Periconception onset diabetes is associated with embryopathy and fetal growth retardation, reproductive tract hyperglycosylation and impaired immune adaptation to pregnancy

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Diabetes has been linked with impaired fertility but the underlying mechanisms are not well defined. Here we use a streptozotocin-induced diabetes mouse model to investigate the cellular and biochemical changes in conceptus and maternal tissues that accompany hyperglycaemia. We report that streptozotocin treatment before conception induces profound intra-cellular protein β-glycosylation (O-GlcNAc) in the oviduct and uterine epithelium, prominent in early pregnancy. Diabetic mice have impaired blastocyst development and reduced embryo implantation rates, and delayed mid-gestation growth and development. Peri-conception changes are accompanied by increased expression of pro-inflammatory cytokine Trail, and a trend towards increased Il1a, Tnf and Ifng in the uterus, and changes in local T-cell dynamics that skew the adaptive immune response to pregnancy, resulting in 60% fewer anti-inflammatory regulatory T-cells within the uterus-draining lymph nodes. Activation of the heat shock chaperones, a mechanism for stress deflection, was evident in the reproductive tract. Additionally, we show that the embryo exhibits elevated hyper-O-GlcNAcylation of both cytoplasmic and nuclear proteins, associated with activation of DNA damage (γH2AX) pathways. These results advance understanding of the impact of peri-conception diabetes, and provide a foundation for designing interventions to support healthy conception without propagation of disease legacy to offspring.

Worldwide, one in seven pregnancies are complicated by diabetes1. Pregnancy in women with diabetes is associated with increased risk of fetal, neonatal and obstetric complications, maternal morbidity and mortality, and a 4- to 10-fold elevated risk that infants will develop diabetes as adults2. Adverse outcomes include fetal and neonatal death, congenital abnormalities, premature delivery and macrosomia, associated with an array of obstetric complications including but not limited to birth trauma, stillbirth, respiratory distress syndrome and neonatal hypoglycaemia. Maternal complications include increased risk of preeclampsia, caesarean section, worsening of pre-existing diabetic complications, and in rare cases, maternal death2,3. The peri-conception phase of early pregnancy is essential for setting in train fetal development and offspring phenotypes, and perturbations at this time can impart an elevated diabetes risk to offspring. In diabetic women, pre-conception treatment to control...
Periconception-onset diabetes impairs pre-implantation embryo development, and increases DNA damage and protein O-GlcNAcylation. To examine the effect of streptozotocin-induced diabetes on early development, embryos were flushed from oviducts recovered on d1.5 and d3.5 p.c. from diabetic female mice. There was no impact of periconception-onset diabetes on the incidence of conception, with all mated mice yielding 2-cell cleavage-stage embryos on d1.5 p.c. (Fig. 1A). The total number of embryos was unchanged (Fig. 1B), however the number of viable embryos was significantly decreased (Fig. 1C) after streptozotocin treatment. At d3.5 p.c., fewer viable embryos were flushed from the uterus (Fig. 1D,E) and many had evidence of arrest at cleavage or morula stages with a 60% reduction in their development to blastocyst (Fig. 1F). γH2AX was used to assess DNA damage in these embryos and RL2 was used to assess β-O-GlcNAc status. Two-cell embryos flushed from the oviduct displayed no change in either DNA damage or O-GlcNAc (Fig. 2A–C). In contrast, O-GlcNAc increases in diabetic complications 12. It is not known whether O-GlcNAc is altered within the reproductive tract in diabetes, whether this is evident during early development, and if maternal hyperglycaemia influences O-GlcNAc in embryos.

During in vitro development, oocytes and zygotes cultured briefly in the absence of glucose are unable to complete embryo compaction, failing to progress beyond the morula stage 13. Hyperglycaemic culture conditions are also toxic to embryos 14, indicating that normal development requires a narrow glucose concentration range. Our previous in vitro studies demonstrate the importance of per-conception hyperglycaemic control for normal development and identified O-GlcNAc as a key regulator 14–17. Notably, exposure to hyperglycaemia caused altered stress deflection in the cumulus oocyte complex via aberrant O-GlcNAc glycosylation of the heat shock chaperones, which prevented normal preimplantation embryo development 15.

