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ORIGINAL ARTICLE

Pregnancy imparts distinct systemic adaptive immune function

Catherine Demery-Poulos[1,2] | Roberto Romero[1,3,4,5,6] | Yi Xu[1,2] | Marcia Arenas-Hernandez[1,2] | Derek Miller[1,2] | Li Tao[1,2] | Jose Galaz[1,2,7] | Marcelo Farias-Jofre[1,2,7] | Gaurav Bhatti[1,2] | Valeria Garcia-Flores[1,2] | Megan Seyerle[8] | Adi L. Tarca[1,2,9] | Nardhy Gomez-Lopez[1,2,10]

[1]Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services (NICHD/NIH/DHHS), Bethesda, Maryland, and Detroit, Detroit, Michigan, USA

[2]Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Michigan, USA

[3]Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, Michigan, USA

[4]Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, Michigan, USA

[5]Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan, USA

[6]Detroit Medical Center, Detroit, Michigan, USA

[7]Division of Obstetrics and Gynecology, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile

[8]Wayne State University School of Medicine, Detroit, Michigan, USA

[9]Department of Computer Science, Wayne State University College of Engineering, Detroit, Michigan, USA

[10]Department of Biochemistry, Microbiology, and Immunology, Wayne State University School of Medicine, Detroit, Michigan, USA

Correspondence
Nardhy Gomez-Lopez, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, 275 E. Hancock, Detroit, MI 48201, USA.
Email: nardhy.gomez-lopez@wayne.edu

Abstract

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

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

Results: Pregnancy was associated with modestly enhanced basal activation of peripheral $CD4^+$ T cells. Both $CD4^+$ and $CD8^+$ T cells from pregnant women showed increased activation-induced proliferation; yet, a reduced proportion of these cells expressed activation markers compared to non-pregnant women. There were no differences in peripheral lymphocyte cytotoxicity between study groups. A greater

proportion of B cells from pregnant women displayed memory-like and activated phenotypes, and such cells exhibited higher activation following stimulation.

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

KEYWORDS
adaptive immunity, B cell, cytotoxicity, flow cytometry, maternal circulation, T cell

1 | INTRODUCTION

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

The conventional belief is that circulating T cells are skewed toward a Th2-like phenotype throughout gestation.[32–37] Accordingly, a number of clinical investigations noted that some autoimmune disorders (e.g., multiple sclerosis and rheumatoid arthritis) are temporarily alleviated during pregnancy.[38–46] This suppression also seems to extend to the maternal-fetal interface where multiple protective mechanisms exist to prevent T-cell activation, such as exhaustion or senescence,[47–49] local silencing of T-cell chemotactic signals and trafficking,[50–52] and expansion of regulatory T cells.[53–63] Importantly, single-cell RNA signatures derived from T cells infiltrating the maternal-fetal interface can be tracked in the maternal circulation and may serve as biomarkers for obstetrical disease.[21,23,64] Hence, investigating the functional status of circulating T cells during pregnancy may provide a window on the events taking place at the maternal-fetal interface. Although a large body of research has focused on examining the phenotypes and functions of T cells and B cells throughout gestation,[3,31,65–68] little is known of the potential pregnancy-specific functional differences in such adaptive immune cells. In the current study, we utilized high-dimensional flow cytometry together with functional assays to characterize T-cell and B-cell cellular responses in the periphery of pregnant and non-pregnant women.

2 | METHODS

2.1 | Human subjects and clinical specimens

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

2.2 | Stimulation of T-cell proliferation

Peripheral blood was obtained by venipuncture and collected in Vacutainer K3 EDTA tubes (BD Biosciences, San Jose, CA, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep density gradient (Axis Shield, Oslo, Norway), per manufacturer instructions. Isolated PBMCs were centrifuged at 300 x *g* for 5 min and resuspended in phosphate-buffered saline (PBS) at a density of 1×10^6 cells/mL. Next, PBMCs were stained with 1 μL/mL CellTrace Violet dye (Thermo Fisher Scientific, Life Technologies Corporation, Carlsbad,

CA, USA) for 20 min at 37°C. The staining reaction was quenched by adding complete RPMI 1640 medium (Thermo Fisher Scientific, Life Technologies Limited, Paisley, UK) [enriched with 5% human serum (Sigma-Aldrich, St Louis, MO, USA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific)] and by allowing the suspension to incubate at room temperature (RT) for 2 min. The PBMCs were then centrifuged at 300 x *g* for 5 min, resuspended in complete RPMI 1640 medium, and counted, using ViaStain AOPI Staining Solution (Nexcelom Bioscience, Lawrence, MA, USA) and a Nexcelom Bioscience Cellometer Auto 2000. An aliquot containing 1×10^6 cells was set aside for basal (day 0) immunophenotyping. The remaining cell suspension volume was divided into control and stimulated samples. Control suspensions were treated with 55 μM 2-mercaptoethanol (Life Technologies Corporation, Grand Island, NY, USA); stimulated solutions were treated with 55 μM 2-mercaptoethanol, Dynabeads™ Human T-activator CD3/CD28 (Thermo Fisher Scientific) at a ratio of 1:1 cells:beads and with 2000 U/mL recombinant human IL-2 (BD Biosciences). Each sample was seeded in triplicate, for control and stimulated cells, at a density of 1×10^5 cells per well in a 96-well U bottom plate. The plate was incubated at 37°C with 5% CO_2 for six days.

2.3 | T-cell phenotyping for basal and proliferated samples

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

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

2.4 | Peripheral lymphocyte cytotoxicity assay

Target K-562 cells (ATCC, Manassas, VA, USA) – myelogenous leukemia cells that lack MHC class I and II expression[69–71] – were cultured in complete RPMI 1640 medium [enriched with 10% fetal bovine serum and 1% Penicillin-Streptomycin], collected, centrifuged at 300 x *g* for 5 min, and resuspended in PBS. Next, cells were incubated with 1 μL/mL carboxyfluorescein diacetate succinimidyl ester (CFSE; Thermo Fisher Scientific) at 37°C with 5% CO_2 for 20 min. To stop the reaction, complete RPMI 1640 medium was added and the suspension was incubated at RT for 2 min. The cells were resuspended in complete RPMI 1640 medium and counted, using ViaStain AOPI Staining Solution and a Nexcelom Bioscience Cellometer Auto 2000.

