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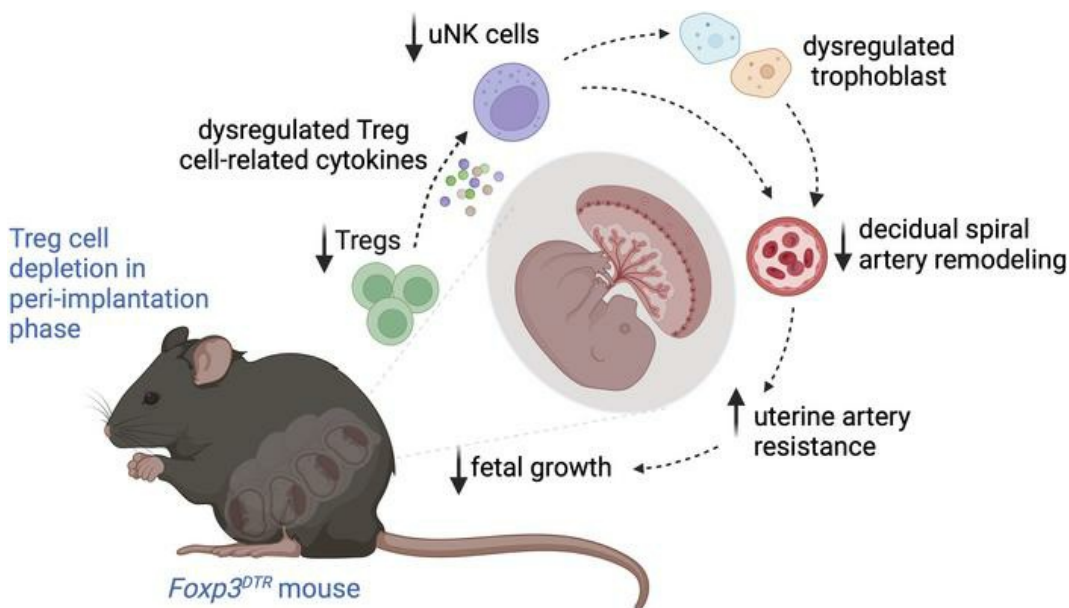
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Graphical abstract

Regulatory T cells promote decidual vascular remodeling and modulate uterine NK cells in pregnant mice

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25 **ABSTRACT**

26 Regulatory T (Treg) cells are essential for maternal immune tolerance of the fetus and placenta. In

27 preeclampsia, aberrant Treg cell tolerance is implicated, but whether and how Treg cells affect the

28 uterine vascular dysfunction thought to precede placental impairment and maternal vasculopathy

29 is unclear. We utilized $Foxp3^{DTR}$ mice to test the hypothesis that Treg cells are essential regulators

30 of decidual spiral artery adaptation to pregnancy. Transient Treg cell depletion during early

31 placental morphogenesis caused impaired remodeling of decidual spiral arteries, altered uterine

32 artery function and led to fewer DBA^+ uterine natural killer (uNK) cells, resulting in late gestation

33 fetal loss and fetal growth restriction. Replacing the Treg cells by transfer from wild-type donors

34 mitigated the impact on uNK cells, vascular remodeling, and fetal loss. RNA sequencing of

35 decidua revealed genes associated with NK cell function and placental extravillous trophoblasts

36 were dysregulated after Treg cell depletion, and normalized by Treg cell replacement. These data

37 implicate Treg cells as essential upstream drivers of uterine vascular adaptation to pregnancy,

38 through a mechanism likely involving phenotypic regulation of uNK cells and trophoblast

39 invasion. The findings provide insight into mechanisms linking impaired adaptive immune

40 tolerance and altered spiral artery remodeling, two hallmark features of preeclampsia.

41

42

2

INTRODUCTION

Regulatory T (Treg) cells are a specialized subset of T cells that suppress inflammation and limit deleterious immune responses to self and foreign antigens (1). Treg cells have a pivotal role in immune homeostasis by modulating the proliferation and activity of effector T and B cells, and natural killer (NK) cells. Treg cells are essential for pregnancy tolerance; sufficient numbers of functionally competent Treg cells must be present in the uterus for successful embryo implantation and placental development (2). Since pregnancy is an inflammatory state and the fetoplacental unit expresses paternally-derived alloantigens (2), the uterine Treg cell pool has important roles in constraining inflammation and preventing aberrant maternal immune reactivity (3-6).

Fewer or functionally impaired Treg cells are a common feature of preeclampsia (7-11)—a serious hypertensive disorder that affects 2-8% of all pregnant women (12, 13). Treg cell deficiency associated with aberrant generation of effector T(eff) cells, including pro-inflammatory Th1 and Th17 cells (8, 14), is evident in peripheral blood and uterine decidual tissue in preeclampsia (7-11), and may originate due to insufficient priming towards paternal alloantigens and/or underlying immune dysfunction (15). Although a definitive causal role for Treg cell deficiency in the placental pathophysiology of these conditions is not proven in humans, studies in rodent models (16, 17) indicate biological plausibility and the significance of paternal factors is consistent with a maladaptive immune response (15, 18).

One candidate mechanism by which Treg cell insufficiency may contribute to defective placentation involves the uterine blood supply (19). Emerging evidence shows Treg cells are essential regulators of systemic vascular homeostasis supporting healthy cardiovascular function

66 in a range of tissues (20, 21). Treg cell insufficiency has been linked to hypertension and aberrant

67 vascular remodeling (22, 23), while treatment interventions to boost Treg cells can reverse

68 cardiovascular dysfunction (24, 25) and mitigate hypertension (24). These considerations raise the

69 prospect that in addition to their immune regulatory roles, Treg cells might facilitate the vascular

70 adaptations required to accommodate pregnancy – but whether and how this occurs is unknown.

71

72 The maternal cardiovascular system must undergo substantial remodeling to support pregnancy

73 and enable optimal placental function. These events commence with local changes to the uterine

74 vasculature during early placental development. Systemic cardiovascular adaptations follow,

75 featuring increased blood volume, enhanced cardiac output, and reduced total peripheral resistance

76 (26). A substantial increase in blood supply to the uterine artery and the placenta supports the

77 increasing nutrient demands of the developing fetus as gestation proceeds (27). Robust fetal

78 growth in late gestation therefore depends on adequate early remodeling and vasodilation of the

79 uterine artery and its network of decidual spiral arteries (27-29).

80

81 Decidual spiral arteries are the terminal branch of the main uterine arteries. Their remodeling

82 involves trophoblast invasion of the decidual vessel wall and a loss of the surrounding smooth

83 muscle cells and elastic lamina in women (30), with similar features during placentation in mice

84 (31). Migrating extravillous trophoblast cells invade both the decidual interstitium and the

85 endovascular space within the spiral arteries to facilitate their transformation from high-resistance

86 vessels to low-resistance, high-capacity vessels. These vessels perfuse the placental intervillous

87 space from which exchange of nutrients and oxygen between maternal and fetal circulations occurs

88 (32) to accommodate escalating fetal demand as pregnancy progresses (33).

89

90   Immune cells, particularly uterine natural killer (uNK) cells, are critical for decidual spiral artery

91   remodeling (34). uNKs are an abundant subset of innate lymphoid cells in the decidua (35) that

92   are essential for decidual vascular remodeling in mice (34, 36, 37), and humans (38). They secrete

93   cytokines such as IFNγ and VEGFA (35, 37, 39, 40) and enzymes that modulate endothelial cell

94   tight junctions, cause smooth muscle cell apoptosis, and promote trophoblast invasion (34, 41).

95   Elevated inflammatory activity impairs uNK cell function, leading to shallow spiral artery

96   remodeling and placental dysfunction (42, 43).

97

98   We previously demonstrated that depletion of Treg cells from pregnant mice causes dysfunction

99   in the main arteries serving the uterus, causing increased resistance and altered regulation of

100   vasoconstriction, in turn affecting systemic blood pressure regulation (19). Additionally, we

101   showed Treg cell deficiency caused by insufficient progesterone signaling elicits impaired

102   decidual spiral artery remodeling (16), building on other studies noting that Treg cell perturbation

103   can be accompanied by uterine vascular changes (2, 19, 44). However, there has been no formal

104   investigation of the specific requirement for Treg cells in remodelling of the decidual spiral arteries

105   or investigation of the cellular mechanisms by which this might occur.

106

107   In this study, we test the hypothesis that Treg cells are essential for decidual spiral artery

108   remodeling in early pregnancy, utilizing $Foxp3^{DTR}$ transgenic mice (45) to allow acute, transient

109   Treg cell depletion. Here we report evidence that Treg cell deficiency is a primary cause of poor

110   remodeling of decidual spiral arteries, causing fetal loss and growth restriction in surviving fetuses,

111   and a mechanism involving dysregulation of uNK cells and altered trophoblast invasion is

112   implicated. Our data provide compelling evidence to link Treg cells and uteroplacental vascular

113   remodeling in an interactive network underpinning establishment and progression of healthy

114   pregnancy.

115

RESULTS

Treg cell depletion during the peri-implantation period causes fetal loss and fetal growth restriction

To evaluate the physiological significance of Treg cells in the peri-implantation phase for pregnancy progression, we administered DT on gestational day (GD)3.5 and 5.5 to selectively and transiently deplete the Treg cell pool, as previously described (19). In $Foxp3^{DTR}$ mice treated with DT, Treg cells (measured as a proportion of CD4$^+$ T cells) were reduced by ≥97% in the uterine-draining, para-aortic lymph nodes (uDLN) by GD6.5, 24 h post-treatment. This site is where Treg cells normally proliferate prior to recruitment into the uterus (46)(Figure 1A, B). In contrast, Treg cells in vehicle-treated control $Foxp3^{DTR}$ mice remained unchanged. Analysis at the critical mid-gestation timepoint of GD10.5, when the mouse placenta is mature and placental access to the maternal blood supply is complete (30, 47), showed partial repopulation of Treg cells in the uDLN, although their proportion remained ~59% less than in control mice (Figure 1B). The majority of Treg cells in the uDLN in mid-gestation were thymic-derived as indicated by NRP1 expression (48), regardless of earlier Treg cell depletion (Figure 1C). Repopulating Treg cells more commonly expressed the proliferation marker Ki67 (Figure 1D), while similar proportions expressed CTLA4, a marker of suppressive competence (49)(Figure 1E). Treg cell deficiency did not cause an increase in CD4$^+$ T cell expression of IFNγ or IL17a (Figure 1F, G). Comparable effects were seen in the spleen, consistent with a systemic impact of DT on Treg cells in $Foxp3^{DTR}$ mice (Supplemental Figure 1). Recovery was faster in the spleen, such that on GD10.5 Treg cells were not different in proportion or phenotype between DT-treated and control mice (Supplemental Figure 1A-D), and IFNγ and IL17a expression were unchanged (Supplemental Figure 1E, F).

139   To evaluate the impact of peri-implantation pregnancy Treg cell depletion on pregnancy success,

140   *Foxp3*[DTR] mice were given DT or vehicle control and birth outcomes were recorded. Additional

141   groups of DT-treated *Foxp3*[DTR] mice were administered wild-type Treg cells (CD4$^+$CD25$^+$ cells)

142   that do not express human DTR and are refractive to DT-induced depletion (45), or conventional

143   CD4$^+$ T cells (Tconv; CD4$^+$CD25$^-$ cells), on GD2.5 and GD4.5. Transferred cells were prepared

144   from the spleens and lymph nodes of pregnant donor mice to ensure hormone and antigen priming,

145   which are important for Treg cell function in pregnancy (50). A subset of dams were allowed to

146   give birth and birth outcomes were evaluated, and for another subset, pregnancy outcomes were

147   evaluated in late gestation (Figure 2A).

148

149   Compared to control dams administered vehicle, Treg cell depletion compromised pup viability at

150   birth (Figure 2D, E) and reduced pup weight (Figure 2F), but had no impact on the proportion of

151   dams with viable pregnancies (defined as 1 or more viable pup), or total pups born (Figure 2B, C).

152   Pre-treatment with wild-type Treg cells in DT-treated *Foxp3*[DTR] mice prevented the shift in

153   pregnancy success, litter size and birth weight, while Tconv cells did not confer the same

154   improvement (Figure 2D-F).

155

156   The poor pregnancy outcomes after peri-implantation Treg cell depletion prompted us to evaluate

157   late gestation fetal and placental parameters. In DT-treated and vehicle control *Foxp3*[DTR] mice

158   autopsied at GD18.5, the rate of viable pregnancy (defined as at least one viable fetus), and total

159   or viable implantation sites per dam (Figure 2G-I) were unchanged. However, DT treatment caused

160   an increase in both the number and the proportion of overt fetal losses (resorptions) per dam

161   compared to control mice (Figure 2J-K), indicating fetal loss occurred after mid-gestation. Pre-

162   treatment with wild-type Treg cells mitigated against Treg cell deficiency (Figure 2J, K), such that

163   resorptions were not different to control mice. In contrast, Tconv cells did not attenuate the effects

164   of Treg cell deficiency (Figure 2J, K).

