Pregnancy imprints regulatory memory that sustains anergy to fetal antigen

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Abstract

Pregnancy is an intricately orchestrated process where immune effector cells with fetal specificity are selectively silenced. This requires the sustained expansion of immune suppressive maternal Foxp3^{+} regulatory T cells (Tregs), because even transient partial ablation triggers fetal-specific effector T cell activation and pregnancy loss[1,2]. In turn, many idiopathic pregnancy complications proposed to stem from disrupted fetal tolerance are associated with blunted maternal Treg expansion[3–5]. Importantly however, the antigen-specificity and cellular origin of maternal Tregs that accumulate during gestation remain undefined. Here we show pregnancy selectively stimulates the accumulation of maternal Foxp3^{+} CD4 cells with fetal-specificity using tetramer-based enrichment that allows the identification of rare endogenous T cells[6]. Interestingly after delivery, fetal-specific Tregs persist at elevated levels, maintain tolerance to pre-existing fetal antigen, and rapidly re-accumulate during subsequent pregnancy. The accelerated expansion of Tregs during secondary pregnancy was driven almost exclusively by proliferation of fetal-specific Foxp3^{+} cells retained from prior pregnancy, while induced Foxp3 expression and proliferation of pre-existing Foxp3^{+} cells each contribute to Treg expansion during primary pregnancy. Furthermore, fetal resorption in secondary compared with primary pregnancy becomes more resilient to partial maternal Foxp3^{+} cell ablation. Thus, pregnancy imprints Foxp3^{+} CD4 cells that sustain protective regulatory memory to fetal antigen. We anticipate these findings will spark further investigation on maternal regulatory T cell specificity that unlocks new strategies for improving pregnancy outcomes and novel approaches for therapeutically exploiting regulatory T cell memory.

The accumulation of maternal Tregs during pregnancy parallels the need for expanded tolerance to encompass "non-self" fetal antigens[3–5,7,8]. However, one consequence of sustained Foxp3^{+} cell expansion is susceptibility to prenatal infection[2]. Given the increasingly recognized importance of Treg specificity in regulating the fluid balance

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between immune activation that maintains host defense and immune suppression that prevents autoimmunity[9–14], we reasoned establishing the specificity of maternal Tregs that expand during pregnancy could unravel ways to dissociate their beneficial and detrimental impacts. Furthermore, extending this analysis postpartum may allow regulatory memory recently described for Tregs responsive to an induced self antigen to be investigated in a more physiological context[15]. To address these questions, we developed a mating strategy where the I-A^b 2W1S$_{52-68}$ peptide becomes a surrogate fetal antigen using male mice (H-2^d; Balb/c or H-2^b C57Bl/6 [B6]) engineered to co-express this peptide with β-actin to impregnate non-2W1S-expressing B6 females[16]. In turn, the high precursor frequency of CD4 cells with 2W1S$_{52-68}$ specificity allows endogenous maternal Tregs to this surrogate fetal antigen to be identified using MHC class II tetramer enrichment[6].

Using this approach, maternal CD4 cells with fetal-2W1S-specificity were found to sharply up-regulate CD44 expression, progressively accumulate throughout pregnancy, and persist at ~10-fold increased levels through day 100 postpartum each compared with non-pregnant controls (Fig. 1a). Maternal 2W1S$^+$ cell expansion was specific to mating with 2W1S-expressing mice because they did not accumulate in females impregnated by non-transgenic Balb/c males (Supplementary Fig. 1). Since seminal fluid also contains cells of paternal origin[17], 2W1S$^+$ cells in female mice rendered infertile with low-dose irradiation were also enumerated. We found that although mating without pregnancy stimulated modest 2W1S$^+$ cell expansion and CD44 up-regulation, the magnitude was sharply reduced compared with pregnant mice (Supplementary Fig. 1). Thus, maternal 2W1S$^+$ CD4 cell expansion during pregnancy reflects an antigen-specific response to cells of fetal origin.

Given the essential requirement for Tregs in maintaining fetal tolerance[2,7,18,19], we investigated Foxp3 expression among maternal cells with fetal-2W1S-specificitiy. Beginning midgestation, 2W1S$^+$ compared with 2W1S$^-$ CD4 cells became enriched for Foxp3 expression in allogeneic (Fig. 1a,b), as well as syngeneic pregnancy (Supplementary Fig. 2). As pregnancy progressed, Foxp3 expression among 2W1S$^+$ cells became progressively more pronounced peaking at ~50% late gestation through the first 48 hours postpartum (E18.5 to PP2) (Fig. 1a,b; Supplementary Fig. 3). Furthermore, 2W1S$^+$Foxp3$^+$ cells, and to a lesser extent 2W1S$^+$Foxp3$^-$ cells, up-regulated the proliferation marker Ki67 that paralleled expanding fetal tissue (Fig. 1c). Reciprocally later after expulsion of the fetus (PP14 to PP100), Ki67 expression among 2W1S$^+$Foxp3$^+$ and 2W1S$^+$Foxp3$^-$ cells became reduced (Fig. 1c). However, despite diminished Ki67 levels, Foxp3 expression among 2W1S$^+$ cells was sustained at ~20% through day 100 postpartum (Fig. 1a,b). Accordingly, maternal Tregs with fetal-specificity selectively accumulate during pregnancy and persist following parturition.

