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Jared M. Pollard, …, Maria-Luisa Alegre, Anita S. Chong

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Pregnancy dedifferentiates memory CD8$^+$ T cells into hypofunctional cells with exhaustion-enriched programs

Jared M. Pollard$^1$, Grace Hynes$^1$, Dengping Yin$^1$, Malay Mandal$^2$, Fotini Gounari$^{2,3}$, Maria-Luisa Alegre$^2$, Anita Chong$^1$

$^1$Section of Transplantation, Department of Surgery, University of Chicago, Chicago IL, USA
$^2$Section of Rheumatology, Department of Medicine, University of Chicago, Chicago IL, USA
$^3$Department of Immunology, Mayo Clinic, Phoenix AZ, USA

Authorship note: JMP an GH are co-first authors. MLA and AC are co-senior authors.
Abstract

Alloreactive memory, unlike naïve, CD8\(^+\) T cells resist transplantation tolerance protocols and are a critical barrier to long-term graft acceptance in the clinic. We here show that semi-allogeneic pregnancy successfully reprogrammed memory fetus/graft-specific CD8\(^+\) T cells (T\(_{FGS}\)) towards hypofunction. Female C57BL/6 mice harboring memory CD8\(^+\) T cells generated by the rejection of BALB/c skin grafts and then mated with BALB/c males achieved rates of pregnancy comparable to naive controls. Post-partum fetus/graft-specific CD8\(^+\) T cells (T\(_{FGS}\)) from skin-sensitized dams upregulated expression of T cell exhaustion (T\(_{EX}\)) markers (Tox, Eomes, PD-1, TIGIT, and Lag3). Transcriptional analysis corroborated an enrichment of canonical T exhaustion (T\(_{EX}\)) genes in post-partum memory T\(_{FGS}\) and additionally, revealed a downregulation of a subset of memory-associated transcripts. Strikingly, pregnancy induced extensive epigenetic modifications of exhaustion- and memory-associated genes in memory T\(_{FGS}\), whereas minimal epigenetic modifications were observed in naive T\(_{FGS}\) cells. Finally, post-partum memory T\(_{FGS}\) durably expressed the exhaustion-enriched phenotype, and their susceptibility to transplantation tolerance was significantly restored compared to memory T\(_{FGS}\). These findings advance the concept of pregnancy as an epigenetic modulator inducing hypofunction in memory CD8\(^+\) T cells that has relevance not only for pregnancy and transplantation tolerance, but also for tumor immunity and chronic infections.
Introduction

Mammalian pregnancy has long been recognized as a model of spontaneous alloantigen-specific tolerance, whereby the maternal adaptive immune system must rapidly regulate responses towards the semi-allogeneic fetus to preserve fetal viability (1, 2). Maternal T cell tolerance is characterized by the upregulation of coinhibitory markers and inhibition of pro-inflammatory cytokine production in CD4$^+$FoxP3$^-$ conventional T cells (T$_{convs}$), as well as the expansion of fetus-specific CD4$^+$FoxP3$^+$ regulatory T cells (T$_{regs}$) that are protective in primary and secondary pregnancies (3-5). Notably, while pregnancy efficiently tolerizes naive fetus-specific T cells, it simultaneously induces humoral sensitization. We showed that pregnancy-induced a state of T cell tolerance that was sufficient to mediate the spontaneous acceptance of subsequently transplanted offspring-matched heart grafts, but only if B cells and fetus-specific antibodies are absent (3). Subsequently, Lewis et al. reported that naive OVA-reactive TCR-transgenic OT-I CD8$^+$ T cells acquired an exhausted transcriptional signature after pregnancy with OVA-expressing progeny (6), while Kinder et al. showed that endogenous OVA-reactive CD8$^+$ T cell expression of PD-1 and Lag-3 acquired during primary pregnancy protected against fetal wastage in a secondary pregnancy (7).

In contrast to pregnancy, semi-allogeneic organ transplants stimulate alloreactive CD4$^+$ and CD8$^+$ T cells that mediate graft rejection, with CD4$^+$ T cells promoting B cell and CD8$^+$ T cell responses as well as secreting pro-inflammatory cytokines and chemokines, and CD8$^+$ T cells play pro-inflammatory and cytotoxic roles (8-11). Alloreactive memory T cells are generated by exposure to allogeneic MHC following transplantation or blood transfusion, or through heterologous immunity, wherein T cells primed by infections or environmental antigens cross-react with donor MHC (12-15). As a result, most humans harbor memory alloreactive T cells, and their frequency increases with age (8). Importantly, memory T cells antagonize therapies that successfully induce transplantation tolerance in naive mice by resisting the induction of cell-intrinsic hypofunction achieved in naive T cells (16-18). Indeed, we recently reported that the presence of memory T cells sensitized to a single donor antigen mediated linked sensitization and were sufficient to prevent co-stimulation blockade-induced transplantation tolerance to a multiple mismatch
allograft (19). Thus, the potent barrier posed by alloreactive memory T cells to transplantation tolerance underscores the critical need to identify mechanisms for tolerizing memory T cell responses (20-23).

The critical role of memory CD8+ T cells in mediating allograft rejection, inducing spontaneous abortions, and antagonizing tolerance, prompted this proof-of-principle study to test whether pregnancy can successfully program hypofunction into memory fetus/graft-specific CD8+ T cells (T_{FGS}) (8-11, 24, 25). We show that despite the presence of rejection-induced memory CD4+, CD8+ and B cell responses, sensitized female mice consistently achieved spontaneous tolerance towards the semi-allogeneic fetus, achieving pregnancy success rates comparable to those of naive mice. We then used high-dimensional multi-omics approaches to show that pregnancy dedifferentiates memory CD8+ T cells into hypofunctional cells with an exhaustion phenotype and reduced expression of a subset of memory genes. The pregnancy-programmed hypofunctional phenotype in memory T_{FGS} was resistant to NFAT inhibition, associated with extensive epigenetic remodeling, persisted post-partum, and manifested as restored susceptibility to co-stimulation blockade-mediated transplantation tolerance. Taken together, our findings highlight the evolutionary robustness of mammalian pregnancy in constraining fully established allogeneic memory responses and introduces a potentially novel hypothesis that successful reprogramming of memory CD8+ T cells towards hypofunction requires the epigenetic imprinting of exhaustion circuits and reduced expression of a subset of memory genes. The conceptual foundation provided here brings us closer to understanding and therapeutically harnessing mechanisms of antigen-specific T cell hypofunction to substantially reduce the barrier that memory CD8+ T cells pose to transplantation tolerance.
Results

Pregnancy successfully constrains immunological memory

To test whether semi-allogeneic pregnancy is possible in females harboring immunological memory to paternal antigens, we sensitized female C57Bl/6 (B6, H-2b) mice with skin transplants (skinTx) from fully-mismatched male BALB/c mice. Female B6 mice rejected BALB/c skin grafts within 10 days (data not shown), and at day \( \geq 30 \) post-transplantation, they were mated with BALB/c males (rejection+pregnancy; R+P). The rates of successful pregnancy, including multiple successive pregnancies, were comparable between R+P and control naive mice mated with BALB/c males (pregnancy only; P) and no differences in resulting viable pups were observed (Figure 1a,b). Thus, pregnancy is able to constrain memory immune responses elicited by the rejection of fully-mismatched skin allografts to permit successful tolerance of the semi-allogeneic fetus.

Pregnancy induces the expression of co-inhibitory molecules in memory \( T_{FGS} \)

To gain insights into pregnancy-imposed hypofunction we tracked a tracer population of endogenous, polyclonal fetus-reactive CD8\(^+\) T cells that recognize the model 2W-OVA antigen expressed by the sensitizing skin and fetus. We sensitized B6 females with 2W-OVA.BALB/c (H-2d) skins and then mated them with 2W-OVA.BALB/c (H-2d) males (Fig 1c). OVA-specific CD8\(^+\) T cells were identified by flow cytometry using double fluorophore-labeled OVA:K\(^b\) tetramers; henceforth, these fetus- and graft-specific T cells are referred to as \( T_{FGS} \) (26, 27). We note that OVA expressed by the skin or F1 fetus is cross-presented by recipient/dam APCs, although it is possible that maternal APCs are cross-decorated with OVA:K\(^b\) complexes from F1 cells, or that microchimeric fetal cells are presenting OVA:K\(^b\) to maternal T cells (7, 28).

