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Supplemental Information

Regulatory T Cells Play a Role in a Subset of Idiopathic Preterm Labor/Birth and Adverse Neonatal Outcomes

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Table S1. Clinical and demographic characteristics of the human study groups utilized for suppressive assays, related to Figure 1.

Clinical characteristics	Preterm (n = 6)	Term (n = 8)	p-value
Maternal age (years; median [IQR])[a]	28.5 (22.3-34.8)	27.5 (25.3-33.3)	0.7
Body mass index (kg/m^2; median [IQR])[a]	33.1 (30.8-42.5)	27 (24.3-31.9)	0.1
Primiparity[b]	16.7% (1/6)	12.5% (1/8)	1
Race/Ethnicity[b]			0.4
Black	83.3% (5/6)	100% (8/8)	
White	16.7% (1/6)	0% (0/8)	
Gestational age at delivery (weeks; median [IQR])[a]	35.5 (34.5-35.9)	40.1 (39.7-40.6)	0.002
Cesarean section[b]	50% (3/6)	37.5% (3/8)	1
Birthweight (grams; median [IQR])[a]	2122.5 (1773.8-2201.3)	3205 (3058.8-3551.3)	0.002

Data are given as median (interquartile range, IQR) and percentage (n/N).
[a]Mann-Whitney U test.
[b]Fisher's exact test

Table S2. Clinical and demographic characteristics of the human study groups utilized for flow cytometry studies, related to Figure 1 and Figure 2.

Clinical characteristics	PTNL (n = 28)	iPTL (n = 19)	iPTL+CI (n = 21)	PTL+AI (n = 13)	TNL (n = 13)	TIL (n = 13)	TIL+CI (n = 19)	TIL+AI (n = 14)	p-value
Maternal age (years; median [IQR])[a]	25 (22-31.8)	23 (20-29)	25 (23-30)	23 (21-25)	27 (25-28)	24 (22-28)	25 (21-33)	23 (21.5-29)	0.2
Body mass index (kg/m^2; median [IQR])[a]	27.9 (24.9-37.6)[c]	25.4 (23.5-32.8)[c]	24.9 (21.8-32.4)[c]	25.8 (24.1-28.3)[c]	28.9 (25-30.4)	25.7 (23.7-31.5)[c]	28.2 (23.8-34.2)	31 (23.6-39.8)	0.7
Primiparity[b]	21.4% (6/28)	31.6% (6/19)	9.5% (2/21)	15.4% (2/13)	15.4% (2/13)	23.1% (3/13)	26.3% (5/19)	28.6% (4/14)	0.7
Race/Ethnicity[b]									0.06
Black	82.1% (23/28)	79% (15/19)	81% (17/21)	92.3% (12/13)	61.5% (8/13)	92.3% (12/13)	94.7% (18/19)	100% (14/14)	
White	14.3% (4/28)	10.5% (2/19)	4.8% (1/21)	0% (0/13)	23.1% (3/13)	7.7% (1/13)	0% (0/19)	0% (0/14)	
Asian	0% (0/28)	10.5% (2/19)	0% (0/21)	0% (0/13)	15.4% (2/13)	0% (0/13)	0% (0/19)	0% (0/14)	
Hispanic	0% (0/28)	0% (0/19)	0% (0/21)	7.7% (1/13)	0% (0/13)	0% (0/13)	0% (0/19)	0% (0/14)	
Other	3.6% (1/28)	0% (0/19)	14.3% (3/21)	0% (0/13)	0% (0/13)	0% (0/13)	5.3% (1/19)	0% (0/14)	
Gestational age at delivery (weeks; median [IQR])[a]	32.4 (28.9-34.7)	35.1 (34.2-36.2)	35.1 (31.9-36.4)	29.3 (22-31.9)	39.1 (39-39.3)	39 (38.6-40.3)	39.1 (38.2-40)	40.7 (39.1-41.1)	<0.001
Cesarean section[b]	100% (28/28)	31.6% (6/19)	9.5% (2/21)	15.4% (2/13)	100% (13/13)	15.4% (2/13)	10.5% (2/19)	21.4% (3/14)	<0.001
Birthweight (grams; median [IQR])[a]	1522.5 (951.8-2202.5)	2395 (2205-2700)	2020 (1755-2355)	1290 (399-1665)	3085 (2915-3615)	3040 (2865-3240)	3170 (2880-3552.5)	3422.5 (3037.5-3670)	<0.001
Maternal inflammatory response (moderate/severe acute chorioamnionitis)[b]	0% (0/28)	0% (0/19)	0% (0/21)	100% (13/13)	0% (0/13)	0% (0/13)	0% (0/19)	100% (14/14)	<0.001
Fetal inflammatory response (moderate/severe acute funisitis)[b]	0% (0/28)	0% (0/19)	0% (0/21)	53.9% (7/13)	0% (0/13)	0% (0/13)	0% (0/19)	14.3% (2/14)	<0.001