Peripheral blood was obtained by venipuncture and collected in Vacutainer K3 EDTA tubes. PBMCs were isolated by Lymphoprep density gradient, per manufacturer instructions. Target (K-562) cells and PBMCs were mixed in sterile FACS tubes in the following ratios (PBMCs:target cells): 0:1 6:1, 12:1, 25:1, and 50:1. The resulting cell suspensions were centrifuged at 300 x *g* for 5 min, resuspended in complete RPMI 1640 culture medium, and transferred to a 96-well U-bottom plate. The plate was centrifuged at 100 x *g* for 2 min and then incubated at 37°C with 5% CO_2 for 4 h. Following incubation, cell suspensions were transferred to FACS tubes, diluted with PBS, and centrifuged at 300 x *g* for 5 min. Cell pellets were resuspended in PBS and incubated with 1 μL/mL 7-aminoactinomycin D (7-AAD; Thermo Fisher Scientific) in the dark at 4°C for 15 min. Cell suspensions were centrifuged at 300 x *g* for 5 min and resuspended in 0.5 mL of stain buffer for analysis by flow cytometry.

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

2.5 | B-cell phenotyping

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

CountBright absolute counting beads were added prior to analysis. Flow cytometry acquisition was performed on a BD LSRFortessa flow cytometer, using FACSDiva software version 6.0. The analysis was performed and the figures were created by using FlowJo software version 10. B-cell subsets were identified based on the gating strategy presented in Supplemental Figure 2.

2.6 | B-cell activation assay

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

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

2.7 | Statistical analysis

Statistical analyses for baseline T-cell phenotyping, stimulated and control proliferated T-cell phenotyping, and B-cell phenotyping were performed by using the R statistical programming language. Linear mixed-effects models[72] were fit for the comparison of stimulated and control T-cell flow cytometry data and between study groups to account for repeated measurements. The data obtained by flow cytometry were modeled as proportions. For T-cell baseline (day 0) phenotyping and B-cell phenotyping, the proportion of cells with a given phenotype was compared between pregnant and non-pregnant study groups, and a p-value < .05 was considered statistically significant. For T-cell proliferated (day 6) phenotyping, involving interactions between control and stimulated samples within both study groups, a false discovery rate-adjusted p-value[73] (q-value) < .05 was considered statistically significant. For heatmap representations of immunophenotyping results, flow cytometry data were transformed into Z-scores by subtracting the mean and dividing by the standard deviation. Of note, the heatmaps were generated to display the proportion of cells with a given phenotype in pregnant vs. non-pregnant women, which included control and stimulated samples for the T-cell proliferation analyses. Phenotypes listed in the heatmap were thus not statistically compared to one another. Statistical analyses for PBMC cytotoxicity and B-cell activation were performed by using the Shapiro-Wilk test for normality followed by the Mann-Whitney U-test and GraphPad Prism software version 9.0.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). A p-value < .05 was considered statistically significant.

3 | RESULTS

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

Pregnancy includes the selective modulation of the adaptive immune system at the maternal-fetal interface[19,48,49,52,62,74–78] and in the periphery.[18,20,21,23,79] Therefore, we first sought to uncover differences in the systemic baseline (day 0) T-cell subset composition as a function of pregnancy. PBMCs were isolated from non-pregnant and pregnant women for phenotyping via flow cytometry (Figure 1A), using the gating strategy presented in Supplemental Figure 1. The relative proportions of $CD4^+$ and $CD8^+$ T cells with the characterized phenotypes in each patient are presented in Figure 1B. While there were no pregnancy-specific differences in the proportions of total $CD4^+$ or $CD8^+$ T cells, pregnancy was associated with a significantly higher basal proportion of $CD4^+$ T cells expressing the early activation marker, CD69 (Figure 1C),[80,81] although the effect size was small. In addition, a significantly higher proportion of cells co-expressed CD69 and the co-inhibitory receptor PD-1[82–84] among $CD4^+$ T cells isolated from pregnant compared to non-pregnant women (Figure 1D). The co-expression of CD69 and PD-1 is likely to be indicative of prolonged T-cell activation.[85,86] These data suggest that pregnancy is associated with a modest enhancement in the baseline activation of peripheral $CD4^+$ T cells.

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

Given the baseline differences in peripheral T-cell activation between non-pregnant and pregnant women, we next considered whether pregnancy was associated with altered function, including proliferative capacity, of such cells. Accordingly, PBMCs isolated from pregnant and non-pregnant women were stimulated with anti-CD3/anti-CD28 and rhIL-2 to assess pregnancy-specific differences in $CD4^+$ (Figure 2A) and $CD8^+$ (Figure 3A) T-cell proliferation. As expected, significant changes in subset proportions (Figure 2B&3B) and absolute numbers (Figure 2C&3C) were observed in T cells derived from non-pregnant and pregnant women following stimulation (Extended Dataset 1); here, we focused on the phenotypic and functional differences between study groups. Both $CD4^+$ (Figure 2C) and $CD8^+$ T cells (Figure 3C) had a significantly higher proliferative capacity, as determined by