165

166   Surviving fetuses from *Foxp3*[DTR] mice treated with DT to elicit Treg cell depletion were growth

167   restricted on GD18.5, compared to vehicle-treated controls. Fetal weight was reduced by 3.1%,

168   crown-to-rump length by 1.9%, abdominal girth by 3.4%, and biparietal diameter by 5.0% (Figure

169   3A, B, H-J). Transfer of wild-type Treg cells to DT-treated mice did not correct late-gestation fetal

170   weight (Figure 3B) but did mitigate other parameters of fetal growth (Figure 3H-J). In contrast,

171   Tconv cells further reduced fetal weight, crown-to-rump length, and biparietal diameter (Figure

172   3B, H, J). When fetal weight distribution was considered (Figure 3E), 27% were below the 10th

173   percentile in DT-treated mice, compared to 21% in dams given DT and Treg cells, and 46% in

174   dams given DT and Tconv cells.

175

176   There was no impact of Treg cell depletion from *Foxp3*[DTR] mice, nor exogenous Treg cell or Tconv

177   cell administration, on placental weight (Figure 3C, F). However, Treg cell depletion reduced the

178   fetal:placental weight ratio, a surrogate measure of placental efficiency (Figure 3D, G). This was

179   unchanged by Treg cell or Tconv transfer (Figure 3D). This indicates that as well as fetal loss,

180   Treg cell depletion impaired placental efficiency in surviving fetuses, in turn affecting fetal

181   growth. Together these data show that Treg cell deficiency, and not off-target effects of DT

182   treatment, are responsible for fetal loss and fetal growth impairment. Consistent with this

183   interpretation, no effect of DT on fetal survival, fetal weight, or growth parameters was seen in

184   wild-type C57BL/6 mice given DT on GD3.5 and 5.5, compared to mice given vehicle

185   (Supplemental Figure 2).

186

187   To further investigate the impacts of Treg cell depletion, placental structure was assessed in late

188   gestation (Supplemental Figure 3A-D). There was no effect of Treg cell depletion on placental

189   structure at GD18.5 as indicated by area of junctional zone (JZ) or labyrinth zone (LZ), the tissues

190   responsible for endocrine function and nutrient transfer respectively (Supplemental Figure 3E-I).

191   The proportion of glycogen cells in the junctional zone was increased following DT treatment to

192   elicit Treg cell depletion compared to control mice. This was prevented by treatment with Treg

193   cells but not Tconv cells (Supplemental Figure 3J).

194

195   *Treg cell depletion disrupts uterine vascular function and decidual spiral artery remodeling*

196   We next investigated the impact of Treg cell depletion on the uterine vasculature in mid-gestation,

197   as this is a critical determinant of placental maturation and capacity to support fetal survival and

198   growth. At GD10.5, *Foxp3*^{DTR} dams given DT to elicit Treg cell depletion had a similar rate of

199   viable pregnancy, number of total and viable implantation sites per dam, and proportion and

200   number of abnormal implantation sites showing early signs of resorption compared to vehicle-

201   treated control *Foxp3*^{DTR} dams. Transfer of Treg cells or Tconv cells did not change these

202   parameters (Supplemental Figure 4A-I).

203

204   When decidual spiral artery remodeling was quantified stereologically in midsagittal sections of

205   implantation sites on GD10.5, impaired spiral artery remodeling was seen after Treg cell depletion

206   (Figure 4A-D). Decidual spiral artery lumen area and lumen diameter were reduced by 29% and

207 15% respectively compared to control (Figure 4A-B, I-J). There was a trend towards an increased

208 relative wall thickness in decidual spiral arteries (vessel:lumen ratio) after Treg cell depletion

209 (Figure 4K; P=0.08). Transfer of wild-type Treg cells normalized decidual spiral artery remodeling

210 (Figure 4C, I-J), but Tconv cells did not (Figure 4D, I-J), and increased the relative wall thickness

211 of vessels, compared to control mice (Figure 4K).

212

213 Smooth muscle cells are normally lost from the arterial vascular media during the spiral artery

214 remodeling process, and smooth muscle actin (SMA) retention indicates impaired remodeling (51).

215 Depletion of Treg cells from $Foxp3^{DTR}$ mice resulted in a 2-fold increase of SMA expression

216 compared to control $Foxp3^{DTR}$ mice (Figure 4E-F, L). Loss of SMA was improved by transfer of

217 Treg cells (Figure 4G, L), but not Tconv cells (Figure 4H, L).

218

219 To determine the impact of Treg cell perturbation in the peri-implantation phase on function of the

220 main uterine arteries in mid-gestation, we used ultrasound biomicroscopy on GD9.5. Treg cell

221 depletion was found to increase uterine artery resistance index by 11.0%, indicating increased

222 resistance to blood flow (Figure 5A, B). Administration of Treg cells, but not Tconv cells,

223 mitigated the increase in uterine artery resistance (Figure 5B). Uterine artery pulsatility index,

224 another measure of resistance, showed an increasing trend following Treg cell depletion (P=0.06;

225 Figure 5C) that was partially improved by Treg cell transfer, but not Tconv cells (Figure 5C).

226 These findings build upon our previous report of altered uterine vascular parameters after Treg

227 cell depletion (19). The effect of Treg cell depletion on uterine artery function was limited to mid-

228 pregnancy – when uterine artery and umbilical artery function were measured on GD17.5, there

229 was no impact of Treg cell depletion or treatment with Treg or Tconv cells (Supplemental Table

230 1).

231

232 *Treg cell depletion modifies uNK cells, key regulators of spiral artery remodeling*

233 Given the central role of uNK cells in spiral artery remodeling, we next investigated whether uNK

234 cells contribute to the mechanism by which Treg cell deficiency causes remodeling impairment.

235 Initially, uNK cell abundance was assessed by histological staining with *Dolichos biflorus*

236 agglutinin (DBA) lectin to identify uNK cells in midsagittal sections of implantation sites. DBA

237 reacts with *N*-acetylgalactosamine (GalNAc) residues present on the cell surface and in granules

238 of uNK cells (52). DBA$^+$ uNK cells, which predominate in pregnancy, produce factors including

239 VEGFA that facilitate spiral artery remodeling by promoting migration of extravillous trophoblasts

240 through the decidua (40), while DBA$^-$ uNK cells produce IFNγ (35, 40). Compared to vehicle-

241 treated control *Foxp3*^{DTR} mice, Treg cell-depleted *Foxp3*^{DTR} mice had substantially less DBA

242 staining in the decidua on GD10.5 (Figure 6A, B, E). DBA$^+$ uNK cell abundance was normalized

243 by Treg cell transfer, but not by Tconv cells (Figure 6A-E). Notably, the total mid-sagittal area of

244 decidual tissue was not affected by Treg cell depletion, indicating the vascular and uNK cell

245 changes were unlikely to be due to defective decidualization (Figure 6F).

246

247 To further understand the impact of Treg cell depletion, uNK cells were investigated by flow

248 cytometry. uNK cells can be classified into three subsets with distinguishing surface phenotypes

249 with potential for distinct roles in angiogenesis and vascular remodeling (53). All three subsets

250 express the NK lineage-defining surface marker NK1.1 (54) and acquire NKp46 [also termed

251 natural cytotoxicity receptor (NCR)1] upon maturation in the decidua (55-58). The uNK cell

252   subsets include: (1) tissue resident (tr)NKs, defined as CD45$^+$NK1.1$^+$CD11B$^{lo/-}$

253   NKp46$^+$CD49a$^+$EOMES$^+$ cells; (2) conventional NK (cNK) defined as CD45$^+$NK1.1$^+$CD11B$^{lo/-}$

254   NKp46$^+$CD49a$^-$ cells, and (3) group 1 innate lymphoid cells (ILC1) defined as

255   CD45$^+$NK1.1$^+$CD11B$^{lo/-}$NKp46$^+$CD49a$^+$EOMES$^-$ cells. cNK cells are the primary source of IFNγ

256   (58) while trNKs produce VEGFA. When trNK, cNK, and ILC1 cells were analyzed in decidua

257   recovered on GD10.5 from vehicle- or DT-treated *Foxp3*^{DTR} mice, as expected the largest

258   population was trNKs, followed by cNKs and then ILC1s (Supplemental Figure 5A-E (58)). Treg

259   cell depletion did not change the proportion of NK1.1$^+$ uNK cells categorised as cNK, ILC1, or

260   trNK compared to vehicle-treated control mice (Supplemental Figure 5C-E). Similarly no change

261   was seen in the proportion of NK1.1$^+$ or NKp46$^+$ NK cells amongst non-T, non-B cells, or the total

262   CD3$^+$ T cells (Supplemental Figure 5F-J).

263

264   *Transcriptomic analysis shows canonical NK cell and T cell pathways are disrupted by Treg cell*

265   *depletion and restored by Treg cell replacement.*

266   The data indicating fewer DBA$^+$ uNK cells compelled us to investigate the impact of Treg cell

267   depletion on gene transcription in the decidua following Treg cell depletion. We performed RNA

268   sequencing on decidual tissue collected at GD10.5 from vehicle-treated (veh), DT-treated

269   *Foxp3*^{DTR} mice (DT), and DT-treated *Foxp3*^{DTR} mice given wild-type Treg cells (DT+Treg; N=5-

270   6 samples per group; see Supplemental Table 2 for RNA-seq metrics). After removal of non- and

271   lowly-expressed genes, 14748 genes were TMM-normalized and subsequent dimensionality

272   reduction via principal component analysis (PCA) identified three distinct clusters corresponding

273   to the three treatment groups (Figure 7A).

274

275   Transcriptomic profiling identified 446 differentially expressed genes (DEGs) following Treg

276   depletion compared to the vehicle-treated group (DT vs veh), including 192 upregulated and 254

277   downregulated genes (all differentially expressed genes detected are listed in the Supporting Data

278   Values file, and the top 50 up- and downregulated genes are listed in Supplemental Table 3 and

279   4). In mice that received Treg cells as well as DT (DT+Treg group) the majority of the 446 DEGs

280   were unchanged and only 35 of the 446 DEGs were differentially expressed (Figure 7B), indicating

281   partial normalization of the transcriptional changes.

282

283   To understand their functional significance, over-representation analysis of the DEGs from these

284   comparisons were performed using Gene Ontology (GO) (59), Kyoto Encyclopaedia of Genes and

285   Genomes (KEGG) (60), Reactome (61), and Ingenuity Pathway Analysis (IPA, QIAGEN)

286   databases (Supplemental Figures 6-8). Pathways regulated by Treg depletion during the peri-

287   implantation phase predominantly reflected immune effector responses, particularly those

288   involving NK cell and T cell activation and cytotoxicity. Notably, GO analysis identified

289   Granzyme-Mediated Programmed Cell Death Signaling Pathway, T Cell Mediated Cytotoxicity,

290   NK Cell Mediated Cytotoxicity, and NK Cell Lectin-like Receptor Binding pathways

291   (Supplemental Figure 6), while KEGG analysis identified pathways including Graft versus Host

292   Disease, Allograft Rejection and Natural Killer Cell-mediated Cytotoxicity (Supplemental Figure

293   7). IPA revealed that Interferon Gamma Signaling and Interferon Alpha/Beta Signaling were

294   amongst the top canonical pathways (Supplemental Figure 8) and biological functions invoking

295   lymphocyte cytotoxicity (Supplemental Figure 9). The logFC of DEGs linked with selected

296   pathways associated with NK cell and T cell activation are shown in Figure 7C.

297

298　Strikingly, transfer of wild-type Treg cells to DT-treated *Foxp3*^{DTR} mice substantially normalised

299　most of the DEGs (Supplemental Table 3 and 4), including many of the DEGs annotated to NK

300　and T cell activation pathways (Figure 7C). Amongst the effector genes upregulated by Treg

301　depletion and downregulated by Treg cell replacement were genes encoding granzyme B (*Gzmb*)

302　and perforin pore-forming proteins (*Prf1*) that mediate lymphocyte cytotoxicity, as well as

303　granzymes associated with remodeling activity *Gzmc, Gzmd, Gzme, Gzmf, Gzmg* (62-64). Certain

304　NK cell-associated transcription factors were upregulated after Treg cell depletion, notably *Eomes*,

305　*Id2*, and *Runx3* which control aspects of NK cell maturation and function. Many genes involved

306　in the interferon response pathway, a key program associated with activation of cNK and ILC1

307　cells, were also upregulated, as were genes *Il15ra, Il2rb*, and *Il2rg* encoding the three components

308　of the trimeric IL15 receptor (IL15Rα, IL15Rβ, and IL2Rγ) that mediates uNK cell responsiveness

309　to the uNK cell activating cytokine IL15.

