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Vascular alterations impede fragile tolerance to pregnancy in type 1 diabetes

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Abstract

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Objective: To determine the impact of autoimmunity in the absence of glycemic alterations on pregnancy in type 1 diabetes (T1D).

Design: Because nonobese diabetic (NOD) mice experience autoimmunity before the onset of hyperglycemia, we studied pregnancy outcomes in prediabetic NOD mice using flow cytometry and enzyme-linked immunosorbent assays. Once we determined that adverse events in pregnancy occurred in euglycemic mice, we performed an exploratory study using electronic health records to better understand pregnancy complications in humans with T1D and normal hemoglobin A1c levels.

Setting: University Medical Center.

Patient(s)/Animal(s): Nonobese diabetic mice and electronic health records from Vanderbilt University Medical Center.

Intervention(s): Nonobese diabetic mice were administered 200 μg of an anti-interleukin 6 (IL-6) antibody every other day starting on day 5 of gestation.

Main Outcome Measure(s): Changes in the number of abnormal and reabsorbed pups in NOD mice and odds of vascular complications in pregnancy in T1D in relation to A1c.

Result(s): Prediabetic NOD mice had increased adverse pregnancy outcomes compared with nonautoimmune mice; blockade of IL-6, which was secreted by endothelial cells, decreased the number of reabsorbed and abnormal fetuses. Similarly, vascular complications were increased in pregnant patients with T1D across all A1c values.

Conclusion(s): The vascular secretion of IL-6 drives adverse pregnancy outcomes in prediabetic NOD mice. Pregnant patients with T1D have increased vascular complications even with normal hemoglobin A1cs, indicating a potential effect of autoimmunity on the placental vasculature.

Keywords

Type 1 diabetes; NOD mice; IL-6; reproductive immunology; autoimmunity

Pregnancy is an incredible test of immune tolerance, requiring the body to accept foreign antigens present in a developing fetus while simultaneously protecting that fetus from microbiologic insult—a feat that mandates carefully timed immune cell activation and senescence (1). Although the multitude of roles played by lymphocytes in pregnancy are still being uncovered, successful pregnancy reflects a balance in which regulatory T cells (Tregs) protect the developing fetus, while activated T cells can initiate labor (2). Immune evolution during pregnancy includes the development of pregnancy-specific regulatory cells, including Tregs that prevent the destruction of male fetuses (3). Given this need for delicate and specific immune regulation, it is striking that patients with autoimmune diseases can still have largely successful pregnancies. It is generally unknown whether autoimmunity in the absence of end-organ damage impedes successful pregnancy, but better understanding may present an important opportunity to further improve pregnancy outcomes.

Type 1 diabetes (T1D) is a prototypical and common autoimmune disease in which pancreatic β cells in the islet of Langerhans are destroyed. Multiple types of lymphocytes collaborate in T1D to cause islet destruction, which contrasts with the protective state

achieved during pregnancy. While CD8+ T cells directly damage islets, B lymphocytes produce autoantibodies that predict the onset of T1D and initiate cytokine-mediated destruction of pancreatic β cells by CD8+ cells (4–6). The collusion between multiple different types of leukocytes presents an environment, even before the onset of overt diabetes, in which Tregs fail to inhibit autoreactivity (7). Similar to pregnancy, novel immune cells can develop throughout the progression of the disease process; in T1D, novel cells are autoreactive instead of protective (8). Despite these challenges to immune tolerance in pregnant patients with T1D, the immune system manages to protect the foreign antigens in a developing fetus even during ongoing autoimmune destruction of pancreatic β cells.

Pregnancy in patients with T1D is associated with increased risks of complications, including preeclampsia, premature delivery, congenital malformations, perinatal death, and perigestational mortality (9). These risks increase with hyperglycemia (10) but arise alongside immune alterations that may independently contribute to these risks. In healthy pregnant individuals, peripheral blood lymphocytes decrease; however, in T1D, patients exhibit similar pregestational and perigestational lymphocyte counts (11). Cord blood from infants delivered by a parent with T1D contains increased Tregs, suggesting an increased need for regulatory cells to dampen the immune response (12). While these findings in peripheral blood illustrate a difference between patients with T1D and healthy patients, it is unknown how these alterations may impact gestation. Using nonobese diabetic (NOD) mice, a mouse model of T1D, we studied the placenta to understand how defective tolerance may impact gestation at the parental-fetal interface. We then used a large electronic health record (EHR) database to explore potential differences in vascular outcomes (including preeclampsia, eclampsia, and HELLP [hemolysis, elevated liver enzyme and low platelet] syndrome) in pregnant patients with and without T1D across early pregnancy hemoglobin A1c levels.

MATERIALS AND METHODS

Animals

C57BL6/J (B6) and NOD/ShiLtJ (NOD) mice were purchased from the Jackson Laboratory. Mice were housed and bred in a specific pathogen-free facility at Vanderbilt University according to the protocols approved in IACUC M1500016–02.

Mating Protocol

Male mice were housed individually. Three days before mating, bedding from male cages was transferred into female cages to induce estrus. In the evening of day 0, 1 female mouse was placed into each individual male cage. Mice were separated the next morning at day 0.5, and mating was confirmed by the presence of a mucous plug. Experiments were performed on day 17.5 of gestation in mice confirmed to be nondiabetic. The gravid uterus was examined for evidence of reabsorbed fetuses as well as for pups that appeared normal or abnormal (small, aberrant in color, or with obvious fetal anomalies).

Interleukin-6 Neutralization

Beginning at gestational day 5.5, pregnant dams were treated every 2 days with 200 μg of an interleukin 6 (IL-6)-neutralizing antibody (αL-6) injected intraperitoneally (clone MP5–20F3 #BE0046, BioXCell, Lebanon, NH).