Interestingly, maternal Tregs with fetal-2W1S-specificity also progressively down-regulated Helios expression that nadired to ~40% Helios^{hi} by late gestation, while the few 2W1S$^+$Foxp3$^+$ cells in non-pregnant mice were uniformly Helios^{hi} (Fig. 1d). Comparatively, Helios expression among bulk maternal Tregs did not shift significantly. Although this discordance in Helios expression may suggest conversion of fetal-specific Foxp3$^-$ into Foxp3$^+$ cells[20], the recent finding that some peripherally induced Tregs also express Helios led us to more definitively investigate the origin of maternal Tregs with fetal-

specificity[21]. In particular, we asked whether mating with 2W1S-expressing males can convert 2W1S$^+$Foxp3$^-$ CD4 cells from Foxp3$^{DTR/DTR}$ donors ablated of Tregs with diphtheria toxin (DT)[22] into Foxp3$^+$ cells after adoptive transfer into virgin Foxp3$^{WT/WT}$ recipient mice. By midgestation, 2W1S$^+$Foxp3$^+$ among Treg-ablated donor Foxp3$^{DTR/DTR}$ cells were readily recovered illustrating induction of maternal Tregs with fetal-specificity (Fig. 1e). This conversion was pregnancy-specific and not due to incomplete donor Treg ablation because Foxp3$^+$ cells were undetectable among Treg-ablated donor cells in unmated control mice (Supplementary Fig. 4). Importantly however, Foxp3$^+$ among Treg-ablated donor CD4 cells was also consistently reduced (by ~50%) compared with either 2W1S$^+$Foxp3$^+$ donor cells in mice without DT treatment or among recipient CD4 cells not susceptible to DT (Fig. 1e). Thus, Foxp3 induction among Foxp3$^-$ precursors and proliferation of pre-existing Foxp3$^+$ cells each contribute to the accumulation of maternal Tregs with fetal-specificity during primary pregnancy.

To further characterize maternal Tregs with specificity to pre-existing fetal antigen that persist postpartum, these cells were tracked during subsequent pregnancy. After secondary mating, maternal Foxp3$^+$ cells with fetal-2W1S-specificity accumulated with accelerated kinetics in an antigen-specific fashion (Fig. 2a; Supplementary Fig. 5). The more rapid expansion of maternal Tregs in separate groups of mice was recapitulated within the same mouse by measuring 2W1S$^+$ Treg accumulation among donor CD4 cells from postpartum mice (secondary expansion) adoptively transferred prior to mating with 2W1S-expressing males, compared with cells in virgin recipient mice (primary expansion) (Fig. 2b). By substituting CD4 cells from postpartum Foxp3$^{DTR/DTR}$ mice for adoptive transfer and using DT to eliminate donor Tregs prior to mating, we also addressed whether the accelerated secondary expansion of maternal Tregs with fetal-2W1S-specificity reflects more vigorous induction among Foxp3$^-$ cells or proliferation of pre-existing Foxp3$^+$ cells. We found in sharp contrast to primary pregnancy, the ablation of donor Tregs from postpartum Foxp3$^{DTR/DTR}$ mice almost uniformly eliminated their expansion in subsequent pregnancy (Fig. 2c). Thus, recurrent pregnancy primes the accelerated accumulation of maternal Foxp3$^+$ cells that expand from pre-existing Tregs retained from prior pregnancy.