310

311　An extensive array of genes known to be expressed by placental trophoblasts were also present

312　among the DEGs (Supplemental Figure 10). A subset of these trophoblast genes selected on the

313　basis of their absence in uterine cells (indicating trophoblast specificity (65)) included *Cdh5*, *Lcp1*,

314　and *Olr1,* all of which are associated with placental glycogen cells (65)(Figure 8, Supplemental

315　Figure 10). Others including *Krt7, Krt8 Psck6*, *Cited4*, and *Hsd11b2* expressed by various

316　extravillous trophoblast lineages (66) were downregulated in decidual tissue after Treg cell

317　depletion (Figure 8). These trophoblast genes were largely normalized after Treg cell replacement

318　(Figure 8, Supplemental Figure 10).

319

320   Notably, there was no evidence of altered expression of hallmark decidualization genes *Prl,*

321   *Igfbp1, Foxo1, Fstl1, Bmp2,* or *Ptgs2*, following Treg depletion as might be expected if decidual

322   development or function was compromised (Supporting Data Values file). These observations

323   clearly point to the significance of Treg cells in constraining uNK cell activation pathways under

324   normal conditions and strongly suggest a role for inappropriately activated uNK cells and altered

325   trophoblast cell invasion or survival in the observed vascular remodeling and fetal loss seen after

326   Treg cell depletion.

327

328   *Treg cell-derived anti-inflammatory cytokines are implicated in uNK cell regulation.*

329   Upstream regulator analysis in IPA was performed to identify factors implicated as potential

330   regulators of the Treg cell depletion-induced decidual gene transcription changes. The predicted

331   upstream regulators included 93 candidate immune regulatory cytokines, cytokine receptors and

332   transcription factors. Around half of these overlapped with genes detected in decidual tissue and

333   some were DEGs regulated by Treg cell depletion and replacement (Supplemental Figure 11).

334   Notably, the predicted regulators included two factors known to be released by Treg cells with

335   potential to modulate uNK cell function, IL10 (activation Z-score=2.1) and EBI3 (a subunit of

336   IL35; activation Z-score=2.6). IL35 is an immunosuppressive cytokine composed of two chains,

337   IL12α (p35) and EBI3 (67), that is produced exclusively by Treg cells to modulate the function of

338   NK cells (54, 68). The upstream regulator analysis also implicated IFNG (activation Z-score=6.1).

339

340   Because these regulators were not detected by RNAseq, presumably due to insufficient sequencing

341   depth, we used qPCR to measure decidual expression of *Il12p35, Ebi3* and *Il10,* as well as *Tgfb1,*

342   another key Treg cell-derived immune regulatory cytokine (67), plus uNK cell factors *Ifng* and

343   *Vegf* involved in vascular remodelling (35, 37, 51). In the same GD10.5 decidual samples utilised

344   in the RNA sequencing analysis, peri-implantation Treg cell depletion resulted in a 62% reduction

345   in *Ebi3* expression and a 58% downregulation of *Il12p35* expression, compared to control

346   *Foxp3*[DTR] mice (Figure 9A-B). Similarly, *Il10* was reduced by 48% and *Tgfb1* was reduced by

347   60% after Treg cell depletion (Figure 9C, D). In contrast decidual *Ifng* was not impacted (Figure

348   9E), but *Vegfa* expression was reduced by 57% compared to control mice (Figure 9H). Decidual

349   expression of *Foxp3* mRNA encoding the Treg cell transcription factor FOXP3 was reduced by

350   71% after earlier Treg cell depletion (Figure 9G), while *Ncr1* encoding the uNK cell maturation

351   marker NKp46 was reduced by 60% (Figure 9F), consistent with the histological finding of fewer

352   uNK cells in decidual tissues.

353

354   **DISCUSSION**

355   There is compelling evidence that dysregulation in the number or function of Treg cells contributes

356   to the pathophysiological origin of preeclampsia and related pregnancy complications (69, 70) but

357   whether and how Treg cells contribute to the uterine and placental dysfunction underpinning these

358   conditions remains unclear. In this study, *Foxp3*[DTR] mice were used to selectively and transiently

359   deplete Treg cells to investigate their role in the critical process of decidual spiral artery

360   remodeling required in early pregnancy for robust placental development and function. Here, we

361   demonstrate that Treg cell depletion causes impaired spiral artery remodeling, resulting in fetal

362   loss and fetal growth restriction. Treg cell depletion was associated with an altered transcriptional

363   profile in the decidua that along with histochemical analysis implicates dysregulation of uNK cells

364   and extravillous trophoblasts in mid-gestation. In particular, depletion of Treg cells caused

365   upregulation of genes involved in NK cell interferon signaling and cytotoxic function, notably

granzyme-mediated programmed cell death signalling. Treg cell replacement experiments largely rescued the reproductive phenotype and reversed the gene expression changes, confirming that Treg cells affect decidual spiral artery remodeling, and supporting the inference of a mechanism involving Treg cell modulation of uNK cell functional status. This requirement for Treg cells provides new insight into immune regulation of uterine vascular remodeling, extending our previous demonstration of a role for Treg cells in regulating the main uterine arteries in pregnancy (19). If a similar effect of Treg cells on uterine vascular remodelling occurs in women, Treg cell deficiency would act to constrain the placental vascular supply, contributing to development of preeclampsia and other pregnancy disorders characterized by impaired placentation including recurrent miscarriage, fetal growth restriction and spontaneous preterm birth (2).

After embryo implantation in mice the uterine lining undergoes decidualization and extravillous trophoblasts invade the decidua both interstitially and endovascularly, to progress remodeling of the decidual spiral arteries and enable placental growth (71, 72) and full access to the maternal blood supply by GD10.5 (30, 47). Remodeling is a process that involves displacement of endothelial cells and smooth muscle cells from the spiral artery wall, converting them to flaccid conduits. uNK cells are the main maternal immune cells present in the decidua at the time of implantation (34, 41). They engage with trophoblasts in clusters around spiral arteries and are paramount in facilitating the remodeling process through secreting cytokines and proteases (37, 39). Treg cells, macrophages, and other immune cells are positioned within the decidual stroma with the potential to interact with uNK cells, as well as trophoblast cells, decidual cells, spiral artery smooth muscle cells, and endothelial cells (2, 32). There is evidence of cross-regulation between these immune cells, such that together they ensure correct uNK cell function (73, 74).

389

390 Our experiments imply that a primary cause of pregnancy loss in late gestation following Treg cell

391 depletion in early pregnancy is placental insufficiency due to inadequate remodeling of decidual

392 spiral arteries in early pregnancy. Spiral arteries from Treg cell-depleted mice had a smaller lumen

393 and cross-sectional area, and failed to exhibit the expected loss of smooth muscle actin. This was

394 accompanied by a trend towards increased relative wall thickness when Treg cells were depleted.

395 Importantly, all of these markers of spiral artery remodeling were normalized when mice were pre-

396 treated with wild-type Treg cells to prevent Treg cell deficiency.

397

398 A combination of immunohistochemical and RNAseq data point to a role for Treg cells in

399 modulating uNK cells. uNK cell subsets can be distinguished by their reactivity to DBA lectin,

400 which detects GalNAc (75), a glycosylated structure acquired during functional maturation after

401 conception (51, 52). DBA$^+$ uNK cells are more abundant than DBA$^-$ uNK cells in the decidua and

402 exhibit pro-angiogenic activity. Treg cell depletion reduced DBA$^+$ uNK cells in the decidua, and

403 this was mitigated by Treg cell replacement. DBA$^+$ uNK cells express higher levels of *Vegfa, Il22,*

404 and *Pgf* compared to DBA$^-$ uNK cells, consistent with a contribution to decidual angiogenesis,

405 while DBA$^-$ uNK cells express more *Ifng*, which also plays a role in remodeling of the decidual

406 vasculature (35, 51).

407

408 We analyzed uNK cells utilizing a flow cytometry panel designed to detect trNK cells, cNK cells

409 and ILC1 cells, without regard to their DBA expression. trNK cells are resident within the decidua

410 and express angiogenic factors, cNK cells are recruited from peripheral blood and are similar to

411 splenic NKs, while ILC1s arise from trNK cells residing in the non-pregnant uterus (76) but do

412   not express the NK cell-specific transcription factor, EOMES (58, 77). Flow cytometry did not

413   reveal any change in the relative proportion of the three uNK subsets in Treg cell deficient mice.

414   We therefore undertook RNAseq to assess the impact of Treg cell depletion on decidual gene

415   expression. We demonstrate that when Treg cells are depleted, transcriptional changes indicating

416   inappropriate NK cell and T cell activation arise, and mostly these were restored to varying degrees

417   by Treg cell pre-treatment.

418

419   Amongst the genes most strongly induced after Treg cell depletion were granzyme serine proteases

420   that mediate NK cell and T cell-mediated cytotoxicity. Granzymes can exert non-cytotoxic

421   enzymatic actions that cause tissue damage, remodel extracellular matrices, and induce pro-

422   inflammatory cytokine release, so a range of consequences of elevated granzymes might contribute

423   to the decidual changes seen after Treg cell depletion. While it is not possible from the data

424   presented here to conclusively define the relative contribution of uNK cells versus T cells to these

425   changes, for several reasons we consider that uNK cells are centrally involved. Previous studies

426   show that T cells are a minor component of the decidual leukocyte population, whereas NK cells

427   predominate. That Treg cell depletion caused the prominent NK gene *Prf1 (*encoding the pore-

428   forming protein perforin-1) and NK cell transcription factors *Eomes, Id2* and *Runx3* to be induced

429   suggests an altered uNK cell transcriptional program. Our analysis did not indicate expansion of

430   effector CD3$^+$ or CD4$^+$ T cells after Treg cell depletion. The decidua has specific mechanisms to

431   exclude effector T cells by epigenetic silencing of chemokine genes *Cxcl9*, *Cxcl10*, and *Ccl5* (78),

432   and no change in expression of these genes was seen after Treg cell depletion. Collectively these

433   data imply that uNK cells contribute to the altered decidual gene expression and the mechanisms

434   underpinning impaired vascular remodelling. Further analysis will be needed to definitively

435 understand how uNK cell functions are affected by Treg cells and how in turn this leads to altered

436 vascular remodelling. It will also be important to evaluate whether specific effector T cell subsets

437 such as CD8^{+} T cells and gamma delta T cells, that can express granzymes and potentially elicit

438 fetal loss (79, 80), might also contribute to the decidual changes we observed after Treg cell

439 depletion.

440

441 The RNAseq data also point to altered decidual trophoblast invasion after Treg cell depletion.

442 Several trophoblast genes were upregulated, including several associated with placental glycogen

443 cells, while genes expressed by other extravillous trophoblast lineages were downregulated. This

444 result is consistent with the histological data showing elevated abundance of glycogen cells, a

445 specific subset of trophoblasts that store glycogen to serve late gestation fetal growth in the phase

446 just prior to parturition (81). Although there were no apparent effects of Treg cell depletion on

447 placental weight in late-gestation, the reduced fetal:placental weight ratio implies impaired

448 placental efficiency. In several mouse models of complicated pregnancy, placental glycogen is not

449 mobilized and instead is retained in the junctional zone (82-84). Retained placental glycogen has

450 also been implicated in humans with preeclampsia and gestational diabetes (84).

451

452 There is extensive evidence of cross-talk between uNK and trophoblasts in the decidua.

453 Trophoblasts influence the maturation of uNK precursor cells into mature uNK and uNK both

454 promote and constrain EVT migration and invasion of spiral arteries (85, 86). The reduced

455 expression of trophoblast genes might imply excessive uNK cell constraint of trophoblast invasion

456 after peri-implantation Treg cell depletion, but whether uNK cell cytotoxicity is involved will

457 require further analysis. Although uNK express granzymes and perforin-1 even under normal

458 circumstances, uNK cells do not exhibit killing of trophoblasts or fetal cells in the manner of

459 peripheral blood NK cells (87), except under very specific circumstances, for example in the event

460 of viral (88) or bacterial infection (89). Given that in the current study, placental integrity was not

461 overtly changed after Treg cell depletion, we consider uNK cell cytotoxic activity to be unlikely

462 and instead, non-cytotoxic effects of uNK granzymes on trophoblast invasion and the decidual

463 vasculature warrant investigation. Single-cell sequencing experiments will be informative in this

464 regard.