Flow Cytometry

Pregnant dams were euthanized before hysterectomy. Individual placentas were dissected from the uterus with the removal of the decidua before manual dissociation and digestion in a solution of Hanks' Balanced Salt Solution + 5% fetal bovine serum (FBS), 1% 0.5-M $CaCl_2$, and 10 mg/mL of collagenase P. The placentas were digested at 37°C for 15 minutes and quenched with Hanks' Balanced Salt Solution + 10% FBS. Splenocytes were collected by manual dissociation of the spleen. Placental cells or splenocytes were stained with the following antibodies: CD45 (30-F11), B220 (RA3–6B2), CD8 (53–6.7), $H2K^d$ (SF1–1.1), CD21 (7G6), CD49b (DX5), and CD31 (MEC13.1) purchased from BD Bioscience (Franklin Lakes, NJ); $H2K^b$ (AF6–88.5.5.3), Foxp3 (FJK-16S), immunoglobulin M (II/41), CD23 (B3B4), CD3 (17A2), TCRβ (H57–597), CD19 (eBio1D3), and CD11B (M1.70) purchased from eBioscience (San Diego, CA); and CD4 (RM4–4), CD19 (6D5), and CD268 (7H22-E16) purchased from BioLegend (San Diego, California). The eBioscience Foxp3 Transcription Factor Staining Kit (#00–5523-00) was used for the detection of Foxp3. Splenocytes were used as compensation controls.

Histopathology

Pregnant dams were euthanized, and individual placentas were dissected from the uterus with the removal of the decidua. The placentas were placed into cassettes in 10% Formalin, processed routinely, and embedded in paraffin, and slides cut at 5 microns were stained with hematoxylin and eosin. Immunohistochemistry for B220 (BD Bioscience #553086) was performed. Three regions of interest 1.2 mm in diameter were randomly selected for each of 3 placentas per group, and the total B220+ cell number was counted.

Cytokine Secretion Assay

Pregnant dams were euthanized, and individual placentas were dissected from the uterus with the removal of the decidua. Manually dissociated placentas were plated overnight in 5% Dulbecco's Modified Eagle's Medium cell culture media (with 10% FBS, penicillin/streptomycin, and β-mercaptoethanol) with 1 μM of R848 (#73782 STEMCELL Technologies, Vancouver, Canada) and 8 μg/mL of αCD3/αCD28 (BD #553057 and #553294). After 12 hours of stimulation, GolgiStop (BD #554724) was added at a concentration of 0.66 μL/mL of cell culture media. Six hours after the addition of GolgiStop, cells were harvested and stained using a Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD #554715). Cells were stained for extracellular markers as listed earlier with IL-6 (MP5–20F3) from eBioscience.

Cytokine Array and Enzyme-Linked Immunosorbent Assay

Pregnant dams were euthanized, and amniotic fluid was collected from healthy-appearing fetuses by inserting a heparinized tube (#02–668-10, ThermoFisher Scientific, Waltham,

MA) into the amniotic sac. Amniotic fluid was collected in microcentrifuge tubes and centrifuged for 8 minutes at 6,800 rcf to pellet red blood cells. Amniotic fluid was stored at −80°C, before analysis for cytokines and IL-6. Samples were sent to Eve Technologies (#MD31 Mouse Cytokine 32-Plex, Calgary, Alberta, Canada) for a cytokine array. The IL-6 level was measured using an IL-6 enzyme-linked immunosorbent assay (ELISA) kit (BD #555240).

Mouse Data Analysis

Mouse data were analyzed with GraphPad Prism version 9.2.0. An unpaired Student's *t* test with Welch's correction was used to compare differences between 2 groups. For multiple groups, a one- or two-way analysis of variance with Šidák's multiple-comparison post hoc test was used. Statistical values with $P \leqq .05$ were considered significant.

EHR Data and Phenotyping

With approval from Vanderbilt University Medical Center (VUMC) (Institutional Review Board #212013), we accessed VUMC's deidentified database of EHRs (>3.1 million patients) and assembled a pregnancy cohort with T1D status and vascular complications during pregnancy. For each patient, we used billing codes that included International Classification of Diseases, 9th/10th Revision, Clinical Modification (ICD-9/10-CM) and Current Procedural Terminology to demarcate the first pregnancy and delivery, ascertain T1D status, and identify vascular complications. The EHRs of this cohort ranged from September 10, 1988 to December 30, 2020.

To demarcate the first pregnancy within an EHR, we required at least 1 billing code for delivery no more than 45 weeks after a billing code indicative of prenatal care. To avoid correlation among outcomes derived from the same individual with multiple pregnancies, we considered only the first pregnancy recorded in the EHR. Next, we used a validated and accurate phenotyping algorithm (13) that incorporated billing codes, clinical laboratories, and diabetes-related medications to ascertain patients with T1D diagnosed before prenatal care as determined by first prenatal billing code. We assigned an A1c value measured closest to and within 3 months of the billing code indicative of prenatal care (14). To identify pregnancies with vascular complications, we required patients to have at least 1 Phecode v2 (15, 16), a condensed and expert curated set of diagnoses mapped from the ICD-9 and ICD-10 codes, for vascular phenotypes as specified in Supplemental Table 1 (available online) occurring between 9 months before delivery and 3 months after delivery. All other billing codes and phecodes used for phenotyping are provided in Supplemental Tables 2 and 3 and at https://github.com/abraham-abin13/a1c_pregnancy_outcomes.git.

Association of the A1c Level With Vascular Outcomes

Our exposure of interest was T1D status before the first recorded pregnancy's delivery date. Our outcomes included a composite of vascular phenotypes (Supplemental Table 1) and preeclampsia, eclampsia, or HELLP syndrome. We assessed for effect modification between T1D and hemoglobin A1c at each participant's first recorded prenatal visit in the EHR using an interaction term. We adjusted for covariates that may have confounded the relationship between T1D and the outcome. These included EHR-collected race (White,

Black, unknown, Asian, or Native American), age at the first pregnancy encounter, and socioeconomic status (17, 18). Further discussion about covariate selection is found in the supplemental methods (available online) (19–32).

