Fetal Sex-Based Differences in Maternal Hormones, Angiogenic Factors, and Immune Mediators During Pregnancy and the Postpartum Period

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Problem
Several pregnancy complications have disparities based on the sex of the fetus. It is unknown whether the sex of the fetus differentially alters the maternal immune milieu, potentially contributing to the observed differences.

Method of study
Using maternal plasma collected during 38 uncomplicated pregnancies (19 males, 19 females), we compared levels of cytokines, sex hormones, and angiogenic factors throughout gestation and postpartum.

Results
Male fetal sex was associated with higher levels of proinflammatory cytokines (G-CSF, IL-12p70, IL-21, and IL-33) and angiogenic factors (PlGF and VEGF-A) compared with female fetal sex at multiple timepoints. Female fetal sex was associated with higher levels of regulatory cytokines (IL-5, IL-9, IL-17, and IL-25). IL-27 increased throughout pregnancy regardless of fetal sex. There was no fetal sex-based difference in analyte concentrations at the postpartum measurement.

Conclusion
Women carrying a male fetus exhibit a more proinflammatory/proangiogenic immune milieu than women carrying a female fetus.

Introduction

Although pregnancy is known to alter the maternal immune milieu,[1–3] it is unknown whether the sex of the fetus results in distinct maternal immune changes throughout the course of pregnancy. This question is relevant given that several observations of disparate outcomes in pregnancy are based on fetal sex.[4,5] Women carrying male fetuses have disproportionate rates of preterm births, higher birth weights, and greater fetal mortality.[6,7] A study of women diagnosed with preeclampsia found that women carrying male fetuses had a significant impairment in vasodilation in response to the potent vasodilator corticotrophin-releasing hormone (CRH).[8] Yet others have found women carrying female fetuses had a higher risk of hypertensive disorders[9] and asthma flares.[10] Fetal sex-based differences in maternal adaptation to pregnancy can have long-term consequences, as seen in studies of the effects

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of fetal sex on maternal nutrient deprivation and excess.[11,12] These observations suggest that differences in the maternal immune response based upon the sex of the fetus are important for both short-term and long-term health outcomes.

Recent studies have assessed mechanisms underlying fetomaternal immunity and their effects on pregnancy outcome based on fetal sex. Histological examination of placentas from babies delivered before 32 weeks found that male placentae had more inflammation, possibly as a result of a more robust maternal immune response to male fetuses.[13] Pathway enrichment analyses of placental tissue mRNA revealed an increase in expression of genes involved in the immune system, inflammation, and graft-versus-host disease during gestation based on male fetal sex,[14] while genes that were increased during gestation when carrying a female fetus were involved in immune regulation and included such genes as *JAK1, IL2RB, CXCL1*, and *IL1RL1*.[15] In addition, mothers with mild asthma who carried a female fetus had increased placental expression of inflammatory and allergic mediators including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8 and IL-5 mRNA.[16] Fetal sex-based immune changes have also been observed on the fetal side. Umbilical cord blood from healthy term male fetuses had greater proinflammatory responses *in vitro* to lipopolysaccharide (LPS) based on measures of IL-1β and IL-6.[17] While these studies represent critical observations, they are based on single time points and do not assess longitudinal changes that occur throughout the course of gestation.

On the basis of these prior observations, we hypothesized that the maternal cytokine and growth factor milieu would be more inflammatory in women carrying male versus female fetuses and that these differences would be apparent as early as the first trimester. We collected plasma from healthy, first-time mothers monthly throughout gestation and at 6 weeks postpartum. We then analyzed plasma for protein concentrations of sex hormones, cytokines, and angiogenic factors.

Materials and methods

Patient Eligibility and Blood Sample Collection

Informed consent was obtained from all patients, and this study was approved by the Mayo Clinic Institutional Review Board, Rochester, Minnesota. Women who were pregnant for the first time were eligible for this study. Women were excluded if they had a known immunodeficiency or autoimmune disease, had undergone a solid organ transplant, had a prior pregnancy termination or miscarriage, were smokers, had hemoglobin levels <9 g/dL, or had undergone fertility treatments. During the study, 10 milliliters (mL) of peripheral blood were collected from each woman at the time of their first prenatal visit (typically 8 weeks from their last menstrual period), every 4 weeks thereafter, and at 6 weeks postpartum (±2 weeks for each of time point). Blood was collected in sodium heparin tubes and separated for plasma and peripheral blood mononuclear cells (PBMCs) over Ficoll-Paque (GE Healthcare) according to the manufacturer's instructions within 18 hr of collection. Samples were collected during 2011 and 2012. Plasma was stored at -80 degrees C until thawing for laboratory analyses.

Laboratory Assays

Hormone concentrations were quantified by enzyme-linked immunosorbent assays (ELISA). ELISA kits for progesterone, estradiol, estrone, and prolactin were purchased from ALPCO (Salem, NH, USA), and an ELISA kit for CRH was purchased from Novatein Biosciences (Woburn, MA, USA). Assays were performed according to manufacturer's instruction. ELISAs were read at 450 nm on a plate reader, and concentrations were calculated using a 6-point standard curve.

MagPlex® Multiplex kits (Millipore, Billerica, MA, USA) were used to determine concentrations of cytokines and angiogenic factors. The Th17 cytokine MagPlex® kit was used to quantify interferon(IFN)-γ, IL-10, (C-C motif) (CC) ligand 20, IL-12p70, IL-13, IL-15, IL-17A, IL-22, IL-9, IL-1β, IL-33, IL-2, IL-21, IL-4, IL-23, IL-5, IL-6, IL-17E/IL-25, IL-27, IL-31, TNF-α, TNF-β, and IL-28A. The Angiogenesis MagPlex® kit was used to quantify epidermal growth factor (EGF), angiopoietin (Ang)-2, granulocyte colony-stimulating factor (G-CSF), bone morphogenetic protein (BMP)-9, endoglin, endothelin-1, leptin, fibroblast growth factor (FGF)-1, FGF-2, follistatin, IL-8, hepatocyte growth factor (HGF), heparin-binding (HB)-EGF, placental growth factor (PlGF), vascular endothelial growth factor (VEGF)-A, VEGF-C, and VEGF-D. Immunoassays were performed according to manufacturer's instruction; however, five washes were utilized instead of two, and samples

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were run in wash buffer instead of sheath fluid. Completing more washes and running data collection on samples in wash buffer was recommended by the company's support team, and we found it to increase bead counts. Multiplex assays were measured using Luminex xPONENT technology (Austin, TX, USA). Concentrations were calculated using MILLIPLEX Analyst 5.1 software (Vigene Tech, Carlisle, MA, USA). All samples were measured in duplicate and averaged to determine concentration.

