (red symbols) women.
960 **(D-G)** Proportion of proliferated $CD8^+$ T cells with the phenotype of **(D)** $CD8^+CD69^+$, **(E)**
961 $CD8^+PD\text{-}1^+$, **(F)** $CD4^+CD69^+PD\text{-}1^+$, and **(G)** $CD8^+CD45RA^+CCR7^-$ (terminal effector
962 memory). Data are presented as box-and-whisker plots where midlines indicate medians,
963 boxes indicate interquartile ranges, and whiskers indicate minimum/maximum ranges. Each
964 dot represents the mean of three biological replicates per sample. Grey lines and asterisks
965 represent within-group differences between control and stimulated samples (i.e., pregnant
966 control vs. pregnant stimulated), while black lines and asterisks represent significant
967 differences between groups after stimulation (i.e., pregnant stimulated vs. non-pregnant
968 stimulated). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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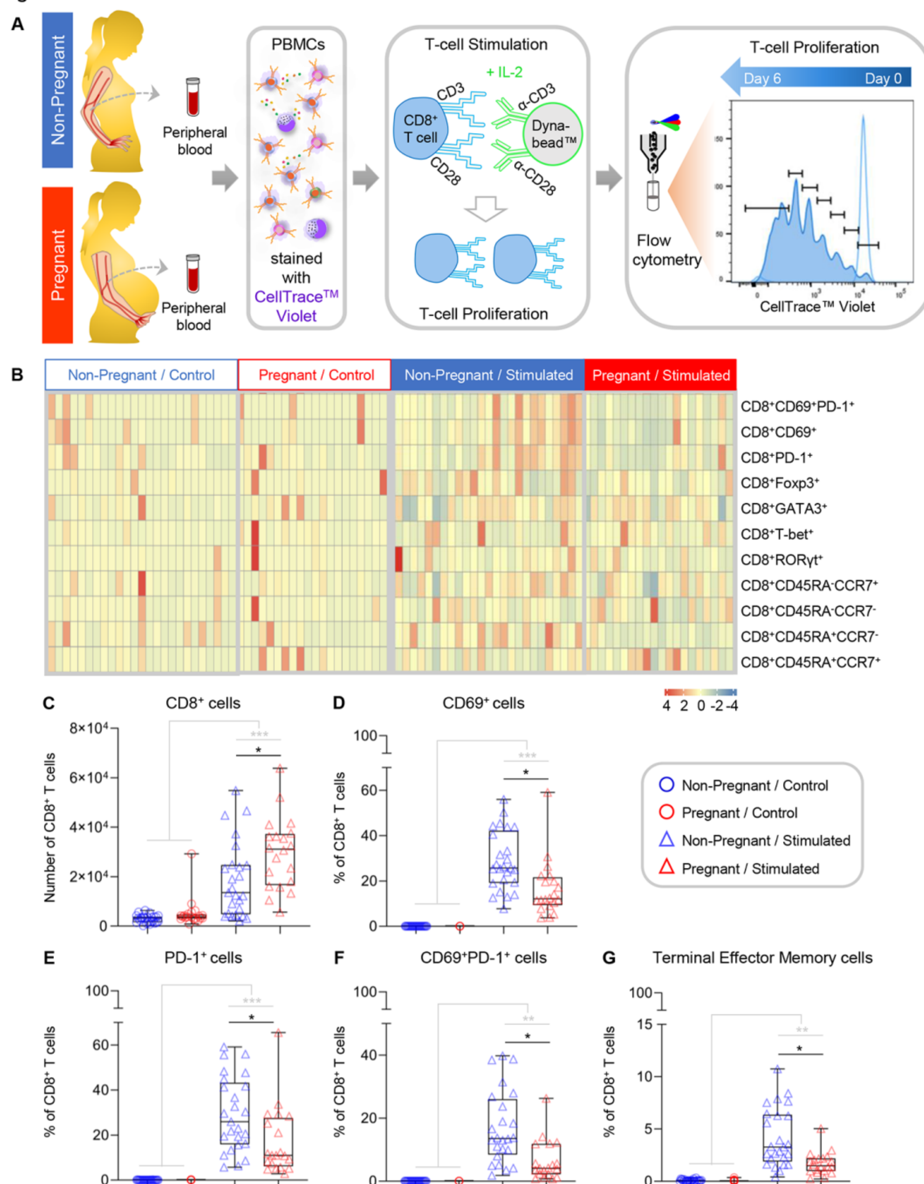