Individuals without T1D were missing hemoglobin A1c values by design. We, therefore, imputed them randomly from a normal distribution that reflected the population mean A1c value among individuals without T1D (mean of 5.3, standard deviation of 0.1) (33, 34). Imputing from a normal distribution enabled computational convergence for regression models. We then performed 35 multiple imputations with chained equations with the exposures, covariates, and outcomes to account for missing covariate data (34). Finally, we used logistic regression to assess the probability of each outcome by T1D status (with A1c fixed to 5.3 in individuals without T1D), modified by A1c, controlled for the aforementioned covariates pooled across the imputed datasets (34). A1c was modeled as a restricted cubic spline with 3 knots given existing evidence that A1c has a nonlinear relationship with vascular outcomes (35). Other continuous covariates were modeled as a linear relationship given the small number of outcomes. Analyses with EHR data were performed using Python v3.8 with Pandas v1.3.4 (36) and NumPy v1.19 (37) and *R Statistical Software* (version 4.1.0). Python and R code is available at https://github.com/abraham-abin13/a1c_pregnancy_outcomes.git.

RESULTS

The Uterine Environment in NOD Mice is Associated With Poor Fetal Outcomes and Abnormal Placental Lymphocytes

We assessed the impact of autoimmunity in gestation by crossing B6 and NOD mice to create genetically identical litters. Nonobese diabetic mice, which spontaneously develop both anti-islet autoimmunity and overt diabetes, were used before the onset of hyperglycemia to isolate the effects of autoimmunity like the presence of autoreactive lymphocytes and autoantibodies. Mice were either paired as B6 females mated with NOD males (B6♀NOD♂) or NOD females mated with B6 males (NOD♀B6♂). Litters from these pairings differ only by the uterine environment in which pups gestate. The NOD (NOD♀B6♂) uterine environment resulted in increased reabsorbed and abnormal pups compared with B6♀NOD♂ litters (Fig. 1A and B). Whereas 98% of B6♀NOD♂ fetuses were normal, only 67% of NOD♀B6♂ fetuses appeared normal. Flow cytometric evaluation of placental immune cells revealed alterations in the immune cell compartment. NOD♀B6♂ placentas had a lower percentage of $CD8^+$ cells of total cells (P=.003) and an even greater decrease in $CD19^+$ cells than B6♀NOD♂ (P=.0002); these findings in allogenic B6♀NOD♂ and NOD♀B6♂ pairings were similar to those in syngenic B6♀B6♂ and NOD♀NOD♂ pairings, respectively (Fig. 1C). Histologic examination corroborated the decrease in B cells associated with the NOD maternal environment (Fig. 1D and E). We assessed whether the alterations in immune cells were due to maternal or fetal abnormalities by staining for the B6 major histocompatibility complex class I, $H2K^b$, on immune cells in allogenic NOD♀B6♂ pairs. In the placenta, as validated with uterine-draining lymph nodes, peripheral blood mononuclear cells, and spleen, all immune cells were H2Kd+, indicating that the cells were maternal and not fetal in origin (Fig. 1F). In summary, we conclude that the changes

in maternal lymphocytes are associated with the abnormal gestational environment in NOD mice that results in larger numbers of abnormal and reabsorbed pups.

NOD Amniotic Fluid Contains Increased IL-6 Secreted by Endothelial Cells

To better understand potential etiologies of adverse pregnancy outcomes in NOD mice, we used a multicytokine array to investigate the cytokines present in amniotic fluid (Fig. 2A). Amniotic fluid from the NOD uterus (both NOD♀NOD♂ and NOD♀2B6♂) contained increased levels of IL-6 family members, including IL-6, leukemia inhibitory factor, and monocyte chemotactic protein-1 (Fig. 2B). Increased IL-6 secretion into NOD amniotic fluid was confirmed by ELISA, with significant increases in NOD♀NOD♂ compared with B6♀B6♂ (P=.0059) as well as NOD♀B6♂ compared with B6♀NOD♂ (P=.05) (Fig. 2C). To identify the specific cell type secreting IL-6, the placentas were activated by R848 and αCD3/αCD28 in cell culture overnight and then incubated with GolgiStop to retain intracellular cytokines. We found that IL-6 was secreted by multiple cell types, notably including Foxp3+ Tregs and CD31+ endothelial cells (Fig. 2D). NOD\B6_ placentas showed increased IL-6 secretion by CD4+ cells, including Foxp3+ Tregs, and by CD31+ endothelial cells (Fig. 2E). Further examination revealed that NOD♀B6♂ endothelial cells were increased in both number and IL-6 mean fluorescence intensity (Fig. 2F). These data identify IL-6 secretion by endothelial cells in the placenta as a possible mechanism of the adverse pregnancy outcomes in NOD mice.

Neutralization of IL-6 Improves Outcomes in NOD Pregnancies

Aberrant IL-6 levels are associated with several pregnancy-related diseases and can be harmful when insufficient or in excess (38–41). Given the increased baseline IL-6 level in NOD amniotic fluid, we investigated the impact of decreasing IL-6 levels by administering an IL-6-neutralizing antibody (αIL-6) throughout gestation. The impact of IL-6 neutralization varied greatly with uterine environment; outcomes worsened for B6♀NOD♂, whereas NOD♀B6♂ litters benefited from decreased IL-6 levels, with fewer reabsorbed fetuses and abnormal pups (Fig. 3A and B). While 64% of fetuses from B6♀NOD♂ pairings treated with αIL-6 appeared normal, 93% of fetuses NOD♀B6♂ treated with αIL-6 appeared normal. The administration of αIL-6 decreased the amniotic fluid IL-6 levels in NOD♀B6♂ to levels similar to untreated B6♀NOD♂ pairings (Fig. 3C). Increased IL-6 levels in NOD♀B6♂ mice are correlated with a significant increase in the IL-6-secreting CD31+ cells compared with those in B6♀NOD♂ pairs (P=.0009) (Fig. 3D), but αIL-6 treatment did not alter the number of CD31+ cells in either B6♀NOD♂ or NOD♀B6♂ pairs. CD31 has been well established as a regulator of leukocyte transmigration that aids in the emigration of inflammatory leukocytes into target tissue (42–44). We found decreased DX5+ natural killer (NK) cells in NOD♀B6♂ placentas compared with those in B6♀NOD♂ placentas (P=.0113) (Fig. 3E); this alteration was partially restored by the administration of αIL-6. We conclude that the increased IL-6 levels from CD31+ cells in NOD mice has a harmful effect on gestation; furthermore, the increased CD31+ cell number is correlated with a decrease in NK cells.