Statistical Analysis

Each woman had samples analyzed on the same plate to minimize plate-to-plate variability. Analytes that were below the detection limit were changed to a concentration of 0, and analytes that were beyond the upper range of detection were assigned the highest detectable limit as described previously.[18] For statistical analysis, samples were divided into gestational intervals: 0 = ≤8 weeks, 1 = 9–12 weeks, 2 = 13–17 weeks, 3 = 18–22 weeks, 4 = 23–27 weeks, 5 = 28–32 weeks, 6 = 33–37 weeks, 7 = delivery (14 days prior to 24 hr after), and 8 = 6 week postpartum. Data were excluded if a sampling occurred twice during a gestational interval to ensure independence of all values (47 of 310 samples were not included). Kruskal–Wallis tests were utilized to assess differences in protein concentrations of analytes across gestational intervals in the entire cohort. Significance was declared at 0.1 for preliminary analysis of differences across gestational intervals in the entire cohort. Longitudinal differences across gestational intervals based on fetal sex were determined using Kruskal–Wallis tests, and significance was declared at 0.001 to account for multiple comparisons. To analyze differences between women carrying male versus female fetuses at each individual gestational interval, Wilcoxon rank-sum tests were conducted, and significance was declared at 0.001 to account for multiple comparisons. Statistical analysis was completed using JMP software version 9 (SAS, Cary, NC).

Pathway Analysis and Enrichment

Cytokines and angiogenic factors that exhibited significant differences according to fetal sex and gestational interval increased or decreased based on fetal sex and gestational interval were analyzed to determine their role in transcription factor networks and biological processes. Network analysis was completed using MetaCore (New York, NY, USA), and pathway enrichment was completed using Ingenuity Pathway Analysis (IPA) (Redwood City, CA, USA). Only interactions based on human data were included. Targets that were significantly different from one sex to the other were matched using comparison analysis. In the pathway enrichment analysis, Fisher's exact test was used to calculate *P*-values that represent the probability of the biological function in our data set being due to chance alone. Differentially expressed proteins with a *P*-value ≤ 0.01 were used to determine differences in the canonical pathways based on the sex of the fetus. Analysis settings included direct and indirect comparisons and a relaxed search filter to maximize possible interactions between proteins.

Results

Patient Demographics

Fifty healthy primigravid women between 18 and 35 years of age consented to participate in the study. Twelve women were excluded from the analysis because of miscarriage (3), voluntary withdrawal (2), or incomplete data collection defined as completing fewer than 50% of study blood draws (7). Of the 38 women available for analysis, 19 carried a male fetus, and 19 carried a female fetus. The mean age of the cohort of 38 women was 28.2 years (range 19-34). There were no significant differences in age between women carrying male fetuses versus female fetuses (average 27.7 versus 28.7 years, respectively; $P = 0.434$).

Variation Based on the Sex of the Fetus In Maternal Cytokines, Growth Factors, Hormones, and Angiogenic Factors

Several notable fetal sex-based differences in maternal cytokines and growth factors were observed during gestation (Fig. 1). Levels of proangiogenic factors and Th1 cytokines were greater in women carrying a male fetus (versus female); specifically, women carrying a male fetus had higher levels of BMP-9, endothelin-1, FGF-2, follistatin, G-CSF, HB-EGF, HGF, IL-12p70, IL-21, IL-33, PlGF, prolactin, and VEGF-A in over 50% of gestational intervals. In addition, women carrying a male fetus had significantly higher levels of angiopoietin-2, BMP-9, follistatin, HB-EGF, HGF,

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Fig. 1 Summary of analytes that showed significant variability based upon the sex of the fetus at different gestational intervals. Median analyte concentrations are plotted on the Y axis (log-transformed in a and b, and linear scale in c, d, e). Gestational intervals are plotted on the X axis, with separation between women carrying female versus male fetuses. Statistical significance was declared at $P = 0.001$ due to multiple comparisons with this analysis, and comparisons between females versus males that reach significance at each gestational time point is indicated with an asterisk (*).

PlGF, and IL-12p70 during the first gestational interval (≤8 weeks). Among women carrying a female fetus, cytokines indicative of a Th2 type immune response were observed; specifically, women carrying a female fetus had higher levels of IL-5, IL-9, IL-17A, and IL-17E/IL-25 in over 50% of gestational intervals. Analytes that were not reported were not significantly different between women carrying a male versus female fetus at any time point. Notably, there were also no significant differences between women carrying a male or female fetus at the 6-week postpartum visit; however, only 15 of 38 women (40.5%) had their postpartum blood draw.

For the entire cohort, plasma levels of estradiol, progesterone, estrone, and IL-27 increased throughout gestation (Fig. 2), with little variability between the women in early gestation and postpartum. IL-27 levels peaked at around 33–37 weeks before returning to low levels after delivery. Plasma levels of sex hormones and IL-27 levels did not differ by fetal sex. Among women pregnant with a female fetus, angiopoietin-2, endoglin, and follistatin levels