969

970 **Fig. 4. Comparison of lymphocyte cytotoxicity between non-pregnant and pregnant**
971 **women. (A)** Peripheral blood samples were collected from non-pregnant (n = 21, indicated in
972 blue) and pregnant (n = 17-20, indicated in red) women to isolate peripheral blood
973 mononuclear cells (PBMCs) for *in vitro* culturing with CFSE-labeled target cells. **(B)** Flow
974 cytometry gating strategy used to identify killed target cells ($CFSE^+7AAD^+$), live target cells
975 ($CFSE^+7AAD^-$), and live PBMCs ($CFSE^-7AAD^-$). **(C)** Percentage of target cells killed
976 (calculated as [$CFSE^+7AAD^+$ / ($CFSE^+7AAD^+$ + $CFSE^+7AAD^-$) * 100]) among ratios of
977 PBMCs:target cells ranging from 0:1 to 50:1 in non-pregnant (blue circles) and pregnant (red

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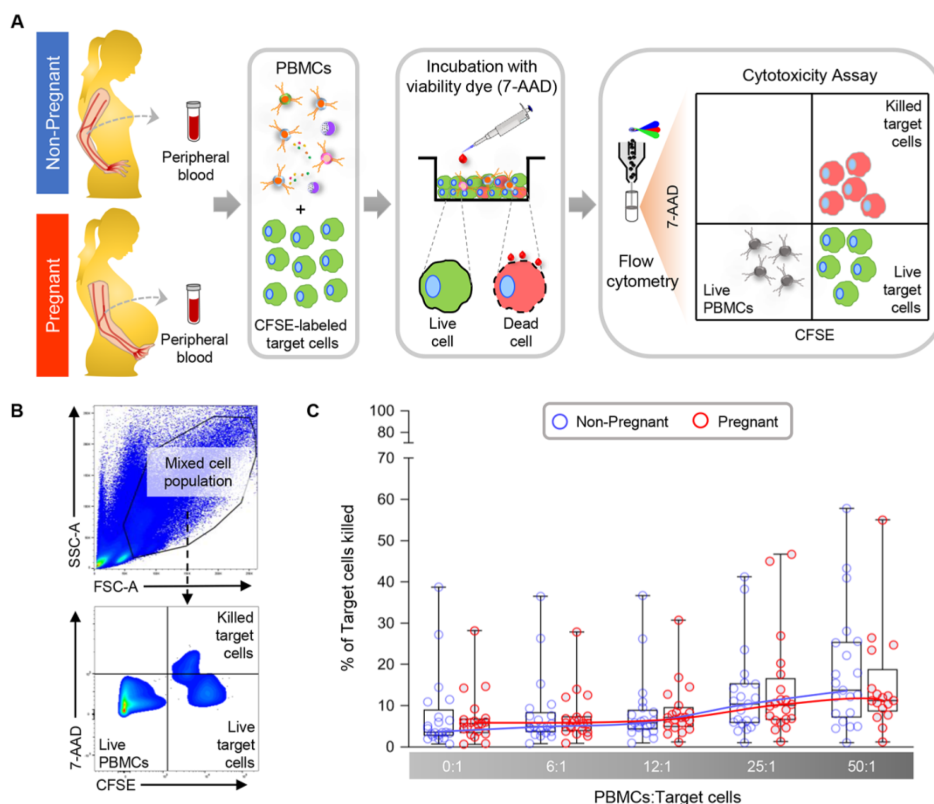
978 circles) women. Data are presented as box-and-whisker plots where midlines indicate
979 medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum
980 ranges. Trend lines for each study group are included.



981

982 **Fig. 5. Comparison of B-cell subset composition and activation between non-pregnant**
983 **and pregnant women. (A)** Peripheral blood samples were collected from non-pregnant (n =
984 25, indicated in blue) and pregnant (n = 19, indicated in red) women to evaluate B-cell
985 phenotypes and activation following anti-human IgM/IgG stimulation. **(B)** Heatmap
986 representation showing the basal proportion of B cells with various immunophenotypes from
987 non-pregnant (indicated in blue) and pregnant (indicated in red) women. The color key
988 indicates the relative proportion of T cells with the various immunophenotypes considered,
989 which were not compared among each other. **(C-E)** Proportion of B cells with the phenotype
990 **(C)** CD19+CD20+CD27^{+}IgG^{+}, **(D)** CD19+CD20+CD38^{+}CD24^{-}, and **(E)**
991 CD19+CD20+CD40^{+}CD138^{-} from non-pregnant (red circles) and pregnant (blue circles)