Patients With T1D, Across all Early Pregnancy Hemoglobin A1cs, Display Increased Vascular Complications in Pregnancy

While the NOD mouse spontaneously develops diabetes like individuals with T1D, our studies identified changes in the placenta and uterine environment before the onset of hyperglycemia. Studies in patients with T1D have reported increased risks of vascular complications like preeclampsia (45), but the impact of normal or target A1c on vascular complications remains poorly understood relative to the general pregnant population. Using the EHR at VUMC, we identified 354 pregnant individuals with and 45,467 without T1D. Among pregnant individuals with T1D, 55 (15.5%) experienced a vascular complication in pregnancy compared with 3,633 (8%) of those without T1D (Supplemental Table 1 and Table 4). Given the reported increased risk of adverse vascular outcomes with higher preconception A1c (9, 10, 46), we hypothesized that hemoglobin A1c early in pregnancy modifies the probability of adverse vascular outcomes. We found that patients with T1D have an increased likelihood of experiencing vascular complications in pregnancy regardless of their A1c (Fig. 4A). Even at a normal A1c level of 5.5, pregnant individuals with T1D had increased odds (2.17; 95% confidence interval [CI], 1.3–3.62) of vascular pathology relative to patients without T1D (Fig. 4B). Furthermore, we found that pregnant individuals with T1D were more likely to experience preeclampsia, eclampsia, or HELLP syndrome relative to patients without T1D regardless of their A1c (Fig. 4C). Notably, the odds of developing preeclampsia, eclampsia, or HELLP syndrome were increased (4.02; 95% CI, 2.2–7.33) even at a normal A1c level of 5.5 (Fig. 4D). Figure 4A and C show the shape of the relationship between A1c and each outcome among individuals with T1D. While we detected increased probabilities of adverse vascular outcomes regardless of A1c, the small cohort size resulted in wide CIs at higher A1c levels. Thus, the probability of adverse vascular outcomes may increase more dramatically at higher A1c values than shown in Figure 4A and C. Even so, the increase in vascular complications at a normal A1c level compared with the general population shows an effect independent of glycemia on pregnancy outcome.

DISCUSSION

This study reveals the precarious nature of pregnancy in T1D. Previous work has failed to tease apart the impacts of hyperglycemia from those attributable to an autoimmune environment; furthermore, previous studies examining peripheral or cord blood lymphocytes revealed cell alterations that were understudied at the actual parental/fetal interface. The mechanism by which patients with T1D experience increased complications in pregnancy remains to be elucidated.

We show that NOD mice have an increased prevalence of abnormal and reabsorbed fetuses. Using flow cytometry, immunohistochemistry, and ELISA, we found that genetically identical fetuses have disparate outcomes determined by the uterine environment, illustrated by alterations in placentallymphocytes and cytokines. The immune cell environment in B6♀NOD♂ and NOD♀B6♂ mice mirror those of their syngenic B6♀B6♂ and NOD♀NOD♂ counterparts (Supplemental Figs. 1 and 2). Notably, NOD♀NOD♂ and NOD♀B6♂ mice had decreased placental B lymphocytes, with deficits in both follicular and marginal zone B

lymphocytes (Supplemental Fig. 3A). In contrast, other organs in NOD mice host expanded marginal zone B lymphocytes that present autoantigen to T cells (47).The absence of these diabetogenic B lymphocytes from the placenta underlies the tolerogenic tendencies of the uterus and gestation-associated lymphocytic changes. Previous work proposed that B cells function in the placenta predominantly as anti-inflammatory secretors of IL-10 (48); the B lymphocytes found in the NOD uterus do not appear to be secreting IL-10 (Supplemental Fig. 3B). As B lymphocytes play an important role in the pathogenesis of T1D, it is tempting to speculate that their absence from the placenta is required for the fragile tolerance developed in pregnancy.

Interestingly, our studies also implicated a common culprit in adverse pregnancy outcomes, IL-6. Both insufficient and excess IL-6 have been associated with poor prognosis, and increased levels of IL-6 family members like leukemia inhibitory factor are required for pregnancy (49). Notably, IL-6 secretion from endothelial cells is increased in patients with preeclampsia (41, 50), which decreases the activity of Foxp3+ CD4+ Tregs (51, 52). Localizing the increased IL-6 secretion to CD31+ cells, which are found to be increased in NOD♀B6♂, may explain the increased levels of IL-6 present in NOD♀B6♂ amniotic fluid. CD31 on endothelial cells aids in leukocyte transmigration; the presence of increased CD31+ cells in NOD mice may promote the translocation of inflammatory cells into the placenta and worsen pregnancy outcomes (42, 44, 53). In contrast, decidual DX5+ NK cells are beneficial in pregnancy and aid in remodeling spiral arteries (54). Nonobese diabetic mice may be subject to a deleterious cycle in which inflammatory IL-6-secreting CD31+ cells increase due to a lack of sufficient NK cells.

Our findings identifying the pathologic effect of IL-6 secretion by endothelial cells in prediabetic NOD mice may reflect the interplay between vascular changes in autoimmune pregnancy and the increased prevalence of preeclampsia in patients with T1D (55). As patients with T1D are not treated with immunosuppressive medications, the impact of autoimmunity on pregnancy is important but previously unknown. When we examined the prevalence of vascular complications in human pregnancy, we noticed an increased proportion of patients with T1D and vascular complications across all A1cs. These data indicate that adverse events in T1D pregnancy cannot purely be tied to glycemic control and suggest a role for abnormal or autoimmunity in pregnancy. While pregnant patients with T1D may have underlying vascular disease that contributes to preeclampsia, we expect the risk of this underlying pathophysiology to be spread across the cohort or even more heavily affecting patients with the highest A1c levels. Our finding of increased risk at patients with target A1cs indicates that the underlying effects of autoimmunity may exacerbate the inflammatory effects of preeclampsia. Further studies may allow for a mechanistic understanding of how vascular complications arise in euglycemic patients with T1D.