We designed a 19-color spectral flow cytometry panel to probe the expression of activation, memory, co-inhibitory and anergy markers by fetus (OVA)-specific \( T_{FGS} \) from naive (N), P, skin rejection (R) and R+P groups (Supplementary Table 1). OVA-specific CD8\(^+\) T cells were analyzed on day 30+ post-skinTx for R mice, or at post-partum day 0-3 for P and R+P mice. We observed a significant increase in
T_{FGS} recovery from R+P mice compared to P mice (Figure 1d, e). Despite this expansion, R+P T_{FGS} displayed elevated expression of multiple coinhibitory markers compared to R T_{FGS}, including PD-1, LAG3, TIGIT, and FR4 (Figure 1f, Extended Data Figure 1a-b). In contrast, P T_{FGS} preferentially upregulated both anergy markers, CD73 and FR4, as well as LAG3 and PD-1, compared to N T_{FGS}. Finally, only post-partum CD8^{+} T_{FGS} exhibited this phenotype in response to pregnancy, as the non-OVA-specific CD8^{+} T cells from P, R and R+P all resembled naïve T cells, thus confirming that the pregnancy-induced phenotype in T_{FGS} is driven by antigen recognition (Figure 1g).

To visualize T_{FGS} phenotypes at single-cell resolution, we used uniform manifold approximation and projection (UMAP) dimensionality reduction and FlowSOM clustering to identify 4 major and 3 minor cell subsets (Figure 1h). As anticipated, N and R T_{FGS} were largely homogenous, with >75% of these cells mapping to Cluster 1 or Cluster 4, respectively (Figure 1i). In contrast, the effect of pregnancy on T_{FGS} was heterogeneous, with ~50% of P and ~25% of R+P T_{FGS} remaining phenotypically similar to N or R T_{FGS}, respectively. Notably, Cluster 5 was identified as a shared cluster induced by pregnancy, comprising ~25% of both P and R+P T_{FGS} and defined by elevated expression of multiple coinhibitory markers, and reduced expression of the proliferation marker Ki67 (Figure 1j, Extended Data Figure 1c). Cluster 7 was unique to R+P T_{FGS} and was similar to Cluster 5 except for reduced CD73 expression. Collectively, these observations support a hypothesis that encounter of alloantigen during pregnancy programs hypofunction in memory T_{FGS} through the induction of higher levels of co-inhibitory exhaustion markers and lower levels of anergy markers compared to post-partum naïve T_{FGS}.

Pregnancy induces both distinct and shared transcriptional modifications in naïve and memory T_{FGS}

We next tested the hypothesis that the difference in phenotypic markers induced by pregnancy was indicative of a broader set of transcriptional modifications induced in memory vs. naïve T_{FGS}. We performed genome-wide transcriptional profiling of flow-sorted T_{FGS} subsets to account for the heterogeneity among pregnancy-modified T_{FGS} while retaining the advantageous sequencing depth of bulk RNA-sequencing.
We sorted OVA-specific T_{FGS} into the 4 predominant phenotypic subsets as illustrated in Fig 1g-h: Cluster 1 (C1, naive-like phenotype), Cluster 4 (C4, rejection-like phenotype), Cluster 5 (C5, shared by P and R+P), and Cluster 7 (C7, unique to R+P) (Figure 2a). The proportions of each cluster in this panel were consistent with our original phenotypic data (Figure 2b-c).

We constructed a heatmap to visualize the transcriptional expression of the markers used in our flow cytometry panel in Figure 1 and observed that the expression patterns in our transcriptional dataset were consistent with phenotypic data (Figure 2d-e). Pregnancy induced comparable levels of Pdcd1, and higher levels of exhaustion-associated transcripts Lag3 and Tigit in R+P C5 and C7 compared to P C5. In contrast, P C5 expressed higher levels of the anergy-associated transcripts, Nt5e (CD73) and Izumo1r (FR4) compared to R+P C7.

We next performed differential expression analysis to visualize the global transcriptional differences via UMAP (Figure 2f). R and N T_{FGS} displayed distinct transcriptional signatures, with the P C1 subset nearly identical to N T_{FGS}, and the R+P C4 subset similar to R T_{FGS}. These data corroborate the phenotyping data (Fig 1h-j) that a subset of T_{FGS} remained unmodified by pregnancy in both P and R+P mice. Differentially expressed genes (DEGs) by Cluster C5 T_{FGS} from P and R+P indicated that they were transcriptionally similar, while R+P C7 vs. R+P C5 T_{FGS} were more similar than initially anticipated based on the phenotypic data, sharing ~60% of their transcriptome respectively (Figure 2g).

To reduce complexity, we henceforward focused our subsequent transcriptional analysis on the post-pregnancy cell clusters P C5 and R+P C5, referring to them P and R+P, respectively (Figure 3a). UMAP confirmed that the transcriptomes of these post-partum P and R+P cells were more similar to each other than before pregnancy (Figure 3b). Visualizing the top DEGs between the 4 experimental groups (Naive, P, R, R+P) by heatmap and K-means clustering identified 4 main DEG clusters (Figure 3c, d). Clusters A and C DEG were upregulated by pregnancy in P and R+P compared to N and R T_{FGS} included T_{EX} genes, Tox, Eomes, Slamf6, Nfatc1/3, Lag3 and Havcr2 (Tim-3). Cluster B DEGs were downregulated in R and P, and even more so in R+P included Tcf7 and Lef1 transcriptional factors that are reduced in exhausted CD8^+ T cells (29-31). Interestingly, DEG Cluster D (n=362) was strongly upregulated in R vs. N
but downregulated in R+P TFGS to levels that approached P TFGS. Metascape analysis categorized these
DEGs as enriched for negative regulation of inflammatory responses, NK cytotoxicity, regulation of
lymphocyte immunity and viral protein interaction genes (Extended Data Figure 2a-e). These DEGs
included critical T cell effector genes (Gzma, Prf1) as well as chemokine genes that control effector T cell
migration to tissue sites (Cxcr3, Ccr5, Ccr6, Ccr2) (32-35). These data raise the possibility that a subset
of memory-associated upregulated transcripts is significantly downregulated by pregnancy.

We next focused our analysis on the unique 817 and 831 DEGs induced by pregnancy, with a 24%
transcriptional overlap (n=196), in R+P and P TFGS, respectively (Figure 3e). Visualizing DEGs unique to
post-partum memory TFGS via heatmap and volcano plots showed comparable numbers upregulated genes
(including exhaustion-associated genes: Ikkf2 (HELIOS), Havcr2 (TIM-3), and downregulated genes
(including memory-associated genes: Lef1, Il7r (CD127), and Tcf7l2 (TCF-4)) (Figure 3f; Extended Data
Figure 3a) (29-31, 36, 37). Metascape pathway analysis of R+P-unique DEGs (vs. R) indicated an
upregulation of the regulatory pathways for cytokine production and T cell differentiation, as well as
downregulation of JAK-STAT and Delta-Notch signaling (Extended Data Figure 3b). In contrast, the
majority of DEGs unique to P TFGS were upregulated, including Nfatc3, Ikkf3, and Runx2, and within the T
cell co-stimulation and cellular response to IL-18 Metascape pathways (Figure 3f; Extended Data Figure
3c).