Expanding this model, the responsiveness of maternal CD4 cells with fetal-specificity was also investigated. We found 2W1S$^+$ cells recovered from mice midgestation or postpartum each compared with non-pregnant controls did not produce appreciable IFN-γ *ex vivo* following stimulation, consistent with previously described anergy of maternal cells with fetal-specificity (Supplementary Fig. 6)[2,23]. Therefore, to more fully evaluate the responsiveness of maternal T cells with fetal-2W1S-specificity, we measured their *in vivo* response to *Listeria monocytogenes* engineered to express 2W1S$_{52-68}$ peptide (Lm-2W1S) that potently stimulates Th1-differentiation in other contexts[24,25]. We found 2W1S$^+$ cells expand and up-regulate T-bet expression each in an antigen-specific fashion in naïve and mice impregnated by 2W1S-expressing males after Lm-2W1S inoculation similar to other intracellular pathogens (Supplementary Fig. 7)[26]. Interestingly however, 2W1S$^+$ cells in pregnant mice where 2W1S represents a surrogate fetal antigen produced only background levels of IFN-γ and other effector cytokines, with reciprocal accumulation of Foxp3$^+$ cells (Fig. 3a; Supplementary Fig. 8). Comparatively, >15% of 2W1S$^+$ cells in Lm-2W1S

inoculated virgin mice were IFN-γ^+ (Fig. 3a). This hypo-responsiveness was specific to fetal-2W1S stimulation because 2W1S$^+$ CD4 cells in mice impregnated with non-2W1S-expressing males produced IFN-γ comparable to non-pregnant controls (Fig. 3a; Supplementary Fig. 8). Given the sustained enrichment of fetal-specific Tregs after delivery (Fig. 1a,b), these studies were extended to investigate whether diminished IFN-γ production among maternal CD4 cells with specificity to pre-existing fetal antigen is similarly maintained. Remarkably, IFN-γ production remained anemic in postpartum mice previously exposed to 2W1S as a fetal antigen, while postpartum mice without prior fetal-2W1S exposure produce IFN-γ comparable to non-pregnant controls (Fig. 3b). Accordingly, pregnancy imprints functional anergy for maternal CD4 cells with fetal-specificity that is sustained postpartum.

To dissociate whether pregnancy-induced T cell anergy was cell-intrinsic or imposed by features associated with the postpartum environment, we measured IFN-γ production by CD4 cells from postpartum or virgin mice each after adoptive transfer into naive recipient mice. We found IFN-γ production by donor postpartum and each group of naïve (donor and recipient) 2W1S$^+$ CD4 cells were similar, and sharply increased compared with 2W1S$^+$ cells in un-manipulated postpartum mice following Lm-2W1S inoculation (Fig. 3b,c). Thus, anergy among maternal CD4 cells with specificity to pre-existing fetal antigen is not cell-intrinsic, but maintained by the postpartum environment.

In complementary studies we addressed the importance of maternal Tregs in sustaining anergy to cells with specificity to pre-existing fetal antigen by investigating the impacts of replacing the entire Treg compartment in postpartum mice previously exposed to fetal-2W1S with naïve Foxp3$^+$ cells from virgin mice. Consistent with recent studies using adoptively transferred Foxp3$^{WT/WT}$ CD4 cells to refill the cellular compartment in Foxp3$^{DTR/DTR}$ mice sustained on DT treatment[2], Tregs from naïve mice efficiently reconstituted Treg-ablated Foxp3$^{DTR/DTR}$ postpartum mice (Fig. 4a). Using this approach, we found replacing maternal Foxp3$^+$ cells in postpartum mice with Tregs from naïve mice restored IFN-γ production for 2W1S$^+$ CD4 cells (Fig. 4b). Furthermore, while only rare 2W1S$^+$Foxp3$^+$ cells that were Helios^{hi} were found among postpartum mice reconstituted with naïve Tregs, a significant proportion of 2W1S$^+$ cells expanded in response to Lm-2W1S in intact postpartum mice remained Foxp3$^+$ (~20%) and Helios^{lo} (~40%) (Fig. 4b). Thus, the muted expansion of naïve Foxp3$^+$ CD4 cells with Lm infection is overcome by pregnancy-induced Treg activation (Fig. 4b; Supplementary Fig. 8)[24]. By extension, the restored responsiveness of postpartum 2W1S$^+$ cells after adoptive transfer into Treg-sufficient naïve mice most likely represents dilution of co-transferred maternal Tregs with fetal-2W1S-specificity (Fig. 3c).

Lastly, to establish how maternal Tregs with fetal-specificity retained postpartum impacts subsequent pregnancy outcomes, the frequency of fetal resorption triggered by partial maternal Foxp3$^+$ cell ablation using Foxp3$^{DTR/WT}$ mice was compared between secondary and primary pregnancy[2]. We found secondary pregnancy became significantly more resilient to partial Treg ablation because fetal resorption was reduced by ~60% compared with primary pregnancy (Fig. 4c). In turn, fetal resorption in Treg-sufficient secondary pregnancy was also significantly reduced from background levels with primary allogeneic pregnancy.