465

466 Decidual gene expression analysis by qPCR revealed a potential role for cytokines TGFβ, IL35,

467 and IL10 in communication between Treg cells and uNK cells. IL35 is produced almost

468 exclusively by Treg cells (90, 91) and has an established role in suppressing effector functions (92,

469 93), including in NK cells (68). IL10 is an anti-inflammatory cytokine produced by both immune

470 and non-immune cells including Treg cells, uNK cells, macrophages, and trophoblast cells (94). It

471 is well established that IL10 is critical for immune tolerance in pregnancy (95), and IL10 null

472 mutant mice exhibit elevated NK1.1$^+$ decidual cell numbers and cytotoxic capacity (96). TGFβ

473 can induce NK cell proliferation, upregulate NKp46 expression (97), and promote a more

474 tolerogenic phenotype in NK cells (98). Reduced expression in each of these cytokines after Treg

475 cell depletion presumably reflects their synthesis in Treg cells, particularly for IL35 which is

476 restricted to this cell lineage. Treg cell release of TGFβ, IL10 and IL35 are implicated in Treg cell-

477 mediated protection of the vasculature in other tissues (99-103). Treg cells constrain vascular

478 inflammation in atherosclerosis (20, 21), but Treg cell-NK cell communication affects vascular

479 biology in other tissues is unknown. Such an interaction is possible given evidence of NK cell-

480 Treg cell interactions in a range of settings (104-106).

481

Decidual gene expression analysis also revealed that *Vegfa* transcription was reduced in Treg cell-depleted mice. Given evidence that production of VEGFA by DBA$^+$ uNK cells is important for angiogenesis and remodeling of the decidual vasculature (39), this finding is consistent with our histochemical data showing decreased DBA$^+$ uNK cells. Our finding that IFNγ remained unchanged after Treg depletion is consistent with Treg cells exerting a greater effect on DBA$^+$ uNK cells producing VEGF, than DBA$^-$ uNK cells secreting IFNγ (39).

488

As well as decidual spiral artery remodeling, Treg cells are implicated in modifying the systemic vasculature and notably the uterine artery. After Treg cell depletion, we saw increased resistance to blood flow in the main uterine arteries in mid-gestation, and this was prevented with Treg cell replacement, confirming our earlier findings. Together with observations that Treg cell deficiency promotes generation of the vasoconstrictor endothelin-1 in uterine arteries (19), this demonstrates that Treg cells are necessary for normal function of the uterine artery in mid-gestation, as well as late gestation (17). The effects of Treg cell depletion on the systemic vasculature may be limited to the window during which Treg cells are deficient, as we found that uterine artery resistance was normalized after Treg cell populations recovered.

498

Collectively our data on structural indicators of placental transport efficiency and compromised fetal growth in late pregnancy indicate a pathological state that is the consequence of early gestation immune perturbation. This sequence of events is reminiscent of other pro-inflammatory perturbations in early pregnancy that manifest as impaired vascular remodeling, altered placental development, followed by fetal loss and or growth restriction in late gestation (30). That all the

23

504   vascular defects, as well as fetal loss, could be rescued by transfer of wild-type Treg cells alleviates

505   concern of off-target effects of DT, and confirms that the consequences of DT treatment can be

506   attributed to Treg cells. Although Treg cell replacement did not improve fetal weight, it did

507   improve parameters of fetal growth including crown-to-rump length, abdominal girth, and

508   biparietal diameter, and independent experiments in C57Bl/6 showed no adverse impact on fetal

509   growth of DT administration. This implies that the fetal weight deficit remaining after Treg

510   replacement is due to incomplete recapitulation of the unperturbed immune state, as opposed to

511   deleterious effects of DT. While these data clearly show an impact of Treg cells on the decidual

512   vasculature and implicate a mediating role for uNK cells, we do not exclude the possibility that

513   other effects of Treg cell depletion, mediated locally in the implantation site or systemically in

514   other tissues, also contribute to fetal loss and growth restriction.

515

516   Impaired vascular remodeling of the maternal spiral arteries is the pathological antecedent of

517   preeclampsia (13), resulting in poor uteroplacental perfusion later in pregnancy and often leading

518   to fetal growth restriction. A possible role of Treg cells in facilitating the spiral artery remodeling

519   process has been speculated, but definitive evidence has been lacking (2, 107). Aberrations in uNK

520   cells have been investigated as a possible contributing factor to impaired spiral artery remodeling

521   in preeclampsia. There are conflicting reports on altered uNK cell numbers in preeclampsia (108-

522   111), indicating the need for further research to resolve these conflicting observations.

523

524   In conclusion, this study provides new evidence demonstrating that Treg cells are essential for

525   remodeling of decidual spiral arteries in early pregnancy and identifies a candidate mechanism

526   involving Treg cell regulation of uNK cells (Figure 10). Given the consistent finding of

527   perturbed Treg cells in preeclampsia and related hypertensive pregnancy complications, these

528   findings indicate that studies to determine effects of Treg cells on spiral artery remodeling in

529   women are warranted and add to the imperative to consider Treg cells as a potential therapeutic

530   target in at-risk women.

531

METHODS

Sex as a biological variable

Our study focused on pregnancy and therefore outcomes were focused on female mice. Male mice were used for mating purposes. Ongoing investigations are examining potential impacts on both male and female offspring.

Animals

*Foxp3*DTR (Forkhead box P3-diphtheria toxin receptor; B6.129(Cg)-*Foxp3tm3(DTR/GFP)Ayr*/J) mice were purchased from Jackson Laboratories (Bar Harbour, Maine USA), and C57Bl/6 (WT) female and BALB/c male mice were purchased from Animal Resources Centre (Western Australia, Australia). Mice were housed and bred in a specific pathogen-free facility. Female mice (8-12 weeks) were housed with proven fertile males, and the presence of a copulatory plug was designated gestational day (GD)0.5. See Supplemental Methods for details.

Treg cell depletion

To induce Treg cell depletion, *Foxp3*DTR mice were injected with DT from *Corynebacterium diphtheria* (Sigma-Aldrich) (37.5 ng/g, i.p) on GD 3.5 and 5.5. Vehicle (PBS)-treated *Foxp3*DTR mice were controls in all experiments. See Supplemental Methods for details.

T cell isolation and adoptive transfer

CD4$^+$CD25$^+$ Treg cells or CD4$^+$CD25$^-$ Tconv cells were isolated from the uterine-draining, para-aortic lymph nodes (uDLN), and the mesenteric, inguinal, and brachial lymph nodes and spleen

554   from pregnant wild-type C57BL/6 mice at GD10.5 to 13.5. Cells were adoptively transferred by

555   i.v. injection into DT-treated *Foxp3*^{DTR} on GD3.5 and 5.5. See Supplemental Methods for details.

556

557   **Flow cytometry**

558   On GD6.5 and GD10.5, single-cell suspensions were prepared from uDLN, mesenteric LN and

559   pooled decidua from *Foxp3*^{DTR} mice. Cells were stained using fluorophore-conjugated antibodies

560   to detect surface and intracellular markers (Supplemental Table 5) and a standardized gating

561   strategy (Supplemental Figure 11 and 12). See Supplemental Methods for details.

562

563   **Ultrasound biomicroscopy**

564   Uterine artery function was assessed on GD9.5 and uterine and umbilical artery function assessed

565   at GD17.5, using an MX550D transducer probe on an ultrasound biomicroscope (model Vevo

566   2100, VisualSonics®, ON, Canada) in *Foxp3*^{DTR} mice. See Supplemental Methods for details.

567

568   **Histology, immunohistochemistry and lectin staining**

569   Mid-sagittal sections from formalin-fixed, paraffin-embedded GD10.5 implantation sites (for

570   decidual spiral artery analysis; Supplemental Figure 13) and GD18.5 placentas were stained with

571   Masson's trichrome. Smooth muscle cells were detected with α-SMA and uNK cells with

572   biotinylated DBA-lectin. See Supplemental Methods for details.

573

574   **RNA sequencing, and data analysis**

575   RNA from decidual tissues were extracted as described previously (12). Library preparation and

576   sequencing on NovaSeq X at 80 million reads per sample was performed by the South Australian

577 Genomics Centre. Sequencing reads were mapped to GRCm38 (mm10) mouse genome and

578 quantified using STAR (112). Limma-voom (113) was then used on TMM normalized reads to

579 identify genes that were differentially expressed (DEGs, FDR <0.1) according to treatment group.

580 Overrepresentation pathway analysis was performed with *clusterProfiler* (114). The Mouse

581 Genomics Informatics resource (65) and mouse placenta single cell sequencing data (66) was used

582 to identify genes enriched in placental trophoblasts. See Supplemental Methods for details and

583 data availability.

584

585 **Statistics**

586 Data were analysed by unpaired t-test or Mann-Whitney U test, or one-way ANOVA or Kruskal-

587 Wallis test, depending on normality of data distribution. Fetal outcome data were analysed by

588 mixed model ANOVA analysis with mother as subject and litter size as covariate. Analysis was

589 performed using GraphPad Prism, SPSS, or R in the case of RNA sequencing data. See

590 Supplemental Methods for details.

591

592 **Study Approval**

593 All experiments were approved by the University of Adelaide Animal Ethics Committee, ethics

594 numbers M-2018-127, M-2017-024 and M-2020-107 in accordance with the Australian Code of

595 Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). See

596 Supplemental Methods for details.

597

598 **Data availability**

599   Values for all data points in graphs are reported in the Supporting Data Values XLS files. Complete

600   R code used to analyze and visualize the RNA-sequencing data have been deposited at

601   https://tranmanhha135.github.io/Treg_uNK/. Raw fastq files have been deposited in the National

602   Center for Biotechnology Information's Gene Expression Omnibus database (GSE267364).

603

AUTHOR CONTRIBUTIONS

604 ASC and SAR designed the studies. SLH, HMG, HYC, HMT, LMM, EAKL and ASC conducted

606 experiments and analysed data. SLH, ASC, HMT and LMM prepared figures. KLF, CTR, and

607 STD provided specific expertise and made substantial contributions to designing experiments and

608 interpreting data. ASC and SAR wrote the manuscript. All authors revised drafts and reviewed the

609 manuscript.

610

623

FIGURE LEGENDS

625

626 **Figure 1: Effect of DT administration to *Foxp3*^DTR mice in the peri-implantation period on**

627 **uterine draining, para-aortic lymph node (uDLN) Treg cell proportion and phenotype in**

628 **mid-gestation.** *Foxp3*^DTR mice were administered PBS (veh) or DT i.p. on GD3.5 and GD5.5.

629 uDLN were recovered on GD6.5 or GD10.5 and the proportion and phenotype of CD4$^+$FOXP3$^+$

630 Treg cells were evaluated using flow cytometry. (**A**) Representative contour plots show the

631 proportion of CD4$^+$FOXP3$^+$ Treg cells in the uDLN on GD6.5 from vehicle-treated (left) and DT-

632 treated (right) *Foxp3*^DTR mice. (**B**) The proportion of CD4$^+$FOXP3$^+$ Treg cells in uDLN at GD6.5

633 and GD10.5. Detailed analysis of Treg cells at GD10.5 shows the proportion of Treg cells

634 expressing NRP1 indicating thymic origin (**C**), proliferation marker Ki67 (**D**), and marker of

635 suppressive competence CTLA4 (**E**). The proportion of IFNγ$^+$CD4$^+$FOXP3$^-$ (Th1 cells; **F**) and

636 IL17a$^+$CD4$^+$FOXP3$^-$ (Th17 cells; **G**) were measured.. N=3-14 mice per group. Data are

637 mean±SEM. Data points are values from individual dams. Analysis was by two-tailed t-test or

638 Mann Whitney U test depending on normality of data distribution for data in (C-G). Data in (B)

639 were analysed using a 1-way ANOVA comparing samples within the same gestational day.

640 *P<0.05; **P<0.01; ***P<0.001.

641

642 **Figure 2: Treg cell depletion in the peri-implantation period results in reduced litter size at**

643 **birth and increased fetal loss in late gestation and is mitigated by Treg cell transfer.** *Foxp3*^DTR

644 mice were administered PBS (veh) or DT i.p. on GD3.5 and GD5.5, then pregnancy outcomes

645 were assessed either at birth (**B-F**), or on GD18.5 (**G-J**). Some mice also received wild-type Treg

646 cells or Tconv cells on GD2.5 and GD4.5. (**A**) Schematic diagram showing the treatment and

647   analysis protocol (created with BioRender.com). (**B**) Proportion of mated mice that delivered at

648   least one viable pup at birth. (**C**) Total number of pups at birth per dam. (**D**) Number of viable

649   pups within 24 h of birth per dam. (**E**) proportion of viable pups within 24 h of birth per dam. (**F**)

650   Pup weight at 24-36 h after birth. (**G**) The proportion of dams carrying a viable pregnancy at

651   GD18.5 (defined as at least one viable implantation site). (**H**) Number of total fetuses per dam at

652   GD18.5. (**I**) Number of viable fetuses per dam at GD18.5. (**J**) Number, and (**K**) proportion of

653   resorbing fetuses per dam at GD18.5. Numbers of mice (**B**) and dams (**G**) in each group are shown

654   in parentheses. Data are mean±SEM. Data points are values from individual dams. Analysis was

655   by chi-squared test (**B** and **G**) or one-way ANOVA with two-tailed posthoc t-test (**C-F**, **H-K**).