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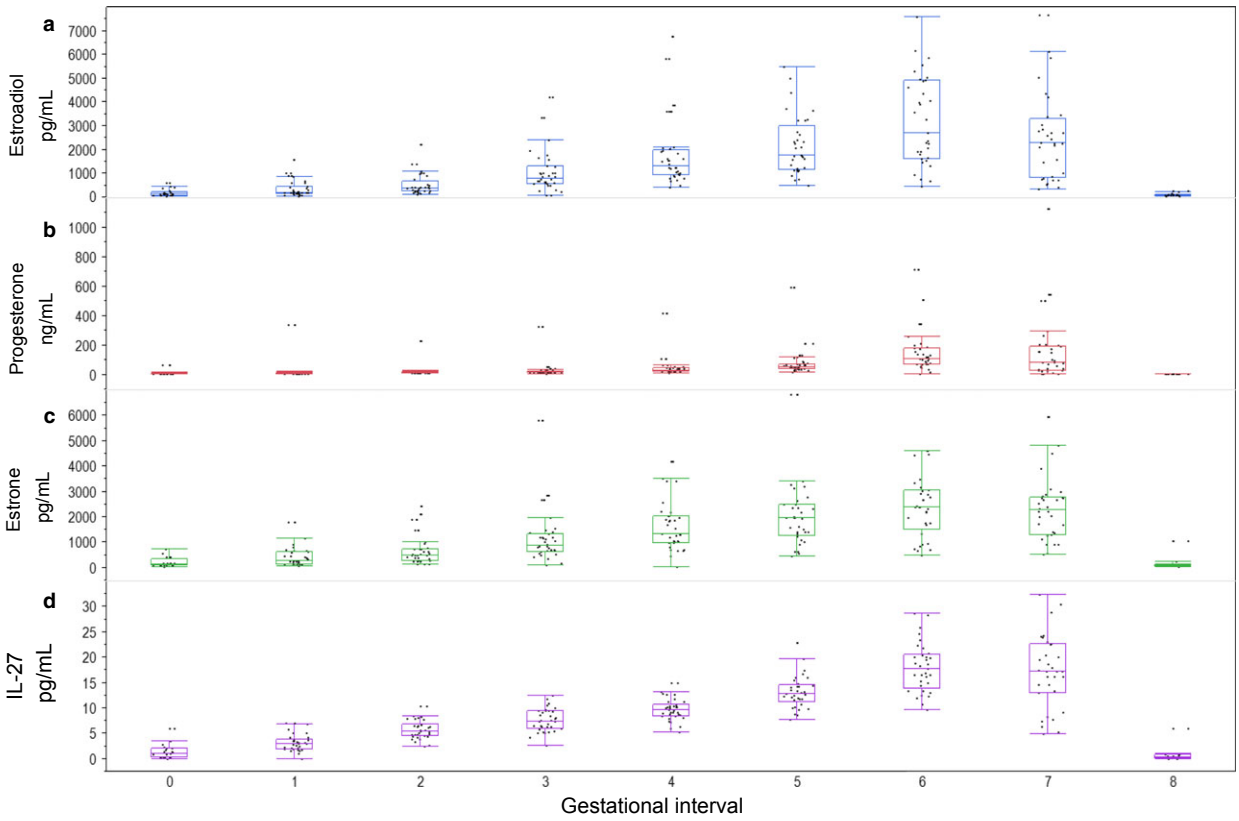

Fig. 2 Progesterone (a), estradiol (b), estrone (c), and IL-27 (d) increase throughout gestation and drop dramatically postpartum regardless of fetal sex ($N = 38$; 310 total samples). Gestational intervals defined as: 0 = ≤8 weeks, 1 = 9–12 weeks, 2 = 13–17 weeks, 3 = 18–22 weeks, 4 = 23–27 weeks, 5 = 28–32 weeks, 6 = 33–37 weeks, 7 = delivery (14 days prior to 24 hr after) and 8 = 6 week postpartum. Results were $P < 0.001$ for all four analytes.

were significantly different longitudinally throughout pregnancy (Fig. 3, $P < 0.0001$). These levels peaked at gestational intervals 2, 7, and 4, respectively. Several of the cytokines and growth factors that showed different patterns of increase based upon fetal sex have known functions in immunity and angiogenesis (Fig. 4).

Pathway Analysis of Fetal Sex-Based Variations in Maternal Proteins During Pregnancy

Using Metacore, we analyzed potential pathways under similar transcriptional control based on the sex of the fetus (Fig. 5). Pathway analysis indicated that the majority of proteins that increased among women carrying a male fetus are under transcriptional regulation of NF-κB, SP1, c-Jun, and CREB1 (Figs S1 and S2). The proteins that increased among women carrying a female fetus are predominantly regulated by transcription factors NFAT and STAT-3 (Fig. S3). Utilizing Ingenuity Pathway Analysis, we compared biological processes based on involvement of cytokines and angiogenic proteins that were statistically significant based on our earlier analysis. Prolactin was excluded from analysis because it is increased by each sex at one gestational interval. The threshold defined as a fold change greater than 2.5, and the association between data and the canonical pathway was measured by Fisher's exact test ($P \leq 0.05$). In the majority of biological processes, proteins that were increased among women carrying a male fetus were highly associated with signaling, development, and inflammatory response pathways (Fig. S4). However, proteins that were increased among women carrying a female fetus were enhanced in pathways important for hematological disease, humoral (Th2) immune responses, and inflammatory disease.

