Decidual-Placental Immune Landscape During Syngeneic Murine Pregnancy

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Adaptive immune system, principally governed by the T cells—dendritic cells (DCs) nexus, is an essential mediator of gestational fetal tolerance and protection against infection. However, the exact composition and dynamics of DCs and T cell subsets in gestational tissues are not well understood. These are controlled in human physiology by a complex interplay of alloantigen distribution and presentation, cellular/humoral active and passive tolerance, hormones/chemokines/angiogenic factors and their gradients, systemic and local microbial communities. Reductive discrimination of these factors in physiology and pathology of model systems and humans requires simplification of the model and increased resolution of interrogative technologies. As a baseline, we have studied the gestational tissue dynamics in the syngeneic C57BL/6 mice, as the simplest immunological environment, and focused on validating the approach to increased data density and computational analysis pipeline afforded by highly polychromatic flow cytometry and machine learning interpretation. We mapped DC and T cell subsets, and comprehensively examined their maternal (decidual)—fetal (placental) interface dynamics. Both frequency and composition of decidual DCs changed across gestation, with a dramatic increase in myeloid DCs in early pregnancy, and exclusion of plasmacytoid DCs. CD4⁺ T cells, in contrast, were lower at all gestational ages and an unusual CD4⁻CD8⁻TCRαβ⁺ group was prominent at mid-pregnancy. Dimensionality reduction with machine learning-aided clustering revealed that CD4⁻CD8⁻ T cells were phenotypically different from CD4⁺ and CD8⁺ T cells. Additionally, divergence between maternal decidual and fetal placental compartment was prominent, with absence of DCs from the placenta, but not decidua or embryo. These results provide a novel framework and a syngeneic baseline on which the specific role of alloantigen/tolerance, polymicrobial environment, and models of pregnancy pathology can be precisely modeled and analyzed.

Keywords: dendritic cells, T cells, t-SNE, decidua, placenta, C57BL/6 mice
INTRODUCTION

Pregnancy is a remarkable challenge that requires coordination of multiple systems, including adaptive and innate immune cells, systemically and at the maternal-fetal interface. Immune cells in pregnancy are responsible for fetal protection from pathogens, establishing and maintaining tolerance for the semi-allogeneic fetus, and directing the placental remodeling of uterine vasculature (1). Critical decisions regarding the type and intensity of adaptive immune response are primarily driven by the decisions reached by T cells interacting with dendritic cells (2), the binary cellular focus of this study. Rich diversity of immune cellular phenotypes and a multitude of influences (chemokines, developmental cytokines, allostimulation in non- syngeneic pregnancies, and microbiome of the reproductive tract) makes this a particularly challenging, yet pivotal, system to study (1, 3, 4). Advances in immunology and a deeper understanding of tissue resident immune cell dynamics in other contexts warrant a re-examination of gestational adaptive immune dynamics (5–7). To manage complexity, and to establish a higher resolution approach to adaptive immune dynamics, we applied high-dimensional flow cytometry and a machine learning pipeline to a simplified model of pregnancy—syngeneic C57BL/6 cross. Herein, allostimulation, complex microbial challenge and polygenic diversity is restricted, thus, allowing us to test the application of high-dimensional immunology in gestational tissues, to set the stage for next generation of complex studies that would reflect human and mouse pregnancy physiology.

DCs (CD11c+) participate in establishment of maternal immunologic tolerance (8) and pregnancy establishment more broadly (1). During embryo implantation DCs may promote angiogenesis, a process necessary for adequate spiral artery remodeling in mice (9, 10). Indeed, depletion of uterine DCs in a CD11c-DTR transgenic model causes failure of decidualization, impaired implantation, and embryonic resorption (8, 11). DCs (identified as CD11c+MHCII+F4/80−) are trapped within the pregnant uterus in mice, inhibiting antigen transfer to the local lymph node, a process necessary for priming of circulating naïve T cells (12), presumably in order to prevent alloantigen anti-fetal response. Despite this trafficking restriction, DCs can prime CD8+ T cells, as CD11c+ depletion abrogated the CD8+ T cells response to infection with Listeria monocytogenes and Plasmodium yoelii in a mouse model (13). Despite these intriguing studies, surface receptor heterogeneity of DCs subsets in peripheral tissues and side-effects of diphertheria toxin receptor (DTR)-based depletion methodology impose significant limitations on interpretation of this data. For one, the CD11c-DTR mouse model is not wholly DCs specific, as certain macrophages, plasmablast, activated T cells, and NK cells, can express DTR and be at least partially depleted, with mouse morbidity and death after repeated DT injections (14, 15).