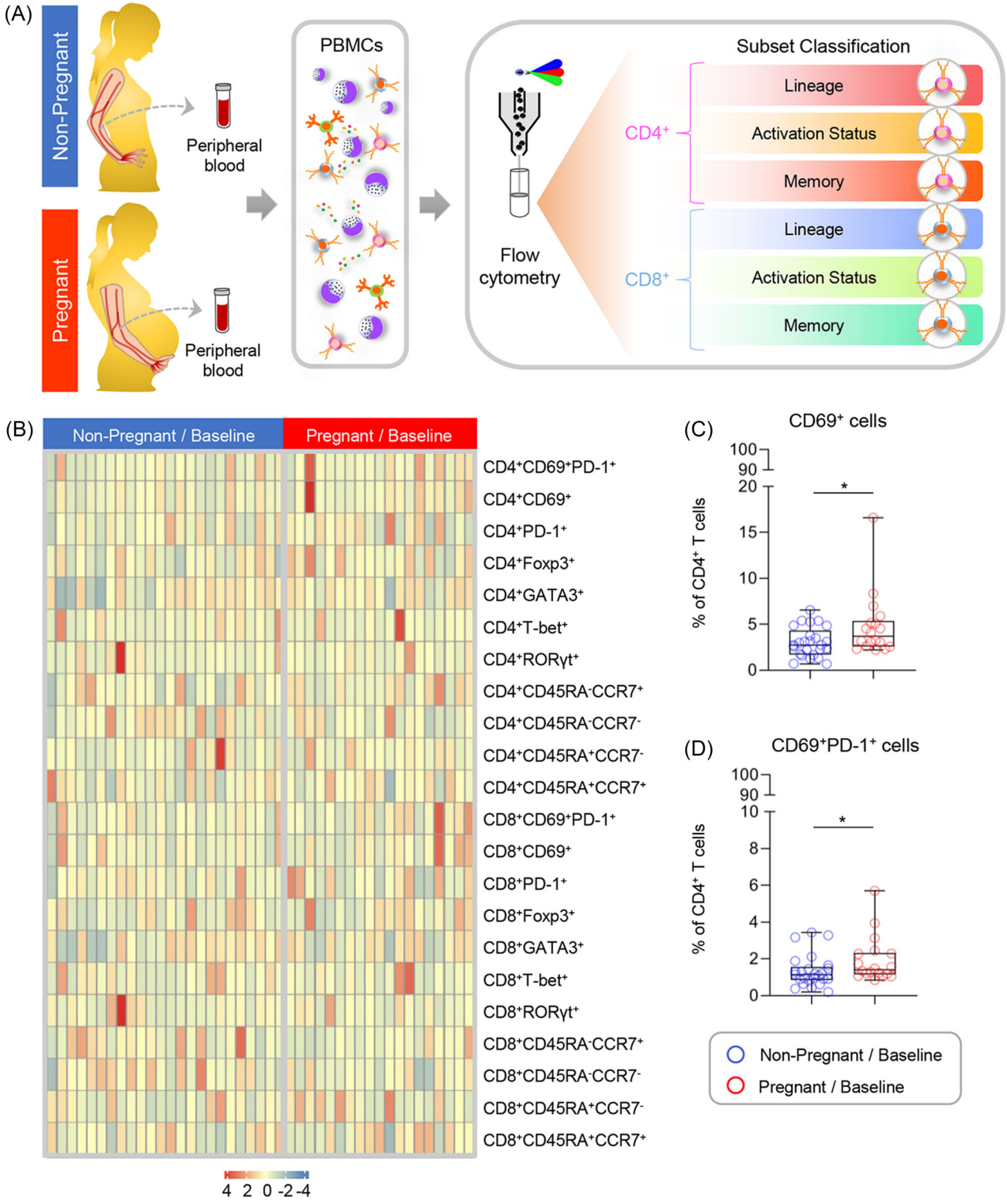

FIGURE 1 Comparison of basal T-cell subset composition between non-pregnant and pregnant women. (A) Peripheral blood samples were collected from non-pregnant (n = 25, indicated in blue) and pregnant (n = 18, indicated in red) women to isolate peripheral blood mononuclear cells (PBMCs) for T-cell phenotyping at baseline (day 0). (B) Heatmap representation showing the basal proportion of T cells with various immunophenotypes from non-pregnant (indicated in blue) and pregnant (indicated in red) women. The color key indicates the relative proportion of T cells with the various immunophenotypes considered, which were not compared among each other. (C) Proportion of $CD4^+$ T cells expressing CD69 and (D) co-expressing CD69 and PD-1 at baseline from non-pregnant (blue circles) and pregnant (red circles) women. Data are presented as box-and-whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum ranges. $^*p < .05$