There is a pressing need to address the mechanisms by which a diabetic environment in utero alters the trajectory of offspring development in order to devise interventions to mitigate the increased risk of fetal, neonatal and obstetric disorders in pregnancies complicated by diabetes 5 and to reduce disease risks for children born to diabetic mothers. We hypothesised that diabetes during the periconception period acts to alter O-GlcNAc abundance and stress deflection pathways important for early development. Furthermore, given the well-described role of O-GlcNAc in immune cell regulation and cytokine signalling 4, the impact of hyperglycaemia on inflammatory parameters 18, and the significance of the maternal immune response for fetal growth and offspring health 20, we predicted a role for the local and systemic immune milieu in mediating the adverse effect of diabetes on early development. Here we utilise the well-defined streptozotocin-induced diabetes mouse model to define the impact of periconception-onset diabetes on biochemical and inflammatory parameters in the reproductive tract, and the consequences for early embryo development and pregnancy progression.

Results

Periconception-onset diabetes alters mid-gestation conceptus development and fetal viability. To examine the effect of streptozotocin-induced periconception-onset diabetes on mid-gestation development, pregnant diabetic female mice were autopsied on d11.5 p.c. There was no difference between treatment groups in the total number of implantation sites (viable and non-viable; Fig. 3A) however due to elevated rates of early fetal resorption the number of viable implantation sites was decreased (Fig. 3B), reflected in decreased reproductive tract weight (Fig. 3C). In viable implantation sites, there was a 30% decrease in the mass of individual conceptus units (fetus plus placenta) (Fig. 3D), and when fetal development was assessed according to Theiler score, a delay corresponding to approximately 24 hours was evident (Fig. 3E).

Periconception-onset diabetes increases protein O-GlcNAcylation and activation of stress deflection pathways in reproductive tract tissues. To examine the effect of streptozotocin-induced periconception-onset diabetes on the abundance of O-GlcNAc in the oviduct (d1.5 p.c.) and uterus (d3.5 p.c.), two antibodies were used to detect O-GlcNAc (Fig. 4, green CTD, red RL2). Treatment significantly increased the
abundance of O-GlcNAc in the luminal epithelium of the oviduct (Fig. 4A–H,Q), with a distinct pattern localising to the cytoplasmic region immediately subjacent to the luminal surface. Although weak staining was similarly localised in controls, the staining intensity was notably higher in treated mice. By d3.5 p.c., when the blastocyst is within the uterus, there was an increased abundance of O-GlcNAc in the uterine luminal epithelium of diabetic females (Fig. 4I–P,R), while no difference was evident in the endometrial stroma (Fig. 4P). To examine if the effect extended throughout the reproductive tract, we also examined the abundance of O-GlcNAc in the ovary in the streptozotocin-treated mice on d1.5 p.c. of pregnancy, but no difference in staining was evident (Suppl. Figure 1).

We also examined the abundance of several heat shock protein family chaperones, which are known to be regulated by glucose (also known as the glucose regulated proteins; Grps) and which we have shown to be targets of O-GlcNAcylation in the cumulus-oocyte complex 15. Endoplasmic-specific heat shock protein HSP90B1 (GRP94) was significantly increased in the oviductal epithelium on d1.5 p.c. particularly in the cytoplasmic region adjacent to the luminal surface (Fig. 5E). This was accompanied by loss of HSP90AA1 (Hsp90a) in the oviductal epithelium of diabetic females (Suppl. Figure 2E,M), and an increase in HSPA5 (GRP78), which is known to be induced by conditions which promote accumulation of unfolded proteins in the endoplasmic reticulum (ER) (Suppl. Figure 2G–L,N). The HSP family of chaperones are a well-characterised target of OGT 21,22, and high levels of co-localisation of HSP90B1 and O-GlcNAc (Fig. 5H–O) were observed, suggesting HSP90 or other ER-localised proteins are targeted.