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992 women. **(F)** Representative flow cytometry gating for B-cell activation assay: viable B cells
993 were identified as $CD19^+$, and then B-cell activation in control (open histograms) and
994 stimulated (filled histograms) samples from non-pregnant (indicated in blue) and pregnant
995 (indicated in red) women was determined as described in the Methods. **(G)** Fold change in B-
996 cell activation in non-pregnant (blue triangles) and pregnant (red triangles) samples after anti-
997 human IgM/IgG stimulation, calculated as the adjusted MFI of IgM/IgG-stimulated samples
998 divided by the adjusted MFI of control samples. Fold changes <1 were considered as "no
999 change" and assigned a value of 1. Data are presented as box-and-whisker plots where
1000 midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate
1001 minimum/maximum ranges. *$p < 0.05$; **$p < 0.01$.
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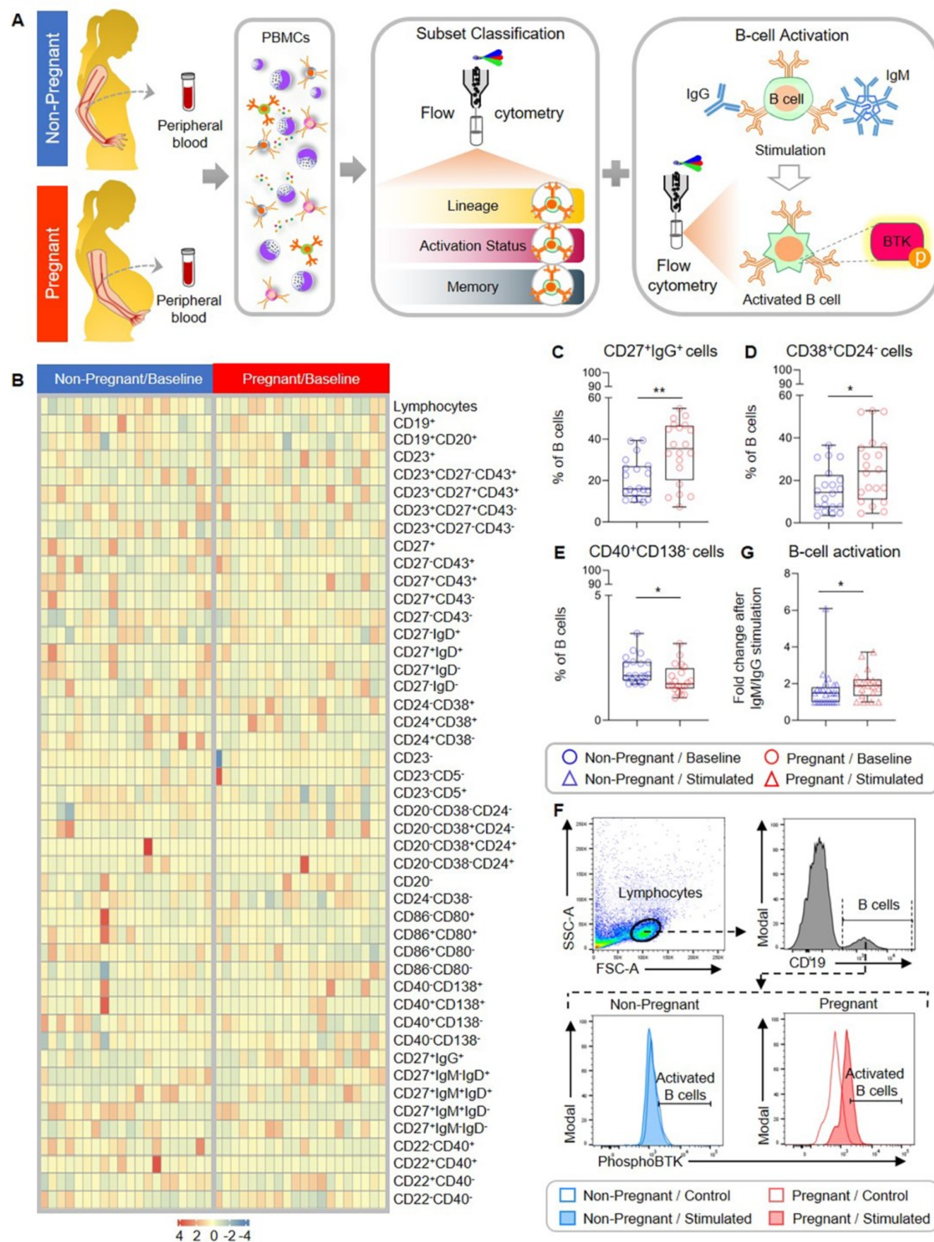

