Title
Exposure to a Wider Variety of Male Antigens May Lead to the Development of Maternal Immune Tolerance

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Publication Date
2023

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

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A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science in Biology

by

Amanda Lauren Reshke

2023

ABSTRACT OF THE THESIS

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the Development of Maternal Immune Tolerance

by

Amanda Lauren Reshke

Master of Science in Biology

University of California, Los Angeles, 2023

Professor Peter N. Nonacs, Chair

Exposure to male antigens has been shown to induce an immune response in women. This response allows for recognition of paternal antigens, and development of maternal immune tolerance for a potential fetus. Prior exposure to a partner's antigens has been shown to decrease risk of pregnancy complications. Humans did not evolve to exclusively have one sexual partner throughout a lifetime. We hypothesize that female physiology may have acquired an adaptation to prevent chronic inflammation, whereby exposure to a wider variety of paternal antigens could lead to the development of protective immune tolerance. In a cohort of pregnant mothers in California we collected peripheral blood, measured T-regs by flow cytometry and measured cytokines by multiplex assay. Using multiple linear regression controlling for gestational age, maternal age, and parity, we found consistent with our hypothesis, that women with more cumulative sexual partners exhibited lower levels of pro-inflammatory cytokines (IL-6 b= -0.02, p= 0.07) and a less pro-inflammatory cytokine balance (IL-6 to IL-10 ratio b= -0.052, p= 0.026). Unexpectedly, we also found that more partners were associated with lower T-reg levels (T-regs b= -1.94, p= 0.045), which could indicate that the physiological pathway to decrease inflammation may occur via a mechanism other than T-regs. Since heightened

inflammation increases risk of pregnancy complications, these findings contribute to our understanding of how life history influences reproductive health and pregnancy outcomes, and imply a possible adaptation for mitigating inflammation to optimize reproductive fitness.

The thesis of Amanda Reshke is approved.

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2023

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INTRODUCTION

The development and maintenance of materno-fetal immune tolerance is highly integral in preventing the immune system from attacking the fetus, and successfully carrying a fetus to term. Male (or paternal) antigens found in sperm play a key role in initiating the development of materno-fetal tolerance, and are integral in priming a mother's body for a semi-allogeneic fetus upon insemination. Prior to first exposure, a woman's adaptive immune system is naive to a partner's unique antigens. Initial exposure to male antigens induces a substantial inflammatory response, and leads to T-regulatory cell expansion (Petroff et al., 2022; Robertson et al., 2009; Shima et al., 2015).

This immune response may be helpful for multiple reasons, including flushing out pathogens that may have been transferred during intercourse. Importantly, this response prepares the body for fetal tolerance if fertilization and implantation are successful (Petroff et al., 2022). This allows for recognition of paternal alloantigens expressed by the embryo, and thus sets the stage for further augmentation of maternal immune tolerance. T-regulatory cells play a key role in this. T-regs mitigate maternal-fetal conflict and facilitate self-tolerance through the suppression of effector T cells, which would otherwise attack the fetus. The development of materno-fetal immune tolerance is required to prevent the maternal immune system from targeting the fetus (La Rocca et al., 2014; Tilburgs & Strominger, 2013).

Prior cumulative vaginal exposure to a partner's paternal antigens has been shown to increase immune tolerance, and decrease risk of pregnancy complications such as preeclampsia (Saftlas et al., 2014). These findings highlight the importance of developing an adaptive immune response to paternal antigens and the subsequent expansion of T-regs, in both maternal and fetal health. Each partner's unique male antigens activate a new immune response in a woman's body. In this study, we investigate whether there may exist a mechanism preventing chronic inflammation in a woman's body via the development of an immune tolerance, which is acquired with an increased number of male sexual partners (and

thus, exposure to a wider variety of male antigens). If this were true, an acquired immune tolerance could mitigate systemic inflammation and therefore decrease a woman's risk of pregnancy complications. Since increased levels of systemic inflammation are strongly associated with a wide range of adverse birth outcomes, such as low birth weight, preterm birth, gestational diabetes, and preeclampsia (Harmon et al., 2016; Pinto et al., 2023; Shafiq et al., 2021), these questions contribute to our understanding of how life history factors can influence reproductive health outcomes.