Periconception-onset diabetes induces proinflammatory cytokine expression in the uterus and imbalance in the T cell response required for immune adaptation to pregnancy. Given the biochemical and growth anomalies described above, and the known role of cytokines and T-cells for implantation success23, we characterised the impact of periconception-onset diabetes on the immune milieu of early pregnancy. In the uterus of diabetic females on the day of implantation (d3.5 p.c.), we demonstrated substantial upregulation of pro-inflammatory cytokines including Trail, Il1b (both P < 0.05), as well as trends towards elevated Il1a, Ifng and Tnf (all P < 0.07) (Fig. 6).

T cell phenotypes and absolute numbers in the spleen and PALN, the latter a major site of maternal-fetal immune regulation and source of T cells recruited to the implantation site, were determined by flow cytometry immediately prior to embryo implantation on d3.5 p.c. We focused on T lymphocytes, particularly regulatory T (Treg) cells, as these are known to be key mediators of maternal immune tolerance required for implantation success24. Total T-cell numbers were decreased in the uterus-draining para-aortic lymph nodes (PALN) of diabetic females (P < 0.05) (Fig. 7A), as were total CD4 T and CD8 T-cells (Fig. 7B). These changes were not evident in the spleen (Fig. 7A and Suppl. Figure 4), indicating an impact of diabetes specific to the immune response to conception, rather than a systemic effect. Despite preferential sparing of the CD4 Foxp3 Treg cell population (%CD4 T cells), there was a trend to reduced total CD4 Foxp3 Treg cells (P = 0.08) (Fig. 7D). Amongst the Treg cells...
peripherally-induced Treg (pTreg) cells and thymically-derived Treg (tTreg) cells can be distinguished by expression of neuropilin-1 (Nrp1) (Fig. 7C). Within the PALN but not the spleen there was a striking shift in the proportion of these two Treg cell subsets in diabetic females, such that mean absolute numbers of Nrp1−pTreg cells were decreased by 60% (P < 0.01) while Nrp1+tTreg cells were not significantly changed (Fig. 7E). This observation indicates substantial impairment of the adaptive immune response to pregnancy in streptozotocin-treated mice.

**Discussion**

The reproductive tract environment at conception profoundly influences the progression of pregnancy and the perinatal and long-term health of infants. During the first days of life, the embryo senses and adapts to the environment within the oviduct and uterus, setting in train a developmental program that will determine fetal growth and influence health trajectory after birth46. For the first time, we have described changes in O-GlcNAcylation of proteins in the early preimplantation embryo caused by streptozotocin-induced diabetes, and demonstrate that the reproductive tract is also prone to altered O-GlcNAc levels. We further demonstrate that this is associated with increases in ER-stress related response mechanisms, evidenced by altered levels of heat-shock pathway.
chaperones, and induces a pro-inflammatory environment accompanied by changes in the local cytokine and immune cell milieu that would impair maternal immune receptivity for implantation and pregnancy progression (Fig. 8).

In this study, we report evidence which supports that the embryo is responding to the external physiological cues of the diabetic state, as indicated by compromised blastocyst development and the increased levels of blastocyst protein O-GlcNAcylation. In other paradigms of hyperglycaemia and hyperinsulinaemia, an abnormal increase in O-GlcNAc has been observed, resulting in a disruption in the balance of protein modification processes involved in controlling cell functions. Dysregulation of O-GlcNAcylation is now an established mechanism causing dysfunction of insulin signalling and glucose toxicity; significant characteristics of type 2 diabetes. Additionally, alteration in O-GlcNAc protein modification is thought to underpin other diabetic-related pathologies, including cardiomyopathy, erectile dysfunction and neurodegenerative diseases, due to a systemic elevation in O-GlcNAcylation. However, this has not previously been explored within the diabetic reproductive tract or in vivo-derived embryos.