Maternal Tregs were essential for these protective effects because wholesale Foxp3^{+} cell ablation using Foxp3[DTR/DTR] mice triggered pervasive fetal resorption equally in secondary and primary allogeneic pregnancy (Fig. 4c). Importantly, fetal wastage with maternal Treg ablation in this context was driven by antigen heterogeneity, and not poor maternal health, because the frequency of resorption was sharply reduced with Treg ablation in mice bearing syngeneic pregnancy (Supplementary Fig. 9).

Together, these findings establish a model whereby pregnancy primes the selective accumulation and activation of maternal Tregs with fetal-specificity (Supplementary Fig. 10), and extend the role of antigen experienced Tregs from primary into subsequent pregnancies[2,7,18]. In this regard while maternal Tregs have been described to expand up to 2-fold when examined in a non-antigen-specific fashion[2–5], our results demonstrate Foxp3^{+} cells with fetal-specificity expand >100-fold through parturition (Fig. 1a; Supplementary Fig. 3). After delivery, maternal Tregs with fetal-specificity are sustained at enriched levels, and are functionally distinct as they re-accumulate with accelerated kinetics and out compete "naïve" Tregs during secondary pregnancy. Similar to discordant functional properties of naïve and activated effector T cells[27], these results uncover the exciting possibility of exploiting antigen-specific "memory" Tregs to dissociate detrimental and beneficial immune responses. Applied to human pregnancy, these data may explain why rates of preeclampsia, and other complications associated with disrupted fetal tolerance, are reduced in secondary compared with primary pregnancy[28]. However, given the increased risk of preeclampsia in recurrent human pregnancy when the inter-pregnancy interval is extended, waning Treg memory similar to other CD4 subsets would not be unexpected[25,29]. Therefore, establishing the durability of pregnancy-induced regulatory memory and characterizing whether this response can be sustained with boosting represent next-steps with exceptional scientific importance.

Methods Summary

Mice

2W1S-expressing and Foxp3[DTR] mice have each been described[2,16,22]. Mated females and the timing of pregnancy was determined by visualization of a copulation plug (E0.5). For infection, Lm-2W1S (10^4 CFUs, see Methods)[24] was intravenously inoculated. Experiments were performed in accordance with University of Minnesota IACUC approved protocols.

Tetramer enrichment and flow cytometry

I-A[b] 2W1S$_{52–68}$ tetramer staining and enrichment have been described[6]. Lymphoid cells from the spleen and lymph nodes were enriched with 2W1S$_{52–68}$ tetramer prior to surface, intracellular, or intranuclear staining. For stimulation, cells were cultured with PMA/ionomycin (5 hours) and Brefeldin A before staining.

Cell transfer and ablation

One mouse equivalent of purified CD4 cells was intravenously transferred into recipient mice one day before mating/infection. Donor Tregs from Foxp3[DTR] mice were ablated in recipient Foxp3[WT] mice using purified DT (two doses 8 hours apart, 0.5 μg/dose). For

ablation of endogenous Tregs, DT was administered daily (0.5 μg first dose, followed by 0.1 μg/dose daily thereafter) beginning midgestation or immediately following donor cell transfer.

Statistics

Data was analyzed using the unpaired (separate groups of mice) or paired (cell subsets within the same mice) Student's *t* test.

Methods

Mice

Balb/c (H-2$^{d/d}$), and C57Bl/6 (B6; H-2$^{b/b}$) or CD45.1 (H-2$^{b/b}$) and CD90.1 (H-2$^{b/b}$) mice each on the B6 background were purchased from The Jackson Laboratory or The National Cancer Institute. Transgenic mice expressing 2W1S$_{52-68}$ antigen behind the β-actin promoter in all cells, and Foxp3$^{DTR/DTR}$ mice where Tregs become susceptible to ablation with low dose diphtheria toxin (DT) have each been described[16,22]. Foxp3$^{DTR/DTR}$ mice on the B6 background were intercrossed with CD45.1 mice to generate CD45.1$^{+/+}$ Foxp3$^{DTR/DTR}$ mice. For mating, 2W1S-expressing males were used either on the B6 background, or backcrossed 5 generations to Balb/c mice, and verified to be H-2$^{d/d}$. Males were introduced to either virgin or postpartum (>14 days) females for 24 hours, and mated mice visualized by a copulation plug representing E0.5. In each experiment, pups were removed within 24 hours after delivery to prevent the potential transfer of fetal antigen through suckling. For sterilization, female mice were sub-lethally irradiated (100 rads) prior to mating.