While we identified gestational complications in the NOD mouse that are correlated with findings in patients with T1D, our studies were limited by several factors. We were unable to capture data from reabsorbed mouse fetuses; our data may represent a survival bias in fetuses that survived to preterm gestation. Furthermore, all placentas from each individual mouse were averaged together; some of the diversity in phenotypes of individual mouse placentas may reflect the diversity in disease onset or severity in adult mice. Because fetal

mice were sacrificed for placental examination, we were unable to follow them to adulthood to monitor for diabetes onset. Autoimmune features are more present in female NOD mice than in male NOD mice, which minimizes the effect of paternal autoimmunity on this study that was designed to investigate the effects of autoimmunity on the uterine environment in the presence of genetically identical pups. Additionally, while we were as restrictive as possible while collecting information from the EHR, we may have an incomplete dataset because of the inconsistent usage of billing codes. Our control group also likely included individuals with other autoimmune conditions or metabolic conditions, which would have biased our effect estimates toward nonsignificance. We have included a detailed discussion of study design and bias considerations inherent to this type of analysis in the supplement. While the low number of included pregnant individuals with T1D and adverse vascular outcomes makes it difficult to accurately assess risk at higher A1c levels, we are confident in the findings at lower A1c levels that are directly related to our hypothesis of an increased risk of vascular complications at lower A1c levels. Regardless, the clinical information we gathered and analyzed revealed a strong signal of an effect that builds on previous knowledge of complications in humans with T1D.

CONCLUSION

In conclusion, our study describes alterations in the placental immune compartment from fetuses gestated in a prediabetic NOD mouse. These alterations coexist with an increase in the inflammatory IL-6 levels secreted by endothelial cells, the alleviation of which allows for better fetal outcomes. Our findings in mice are correlated with clinical information from patients showing an increase in vascular complications even with normoglycemic A1c levels. This work sets the stage for further investigation of the mechanism of collaborative immune and vascular dysfunction in pregnancy in T1D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

Flow cytometry experiments were performed in the VUMC Flow Cytometry Shared Resource.

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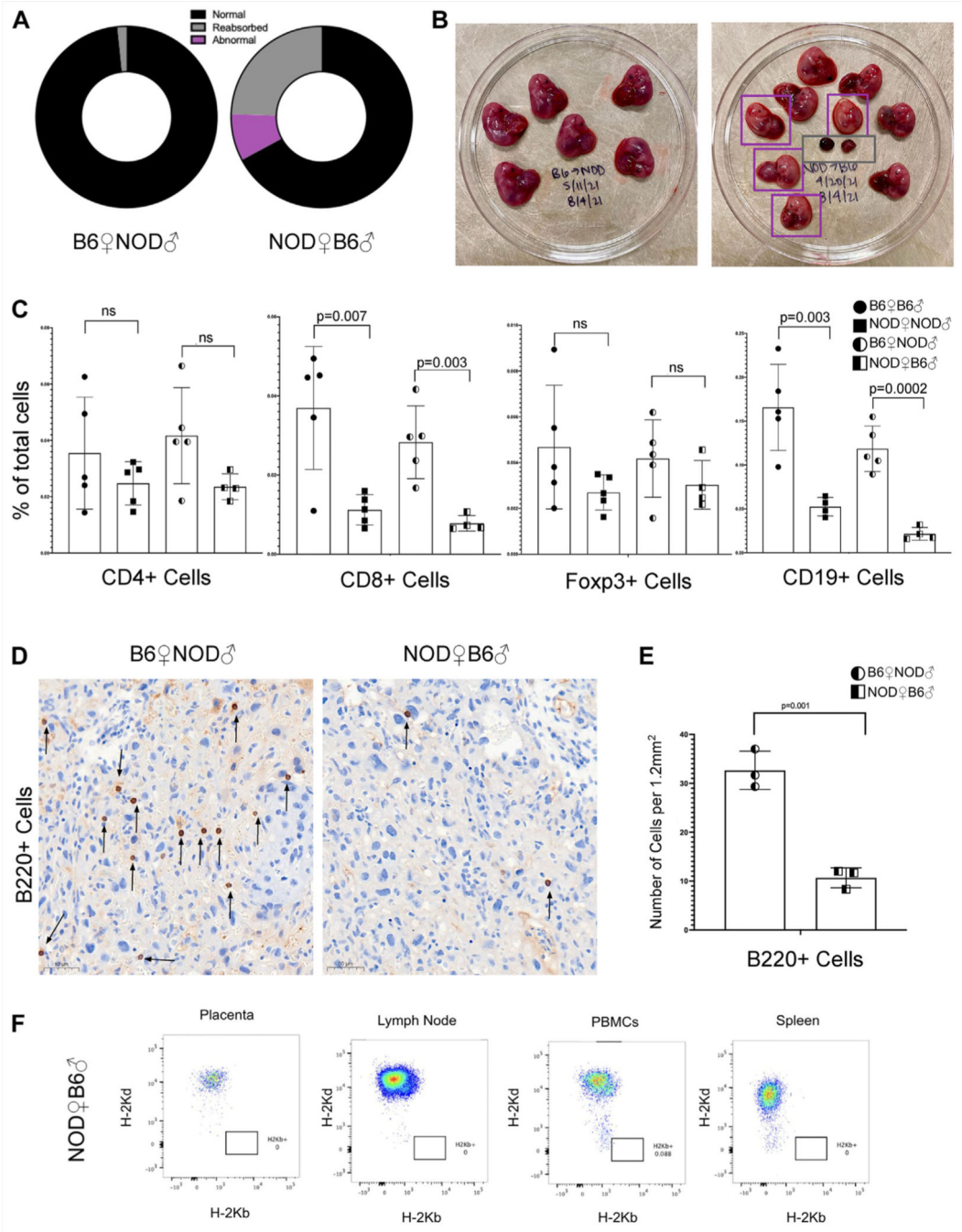

FIGURE 1.