Finally, we examined the set of 196 shared DEGs induced by pregnancy in both memory and naive
TFGS, with the majority of these shared DEGs being upregulated (168 genes) (Figure 3f). Metascape
pathway analysis revealed an enrichment in regulation of cytokine production, and of T cell activation and
differentiation (Extended Data Figure 3d). Notable examples included upregulated Tox, Nfatc1, Il10, Il21
and Tnfsf4, and downregulated Ccr7 and Satb1 (Extended Data Figure 3e). In contrast, R TFGS displayed
distinct transcriptional signatures, with many of the upregulated genes classified in T cell activation and
effector function pathways (Extended Data Figure 4a-b). Taken together, these data show that the
induction of T cell hypofunction by pregnancy results in shared and distinct transcriptional changes in
memory vs. naive TFGS.
Pregnancy elicits an exhausted transcriptional signature in memory T\textsubscript{FGS}

Lewis et al. (6) recently reported that pregnancy-induced hypofunction in naive OT-I cells was associated with a transcriptional state of exhaustion, prompting us to test whether memory T\textsubscript{FGS} could be similarly reprogrammed into exhaustion. To this end, we ranked the DEGs induced by pregnancy in memory or naive T\textsubscript{FGS}, comparing them to hallmark gene sets of exhausted CD8\textsuperscript{+} T cells (T\textsubscript{EX}) during chronic infection by Gene Set Enrichment Analysis (GSEA) (Figure 4a) (38). Indeed, we observed a significant enrichment in both upregulated and downregulated T\textsubscript{EX} signatures in R+P T\textsubscript{FGS}. Notably, Tox and Tigit were identified as part of the leading edge of upregulated genes, while Satb1 and IL-7r were in the leading edge of downregulated genes. In contrast, GSEA of the DEGs induced in P T\textsubscript{FGS} revealed a significant enrichment of only the upregulated T\textsubscript{EX} signature. As a control, we also ran GSEA on the DEGs of rejection (R vs. N T\textsubscript{FGS}) and showed that there was no enrichment for any exhaustion gene sets (Extended Data Figure 4c). Taken together, these GSEA analyses supported a more enriched transcriptional response towards exhaustion in post-partum memory compared to naive T\textsubscript{FGS}, consistent with the hypothesis that more co-inhibition is required to constrain memory T\textsubscript{FGS}.

We performed GSEA analyses on the distinct and shared DEGs induced by pregnancy in naive and memory T\textsubscript{FGS}, and compared them with multiple additional T\textsubscript{EX} gene sets from cancer, chronic infection, and pregnancy (Figure 4c-d, Extended Data Figure 5) (38-40). We corroborated the observation that pregnancy-induced DEGs unique to R+P were enriched for both up- and down-regulated T\textsubscript{EX} genes, whereas only upregulated T\textsubscript{EX} transcripts were enriched in P. Furthermore, even within the 196 pregnancy-induced gene set shared by naive and memory T\textsubscript{FGS}, we observed a statistically significant trend that the relative magnitude of transcriptional change was greater in R+P vs. P T\textsubscript{FGS} (Figure 4d). Notable examples of DEGs following this trend included Tox, Tigit, Il10, IL-21, and Satb1. In contrast, only a small subset of genes was more upregulated in P, including Pdcd1 (PD-1) and Tnfsf4 (OX40L).
Taken together, our RNA-seq data confirm that pregnancy induces clearly distinct global transcription signatures in memory vs. naive T\textsubscript{FGS}, with a significantly higher level and more extensive expression of exhaustion-associated transcripts in memory T\textsubscript{FGS}.

*Pregnancy induces distinct phenotypes of hypofunction in memory vs. naïve T\textsubscript{FGS}*

We validated our transcriptional findings by developing a larger 23-color spectral flow cytometry panel to assess the phenotypic expression of additional markers identified in our transcriptional analysis (Supplementary Table 1). This panel more readily captured differences between P and R+P T\textsubscript{FGS}, as illustrated by radar plot and UMAP+FlowSOM (Figure 5a-c), that were not observed in the non-OVA-specific CD8\textsuperscript{+} T cells (Extended Data Figure 6a). P T\textsubscript{FGS} (Cluster E) preferentially upregulated the anergy markers, FR4 and CD73, and more modestly upregulated T\textsubscript{EX} markers, Tox and Eomes, compared to R+P T\textsubscript{FGS} (Figure 5b-d, Extended Data Figure 6b-g). The majority of R+P T\textsubscript{FGS} mapped to Clusters C and D, which were characterized by a significantly more robust expression of Tox and Eomes compared to P T\textsubscript{FGS}. These data highlight the distinct gradation of phenotypic exhaustion markers induced by pregnancy in memory vs. naïve CD8\textsuperscript{+} T\textsubscript{FGS}.

Observations by Lewis et al. (6) and our observation of induced expression of NFAT in memory vs. naïve T\textsubscript{FGS} prompted us to test whether the phenotypic profiles of exhaustion was dependent on NFAT signaling. We show that treatment with FK506, a pharmacological inhibitor of NFAT, during pregnancy significantly reduced the expression of exhaustion markers, PD-1, Tox, NFATc1, Tigit, SLAMF6, and the anergy marker CD73, in P T\textsubscript{FGS} consistent with Lewis et al. (6) (Figure 5e, Extended Data Figure 7a-c). Notably, these markers were not significantly inhibited in R+P T\textsubscript{FGS}, suggesting that the expression of exhaustion/anergy markers in P T\textsubscript{FGS} is partially dependent on NFAT signaling whereas their expression by R+P T\textsubscript{FGS} is NFAT-independent. This underscores another difference in how pregnancy affects naïve vs. memory T\textsubscript{FGS} and raises the possibility of differential epigenetic modification driving the T\textsubscript{EX} phenotype in R+P T\textsubscript{FGS}. 
Pregnancy programs extensive exhaustion-associated chromatin remodeling in memory $T_{\text{FGS}}$, but not in naive $T_{\text{FGS}}$

Because CD8$^+$ T cells undergo epigenetic modifications during the differentiation into effector/memory and exhausted/hypofunctional T cells (37, 41-45), we hypothesized that pregnancy would epigenetically program memory and naive $T_{\text{FGS}}$ to sustain their states of hypofunction. We used the same sorting strategy described for RNA-Seq as defined in Figure 2 on fetus/graft-specific T cells to perform the Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-Seq). Chromatin accessibility heatmaps provided a visualization of global differences between $T_{\text{FGS}}$ subsets, while pie charts show comparable genomic distribution of the reproducible ATAC-Seq peaks identified for each $T_{\text{FGS}}$ cluster (Extended Data Figure 8a-b). An Upset plot showed the total number of reproducible peaks shared by various combinations of $T_{\text{FGS}}$ subsets, noting unique peaks present only in R+P C5 and/or C7 $T_{\text{FGS}}$ (Extended Data Figure 8c).

By visualizing the Differentially Accessible Peaks (DAPs) using UMAP and heatmap with K-means clustering, we show that N vs. R $T_{\text{FGS}}$ had distinct chromatin accessibility profiles (K-means clusters A-B) consistent with the acquisition of a memory T cell epigenome (Extended Data Figure 9a-d). These clusters grouped loci that were remodeled in R and more extensively in post-partum memory (R+P) $T_{\text{FGS}}$. In contrast, K-means cluster C grouped loci that were closed in N, P, R, and the R+P C4 (PD-1$^{\text{neg}}$) subsets, but significantly opened in R+P C5 and C7 $T_{\text{FGS}}$. Finally, K-means cluster D loci were open in N, P, and R subsets but closed in R+P $T_{\text{FGS}}$ (46-49). Collectively, these observations support the hypothesis that pregnancy imposed more extensive epigenetic modulation in memory vs. naive $T_{\text{FGS}}$.

To more rigorously address the hypothesis that epigenetic modifications in R+P but not P $T_{\text{FGS}}$ occurred during pregnancy, we leveraged our RNA-Seq dataset from Fig 3c to assess chromatin remodeling associated with pregnancy-induced DEGs in P or R+P $T_{\text{FGS}}$. At the loci of all DEGs (n=831) uniquely induced in naive $T_{\text{FGS}}$ by pregnancy, we observed no significant change in chromatin accessibility.
In contrast, significant increases and decreases in chromatin accessibility in the DEGs (n=817) induced by pregnancy in memory T\textsubscript{FGS}, corresponding to transcriptional up- and down-regulation, respectively (Figure 6b). These observations support the hypothesis that exhaustion transcriptome was associated with extensive pregnancy-mediated chromatin remodeling uniquely in memory T\textsubscript{FGS}, while the exhaustion transcriptome in naive T\textsubscript{FGS} required minimal chromatin remodeling.