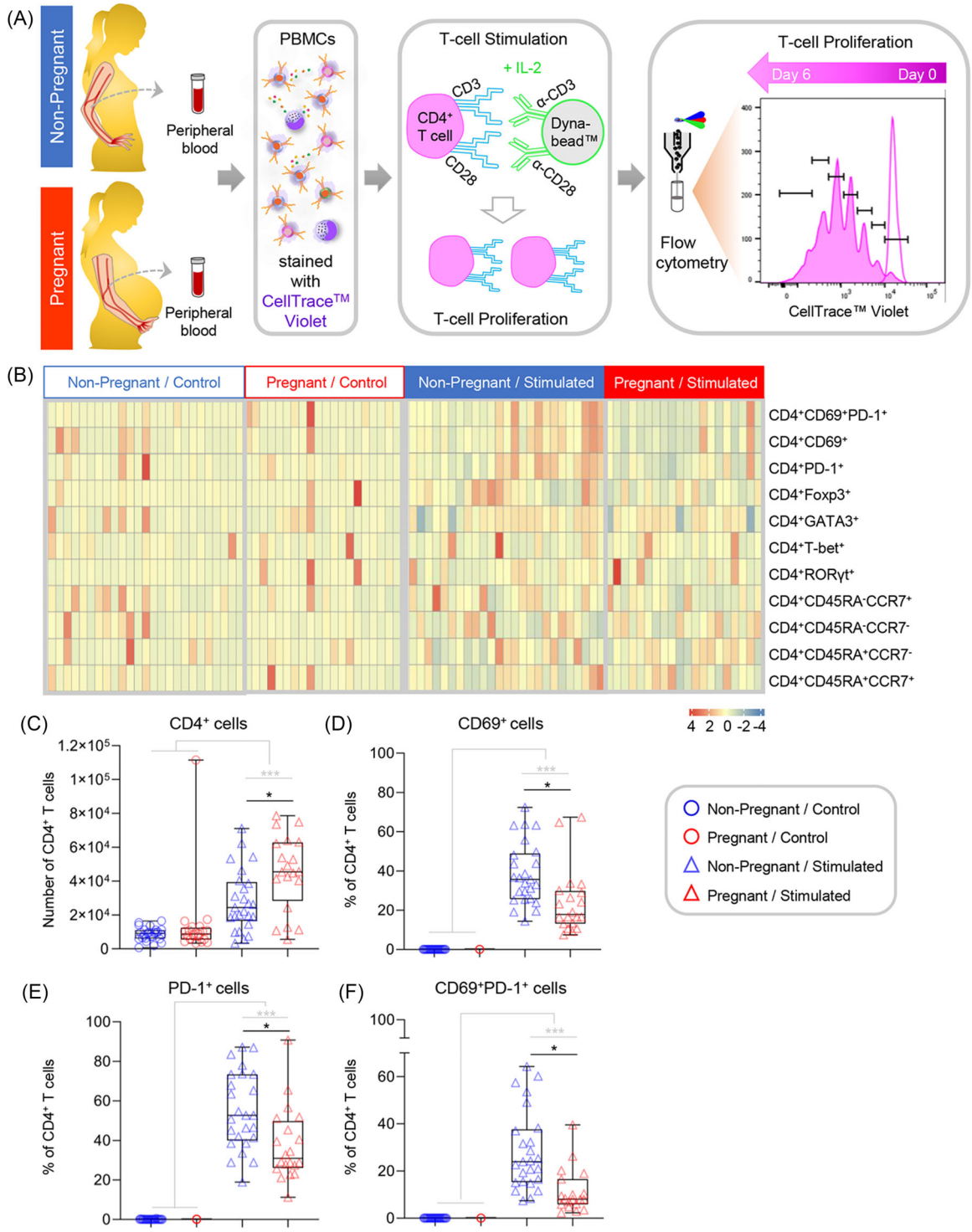

FIGURE 2 Comparison of $CD4^+$ T-cell proliferation between non-pregnant and pregnant women. (A) Peripheral blood samples were collected from non-pregnant (n = 25, indicated in blue) and pregnant (n = 20, indicated in red) women to isolate peripheral blood mononuclear cells (PBMCs) for in vitro stimulation with anti-CD3/anti-CD28 and recombinant human IL-2. Cells were cultured for 6 days prior to phenotyping. Controls were cultured in parallel without stimulation. (B) Heatmap representation showing the proportion of $CD4^+$ T cells with various immunophenotypes from non-pregnant (indicated in blue) and pregnant (indicated in red) women with (stimulated) or without (control) stimulation. The color key indicates the relative proportion of T cells with the various immunophenotypes considered, which were not compared among each other. (C) Absolute number of $CD4^+$ T cells in control and proliferated samples from non-pregnant (blue symbols) and pregnant (red symbols) women. (D-F) Proportion of proliferated $CD4^+$ T cells with the phenotype of (D) $CD4^+CD69^+$, (E) $CD4^+PD\text{-}1^+$, and (F) $CD4^+CD69^+PD\text{-}1^+$. Data are presented as box-and-whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum ranges. Each dot represents the mean of three biological replicates per sample. Grey lines and asterisks represent within-group differences between control and stimulated samples (i.e., pregnant control vs. pregnant stimulated), while black lines and asterisks represent significant differences between groups after stimulation (i.e., pregnant stimulated vs. non-pregnant stimulated). $^*p < .05$; $^{***}p < .001$