We observed elevated DNA damage in diabetic embryos, with distinct co-localisation between the DNA-damage histone marker γH2AX (phosphorylation at Serine 139), and O-GlcNAcylation. In response to DNA damage, multiple repair factors relocate to the sites of damage to activate repair and cell cycle checkpoints. The dynamic relocation of DNA repair factors is mediated by DNA damage-induced post-translational modifications on histones and their binding partners at, or adjacent to, the sites of DNA damage, one of which includes the phosphorylation of histone H2AX (aka γH2AX). A recent report describes O-GlcNAc transferase (OGT) locating to the site of DNA damage, with O-GlcNAc glycosylation of histone H2AX reducing the phosphorylation events, and helping cells recover from DNA damage. In addition to the targeting of histone H2AX, there are many other nuclear targets for O-GlcNAc glycosylation and provide plausible mechanism by which an epigenetic change may underpin the transmission of diabetes from mother to offspring in utero.

Several studies describe the impact of environmental perturbation in the conception phase on early embryo biochemistry and molecular biology, but how these link to long-term programming of offspring is not clear. The immunological state within the reproductive tract shapes embryonic programming, in part through direct effects of cytokines on the embryo and in part through influencing maternal tract receptivity and placental morphogenesis. Immune-modulating cytokines are pivotal for mediating communication between the maternal tract and the embryo. Compelling evidence shows that cytokines emanating from the oviduct and uterus can ‘fine-tune’ embryo development during the peri-conception period, influencing a range of cellular events from cell survival and metabolism, through division and differentiation, and potentially impact long-term impact

Figure 3. Periconception-onset diabetes causes midgestation fetal loss, impaired fetal growth and delayed fetal development. Control (black) and diabetic (grey) female mice were naturally mated and conceptus tissue was assessed on d11.5 p.c. Total implantation site number (A), percent viable implantation sites (B), reproductive tract weight (C), whole implantation site weight (D), and Thézé developmental score (E) were assessed (n = 15–16 mated females per treatment). Data is presented as mean (D) or mean + SEM and effects of treatment were analysed by unpaired Student’s t-test. *P < 0.05.
through epigenetic remodeling⁷. The relative balance between survival agents and apoptosis-inducing agents influence the course of preimplantation development, causing embryos to adapt to varying maternal environments. In a healthy state, embryotrophic factors protect embryos from cell stress and support them to thrive⁸. In contrast, with excessive inflammation, embryotoxic cytokines such as TRAIL, TNF and IFNG elicit cell stress in embryos and depending on their levels, can cause embryo developmental arrest and demise⁹. Our observation of elevated expression of embryotoxic cytokines TRAIL, and a trend towards increased IL1A, TNF and IFNG in
hyperglycemic conditions, provides a direct mechanism contributing to the impact of diabetes on altered developmental trajectory of offspring, and support earlier reports of elevated uterine TNF and reduced TGFB2 and LIF accompanied by impaired blastocyst development in diabetic rat and mouse models39–41.

In addition to the altered uterine cytokine profile, we found striking changes in the immune cells of the local lymph nodes in diabetic females at implantation. As the developing fetus expresses paternal transplantation antigens, is it susceptible to a potentially detrimental maternal immune response. In successful pregnancy, the effects

Figure 5. Periconception-onset diabetes is associated with elevated GRP94 abundance, and GRP94-O-GlcNAc co-localisation in the oviduct. Control (A–C, H–K, black) and diabetic (D–F, L–O, grey) female mice were hyperstimulated and mated, then oviducts were recovered on day 1.5 pc and fixed for immunohistochemical analysis of GRP94 (red, A–F, I, M) and O-GlcNAc (CTD antibody, green, J, N) and counterstained with DAPI (blue). Scale bar represents 50 μm. Image analysis (G) was performed on oviductal luminal epithelium using FIJI in 20 sections per mouse from 6 mice per treatment. Data is presented as mean ± SEM staining intensity and effects of treatment were analysed by unpaired Student’s t-test. *P < 0.05.
Periconception-onset diabetes is associated with pro-inflammatory cytokine expression in the uterus in early pregnancy. Control (black) and diabetic (grey) female mice were hormone-stimulated and mated, then the uterus was collected on d3.5 p.c. for cytokine analysis. mRNAs for proinflammatory cytokines (A) tumor necrosis factor-related apoptosis-inducing ligand (Storm), (B) interleukin 1α (Il1a), (C) interleukin 1β (Il1b), (D) interleukin 6 (Il6), (E) chemokine (C-X-C motif) ligand 1 (Cxcl1), (F) interferon gamma (Ifng) and (G) tumor necrosis factor (Tnf) and (H) anti-inflammatory cytokine interleukin 10 (Il10), were quantified by qPCR. Data is presented as mean + SEM from 5–7 mice per treatment group and effects of treatment were analysed by unpaired Student’s t-test. *P ≤ 0.05.