Multiple T cells subsets perform a variety of functions (16, 17) in pregnancy. Regulatory T cells (Tregs) maintain tolerance toward the semi-allogeneic fetus in mice (18, 19). Tregs deficiency impedes implantation in mice either due to implantation failure or embryo resorption (16, 20). Tregs during pregnancy are enriched primarily at the maternal-fetal interface, but not in the circulation, highlighting the distinct phenotype and function of decidual T cells (21). Furthermore, type 1 helper (Th1) cells and cytotoxic T cells (CTLs) are actively excluded from mouse decidua, due to an epigenetic chemokine gene silencing program rather than an alloantigen-specific manner (22). However, comprehensive assessment of decidual T cell subset dynamics in the syngeneic breeding mouse model is still lacking (16). Advent of highly polychromatic flow cytometry and computational analysis methods allows for a redefinition of immune system dynamics during pregnancy given complex data in multiple models (23).

In this study, although no allo-reactivity or complex polymicrobial environment is present, the dynamics of T-DCs in this system may reveal the influence of other gestational factors (hormonal, pregnancy stage-specific chemokines, or others). Therefore, we focused on the following questions: (a) Does application of high-dimensional flow cytometry and machine learning bring a more nuanced insight into the dynamics of DC/T cells in gestational tissues? (b) What is the population dynamics of DC/T cell subsets in the uterus, decidua and placenta across syngeneic mouse gestation? This study, while not reflective of a physiological pregnancy, provides a baseline that integrated with allogenic and microbial influences will increasingly provide more detailed, complex and faithful representations of adaptive immunology of pregnancy.

MATERIALS AND METHODS

Mice

Female and male C57BL/6J (B6) mice were purchased from Jackson laboratory (Bar Harbor, ME, cat# 000664). The mice were housed in specific pathogen free facility at the Biotron in ventilated micro-isolator cages at University of Wisconsin-Madison. All caging equipment, bedding and enrichment items are sterile and mice are provided with irradiated feed and acidified water. The breeding and timed mating were set up and maintained by trained staff. The protocol was approved the Institutional Animal Care and Use Committee at University of Wisconsin-Madison. Female mice (6–13 weeks) were used for timed mating and experiments. The day when a vaginal plug was detected in a timed mating was counted as gestational day 0.5. Virgin mice (6–13 weeks) and the mice at various specified gestational day (early: 6, 7, 8, mid:12, 13, 14, late: 16, 17, 18 days) were sacrificed, and gestational day of each embryo decidua/placenta/uterus saved for analysis.

In this study, estrous stage was not assessed in the virgin mouse group. In order to keep the experiments consistent, the mice used for timed mating and virgin controls were roomed together for weeks, with likely estrus synchrony as occurs in co-housed mice. Still, virgin mice from different individual experiment are likely to be in different stages of estrous, contributing some immune cell variability to the virgin uterus “baseline” studies.

Tissue Processing/Immune Cells Isolation

Mouse decidua, placenta, embryos (GD6-8 only), and uteri (virgin only) were collected (Supplementary Figure 5 shows the
Flow Cytometry Labeling and Analysis

Single cell suspensions were first labeled with LIVE/DEAD® fixable blue stain (Invitrogen, cat# L34962) and subsequently a cocktail of fluorochrome-conjugated monoclonal antibodies (list in Table 1) according to the manufacturer’s instructions. Briefly, antibodies were diluted in BD Horizon BrilliantTM Stain Buffer (BD Biosciences, San Jose, CA, cat#566349) and used to label cells for 30 min, washed, and fixed with 4% formaldehyde (TED PELLA, Inc., cat# 1805) for 5 min before washout using stain buffer (BD, cat#554656). Transcription factor assessment for intracellular staining was done using BD Transcription Factor Buffer Set (BD, cat# 562574). These specimens were then processed using gentleMACSTM C tube (Miltenyi Biotec Inc., San Diego, CA, cat# 120-005-331), and a specially adapted tissue dissociation program (clockwise spin of 100 rpm for 1 min, then counter-clockwise spin of 100 rpm for 1 min, followed by clockwise spin of 1,000 rpm for 5 s, loop 5 times, finally counter-clockwise of 100 rpm for 1 min and then clockwise spin of 100 rpm for 1 min, all steps were within 37°C) run in gentleMACSTM Dissociator for 30 min (Miltenyi Biotec Inc. San Diego, CA, cat# 130-096-427). Spleen, thymus, and Peyer’s patches (used as control tissue) were mechanically dissociated in RMPI 1640 containing 10% heated FBS in gentleMACSTM C tube, by running corresponding standard programs for different tissue types in gentleMACSTM Dissociator.