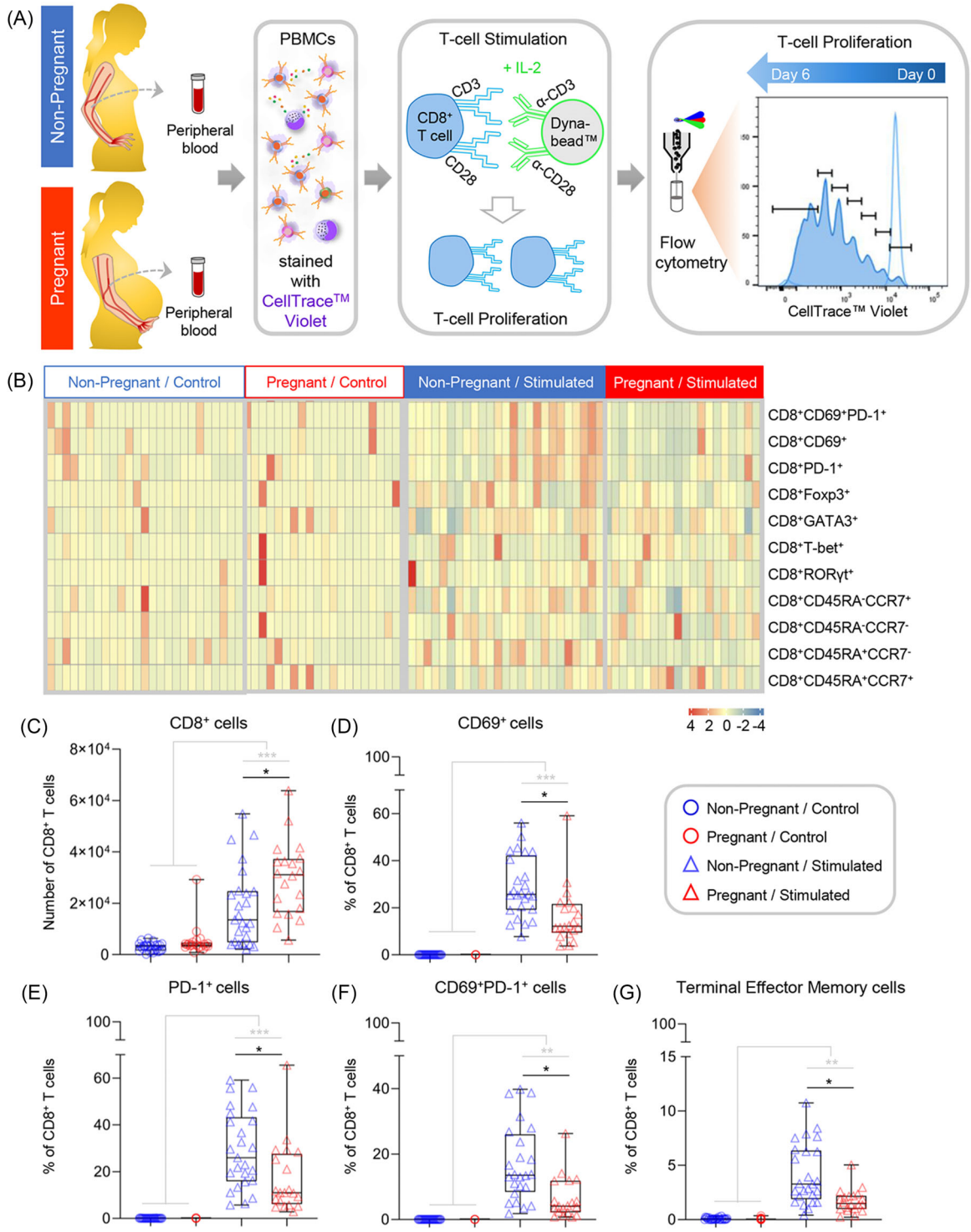

FIGURE 3 Comparison of $CD8^+$ T-cell proliferation between non-pregnant and pregnant women. (A) Peripheral blood samples were collected from non-pregnant (n = 25, indicated in blue) and pregnant (n = 20, indicated in red) women to isolate peripheral blood mononuclear cells (PBMCs) for in vitro stimulation with anti-CD3/anti-CD28 and recombinant human IL-2. Cells were cultured for 6 days prior to phenotyping. Controls were cultured in parallel without stimulation. (B) Heatmap representation showing the proportion of $CD8^+$ T cells with various immunophenotypes from non-pregnant (indicated in blue) and pregnant (indicated in red) women with (stimulated) or without (control) stimulation. The color key indicates the relative proportion of T cells with the various immunophenotypes considered, which were not compared among each other. (C) Absolute number of $CD8^+$ T cells in control and proliferated samples from non-pregnant (blue symbols) and pregnant (red symbols) women. (D-G) Proportion of proliferated $CD8^+$ T cells with the phenotype of (D) $CD8^+CD69^+$, (E) $CD8^+PD\text{-}1^+$, (F) $CD4^+CD69^+PD\text{-}1^+$, and (G) $CD8^+CD45RA^+CCR7^-$ (terminal effector memory). Data are presented as box-and-whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum ranges. Each dot represents the mean of three biological replicates per sample. Grey lines and asterisks represent within-group differences between control and stimulated samples (i.e., pregnant control vs. pregnant stimulated), while black lines and asterisks represent significant differences between groups after stimulation (i.e., pregnant stimulated vs. non-pregnant stimulated). $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$

absolute cell counts, in pregnant compared to non-pregnant women. Next, we analyzed the proliferated $CD4^+$ (Figure 2B) and $CD8^+$ (Figure 3B) T-cell subset composition of the two study groups relative to controls that were cultured under identical conditions without stimulation. Strikingly, we found that the proportion of T cells expressing the activation marker CD69 was significantly reduced in stimulated $CD4^+$ (Figure 2D) and $CD8^+$ (Figure 3D) T cells from pregnant compared to non-pregnant women. Furthermore, a significantly decreased proportion of $CD4^+$ (Figure 2E) and $CD8^+$ (Figure 3E) T cells expressed PD-1 in pregnant compared to non-pregnant women following stimulation. The proportion of cells co-expressing CD69 and PD-1 was also found to be significantly lower in $CD4^+$ (Figure 2F) and $CD8^+$ (Figure 3F) T cells from pregnant women. Finally, a significantly lower proportion of $CD8^+$ terminally differentiated effector memory T cells ($CD45RA^+CCR7^-$), characterized by low proliferative capacity and rapid effector function,[87,88] was observed in pregnant compared to non-pregnant women (Figure 3G). Taken together, these results demonstrate that $CD4^+$ and $CD8^+$ T cells isolated from pregnant women have an increased capacity for proliferation; however, when proliferated, pregnancy-derived T cells show a reduced proportion of cells expressing CD69 and PD-1, suggesting that pregnancy modulates T-cell responses.

3.3 | Pregnancy does not alter peripheral lymphocyte cytotoxicity

Having observed pregnancy-specific differences in lymphocyte activation status, we wondered whether the cytotoxicity of circulating lymphocytes would differ based on pregnancy status. Cytotoxic lymphocytes directly kill target cells through the release of granules, which represents an important mechanism of defense against viruses and intracellular bacteria.[89,90] Hence, we isolated PBMCs from pregnant and non-pregnant women and incubated them with CFSE-labeled target cells (Figure 4A). Flow cytometry was used to quantify the number of killed target cells (Figure 4B). No significant differences were found between the pregnant and non-pregnant study groups among the various ratios of PBMCs:target cells evaluated (Figure 4C), indicating that peripheral lymphocytes from both study groups were able to display comparable cytotoxic activity.

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

After evaluating functional differences in peripheral T cells in the context of pregnancy, we next focused on the second cellular component of adaptive immunity, B cells.[65] During gestation, B cells are necessary for immune regulation and the promotion of humoral immunity,[65] including the production of protective antibodies specific for paternal antigens.[91,92] However, the pregnancy-specific cellular responses exhibited by circulating B cells require further investigation. Hence, PBMCs were isolated from pregnant and non-pregnant women to evaluate differences in B-cell phenotypes as well as B-cell functionality (Figure 5A). First, flow cytometry was used to evaluate differences in B-cell phenotypes following the gating strategy presented in Supplemental Figure 2. Several B-cell subsets displayed distinct modulation in pregnant compared to non-pregnant women (Figure 5B). The proportion of memory-like $CD27^+IgG^+$ B cells (Figure 5C) was found to be elevated during pregnancy, as was the proportion of B cells with an activated $CD38^+CD24^-$ phenotype (Figure 5D). By contrast, the proportion of B cells displaying a $CD40^+CD138^-$ phenotype was increased in non-pregnant women compared to the pregnant study group (Figure 5E). Next, PBMCs were stimulated with anti-human IgM and anti-human IgG, and then flow cytometry was utilized to quantify downstream B-cell receptor activation (Figure 5F). Consistent with the increased proportion of B cells displaying a $CD38^+$ activated phenotype, we found that there was a significantly higher fold-change in activation following anti-human IgM/IgG stimulation by B cells isolated from pregnant compared to non-pregnant women (Figure 5G). This finding suggests that pregnancy enhances circulating B-cell responses.

4 | DISCUSSION

Herein, we evaluated the phenotypes and functions of peripheral T and B cells in pregnant compared to non-pregnant women, as these adaptive immune cells play a critical role in maintaining maternal-fetal tolerance.[62,93–98] First, we showed that pregnancy is associated with modestly enhanced basal activation of peripheral $CD4^+$ T cells. Interestingly, both $CD4^+$ and $CD8^+$ T cells derived from pregnant women showed increased activation-induced proliferation; yet, a reduced proportion of these cells expressed markers of activation compared to T cells from non-pregnant women. No differences were observed in peripheral lymphocyte cytotoxicity between the study groups. Finally, a greater proportion of B cells from pregnant women displayed memory-like and activated phenotypes, and such cells exhibited higher activation following stimulation. Taken together, these data indicate generalized T- and B-cell activation in pregnancy, with a restricted T-cell response to stimulation that may foster systemic maternal-fetal tolerance.