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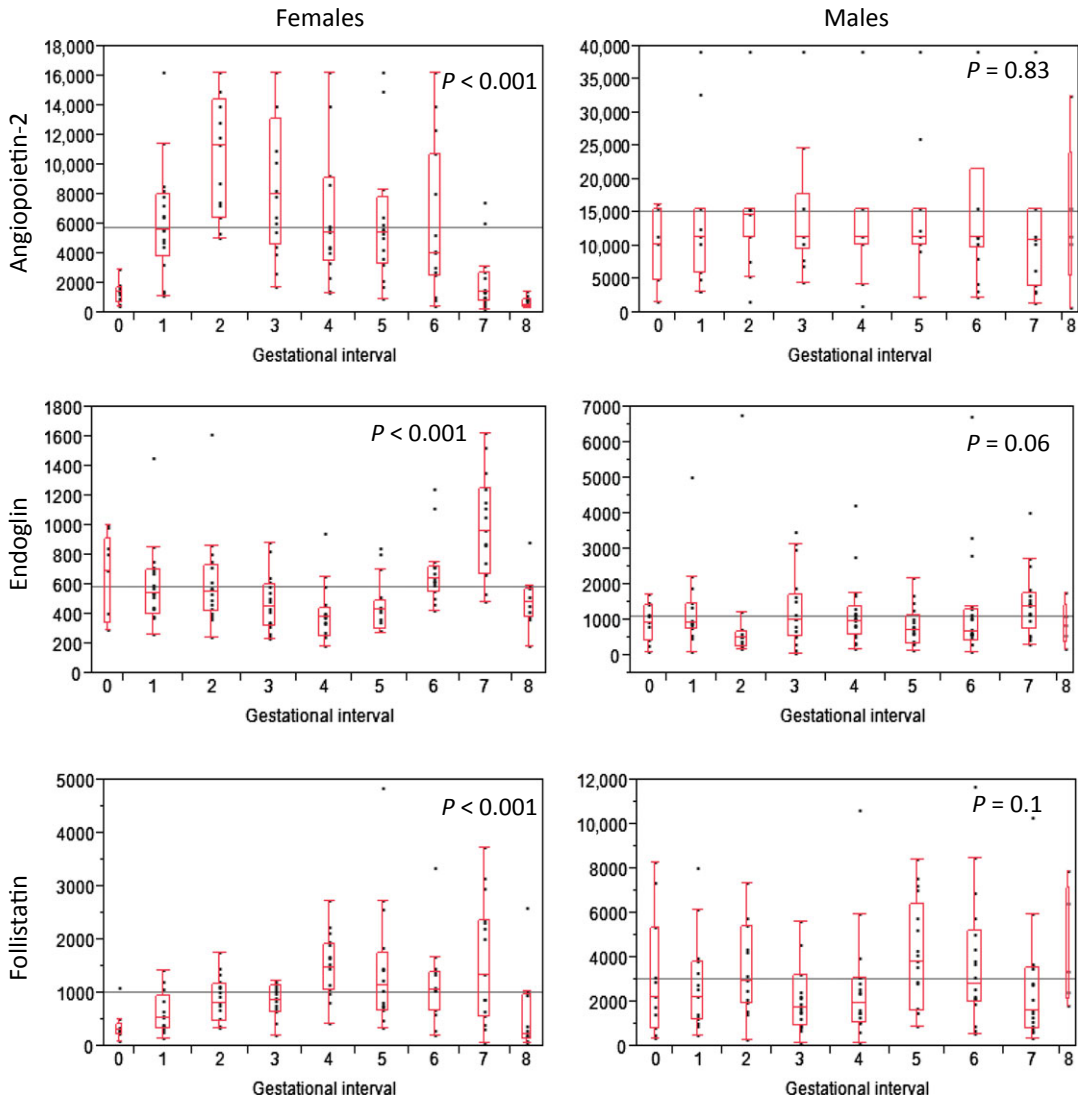

Fig. 3 Boxplot of angiogenesis proteins that differ longitudinally through gestation based on fetal sex. The horizontal line represents the grand mean of the cohort, pooled over time.

Discussion

Male fetuses have disproportionate rates of preterm births, higher birth weights, and greater fetal mortality.[6,7] In our cohort of 38 women, we show that the maternal hormonal and immune milieu undergoes many changes during pregnancy that vary based on fetal sex. The hormonal and immune changes among women carrying a male fetus were characterized by increases in levels of proinflammatory cytokines and proangiogenic growth factors, while the hormonal and immune changes among women carrying a female fetus were characterized by increases in the expression of regulatory cytokines. These findings may explain the disparate pregnancy outcomes based on fetal sex and are consistent with our hypothesis that women carrying a male fetus would have a cytokine and growth factor milieu that is biased toward a Th1 inflammatory response as compared to women carrying a female fetus.

Compared to women pregnant with a female fetus, women carrying a male fetus had higher levels of inflammatory cytokines at multiple time

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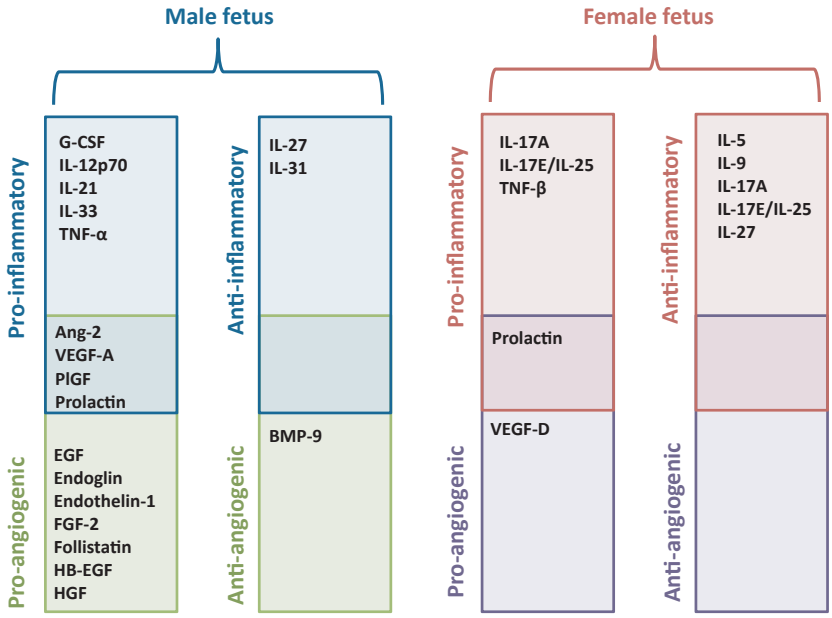

Fig. 4 Summary of cytokines and growth factors that showed differences based specifically on the sex of the fetus through multiple time points during pregnancy, along with predominant function based upon current literature. This is an oversimplified representation of the function of these cytokines and growth factors, as many of these factors, especially IL-17 family members, may have different context-dependent roles.

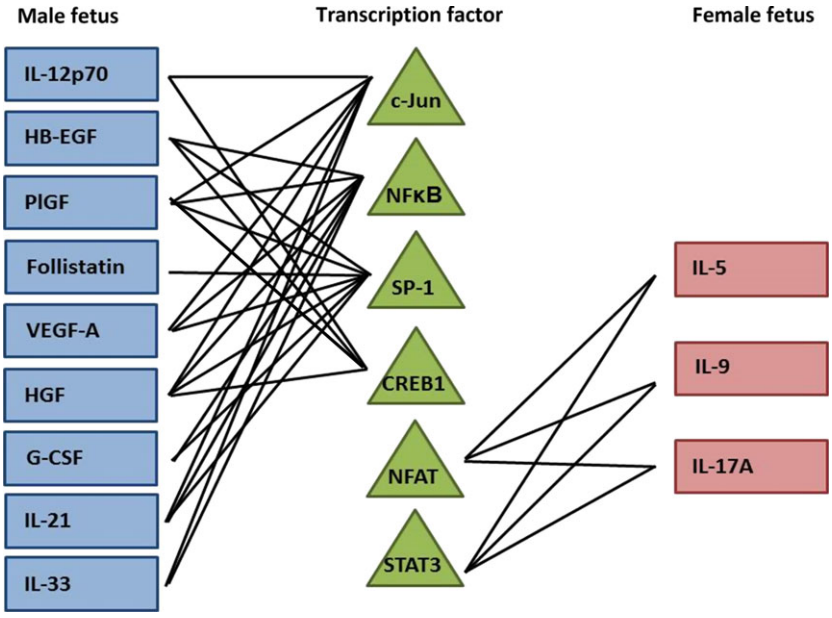

Fig. 5 Summary of transcription factor regulation of proteins showing different maternal levels based on fetal sex. Cytokines and angiogenic factors upregulated during gestation with a male fetus predominantly utilize transcription factors NF-κB, c-Jun, SP-1 and CREB-1, while female fetuses predominantly utilize NF-AT and STAT3.