After dissociation, homogenates were filtered through 70 µm cell strainer, and red cells of splenic or thymic (as needed) specimens were lysed with ACK lysis buffer (Life Technologies, cat# A10492-01). Single cell suspension obtained was used for downstream applications.

Table 1

| Antibody | Clone | Fluorochrome | Supplier |
|----------|-------|--------------|----------|
| CLEC9A   | 104B  | BB515        | BD Bioscience |
| CD25     | PC61  | BB515        | BD Bioscience |
| I-A/I-E  | M5/114.15.2 | PerCP-Cy5.5        | BD Bioscience |
| CD44     | IM7   | PerCP-Cy5.5  | BD Bioscience |
| CD14     | RmC5-3 | PE           | BD Bioscience |
| CD69     | H1.2F3 | PE           | BD Bioscience |
| CD80     | 16-10A1 | PE-CF594     | BD Bioscience |
| CD62L    | MEL-14 | PE-CF594     | BD Bioscience |
| CD8a     | 53-6.7 | PE-Cy5       | BD Bioscience |
| CD9      | 145-2C11 | PE-Cy7      | BD Bioscience |
| CD80     | 1A8   | PE-Cy7       | BD Bioscience |
| B200     | RA3-6B2 | PE-Cy7      | BD Bioscience |
| CD209    | 5H10  | APC          | BD Bioscience |
| CD122    | TM-Beta 1 | APC    | BD Bioscience |
| L-y-6G   | RB6-8C5 | AF700    | BD Bioscience |
| TCP8     | H57-597 | AF700      | BD Bioscience |
| B220     | Ra3-6b2 | APC-Cy7     | BD Bioscience |
| CD11c    | HL3   | BV421        | BD Bioscience |
| CD198    | 140706 | BV421       | BD Bioscience |
| F4/80 lce | 6f12  | BV510        | BD Bioscience |
| receptor |       |              |          |
| CD127    | SB/199 | BV510        | BD Bioscience |
| CD11b    | M1/70  | BV605        | BD Bioscience |
| I-A/I-E  | M5/114.15.2 | BV605      | BD Bioscience |
| BST2     | 927   | BV605        | BioLegend |
| CD19     | D3    | BV395        | BD Bioscience |
| CD4      | GK1.5  | BV496        | BD Bioscience |

Data Analysis

Manual gating analysis was performed using FlowJo v.10.3 (Flowjo LLC, Ashland, OR). Dimensionality reduction was performed using the t-SNE algorithm, followed by DensVM clustering, both part of the open-source R package, Cytofkit (github.com/JinmiaoChenLab/cytofkit) (25). Briefly, data files were pre-gated to exclude dead cells and irrelevant lineage populations and concatenated using FlowJo. Concatenated files were then entered into the R/Cytofkit analysis pipeline via the GUI interface, and parameters of interest selected. Newly derived t-SNE and DensVM coordinates were added to original data matrices, exported, and analyzed in FlowJo. Cluster frequencies and mean fluorescence intensity (MFI) values were calculated using FlowJo and exported into Excel and JMP Pro 13 (SAS, Cary, NC, USA) for analysis. Heatmaps for MFI (z-score normalized) and cluster frequencies were constructed in JMP Pro 13.

The data from each gestational day were analyzed separately, when we created the figure and analyzed statistics, we combined the data of early pregnancy GD 6, 7, 8 together, middle pregnancy GD 12, 13, 14 together, late pregnancy GD16, 17, 18 together, respectively. Manual statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVA followed by Tukey’s multiple-testing adjusted post-hoc analysis was used to determine statistical significance (p < 0.05), all statistical analysis details are
shown in Supplementary Tables 4–8. All data are represented as box plots with minimum to maximum, showing all points.

RESULTS

Dramatic Remodeling of DC Compartment Across Pregnancy

To examine the composition of DC subsets across gestational age, we validated a comprehensive, polychromatic (16-marker) panel (Table 1). We monitored overall DCs (Figures 1A,B, lin1-: CD3neg NK1.1neg CD19neg Ly-6Gneg, CD11c+ I-A/I-E+ cells) and subsets (Figure 1C). DCs in control tissues (spleen, Peyer’s patches, and thymus) were examined in every experiment for validation of staining strategies and to monitor technical variability (Supplementary Figure 1, 16 independent experiments).