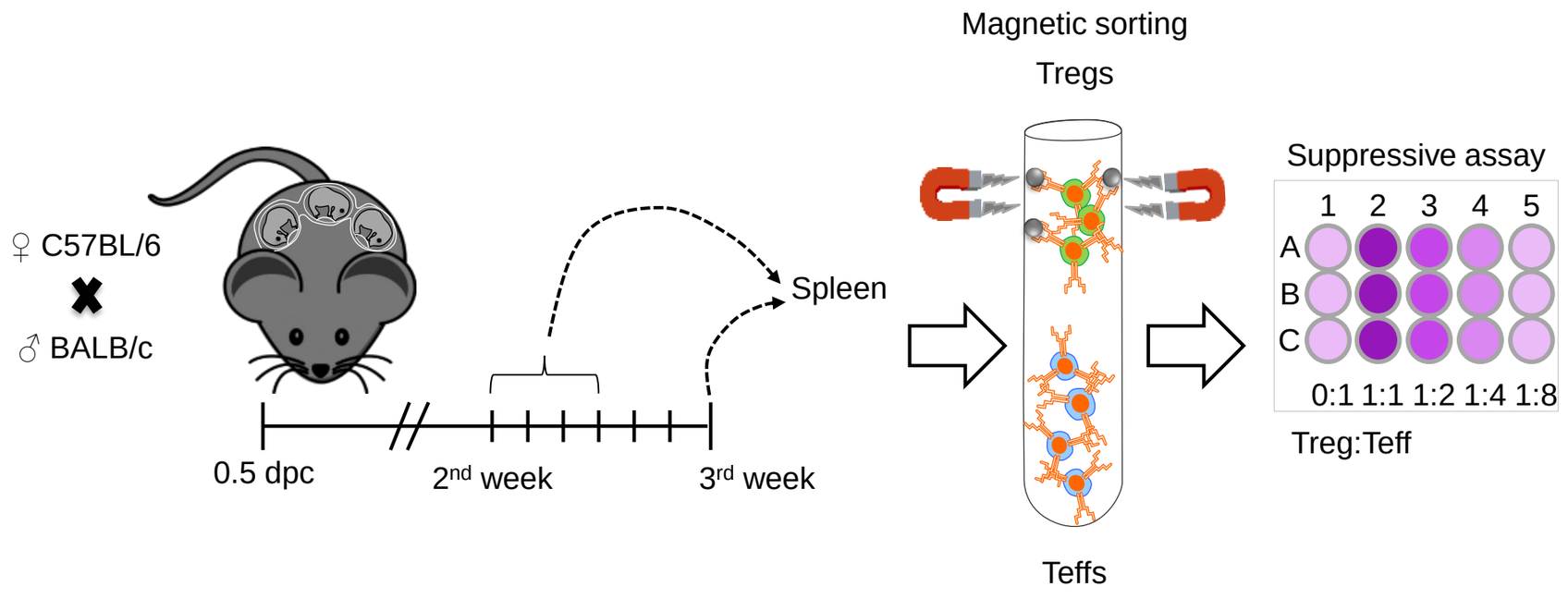
Data are given as median (interquartile range, IQR) and percentage (n/N). PTNL = preterm without labor; iPTL = idiopathic preterm labor; PTL+AI = preterm labor associated with intra-amniotic inflammation/infection; TNL = term without labor; TIL = term with labor; CI = chronic inflammatory lesions of the placenta; AI = acute inflammatory lesions of the placenta.

[a]Kruskal-Wallis test.

[b]Fisher's test with Monte-Carlo simulation.