In a cohort of pregnant mothers in California, we collected peripheral blood, and measured T-regs by flow cytometry and cytokines by multiplex assay to identify whether exposure to a wider variety of male antigens leads to the development of immune tolerance in maternal systemic physiology. We used the lifetime number of sexual partners as a proxy to represent each mother's prior exposure to unique male antigens. Many studies have investigated associations between pregnancy outcomes and prior cumulative male antigen exposure from the father of the fetus. To our knowledge, this is one of few other studies looking at possible immune tolerance in correlation with the number of lifetime sexual partners. The goal of this study is to contribute to the growing literature on female health and reproductive life history, and consider how these findings may be viewed from an evolutionary perspective.

MATERIALS AND METHODS

Participants

Data for this project were procured from Wave 2 of the Mothers' Cultural Experiences (MCE) study, an NIH-funded cohort study of Latina pregnant women in Southern California whose overarching goal is to examine the links between socio-cultural and environmental stressors with maternal-fetal and postnatal health and development (grant numbers K01DK105110 and RO3DK125524 to MMF). MCE involved two waves. Wave 1 was a cross-sectional study of pregnant and postpartum women. Wave 2 was a prospective, longitudinal study that followed

women recruited during early pregnancy through 18-months postpartum. Eligibility for MCE Wave 2 included women who were aged 18 years or older, English or Spanish speaking, self-identified as Latina, Hispanic, Chicana, or Mexican. Ethnicity eligibility was restricted due to study goals unrelated to the current project. MCE Wave 2 participants were recruited in prenatal clinic waiting rooms, gave informed written consent,and received modest compensation. Human subjects ethics approval was received from the institutional review boards of participating institutions. All protocols comply with the tenets of the Declaration of Helsinki.

Protocol

This project uses data from the two MCE Wave 2 prenatal assessments occurring around 12 weeks' and 24 weeks' gestational ages. Timepoint 1 gestational ages ranged 5-19 weeks and timepoint 2 gestational ages ranged from 20-33 weeks, plus one individual whose Timepoint 2 assessment occurred outside the target window at 16.6 weeks due to a scheduling error (her Timepoint 1 was in-range at 7.6 weeks). Inclusion of this individual's data did not meaningfully change our results, so we elected to include this participant in analyses to maximize sample size. For this study, biosamples from only Timepoint 1 were used, and cumulative sexual partner data was collected in Timepoint 2. At each prenatal assessment, participants completed a written questionnaire and gave specimens of saliva, urine, and peripheral blood. Demographic and health history data are self-reported, deriving from these questionnaires. The blood samples relevant to the current project were drawn by antecubital venipuncture into sodium heparinized vacutainers (Becton, Dickinson, and Company, Franklin Lakes, NJ) by hospital or clinic phlebotomists. The sodium heparin tubes were drawn first, before any other labs. These samples were kept at room temperature and transported immediately to the Cousins Center laboratory at the University of California Los Angeles, arriving within a few hours of the blood draw. Peripheral blood mononuclear cells (PBMCs) were isolated following standard protocol, aliquoted, and stored at -80°C until analysis. The number of male sexual partners was assessed

with questions preceded by the instructions, “Please answer these questions honestly” or “Por favor responda a estas preguntas honestamente.” This was followed by the prompt, “During your entire life, with how many men have you had sex (intercourse)?” or, “Durante toda su vida, ¿con cuántos hombres has tenido relaciones sexuales (coito)?”

Staining and Flow Cytometry

To quantify T-cell populations, PBMCs were stained using antibodies from BioLegend, including CD3-Brilliant Violet 650, CD4-APC-Cyanine7, CD25-Brilliant Violet 421, CD127-Brilliant Violet 605, FoxP3-PE. Stained cells were then quantified by color flow cytometry using 4 laser AttuneNxT Accoustic Focusing cytometer (Invitrogen) using the AttuneNxT software. Cell populations were identified using the FlowJo software package (Tree Star, Ashland, OR, USA). The gating strategy for the cell populations of interest is provided in Figure 4. The positivity borderline was determined from fluorescence minus one (FMO) control tubes. T-regs are defined as $CD3^{+}CD4^{+}CD25^{hi}CD127^{lo}FoxP3$.