Adverse pregnancy outcomes in NOD pregnancy and the associated pregnancy immune phenotypes for B6 and NOD crosses. (**A**) Composite pregnancy outcomes for B6♀NOD♂ and NOD♀B6♂ mice. Of 18 B6♀NOD♂ litters, 2 of 123 fetuses are reabsorbed, and no fetuses are abnormal. Of 17 NOD♀B6♂ litters, 35 of 143 fetuses are reabsorbed, and 12 of 143 fetuses are abnormal. (**B**) Representative images of B6♀NOD♂ (*left*) and NOD♀B6♂ (*right*) litters. Outlined in purple are 4 abnormal fetuses (*small* and *pale*); additionally, 2 reabsorbed fetuses are outlined in gray. (**C**) Immune cells as the percentage of total cells

in the B6✝NOD♂ (n = 5) and NOD✝B6♂ (n = 4) placenta, shown with B6♀B6♂ and NOD♀NOD♂ pairings. As a percentage of total cells, NOD♀B6♂ litters have decreased CD8+ cells (P=.003) and CD19+ cells (P=.0002). Each dot represents 1 mouse pregnancy with an average of all placentas analyzed from that mouse. (**D**) Immunohistochemical staining of B6♀NOD♂ (*left*) and NOD♀B6♂ (*right*) placentas for B220. *Arrows* illustrate positive cells. (**E**) Graphical representation of the number of B220+ cells per 1.2-mm^2field of view. Each point represents the average of 3 random 1.2-mm^2 circles per placenta (n = 3). Nonobese diabetic mice have decreased B220+ B cells. (**F**) Lymphocytes in the placentas of NOD♀B6♂ mice are maternal. Flow plots from an NOD♀B6♂ litter with H2kd (NOD major histocompatibility complex class I) vs. H2Kb (B6 major histocompatibility complex class I) show that in the placenta, lymph nodes, peripheral blood mononuclear cells, and spleen, all cells are H2Kd+ and, therefore, maternally derived. NOD = nonobese diabetic.

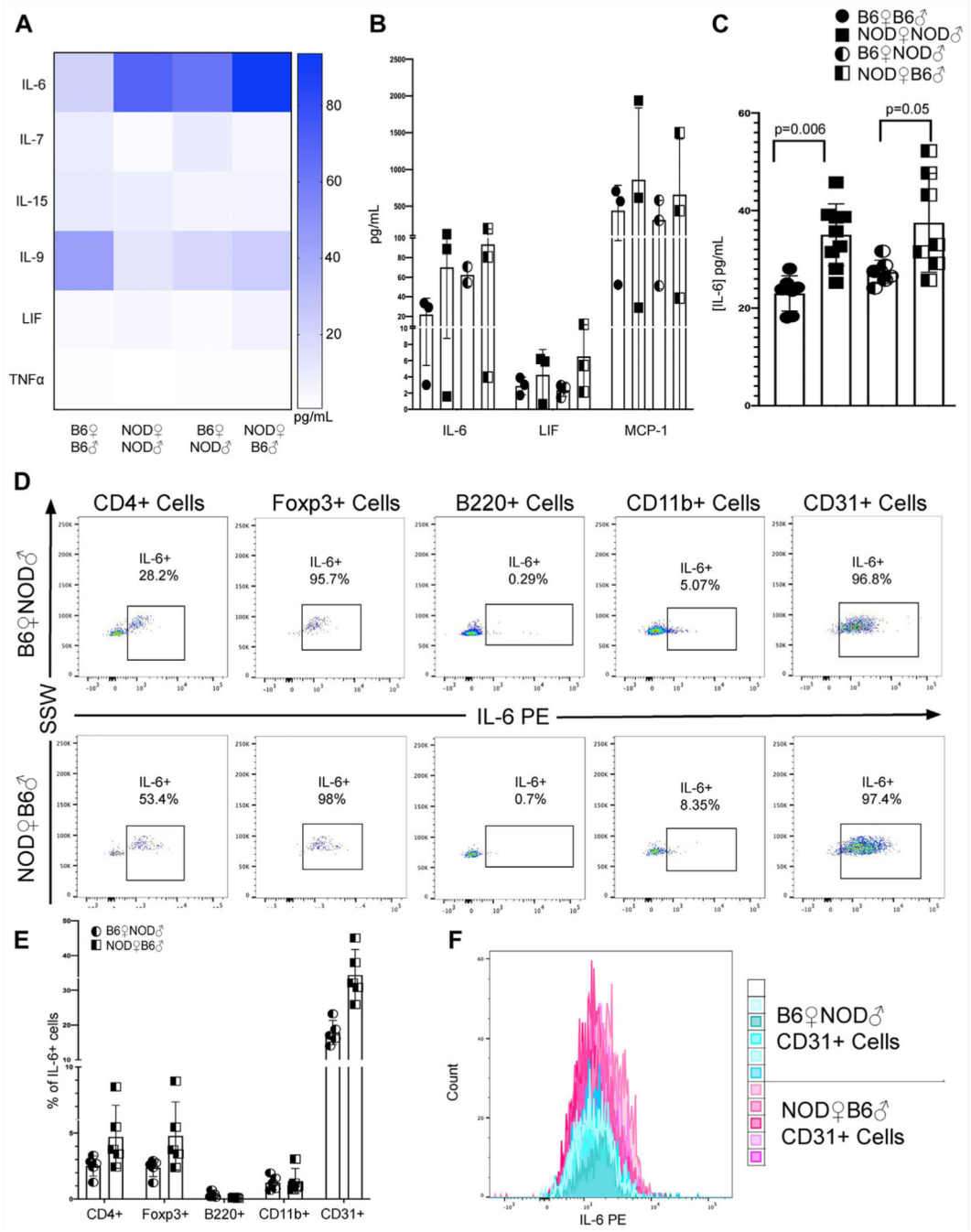

FIGURE 2.