Tetramer enrichment and flow cytometry

PE or APC conjugated MHC class II I-A^b 2W1S$_{52-68}$ tetramer, and their use with anti-fluorophore conjugated magnetic beads (Miltenyi Biotec) for enrichment have been described[6,30]. For enumerating total 2W1S$^+$ cells, all nucleated cells from secondary lymphoid tissue (spleen and axillary, brachial, cervical, inguinal, mesenteric, pancreatic, paraaortic/uterine lymph nodes) were collected, enriched using I-A^b 2W1S$_{52-68}$ tetramer, and stained for cell-surface CD4, CD8α, CD25, CD45.1, CD45.2, CD90.1, CD90.2, CD44, CD11b, CD11c, B220, F4/80, intracellular IFN-γ, IL-4, IL-10, IL-17A, or intranuclear Foxp3, Helios, Ki67, or T-bet expression using commercially available antibodies and cell permeabilization reagents (BD PharMingen or eBioscience). For stimulation, cells were cultured with PMA/ionomycin for 5 hours in media supplemented with Brefeldin A before tetramer staining.

Cell transfer and ablation

For adoptive transfer, CD4 cells in the spleen and lymph nodes were first purified by negative selection (Miltenyi Biotech), and one mouse equivalent donor cells intravenously transferred into recipient mice one day before mating and/or infection. For ablation of donor Foxp3$^{DTR/DTR}$ cells, recipient Foxp3$^{WT/WT}$ mice were treated with purified DT (Sigma Chemicals, two doses 8 hours apart, 0.5 μg/dose). For ablation of endogenous Tregs in Foxp3$^{DTR/WT}$ or Foxp3$^{DTR/DTR}$ mice during pregnancy, purified DT was administered daily

(0.5μg first dose, followed by 0.1 μg/dose thereafter beginning midgestation) for 4 consecutive days. For Foxp3^{+} cell reconstitution requiring sustained ablation of endogenous Tregs in Foxp3[DTR/DTR] recipient mice, purified DT was administered daily (0.5 μg first dose, followed by 0.1 μg/dose thereafter) immediately following transfer of purified donor CD4 cells as described[2].

Bacteria

Listeria monocytogenes (Lm) was engineered to stably express and secrete $2W1S_{52-68}$ antigen by sub-cloning the *hly* promoter, signal sequence, $2W1S_{52-68}$ and ovallbumin coding sequence from the pAM401-based expression contstruct[24,31] into the temperature sensitive plasmid pKSV7[32]. This pKSV7-based plasmid containing the *hly* promoter, signal sequence, $2W1S_{52-68}$ and ovallbumin coding sequence was used to electroporate Lm-OVA[33], and transformants selected by resistance to chloramphenicol (10 μg/ml final concentration) at room temperature. Individual clones were then passaged five times at 40oC in brain heart infusion media (Becton Dickinson) with chloramphenicol selection (plasmid integration into the Lm genome), followed by passage without antibiotic selection (plasmid excision from the Lm genome). Lm clones where double homologous recombination had occurred were initially screened by replica plating for sensitivity to chloramphenicol, and a single clone (Lm-2W1S) verified by PCR and DNA sequencing using previously described methods[34]. For infection, Lm-2W1S was grown to early log phase (OD_{600} 0.1) in brain heart infusion media, washed and diluted with sterile saline, and then intravenously inoculated via the lateral tail vein (10^4 CFUs per mouse). Five days thereafter, the antigen-specific CD4 cell response in the spleen and lymph nodes was investigated with I-A^b $2W1S_{52-68}$ tetramer staining.