Multiplex Assays

Cytokines were measured from non-fasting morning blood samples. Blood used for cytokine assays was collected by antecubital venipuncture into EDTA-treated tubes and kept at refrigerator temperature until plasma extraction within a few hours, and the plasma aliquots were frozen at -80c until assay. A multiplex assay was used to measure concentrations of the cytokines (Meso Scale Discovery, Meso Scale Diagnostics LLC, Rockville, MD). The minimum detectable amount for cytokines were as follows: IL-6 0.06 pg/mL, IL-10 0.03 pg/mL.

Statistical Methods

We used linear regression models to assess the association of a woman’s cumulative number of sexual partners with their concurrent immune response, and we used R version 3.6.2 for analysis.

Control variables were included based on their hypothesized role in exerting direct and independent effects on maternal immune function during pregnancy. For this reason, gestational age at sample collection (in weeks), parity, and maternal age were included as covariates. T-reg and cytokine levels have been shown to vary by gestational age and increase over pregnancy, and nulliparous women could plausibly have lower T-reg cell percentages than parous women (Wegienka et al., 2011).

RESULTS

From our original cohort of 107 women, our final analytic cohort included 54 women, after omitting women who were missing cumulative partner data. The large attrition was due to the onset of the covid 19 pandemic since partner history was asked at a follow-up, in person assessment that many women had not yet had when the pandemic started. To investigate levels of systemic inflammation in mothers, we quantified cytokines IL-6 and IL-10. We also quantified T-reg levels, since T-regs are integral in the development and maintenance of materno-fetal tolerance. **Table 1** shows the results of bivariate analyses using the number of cumulative partners as a predictor, with cytokine and T-reg levels as outcome variables. With these unadjusted models, we found negative correlations for all biomarkers (IL-6 b= -0.018, *p*= 0.102; IL-6/IL-10 b= -0.05, *p*= 0.041; and Tregs b=-1.92, *p*= 0.045). Both the IL-6/IL-10 cytokine balance and T-reg levels showed statistically significant correlations with the number of cumulative partners, whereas IL-6 by itself did not show a significant correlation.

When controlling for gestational age at sample collection, maternal age, and parity in our multivariable linear regression analyses, we again found that pro-inflammatory cytokine levels were negatively correlated with the number of cumulative sexual partners, although it was not statistically significant (IL-6 b= -0.02, *p*= 0.07) (**Table 2**). There was also a less inflammatory cytokine balance associated with the number of cumulative partners (IL-6 to IL-10 ratio b= -0.052, *p*= 0.026). Levels of $FoxP3^{+}$ T-reg cells were again found to be negatively correlated with

the number of partners (T-reg b= -1.94, *p*= 0.045). **Figures 1-3** show scatterplots of our multivariate linear regression analyses.

Table 1. Number of cumulative partners as a predictor of cytokine and T-reg levels

Unadjusted models

	IL-6		IL-6/IL-10		T-regs	
	b	*p*	b	*p*	b	*p*
Number of cumulative partners	-0.018	0.102	-0.05	**0.041***	-1.92	**0.045***
	Model *p*-value: 0.102		Model *p*-value: 0.041		Model *p*-value: 0.045	
	R^2 = 0.03		R^2 = 0.06		R^2 = 0.06	

Caption: Data were analyzed via bivariate analysis. *p*-values < 0.05 were considered significant and are bolded.

Table 2. Number of cumulative partners as a predictor of cytokine and T-reg levels, adjusted for gestational age, maternal age and parity at the time of biosample collection

Adjusted models

	IL-6		IL-6/IL-10		T-regs	
	b	*p*	b	*p*	b	*p*
Number of cumulative partners	-0.02	0.07	-0.05	**0.026***	-1.94	**0.045***
	Model *p*-value: 0.162		Model *p*-value: 0.103		Model *p*-value: 0.15	
	R^2 = 0.05		R^2 = 0.08		R^2 = 0.06	

Caption: Data were analyzed via multiple linear regression. *p*-values < 0.05 were considered significant and are bolded.