points during gestation. Specifically, male fetuses were associated with higher levels of IL-12p70, IL-21, IL-33, and G-CSF in maternal plasma during pregnancy, with many of these proteins increased above females as early as 6 weeks post-conception. These cytokines are typical of a proinflammatory, Th1 T cell response.[19] IL-12p70, produced by monocytes, is involved in the differentiation of naïve T cells into Th1-biased T cells. IL-12p70 has previously been shown to increase in pregnant women with preeclampsia.[20] In neonatal immunity, IL-21 drives a Th1 response that is hypothesized to

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decrease infectious disease susceptibility of newborns.[21] IL-33 is an IL-1 family member involved in innate immunity and inflammation.[22] This immune milieu with high levels of IL-12, IL-21, and IL-33 could account for disparate outcomes in prenatal infectious disease. In addition, human miscarriage is associated with a Th1-biased, proinflammatory response at the maternal-fetal interface.[23] Although not addressed in this study, one could hypothesize that there may be a higher incidence of miscarriages when women are carrying a male fetus versus a female. Further studies must be completed to test this hypothesis.

Angiogenesis is required during placentation to supply essential nutrients and oxygen to the developing fetus. An appropriate balance between proangiogenic and anti-angiogenic factors is essential to develop adequate, but not pathologically aggressive, placental invasion. Adding an additional layer of complexity, many angiogenic factors have immunemodulating properties;[24] therefore, we considered the role of angiogenesis proteins along with recognized immunoregulatory molecules. We found that angiogenic growth factors such as PlGF and VEGF-A were increased in the blood of women carrying a male fetus, where higher levels of inflammatory cytokines were observed. PlGF has also been associated with adaptation to inflammation in other settings (e.g. sepsis[25]), suggesting that its relative increase with male fetuses may also be adaptive. Pregnancy complications, such as ectopic pregnancies and threatened abortions, correlate with a decrease in PlGF and an increase in VEGF-A expression in plasma.[26] PlGF has been suggested as a biomarker for preeclampsia as reduced levels of this growth factor occur weeks before clinical onset of disease.[27]

Levels of BMP-9, HB-EGF, and HGF, factors also involved in angiogenesis, were also increased in pregnancies with male fetuses. BMP-9 binds to activin receptor-like kinase (ALK1) with high affinity which effectively inhibits angiogenesis.[28] However, in combination with transforming growth factor (TGF)–β, a cytokine expressed at high levels in the placenta,[29] BMP-9 binding to ALK1 improved the angiogenic response of endothelial cells.[29] HB-EGF production is regulated by estradiol and progesterone in the endometrium prior to conception and blocking it negatively affected uterine receptivity during implantation.[30] Syncytiotrophoblasts and extravillous trophoblasts express HGF, which is embryonic lethal when mutated in murine models.[31,32] Taken together, our results suggest that higher levels of inflammatory cytokines coupled with higher levels of proangiogenic growth factors observed with male fetuses could reflect the normal adaptation to a greater immunologic challenge for the mother compared with that induced by female fetuses.

In our cohort, female fetuses induced a type 2-like regulatory maternal response with increased levels of IL-5, IL-9, IL-17, and IL-25.[33] Research into the role of IL-5 in pregnancy is limited, but an IL-5 knockout mouse model showed that this cytokine regulates eosinophilia and endometrial tissue remodeling.[34] Although little has been published on IL-9 in human pregnancy, it has been shown to promote T-cell proliferation of CD4+, but not CD8+ cells, and can stimulate a Th17 autoimmune response.[35,36] IL-17 is a proinflammatory cytokine that has an important role in the pathogenesis of chronic inflammation, autoimmune diseases and pregnancy through the induction of anti-inflammatory cytokines IL-6 and IL-10.[37] In healthy pregnancies, IL-17 levels in maternal serum have been reported to increase throughout gestation, with the highest levels of expression during the third trimester.[38] Others have found that IL-17 producing T cells do not change in peripheral blood during normal human pregnancies, only in the decidua.[39] Levels of IL-17E/IL-25 have not been documented during pregnancy to our knowledge, but the cytokine is known to initiate a type 2 immune response through the alternative activation of macrophages and expansion of innate lymphoid cells.[40–42] Lymphocyte effector functions guided by nuclear factor of activated T cells (NFAT)[43] may be in part responsible for the maternal response to female fetuses. Based on numerous prior experimental results, successful pregnancy has previously been characterized as a type 2 immune-biased phenomenon.[44–46] Our results appear consistent with that view for women carrying a female fetus.

At the 6-week postpartum visit, we saw no significant differences between women carrying a male fetus versus women carrying a female fetus, which increases the probability that our results are due to fetal sex and not individual variation. However, only 15 of the 37 (40.6%) women on our study came in for their postpartum blood draw. Because of the nature of designing studies with a vulnerable (and often very busy) population, our sample size was limited,

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and it was difficult to achieve compliance for monthly blood draw. Even with these limitations, our study is one of the first to look at maternal immune based differences longitudinally throughout pregnancy based on the sex of the fetus. We cannot rule out that some differences in maternal cytokine milieu persist for up to 6 weeks and beyond after pregnancy. Obtaining pre-pregnancy cytokine/growth factor profiles on women, followed by a longitudinal study that includes timepoints later than 6 weeks postpartum, could address this limitation in our study.