We observed a pregnancy-specific increase in the basal proportion of activated peripheral $CD4^+$ T cells, as indicated by the expression of the early activation marker CD69. In line with this finding, a higher baseline proportion of $CD4^+CD69^+$, but not $CD8^+CD69^+$, T cells has been reported in C57BL/6 mice in late pregnancy relative to non-pregnant mice.[99] We also detected a modest pregnancy-specific increase in the proportion of peripheral $CD4^+$ T cells co-expressing CD69 and PD-1, of which the latter is typically regarded as a co-inhibitory receptor.[82,100,101] Yet, PD-1 expression is upregulated within 24 - 48 hours of T-cell activation,[85] potentially as a mechanism to limit excessive responses and tissue damage.[86] Thus, the co-expression of CD69 and PD-1 likely indicates prolonged T-cell activation, as would be expected following chronic antigen exposure. Considering the presence of fetal antigens in the maternal circulation,[53,102,103] it is tempting to suggest that the increased

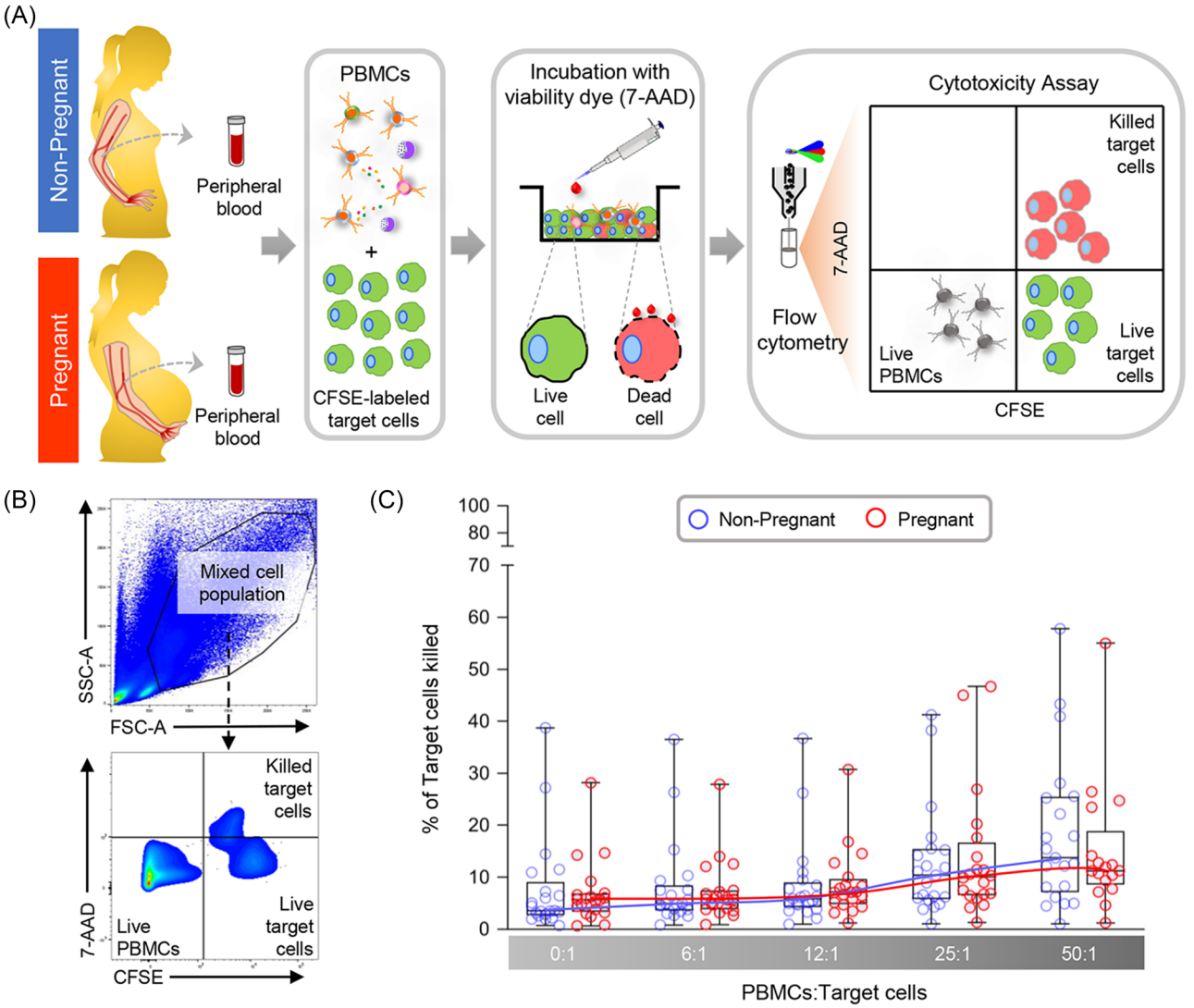

FIGURE 4 Comparison of lymphocyte cytotoxicity between non-pregnant and pregnant women. (A) Peripheral blood samples were collected from non-pregnant (n = 21, indicated in blue) and pregnant (n = 17–20, indicated in red) women to isolate peripheral blood mononuclear cells (PBMCs) for in vitro culturing with CFSE-labeled target cells. (B) Flow cytometry gating strategy used to identify killed target cells ($CFSE^+7AAD^+$), live target cells ($CFSE^+7AAD^-$), and live PBMCs ($CFSE^-7AAD^-$). (C) Percentage of target cells killed (calculated as [$CFSE^+7AAD^+$ / ($CFSE^+7AAD^+$ + $CFSE^+7AAD^-$) * 100]) among ratios of PBMCs:target cells ranging from 0:1 to 50:1 in non-pregnant (blue circles) and pregnant (red circles) women. Data are presented as box-and-whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum ranges. Trend lines for each study group are included.

proportion of $CD4^+CD69^+PD\text{-}1^+$ T cells in pregnant women may reflect repeated exposure to such fetal antigens.[102–105] Indeed, prior studies in mice have demonstrated that innate immune cells in the periphery interact with fetal antigens throughout pregnancy, which was replicated in vitro by using human innate immune cells from the second and third trimesters.[103] Furthermore, cell-free fetal DNA (cffDNA) concentrations have been shown to increase in the maternal circulation in late gestation, which coincides with a pro-inflammatory shift in maternal immunity prior to parturition.[106–109] Specifically, cffDNA has been demonstrated to stimulate a monocyte response in the third trimester capable of activating bystander T cells.[109] Moreover, phenotyping and omics studies have provided evidence of T-cell activation that occurs during labor,[19,21,23,110] and T-cell responses in late pregnancy have been associated with the increased expression of activation markers.[111–113] In support of these concepts, the samples herein were obtained from pregnant women in late gestation and close to delivery. Of note, parity information was not available for the control/non-pregnant study participants, so analyses accounting for both pregnancy status and parity were not performed. Collectively, these data suggest the possibility that the presence of or increases in the circulating concentrations of fetal antigens and cffDNA may contribute to the modest increase in basal activation of peripheral $CD4^+$ T cells observed in pregnancy.