DISCUSSION

This study investigates the association of immune tolerance with male antigen exposure (using the number of cumulative male sexual partners as a proxy). Other studies have found that inflammation and risk of pregnancy complications are negatively correlated with cumulative exposure to the fathering partner's antigens. This study, however, suggests that a certain level

of immune tolerance may not only result from cumulative exposure to one partner's antigens, but may also arise with exposure to an increased variety of male antigens.

Other studies that have looked at associations between cumulative number of lifetime sexual partners and inflammation in female reproductive physiology have found that some women can have significantly increased inflammation in certain circumstances. These include instances of STI transmission or complications resulting from untreated STIs, such as Pelvic Inflammatory Disease (PID). Young women between ages 15-25 are disproportionately at risk for PID compared to older women, and PID is characterized by significant inflammation of the upper female reproductive tract (including the uterus, ovaries, and fallopian tube) due to infection (Tabacco et al., 2018).

This conflicts with our findings that inflammation may decrease as a result of increased cumulative male sexual partners and male antigens. However, there has not been a study that investigated the correlation between PID and cumulative exposure to male antigens that we know of. Approximately 85% of PID cases have been documented as complications of bacterial STIs (Jennings & Krywko, 2023), and in other cases, PID has been linked to non-STI related bacteria (Goller et al., 2017). Considering this, it appears that the cumulative number of male sexual partners can lead to different inflammatory outcomes, depending on the circumstances. If an STI is transmitted, an individual is more at risk of increased inflammation due to the pathogen itself, as well as possible PID resulting from untreated infection. However, in cases unaccompanied by infections and/or PID, our results indicate that more cumulative male partners and exposure to a wider variety of male antigens may decrease systemic inflammation and lead to an acquired immune tolerance.

IL6 is recognized as a proinflammatory cytokine, and is normally elevated during healthy parturition. However, unusually high levels of IL6 expressed during earlier timepoints in pregnancy have been associated with adverse pregnancy outcomes, such as preterm birth (Menon et al., 2011; Omere et al., 2020). IL10 is recognized as an anti-inflammatory cytokine.

Together, the ratio has been indicative of inflammation in the body, with higher IL6/IL10 ratios indicative of increased inflammation. A decreased IL6/IL10 ratio is indicative of a less inflammatory cytokine balance, and is generally associated with better outcomes (Ragsdale et al., 2019). Our findings showed a negative association between the number of partners and IL6, and a lower IL6/IL10 ratio was associated with having more cumulative male sexual partners. Overall, this demonstrates less inflammation and a less inflammatory cytokine balance with an increased number of partners.

Although we observed that inflammatory cytokine balances were negatively associated with cumulative male partners, we also found a negative association between T-regs and the number of cumulative partners. These findings are interesting, especially because T-regs mediate immune tolerance (La Rocca et al., 2014). We would have expected a positive association between T-regs and cumulative partners, as it was presumed to be a mechanism through which the decrease in inflammatory cytokines could occur. Looking into the characteristics of these T-regs and exploring different mechanisms that could lead to a lower inflammatory cytokine balance would be intriguing, and worth further exploration. More evidence is needed to elucidate these mechanisms through which increased immune tolerance may occur.

Our findings indicate the possible existence of a mechanism promoting the development of immune tolerance in women with more cumulative male partners. Since exposure to a unique partner's antigens induces a new immune response, the development of an immune tolerance acquired with more sexual partners could prevent chronic systemic inflammation, and could therefore be very beneficial for maternal health.