Data acquisition and analysis

Cells stained with fluorochrome-conjugated tetramer and antibody were acquired on a FACSCanto cytometer (Becton Dickinson), and analyzed using FlowJo (TreeStar) software. The number and percent cells were then analyzed and found to be normally distributed, and thereafter the difference between separate groups of mice were analyzed using an unpaired Student's *t* test, while differences between individual cell subsets within the same mouse were analyzed using the paired Student's *t* test (Prism, GraphPad). For all analysis, *P* < 0.05 was taken as statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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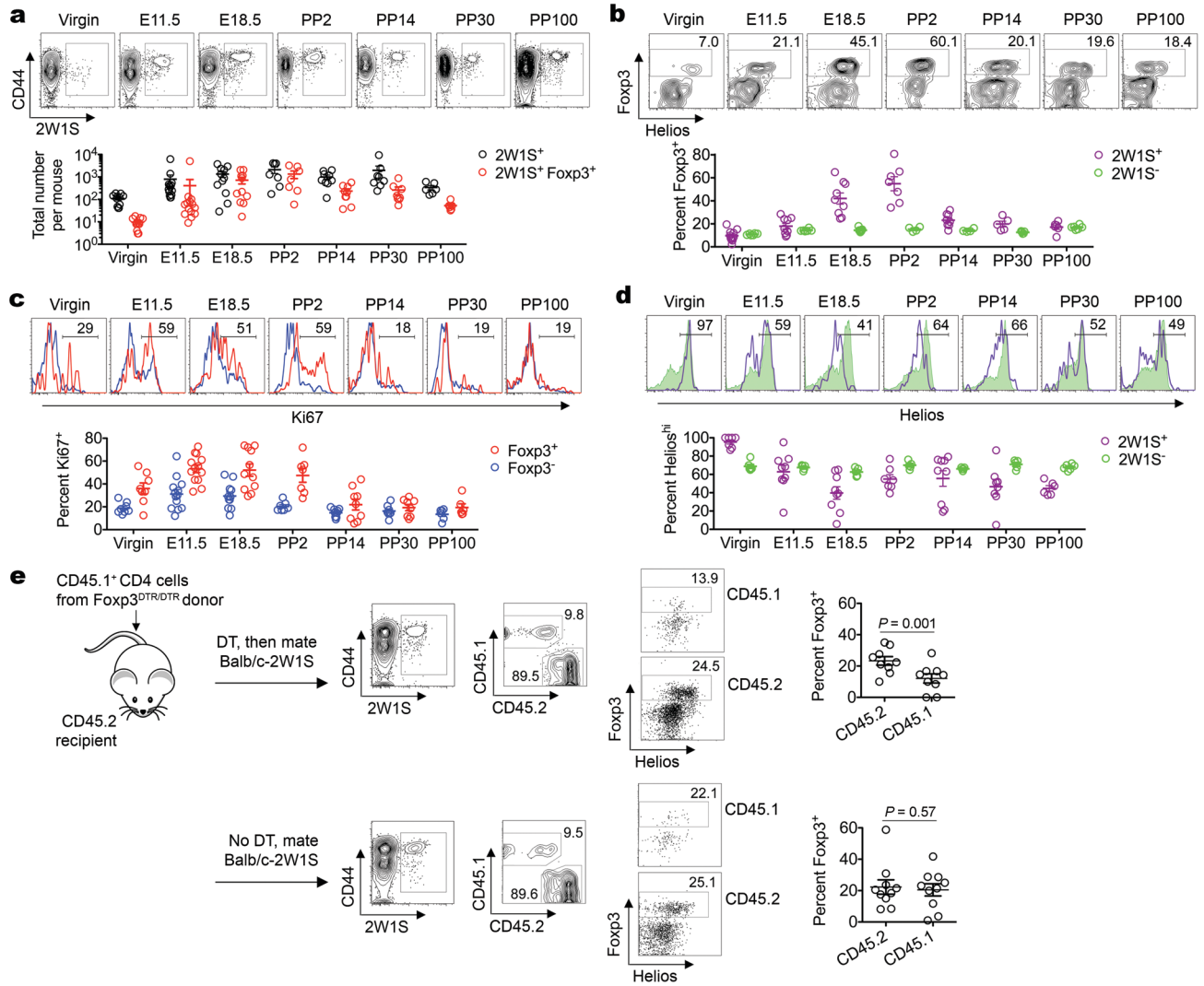
Figure 1. Accumulation of maternal CD4 and Foxp3^{+} Tregs with fetal-specificity during gestation

a, Total 2W1S[+] or 2W1S[+]Foxp3^{+} CD4 cells in B6 females impregnated by Balb/c-2W1S males. **b,** Percent Foxp3^{+} among 2W1S[+] or 2W1S^{−} CD4 cells. **c,** Percent Ki67^{+} among 2W1S[+]Foxp3^{+} or 2W1S[+]Foxp3^{−} CD4 cells. **d,** Percent Helios[hi] among 2W1S[+]Foxp3^{+}or 2W1S[−]Foxp3^{+} CD4 cells. **e,** Percent Foxp3^{+} among Foxp3[DTR/DTR] donor (CD45.1[+]) or Foxp3[WT/WT] recipient (CD45.2[+]) 2W1S^{+} CD4 cells midgestation (E11.5) by Balb/c-2W1S males, with DT treatment (top) or no DT controls (bottom). Bar, mean ± one standard error.
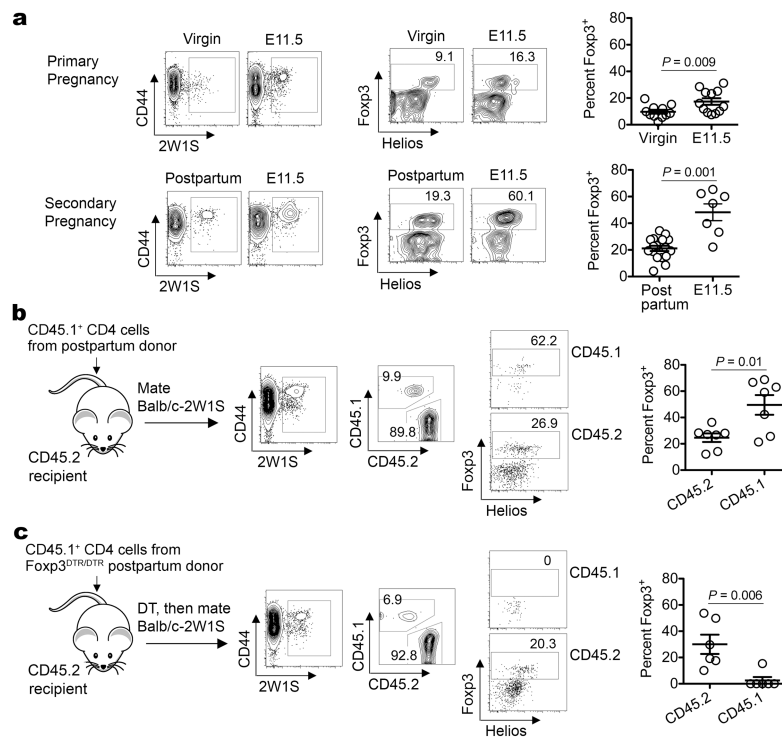
Figure 2. Accelerated expansion of maternal Tregs with fetal-specificity during secondary pregnancy