1004

1005 Maternal peripheral T cells and B cells display distinct responses during pregnancy.

1006 Pregnancy drives enhanced activation and proliferative capacity of T cells; yet, these cells

1007 exhibit diminished activation in response to stimulation. Moreover, pregnancy is

1008 accompanied by increased memory-like and activated B cells.

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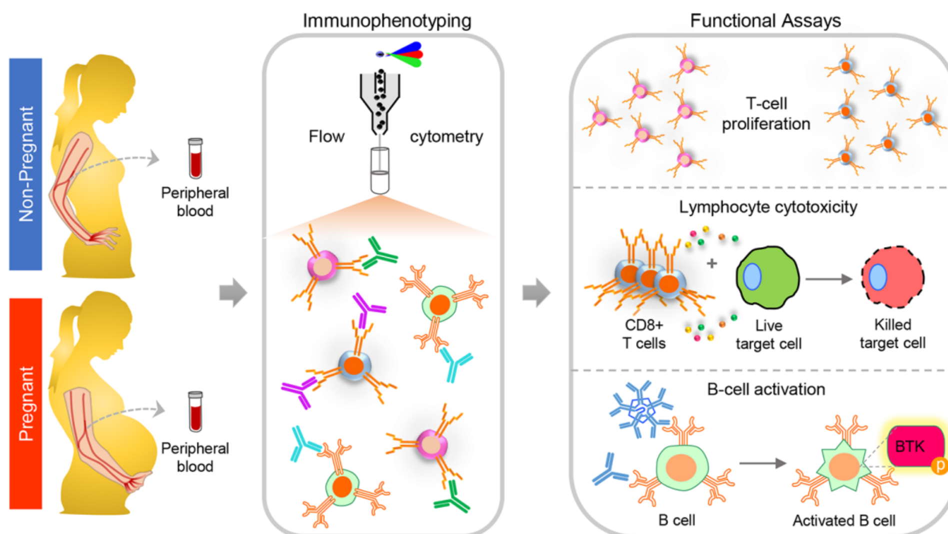

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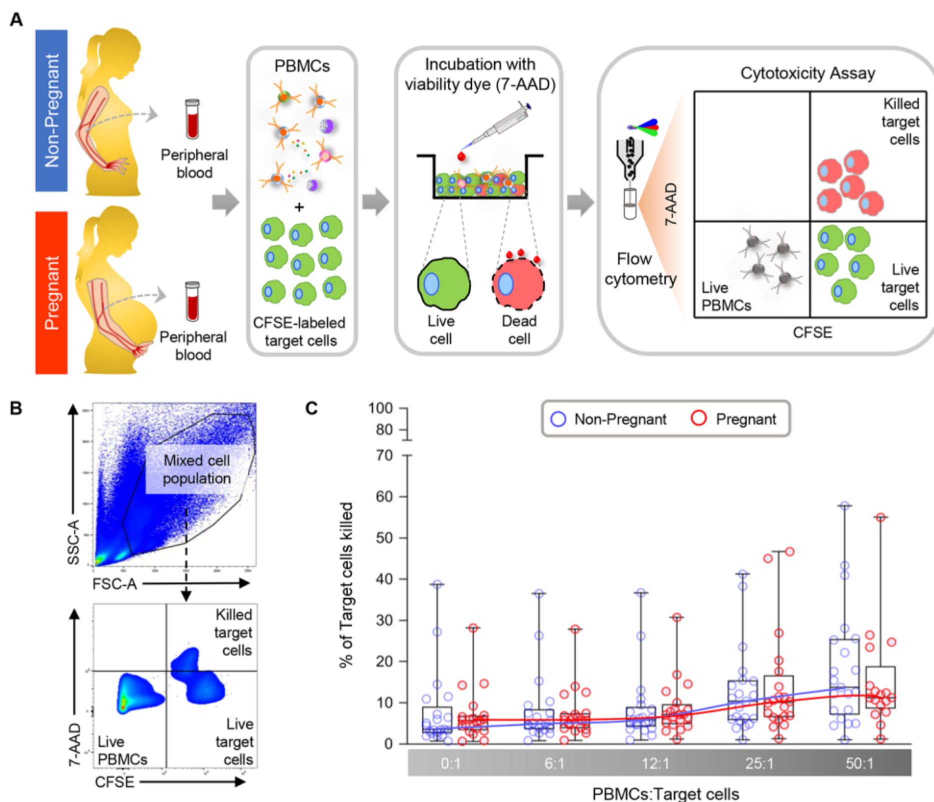
Figure 4