We detected a sharp rise in proportion of DCs (CD11c+ I-A/I-E+, % of live lin-, Figure 1D) in decidua of early gestational age (GD6–8, p < 0.0001), with subsequent decline starting in middle gestational age (GD12–14, p < 0.0005) with further decline in late gestational age (GD16–18, p < 0.0001). In contrast, on the fetal side of the interface (placenta), DCs were entirely excluded during middle (p < 0.0001) and late pregnancy (p < 0.0001) compared to their proportion in the embryo (GD6–8). In addition, compared with the virgin uterus, the DCs also increase in the embryo (GD6–8; p < 0.0005). Note that the immune cells isolated from the placenta and embryos are fetal in origin and reflect development of fetal nascent immune system. Taken together, DCs are dramatically expanded in early pregnancy decidua but entirely excluded from the placental compartment.

As CD11c+ I-A/I-E+ compartment includes both myeloid and lymphoid DCs, we investigated gestational subset dynamics: (a) Myeloid DCs, defined as lin1-CD11c+ I-A/I-E+B220neg CD11b+ CD8αneg (Figures 1C,E); and (b) Lymphoid CD8α+ DCs, defined as lin1-CD11c+ I-A/I-E+B220neg CD11b+ CD8α+ (Figures 1C,F). Interestingly, myeloid DCs predominate in the decidua, while CD8α+ DCs were a proportionally small subset, especially in light of their considerable proportion in non-gestational control tissues (Figures 1E, Supplementary Figure 1B). Myeloid DCs, in the decidua, showed a bimodal pattern, with early increase (p < 0.05), mid-pregnancy dip (p < 0.05) and late recovery of myeloid subsets (p < 0.005, Figure 1E). In the fetal compartment, early myeloid DCs are identified in the embryo (GD6–8). Finally, CD8α DCs were low and not significantly different across gestational reproductive tissues tested (Figure 1F).

Plasmacytoid DCs Are Excluded From Decidua

Gestational dynamics of plasmacytoid DCs (PDCs, lin1- I-A/I-E+B220neg CD11bneg, Figures 2A,B), and their subsets based on CD8α: CD8α+ and CD8αneg PDCs (Figure 2A) were also analyzed PDCs were largely BST2 positive (Figure 2A, right
FIGURE 1 | mouse gestation. (C) The subset of DCs, Myeloid DCs (lin1-CD11c+I-A/I-E+B220negCD11b+CD8a+ ) and CD8a+ DCs (lin1-CD11c+I-A/I-E+B220negCD11b+CD8a+), were further identified in murine virgin uterus, decidua, placenta across gestation age. (D) Proportions of CD11c+I-A/I-E+DCs out of lin1-cells in the murine virgin uterus, decidua, placenta, and embryo across gestation age. (E) Proportions of myeloid DCs out of CD11c+I-A/I-E+DCs in the virgin uterus, decidua, placenta, and embryo. (F) Proportions of CD8α+DCs out of CD11c+I-A/I-E+DCs in murine virgin uterus, decidua, placenta, and embryo across gestation age. These gating strategy figures were not obtained from the same experiment. n = 14–16 (16 independent experiments), the exact number of animals used were summarized in Supplementary Table 3. The data of virgin uterus were compared with decidua group or placenta (include embryo) group, respectively. Statistical analysis was performed using ANOVA followed by post-hoc Tukey analysis. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00001.

Gestational Redistribution of T Cell Subsets

Dominant function of DCs is antigen presentation to T cells and instruct their subset differentiation (2). As we found complex DC dynamics during pregnancy, we hypothesized that T cell and their subsets also follow a characteristic dynamic across gestation, even in a syngeneic context. Therefore, we validated a 16-marker αβ cells panel (Table 1) to examine T cell subsets composition at the maternal-fetal interface across gestation (Figures 3A–C). We monitored T cells overall (Figures 3A,B, Iin2-: Ly-6GnegB220negI-A/I-Eneg, TCRβ+cells) and examined subsets at maternal-fetal interface across gestation. Similar to DCs, T cells in control tissues including spleen, Peyer’s patches, and thymus were determined in every analysis for validation of staining strategies and to monitor experimental variability (Supplementary Figure 3, 16 independent experiments).

In early gestation, we found a decrease in decidual αβ T cells proportion compared to virgin uterus, followed by recovery and increase toward the end of pregnancy (p < 0.05–0.0001, depending on time point, Figure 3D), and the αβ T cells proportion of middle and late pregnancy in decidua are higher than that of virgin uterus. Overall T cell dynamics was opposite that of CD11c+ I-A/I-E+ DCs in decidua (Figures 1D, 3D).

