Pregnancy imprints regulatory memory that sustains anergy to fetal antigen

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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 (Treg cells), because even transient partial ablation triggers fetal-specific effector T-cell activation and pregnancy loss3,4. In turn, many idiopathic pregnancy complications proposed to originate from disrupted fetal tolerance are associated with blunted maternal Treg expansion5–9. Importantly, however, the antigen specificity and cellular origin of maternal Treg cells that accumulate during gestation remain incompletely defined. Here we show that 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 cells6. Interestingly, after delivery, fetal-specific Treg cells persist at elevated levels, maintain tolerance to pre-existing fetal antigen, and rapidly re-accumulate during subsequent pregnancy. The accelerated expansion of Treg cells during secondary pregnancy was driven almost exclusively by proliferation of fetal-specific FOXP3+ cells retained from prior pregnancy, whereas 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 ablation. Thus, pregnancy imprints FOXP3+ CD4 cells that sustain protective regulatory memory to fetal antigen. We anticipate that 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 Treg cell memory.

The accumulation of maternal Treg cells during pregnancy parallels the need for expanded tolerance to encompass ‘non-self’ fetal antigens4–5,22. However, one consequence of sustained FOXP3+ cell expansion is susceptibility to prenatal infection2. Given the increasingly recognized importance of Treg specificity in regulating the fluid balance between immune activation that maintains host defense and immune suppression that prevents autoimmunity4–11, we reasoned that establishing the specificity of maternal Treg cells that expand during pregnancy could unravel ways to dissociate their beneficial and detrimental impacts. Furthermore, extending this analysis post-partum may allow the regulatory memory recently described for Treg cells responsive to an induced self antigen to be investigated in a more physiological context13. To address these questions, we developed a mating strategy where the I-A\(^b\) 2W1S55–68 peptide (a variant of peptide residues 55–68 of the alpha chain of the mouse major histocompatibility complex (MHC) class II, I-E\(^b\)) becomes a surrogate fetal antigen using male mice (H-2\(^d\); Balb/c or H-2b C57BL/6 [B6]) engineered to co-express maternal FOXP3+ females16. In turn, the high precursor frequency of CD4 cells with fetal-2W1S specificity allows endogenous maternal Treg cells to this surrogate fetal antigen to be identified using MHC class II tetramer enrichment14.

Using this approach, maternal CD4 cells with fetal-2W1S specificity were found to sharply upregulate CD44 expression, progressively accumulate throughout pregnancy, and persist at approximately ten-fold increased levels through day 100 post-partum 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). Because seminal fluid also contains cells of paternal origin17, 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 upregulation, the magnitude was markedly 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 Treg cell in maintaining fetal tolerance2–7, we investigated FOXP3 expression among maternal cells with fetal-2W1S specificity. Beginning mid-gestation, 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 around 50% late gestation through to the first 48 h post-partum (embryonic day 18.5 (E18.5) to post-partum day 2 (PP2)) (Fig. 1a, b and Supplementary Fig. 3). Furthermore, 2W1S+ FOXP3+ cells, and to a lesser extent 2W1S− FOXP3+ cells, upregulated the proliferation marker Ki67 that paralleled expanding fetal tissue (Fig. 1c). Reciprocally 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 post-partum (Fig. 1a, b). Accordingly, maternal Treg cells with fetal specificity selectively accumulate during pregnancy and persist following parturition.