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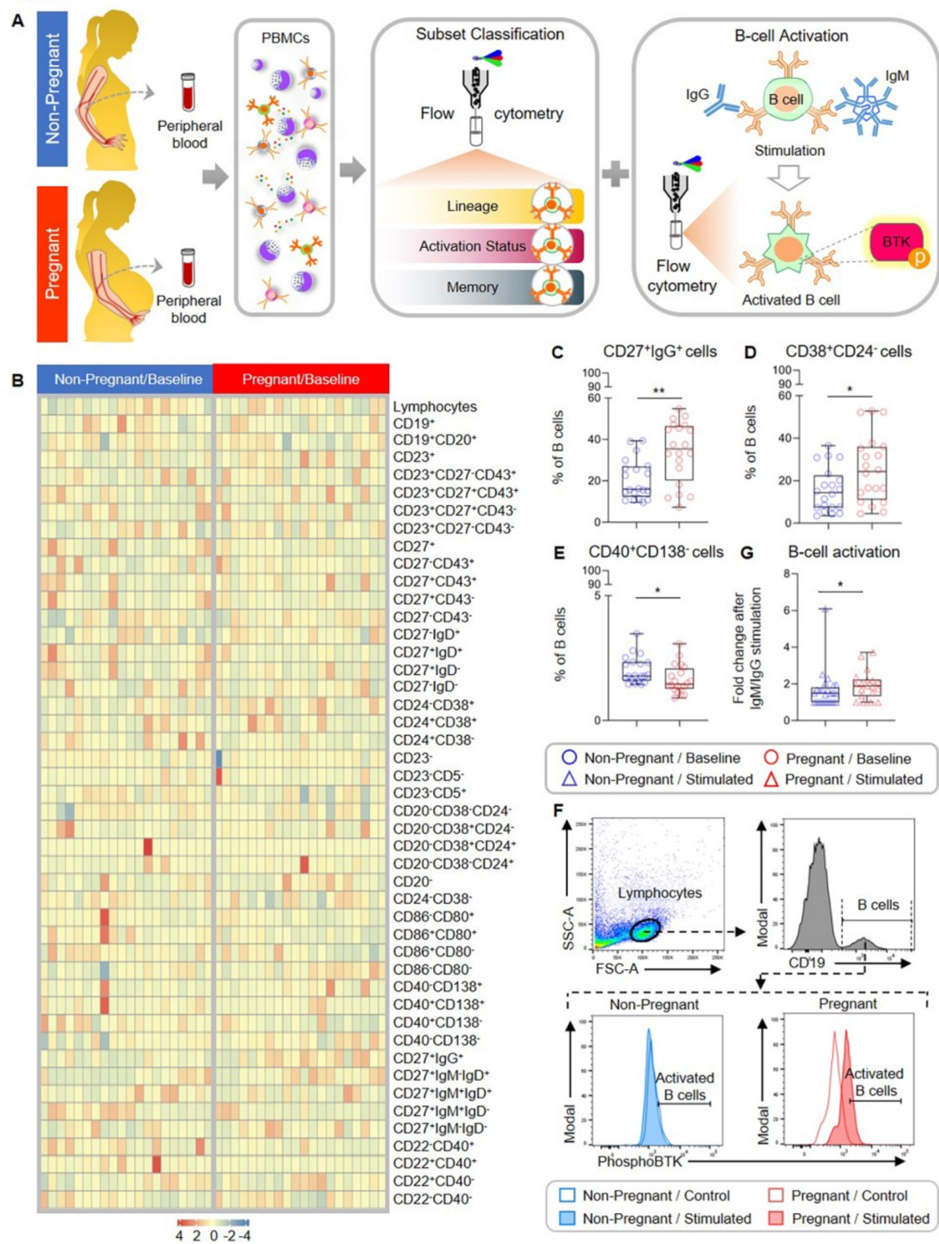

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