[c]Some missing data

A



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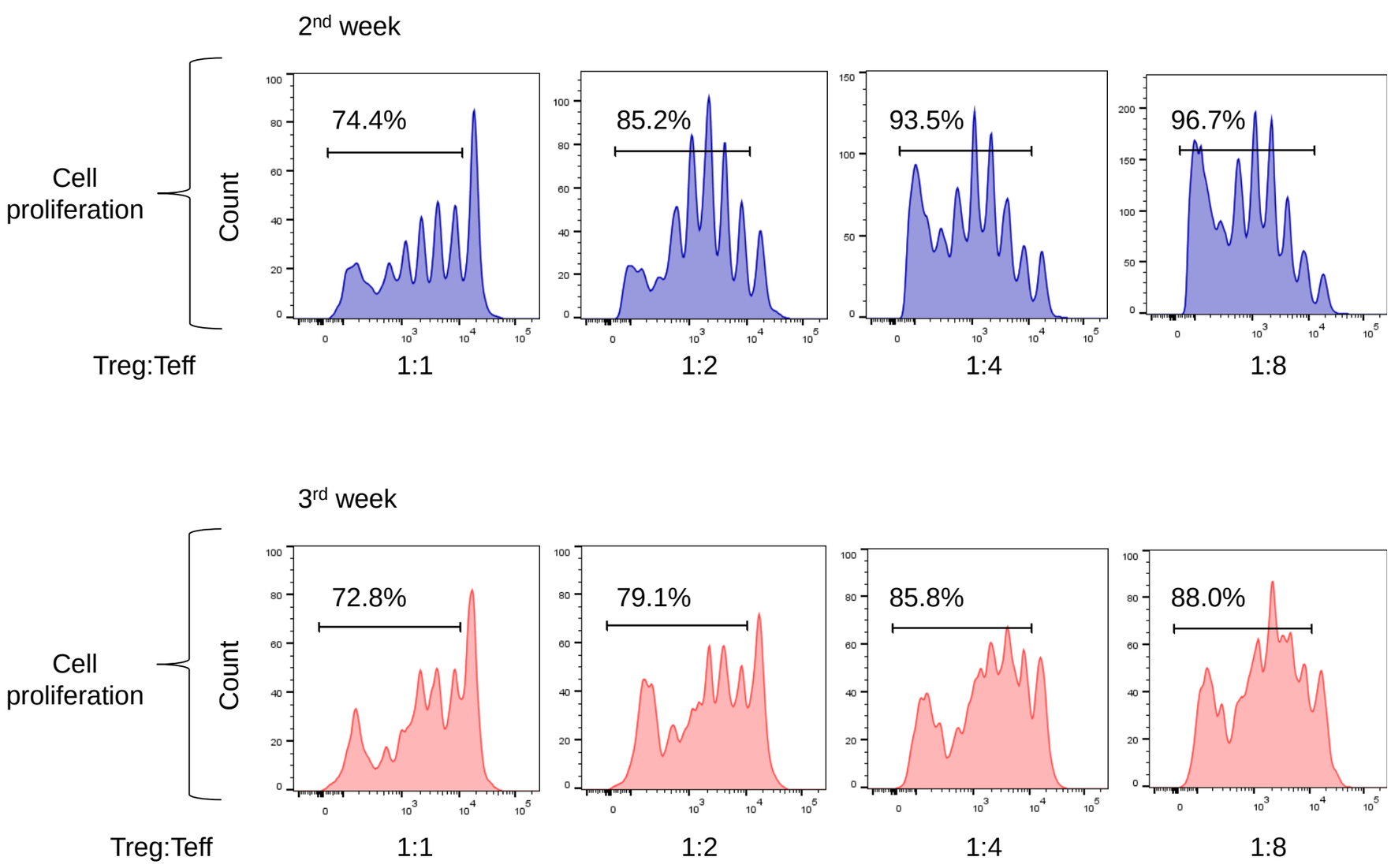

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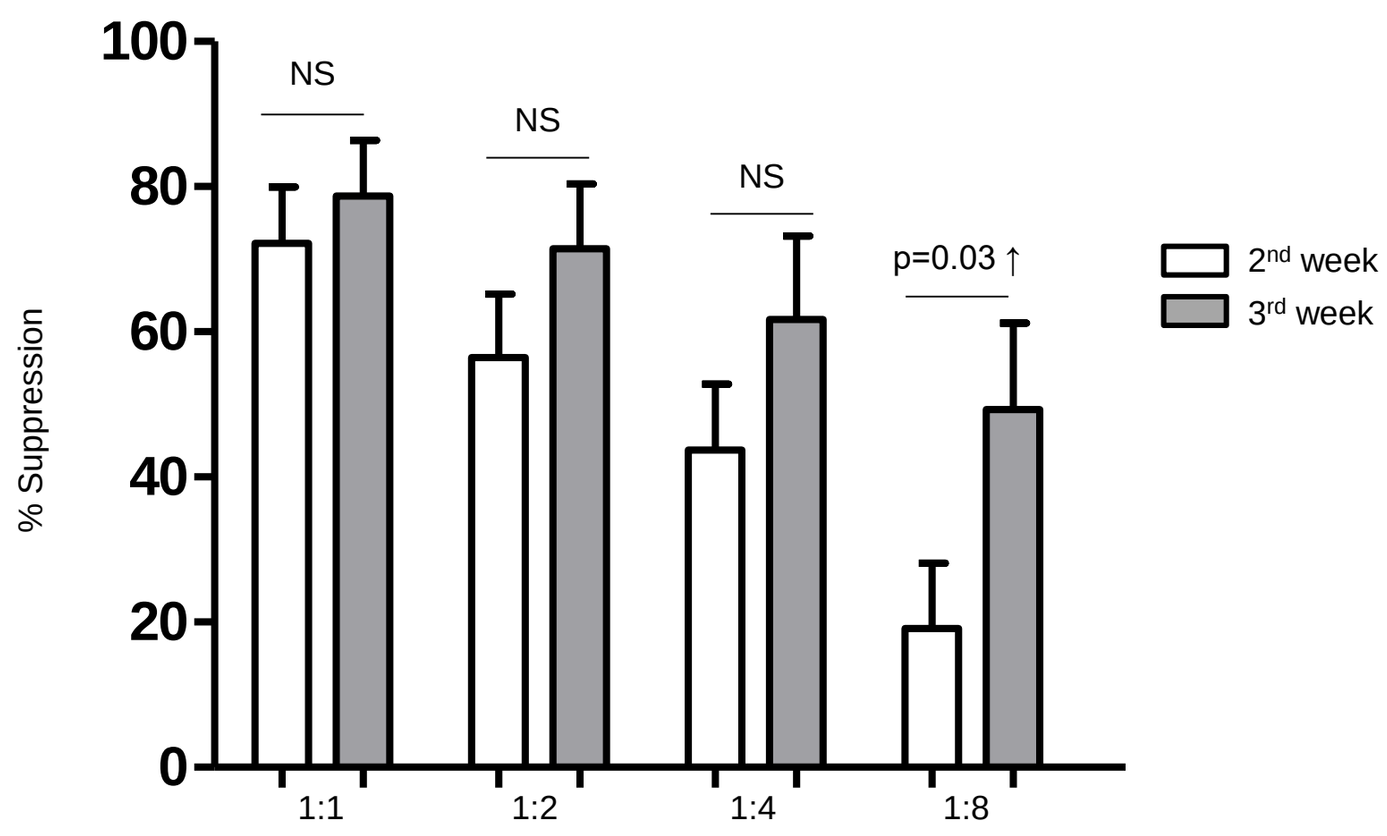