a, Percent Foxp3$^+$ among virgin (primary pregnancy) or postpartum (secondary pregnancy) females before mating or midgestation (E11.5) by Balb/c-2W1S males. **b,** Percent Foxp3$^+$ among postpartum donor (CD45.1$^+$) or naïve recipient (CD45.2$^+$) 2W1S$^+$ CD4 cells midgestation by Balb/c-2W1S males. **c,** Percent Foxp3$^+$ among Treg-ablated Foxp3$^{DTR/DTR}$ postpartum donor (CD45.1$^+$) or naïve recipient (CD45.2$^+$) 2W1S$^+$ CD4 cells midgestation. Bar, mean ± one standard error.
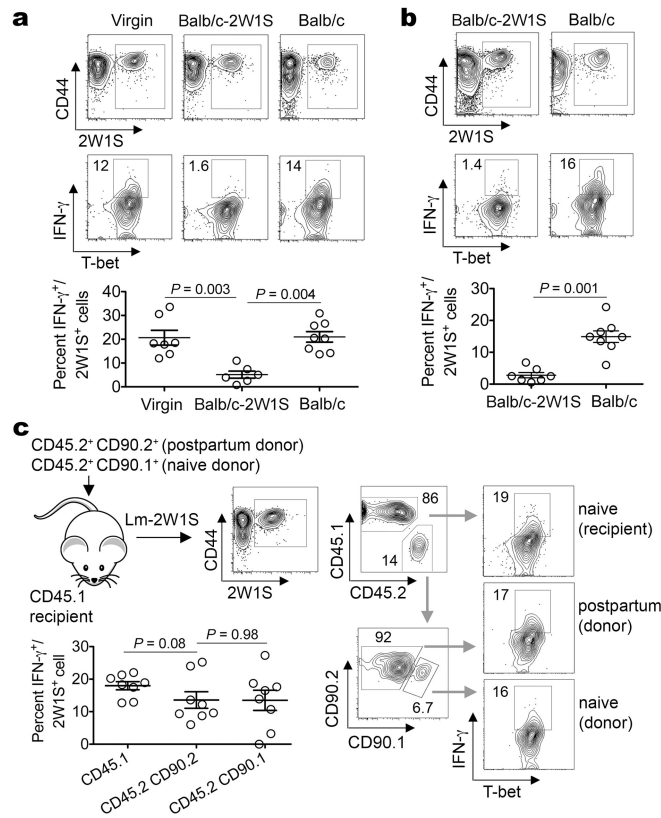
Figure 3. The postpartum environment maintains anergy for maternal CD4 cells with pre-existing fetal-specificity