Supporting this conclusion, chromatin accessibility of the 196 shared DEGs induced by pregnancy and enriched for T\textsubscript{EX} in R+P and P T\textsubscript{FGS} was also significantly changed in R+P vs. R T\textsubscript{FGS}, but not in P vs. N T\textsubscript{FGS} (Figure 6c, d). Notably, pregnancy-mediated chromatin remodeling remained detectable at distances of up to 100kb from the transcription start sites of these loci, supporting the possibility of both proximal remodeling of the locus itself and distal enhancer remodeling (Figure 6e). These differences are readily apparent when visualizing individual exhaustion-associated loci such as Tox and Maf, where multiple open peaks were present in R+P T\textsubscript{FGS} but not in R or P T\textsubscript{FGS} (Figure 6f-g) (36, 37, 40, 50, 51).

Chromatin accessibility of Satb1 was reduced in R+P, consistent with reduced transcription and its ability to repress PD-1 expression in CD8\textsuperscript{+} T cells (52). Notably, increased chromatin accessibility in R and decreased in R+P T\textsubscript{FGS} were also observed for T cell effector/memory genes, Prf1, Ccl5, Ifngr1, FasL, and Gata3, which were transcriptionally downregulated (Cluster D, Figure 3d) in post-partum memory T\textsubscript{FGS} (Figure 6i-k, Extended Data Figure 10a-b) (29, 30, 48, 53).

Finally, HOMER de-novo motif analysis was used to search for enrichment of conserved transcription factor DNA binding motifs associated with T cell function and differentiation among the DAPs in R+P vs. R T\textsubscript{FGS} (Extended Data Figure 11). This analysis identified in R+P vs. R T\textsubscript{FGS}, key motifs closing for Lef1, Tcf7, Tcf4, Tcf12, Batf, and motifs opening for Nfatc1, Tbx21, Eomes, Runx, and Jun, that have been implicated in T cell exhaustion (48). Together, these data support the conclusion of extensive epigenetic modification in post-partum memory T\textsubscript{FGS} at loci involved in T cell exhaustion and in a subset of the memory T cell signature.
Pregnancy programs sustained hypofunction in memory $T_{FGS}$

Because T cell exhaustion is diminished upon antigen deprivation, we tested whether the exhaustion phenotype induced by pregnancy was persistent in P and R+P $T_{FGS}$ (45, 54). On post-skin transplant (D30-60) or post-partum day 30-37, the expression of CD44 was significantly increased, and CD62L significantly reduced (Extended Data Figure 12a, b). The levels of exhaustion markers Tox, Tigit and PD-1 by R+P $T_{FGS}$ remained significantly elevated compared to R or P $T_{FGS}$ (Fig 7a). In contrast, the expression of, NFAT and FR4 was comparable in R+P and P $T_{FGS}$, while CD73 highest in P $T_{FGS}$ (Fig 7a; Extended Data Figure 12c). These data suggest that pregnancy-induced exhaustion was persistent especially in post-partum memory compared to naive $T_{FGS}$.

We next quantified the in vitro cytokine production capability of CD8$^+$ T cells following stimulation with allogeneic APCs. As expected, ~12% and 30% of R $T_{FGS}$ produced TNFα and IFNγ, respectively, which is significantly higher than N $T_{FGS}$ (Fig 7b; Extended Data Figure 13). P $T_{FGS}$ exhibited minimal TNFα and IFNγ production, remaining comparable to N $T_{FGS}$. Notably, TNFα production was significantly reduced in R+P $T_{FGS}$ compared to R $T_{FGS}$, however, the ability to IFNγ was not significantly altered (Figure 7b).

Finally, we tested whether the recall encounter of fetal antigens by memory T cells during pregnancy resulted in a persistent hyporesponsive state in the context of offspring-matched heart transplantation. To avoid the humoral sensitization that is simultaneously elicited by pregnancy and that we have previously shown as sufficient to mediate rejection of F1-heart grafts (3), we used an adoptive transfer (AdTr) approach whereby CD8$^+$ T cells purified from R or R+P (post-partum day 0-10) mice were injected into naive B6 hosts. Following AdTr of CD8$^+$ T cells, B6 hosts were transplanted with an F1 heart graft (B6xBALB/c) and received anti-CD154 and donor splenocyte transfusion (DST) (anti-CD154/DST; Figure 1d-e), a therapy that induces long-term graft acceptance in naive hosts. Consistent with previous reports (17, 18), memory CD8$^+$ T cells from R mice prevented stable graft acceptance. Remarkably, anti-CD154/DST treatment induced a significant extension of allograft survival in recipients of R+P CD8$^+$ T cells.
(Figure 7c-d; Supplementary Table 2). Thus, pregnancy enforces a cell-intrinsic state of hypofunction in post-partum memory $T_{FGS}$ that manifests as restored susceptibility to anti-CD154/DST-induced tolerance of offspring-matched heart grafts.
Discussion

Most studies of T cell tolerance to the semi-allogeneic fetus investigate the immunological effects of pregnancy in naive mice or those sensitized by prior pregnancy; in contrast, we show that the processes evoked during pregnancy are capable of restraining alloreactive memory T and B cell responses generated by skin graft rejection to allow for full-term delivery of viable semi-allogeneic offspring. The potential mechanisms mediating the reprogramming of memory CD8$^+$ T cells to hypofunction by pregnancy are suggested by their phenotypic and transcriptional signatures, which illustrated the differential impact pregnancy had on memory vs. naive TFGS. Post-partum memory TFGS had significantly higher transcriptional and phenotypic expression of exhaustion markers, Tox, Eomes, PD-1, and Tigit, whereas post-partum naive TFGS preferentially expressed the anergy markers, FR4 and CD73. GSEA analysis confirmed that pregnancy-induced transcripts in R+P were significantly enriched for canonical CD8$^+$ T cell exhaustion signatures that were up- and down-regulated in CD8$^+$ T cells infiltrating tumors or in chronic infection. In contrast, P TFGS were enriched for only upregulated transcripts associated with exhaustion. Additionally, even within the shared 196 DEGs induced by pregnancy in both naive and memory TFGS, the magnitude of up- or downregulation was significantly greater in R+P compared to P TFGS. We hypothesize that the higher levels of exhaustion and co-inhibitory markers are required to successfully restrain memory T cells, which have lower levels of activation thresholds due to increased TCR avidity and epigenetic programming (55, 56).

Changes in chromatin accessibility are the result of histone methylation, acetylation and phosphorylation that allow for increased or reduced transcriptional factor binding and subsequent gene transcription (57). We observed that pregnancy uniquely induced chromatin remodeling in memory CD8$^+$ TFGS, whereas naive TFGS remained largely epigenetically unmodified by pregnancy, even at shared exhaustion-associated loci induced transcriptionally by pregnancy. Additionally, pregnancy-induced opening of chromatin in post-partum memory TFGS was enriched for transcription factor motifs implicated in both early- and late-stage T cell exhaustion, including Tbx21, Eomes, and Jun (48). These observations are congruent with the significant increase in transcription of exhaustion-associated genes in post-partum
memory TFGS. That epigenetic modification enforces the hypofunctional state may provide an explanation
for the resistance to NFAT inhibition seen in R+P compared to P TFGS. The basis for why pregnancy has
distinct chromatin remodeling effects in R+P vs. P TFGS is unclear, but we speculate that it may be due to
intrinsically distinct epigenetic landscape in N vs. R TFGS from which R+P and P TFGS are derived.