656   *P<0.05; **P<0.01; ***P<0.001.

657

658

659   **Figure 3: Treg cell depletion in the peri-implantation period causes fetal growth restriction**

660   **that is mitigated by Treg cell transfer.** *Foxp3*^{DTR} mice were administered PBS (veh) or DT i.p.

661   on GD3.5 and GD5.5, then fetal and placental development were assessed on GD18.5. Some mice

662   also received wild-type Treg cells or Tconv cells on GD2.5 and GD4.5. (**A**) Representative images

663   of fetuses, (**B**) fetal weight, (**C**) placental weight, and (**D**) fetal to placental weight ratio. The

664   distribution of (**E**) fetal weights, (**F**) placental weights, and (**G**) fetal to placental weight ratio were

665   calculated. (**H**) Fetal crown-to-rump length, (**I**) abdominal girth, and (**J**) biparietal diameter.

666   Vertical dashed line represents the 10th centile of the curve (1.15 g, 0.08 g and 11.45 in E, F and

667   G, respectively). N= 2-11 fetuses from 14-19 dams per group. Fetal and placental data are shown

668   as violin plots with median and quartile values marked. Analysis was by mixed model ANOVA

669   with mother as subject and litter size as covariate. *P<0.05; **P<0.01; ***P<0.001. Scale bar = 1

670   cm.

671

672   **Figure 4: Treg cell depletion impairs spiral artery remodeling in mid-gestation.** Pregnant

673   *Foxp3*^{DTR} were administered PBS (veh) or DT i.p. on GD3.5 and GD5.5, then tissues were

674   collected on GD10.5. Some mice also received wild-type Treg cells or Tconv cells on GD2.5 and

675   GD4.5, then tissues were collected on GD10.5. Representative images of mid-sagittal sections of

676   uterus stained with Masson's trichrome (**A-D**), or to detect α-smooth muscle actin (**E-H**). Black

677   arrowheads indicate spiral arteries, black arrows indicate α-smooth muscle actin-positive cells.

678   Parameters including (**I**) average lumen area of spiral arteries, (**J**) lumen diameter, (**K**) the vessel

679   to lumen area ratio, and (**L**) proportion of decidual cells positive for α-smooth muscle actin, are

680   shown. N=2 implantation sites per dam from 8-12 dams per group. Data are mean±SEM. Data

681   points are average values for individual dams. Analysis was by one-way ANOVA with two-tailed

682   posthoc t-test. *P<0.05; **P<0.01; ****P<0.0001. Scale bars; A-D = 1 mm; insets = 50 μm; E-H

683   = 50 μm.

684

685   **Figure 5: Uterine artery resistance in mid-gestation is increased by Treg cell depletion and**

686   **mitigated by Treg cell replacement.** Pregnant *Foxp3*^{DTR} mice were administered PBS (veh) or

687   DT i.p. on GD3.5 and GD5.5, then tissues were collected on GD10.5. Some mice also received

688   wild-type Treg cells or Tconv cells on GD2.5 and GD4.5. Measurements were taken on GD9.5.

689   (**A**) Representative waveforms of uterine arteries. (**B**) Resistance index and (**C**) pulsatility index

690   were calculated. N=5-8 dams per group. Data are mean±SEM. Data points are average values for

691   individual dams. Analysis was by one-way ANOVA with two-tailed posthoc t-test. EDV indicates

692 end-diastolic velocity; PSV, peak systolic velocity and TAV, time-averaged velocity. *P<0.05;

693 **P<0.01.

694

695 **Figure 6: Treg cell depletion causes a reduction in DBA⁺ uNK cells in mid-gestation that is**

696 **mitigated by Treg cell transfer.** Pregnant *Foxp3*[DTR] mice were administered PBS (veh) or DT

697 i.p. on GD3.5 and GD5.5, then tissues were collected on GD10.5. Some mice also received wild-

698 type Treg cells or Tconv cells on GD2.5 and GD4.5. Tissues were collected on GD10.5. (**A-D**)

699 Decidual tissue sections were labelled with biotinylated *Dolichos biflorus* agglutinin (DBA)-lectin

700 to detect the DBA⁺ subset of uNK cells that predominates in pregnancy (brown stain, arrows). (**E**)

701 The percent positivity for DBA⁺ uNK cells was quantified. (**F**) The decidual region of each section

702 (marked, dotted line in A) was identified and measured. N=2 implantation sites per dam, 8-12

703 dams per group. Data are mean±SEM. Data points are average values for individual dams.

704 Analysis was by one-way ANOVA with two-tailed posthoc t-test. ****P<0.0001. Scale bars:

705 outset = 500 µm; insets = 100 µm.

706

707 **Figure 7: Treg cell depletion causes a perturbation in decidual transcription profile in**

708 **midgestation that is mitigated by Treg cell transfer.** Pregnant *Foxp3*[DTR] mice were administered

709 PBS (veh; n=6) or DT (n=5) i.p. on GD3.5 and GD5.5, and decidual tissue was collected on

710 GD10.5. Some mice (n=5) also received wild-type Treg cells on GD2.5 and GD4.5. (**A**) Principle

711 component analysis (PCA) of filtered genes, illustrating gene expression patterns in individual

712 samples. (**B**) The number of DEGs (FDR<0.1) that overlap between DT-treated mice compared to

713 PBS vehicle control (purple) and DT+Treg–treated mice compared to DT-treated mice (green).

714 (**C**) Functional heatmap of DEGs (FDR<0.1) and their relationship to enriched terms/pathways

715   identified using Gene Ontology (GO, FDR < 0.05), Kyoto Encyclopedia of Genes and Genomes

716   (KEGG, FDR<0.2), Reactome (FDR < 0.1), and Ingenuity Pathway Analysis (IPA, P<0.05)

717   databases.

718

719   **Figure 8: Treg cell depletion causes a perturbation in decidual trophoblast genes in**

720   **midgestation that is mitigated by Treg cell transfer.** Pregnant $Foxp3^{DTR}$ mice were

721   administered PBS (veh; n=6) or DT (n=5) i.p. on GD3.5 and GD5.5, and decidual tissue was

722   collected on GD10.5. Some mice (n=5) also received wild-type Treg cells on GD2.5 and GD4.5.

723   Functional heatmap of DEGs (FDR <0.1) identified as indicative of altered extravillous

724   trophoblasts on the basis of both: (1) reported expression in specific mouse trophoblast cell types

725   (colour coded, LHS) (extracted from published single cell RNA-seq data (66)); and (2) expression

726   in mouse placenta but not mouse uterus, according to Mouse Genomics Informatics database (see

727   Materials and Methods for details). Cell labels indicate the FDR-adjusted P value (FDR) of DEGs

728   present in the RNA-seq dataset. *FDR <0.1; **FDR <0.05; FDR <0.01. 1' P-TGC, primary

729   parietal trophoblast giant cell; 2' P-TGC, secondary parietal trophoblast giant cells; EPC,

730   ectoplacental cone; Gly-T, glycogen trophoblast cells; LaTP, labyrinthine trophoblast; S-TGC,

731   sinusoid trophoblast giant cell; Spa-TGC, spiral artery-associated trophoblast giant cell; SpT,

732   spongiotrophoblast cell; SynT1, multinucleated syncytiotrophoblast cells; TSC, trophoblast stem

733   cell; ExE, extraembryonic ectoderm.

734

735   **Figure 9: Peri-implantation Treg depletion elicits reduction of anti-inflammatory**

736   **decidual gene transcripts.** Pregnant $Foxp3^{DTR}$ mice were given PBS (veh) or DT on GD3.5 and

737   GD5.5, then tissues were collected on GD10.5. (**A**) *Ebi3* and (**B**) *Il12p35* genes encoding IL35,

738   (**C**) *Il10*, (**D**) *Tgfb1,* (**E**) *Ncr1* encoding NKp46, (**F**) *Foxp3* encoding FOXP3 transcription factor

739   necessary for Treg cell development, and (**G**) *Vegfa*, were quantified by qPCR. N=9-10 dams per

740   group. Data are presented as mean±SEM. Each data point represents the average of two decidua

741   per dam. Analysis was by unpaired t-test. *P<0.05; **P<0.01; ***P<0.001.

742   Pregnant *Foxp3*^{DTR} mice were given PBS (veh) or DT on GD3.5 and GD5.5, then tissues were

743   collected on GD10.5. (A) *Ebi3* and (B) *Il12p35* genes encoding IL35, (C) *Il10*, (D) *Tgfb1,* (E)

744   *Ncr1* encoding NKp46, (F) *Foxp3* encoding FOXP3 transcription factor necessary for Treg cell

745   development, and (G) *Vegfa*, were quantified by qPCR. N=9-10 dams per group. Data are

746   mean±SEM. Each data point represents the average of two decidua per dam. Analysis was by

747   unpaired t-test. *P<0.05; **P<0.01; ***P<0.001.

748

749   **Figure 10: Schematic illustration of current working hypothesis on mechanisms of Treg cell-**

750   **mediated regulation of decidual spiral artery remodeling.** Treg cells modulate the decidual

751   microenvironment to facilitate decidual spiral artery remodelling in early pregnancy in mice. When

752   Treg cells are deficient, spiral artery remodeling is impaired. In turn this causes fetal growth

753   restriction and late gestation fetal loss that is exacerbated by increased resistance to blood flow in

754   the uterine arteries. Treg cell support of spiral artery remodelling is likely to be mediated through

755   Treg cell effects in a decidual network involving uNK cells and trophoblasts. Our data show

756   reduced numbers of DBA⁺ uNK cells, and plus attenuation of genes associated with uNK cell

757   function and extravillous trophoblast invasion. Since uNK cells are known to be essential for spiral

758   artery remodelling through IFNγ and VEGF production, and Treg cells produce cytokines TGFβ,

759   IL10, and IL35 known to regulate uNK cell function, a direct effect of Treg cells on uNK cells is

760   implicated. Altered extravillous trophoblast invasion and/or survival in the decidua may also be

739   necessary for Treg cell development, and (**G**) *Vegfa*, were quantified by qPCR. N=9-10 dams per

740   group. Data are presented as mean±SEM. Each data point represents the average of two decidua

741   per dam. Analysis was by unpaired t-test. *P<0.05; **P<0.01; ***P<0.001.

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745   development, and (G) *Vegfa*, were quantified by qPCR. N=9-10 dams per group. Data are

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756   reduced numbers of DBA^+ uNK cells, and plus attenuation of genes associated with uNK cell

757   function and extravillous trophoblast invasion. Since uNK cells are known to be essential for spiral

758   artery remodelling through IFNγ and VEGF production, and Treg cells produce cytokines TGFβ,

759   IL10, and IL35 known to regulate uNK cell function, a direct effect of Treg cells on uNK cells is

760   implicated. Altered extravillous trophoblast invasion and/or survival in the decidua may also be

761   involved, since extravillous trophoblasts interact with uNK cells and contribute to spiral artery

762   remodelling. See text for details. Created with BioRender.com.