Herein, we also found that $CD4^+$ and $CD8^+$ T cells from pregnant women displayed greater proliferation in response to in vitro stimulation than those from non-pregnant women. In support of this finding,

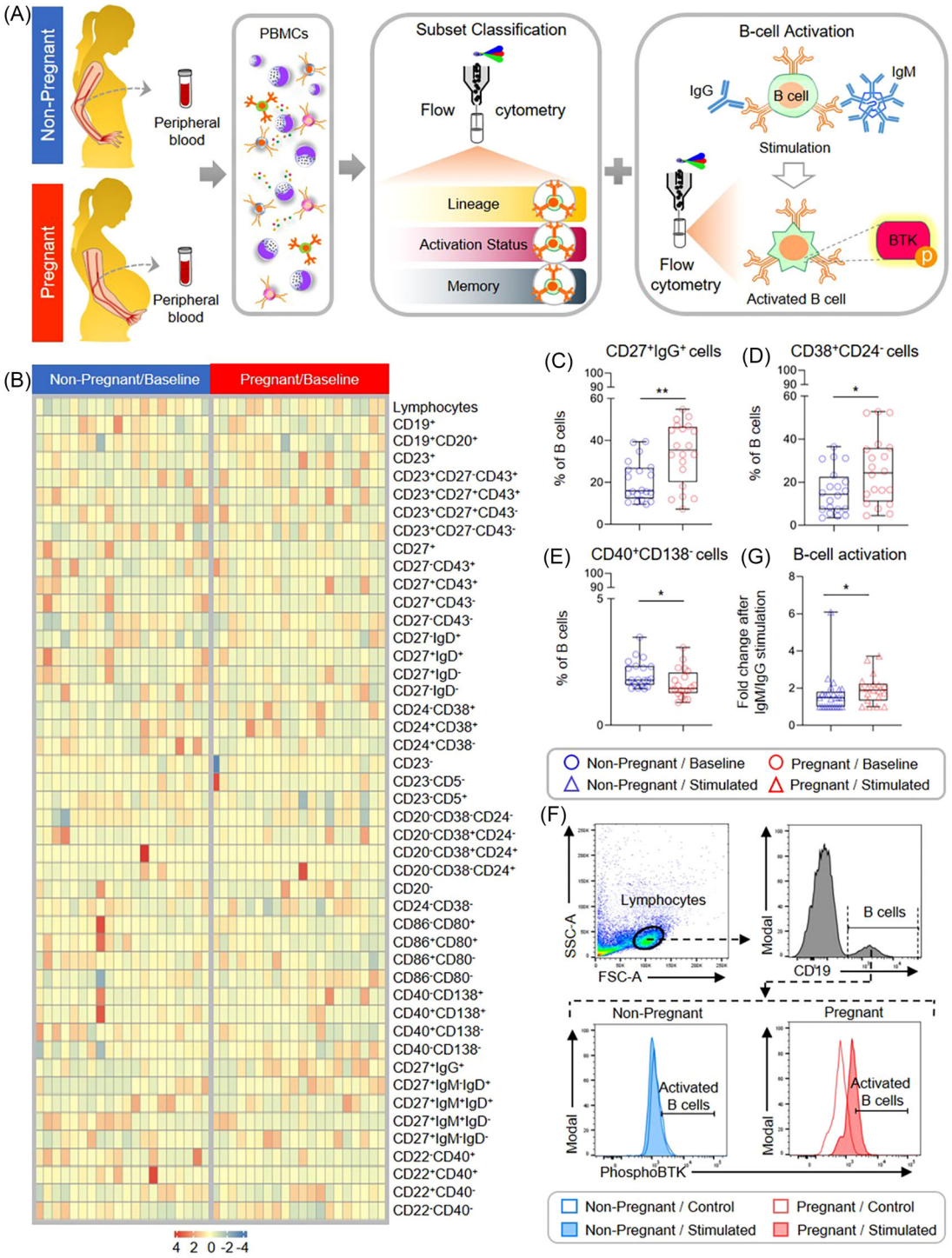

FIGURE 5 Comparison of B-cell subset composition and activation between non-pregnant and pregnant women. (A) Peripheral blood samples were collected from non-pregnant [n = 20 (phenotyping) - 25 (activation), indicated in blue] and pregnant (n = 19, indicated in red) women to evaluate B-cell phenotypes and activation following anti-human IgM/IgG stimulation. (B) Heatmap representation showing the basal proportion of B cells with various immunophenotypes from non-pregnant (indicated in blue) and pregnant (indicated in red) women. The color key indicates the relative proportion of B cells with the various immunophenotypes considered, which were not compared among each other. (C-E) Proportion of B cells with the phenotype (C) $CD19^+CD20^+CD27^+IgG^+$, (D) $CD19^+CD20^+CD38^+CD24^-$, and (E) $CD19^+CD20^+CD40^+CD138^-$ from non-pregnant (red circles) and pregnant (blue circles) women. (F) Representative flow cytometry gating strategy for B-cell activation assay: viable B cells were identified as $CD19^+$, and then B-cell activation in control (open histograms) and stimulated (filled histograms) samples from non-pregnant (indicated in blue) and pregnant (indicated in red) women was determined as described in the Methods. (G) Fold change in B-cell activation in non-pregnant (blue triangles) and pregnant (red triangles) samples after anti-human IgM/IgG stimulation, calculated as the adjusted MFI of IgM/IgG-stimulated samples divided by the adjusted MFI of control samples. Fold changes < 1 were considered as "no change" and assigned a value of 1. Data are presented as box-and-whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum ranges. $^*p < .05$; $^{**}p < .01$

increased proliferation of CD4+ and CD8+ T cells as a function of pregnancy has also been reported in mice.[114] One of the most prominent findings in the current study was that, by contrast with the baseline differences in T-cell subset composition, the proportions of proliferated CD4+ and CD8+ T-cell populations expressing the activation markers CD69 and PD-1 were reduced in pregnant women. In mice, an increase in the proliferation of CD4+ and CD8+ pregnancy-derived T cells following the blockade of PD-1 has been reported[114]; therefore, the reduced proportion of peripheral T cells expressing PD-1 in pregnant women may have contributed to the higher proliferation of pregnancy-derived CD4+ and CD8+ T cells observed in this study. PD-1 is well-studied for its role in cancer and the therapeutic potential of inhibiting this pathway,[115,116] and the co-expression of PD-1 and CD69 has been reported in activated CD4+ and CD8+ T[117] cells and in NK[118] cells isolated from cancer patients. Of note, CD69 has also been demonstrated to play a role in immune[119,120] and metabolic[121,122] regulation, indicating it may be more than a marker of activation.[123] Yet, additional experiments are needed to define the functional implications of this phenotype in the context of pregnancy.