It is known that humans did not evolve to be entirely sexually monogamous. Early hominins were even more sexually dimorphic than Homo sapiens and thus are assumed to have had an evolutionary history of being less pair-bonded, before beginning a trajectory towards less sexual dimorphism (Immerman & Mackey, 2003). From an evolutionary

perspective, our findings could suggest a possible adaptive mechanism for preventing chronic inflammation, that we speculate could have deep phylogenetic roots in the Hominin lineage, whereby exposure to a wider variety of male antigens could lead to the development of a certain level of protective immune tolerance. If this adaptation existed it would be beneficial in mitigating inflammation, therefore decreasing pregnancy complications and increasing reproductive fitness. Other animals such as dogs, cats, mice, and bovines have been found to have IL-6, IL-10, and FoxP3 T-regs (Choi et al., 2021; Moreau & Meurens, 2017; Wypij & Pondenis, 2014). Considering that other mammals are viviparous and must also maintain materno-fetal tolerance during pregnancy, this shared physiology across species may indicate that other mammals could be capable of developing a similar immune tolerance with cumulative sexual partners. More studies would be needed to clarify whether a comparable effect exists in other species.

In this analysis, we used the number of lifetime sexual partners as a proxy for male antigen exposure but we did not have data on when or if protection was used in some cases, due to practical limitations of the study. Here, we demonstrate for the first time to our knowledge that an increased number of lifetime partners, and thus we presume male antigen exposure, may lead to increased immune tolerance by decreasing a pro-inflammatory cytokine balance. Since heightened inflammation increases risk of pregnancy complications, these findings contribute to our understanding of how life history influences reproductive health and pregnancy outcomes, and may imply a possible mechanism that mitigates maternal inflammation to optimize reproductive fitness.

FIGURES

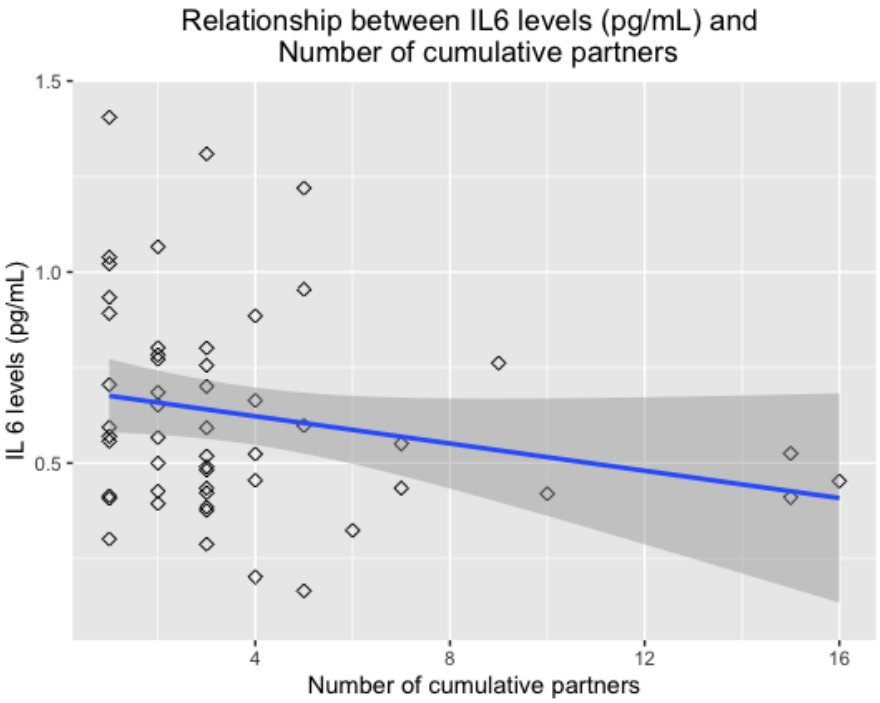

Figure 1. Number of cumulative partners as a predictor of IL-6 levels. This model is adjusted for gestational age, maternal age and parity. The gray-shaded area represents the 95% confidence interval.