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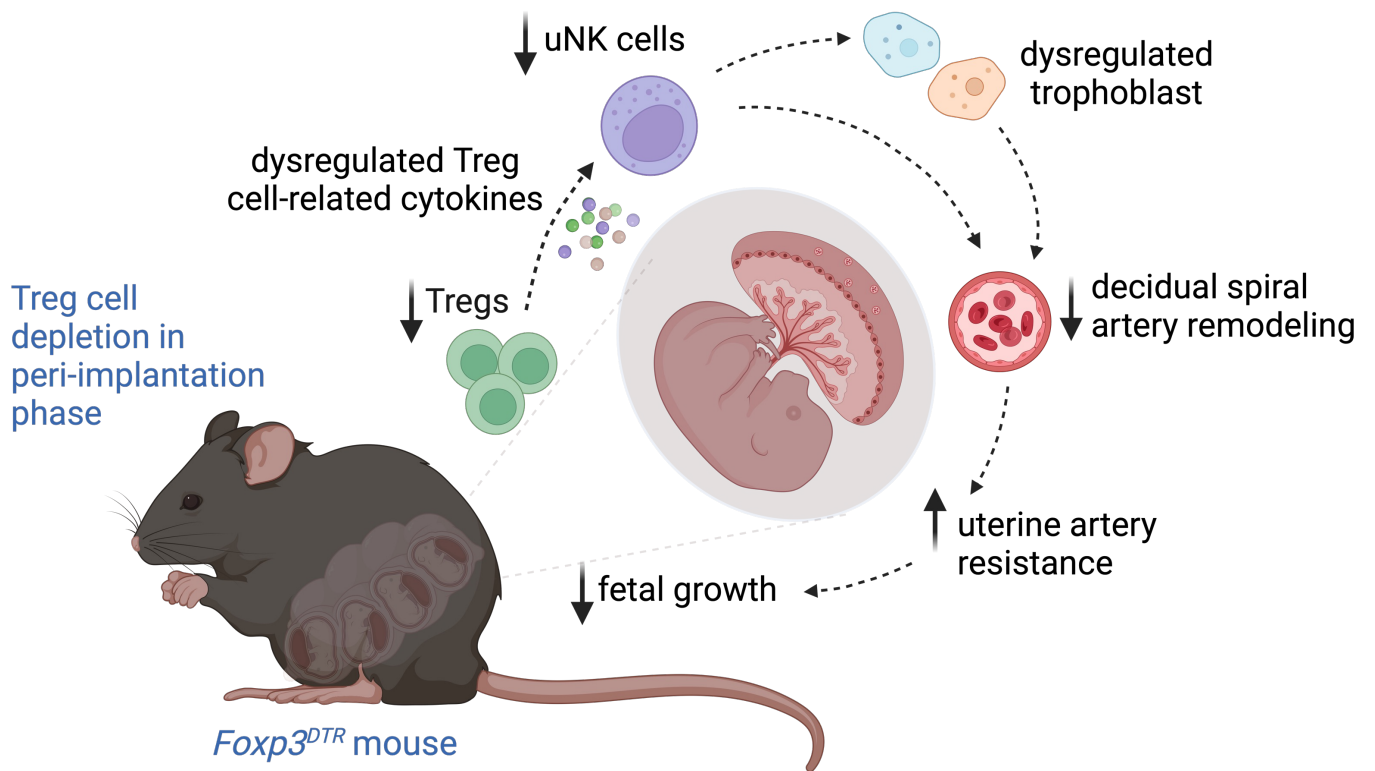1065 1999;434(3):207-11.
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↓ uNK cells

dysregulated trophoblast

dysregulated Treg cell-related cytokines

Treg cell depletion in peri-implantation phase

↓ Tregs

↓ decidual spiral artery remodeling

↑ uterine artery resistance

Foxp3^{DTR} mouse

↓ fetal growth

Graphical Abstract

Created with BioRender.com
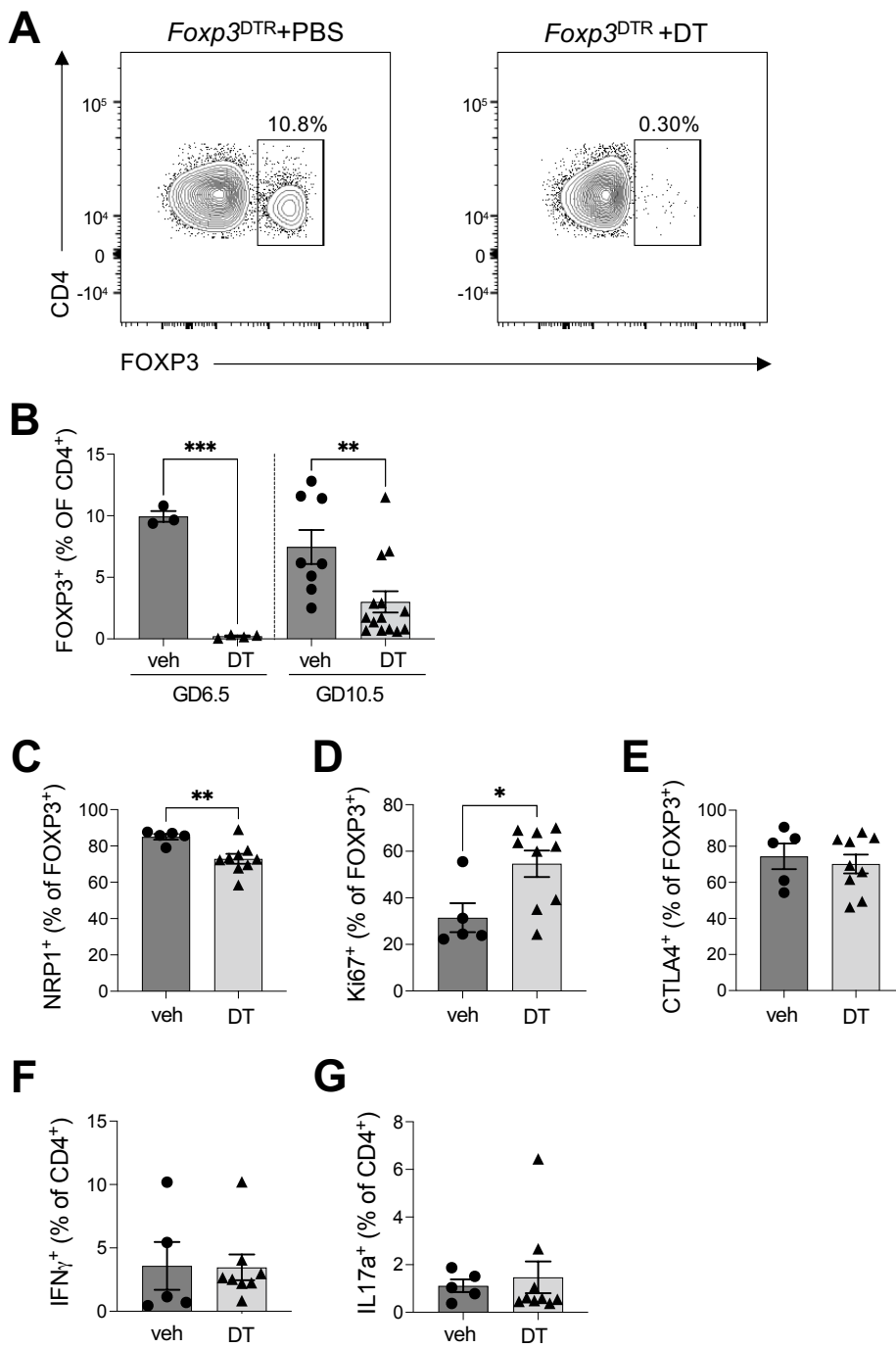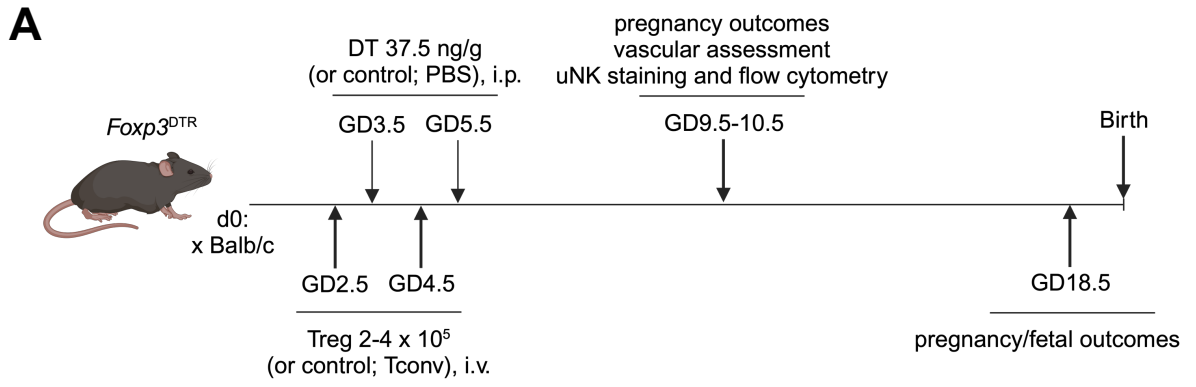
1

FIGURE 1

FIGURE 2

FIGURE 3

Foxp3^{DTR} +PBS+veh   Foxp3^{DTR} +DT+veh   Foxp3^{DTR} +DT+Treg   Foxp3^{DTR} +DT+Tconv

FIGURE 4

Foxp3[DTR] +PBS+veh · Foxp3[DTR] +DT+veh · Foxp3[DTR] +DT+Treg · Foxp3[DTR] +DT+Tconv

FIGURE 4

A

Foxp3^{DTR} +PBS+veh   *Foxp3*^{DTR} +DT+veh   *Foxp3*^{DTR} +DT+Treg   *Foxp3*^{DTR} +DT+Tconv