Figure S1. Suppressive function of murine regulatory T cells during the 2nd or 3rd week of pregnancy, related to Figure 3. (A) The spleen was collected from C57BL/6 dams in the 2nd or 3rd week of pregnancy for the magnetic isolation of splenic regulatory T cells (Tregs) and effector T cells (Teffs or Teff cells). Tregs were co-cultured with Teff cells at a 0:1, 1:1, 1:2, 1:4, or 1:8 Treg:Teff ratio for 96 h and Teff cell proliferation was measured by flow cytometry using CellTrace Violet. **(B)** Representative plots showing the proliferation of Teff cells in the 2nd week of pregnancy. **(C)** Representative plots showing the proliferation of Teff cells in the 3rd week of pregnancy. **(D)** Percentage of splenic Treg suppression of Teff cells in the 2nd or 3rd week of pregnancy (n = 4-6 per group). Data are shown as means ± SEM. Statistical analysis was performed using Mann-Whitney U tests.

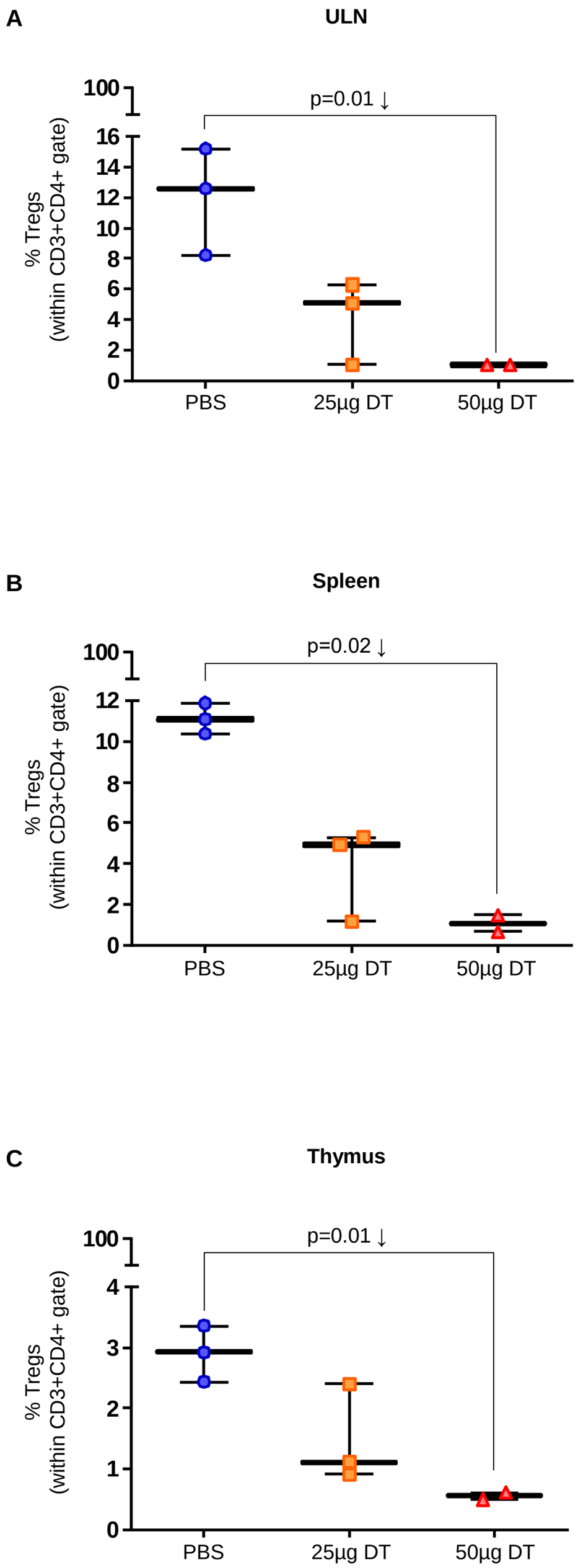

Figure S2. Depletion of regulatory T cells in non-pregnant *Foxp3*^{DTR} mice, related to Figure 3. *Foxp3*^{DTR} naïve females underwent partial or total regulatory T cell (Treg) depletion. Controls were injected with sterile 1X PBS. Mice were euthanized approximately 4 h after the second DT or PBS injection and tissues were collected for determination of Tregs. The frequencies of Tregs in the **(A)** uterine-draining lymph nodes (ULN), **(B)** spleen, and **(C)** thymus of non-Treg-depleted-, partially Treg-depleted-, and totally Treg-depleted-*Foxp3*^{DTR} mice (n = 2-3 per group). Data are shown as medians with minimum/maximum ranges. Statistical analysis was performed using Kruskal-Wallis tests with correction for multiple comparisons.