Interestingly, maternal Treg cells with fetal-2W1S specificity also progressively downregulated Helios (also known as IKZF2) expression that dropped to its lowest level of ~40% Helios4 by late gestation, whereas the few 2W1S− FOXP3+ cells in non-pregnant mice were uniformly Helios+ (Fig. 1d). Comparatively, Helios expression among bulk maternal Treg cells did not shift significantly. Although this discordance in Helios expression may suggest conversion of fetal-specific FOXP3+ cells into FOXP3− cells20, the recent finding that some peripherally induced Treg cells also express Helios led us to more definitively investigate the origin of maternal Treg cells with fetal specificity4,15. In particular, we asked whether mating with 2W1S-expressing males can convert 2W1S− FOXP3− CD4 cells from Foxp3− DTR/DTR donors ablated of Treg cells with diphtheria toxin22 (Foxp3\(^{DTR/DTR}\)) into FOXP3+ cells after adoptive transfer into virgin Foxp3\(^{WT/WT}\) recipient mice. By mid-gestation, 2W1S− FOXP3− among Treg cells ablated donor Foxp3\(^{DTR/DTR}\).
cells were readily recovered, illustrating induction of maternal $T_{reg}$ cells with fetal specificity (Fig. 1e). This conversion was pregnancy-specific and not due to incomplete donor $T_{reg}$ ablation because FOXP3$^+$ cells were undetectable among $T_{reg}$-ablated donor cells in unmated control mice (Supplementary Fig. 4). Importantly, however, FOXP3$^+$ among $T_{reg}$-ablated donor CD4 cells was also consistently reduced (by $\sim$50%) compared with either 2W1S$^+$ FOXP3$^+$ donor cells in mice without diphtheria toxin treatment or among recipient CD4 cells not susceptible to diphtheria toxin (Fig. 1e). Thus, FOXP3 induction among FOXP3$^+$ precursors and proliferation of pre-existing FOXP3$^+$ cells each contribute to the accumulation of maternal $T_{reg}$ cells with fetal specificity during primary pregnancy.

To further characterize maternal $T_{reg}$ cells with specificity to pre-existing fetal antigen that persist post-partum, 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 and Supplementary Fig. 5). The more rapid expansion of maternal $T_{reg}$ cells in separate groups of mice was recapitulated within the same mouse by measuring 2W1S$^+$ FOXP3$^+$ or 2W1S$^+$ FOXP3$^+$ CD4 cells. e. Percentage FOXP3$^+$ among Foxp3$^{DTR/DTR}$ donor (CD45.1$^+$) or Foxp3$^{WT/WT}$ recipient (CD45.2$^+$) 2W1S$^+$ CD4 cells mid-gestation (E11.5) by Balb/c-2W1S males, with diphtheria toxin (DT) treatment (top) or no diphtheria toxin controls (bottom). Bars, means $\pm$ one standard error.

Figure 1 | Accumulation of maternal CD4 and FOXP3$^+$ $T_{reg}$ cells with fetal specificity during gestation. a, Total 2W1S$^+$ or 2W1S$^+$ FOXP3$^+$ CD4 cells in B6 females impregnated by Balb/c-2W1S males. b, Percentage FOXP3$^+$ among 2W1S$^+$ or 2W1S$^+$ CD4 cells. c, Percentage Ki67$^+$ among 2W1S$^+$ or 2W1S$^+$ FOXP3$^+$ CD4 cells. d, Percentage Helios$^{hi}$ among 2W1S$^+$ FOXP3$^+$ or 2W1S$^+$ FOXP3$^+$ CD4 cells. e, Percentage FOXP3$^+$ among Foxp3$^{DTR/DTR}$ donor (CD45.1$^+$) or Foxp3$^{WT/WT}$ recipient (CD45.2$^+$) 2W1S$^+$ CD4 cells mid-gestation (E11.5) by Balb/c-2W1S males, with diphtheria toxin (DT) treatment (top) or no diphtheria toxin controls (bottom). Bars, means $\pm$ one standard error.
following stimulation, consistent with previously described anergy of maternal cells with fetal specificity (Supplementary Fig. 6). 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 the 2W1S\textsubscript{55-68} peptide (Lm-2W1S) that potently stimulates T\textsubscript{H}1-differentiation in other contexts\textsuperscript{24,25}. We found that 2W1S\textsuperscript{+} cells expand and upregulate T-bet (also known as TBX21) expression each in an antigen-specific fashion in both naive mice and mice impregnated by 2W1S-expressing males after Lm-2W1S inoculation, similar to other intracellular pathogens (Supplementary Fig. 7). Interestingly, however, 2W1S\textsuperscript{+} cells in pregnant mice where 2W1S represents a surrogate fetal antigen produced only background levels of IFN-\(\gamma\) and other effector cytokines, with reciprocal accumulation of FOXP3\textsuperscript{+} cells (Fig. 3a and Supplementary Fig. 8). Comparatively, >15% of 2W1S\textsuperscript{+} cells in Lm-2W1S-inoculated virgin mice were IFN-\(\gamma\)\textsuperscript{+} (Fig. 3a). This hypo-responsiveness was specific to fetal-2W1S stimulation, because 2W1S\textsuperscript{+} CD4 cells in mice impregnated with non-2W1S-expressing males produced IFN-\(\gamma\) levels comparable to non-pregnant controls (Fig. 3a and Supplementary Fig. 8). Given the sustained enrichment of fetal-specific T\textsubscript{reg} cells after delivery (Fig. 1a, b), these studies were extended to investigate whether diminished IFN-\(\gamma\) production among maternal CD4 cells with specificity to pre-existing fetal antigen is similarly maintained. Remarkably, IFN-\(\gamma\) production remained anaemic in post-partum mice previously exposed to 2W1S as a fetal antigen, whereas post-partum mice without prior fetal-2W1S exposure produce IFN-\(\gamma\) comparable to non-pregnant controls (Fig. 3b). Accordingly, pregnancy imprints functional anergy for maternal CD4 cells with fetal specificity that is sustained post-partum.