Major lineages of αβ T cells across pregnancy, including a) CD4- (Figure 3C); b) CD8- (Figure 3C); c) CD4-CD8- T cells (Figure 3C) were further studied. We found that decidual and placental CD8+ T cells proportion was stable (Figure 3E).
Interestingly, CD4+ T cells were lower in decidua throughout the gestation and in fetal tissues (early embryo, mid-gestation placenta) compared to that of virgin uterus ($p < 0.05–0.005$, Figure 3F). Finally, we were surprised to find that CD4-CD8- T cells were dramatically increased in decidua ($p < 0.05$) and placenta ($p < 0.0005$) of middle pregnancy (GD12-14) compared with maternal uterus (Figure 3G) and all control tissues (Supplementary Figure 3A).

Next, we examined the dynamics of CD4+ lineage subsets. We differentiated naïve (CD62L+CD44<), memory (CD62L<CD44+), and activated (CD69+) CD4+αβT cells (Figures 4A,B). In the decidua, we detected an increase of naïve CD4+ T cells proportion in late compared to early pregnancy (Figure 4C, $p < 0.05$); while activated CD4+ T cells proportion was lower in middle and late pregnancy (Figure 4E, $p < 0.05$ and $p < 0.0005$, respectively), compared to virgin uterus. Amongst fetal cells, naïve CD4+ T cells predominated in placenta (Figure 4C, $p < 0.0005$), while activated CD4+ T cells were proportionally low/excluded (Figure 4E, $p < 0.0005$). Memory CD4+ T cells were stable across gestation in the decidua, but low in placenta of late pregnancy (Figure 4D, $p < 0.005$). Decidual CD4+ cells with a "regulatory" phenotype (Tregs, CD127lowCD25+CD44+, Supplementary Figure 4), were low at late pregnancy in decidual and across gestation in placental locations (Figure 4F). Tregs phenotypes also showed distinct dynamics, with conventional Tregs decreased in late pregnancy decidua and very low in the placenta (CD62L<CD69+, Supplementary Figure 4, Figure 4G, $p < 0.05–0.005$). On the other hand, precursor-type Tregs proportion (CD62L+CD69<, Supplementary Figure 4) was stable across tissues and time (Figure 4H).

Similarly, CD8+ T cells lineage subsets with naïve (CD62L+CD44<, Figure 5A), memory (CD62L<CD44+, Figure 5A), and activated phenotypes (CD69+, Figure 5B) were investigated. In the maternal compartment, the naïve CD8+ T cells were stable in early and middle pregnancy compared with virgin uterus, but expanded in late gestational age (Figure 5C, $p < 0.1$, $p < 0.005$, virgin uterus and decidua in early gestational age, respectively). Corresponding decline in memory (Figure 5D, $p < 0.1$) and activated (Figure 5E, $p < 0.0001$) CD8+ proportion was seen at late gestational age. In the fetal compartment, the proportion of memory CD8+ T cells was lower in early embryo (Figure 5D, $p < 0.1$), and placenta excluded CD8+ cells with a memory (Figure 5D, $p < 0.0001$) and activated (Figure 5E, $p < 0.0001$) phenotype.

Interface DC- and T-Subsets Visualized by Dimensionality Reduction and Machine Learning

Given the complexity of DCs and T cells compartments, and difficulty in assigning subset phenotypes by 2-dimensional gating section through 18-dimensional parameter space, we employed dimensionality reduction and machine learning (DensVM) methods to parse the full dataset.

First, we examined the lin1-I-A/E+ compartment from coded/concatenated data of a single experiment that included...
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FIGURE 4 | Gating strategy and dynamics of CD4+ T cells subsets. (A) Gating strategy of Naïve CD4+ T cells, defined as lin2- (Ly6G<sup>neg</sup>B220<sup>neg</sup>I-A/I-E<sup>neg</sup>) TCR<sub>β</sub>+, CD4<sup>+</sup>CD62L<sup>+</sup>CD44<sup>neg</sup>, and memory CD4+ T cells, defined as lin2- (Ly6G<sup>neg</sup>B220<sup>neg</sup>I-A/I-E<sup>neg</sup>) TCR<sub>β</sub>+, CD4<sup>+</sup>CD62L<sup>neg</sup>CD44<sup>+</sup>. (B) Gating strategy of activated CD4+ T cells, defined as lin2- (Ly6G<sup>neg</sup>B220<sup>neg</sup>I-A/I-E<sup>neg</sup>) TCR<sub>β</sub>+, CD4<sup>+</sup>CD69+. (C–E) Proportions of Naïve CD4+ T cells, memory CD4+ T cells, and activated CD4+ T cells in murine virgin uterus, decidua, placenta, and embryo across gestational age. (Continued)
FIGURE 5 | Gating strategy and dynamic change of CD8+ T cells subsets. (A) Gating strategy of Naïve CD8+ T cells, defined as lin2-(Ly6Gneg B220neg I-A/I-Eneg) TCRβ+ CD8+ CD62L+ CD44neg, and memory CD8+ T cells, defined as lin2-(Ly6Gneg B220neg I-A/I-Eneg) TCRβ+ CD8+ CD62Lneg CD44+.