Cytokine analysis reveals increased interleukin-6 (IL-6) levels in NOD litters. (**A**) Selected cytokines from an unbiased cytokine array showing levels in amniotic fluid in picograms per milliliter. (**B**) Graphical representation of the levels of IL-6 family members from cytokine array (n = 3 per mouse pairing). NOD♀NOD♂ and NOD♀B6♂ litters have increases in IL-6, leukemia inhibitory factor, and monocyte chemotactic protein-1 levels. (**C**) The increase in the IL-6 level in amniotic fluid from NOD mice is confirmed via enzyme-linked immunosorbent assay, where each point represents amniotic fluid from 1 mouse uterus.

(**D**) The placentas from B6♀NOD♂ and NOD♀B6♂ litters are stimulated with R848 and αCD3/αCD28 overnight and then incubated with GolgiStop for 4 hours to capture cytokine secretion. Interleukin-6 secretion from CD4+, Foxp3+, B220+, CD11b+, and CD31+ cells shows that the most IL-6 secretion is coming from CD31+ endothelial cells. (**E**) Percentage of CD4+, Foxp3+, B220+, CD11b+, and CD31+ of IL-6-secreting cells, where each point represents 1 placenta. Of IL-6-secreting cells, CD31+ cells had the largest percentage in both B6♀NOD♂ and NOD♀B6♂ litters. (**F**) The histogram of all placentas per B6♀NOD♂ litter (*blues*) and NOD♀B6♂ litter (*pinks*) shows that on average, NOD♀B6♂CD31+ cells have an increase in stored IL-6. IL = interleukin; NOD = nonobese diabetic; SSW = side scatter width; TNFα= tumor necrosis factor-α.

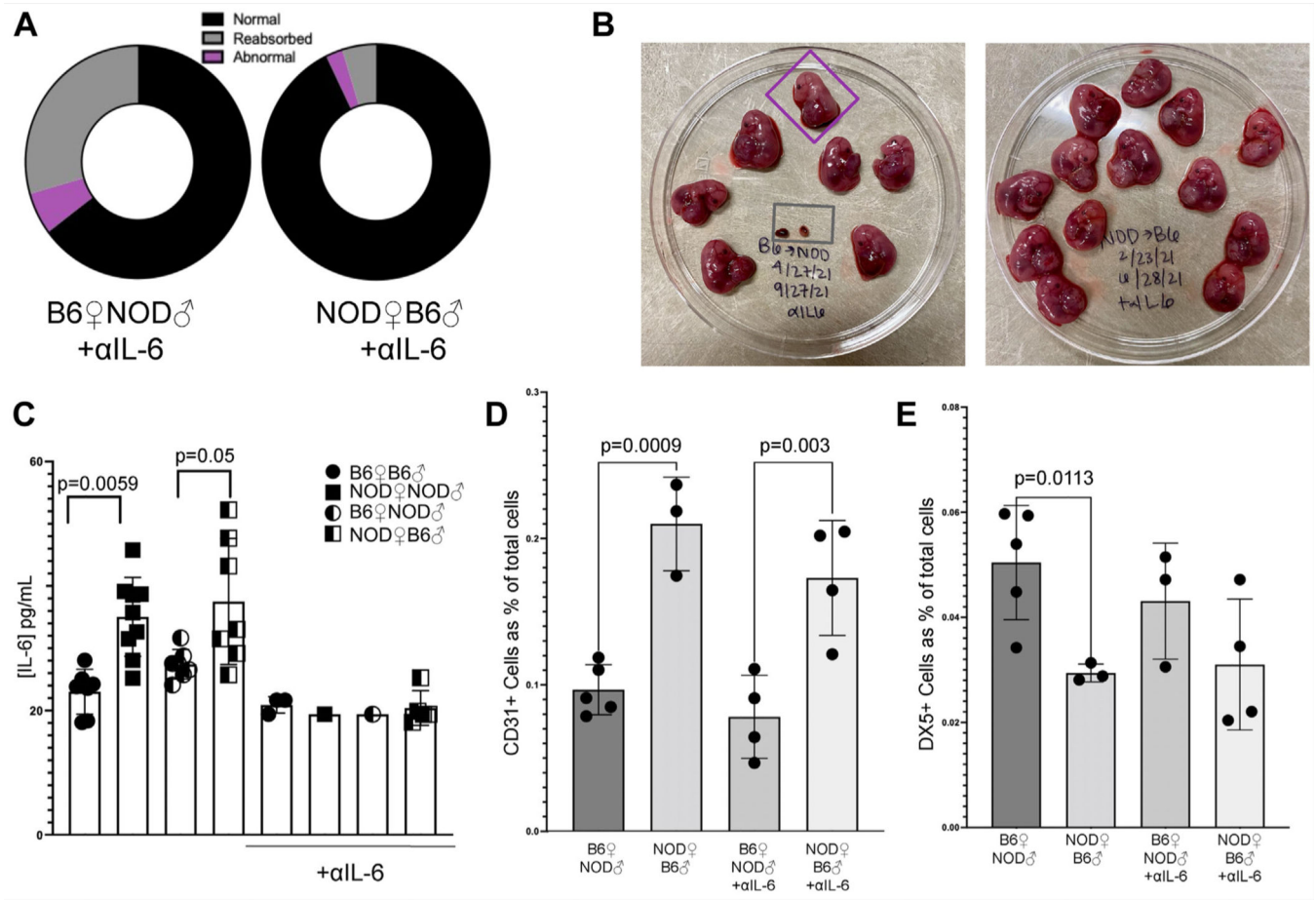

FIGURE 3.