a, IFN-γ producing 2W1S+ CD4 cells five days after Lm-2W1S inoculation in virgin, or pregnant females midgestation by Balb/c-2W1S or Balb/c males. **b,** IFN-γ producing 2W1S+ CD4 cells five days after Lm-2W1S inoculation in postpartum mice previously impregnated by Balb/c-2W1S or Balb/c males. **c,** IFN-γ producing postpartum donor (CD45.2+CD90.2+), naive donor (CD45.2+CD90.1+), or naive recipient (CD45.1+) CD4 cells five days after Lm-2W1S inoculation and stimulation with PMA/ionomycin. Bar, mean ± one standard error.
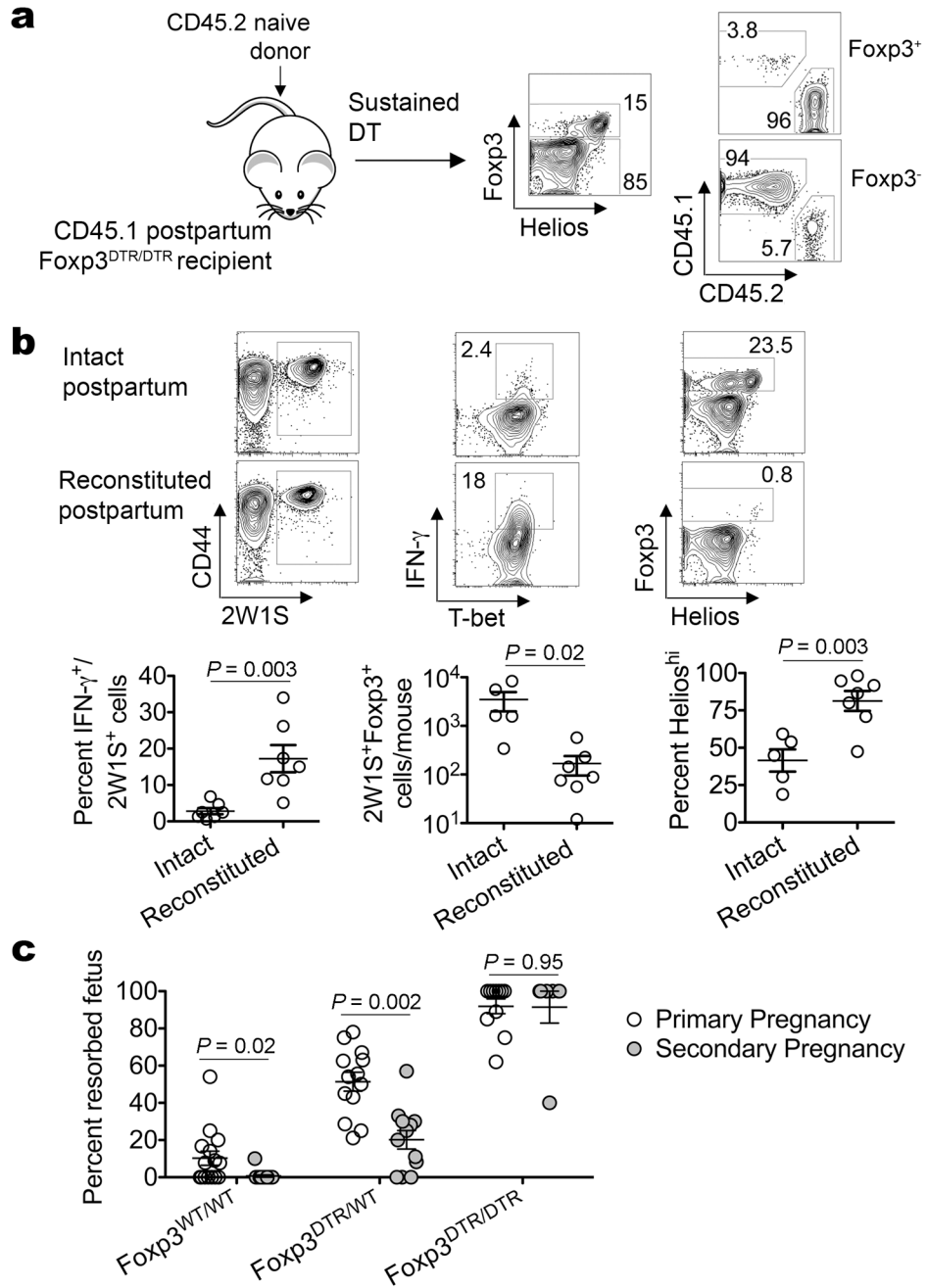
Figure 4. Maternal postpartum Tregs mitigate IFN-γ responsiveness and mediate resiliency to fetal resorption in secondary pregnancy

a, Representative plots illustrating the majority (>96%) of Foxp3^{+} cells are derived from adoptively transferred CD4 in DT treated Foxp3[DTR/DTR] mice. **b,** IFN-γ producing 2W1S^{+} cells among CD45.1[+]CD45.2^{−} cells, accumulation of 2W1S[+]Foxp3^{+} cells, and Helios expression among 2W1S[+]Foxp3^{+} Tregs five days after Lm-2W1S inoculation. **c,** Percent fetal resorption during primary (open) or secondary (shaded) allogeneic pregnancy for

Foxp3$^{WT/WT}$, Foxp3$^{DTR/WT}$, or Foxp3$^{DTR/DTR}$ females five days after DT initiation beginning midgestation. Bar, mean ± one standard error.