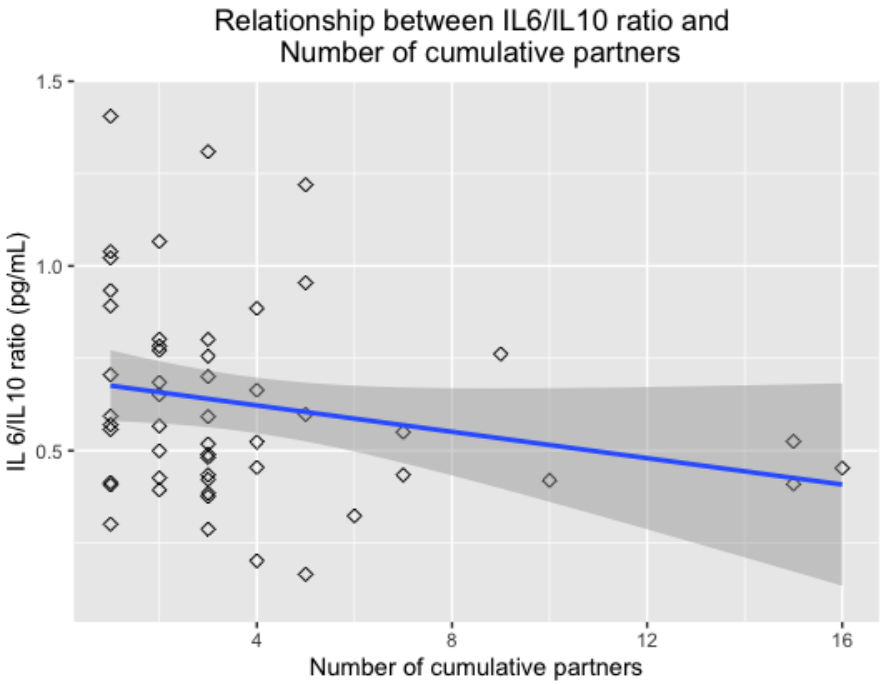

Figure 2. Number of cumulative partners as a predictor of the IL-6 / IL-10 cytokine balance. This model is adjusted for gestational age, maternal age and parity. The gray-shaded area represents the 95% confidence interval.

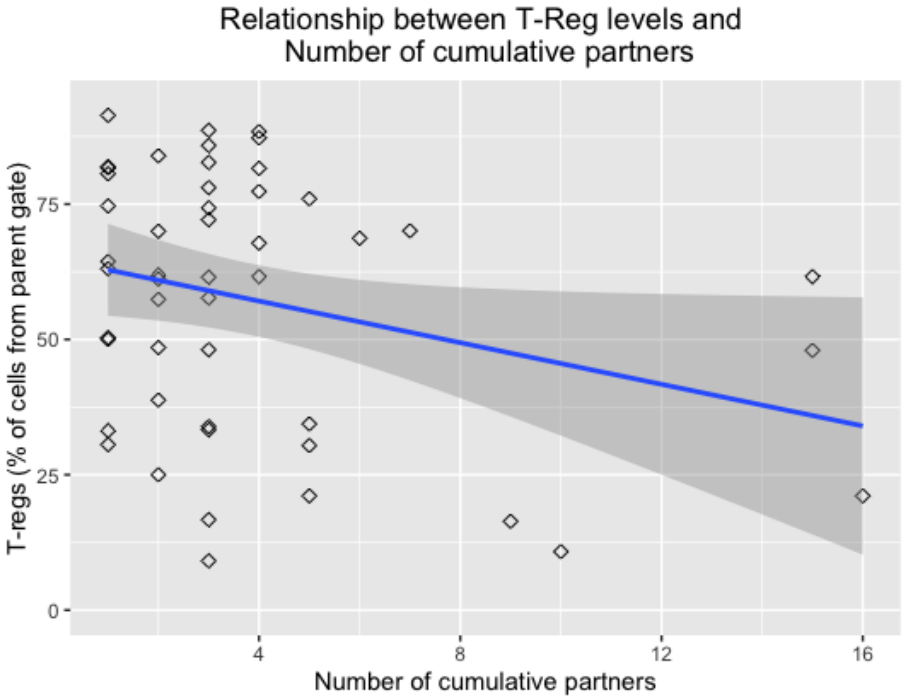

Figure 3. Number of cumulative partners as a predictor of T-reg levels. This model is adjusted for gestational age, maternal age and parity. The gray-shaded area represents the 95% confidence interval.