We found angiopoietin-2 (Ang-2), endoglin, and follistatin levels were variable during the course of pregnancy in women carrying a female fetus but not a male fetus. All three have been studied in outcomes of pregnancy. Regarding Ang-2, serum taken from pregnant women showed a shorter time to delivery when Ang-2 levels were greater than 4 ng/mL.[47] Concerning endoglin, serum from women diagnosed with preeclampsia showed elevated levels of soluble endoglin, which directly correlated with disease severity.[48] As none of the women followed in our study had preeclampsia, our data suggest that endoglin plays a supportive, tightly regulated role in pregnancy, especially considering the relative lack of longitudinal variability observed with male fetuses. Finally, follistatin has been suggested as a predictor of ectopic pregnancies or missed abortions.[49] Although our cohort is fairly small, the differences were statistically significant, and future research involving these factors and pregnancy outcomes should consider the possibility of differences based on the sex of the fetus.

Pregnancy is not a static immunologic event for the mother. Rather, the immunologic changes are dynamic, characterized by widespread alterations in hormones, immune cells, cytokines, and angiogenic factors. We noted that IL-27 increased with estrogens and progesterone throughout gestation and was highest at parturition. A novel finding of our study was the determination that IL-27 increased throughout gestation, peaking at around 33–37 weeks before returning to low levels after delivery. IL-27 is secreted by antigen-presenting cells and has been shown to regulate inflammation during pregnancy,[39] to control the intensity and duration of immune responses,[50,51] and to induce Th1 differentiation of naïve CD4+ T cells, while attenuating proinflammatory cytokine production through STAT transcription factors.[52–54] Our data suggest that rising levels of IL-27 may reflect a change of the maternal immune milieu toward Th1 polarization near the end of pregnancy in women carrying both male and female fetuses. Alternatively, IL-27 could increase as a compensatory mechanism to adapt to rising levels of other proinflammatory mediators in an otherwise immune-privileged condition.[55] These results will require validation in larger series.

Our findings of divergent maternal immune responses based upon fetal sex may have applications beyond fetomaternal tolerance. Like pregnancy, the success of organ and tissue transplantation requires tolerance induction toward allogeneic stimuli. Although preliminary, our results delineate pathways that could be further studied to explore outcomes of transplantation that also show disproportionately poorer outcomes when female versus male immune responses are invoked. Perhaps not unlike maternal immune responses to male pregnancies, an increase in immune-related late effects has been observed in male recipients of female hematopoietic cell transplants (HCT). More extensive chronic graft-versus-host disease has been observed in HCT recipients receiving grafts from female donors,[56,57] while reduced cancer relapse rates have been observed in female-to-male transplants.[58–60] Differences in outcomes in female-to-male transplants are often related to minor histocompatibility antigens involving the Y chromosome,[61,62] but additional sex-based differences in immune response (e.g., B7-H1-dependent regulatory T-cell responses in females[63]) are possible. A deeper understanding of female-to-male alloimmune reactions may help identify new molecular targets for sex-based immune modulation.

In conclusion, we have found that the maternal immune milieu undergoes many changes throughout gestation. Several of these changes appear to be divergent based on the sex of the fetus: male fetuses are associated with an increase in the levels of proinflammatory cytokines and proangiogenic growth factors, while female fetuses are associated with an increase the expression of regulatory cytokines. These results suggest that tolerance induction programs may be divergent based upon fetal sex, although these results do not define the mechanism by which such adaptation occurs. Future mechanistic studies should aim for a deeper understanding of fetomaternal tolerance as it relates to fetal sex.