We observed lymphopenia in the PALNs draining the uterus in diabetic females, with less than half the expected CD4+ and CD8+ T cell population at the time of embryo implantation, when T-cell proliferation to generate the facilitating maternal immune response to pregnancy is normally initiated. Two main subsets of Treg cells exist, both with recognised roles in mediating maternal tolerance during pregnancy; tTreg cells, derived from the thymus, and pTreg cells, induced via the peripheral conversion of conventional CD4+ T cells. pTreg cell induction during early pregnancy is particularly critical and their genetic ablation disrupts placental morphogenesis and causes fetal loss in mice. In human, perturbations in Treg cells and tolerance induction are associated with fetal growth restriction, preclampsia and preterm birth.

The mechanism by which diabetes induces inflammatory cytokines in the female reproductive tract is not clear, but this is a well-known consequence of diabetes-induced hyperglycaemia in other tissues (reviewed in refs). One biologically plausible mechanism is that elevated stress response in oviductal and uterine epithelial cells secondary to hyperglycaemia promotes their release of TRAIL and other pro-inflammatory cytokines. Based on previous studies, it seems likely that hyperglycaemic is the cause of the inflammatory response. However, a limitation of the streptozotocin model is that direct toxic effects of the streptozotocin cannot be excluded, although this seems highly unlikely given that the half-life of this chemical is 15–30 minutes and cytokine expression was quantified at least 9–12 days after streptozotocin administration. Consistent with a role for the stress response, we found distinct changes in the abundance and localisation of stress deflection proteins GRP94 (HSP90B1), HSP90AA1 and HSPA5 in the luminal epithelium of the oviduct in early pregnancy. The pattern of regulation described is consistent with activation of ER stress and the unfolded protein response (UPR), reported...
Figure 7. Periconception-onset diabetes diminishes total leukocytes and perturbs Foxp3+ Treg cell frequency in the uterus-draining PALN in early pregnancy. Control and diabetic female mice were hormone-stimulated and mated, then on day 3.5 of pregnancy, leukocyte subsets were quantified by flow cytometry. Total cell numbers in PALNs and spleens (A), and proportion and number of CD4+ and CD8+ T cells (B), Treg cells (CD4+ Foxp3+) (D), peripherally-induced Treg cells (pTreg; CD4+ Foxp3+ Nrp1−) and thymus-derived Treg cells (tTreg; CD4+ Foxp3+ Nrp1+) (E) in PALNs are depicted. (C) Representative flow cytometric analysis of Foxp3 staining in CD4+ T cells and Nrp1 staining in CD4+ Foxp3+ Treg cells, in PALNs, and representative histogram depicting mean fluorescence intensity (MFI) of Nrp1 in Treg cells. (A,B,D,E) n = 6–8 mice per treatment group. Data are presented as mean ± SEM and effects of treatment were analysed by unpaired Student’s t-test (*p < 0.05, **p < 0.01).
in diabetes previously\(^{58}\). Of particular interest is the downregulation of HSP90\(\alpha\), which is known to be important for processing of antigens in the ER in preparation for presentation via MHC Class I\(^{59}\). Given the importance for appropriate paternal antigen presentation for the activation of a tolerant maternal immune response in early pregnancy\(^{60}\), we speculate that the shift in the HSP chaperone proteins may disrupt the immune cell cross-talk required to establish a healthy pregnancy. Excitingly, pharmacological interventions to suppress stress deflecting pathways have been demonstrated to be effective in pregnancy\(^{61}\), so may form future therapeutic targets for consideration in maternal diabetes.