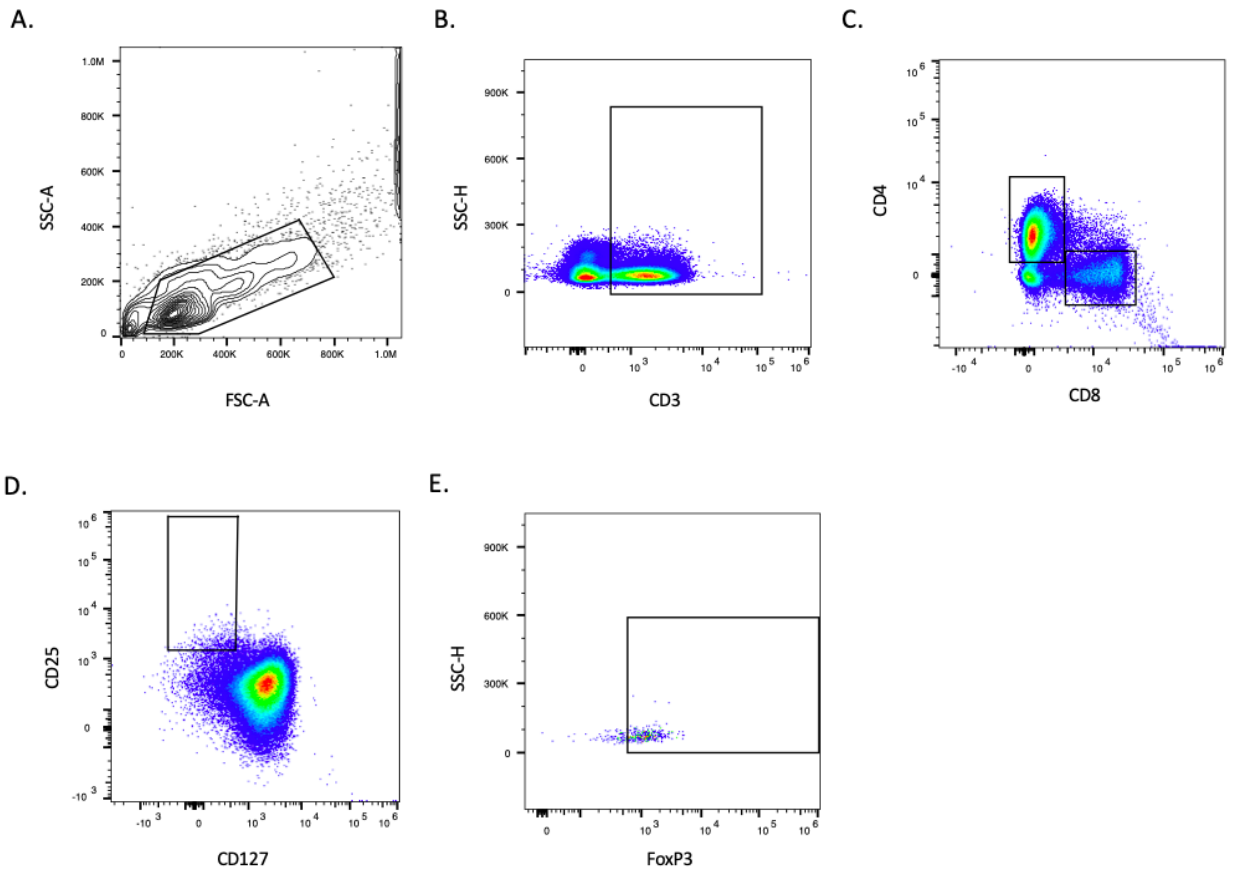

Figure 4. Definition of T-cell subsets and gating strategy.
(**A**) Selection of live lymphocytes after gating for single and living cells. (**B**) Selection of CD3+ lymphocytes within the lymphocyte gate. (**C**) Selection of CD4+ and CD8+ lymphocytes within the CD3+ lymphocyte gate. (**D**) Selection of $CD25^{hi}CD127^{lo}$ cells within the CD4+ lymphocyte gate. (**E**) Selection of FoxP3+ T-regulatory (Tregs) cells within the $CD25^{hi}CD127^{lo}$ gate. This figure was created and provided by Dr. Kyle Wiley and appears in the supplementary materials of his manuscript titled *"Regulatory T-cell phenotypes in prenatal psychological distress"* currently submitted to the journal *Brain, Behavior and Immunity*.

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