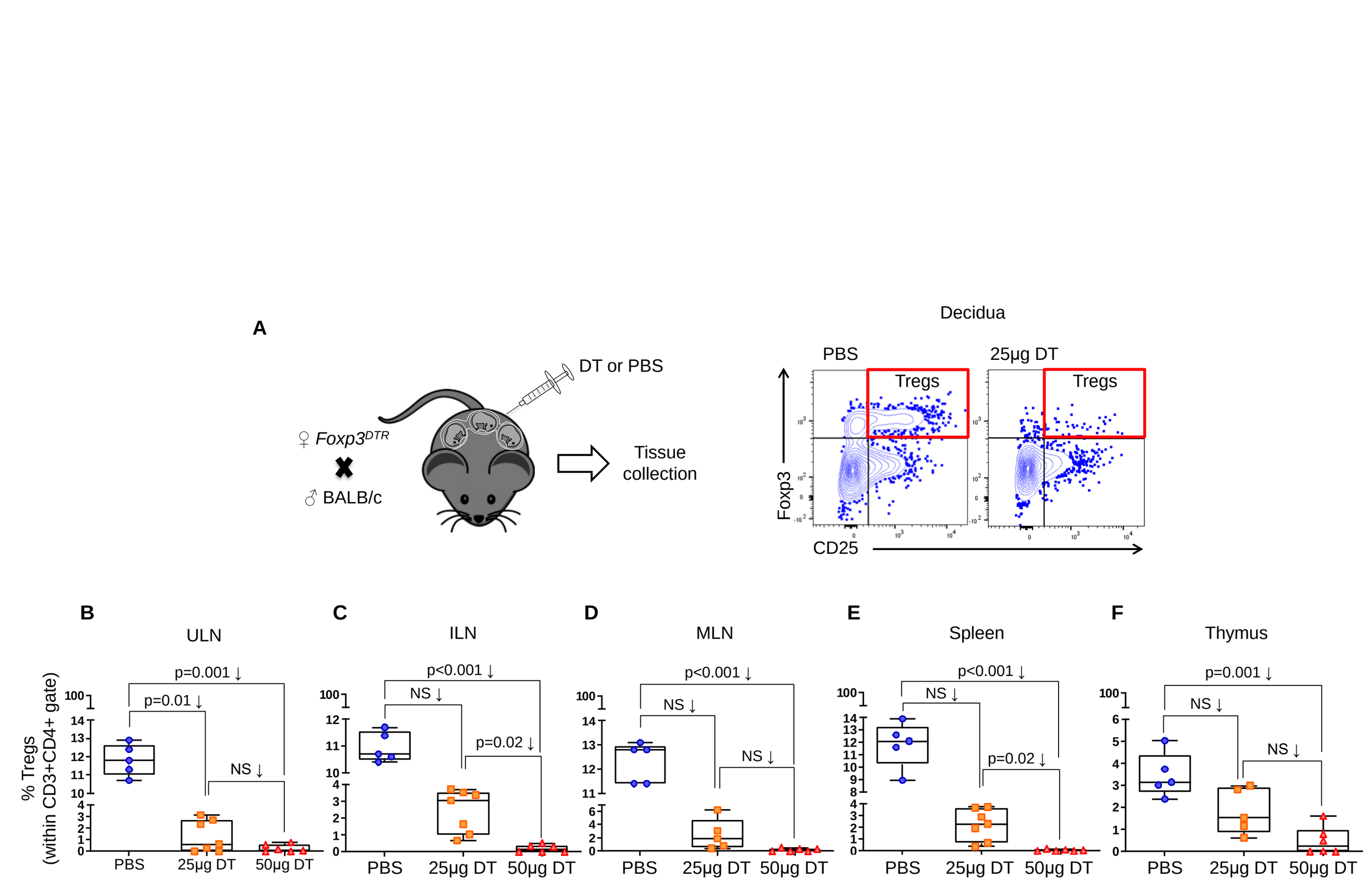

Figure S3. Depletion of regulatory T cells in pregnant *Foxp3^{DTR}* mice, related to Figure 3. (A) *Foxp3^{DTR}* dams underwent partial or total regulatory T cell (Treg) depletion. Controls were injected with sterile 1X PBS. Mice were euthanized approximately 4 h after the second DT or PBS injection and tissues were collected for determination of Tregs. Representative gating strategy of Treg depletion in the decidua is shown. The frequencies of Tregs in the **(B)** uterine-draining lymph nodes (ULN), **(C)** inguinal lymph nodes (ILN), **(D)** mesenteric lymph nodes (MLN), **(E)** spleen, and **(F)** thymus of non-Treg-depleted-, partially Treg-depleted-, and totally Treg-depleted-*Foxp3^{DTR}* dams (n = 5-7 per group). Data are shown as medians with interquartile ranges and minimum/maximum ranges. Statistical analysis was performed using Kruskal-Wallis tests with correction for multiple comparisons.