In this regard, increased expression of CD69 by peripheral T cells has been described in patients with a history of recurrent spontaneous abortion,[124,125] and the basal and stimulated CD69 expression was higher in women with miscarriage than in those with normal pregnancy.[125] Furthermore, increased CD69 expression by peripheral CD8+ T cells has been reported in patients with cardiac[126] and renal[127] allograft rejection, and thus proposed as a biomarker for transplant rejection. Together, these studies suggest that the strong upregulation of this activation marker in response to a stimulus can indicate adverse consequences for pregnancy. Indeed, the in vivo activation of T cells with an anti-CD3ε antibody in late and mid pregnancy has been shown to cause systemic inflammation and preterm labor and birth[19] as well as pregnancy loss (Gomez-Lopez et al., unpublished data), respectively. In this murine model, the systemic inflammatory response also extended to the amniotic cavity and resulted in fetal growth restriction,[19] indicating that the systemic over-activation of maternal T cells in pregnancy can be detrimental to the fetus. Thus, the lower proportion of pregnancy-derived CD69+PD-1+ peripheral T cells following stimulation observed herein may indicate a higher threshold for T-cell activation as a mechanism to preserve systemic immune homeostasis.

In addition to the protective mechanism proposed above, the diminished activation of circulating maternal T cells observed in the current study may also allow them to retain memory and proliferative functions for a longer duration.[49,128] This concept is in line with our finding that a lower proportion of terminally differentiated effector memory cells was observed in proliferated T cells from pregnant compared to non-pregnant women. Terminally differentiated CD8+ effector memory T cells display greater effector functions but lower memory and proliferative capabilities and are considered to be short-lived.[129–132] The reduced proportion of T cells expressing activation and terminal effector memory phenotypes following stimulation may reflect a more stringent use of effector functions by T cells during pregnancy. That is, we observed fewer T cells to be activated or terminally differentiated following stimulation in the context of pregnancy, which could reflect a diminished tendency to display effector functions by this peripheral T-cell population.

In line with the above concept, it is reasonable to propose that maternal peripheral T-cell responses are controlled in an antigen-specific manner,[67] which could be a useful feature for avoiding unnecessary T-cell activation that could adversely affect pregnancy. Herein, we utilized a form of T-cell stimulation that bypasses antigen recognition to directly stimulate the T-cell and costimulatory receptors. Yet, prior in vitro studies evaluating the response to influenza A viral stimulation in PBMCs have demonstrated a pregnancy-specific attenuation of the release of pro-inflammatory cytokines such as IFNα[133,134] and IL-2.[134] Futhermore, we have recently shown a reduction in the proportions of pro-inflammatory T-cell subsets, such as Th1 and Tc17, in pregnant women with asymptomatic SARS-CoV-2 infection relative to healthy controls.[135] On the other hand, in vitro stimulation of PBMCs from pregnant women with SARS-CoV-2 proteins results in enhanced T-cell activation 158. Taken together, these data suggest that stimulation with antigens of differing nature/pathogenicity can elicit distinct T-cell responses in the maternal circulation.

In this study, we observed comparable cytotoxic activity by PBMCs from pregnant and non-pregnant women, as reported previously.[136] In the periphery, both T and NK cells are capable of cytotoxic activity,[137] and T cells have been reported to be more prevalent in the maternal periphery.[138] However, the assay used herein relies on C-type lectin-like receptor NKG2D-mediated cytotoxicity[139]; although the CD8+ T and NK cells express this activation receptor,[140] NKG2D signaling in isolation is only sufficient to activate NK cells, as CD8+ T cells require simultaneous stimulation of the T-cell receptor and by cytokines.[139] Despite this limitation, the data indicate comparable peripheral lymphocyte cytotoxicity upon exposure to non-self-antigens between pregnant and non-pregnant women.

While a large body of work has considered the role of T cells in establishing and maintaining maternal-fetal tolerance, the second cellular component of adaptive immunity – B cells – is also critical to establishing and maintaining tolerance throughout gestation.[31,65,92,141,142] Prior studies have demonstrated that IgG immunoglobulins contained in maternal serum prevent maternal lymphocytes from mounting a cytotoxic response against cultured trophoblasts.[91,143] Indeed, spontaneous recurrent abortions are characterized by a lack of protective maternal antibodies directed toward paternal HLA antigens.[144–147] Protective antibodies bind their antigens with high affinity but are unable to initiate downstream immune responses such as complement activation and cytotoxicity.[148] By contrast, natural autoantibodies, produced by B1a cells,[65,149,150] are associated with a range of obstetrical complications that includesintrauterine fetal demise and preeclampsia.[151–154] Accordingly, the circulating proportion of B1, but not B2, cells has been reported to decrease throughout gestation,[30] and B-cell subset composition at the maternal-fetal interface is altered by the process of labor, preterm birth, or chronic histologic chorioamnionitis.[31] Herein, we considered alterations in peripheral B-cell subset composition as a function of pregnancy itself and reported increased frequencies of memory-like CD27+IgG+ B cells and activated CD38+CD24− B cells in pregnant women. Notably, the latter finding is consistent with the observed greater responses

to in vitro IgM/IgG stimulation in pregnancy-derived B cells, given that CD38 ligation has been linked to Bruton tyrosine kinase (BTK) phosphorylation.[155] Higher median peripheral concentrations of B-cell activating factor (BAFF) have been reported in pregnant compared to non-pregnant women, suggesting that BAFF may prime B cells and thus contribute to the pregnancy-specific increase in activation displayed by these cells.[156] In this study, we showed that peripheral B cells display a heightened response to stimulation during gestation, which could provide a more efficient cellular immune response to insults.

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

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Roberto Romero https://orcid.org/0000-0002-4448-5121
Li Tao https://orcid.org/0000-0003-1531-358X
Nardhy Gomez-Lopez https://orcid.org/0000-0002-3406-5262

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SUPPORTING INFORMATION

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