Our observations also support the hypothesis that the partial reduction of the memory transcriptome
and epigenome contributes to the hypofunctional state of R+P TFGS. In addition to the upregulation of DEGs
negatively regulating cytokine production (Havcr2, Pdcd1lg2, Tgfb3), multiple genes involved in the rapid
response upon antigen reencounter were downregulated in R+P vs. R TFGS. These include genes encoding
effector molecules (CD48, Prf1, Fasl, Fcgr2b, Klr family), chemokine/chemokine receptors (Ccl6, Ccl9,
Ccl5, Ccr2, Ccr3, Cxcr3, Cx3cr1, Xcr1), and cytokine/ cytokine receptors (Il18r1, Il18r1, Ifngr1, Il2rb) (34,
45, 53, 55). Furthermore, a subset of these genes (Ccl5, Gata3, Ifnr1, Prf1 and Fasl) underwent chromatin
remodeling following both rejection that was reversed by pregnancy. These data raise the tantalizing
possibility that pregnancy utilizes targeted epigenetic modifications in memory TFGS not only to induce
transcriptional exhaustion but also to dedifferentiate TFGS from memory/effector programming.

Memory CD8\(^+\) TFGS generated following rejection of allogeneic skin grafts exhibit increased
production of TNF\(\alpha\) and IFN\(\gamma\) and resistance to co-stimulation blockade-mediated acceptance of heart
allografts compared to naive TFGS (3, 16-18). Post-partum memory CD8\(^+\) T cells exhibited significantly
reduced ability to produce TNF\(\alpha\), but retained their ability to produce IFN\(\gamma\), relative to R CD8\(^+\) T cells. The
physiological roles of uterine NK cells producing IFN\(\gamma\) in promoting pregnancy, vascular/tissue remodeling
and preventing excessive trophoblast invasion have been described (58, 59). Furthermore, TNF\(\alpha\)
combined with high doses of IFN\(\gamma\) is compatible with healthy pregnancy, and "controlled" levels of Th1
cells and TNF\(\alpha\) may have essential roles in successfully pregnancy (58, 60, 61). Thus, we speculate that
the ability to produce IFN\(\gamma\) and TNF\(\alpha\) by T cells may be preserved in pregnancy. It is notable that IFN\(\gamma\)
plays a non-redundant role in allograft tolerance, as mice deficient in IFN\(\gamma\) fail to develop tolerance with no
defects in acute rejection (62, 63). Indeed, we show that pregnancy was able to relieve the barrier memory
CD8\(^+\) T cells normally pose to transplantation tolerance, as evidenced by the enhanced survival of
subsequent offspring-matched heart grafts under co-stimulation blockade in recipients that received R+P vs. R CD8^+ T_{FGS} cells. These observations provide proof-of-concept that memory CD8^+ T cells, which heretofore were considered an insurmountable barrier to clinical transplantation tolerance, can be reprogrammed to hypofunction and susceptibility to anti-CD154/DST-induced graft acceptance.

There are several limitations to this study. Firstly, we introduced a model OVA antigen to the semi-allogeneic fetus and allograft to enable tracking endogenous polyclonal fetus/graft specific CD8^+ T cells. It is possible that the highly immunogenic OVA may be immunodominant over “true” alloantigens and elicit higher avidity T cell responses than observed for alloreactive T cells. Secondly, our data suggest that the expression of exhaustion transcriptome and markers, as well as the partial reversal of the memory phenotype contributes to the hypofunctional state of post-partum memory T_{FGS}. However, definitive necessity and sufficiency studies are necessary. Thirdly, a mechanistic explanation for why memory but not naive T_{FGS} undergo such extensive chromatin remodeling during pregnancy is lacking, and necessity of this remodeling for the maintenance of the hypofunction state in post-partum memory T_{FGS} has not be demonstrated. Finally, while sensitivity to co-stimulation blockade is significantly restored, all the F1 heart grafts ultimately rejected. Post-partum memory T_{FGS} retained the ability to produce IFNγ and TNFα, and a subset of R T_{FGS} were unmodified by pregnancy. These observations suggest that additional memory programs have to be constrained to achieve comparable states of hypofunction as observed in post-partum naive T_{FGS} (3).

Pregnancy is an immunological paradox, wherein the conflict between the preservation of robust immunity towards foreign pathogens and tolerance to the semi-allogeneic fetus must be simultaneously resolved to preserve the survival of the species. Furthermore, memory fetus-specific T cells must be constrained. The imperative to preserve fetal viability underscores the necessity of multiple conserved and redundant mechanisms for controlling both naive and memory T cells. Our studies reveal a potentially novel endogenous mechanism for the reprogramming of antigen-specific memory T cells towards exhaustion and hypofunction (Extended Data Figure 14). This mechanistic insight is critically relevant for understanding semi-allogeneic pregnancy as well as the successful induction of transplantation tolerance.
in the clinic, where no conceptual framework for reprogramming of memory donor-specific T cells has yet been identified (16-18). In addition, viewing CD8+ T cell exhaustion/hypofunction through the lens of pregnancy potentially solves the seemingly counterintuitive evolutionary puzzle of why exhaustion is so quickly induced when T cells are exposed to chronic infections or tumors, which is often detrimental to the host (64). We theorize that this timeline is imposed by mammalian pregnancy requiring a rapid restraint of fetus-specific alloreactive T cells to preserve fetal viability. Moreover, while the phenotype and transcriptome of exhaustion was initially discovered in the context of chronic infection and tumors, we posit that this phenomenon should be re-evaluated from the perspective that exhaustion pathways developed due to the stringent need to preserve the semi-allogeneic fetus, and these mechanisms have been subsequently hijacked by chronic infections and tumors. Thus, insights into how exhaustion is programmed into memory T_FGS during pregnancy are relevant not only to addressing problems related to high-risk pregnancies and transplantation tolerance, but also to broader clinical issues such as autoimmunity, chronic infection, and cancer, where controlling T cell hypofunction is also desirable.
Methods

Sex as a biological variable: This study’s main focus is the effect of pregnancy on the maternal immune system. The investigation of pregnancy justifies and necessitates the use of exclusively female mice in this study.

Mice. Eight- to twelve-week-old female C57Bl/6 (B6, H-2\(^{b}\)) mice were purchased from Harlan Laboratories. Act-2W-OVA transgenic mice on a B/6 background (2W-OVA.C57BL/6) were a gift from James Moon (Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts, USA). Donor/paternal 2W-OVA.BALB/c (2W-OVA.B/c, H-2\(^{d}\)) mice were backcrossed from 2W-OVA.B/6 mice for >10 generations. For semi-allogeneic pregnancies, a harem breeding involving one male 2W-OVA.BALB/c with 4 virgin or post-partum B/6 females. Approximately 50% of F1 from this mating were confirmed to be 2W-OVA-positive, and 2W-OVA.F1 (B6x2W-OVA.B/c) mice were used as heart donors.

Adoptive transfer, heart, and skin transplantation. For adoptive transfer experiments, \(\sim 4 - 12 \times 10^5\) CD45.2\(^+\) CD8\(^+\) T cells, isolated via magnetic enrichment, were transferred retro-orbitally (r.o.) into naive CD45.1\(^+\) C57BL/6 hosts one day prior to heart transplantation. See below for T cell enrichment description. Heterotopic heart transplantations were performed as previously described (65), by grafting 2W-OVA.F1 (B6x2W-OVA.B/c) hearts onto the inferior vena cava and aorta of female recipients. Tolerance (CoB/DST) was induced with a combination of anti-CD154 (MR1, BioXCell) at a dose of 500\(\mu\)g on day 0 (i.v.), and 250\(\mu\)g on days 7 and 14 (i.p.) post-transplantation, in combination with \(2 \times 10^7\) donor spleen cells on day 0. Graft survival was assessed by palpation 2-3 times per week, and the day of rejection was defined as the last day of detectable heartbeat. Flank skin from 2W-OVA.BALB/c was transplanted onto the B/6 mice.

FK506 injection. FK506 was injected daily (1 mg/kg i.p.) into pregnant mice beginning 5 days after the first observation of a copulation plug and ending on the date of euthanasia (day 0-3 post-delivery).
**T Cell enrichment.** Single-cell suspensions from spleens and pooled LNs (brachial, inguinal, and axillary) of individual mice were prepared for each experiment (see below). For flow cytometry and cell sorting assays, Pan-T lymphocytes were enriched with Pan-T cell isolation kit II (Miltenyi Biotech). For CD8⁺ T cell adoptive transfer experiments, the CD8α⁺ T Cell isolation kit (Miltenyi Biotech) was used instead. Samples were passed through LS columns on a QuadroMACS separator (Miltenyi Biotech) in MACS buffer (2%FBS + 2mM EDTA).