(B) Gating strategy of activated CD8+ T cells, defined as lin2-(Ly6Gneg B220neg I-A/I-Eneg) TCRβ+ CD8+ CD69+.

(C–E) Proportions of Naïve CD8+ T cells, memory CD8+ T cells, and activated CD8+ T cells in murine virgin uterus, decidua, placenta, and embryo across gestational age. n = 11–15 (15 independent experiments), the exact number of animals was summarized in Supplementary Table 3. The data of virgin uterus were compared with decidua group or placenta (include embryo) group, respectively. Statistical analysis was performed using ANOVA followed by post-hoc Tukey analysis. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.

FIGURE 6 | Visualization of dendritic cell diversity. To avoid the cross-batch effect, we used a single experiment including 15 samples of virgin uterus, decidua, and placenta across the whole murine pregnancy. (A) t-SNE map generated from pre-gated lin1-I-A/I-E+ cells from murine virgin uterus, decidua, and placenta across gestational age data sets (top) and manually gated subsets overlaid onto total lin1-I-A/I-E+ cells (bottom). (B) Hierarchical clustering of median surface marker expression levels of clusters identified by DensVM. Total of 16 clusters were categorized in 6 groups, group A–F. (C) Pie chart of different group proportions of total DC population. (D) Hierarchical clustering of cluster frequency within lin1-I-A/I-E+ from murine virgin uterus, decidua, and placenta across gestational age. (E) Separate visualization of cluster in different murine tissues across gestation using t-SNE map generated from the merged data set.

cluster similarity (Figures 7B,C, Supplementary Table 2). Thus subdivided, groups A, B, C, D, E consisted of 14.1, 20.9, 16.6, 16.5, 31.9%, respectively (Figure 7C, Table 3).
TABLE 2 | Presumed classification of I-A/E+ clusters (Figure 6B).

| Cluster | Phenotype | Presumed name |
|---------|-----------|---------------|
| 1       | BST2- B20+ CD14+/− CD8a− CLEC9A− CD209− CD80− CD11b− F4/80− CD11c− | Undefined |
| 2       | BST2+ B20+ CD14+ CD8a− CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Undefined |
| 3       | BST2+ B20+ CD14+ CD8a+ CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Myeloid DCs |
| 4       | BST2+ B20+ CD14+ CD8a+ CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Undefined |
| 5       | BST2+ B20+ CD14+ CD8a+ CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Undefined |
| 6       | BST2+ B20+ CD14+ CD8a+ CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Myeloid DCs |
| 7       | BST2+ B20+ CD14+ CD8a+ CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Undefined |
| 8       | BST2+ B20+ CD14+ CD8a+ CLEC9A− CD209+ CD80+ CD11b− F4/80− CD11c− | Undefined |
| 9       | BST2+ B20+ CD14+ CD8a− CLEC9A− CD209− CD80+ CD11b− F4/80− CD11c− | Undefined |
| 10      | BST2+ B20+ CD14+ CD8a− CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Undefined |
| 11      | BST2+ B20+ CD14+ CD8a− CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Undefined |
| 12      | BST2+ B20+ CD14+ CD8a− CLEC9A+ CD209+ CD80+ CD11b− F4/80− CD11c− | Undefined |
| 13      | BST2+ B20+ CD14+ CD8a+ CLEC9A− CD209+ CD80+ CD11b+ F4/80+ CD11c+ | Undefined |
| 14      | BST2+ B20+ CD14+ CD8a− CLEC9A− CD209+ CD80+ CD11b− F4/80− CD11c− | PDCs |
| 15      | BST2+ B20+ CD14+ CD8a+ CLEC9A+ CD209+ CD80+ CD11b+ F4/80+ CD11c+ | PDCs |
| 16      | BST2+ B20+ CD14+ CD8a+ CLEC9A+ CD209+ CD80+ CD11b− F4/80− CD11c− | Undefined |

Matching the cluster markers profile and manual characterization of T cell subsets, we identified cluster 1 (Group A) as naïve CD8+ T cells, cluster 3 (group B) as memory CD8+ T cells, clusters 4 and 6 (group D) as naïve CD4+ T cells and clusters 9 and 13 (group C) as memory CD4+ T cells. Interestingly, in this analysis, similar to the classical CD4/CD8 gating, we find a group of broad and populous clusters that are CD4-CD8-, prevalent in mid-late pregnancy, and with a broadly distinct phenotype compared with groups A-D (group E, Figures 7C,D). Similar to DCs, T cells distribution also exhibited a tissue and time signature specific to pregnancy (Figure 7D). These were recapitulated on t-SNE maps of individual samples (Figure 7E).