Impact of treatment with 200 μg of an interleukin-6 (IL-6)-neutralizing antibody (αIL-6) every other day starting on day 5 of gestation. (**A**) B6♀NOD♂ and NOD♀B6♂ mice that were treated with anti-IL-6 have vastly different responses to treatment. Of 5 B6♀NOD♂+αIL-6 litters, 10 of 34 fetuses are reabsorbed, and 2 of 34 fetuses are abnormal. Of 10 NOD♀B6♂+αIL-6 litters, 4 of 83 fetuses are reabsorbed, and 2 of 83 fetuses are abnormal. B6♀NOD♂ + αIL-6 litters have increased reabsorbed and abnormal fetuses, both relative to B6♀NOD♂ and NOD♀B6♂ + aIL-6 litters. (**B**) Representative images of B6♀NOD♂ and NOD♀B6♂ litters after anti-IL-6 treatment. The pictured B6♀NOD♂ litter has 1 abnormal fetus, outlined in purple, and 2 reabsorbed fetuses, outlined in gray. (**C**) The administration of αIL-6 decreases the levels of IL-6 present in amniotic fluid, where each point represents amniotic fluid from 1 mouse uterus. (**D**) Both NOD♀B6♂ without treatment and treated with αIL-6 have a significant increase in placental CD31+ endothelial cells relative to B6♀NOD♂ mice. Each dot represents 1 mouse pregnancy with an average of all placentas analyzed from that mouse. (**E**) NOD♀B6♂ mice have significantly fewer placental DX5+ NK cells relative to B6♀NOD♂ mice, which may have normalized with αIL-6 treatment. Each dot represents 1 mouse with an average of all placentas analyzed from that mouse. NOD = nonobese diabetic.

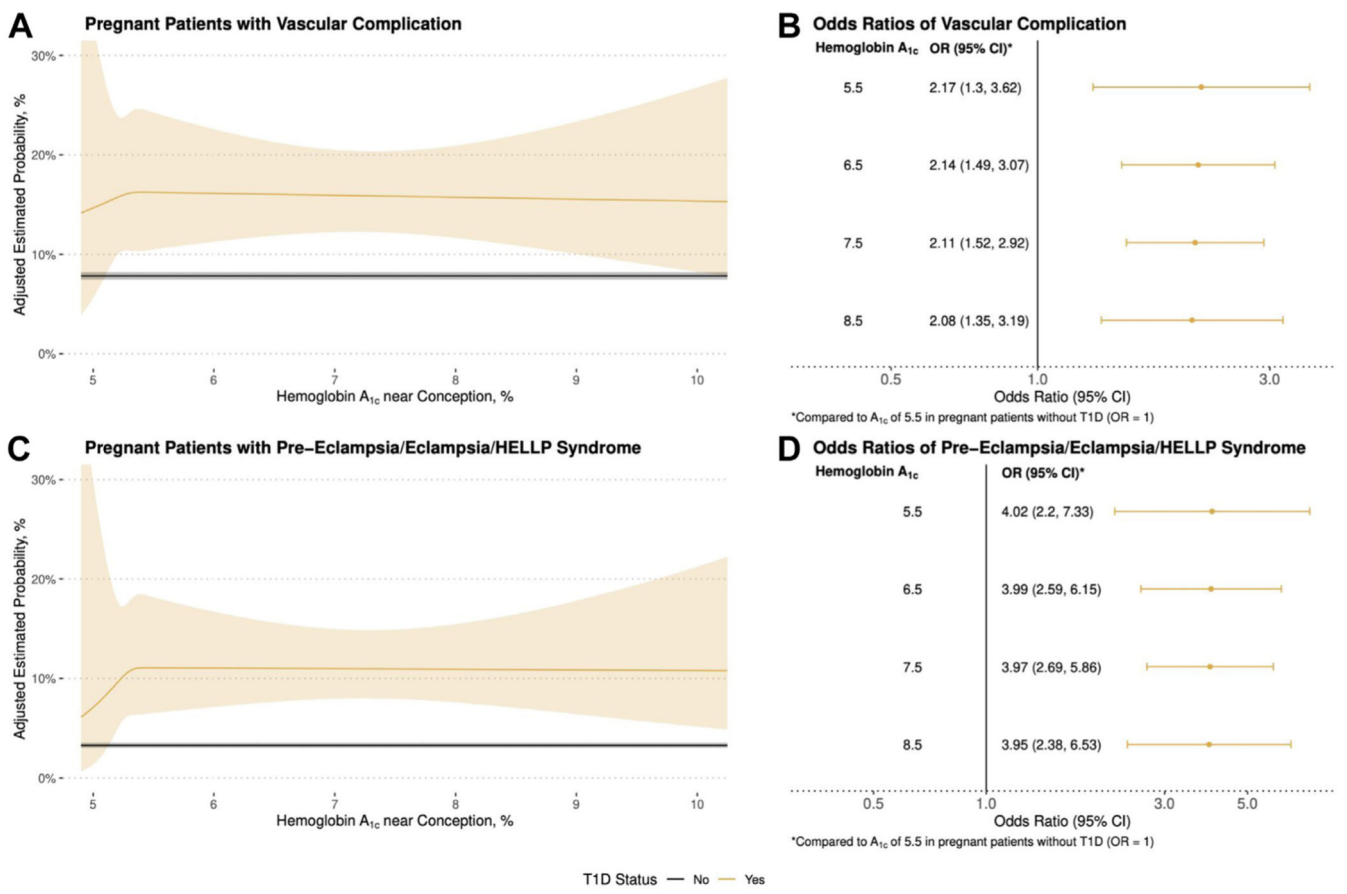

FIGURE 4.

Electronic health record data illustrating differences in vascular outcomes in patients with type 1 diabetes (T1D) compared with the general population regardless of A1c. (**A**) Proportion of pregnant patients with a vascular complication in the general population (*black line*) and with T1D (gold line with 95% confidence interval [CI]). (**B**) When stratified by hemoglobin A1c at the first recorded antenatal visit, patients with T1D have an increased odds ratio (OR) of a vascular complication across all A1c values. The ORs are calculated for a pregnant person with T1D and A1c levels of 5.5, 6.5, 7.5, and 8.5. relative to a pregnant person without T1D with an A1c level of 5.5. (**C**) Proportion of patients with phecodes for preeclampsia, eclampsia, or HELLP syndrome in the general population (*black line*) and with T1D (*gold line* with 95% CI). (**D**) When stratified by hemoglobin A1c near conception, patients with T1D have an increased OR of preeclampsia, eclampsia, or HELLP syndrome across all A1c values. The ORs are calculated for a pregnant person with T1D and A1c levels of 5.5, 6.5, 7.5, and 8.5. relative to a pregnant person without T1D with an A1c level of 5.5. CI = confidence interval; HELLP = hemolysis, elevated liver enzymes, low platelets; OR = odds ratios; T1D = type 1 diabetes.