**Cell harvest and fluorescent staining for flow cytometry and cell sorting.** Spleens and LNs were harvested and passed through a 40μm cell strainer (Corning Inc., USA), followed by lysis of red blood cells via 2-minute incubation with ammonium chloride-potassium (ACK) lysis buffer (Quality Biological). After magnetic enrichment for T cells (see above), approximately 2x10⁷ cells were stained with a fixable live/dead stain (Invitrogen), followed by tetramer staining. Tetramer staining was performed for 35-45 min at room temperature with PE- and APC-conjugated OVA (SIINFEKL):H-2Kb tetramers (NIH Tetramer Core Facility). The cells were then stained for extracellular antibodies for 15-20min at 4°C. Samples were fixed with the Invitrogen Fix/Perm buffer kit according to the manufacturer’s instruction. Finally, fixed and permeabilized samples were stained for intracellular markers overnight. For phenotypic analysis, samples were acquired via flow cytometry after fixation and intracellular staining. For cell sorting, samples were sorted into RPMI after extracellular staining.

**In vitro stimulation and staining for IFNγ and TNFα.** Splenocyte stimulators from 2W-OVA.F1 mice were treated with ACK lysing buffer (Quality biological), followed by 30min incubation with anti-CD90.2 (53-2.1, BD Biosciences) to deplete T cells. Labeled T cells were depleted with two consecutive 35min incubations with rabbit complement (Cedarlane) at 37°C, and then incubated overnight with 20μg/ml LPS. 1 x 10⁶ responder cells (Pan-T enriched splenocytes) were plated with 0.5 x 10⁶ stimulators (T-depleted APC’s) in triplicate in a 96-well plate (Corning) and incubated at 37°C overnight. Next, Golgi Plug (BD Biosciences) was added at 1:1000 and incubated for an additional 6h at 37°C. Live/Dead and extracellular staining were performed for 10min and 15min (respectively) on ice, and cells were then fixed with BD
Cytofix/Cytoperm according to the manufacturer’s instruction (BD Biosciences). Finally, cells were stained for intracellular IFNγ and TNFα and acquired via flow cytometry.

**Flow cytometry and cell sorting acquisition and analysis.** Flow cytometry samples for phenotypic panels and in-vitro cytokine stimulation assays were acquired on a Cytek Aurora flow cytometer (5 lasers, 16UV-16V-14B-10YG-8R). For cell sorting, samples were acquired and sorted on a BD Aria II 4-15 (70μm nozzle), BD Aria Fusion 5-18 (70μm nozzle), or the Invitrogen Bigfoot (100μm nozzle). The associated software for each cytometer is as follows: Aurora is Cytek SpectroFlo, Aria and Aria Fusion are BD FACSDiva, and Bigfoot is Invitrogen Sasquatch Software (SQS). Data were analyzed and visualized with FlowJo software v10.8.1 (FlowJo, LLC).

**Fluorescent antibodies for flow cytometry and cell sorting.** Fluorochrome-conjugated antibodies were used to select and sort cell subsets, analyze T cell phenotypes, and to determine cytokine production. The following antibodies were used in this study, separated by manufacturer (clone is indicated in parentheses).

- **Biolegend:** Ki67-PacificBlue (16A8), CD62L-BV510 (MEL-14), CD73-BV605 (TY/11.8), CD44-FITC (IM7), PD1-PEDazzle594 (RMP1-30), TIGIT-PECy7 (1G9), LAG3-BV785 (C9B7W), IFNγ (XMG1.2), TNF-α-PECy7 (MP6-XT22), SATB1-AlexaFluor594 (O96C6), TIM3-APC/Fire750 (B8.2C12), OX40-BV711 (OX-86), OX40L-PECy7 (RM134L), Tim3-PerCP/Cy5.5 (B8.2C12), CD8-FITC (53-6.7), CD90.2-PECy7 (30-H12), CD90.2-PerCP/Cy5 (53-2.1), CD4-APCCy7 (RM4-5).
- **BD Biosciences:** CD44-BUV496 (GK1.5), CD19-BUV661 (1D3), CD11c-BUV661 (N418), F4/80-BUV661 (T45-2342), NK1.1-BUV661 (PK136), TER119-BUV661 (TER-119), CD127-BUV737 (SB/199), CD8-BUV805 (53-6.7), FR4-BV421 (12A5), CTLA4-APC700 (UC10-4F10-11), NK1.1-eFluor450 (PK136), Ter-119-eFluor450 (Ter-119), Rorty-BV650 (Q31-378), CD62L-BV605 (MEL-14).
- **Invitrogen:** FoxP3-AlexaFluor532 (FJK-16s), CD44-BUV737 (IM7), PD1-SB780 (J43), TOX-eFluor660 (TXRX10), EOMES-PerCP/eFluor710 (Dan11mag), F4/80-eFluor450 (BM8), CD49b-eFluor450 (DX5), CD11c-eFluor450 (N418), PD1-PerCP-e710 (J43).
- **Santa Cruz Biotechnologies:** NFATc1-AlexaFluor488 (7A6), CD30L-AlexaFluor680 (RM153).
RNA-Sequencing Data Collection and Processing. RNA-Seq libraries were generated and amplified according to the SmartSeq2 protocol (66). 200 live cells per sample/subset were sorted into 96-well optical PCR plates (Thomas Scientific) containing 4μl of lysis buffer at 4°C. cDNA sequencing libraries were generated using Nextera XT DNA Library Prep Kit and Nextera XT Index Kit (Illumina). All libraries were sequenced in the same run on a NovaSeq 6000 in a 150 bp/150 bp paired-end configuration. An average of $\sim 55 \times 10^6$ paired reads was generated per sample.

RNA-Sequencing Data and Processing. Raw RNA-Seq reads were trimmed for adapter content and filtered for truncated reads using Cutadapt v3.4 (67). Paired-end reads were aligned using STAR v2.6.1b (68) against the GRCm39 (mm39) reference genome and transcriptome annotations, and non-uniquely mapping reads were removed. Per-sample read counts for each gene were quantified sample using featureCounts v2.0.1 (69).

ATAC sequencing. Chromatin profiling was performed by ATAC-seq as described previously (70, 71). In brief, ~3,000-50,000 sorted cells were washed in cold PBS and lysed to isolate intact nuclei. Transposition was performed at 37°C for 30min with the Tagment DNA Enzyme and Buffer kit (Illumina). After purification of the transposed DNA with the MinElute PCR purification kit (Qiagen), material was amplified via PCR for 13-14 cycles with Nextera XT Index primers (Illumina). Final product was purified again via MinElute PCR purification kit (Qiagen). Libraries were sequenced in the same run on a NovaSeq 6000 in a 150 bp/150 bp paired-end configuration. An average of $75 \times 10^6$ paired reads was generated per sample.

ATAC-Sequencing Data and Processing. Raw ATAC-Seq reads were trimmed for adapter content and filtered for truncated reads using Cutadapt v3.4 (67). Paired-end reads were aligned using Bowtie2 v2.2.9 (72) against the GRCm39 (mm39) reference genome. Non-uniquely mapping reads and PCR duplicates were filtered with Bamtools v2.5 and Picard v2.21.8, respectively (73, 74). Peaks corresponding to ATAC-Seq cut sites for each sample were called using Genrich v0.6.1 in ATAC-Seq mode (https://github.com/jsh58/Genrich). Finally, reproducibly identifiable peaks for each experimental group were identified via ChIP-R v1.2.0 (75).
Processing of ATAC-Seq peak set for differential accessibility analysis. Reproducibly identifiable peaks across all experimental groups were merged into a single reference peak set using Bedtools v2.27.1 (76). Multibam summary v3.5.1 from the Deeptools suite (77) was used to generate per-sample read counts at each peak from the reference peak set. The read counts data was then imported into R v4.1.0, and each peak was assigned to a single gene via nearest TSS using GenomicRanges v1.46.1, ChIPpeakAnno v3.28.1, and the Org.mm.eg.db v3.14.0 genomic annotation object (78, 79).