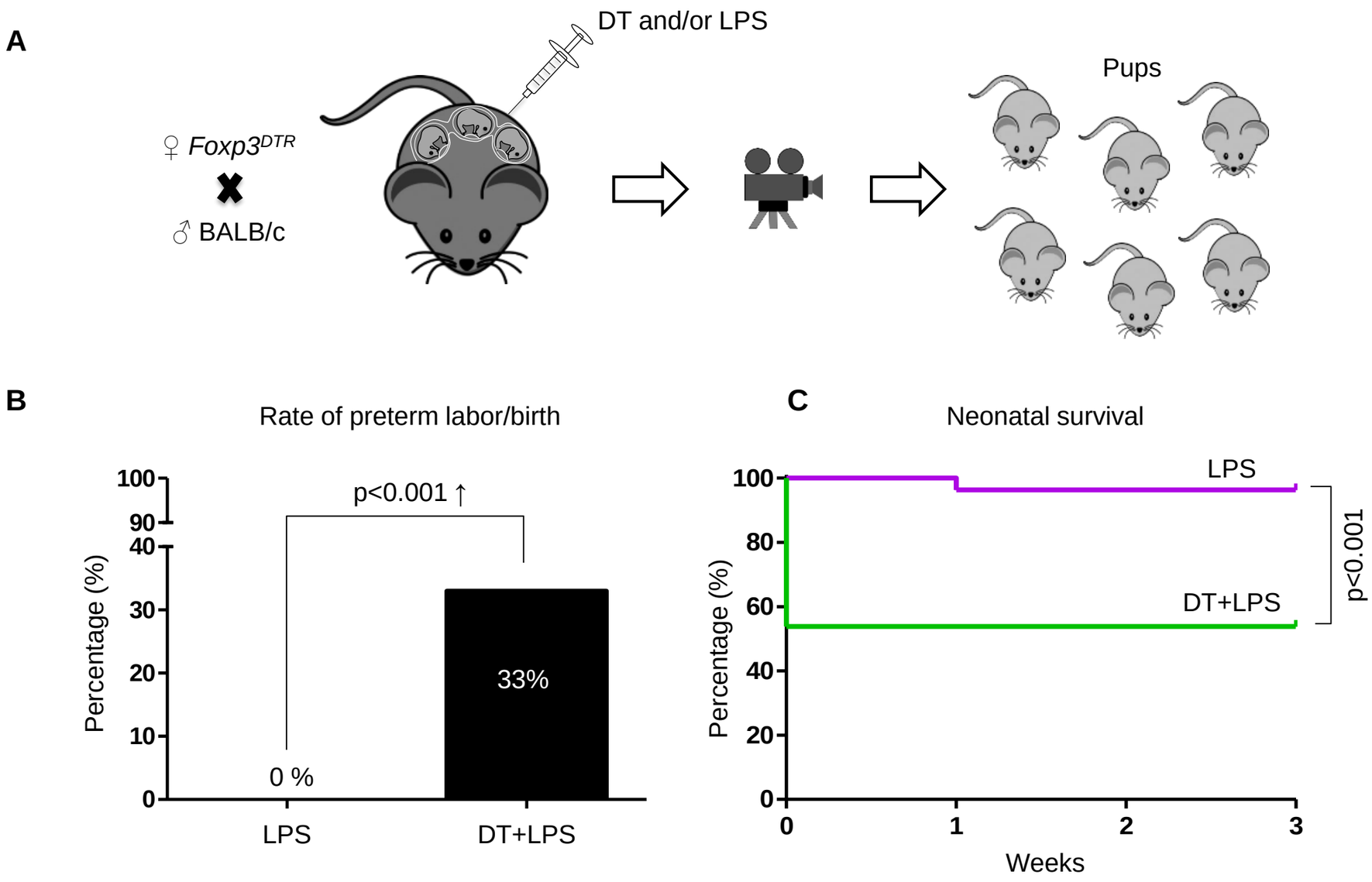

Figure S4. Depletion of Tregs increases the susceptibility to endotoxin-induced preterm birth, related to Figure 3. (A) *Foxp3*^{DTR} dams underwent partial regulatory T cell (Treg) depletion. On 16.5 dpc, a single intraperitoneal injection of 2 μg/200 μL of lipopolysaccharide (LPS; endotoxin) was given. Controls also included *Foxp3*^{DTR} dams injected with DT only on 14.5 and 15.5 dpc (data not shown). **(B)** Preterm birth rates of non-Treg-depleted- or partially Treg-depleted-*Foxp3*^{DTR} dams injected with LPS (n = 9 per group). Data are represented as means. **(C)** Percentage of survival from birth until 3 weeks postpartum for neonates born to non-Treg-depleted- or partially Treg-depleted- *Foxp3*^{DTR} dams injected with LPS (n = 5-8 per group). Statistical analyses were performed using the Fisher's exact test for rate of preterm birth or Mantel-Cox test for survival curves.