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References

1 Ostensen M, Villiger PM: Immunology of pregnancy-pregnancy as a remission inducing agent in rheumatoid arthritis. *Transpl Immunol* 2002; 9:155–160.
2 Curry AE, Vogel I, Skogstrand K, Drews C, Schendel DE, Flanders WD, Hougaard DM, Thorsen P: Maternal plasma cytokines in early- and mid-gestation of normal human pregnancy and their association with maternal factors. *J Reprod Immunol* 2008; 77:152–160.
3 Anderson BL, Mendez-Figueroa H, Dahlke JD, Raker C, Hillier SL, Cu-Uvin S: Pregnancy-induced changes in immune protection of the genital tract: defining normal. *Am J Obstet Gynecol* 2013; 208:321.e321–329.
4 Soldin OP, Mattison DR: Sex differences in pharmacokinetics and pharmacodynamics. *Clin Pharmacokinet* 2009; 48:143–157.
5 Pennell LM, Galligan CL, Fish EN: Sex affects immunity. *J Autoimmun* 2012; 38:J282–291.
6 Zeitlin J, Saurel-Cubizolles MJ, De Mouzon J, Rivera L, Ancel PY, Blondel B, Kaminski M: Fetal sex and preterm birth: are males at greater risk? *Hum Reprod* 2002; 17:2762–2768.
7 Di Renzo GC, Rosati A, Sarti RD, Cruciani L, Cutuli AM: Does fetal sex affect pregnancy outcome? *Gend Med* 2007; 4:19–30.
8 Stark MJ, Dierkx L, Clifton VL, Wright IM: Alterations in the maternal peripheral microvascular response in pregnancies complicated by preeclampsia and the impact of fetal sex. *J Soc Gynecol Investig* 2006; 13:573–578.
9 Shiozaki A, Matsuda Y, Satoh S, Saito S: Impact of fetal sex in pregnancy-induced hypertension and preeclampsia in Japan. *J Reprod Immunol* 2011; 89:133–139.
10 Clifton V: Maternal asthma during pregnancy and fetal outcomes: potential mechanisms and possible solutions. *Curr Opin Allergy Clin Immunol* 2006; 6:307–311.
11 Mingrone G, Manco M, Mora ME, Guidone C, Iaconelli A, Gniuli D, Leccesi L, Chiellini C, Ghirlanda G: Influence of maternal obesity on insulin sensitivity and secretion in offspring. *Diabetes Care* 2008; 31:1872–1876.
12 van Abeelen AF, de Rooij SR, Osmond C, Painter RC, Veenendaal MV, Bossuyt PM, Elias SG, Grobbee DE, van der Schouw YT, Barker DJ, Roseboom TJ: The sex-specific effects of famine on the association between placental size and later hypertension. *Placenta* 2011; 32:694–698.
13 Ghidini A, Salafia CM: Gender differences of placental dysfunction in severe prematurity. *BJOG* 2005; 112:140–144.
14 Cvitic S, Longtine MS, Hackl H, Wagner K, Nelson MD, Desoye G, Hiden U: The human placental sexome differs between trophoblast epithelium and villous vessel endothelium. *PLoS ONE* 2013; 8: e79233.
15 Sood R, Zehnder JL, Druzin ML, Brown PO: Gene expression patterns in human placenta. *Proc Natl Acad Sci U S A* 2006; 103:5478–5483.
16 Scott NM, Hodyl NA, Murphy VE, Osei-Kumah A, Wyper H, Hodgson DM, Smith R, Clifton VL: Placental cytokine expression covaries with maternal asthma severity and fetal sex. *J Immunol* 2009; 182:1411–1420.
17 Kim-Fine S, Regnault TR, Lee JS, Gimbel SA, Greenspoon JA, Fairbairn J, Summers K, De Vrijer B: Male gender promotes an increased inflammatory response to lipopolysaccharide in umbilical vein blood. *J Matern Fetal Neonatal Med* 2012; 25:2470–2474.
18 Potter DM, Butterfield LH, Divito SJ, Sander CA, Kirkwood JM: Pitfalls in retrospective analyses of biomarkers: a case study with metastatic melanoma patients. *J Immunol Methods* 2012; 376:108–112.
19 D'Acquisto F, Maione F, Pederzoli-Ribeil M: From IL-15 to IL-33: the never-ending list of new players in inflammation. Is it time to forget the humble aspirin and move ahead? *Biochem Pharmacol* 2010; 79:525–534.
20 Germain SJ, Sacks GP, Sooranna SR, Sargent IL, Redman CW: Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *J Immunol* 2007; 178:5949–5956.
21 Doganci A, Birkholz J, Gehring S, Puhl AG, Zepp F, Meyer CU: In the presence of IL-21 human cord blood T cells differentiate to IL-10-producing Th1 but not Th17 or Th2 cells. *Int Immunol* 2013; 25:157–169.
22 Garlanda C, Dinarello CA, Mantovani A: The interleukin-1 family: back to the future. *Immunity* 2013; 39:1003–1018.
23 Jin LP, Fan DX, Zhang T, Guo PF, Li DJ: The costimulatory signal upregulation is associated with Th1 bias at the maternal-fetal interface in human miscarriage. *Am J Reprod Immunol* 2011; 66:270–278.
24 Galvao AM, Ferreira-Dias G, Skarzynski DJ: Cytokines and angiogenesis in the corpus luteum. *Mediators Inflamm* 2013; 2013:420186.
25 Yano K, Okada Y, Beldi G, Shih SC, Bodyak N, Okada H, Kang PM, Luscinskas W, Robson SC, Carmeliet P, Karumanchi SA, Aird WC: Elevated levels of placental growth factor represent an adaptive host response in sepsis. *J Exp Med* 2008; 205:2623–2631.
26 Patrelli TS, Gizzo S, Plebani M, Basso D, Capobianco G, Bartolucci C, Modena AB, Rondinelli M, Nardelli GB: The trend of VEGF-A and PlGF in pregnant patients: a perspective case-control study on 214 women. *Clin Exp Obstet Gynecol* 2012; 39:57–64.
27 Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA: Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004; 350:672–683.
28 Scharpfenecker M, van Dinther M, Liu Z, van Bezooijen RL, Zhao Q, Pukac L, Lowik CW, ten Dijke P: BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J Cell Sci* 2007; 120:964–972.
29 Cunha SI, Pardali E, Thorikay M, Anderberg C, Hawinkels L, Goumans MJ, Seehra J, Heldin CH, ten Dijke P, Pietras K: Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. *J Exp Med* 2010; 207:85–100.