Taken together, the use of tSNE/densVM pipeline allowed us to visualize the complex manual analysis in a simple and concise fashion. Furthermore, clustering analysis revealed unexpected, broad differences in the phenotype of CD4-CD8-T cells compared with conventional CD4+ and CD8+ T cells in the decidua. Finally, a tissue- and time-dependent signature of gestational specimens is evident.

DISCUSSION

Adaptive immune cells at the maternal-fetal interface are essential immune mediators necessary for a successful pregnancy. The composition and function of interface immune cells changes throughout the pregnancy to adjust to different pregnancy challenges. Consequently, dysregulation of adaptive immune...
TABLE 3 | Presumed classification of TCRδ+ clusters (Figure 7B).

| Cluster | Phenotype | Presumed name |
|---------|-----------|---------------|
| 1       | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122+ | Naïve CD8+ T cells |
| 2       | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122+ | Undefined |
| 3       | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122+ | Memory CD8+ T cells |
| 4       | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122+ | Naïve CD4+ T cells |
| 5       | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122INTER | Undefined |
| 6       | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122INTER | Naïve CD4+ T cells |
| 7       | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122INTER | Double negative T cells |
| 8       | CD4+ CD45INTER CD8α+ CD62L+ CD127INTER CD25+ CD196+ CD69+ CD122INTER | Undefined |
| 9       | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122INTER | Memory CD4+ T cells |
| 10      | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122INTER | Double negative T cells |
| 11      | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122INTER | Double negative T cells |
| 12      | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122INTER | Double negative T cells |
| 13      | CD4+ CD45INTER CD8α+ CD62L+ CD127+ CD25+ CD196+ CD69+ CD122INTER | Memory CD4+ T cells |

The overall αβ T cell dynamics was opposite to that of CD11c+I-A/I+DCs in decidua (Figures 1D, 3D), suggesting the possible interaction of CD11c+I-A/I+DCs and αβ T cells in the decidua during pregnancy. Early, and massive DC accumulation, may physically displace T cells in early pregnancy, alternatively, T cells population increase in late pregnancy could be in response to diminished DC inhibition or local priming. Consistent to our data in mice (Figure 3D), Vassiliadou and Bulmer also showed that T cells decreased in early pregnancy while CD8α+DCs showed the opposite trend in early pregnancy in decidua (Figures 1E,F), helping to explain the known Th2 bias of pregnancy. Third, while we found there was very few PDCs (I-A/I- E B220+) in decidua, they were present in the placenta (Figure 2C). As CD11c+I-A/I+E+DCs were completely excluded from placenta (Figure 1D), PDC exclusion of CD11c+I-A/I-from decidua is likely functionally meaningful. Outside of the utero-placental compartment (mouse gestational para-aortic lymph nodes), conventional DCs (CD11c+) are reduced while PDCs (CD11c-CD20+PDCA-1+) increased from E8.5 early to E16.5 late pregnancy (33), possibly reflecting trapping of conventional DCs within the decidua and export of PDCs.
CD4-CD8-T cells have been reported to play important roles in many tissue of different species. For example, αβTCR+CD3+CD4−CD8− T cells have been shown to inhibit a variety of immune responses by directing killing of effector T cells in an antigen specific manner in both human and mice (35). Lung CD4-CD8-double negative T cells are primarily responsible for producing IL-17A and IFN-γ during respiratory murine infection with Francisella Tularensis live vaccine strain (36). It is interesting that we find accumulation of CD4-CD8-T cells in decidua and placenta. To our knowledge, this specific subpopulation of T cells has not been reported in the mouse decidua or placenta, although there is a study by Joansson and Lycke that detected αβTCR+CD4-CD8-T cells (CD3+αβTCRintCD4−CD8−B220low) as dominant lymphocytes in the mouse female genital tract (37). We are in the process of investigating this curious finding further.

Tregs play an essential role in maintaining pregnancy (38, 39), and prior studies showed that Tregs (CD4+CD25high) proportion is higher in decidua than periphery in early pregnancy in humans (38, 39). Our model is syngeneic and consistent with prior studies that CD4+CD25+ were not dramatically altered in pregnant mice (40), suggesting a primary role in control of allosresponse (41). We show that proportion of Tregs in syngeneic C57BL/6 pregnancy significantly declined at late gestation in decidua and was low in placenta consistent with the prior work using Foxp3 as the Tregs-defining marker (42).