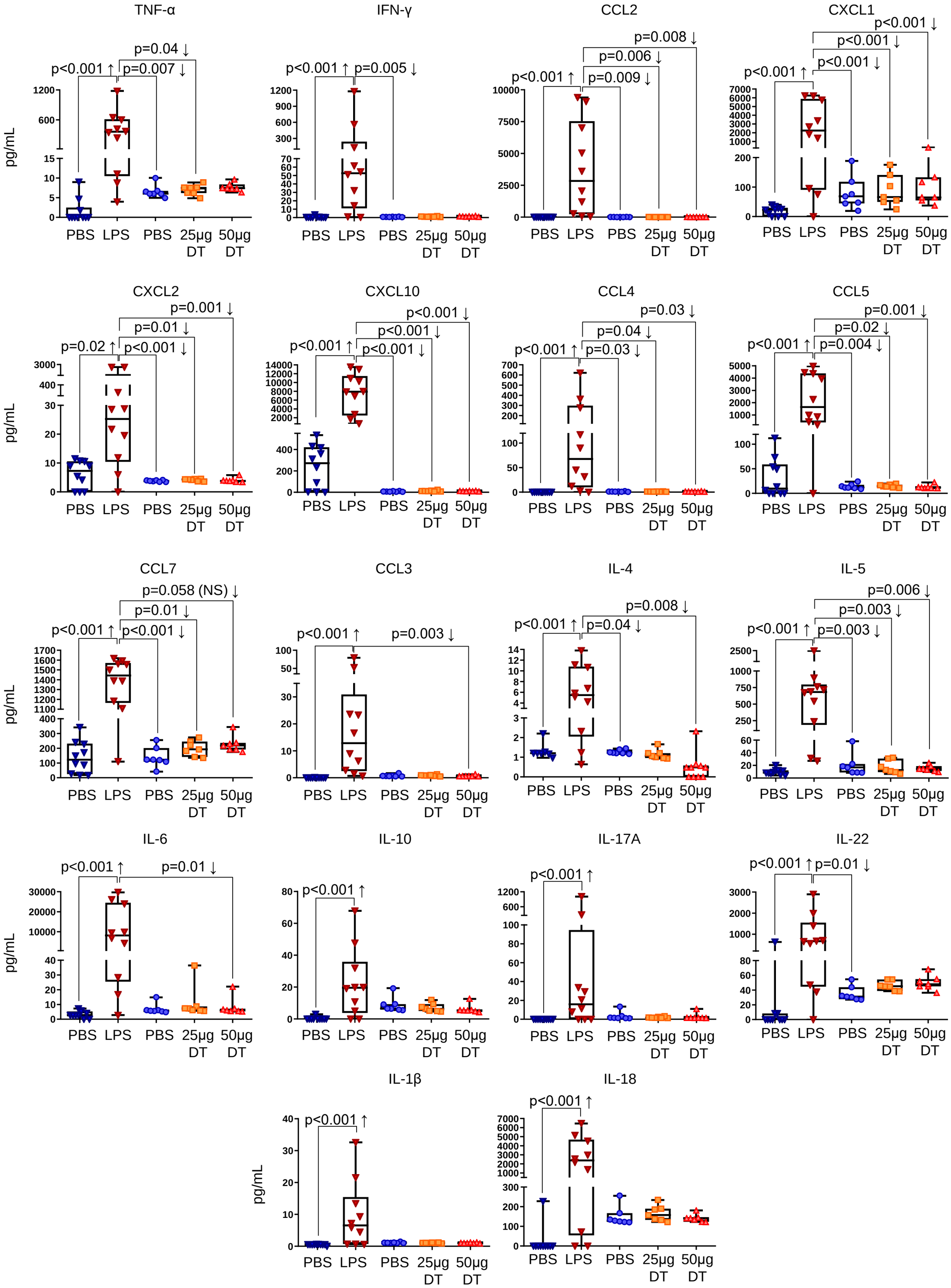

Figure S5. Comparison of the systemic acute proinflammatory response in the endotoxin-induced preterm birth model and the Treg-depletion induced preterm birth model, related to Figure 6. C57BL/6 dams were intraperitoneally injected with 10 μg/200 μL of lipopolysaccharide (LPS; endotoxin) or sterile 1X PBS on 16.5 dpc. Mice were euthanized on 17.5 dpc and maternal plasma was collected for cytokine and chemokine determination (n = 10 per group). *Foxp3*^{DTR} dams underwent partial or total regulatory T cell (Treg) depletion. Non-Treg-depleted controls were injected with sterile 1X PBS. Mice were euthanized approximately 4 h after the second injection and maternal plasma was collected for cytokine and chemokine determination (n = 7 per group). Data are shown as medians with interquartile ranges and minimum/maximum ranges. Statistical analysis was performed using Kruskal-Wallis or ANOVA tests with correction for multiple comparisons.