Sequencing Data Analysis and Visualization. After completing data preprocessing as described above, the DESeq v1.34.0 package was used to conduct differential expression/accessibility analysis on sequencing datasets (80). For both RNA-Seq and ATAC-Seq, the threshold for determining differential expression/accessibility was FDR \( p_{\text{adj}} < 0.1 \) and absolute value of log2 fold-change >0.9. In addition to DESeq2, we used current versions of the following packages for analysis and visualization (with description of purpose in parentheses). Viridis and RColorBrewer (color scale creation). Gplots, ggplot2 and ggrepel (graphing data and generating heatmaps). Uwot and VennDiagram (UMAP and Venn graphs, respectively). Tidyverse suite (dataset manipulation).

ATAC-Seq motif analysis and locus visualization. Motif analysis was performed by identifying unique and common peak sets between two experimental groups (using the reproducible peaks for each group as described above). These peak sets were then analyzed via HOMER de-novo motif analysis (81) to search for significantly enriched motifs associated with ATAC-Seq cut sites and annotate these motifs to possible transcription factor targets. Individual loci were visualized by generating bigwig files for each sample and importing them into IGV v2.12.3 (82). A single track for each experimental group was created by summing the read counts of two representative samples from each group.

Gene Set Enrichment Analysis. GSEA software (4.0.3) was downloaded from the Broad Institute (https://www.gsea-msigdb.org/gsea/index.jsp), and pre-ranked GSEA was performed on the selected gene sets in this study. Gene set files were downloaded from the Molecular Signatures Database or prepared manually as gene matrix expression files (.GMX), using DESeq2 on published RNA-Seq data. Ranked
gene lists for our transcriptional data were generated from by arranging genes based on the Change Metric (fold change × −log₁₀ p_adj) from high to low. The change metric combines both significance and intensity of expression changes, while preserving the direction (up- or -downregulation) with positive or negative values.

**Pathway Analysis.** Two lists of DEGs (or differentially accessible peaks) were created for each pairwise comparison, one for upregulated/opened regions, and one for downregulated/closed regions. The ENSEMBL gene IDs of each list were then uploaded to Metascape Pathway analysis (83) to calculate the enrichment and significance of functional gene pathways from Gene Ontology (GO), Kegg, Reactome, or WikiPathways databases (primarily GO).

**Computational resources.** All data preprocessing for both ATAC-Seq and RNA-Seq (adapter trimming, alignment, filtering, generation of read-counts, and peak-calling) was performed on the Midway2 high-performance compute cluster, which is maintained by the University of Chicago Research Computing Center.

**Statistics.** Statistical significance analyses were performed using GraphPad Prism version 9.2.0. Sample size of >5 animals per experiment were chosen to ensure adequate power. Graft survival significance was assessed using a Kaplan-Meier/Mantel-Cox log rank test. P values <0.05 were considered to indicate a significant difference. To calculate differences between experimental animals, we used Kruskal-Wallis test (ANOVA) with Dunn’s post hoc test for pairwise multiple comparisons, one-way ANOVA with Tukey’s post-hoc test, or Welch’s unpaired t test (specific tests for each subfigure are indicated in the figure legends). Asterisks used to indicate significance correspond to the following: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

**Study approval:** All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago and adhered to the standards of the NIH Guide for the Care and Use of Laboratory Animals.
Data availability: The RNA-seq and ATAC-seq data have been deposited as a SuperSeries in the Gene Expression Omnibus (Accession code GSE216302). All scripts used for data analysis have been uploaded to GitHub at https://github.com/jardplard/Chong_CD8_Pregnancy. This repository includes the supporting data values for additional graphs displayed in this manuscript that were not generated from the scripts mentioned above (“Merged Source Data.xls”). Additional information and materials will be made available upon request.

Code availability: All code was generated based on publicly available software packages; scripts used for data analysis have been uploaded to GitHub at https://github.com/jardplard/Chong_CD8_Pregnancy.

Conflict of interest: The authors have declared that no conflict of interest exists.

Author Contributions: Authorship order was made after discussion between co-first and co-senior authors, with additional recommendation from three anonymous faculty at the University of Chicago. JMP designed and performed the experiments and analyzed data, including pipeline design and script writing for ATAC-Seq and RNA-Seq preprocessing and downstream analyses. JMP wrote the manuscript and generated figures, with guidance from ASC and MLA. GH also performed experiments that generated flow cytometry data, and analyzed RNA-seq and ATAC-seq data, generated figures, and edited the manuscript. DY and ASC conceived the initial project, and DY performed skin and heart transplantations. MM and FG provided supervision on ATAC-Seq and integrated bioinformatics analysis. All authors provided feedback on figure layout and manuscript content.

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Resources Center at The University of Chicago for their assistance. The $T_{EX}$ datasets used for GSEA analysis were generously provided by Dr. Paige Porrett (University of Alabama, Birmingham, AL) (6).
Figures and Legends