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

In complementary studies we addressed the importance of maternal T\textsubscript{reg} cells in sustaining anergy to cells with specificity to pre-existing fetal antigen by investigating the effect of replacing the entire T\textsubscript{reg} compartment in post-partum mice previously exposed to fetal-2W1S with naive FOXP3\textsuperscript{+} cells from virgin mice. Consistent with recent studies using adoptively transferred Foxp3\textsuperscript{WT/WT} CD4 cells to refill the cellular compartment in Foxp3\textsuperscript{DTR/DTR} mice sustained on diphtheria toxin treatment, T\textsubscript{reg} cells from naive mice efficiently reconstituted Foxp3\textsuperscript{DTR/DTR} post-partum mice (Fig. 4a). Using this approach, we found that replacing maternal FOXP3\textsuperscript{+} cells in post-partum mice with T\textsubscript{reg} cells from naive mice restored IFN-\(\gamma\) production for 2W1S\textsuperscript{+} CD4 cells (Fig. 4b). Furthermore, whereas only rare 2W1S\textsuperscript{+} FOXP3\textsuperscript{+} cells that were Helios\textsuperscript{lo} were found among post-partum mice reconstituted with naive T\textsubscript{reg} cells, a significant proportion of 2W1S\textsuperscript{+} cells expanded in response to Lm-2W1S in intact post-partum mice remained FOXP3\textsuperscript{+} (~20%) and Helios\textsuperscript{lo} (~40%) (Fig. 4b). Thus, the muted expansion of naive FOXP3\textsuperscript{+} CD4 cells with L. monocytogenes infection is overcome by pregnancy-induced T\textsubscript{reg} activation (Fig. 4b and
Together, these findings establish a model whereby pregnancy primes the selective accumulation and activation of maternal Treg cells with fetal specificity (Supplementary Fig. 10), and extend the role of antigen-specific maternal Treg cells to mediate resiliency in secondary pregnancy. After delivery, maternal Treg cells with fetal specificity are sustained at enriched levels, and are functionally distinct as they re-accumulate and mediate resiliency to fetal resorption in secondary pregnancy. Similar to discordant functional properties of maternal Treg cells in primary versus secondary pregnancy, these results demonstrate that FOXP3+ cells among CD45.1+CD45.2+ cells, accumulation of 2W1S+FOXP3+ cells, and Helios expression among 2W1S+FOXP3+ Treg cells 5 days after Lm-2W1S inoculation. c. Percentage fetal resorption during primary (open) or secondary (shaded) allogeneic pregnancy for Foxp3DTR/+ WT/WT, Foxp3DTR/+ DTR/WT or Foxp3DTR/+ DTR/DTR females 5 days after diphtheria toxin initiation beginning mid-gestation. Bars, means ± one standard error.

Supplementary Fig. 8)24. By extension, the restored responsiveness of post-partum 2W1S+ cells after adoptive transfer into Treg-sufficient naive mice most likely represents dilution of co-transferred maternal Treg cells with fetal-2W1S specificity (Fig. 3c).