In conclusion, we have identified that the pre-implantation embryo and reproductive tract are dramatically altered by streptozotocin-induced periconception-onset diabetes. We report that the embryo is susceptible to hyper-O-GlcNAcylation of both cytoplasmic and nuclear proteins, and this accompanies DNA damage. We show changes to cellular function of the oviductal and uterine epithelium during early pregnancy, including disruption of the heat shock pathway and upregulation of a number of pro-inflammatory cytokines that amplify cell stress in embryos and cause changes to the local immune environment. Each of these pathophysiological changes presumably synergise to impact the events of embryo implantation and robust placentation required for optimal fetal growth. This work identifies molecular and physiological disruptions to the embryo, female reproductive tract and immune system in early pregnancy, all of which we speculate could also occur in diabetic women. If aspects of this pathophysiological response are recapitulated in women, this would imply that reduced fertility in diabetic women may be secondary to a shift in the per-conception stress response, and cytokine and immune environment. If indeed evidence for a periconception impact of maternal diabetes continues to build, preconception screening and treatment interventions will likely have a greater clinical impact than interventions in later pregnancy.

**Materials and Methods**

**Animal handling and models.** Mice were purchased from Animal Resources Centre, Perth, maintained in 14 h/10 h light dark conditions and given water and rodent chow *ad libitum*. All experiments were approved by The University of Adelaide Animal Ethics Committee (M-2013–233/172) and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Eight week old female C57BL/6 mice were treated with 180 mg/kg streptozotocin dissolved in citrate buffer (pH 4.5). Blood glucose was monitored every two days by tail prick (Accu-Chek, Roche, Sydney, NSW, Australia). When blood glucose reached 14 mmol/L (6–9 days following injection), mice were available for experiments. For analysis on day (d) 1.3 post-coitus (p.c.) and d3.5 p.c., female mice were administered (i.p.) 5 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet, Boxmeer, The Netherlands). Forty-six hours post-eCG injection, mice were administered 5 IU human chorionic gonadotrophin (hCG; hCG/Pregnyl; Merck, Kilsyth, VIC, Australia) and mated with males of the same strain. The following morning, considered d0.5 p.c., mice were assessed to confirm the presence of copulation plugs and then culled via cervical dislocation on d1.5 or d3.5 p.c. For mid-gestation pregnancy parameters, female mice were placed with males without prior hormone treatment and assessed daily for copulation plugs then killed and autopsied on d11.5 p.c.

**Tissue and embryo collection.** Unless otherwise specified, all reagents and antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA). Embryos and reproductive tracts were collected into Research Vitro Wash (Cook Medical Pty. Ltd.) on warming stages calibrated to 37 °C. On d1.5 and 3.5 p.c., embryos were flushed into media followed by fixation (30 min, 4% paraformaldehyde in phosphate-buffered saline (PBS) + 3 mg/ml polyvinylalcohol). Oviducts/uteri were snap frozen in liquid nitrogen for qPCR analysis or fixed O/N (as above) for histological analysis. On d3.5 p.c., spleen and uterus-draining para-aortic lymph nodes (PALN) from females confirmed pregnant by embryo flushing, were harvested for flow cytometry analysis. On d11.5 p.c., reproductive tracts and implantation sites were dissected and weighed, and embryos staged using the Theiler system.

**Immunohistochemistry, confocal microscopy and image analysis.** Embryos: Following fixation, embryos were analysed by immunohistochemistry for DNA damage repair (anti-\(\gamma\)H2AX primary antibody; Cell

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**Figure 8.** Periconception-onset diabetes alters the reproductive tract environment in early pregnancy. A schematic summary of the changes seen in early pregnancy in the streptozotocin-induced diabetic model.
All data were analysed using FlowJo software (TreeStar, Inc., Ashland, OR). 

Instructions, and anti-Foxp3 APC (FJK-16s, eBioscience) antibody. Data were acquired on a BD FACSCantoII.

Intracellular staining was performed using Foxp3 Staining Buffer Set (eBioscience, San Diego, CA), as per the manufacturer’s instructions. 