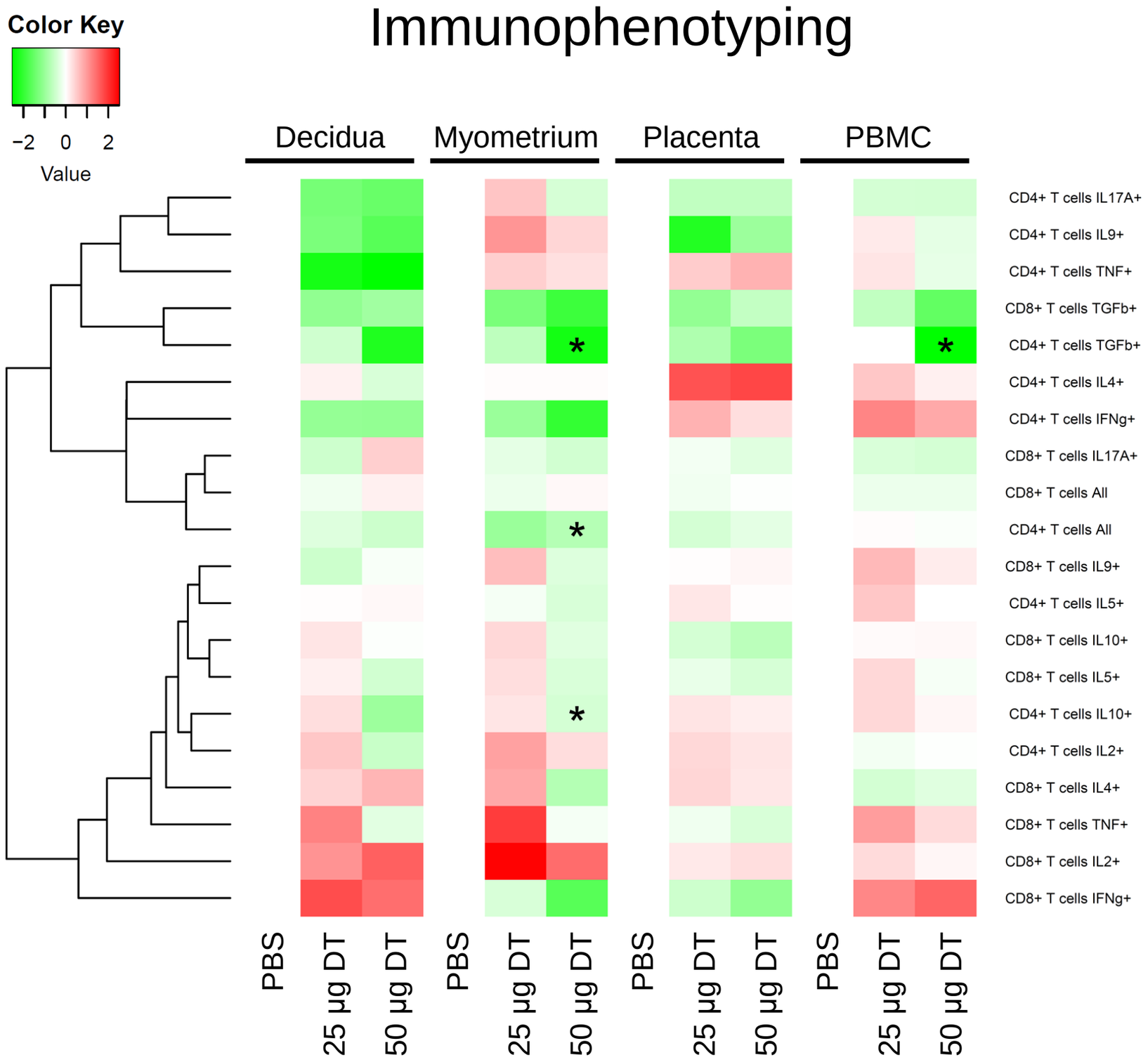

Figure S6. Depletion of Tregs is associated with altered local and systemic T-cell responses, related to Figure 7. Foxp3^{DTR} dams underwent partial or total regulatory T cell (Treg) depletion. Controls were injected with sterile 1X PBS. Mice were euthanized approximately 4 h after the second injection and the decidua, myometrium, placenta, and peripheral blood were collected for immunophenotyping (n = 5-7 per group). Heatmap visualization of changes in the frequencies of T-cell subsets in the decidua, myometrium, placenta, and peripheral blood of partially and totally Treg-depleted Foxp3^{DTR} dams relative to non-Treg-depleted controls. Red indicates increased frequency and green indicates decreased frequency. Statistical analysis was performed using t-tests with false discovery rate adjustment. Asterisks indicate significant differences compared to controls after adjustment.