Different subsets of T cells may be primed by different subsets of DCs, since myeloid but not lymphoid DCs cross-prime CTLs (CD8+) in mice (43). In decidua, our data did not demonstrate prominent change in CTL (CD8+T cells) or lymphoid (CD8α) DC frequency across gestation, however, their functional responsiveness was not investigated. Placenta, in contrast, demonstrated accumulation of CD8α T cells in late pregnancy, and a non-significant trend toward increase in late gestation lymphoid DCs, suggesting that CD8+ T cells at the fetal side may be primed by the lymphoid DCs although very few are present.

Our data demonstrates the divergence of proportion and dynamics of adaptive immune cells subsets in maternal decidua and fetal placenta. As noted, DCs predominately accumulate in the decidua while exclude in placenta, naïve but not active CD4+ T cells predominated in placenta, indicating the majority of nascent fetal CD4 lymphocytes have not encounter antigen/antigen-presenting cells (16). These findings were vividly demonstrated by the machine learning aided clustering of decidual and placental samples (Figures 6D, 7D). Finally, although we have kept the virgin mice during rearing in the same cage as the experimental animals, variability in this group is a limitation and to be expected as we have not explicitly tested the estrous stage of the isolated virgin uteri.

The high heterogeneity of DCs and T cells required high-dimensional flow cytometry with multiple markers to define the specific subsets as precisely as possible. Dimensionality reduction/machine learning algorithms promises the simplicity and standardization of high dimensional data in an unbiased fashion (44). Therefore, in addition to the manual analysis, we also employed operator-independent dimensionality reduction and machine learning algorithms for cellular subset identification and tracking their gestational dynamics to verify manual analysis and explore their future application in evaluating the maternal-fetal immunome. Computational methods not only verified our manual analysis such as that cluster 3 (identified as myeloid DCs, group E, Figure 6B) showed the same dynamics (Figure 6D) with myeloid DCs manual gating (Figure 1E). It also identified previous unknown subsets. Amongst MHC Class II expressing cells (I-A/I-E+), cluster 9 (Figure 6B) is lin-I-A/I-E+B220+CD14+CD80intCD8αlowCD11bseg F4/80negCD11cneg, which does not match known systemic DC subsets, and may be an intermediate subset in development (pre-PDCs?). For TCRαβ+ population, the clusters 11, 12 (group E) are lin-TCRαβ+CD4negCD8neg CD62LnegCD44+CD127+ CD196+CD69+CD122+, and belongs to the unusual CD4-CD8-T cell population. Overall, little is known about double negative T cells in other tissues and even less in decidua (35). Furthermore, as demonstrated by the expression heatmap, CD4-CD8-T cells were very different in their overall expression of these non-TCR receptors, suggesting an alternate developmental/activation path.

Within the syngeneic pregnancy model, the complex and divergent patterns of multiple DC- and T-cell subsets reveals normative information on remodeling of DCs and T cells compartment at the maternal-fetal interface, with dramatic gestational dynamics implying their complex roles at different developmental times. Dimensionality reduction and DensVM cluster on t-SNE map allowed us to more clearly visualize the known and novel clusters in an unbiased and standardized fashion. These results provide a new normative framework for studies of pregnancy immunology in a reproducible fashion, with a focus on discovery of novel phenotypes.

AUTHOR CONTRIBUTIONS

YL and AS designed the research. YL performed the majority of experiments with GL, YS, MC, SF, and NK. JV and PL assisted with machine learning analysis. YL and AS wrote the manuscript. AS supervised the project.

FUNDING

YL was supported by AAI Careers in Immunology Fellowship (to AS). JV was supported by NIH TEAM-Science (R25GM083252), and UW SciMed GRS Fellowship. MC was supported by UW WISE Summer Research Grant. AS and this research project were supported by grant K12HD000849-28 awarded to the Reproductive Scientist Development Program by the Eunice Kennedy Shriver National Institute of Child Health & Human Development and March of Dimes Basil O’Connor Starter Scholar Award (5-FY18-541). Additional research support (to AS) was provided by March of Dimes, American Society for Reproductive Medicine and Burroughs Wellcome Fund, as part of the Reproductive Scientist Development Program. UWCCC Flow Core Grant (1S100OD018202-01).
ACKNOWLEDGMENTS

We would like to thank T. Golos, L. Reyes, M. Patankar, C. T. Tyler for their review of manuscript and helpful suggestions; D. Sheerar and R. Sheridan from UWCCC Flow lab for technical support.

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SUPPLEMENTARY MATERIAL

The Supplemental Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02087/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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