First-strand cDNA was synthesized in triplicate for each sample on a Rotor-Gene 6000 (Corbett Life Science, Sydney, NSW, Australia) and synthesized by Geneworks (Hindmarsh, SA, Australia). qPCR data were natural log-transformed where necessary to achieve equal variances before analysis. Embryo rate exclusion was enabled by addition of BD Horizon™ Fixable Viability Stain 620 (Becton Dickinson (BD), Franklin Lakes, NJ). $10^6$ cells were stained in PBS (0.05% sodium azide, 0.1% BSA (Sigma)). Fc receptors were blocked with anti-CD16/CD32 Fc Block™ (Mouse Fc Block™; BD) before surface staining with antibodies; anti-CD4 APC-Cy7 (GK1.5, BD), anti-CD8 PE-Cy7 (53–6.7, BD) and Nrp1 BV421 (3E12, Biolegend, San Diego, CA). Intracellular staining was performed using Foxp3 Staining Buffer Set (eBioscience, San Diego, CA), as per the manufacturer’s instructions, and anti-Foxp3 APC (FJK-16s, eBioscience) antibody. Data were acquired on a BD FACSCantoII. All data were analysed using FlowJo software (TreeStar, Inc., Ashland, OR).

Flow cytometry. Cell suspensions were generated from spleens and PALNs by gentle homogenization in RPMI 1640 (Gibco™, Life Technologies, Invitrogen, Australia Pty. Ltd.). Erythrocytes were lysed in splenocyte suspensions using red blood cell lysis buffer (155 mM NH$_4$Cl, 10 mM KHCO$_3$, and 0.1 mM EDTA). Dead cell exclusion was enabled by addition of BD Horizon™ Fixable Viability Stain 620 (Becton Dickinson (BD), Franklin Lakes, NJ). $10^6$ cells were stained in PBS (0.05% sodium azide, 0.1% BSA (Sigma)). Fc receptors were blocked with anti-CD16/CD32 Fc Block™ (Mouse Fc Block™; BD) before surface staining with antibodies; anti-CD4 APC-Cy7 (GK1.5, BD), anti-CD8 PE-Cy7 (53–6.7, BD) and Nrp1 BV421 (3E12, Biologic, San Diego, CA). Intracellular staining was performed using Foxp3 Staining Buffer Set (eBioscience, San Diego, CA), as per the manufacturer’s instructions, and anti-Foxp3 APC (FJK-16s, eBioscience) antibody. Data were acquired on a BD FACSCantoII. All data were analysed using FlowJo software (TreeStar, Inc., Ashland, OR).

Statistical Analysis. Data are presented as mean ± SEM, and effects of treatment were analysed by one-way Student’s T test using GraphPad Prism Version 7 and SPSS (Version 22) for Windows as described in the figure legends. Implantation size weight was expressed as estimated marginal means and analysed by mixed model linear repeated-measures analysis of variance and post-hoc least significant difference test, with mother as subject. qPCR data were natural log-transformed where necessary to achieve equal variances before analysis. Embryo rate (%) data was arcsine transformed for analysis. Statistically significant difference was concluded when $P < 0.05$. All data is generated from $>3$ experimental replicates, unless stated otherwise.

Data Availability. All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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**Author Contributions**

H.M.B. conceived and performed experiments, obtained funding, analysed data and wrote manuscript. E.S.G. and T.C.Y.T. performed experiments. M.B.G. and A.R.R. contributed to experimental design, interpretation of findings and manuscript preparation. L.H. and R.J.N. provided clinical mentorship and advice. N.H.P. and S.A.R. provided advice on experimental design and data interpretation, and revised the manuscript. J.G.T. co-funded research and provided support and guidance on all aspects of the research. All authors contributed to the manuscript preparation and approved the final version. H.M.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. H.M.B. is supported by the University of Adelaide Beacon Postdoctoral Fellowship. A.R.R. is supported by a Career Development Fellowship frim the National Health and Medical Research Council of Australia (NHMRC). J.G.T. is supported by a Senior Research Fellowship from the NHMRC. Research was funded by the Robinson Research Institute (University of Adelaide) and the A.R.C. Centre of Excellence for Nanoscale BioPhotonics.

**Additional Information**

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