B

C

FIGURE 5

A *Foxp3*^{DTR} +PBS+veh

B *Foxp3*^{DTR} +DT+veh

C *Foxp3*^{DTR} +DT+Tregs

D *Foxp3*^{DTR} +DT+Tconv

E

% DBA-lectin positivity

F

decidual area (mm²)
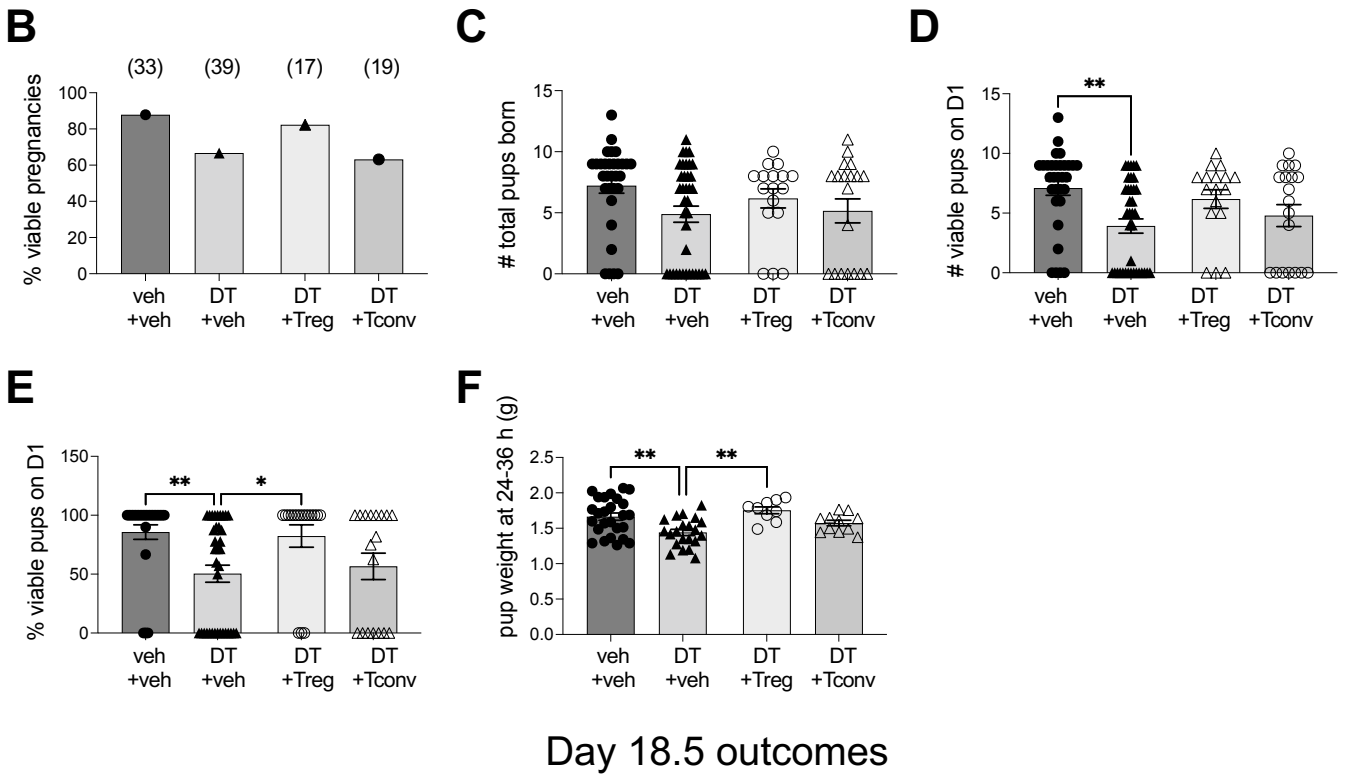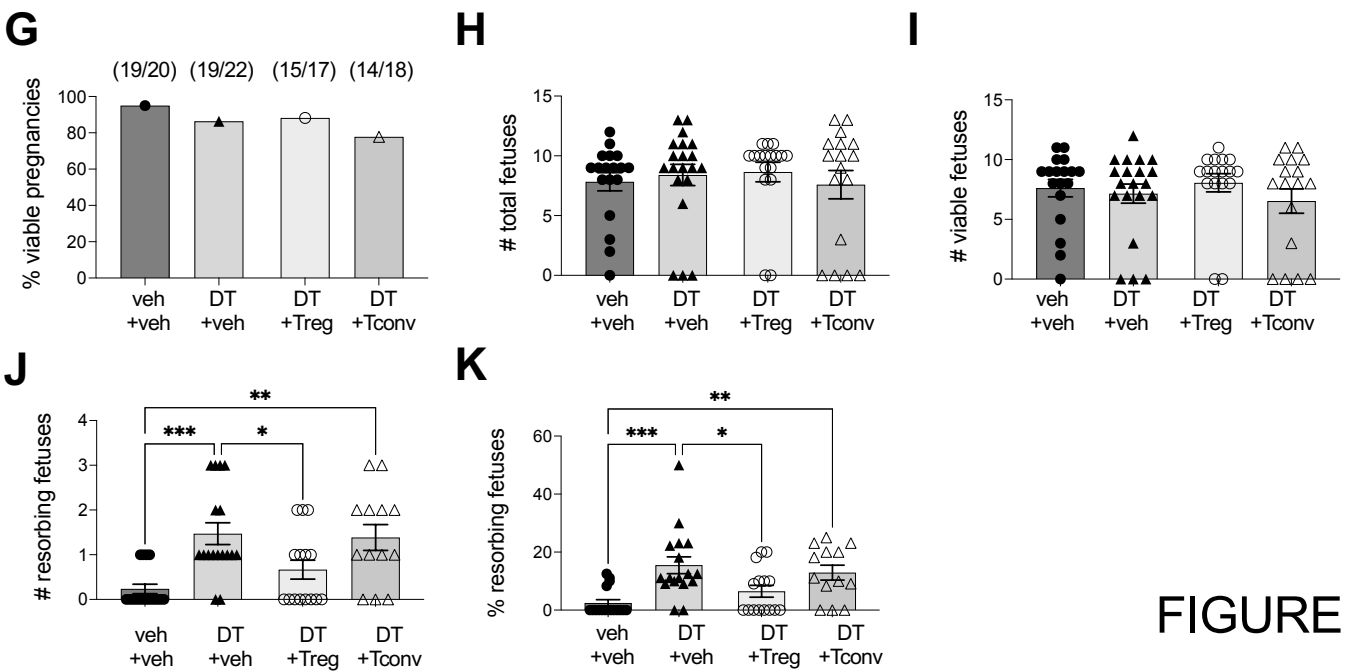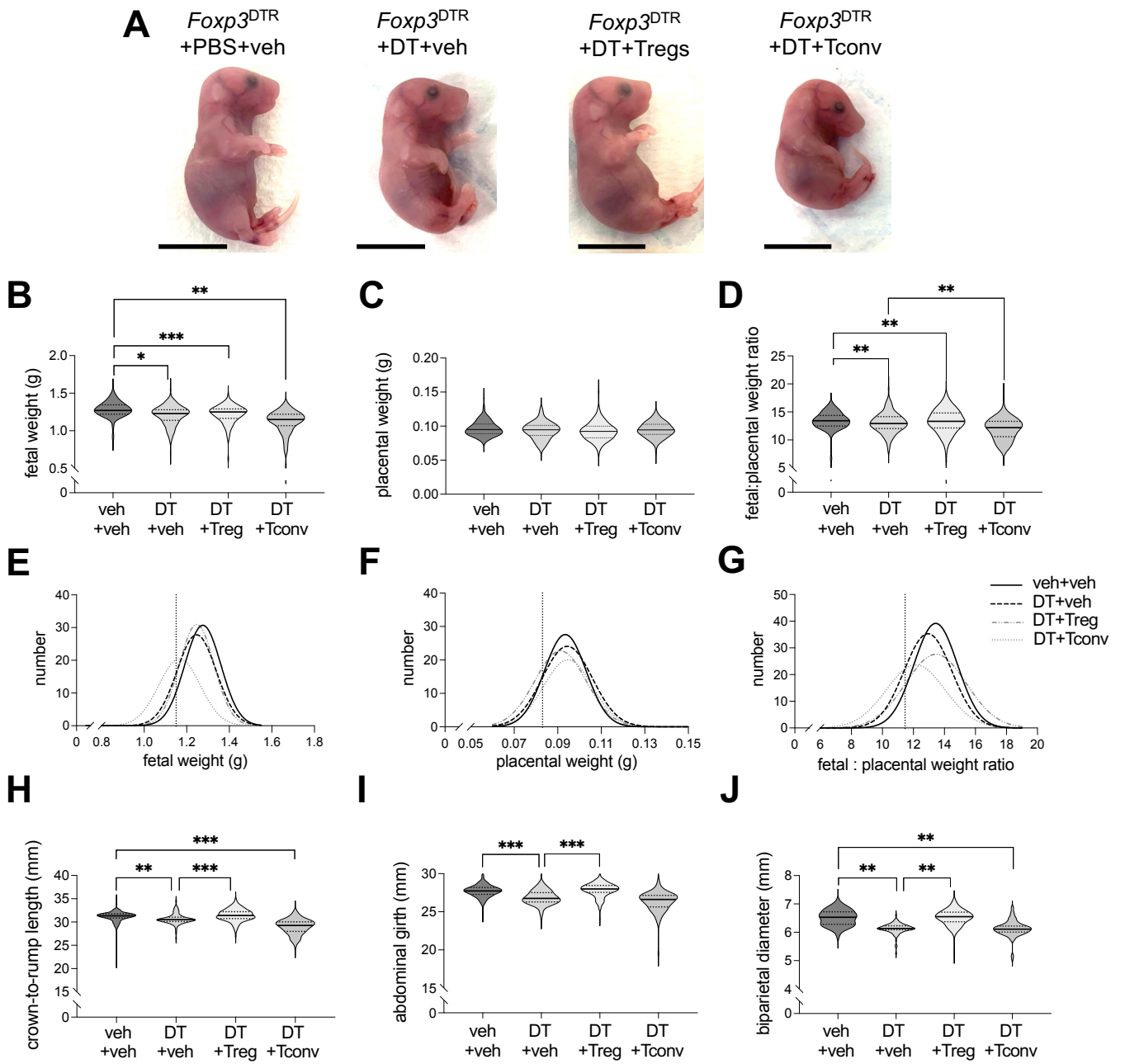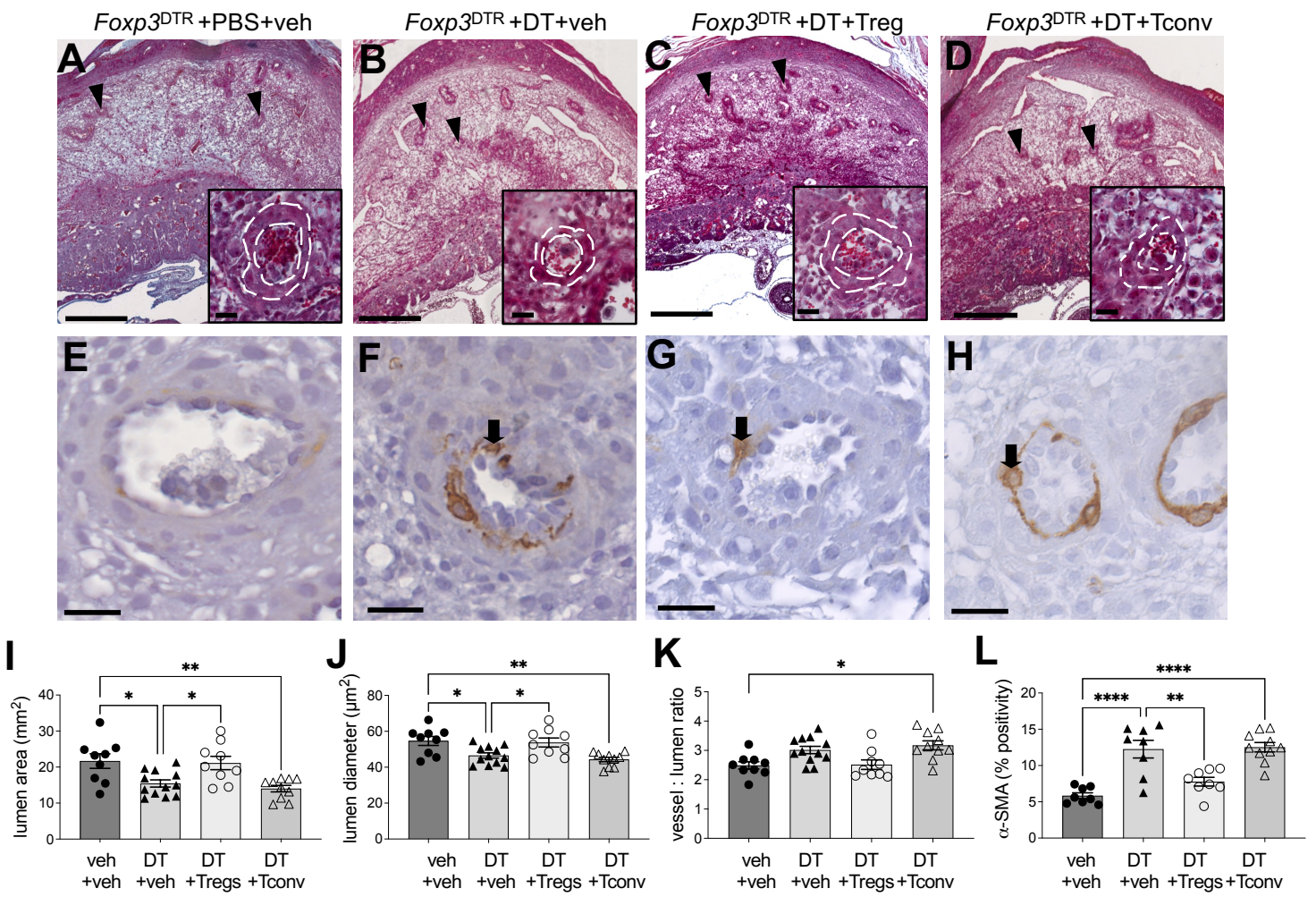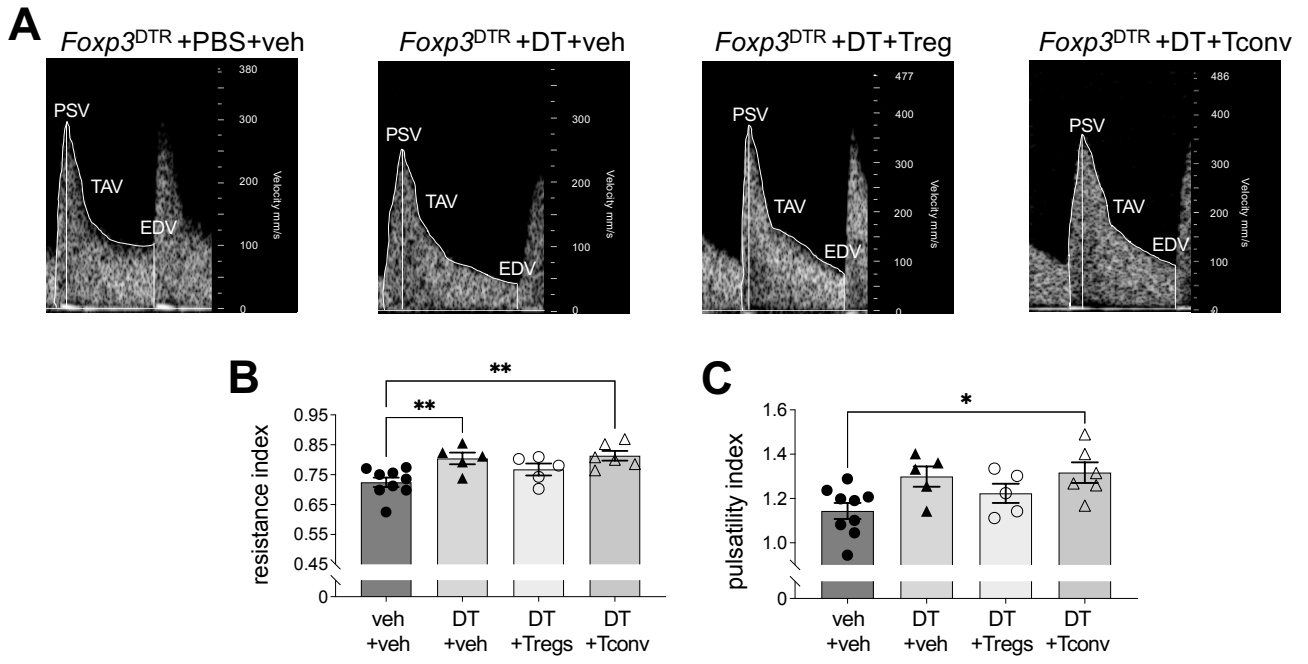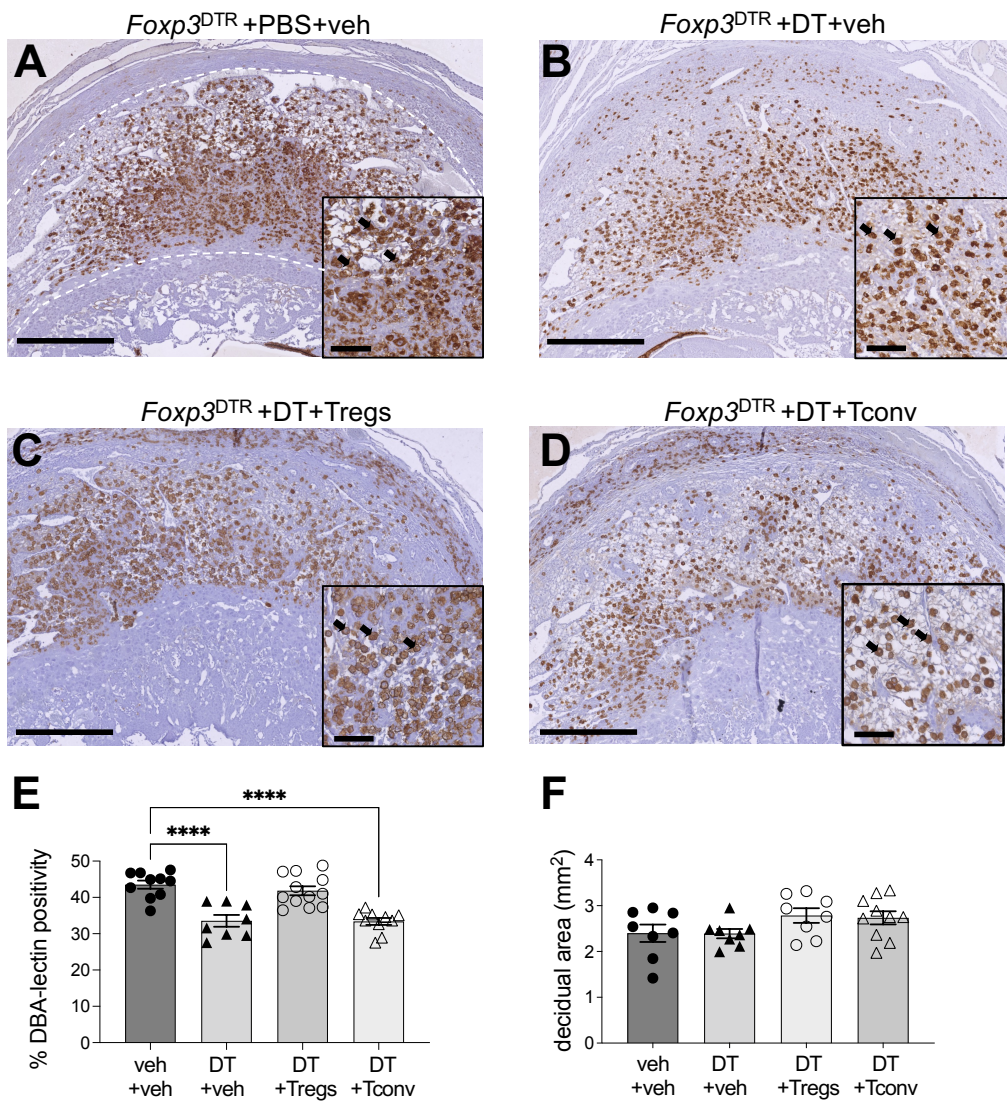