Lastly, to establish how maternal Treg cells with fetal specificity retained post-partum have an effect on subsequent pregnancy outcomes, the frequency of fetal resorption triggered by partial maternal FOXP3+ cell ablation using Foxp3DTR/WT mice was compared between secondary and primary pregnancy. 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 Foxp3WT/WT mice during secondary pregnancy was also significantly reduced from background levels compared with primary allogeneic pregnancy. Maternal Treg cells were essential for these protective effects because wholesale FOXP3+ cell ablation using Foxp3DTR/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 Treg cells with fetal specificity (Supplementary Fig. 10), and extend the role of antigen-experienced Treg cells from primary into subsequent pregnancies2,7,18.

In this regard, whereas maternal Treg cells have been described to expand up to twofold when examined in a non-antigen-specific fashion2,3, our results demonstrate that FOXP3+ cells with fetal specificity expand >100-fold through parturition (Fig. 1a and Supplementary Fig. 3). After delivery, maternal Treg cells with fetal specificity are sustained at enriched levels, and are functionally distinct as they re-accumulate with accelerated kinetics and out-compete ‘naive’ Treg cells during secondary pregnancy. Similar to discordant functional properties of naive and activated effector T cells7, these results uncover the exciting possibility of exploiting antigen-specific ‘memory’ Treg cells to dissociate detrimental and beneficial immune responses. Applied to human pregnancy, these data may explain why rates of pre-eclampsia, and other complications associated with disrupted fetal tolerance, are reduced in secondary compared with primary pregnancy24. However, given the increased risk of pre-eclampsia in recurrent human pregnancy when the inter-pregnancy interval is extended, waning Treg memory similar
to other CD4 subsets would not be unexpected\textsuperscript{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 Foxp\textsuperscript{3\textit{DTR}} mice have each been described\textsuperscript{16,22}. Mated females and the timing of pregnancy was determined by visualization of a copulation plug (E0.5). For infection, Lm-2W1S (10\textsuperscript{6} colony-forming units, see Methods\textsuperscript{34}) was inoculated intravenously. Experiments were performed in accordance with University of Minnesota IACUC approved protocols.

**Tetramer enrichment and flow cytometry.** I-\textit{A}\textsuperscript{b} 2W1S\textsubscript{55–68} tetramer staining and enrichment have been described\textsuperscript{2}. Lymphoid cells from the spleen and lymph nodes were enriched with 2W1S\textsubscript{55–68} tetramer before surface, intracellular or intranuclear staining. For stimulation, cells were cultured with phorbol myristate acetate plus ionomycin (5 h) before staining.

**Cell transfer and ablation.** One mouse equivalent of purified CD4 cells was intravenously transferred into recipient mice 1 day before mating or infection. Donor T\textsubscript{reg} cells from Foxp\textsuperscript{3\textit{DTR}} mice were ablated in recipient Foxp\textsuperscript{3\textit{DTR}} mice using purified diphtheria toxin (two doses 8 h apart, 0.5 µg per dose). For ablation of endogenous T\textsubscript{reg} cells, diphtheria toxin was administered daily (0.5 µg first dose, followed by 0.1 µg per dose daily thereafter) beginning mid-gestation or immediately following donor-cell transfer.

**Statistics.** Data were analysed using the unpaired (separate groups of mice) or paired (cell subsets within the same mouse) Student’s t test.