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30 Lessey BA, Gui Y, Apparao KB, Young SL, Mulholland J: Regulated expression of heparin-binding EGF-like growth factor (HB-EGF) in the human endometrium: a potential paracrine role during implantation. *Mol Reprod Dev* 2002; 62:446–455.
31 Wolf HK, Zarnegar R, Oliver L, Michalopoulos GK: Hepatocyte growth factor in human placenta and trophoblastic disease. *Am J Pathol* 1991; 138:1035–1043.
32 Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T, Kitamura N: Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* 1995; 373:702–705.
33 Barlow JL, McKenzie AN: IL-25: a key requirement for the regulation of type-2 immunity. *BioFactors* 2009; 35:178–182.
34 Robertson SA, Mau VJ, Young IG, Matthaei KI: Uterine eosinophils and reproductive performance in interleukin 5-deficient mice. *J Reprod Fertil* 2000; 120:423–432.
35 Schmitt E, Van Brandwijk R, Van Snick J, Siebold B, Rude E: TCGF III/P40 is produced by naive murine CD4 + T cells but is not a general T cell growth factor. *Eur J Immunol* 1989; 19:2167–2170.
36 Elyaman W, Bradshaw EM, Uyttenhove C, Dardalhon V, Awasthi A, Imitola J, Bettelli E, Oukka M, van Snick J, Renauld JC, Kuchroo VK, Khoury SJ: IL-9 induces differentiation of TH17 cells and enhances function of FoxP3 + natural regulatory T cells. *Proc Natl Acad Sci U S A* 2009; 106:12885–12890.
37 Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP: IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 1998; 160:3513–3521.
38 Martinez-Garcia EA, Chavez-Robles B, Sanchez-Hernandez PE, Nunez-Atahualpa L, Martin-Maquez BT, Munoz-Gomez A, Gonzalez-Lopez L, Gamez-Nava JI, Salazar-Paramo M, Davalos-Rodriguez I, Petri MH, Zuniga-Tamayo D, Vargas-Ramirez R, Vazquez-Del Mercado M: IL-17 increased in the third trimester in healthy women with term labor. *Am J Reprod Immunol* 2011; 65:99–103.
39 Nakashima A, Ito M, Yoneda S, Shiozaki A, Hidaka T, Saito S: Circulating and decidual Th17 cell levels in healthy pregnancy. *Am J Reprod Immunol* 2010; 63:104–109.
40 Yang Z, Grinchuk V, Urban JF Jr, Bohl J, Sun R, Notari L, Yan S, Ramalingam T, Keegan AD, Wynn TA, Shea-Donohue T, Zhao A: Macrophages as IL-25/IL-33-responsive cells play an important role in the induction of type 2 immunity. *PLoS ONE* 2013; 8: e59441.
41 Saenz SA, Siracusa MC, Perrigoue JG, Spencer SP, Urban JF Jr, Tocker JE, Budelsky AL, Kleinschek MA, Kastelein RA, Kambayashi T, Bhandoola A, Artis D: IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 2010; 464:1362–1366.
42 Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, Bucks C, Kane CM, Fallon PG, Pannell R, Jolin HE, McKenzie AN: Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 2010; 464:1367–1370.
43 Hermann-Kleiter N, Baier G: NFAT pulls the strings during CD4 + T helper cell effector functions. *Blood* 2010; 115:2989–2997.
44 Wegmann TG, Lin H, Guilbert L, Mosmann TR: Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* 1993; 14:353–356.
45 Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG: Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *J Immunol* 1993; 151:4562–4573.
46 Krasnow JS, Tollerud DJ, Naus G, DeLoia JA: Endometrial Th2 cytokine expression throughout the menstrual cycle and early pregnancy. *Hum Reprod* 1996; 11:1747–1754.
47 Polyzou EN, Evangelinakis NE, Pistiki A, Kotsaki A, Siristatidis CS, Chrelias CG, Salamalekis E, Kassanos DP, Giamarellos-Bourboulis EJ: Angiopoietin-2 primes infection-induced preterm delivery. *PLoS ONE* 2014; 9:e86523.
48 Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke FW, Romero R, Sukhatme VP, Letarte M, Karumanchi SA: Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006; 12:642–649.
49 Daponte A, Deligeoroglou E, Garas A, Pournaras S, Hadjichristodoulou C, Messinis IE: Activin A and follistatin as biomarkers for ectopic pregnancy and missed abortion. *Dis Markers* 2013; 35:497–503.
50 Hunter CA: New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 2005; 5:521–531.
51 Colgan J, Rothman P: All in the family: IL-27 suppression of T(H)-17 cells. *Nat Immunol* 2006; 7:899–901.
52 Takeda A, Hamano S, Yamanaka A, Hanada T, Ishibashi T, Mak TW, Yoshimura A, Yoshida H: Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J Immunol* 2003; 170:4886–4890.
53 Kamiya S, Owaki T, Morishima N, Fukai F, Mizuguchi J, Yoshimoto T: An indispensable role for STAT1 in IL-27-induced T-bet expression but not proliferation of naive CD4 + T cells. *J Immunol* 2004; 173:3871–3877.
54 Yoshimura T, Takeda A, Hamano S, Miyazaki Y, Kinjyo I, Ishibashi T, Yoshimura A, Yoshida H: Two-sided roles of IL-27: induction of Th1 differentiation on naive CD4 + T cells versus suppression of proinflammatory cytokine production including IL-23-induced IL-17 on activated CD4 + T cells partially through STAT3-dependent mechanism. *J Immunol* 2006; 177:5377–5385.
55 Amadi-Obi A, Yu CR, Liu X, Mahdi RM, Clarke GL, Nussenblatt RB, Gery I, Lee YS, Egwuagu CE: TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat Med* 2007; 13:711–718.
56 Kollman C, Howe CW, Anasetti C, Antin JH, Davies SM, Filipovich AH, Hegland J, Kamani N, Kernan NA, King R, Ratanatharathorn V, Weisdorf D, Confer DL: Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: the effect of donor age. *Blood* 2001; 98:43–51.
57 Remberger M, Kumlien G, Aschan J, Barkholt L, Hentschke P, Ljungman P, Mattsson J, Svennilson J, Ringden O: Risk factors for moderate-to-severe chronic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2002; 8:674–682.
58 Gratwohl A, Hermans J, Niederwieser D, van Biezen A, van Houwelingen HC, Apperley J: Female donors influence transplant-related mortality and relapse incidence in male recipients of sibling blood and marrow transplants. *Hematol J* 2001; 2:363–370.
59 Randolph SS, Gooley TA, Warren EH, Appelbaum FR, Riddell SR: Female donors contribute to a selective graft-versus-leukemia effect in male recipients of HLA-matched, related hematopoietic stem cell transplants. *Blood* 2004; 103:347–352.
60 Gahrton G, Iacobelli S, Apperley J, Bandini G, Bjorkstrand B, Blade J, Boiron JM, Cavo M, Cornelissen J, Corradini P, Kroger N, Ljungman P, Michallet M, Russell NH, Samson D, Schattenberg A,

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Sirohi B, Verdonck LF, Volin L, Zander A, Niederwieser D: The impact of donor gender on outcome of allogeneic hematopoietic stem cell transplantation for multiple myeloma: reduced relapse risk in female to male transplants. *Bone Marrow Transplant* 2005; 35:609–617.

61 -Stern M, Passweg JR, Locasciulli A, Socie G, Schrezenmeier H, Bekassy AN, Fuehrer M, Hows J, Korthof ET, McCann S, Tichelli A, Zoumbos NC, Marsh JC, Bacigalupo A, Gratwohl A: Influence of donor/recipient sex matching on outcome of allogeneic hematopoietic stem cell transplantation for aplastic anemia. *Transplantation* 2006; 82:218–226.

62 Ofran Y, Kim HT, Brusic V, Blake L, Mandrell M, Wu CJ, Sarantopoulos S, Bellucci R, Keskin DB, Soiffer RJ, Antin JH, Ritz J: Diverse patterns of T-cell response against multiple newly identified human Y chromosome-encoded minor histocompatibility epitopes. *Clin Cancer Res* 2010; 16:1642–1651.

63 Lin PY, Sun L, Thibodeaux SR, Ludwig SM, Vadlamudi RK, Hurez VJ, Bahar R, Kious MJ, Livi CB, Wall SR, Chen L, Zhang B, Shin T, Curiel TJ: B7-H1-dependent sex-related differences in tumor immunity and immunotherapy responses. *J Immunol* 2010; 185:2747–2753.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Transcriptional regulation of proteins increased in pregnancies with male fetuses, part 1.

Figure S2. Transcriptional regulation of proteins increased in pregnancies with male fetuses, part 2.

Figure S3. Transcriptional regulation of proteins increased in pregnancies with female fetuses. Pathway analysis shows that extracellular proteins which are increased in the systemic maternal immune system due to a female fetus have shared transcriptional regulation through STAT3 and NF-AT.

Figure S4. Top biological processes (Ingenuity Pathway Analysis) of proteins that differ between male and female fetuses during pregnancy.

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