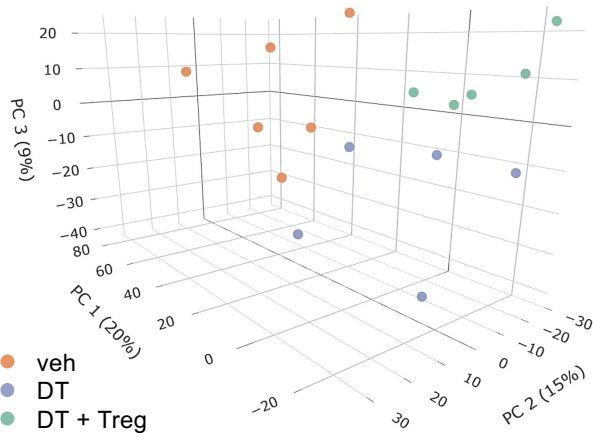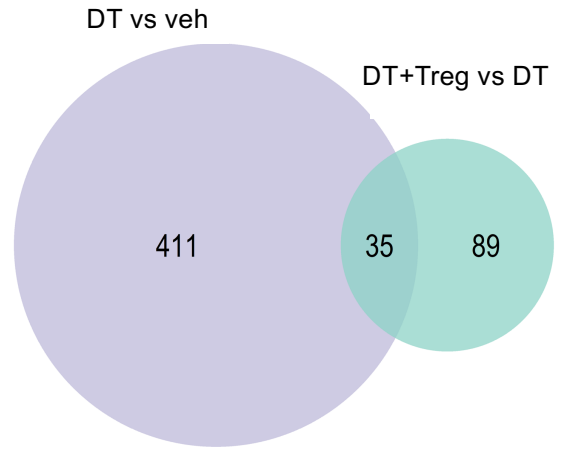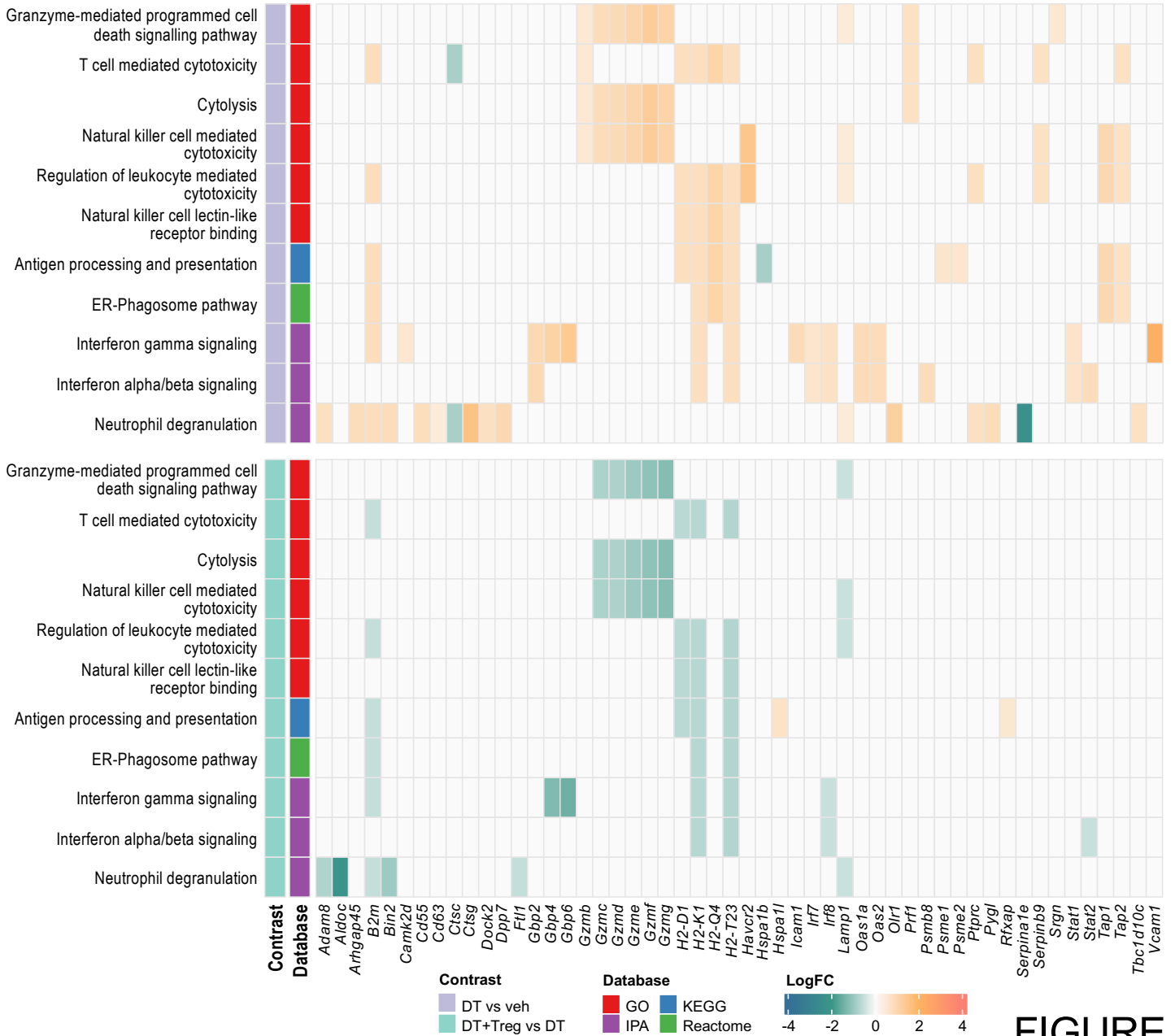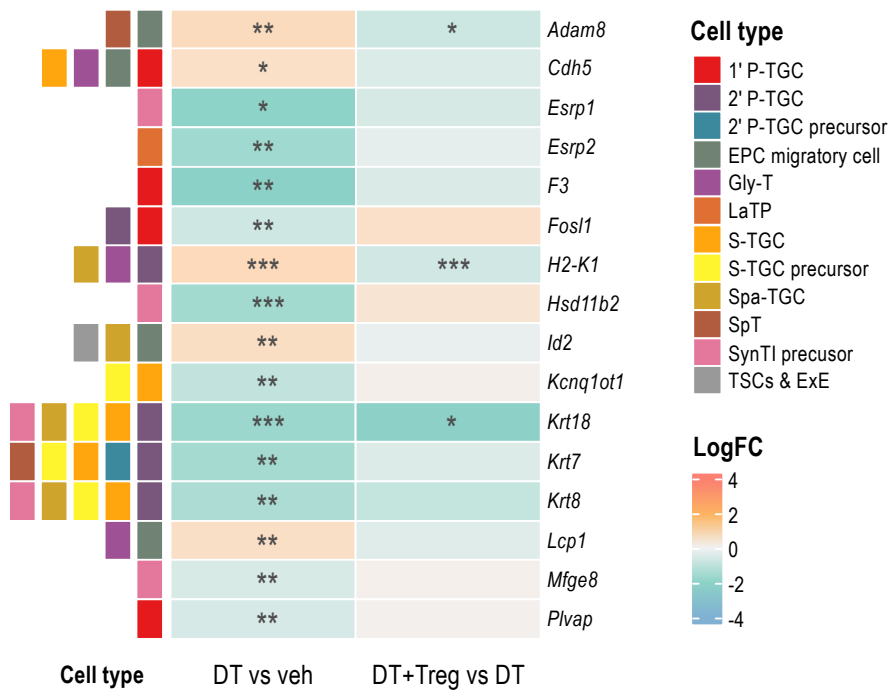
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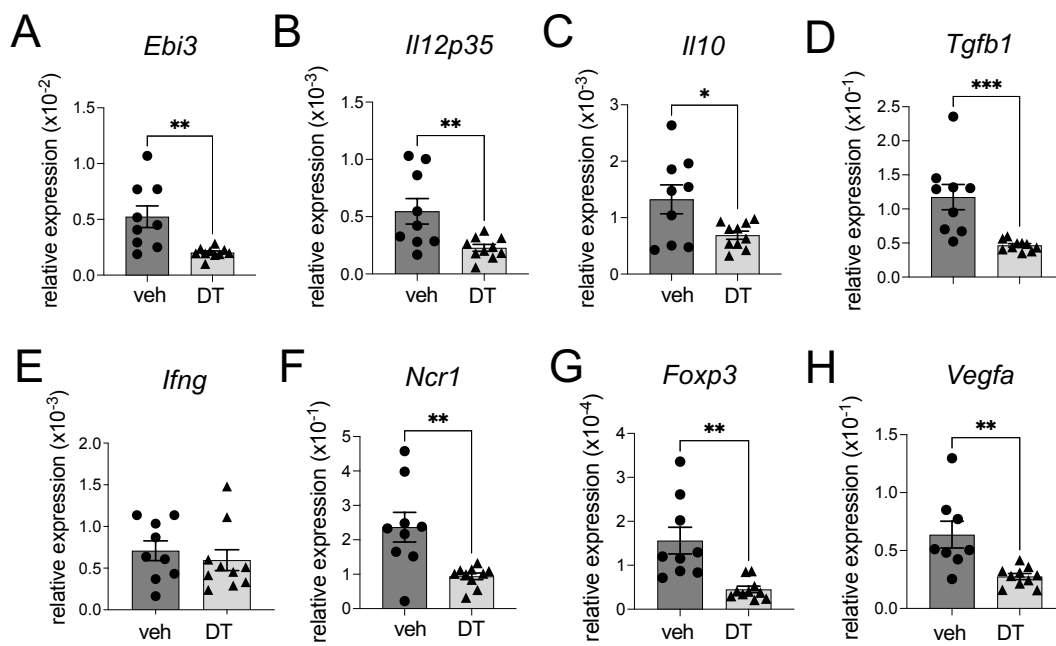
FIGURE 7

FIGURE 8

FIGURE 9

UTERUS

control
NORMAL REMODELLING

depleted of Treg cells
IMPAIRED REMODELLING

FIGURE 10