**Full Methods** and any associated references are available in the online version of the paper.

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METHODS

Mice. C57BL/6 (B6; H-2\textsuperscript{b/b}), or CD45.1 (H-2\textsuperscript{b/b}) and CD90.1 (H-2\textsuperscript{b/b}) mice on the B6 background, and Balb/c (H-2\textsuperscript{d/d}) mice, were purchased from The Jackson Laboratory or The National Cancer Institute. Transgenic mice expressing 2W1S\textsubscript{55–68} antigen behind the β-actin promoter in all cells, and Foxp\textsuperscript{3\textsubscript{DTR/DTR}} mice where T\textsubscript{reg} cells become susceptible to ablation with low-dose diphtheria toxin have each been described\textsuperscript{14,22}. Foxp\textsuperscript{3\textsubscript{DTR/DTR}} mice on the B6 background were intercrossed with CD45.1 mice to generate CD45.1\textsuperscript{+/−}/Foxp\textsuperscript{3\textsubscript{DTR/DTR}} mice. For mating, 2W1S-expressing males were used either on the B6 background, or backcrossed five generations to Balb/c mice, and verified to be H-2\textsuperscript{b/b}. Males were introduced to either virgin or post-partum (≥14 days) females for 24 h, and mated mice visualized by a copulation plug representing E0.5. In each experiment, pups were removed within 24 h after delivery to prevent the potential transfer of fetal antigen through suckling. For sterilization, female mice were sub-lethally irradiated (100 rads) before mating.

Tetramer enrichment and flow cytometry. Phycoerythrin or allophycocyanin-conjugated MHC class II 1-A\textsuperscript{b} 2W1S\textsubscript{55–68} tetramer, and their use with anti-fluorophore-conjugated magnetic beads (Miltenyi Biotec) for enrichment have been described\textsuperscript{30}. For enumerating total 2W1S\textsuperscript{+} cells, all nucleated cells from secondary lymphoid tissue (spleen and axillary, brachial, cervical, inguinal, mesenteric, pancreatic, para-aortic/uterine lymph nodes) were collected, enriched, stained for cell-surface CD4, CD8, 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 phorbol myristate acetate/ionomycin for 5 h 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 Biotec), and one mouse equivalent of donor cells intravenously transferred into recipient mice 1 day before mating and/or infection. For ablation of donor Foxp\textsuperscript{3\textsubscript{DTR/DTR}} cells, recipient Foxp\textsuperscript{3\textsubscript{DTR/GT}} mice were treated with purified diphtheria toxin (Sigma Chemicals, two doses 8 h apart, 0.5 µg per dose). For ablation of endogenous T\textsubscript{reg} cells in Foxp\textsuperscript{3\textsubscript{DTR/WT}} or Foxp\textsuperscript{3\textsubscript{DTR/DTR}} mice during pregnancy, purified diphtheria toxin was administered daily (0.5 µg first dose, followed by 0.1 µg per dose thereafter) after beginning mid-gestation for 4 consecutive days. For FOXP3\textsuperscript{−} cell reconstitution required sustained ablation of endogenous T\textsubscript{reg} cells in Foxp\textsuperscript{3\textsubscript{DTR/DTR}} recipient mice, purified diphtheria toxin was administered daily (0.5 µg first dose, followed by 0.1 µg per dose thereafter) immediately following transfer of purified donor CD4 cells as described\textsuperscript{13}.

Bacteria. Listeria monocytogenes was engineered to stably express and secrete 2W1S\textsubscript{55–68} antigen by sub-cloning the hly promoter, signal sequence, 2W1S\textsubscript{55–68} and ovalbumin coding sequence from the pAM401-based expression construct\textsuperscript{131} into the temperature sensitive plasmid pKSV7\textsuperscript{32}. This pKSV7-based plasmid containing the hly promoter, signal sequence, 2W1S\textsubscript{55–68} and ovalbumin coding sequence was used to electroporate Lm-OVA\textsuperscript{33}, and transformants selected by resistance to chloramphenicol (10 µg ml\textsuperscript{−1} final concentration) at room temperature. Individual clones were then passaged five times at 40 °C in brain heart infusion media (Becton Dickinson) with chloramphenicol selection (plasmid integration into the L. monocytogenes genome), followed by passage without antibiotic selection (plasmid excision from the L. monocytogenes genome). L. monocytogenes 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\textsuperscript{34}. For infection, Lm-2W1S was grown to early log phase (OD\textsubscript{600 nm} 0.1) in brain heart infusion media, washed and diluted with sterile saline, and then inoculated intravenously in the lateral tail vein (10\textsuperscript{5} colony-forming units per mouse). Five days thereafter, the antigen-specific CD4 cell response in the spleen and lymph nodes was investigated with 1-A\textsuperscript{b} 2W1S\textsubscript{55–68} tetramer staining.

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