(a) % Successful Pregnancy
(b) Litter Size
(c) Diagram of experimental setup
(d) Flow cytometry plots
(e) Heatmap of total OVA+CD8+ cells
(f) Hexagon plot of immune cell expression
(g) Spider plot of immune cell expression
(h) UMAP plots of cell clusters
(i) Bar chart showing % FlowSOM Cluster
(j) UMAP plots of expression levels
Figure 1: Pregnancy induces a hypofunctional phenotype in memory OVA-specific TFGS. a, Bar graph showing percentage of Naive vs. Sensitized female mice achieving successful full-term pregnancies; $n = 35-49$ mated mice per group. Additionally, there was a 100% success rate in sensitized mice subjected to a second pregnancy ($n=12$). P values were determined by Chi-square ($\chi^2$) Test of Independence. b, Bar graph showing number of viable pups at birth (litter sizes) of Naive vs. Sensitized female mice achieving successful full-term pregnancies; $n = 31-39$ per group. Each dot indicates individual mice. c, Experimental design. Female B6 mice were mated with transgenic 2W-OVA.B/c mice, with or without sensitization to 2W-OVA.B/c via skin graft 30 days prior (R+P and P, respectively). Unmated mice with or without skin graft rejection were included as controls (naïve (N) and rejection (R), respectively). d, Representative pseudocolor plots showing OVA:Kb-specific CD8+ T cells (TFGS). Each dot indicates individual mice. e, Normalized total recovery of TFGS cells at post-partum day 0-3. Data acquired from 2 or more biologically independent experiments; $n = 20-38$ per group. P values were determined by Kruskal-Wallis test with Dunn’s post hoc test. f-g, Radar plot showing phenotypic profile of (f) TFGS or (g) non-TFGS based on markers of activation, memory, and co-inhibition. Data are normalized to the highest and lowest MFI for each marker expressed by TFGS or non-TFGS from all 4 experimental groups. Symbols color coded as in d. Expression is represented as normalized percentage of the highest/lowest-expressing group (based on all OVA+TFGS and non- TFGS) for each marker. h, UMAP with experimental groups (left) and FlowSOM clustering (right) reveals distinct phenotypic subsets in TFGS. i, Stacked bar graph showing FlowSOM cluster distributions for each experimental group. j, UMAP with heatmap overlays to show expression of each phenotypic marker on TFGS at single-cell resolution. Data represent mean ± SEM. Gating strategy, statistical analysis and representative histograms of this flow dataset are in Extended Data Figure 1a-c. ns: not significant; *P<0.05; ****P<0.0001.
Figure 2: Pregnancy induces broad transcriptional modification in memory OVA-specific T_FGS. a, Sorting strategy for OVA:K^b-specific T_FGS from each experimental group into their most prevalent phenotypic subsets as defined by FlowSOM (in Fig 1i). T_FGS were acquired and sorted from 2 biologically independent experiments; n = 3-5 per group, with technical replicates for each biological sample. b, Gating strategy for each cluster of OVA:K^b-specific T_FGS. c, Representative plots showing the distributions of Cluster 1+4, Cluster 5, and Cluster 7 within bulk or OVA:K^b-specific R+P CD8^+ T cells. Percentage of each cell cluster is comparable to the distribution of our FlowSOM analysis in Figure 1h for the experimental groups. d-e, Row-normalized RNA-seq expression and box plots of normalized RNA-seq read counts for key exhaustion and anergy markers corresponding to Fig 1i. Each dot in box plots or UMAP, and each column in the heatmap, indicates individual mice. P values (in e) were determined by Kruskal-Wallis test with Dunn’s post hoc test. ns: not significant; *P<0.05; ***P<0.001; ****P<0.0001. f, UMAP comparing all T_FGS subsets analyzed by RNA-seq. g, Venn diagram of DEGs unique to R+P C7 T_FGS, R+P C5 T_FGS, and shared between both subsets.
Figure 3: Post-partum memory and naive OVA-specific T_{FGS} acquire distinct and shared transcriptional signatures. a. OVA:K^b-specific T_{FGS} from each experimental group; N, R, P (C5) and R+P (C5) b. UMAP plot comparing transcriptional profiles among T_{FGS} subsets. c-d, Row-normalized RNA-seq expression of the top differentially expressed genes (n=1894) (c), organized by K-means clustering into Clusters A-D, indicated by right-side column. The total number and examples of DEGs in each K-means cluster are listed on the right. d, Box plots visualizing relative expression of DEGs in each K-Means cluster identified in (c). Minimum criteria for DEGs shown in this figure was both q<0.1 and log_2 fold-change>0.9. P values (in d) were determined by Kruskal-Wallis test with Dunn’s post hoc test. ns: not significant; ****P<0.0001. e. Venn diagram and f, row-normalized RNA-seq expression of DEGs induced by pregnancy in only R+P (n=635 DEGs), only P (n=621 DEGs) or both R+P and P T_{FGS} (n=196 DEGs). Each dot in UMAP or box plots, and each column in the heatmap, indicates individual mice.
Figure 4: Post-partum memory OVA-specific T<sub>FGS</sub> acquire a transcriptional signature of exhaustion. a, GSEA curves showing enrichment of the exhausted T cell signature (chronic viral infection (38)) in R+P vs. R and P vs. N DEGs. NES, Normalized Enrichment Score. b, Summary of GSEA analysis comparing DEGs unique to R+P vs. R (left) or P vs. N (right) or c, shared DEGs by R+P and P to published gene sets of exhaustion (6, 38-40). d, Dot plot comparing magnitude of up- or downregulation for shared DEG’s between R+P and P T<sub>FGS</sub> using the Change Metric (C.M.), a single statistic that merges FDR-corrected p-value and log fold change (±log<sub>2</sub>(FC) x -log(FDR)). P-values were calculated with the Wilcoxon matched-pairs signed rank test comparing R+P vs. P T<sub>FGS</sub>. P(upregulation)<0.0001 | P(downregulation)=0.0032 |.
a

OX40  NFATc1  TOX
CD44  EOMES
CD62L  SATB1
FR4  CD73
SLAMF6

b

UMAP Y
UMAP X

FlowSOM A  FlowSOM B  FlowSOM C  FlowSOM D  FlowSOM E

FlowSOM E
FlowSOM C
FlowSOM B
FlowSOM D
FlowSOM A

FlowSOM Cluster

% FlowSOM Cluster

0  25  50  75  100

N  P  R  R+P

c

d

TOX
EOMES
NFATc1
SLAMF6

PD-1
CD73
FR4
OVA:K\(^{\circ}\)

Low
High

UMAP Y
UMAP X

e

% PD-1\(^{-}\) – OVA:K\(^{\circ}\) – CD8\(^{+}\)

% TOX\(^{-}\) – OVA:K\(^{\circ}\) – CD8\(^{+}\)

% TIGIT\(^{-}\) – OVA:K\(^{\circ}\) – CD8\(^{+}\)

% SLAMF6\(^{-}\) – OVA:K\(^{\circ}\) – CD8\(^{+}\)

Group: N  P  R  R+P
FK506: -  -  +  +
Figure 5: Pregnancy programs distinct exhaustion phenotypes in memory vs. naive OVA-specific T_{FGS}. a-e, Flow cytometry panel based on RNA-seq results confirms phenotypic exhaustion in post-partum T_{FGS}. a, Radar plot presenting normalized expression of phenotypic markers (based on highest and lowest MFI for each marker expressed by T_{FGS} and non-T_{FGS} from all 4 experimental groups) demonstrates enhanced separation between R+P and P T_{FGS}. b-c, UMAP and FlowSOM reveal distinct clusters for R+P and P T_{FGS} driven by phenotypic differences in TOX, EOMES, FR4, and CD73. d, UMAP with heatmap overlays were generated to visualize phenotypic differences between T_{FGS} subsets. e, Expression levels of PD-1, TOX, TIGIT and SLAMF6 by memory vs. naive T_{FGS} from dams treated with FK506, an inhibitor of NFAT. P values were determined by one-way ANOVA; *P<0.05; **P<0.01; ****P<0.0001.
Figure 6: Pregnancy alters the chromatin state of memory but not naive OVA-specific T_{FGS}. a-b, T_{FGS} subsets were acquired and sorted for ATAC-Seq as in Figure 2a. Box plots visualizing chromatin accessibility at DEGs unique to P vs. N (a), or unique to R+P vs. R (b). P values (for a-b) were determined by Welch’s t-test. c-d, UMAP and box plots of chromatin accessibility at the 196 DEGs shared by P and R+P vs. R T_{FGS}. Data acquired from ≥2 biologically independent experiments with n = 3-4 per group. P values (for d) were determined by Welch’s t-test. e, Bar plots visualizing the mean fold-change of distal ATAC-seq peaks within 0-50kb (left) or 50-100kb (right) of the TSS of shared pregnancy-induced DEGs. P vs. N T_{FGS} (blue) or R+P vs. R T_{FGS} (red). P values (for e) were determined by Welch’s t-test. f-h, ATAC-Seq tracks at the Tox, Maf and Satb1 loci, and i-k, Ccl5, Ifngr1 and Prf1. Peaks uniquely induced in R and reversed in R+P T_{FGS} are highlighted in gray. Each dot in box plots or UMAP indicates individual mice. ns: not significant; *P<0.05; **P<0.01, ***P<0.001; ****P<0.0001.
Figure 7: Pregnancy induces in memory OVA-specific TFGS a sustained exhausted phenotype and restores susceptibility to co-stimulation blockade-induced acceptance of fetus-matched heart allografts. a, Percentage of OVA-specific TFGS from P and R+P (both at post-partum day 30), Naive (N) or R (day 30-60 post-skin transplant) expressing Tox, Tigit, PD-1, NFATc1 and CD73. b, Bar graphs visualizing IFNγ (left) and TNFα (right) production of CD8⁺ T EM cells (CD44⁺CD62L⁻) after overnight in vitro stimulation with activated F1 APCs. Data were acquired from 2 or more biologically independent experiments; n = 4-13 per group. Data represent mean ± SEM. P values were determined by one-way ANOVA (for a), and Kruskal-Wallis test with Dunn’s post hoc test (for b). c, Experimental design for adoptive transfer (AdTr) of CD8⁺ T cells from R or R+P mice (harvested on post-partum day 0-10) into
naive CD45.1 B6 mice. 1 day after AdTr, these and PBS-control mice received allogeneic 2W-OVA,F1
(2W-OVA.B/c x B6) heart transplantation with anti-CD154/DST tolerance induction. d, Percentage of 2W-
OVA,F1 heart graft survival among AdTr recipients; n = 6-7 per group. Each dot indicates individual mice.
P values were determined by Mantel-Cox log-rank test. ns: not significant; *P<0.05; **P<0.01, ***P<0.001;
****P<0.0001.
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